Consejo Superior de Investigaciones Científicas

Estación Experimental del Zaidín

UNIVERSIDAD DE GRANADA

Bacterial gene regulation mediated by extracytoplasmic function (ECF) sigma factors: ECF as target for developing new antimicrobial compounds

Programa de Doctorado de Biología Fundamental y de Sistemas

Tesis Doctoral

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2019

Editor: Universidad de Granada. Tesis Doctorales
Autor: Joaquín Rodrigo Otero Asman
ISBN: 978-84-1306-237-2
URI: http://hdl.handle.net/10481/56224

Bacterial gene regulation mediated by extracytoplasmic function (ECF) sigma factors: ECF as target for developing new antimicrobial compounds

Memoria que presenta el Licenciado en Biología

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para aspirar al Título de Doctor

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EEZ-CSIC/Universidad de Granada

2019

Esta tesis doctoral ha sido realizada en el Grupo de Microbiología Ambiental y Biodegradación perteneciente al Departamento de Protección Ambiental de la Estación Experimental del Zaidín (CSIC), por Joaquín Rodrigo Otero Asman, cuya investigación ha sido financiada por los proyectos SAF2012-31919 con la ayuda FPI asociada BES-2013-066301 y SAF2015-68873-P del Ministerio de Economía Industria y Competitividad.

Parte de los resultados obtenidos durante esta tesis doctoral han sido presentados en los siguientes congresos y publicaciones:

Publicación

Quesada, J. M., Otero-Asman, J. R., Bastiaansen, K. C., Civantos, C. & Llamas, M. A. The activity of the *Pseudomonas aeruginosa* virulence regulator σ^{VreI} is modulated by the anti-σ factor VreR and the transcription factor PhoB. *Frontiers in microbiology* **7**, 1159, doi:10.3389/fmicb.2016.01159 (2016).

Trabajo en revisión:

Otero-Asman, J.R., García-García, A.I., Civantos, C., Quesada, J.M. & Llamas, M.A. *Pseudomonas aeruginosa* possesses three distinct systems for sensing and using the host molecule heme. Plos Pathogens.

Trabajos en proceso:

The alternative sigma factor σ^{VreI} is active during infection and contributes to the *Pseudomonas aeruginosa* phosphate starvation-induced virulence.

Identification of new proteases involved in the control of cell-surface signaling activity and Pseudomonas aeruginosa virulence.

Congresos

Focused Meeting Liverpool 2017: 16TH International Conference on Pseudomonas. Presentando el póster "*The Pseudomonas aeruginosa PUMA3 cell-surface signalling system is induced in response to the host*".

2ND Young researchers' Science Syposium 2017 Granada. Presentando la charla "*The Pseudomonas aeruginosa PUMA3 virulence system is induced in response to the host*".

1ST Young researchers' Science Syposium 2016 Granada. Presentando la charla "*Study of the Pseudomonas aeruginosa PUMA3 virulence system during the infection of zebra fish embryo*".

FEMS Valencia 2017 7TH Congress of European Microbiologists. Participando en el póster "*Refinement of the regulon of the Pseudomonas aeruginosa virulence regulator σvreI by rna-seq*".

FEMS Maastricht 2015 6TH Congress of European Microbiologists. Presentando el póster "*New insights in the activation of the Pseudomonas aeruginosa virulence regulator σVreI and the PUMA3 Cell-Surface Signaling System*"

XXXVII Congreso de la Sociedad Española de Bioquíma y Biología Molecular, Granada Septiembre 2014. Presentando el póster "*Unravelling the molecular mechanism activating ECF sigma factor in Pseudomonas aeruginosa*"

El doctorando **JOAQUÍN RODRIGO OTERO ASMAN** y la directora de la tesis **MARIAN LLAMAS LORENTE** garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de la directora de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

marzo de 2019

Directora de Tesis | Doctorando

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LIST OF ABREVIATIONS

ASD	Anti-σ domain
CF	Cystic fibrosis
CFU	Colony forming units
c-di-AMP	Cyclic-di-AMP
CSS	Cell-surface signaling
FBS	Fetal bovine serum
ECF	Extracytoplasmic function
GFP	Green fluorescent protein
HK	Histidine kinase
Hpi	Hours post-infection
HSL	Homoserine lactone
HTH	Helix-turn-helix
LTTR	Lysr type transcriptional regulator
OMP	Outer membrane porin
Pi	Inorganic phosphate
(p)ppGpp	Guanosine penta or tetraphosphate
PQS	Pseudomonas quinolone signal
PSS	Partner switching systems
PTM	Post-translational modification
QS	Quorum sensing
RBP	RNA binding proteins
RBS	Ribosome binding site
RFP	Red fluorescent protein
RIP	Regulated intramembrane proteolysis
RNAP	RNA polymerase
RNAPc	Core RNA polymerase
RR	Response regulator
SAM	S-adenosylmethionine
SD	Signaling domain
sRNA	Small RNA
T2SS	Type II secretion system
T3SS	Type III secretion system
T4SS	Type IV secretion system
TBDR	TonB-dependent receptor
TCCF	Total corrected cellular fluorescence
TCS	Two-component systems
TF	Transcription factor
TRNA	RNA thermomether
TPS	Two-partner secretion
ZAS	Zinc-dependent anti-sigma

Summary/Resumen

THESIS SUMMARY

Bacterial signaling systems are crucial for bacterial adaptation to the environment and the host. These systems allow bacteria to adapt to changing circumstances by generating a specific response that usually results in the modulation of gene expression. Bacterial gene expression is mainly regulated at the transcriptional level, through modification of promoter recognition by the RNA polymerase (RNAP). The bacterial RNAP is composed of a 'core' and a dissociable sigma (σ) subunit that contains most promoter recognition determinants. All bacteria contain a primary σ factor that triggers expression of general functions (Ishihama, 2000). Furthermore, most bacteria, especially those with complex lifestyles able to colonize many different niches, contain several alternative σ factors that promote expression of functions required only under specific circumstances (Ishihama, 2000). The most abundant and diverse group of alternative σ factors is the extracytoplasmic function σ (σ^{ECF}) factor group, a name that refers to their initial discovery as regulators of processes that take place outside the bacterial cytoplasm (i.e. secretion, iron transport or the response to periplasmic stress) (Lonetto et al., 1994). Synthesis and activation of σ^{ECF} factors are tightly regulated processes. σ^{ECF} factor activity is controlled by an anti-σ factor that binds to and keep sequestered the σ^{ECF} factor, which is only released and activated in response to an inducing signal. The functional unit of e σ^{ECF}-dependent signaling is thus formed by the σ^{ECF} and its cognate anti-σ factor and these systems represent a fundamental mechanism of bacterial signal transduction.

This Thesis has focused on the study of the signaling mediated by σ^{ECF}/anti-σ factor systems in the human pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is a free-living bacterium usually found in soil and water. This bacterium also colonizes the surfaces of plants of which it can be an occasional pathogen. The clinical relevance of *P. aeruginosa* lies in its ability to cause life-threatening acute and chronic infections in humans, especially in immunocompromised, cancer, burn, and cystic fibrosis patients (Juhas, 2015). This bacterium is moreover one of the leading causes of nosocomial infections affecting hospitalized patients and mortality associated with hospital-acquired *P. aeruginosa* infections, like ventilator-associated pneumonia or bacteremia, is above 35% (Lynch et al., 2017). Treatment of *P. aeruginosa* infections can be particularly challenging because this bacterium is intrinsically resistant to multiple antibiotics and can easily acquire new resistances (Breidenstein et al., 2011). In fact, antibiotic resistance among *P. aeruginosa* has increased worldwide in the last three decades and the World Health Organisation (WHO) has declared this bacterium the second priority pathogen for research and development of new strategies to fight it (WHO, 2017).

P. aeruginosa contains many virulence factors that enable the bacterium to infect essentially any mammalian organ or tissue (Lyczak et al., 2000; Klockgether and Tummler, 2017). Central to the infectious process is the ability of *P. aeruginosa* to transition from a free-living lifestyle to the life in the human body, and to detect and adapt to changing environments encountered in this host. To this end, *P. aeruginosa* has evolved different signal transduction mechanisms that form complex regulatory networks responsible for phenotypic adaptation and virulence (Balasubramanian et al., 2013). This includes one and two component systems, chemosensory pathways, quorum sensing systems and σ^{ECF} factors (Bashyam and Hasnain, 2004; Chen et al., 2004; Ulrich et al., 2005; Williams and Camara, 2009; Llamas et al., 2014; Chevalier et al., 2018; Matilla and Krell, 2018). Most *P. aeruginosa* σ^{ECF} factors regulate iron uptake in response to specific iron carriers produced by the bacterium itself, by other microorganisms with which *P. aeruginosa* shares niche, or present in the host (Llamas et al., 2014; Chevalier et al., 2018). Because iron is involved in important metabolic reactions, this metal is an essential element for the survival of almost all organisms, and iron acquisition during infection is crucial for pathogens to colonize and survive in the host (Schaible and Kaufmann, 2004; Wandersman and Delepelaire, 2004; Cassat and Skaar, 2013; Parrow et al., 2013). Moreover, several *P. aeruginosa* σ^{ECF} factors mediate transcription of important virulence factors like the exopolysaccharide alginate, responsible of the mucoid phenotype needed for the establishment of chronic infections. Or the

protease PrpL that is able to hydrolyse host molecules like casein, lactoferrin, elastin, and decorin, producing subproducts that are used by *P. aeruginosa* as sources or iron, carbon or nitrogen (Lamont et al., 2002). Another example is the secreted exotoxin A (ToxA), which enters into host cells inducing apoptosis (Lamont et al., 2002). Synthesis of secretion systems and secreted proteins needed for adhesion of the pathogen to host cells or injection of toxins is also regulated by σ^{ECF} factors in *P. aeruginosa* (Llamas et al., 2009; Llamas et al., 2014; Chevalier et al., 2018). These σ factors furthermore regulate the response of *P. aeruginosa* to the oxidative stress produced by neutrophils and macrophages or by compounds that affect the integrity of the bacterial membrane (Ramsey and Wozniak, 2005; Chevalier et al., 2018).

The anti-σ factors that control σ^{ECF} factor activation in *P. aeruginosa* are cytoplasmic membrane-anchored proteins that contain a small cytosolic N-terminal domain and a large periplasmic C-terminal domain separated by a single transmembrane segment (Llamas et al., 2014; Chevalier et al., 2018). The cytosolic domain is the domain that binds to and prevents the association of the σ^{ECF} factor with the RNAP in absence of the inducing signal while the C-domain is the domain that receives the signal. Thus, σ^{ECF} factors of *P. aeruginosa* usually respond to signals generated in the periplasm or the bacterial outer membrane. Several *P. aeruginosa* σ^{ECF}/anti-σ pairs are functionally, and often also genomically, associated with an outer membrane receptor that belongs to a subgroup of the TonB dependent receptor (TBDR) family. Together, these three proteins form a signal transduction system known as cell surface signaling (CSS) system. Presence of the receptor allows the detection of extracellular signals that do not necessarily have to enter into the cell to produce a response (Llamas et al., 2014). CSS receptors are composed by an outer membrane-anchored C-terminal domain and a small N-terminal domain located in the periplasm known as signaling domain (SD) (Noinaj et al., 2010). The SD is the domain that transmits the presence of the signal to the anti-σ factor, which initiates the CSS signal transduction cascade (Enz et al., 2003b). Activation of *P. aeruginosa* σ^{ECF} factors, including both those that respond to periplasmic signals and those that respond to signals detected at the outer membrane via a CSS receptor, requires the regulated proteolysis of the anti-σ factor (Qiu et al., 2007; Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2015b). Some proteases involved in this process have been identified, the RseP protease being a common component of the proteolysis of transmembrane anti-σ factors in *P. aeruginosa* (and other bacteria). This cytoplasmic membrane-anchored protease cleaves within the transmembrane segment of the anti-σ factor in response to the inducing signal, which produces the release of the σ^{ECF} factor in the cytosol allowing its binding to the RNAP and the transcription of the signal responsive genes (Llamas et al., 2014).

The σ^{VreI} factor is one of the σ^{ECF} factors that regulates virulence in *P. aeruginosa* and has been a subject of study in this Thesis. This σ factor is encoded by the *vreAIR* operon together with an anti-σ factor (VreR) and a receptor-like protein (VreA) that shows similarities to CSS receptors but also important differences (Llamas et al., 2009). Previous transcriptomic analyses determined that σ^{VreI} promotes production of several virulent determinants (Llamas et al., 2009). This σ factor was originally classified as an iron starvation σ^{ECF} factor; however, a recent study showed that σ^{VreI} is not expressed under iron starvation but under inorganic phosphate (Pi) starvation conditions (Faure et al., 2013). Intriguingly, the genes of the σ^{VreI} regulon are also expressed in low Pi despite the fact that the σ^{VreI} repressor, the anti-σ factor VreR, is also produced in this condition (Faure et al., 2013). The study presented in **chapter 2** was performed to understand how σ^{VreI} activity is modulated in low Pi conditions. Our analyses show that although σ^{VreI} is partially active under Pi starvation, maximal transcription of the σ^{VreI} regulon genes requires the removal of VreR, which strongly suggests that an additional signal is required for complete σ^{VreI} activation. Furthermore, we demonstrate that the activity of σ^{VreI} is modulated not only by VreR but also by the transcription factor PhoB. Presence of this regulator is an absolute requirement for σ^{VreI} to complex with the DNA and initiate transcription of the σ^{VreI} regulon genes. By site-directed mutagenesis, we have determined the binding sites of PhoB

and σ^{VreI} in the promoter of the σ^{VreI} regulon genes. Pi starvation is a physiological condition often encountered by pathogens in the host environment that in *P. aeruginosa* is known to induce a virulent phenotype. In **chapter 3**, we have analyzed the contribution of σ^{VreI} and VreR to this phenotype. By virulence assays using zebrafish (*Danio rerio*) embryos and human epithelial cells as hosts, we show that the *vreI* mutation reduces the virulence and cytotoxicity of this pathogen. Surprisingly, a *vreR* mutant showed a similar phenotype. Transcriptomic analyses by RNA-seq show that VreR regulates gene expression not only in a σ^{VreI}-dependent, but also in a σ^{VreI}-independent manner that could explain the reduced virulence observed in the *vreR* mutant. Importantly, we have determined that σ^{VreI} is active *in vivo* during infection.

Besides, in **chapter 4** we have identified a not previously characterized *P. aeruginosa* CSS system, which we have named Hxu, which responds to heme. Because most of the iron in mammals is complexed within heme, this host molecule represents an important source of iron for pathogens during infection. Together with the previously identified Phu and Has systems (Ochsner et al., 2000), Hxu represents the third heme system of *P. aeruginosa* and this bacterium the only pathogen in which three heme systems have been reported to date. We have determined that these three heme systems are not redundant because they function in response to different concentrations of heme and present connections that allow them to function more efficiently. Moreover, we show that the Has and Hxu systems are CSS systems that respond to heme-hemophore complexes and free heme, respectively, and have identified the molecular mechanism triggering their activation. We propose that *P. aeruginosa* uses heme not only as a source of iron, but also as a signal molecule to detect the host.

Finally, in **chapter 5** we have analyzed the molecular mechanism that activates CSS systems in *Pseudomonas*, especially the proteolytic cascade that processes the anti-σ factor. By *in silico* and random transposon mutagenesis analyses, we have identified four new proteases that, together with RseP, directly or indirectly participate in *P. aeruginosa* σ^{ECF} factors activation. This includes two proteases that function in the periplasm, Prc and CtpA, and two that act in the cytosol, PepA and FtsH. We show that CtpA and Prc modulate the stability of the periplasmic domain of the anti-σ factor and fine-tune the response of the CSS system to the inducing signal. The cytosolic protease PepA seems to function as an inhibitor of the CSS σ^{ECF} factor because its absence increases both the activity and the stability of the σ factor. FtsH seems to activate the function of the σ^{ECF} factor although the role of this protease in the signaling cascade has not yet been determined. Furthermore, our virulence assays show that mutants in the *rseP* and *ctpA* proteases are attenuated in virulence while the *prc* mutation increases the pathogenicity of *P. aeruginosa* probably by increasing the production of outer membrane vesicles, which are used by this pathogen to release virulence factors into host cells (Schwechheimer and Kuehn, 2015).

In summary, in this Thesis we have identified new and important characteristics of the σ^{ECF}-mediated signaling in *P. aeruginosa*. The results presented in this Thesis have considerably advanced our knowledge of how this human pathogen is able to detect and respond to the host. Given that the inhibition of bacterial signaling systems has been proposed as a promising strategy to combat pathogens, the work presented in this Thesis provides new perspectives that could be used to achieve this objective.

RESUMEN TESIS

Los sistemas de señalización celular bacterianos son fundamentales en la adaptación de las bacterias a su medio ambiente y al hospedador en el caso de patógenos. Estos sistemas permiten a las bacterias identificar cambios en su entorno y adaptarse a ellos mediante la generación de una respuesta específica que suele producirse a través de la modificación de la expresión génica. La expresión génica bacteriana está regulada principalmente a nivel del inicio de la transcripción mediante la modificación del reconocimiento del promotor por la RNA polimerasa (RNAP). La RNAP bacteriana está formada por un 'core' o núcleo y por una subunidad sigma (σ) disociable que es la que contiene los determinantes para el reconocimiento del promotor. Todas las bacterias tienen un factor σ primario encargado de la expresión de las funciones generales de la bacteria (Ishihama, 2000). Además, la mayoría de las bacterias, especialmente aquellas con estilos de vida complejos capaces de colonizar muchos ambientes distintos, contienen varios factores σ alternativos que producen la expresión de funciones requeridas sólo en circunstancias específicas (Ishihama, 2000). El grupo más abundante y diverso de factores σ alternativos consiste en los denominados factores σ de función extracitoplásmica (σ^{ECF}), un nombre que hace referencia a su descubrimiento inicial como reguladores de procesos que se producen fuera del citoplasma bacteriano como secreción, transporte de hierro o respuesta a estrés periplásmico (Lonetto et al., 1994). Tanto la síntesis como la activación de los factores σ^{ECF} son procesos altamente regulados. La actividad de los factores σ^{ECF} está controlada por factores anti-σ que se unen y mantienen secuestrado el factor σ^{ECF}, el cual sólo se libera y activa en presencia de una señal inductora. La unidad funcional de la señalización dependiente de σ^{ECF} está por tanto formada por el factor σ^{ECF} y su factor anti-σ, y estos sistemas representan un mecanismo fundamental en la transducción de señales en bacterias.

Esta Tesis doctoral se ha centrado en el estudio de la señalización celular mediada por sistemas σ^{ECF}/anti-σ en el patógeno de humanos *Pseudomonas aeruginosa*. *P. aeruginosa* es una bacteria de vida libre que se encuentra comúnmente en el suelo y el agua. Esta bacteria también coloniza las superficies de las plantas de las cuales puede ser un patógeno ocasional. La importancia clínica de *P. aeruginosa* radica en su capacidad de causar una gran cantidad de infecciones agudas y crónicas en humanos que ponen en peligro la vida, especialmente en pacientes inmunocomprometidos, con cáncer, con quemados o con fibrosis quística (Juhas, 2015). Además, *P. aeruginosa* es una de las causas principales de las infecciones nosocomiales que afectan a pacientes hospitalizados, y la mortalidad asociada con infecciones por esta bacteria adquiridas en el hospital, como neumonía o bacteremia, está por encima del 35% (Lynch et al., 2017). El tratamiento de las infecciones producidas por *P. aeruginosa* no siempre es efectivo ya que esta bacteria es intrínsecamente resistente a muchos antibióticos y es capaz de adquirir fácilmente nuevas resistencias (Breidenstein et al., 2011). De hecho, la resistencia a antibióticos entre cepas de *P. aeruginosa* se ha incrementado a nivel mundial en las últimas tres décadas por lo que la Organización Mundial de la Salud (OMS) ha declarado a esta bacteria como el segundo patógeno prioritario para la investigación y el desarrollo de nuevas estrategias para combatirlo (WHO, 2017).

P. aeruginosa contiene muchos factores de virulencia que le permiten infectar básicamente cualquier órgano o tejido de mamíferos (Lyczak et al., 2000; Klockgether and Tummler, 2017). Un elemento central del proceso infeccioso es la capacidad de *P. aeruginosa* de producir la transición desde un estilo de vida libre a la vida en el cuerpo humano, y de detectar y adaptarse a los entornos cambiantes con los que se encuentra en este hospedador. Para ello *P. aeruginosa* contiene multitud de mecanismos de transducción de señales que forman complejas redes reguladoras responsables de la adaptación fenotípica y de la virulencia (Balasubramanian et al., 2013). Esto incluye sistemas reguladores de uno y dos componentes, vías quimiosensoriales, sistemas de 'quorum sensing' y factores σ^{ECF} (Bashyam and Hasnain, 2004; Chen et al., 2004; Ulrich et al., 2005; Williams and Camara,

2009; Llamas et al., 2014; Chevalier et al., 2018; Matilla and Krell, 2018). Muchos de los factores σ^{ECF} de *P. aeruginosa* regulan la captación de hierro en respuesta a quelantes o transportadores de hierro específicos producidos tanto por la propia bacteria, por otros microorganismos con los que *P. aeruginosa* comparte nicho, o presentes en el hospedador (Llamas et al., 2014; Chevalier et al., 2018). Dada su participación en importantes reacciones metabólicas, el hierro es un elemento esencial para la supervivencia de prácticamente todos los organismos y la captación de hierro durante la infección es clave para que un patógeno sea capaz de colonizar y sobrevivir en el huésped (Schaible and Kaufmann, 2004; Wandersman and Delepelaire, 2004; Cassat and Skaar, 2013; Parrow et al., 2013). Además, varios factores σ^{ECF} de *P. aeruginosa* median la trascripción de importantes factores de virulencia, como la del exopolisacárido alginato responsable del fenotipo mucoide de *P. aeruginosa* necesario para el establecimiento de infecciones crónicas (Ramsey and Wozniak, 2005). O la de proteasas como PrpL que hidroliza moléculas del huésped como la caseína, la lactoferrina, la elastina o la decorina, produciendo subproductos que pueden ser utilizados por *P. aeruginosa* como fuente de hierro, carbono o nitrógeno (Lamont et al., 2002). También regulan la producción de toxinas como la exotoxina A (ToxA) que tras ser secretada por la bacteria entra en las células del hospedador induciendo apoptosis (muerte celular programada) (Lamont et al., 2002). La síntesis de sistemas de secreción necesarios para la adhesión del patógeno a células del hospedador y la inyección de toxinas directamente en dichas células también esta regulada por factores σ^{ECF} en *P. aeruginosa* (Llamas et al., 2009; Llamas et al., 2014; Chevalier et al., 2018). Estos factores σ también regulan las respuestas al estrés oxidativo producido por el ataque de macrófagos y neutrófilos o por compuestos que afectan la integridad de la membrana celular bacteriana (Ramsey and Wozniak, 2005; Chevalier et al., 2018).

Los factores anti-σ que controlan la actividad de los factores σ^{ECF} de *P. aeruginosa* son proteínas ancladas a la membrana citoplásmica que presentan un pequeño dominio citosólico N-terminal y un dominio periplásmico C-terminal de mayor tamaño separados por un único segmento transmembrana (Llamas et al., 2014; Chevalier et al., 2018). El dominio citosólico es el que une e impide la unión del factor σ^{ECF} a la RNAP en ausencia de la señal inductora mientras que el dominio periplásmico es el que recibe la señal. Por tanto, los factores σ^{ECF} de *P. aeruginosa* generalmente responden a señales detectadas en el periplasma o en la membrana externa bacteriana. Una gran cantidad de sistemas σ^{ECF}/anti-σ de *P. aeruginosa* están asociados funcional, y normalmente también genómicamente, con un receptor de la membrana externa perteneciente a un subgrupo de la familia de receptores dependientes de TonB (TBDR por su nombre en inglés 'TonB-dependent receptors'). Estas tres proteínas forman un sistema conocido como sistema de señalización de la superficie celular (CSS por su nombre en inglés 'cell-surface signaling') que le permite a la bacteria detectar señales presentes en el medio extracelular sin que sea necesaria su entrada en la célula para producir una respuesta (Llamas et al., 2014). Los receptores CSS están constituidos por un dominio C-terminal insertado en la membrana externa y por un pequeño dominio N-terminal localizado en el periplasma y denominado dominio señalizador (SD por su nombre en inglés 'signaling domain') (Noinaj et al., 2010). El SD es el dominio que transmite la presencia de la señal al factor anti-σ, iniciando así la cascada de señalización CSS (Braun and Endriss, 2007). La activación de los factores σ^{ECF} de *P. aeruginosa*, tanto de los que responden a señales detectadas en el periplasma como en la membrana externa a través de un receptor CSS, se produce mediante la proteólisis regulada y secuencial del factor anti-σ (Qiu et al., 2007; Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2015b). Varias proteasas implicadas en este proceso han sido identificadas siendo la proteasa RseP un elemento común en la proteólisis de los factores anti-σ transmembrana de *P. aeruginosa* (y de muchas otras bacterias). Esta proteasa localizada en la membrana citoplásmica bacteriana corta el segmento transmembrana del factor anti-σ en respuesta a la señal inductora, lo que produce la liberación del factor σ^{ECF} en el citosol permitiendo su unión a la RNAP y la transcripción de los genes de respuesta a la señal (Llamas et al., 2014).

El factor σ^{VreI} es uno de los factores σ^{ECF} de *P. aeruginosa* que regula virulencia y ha sido objeto de estudio de esta Tesis doctoral. Este factor σ esta codificado por el operon *vreAIR* que también codifica un factor anti-σ (VreR) y un receptor (VreA) que presenta algunas similitudes con receptores tipo CSS aunque también importantes diferencias (Llamas et al., 2009). Análisis previos de transcriptómica determinaron que σ^{VreI} promueve la expresión de determinantes de virulencia (Llamas et al., 2009). Este factor σ fue originalmente clasificado como un factor σ^{ECF} de respuesta a inanición de hierro. Sin embargo, un estudio reciente ha demostrado que σ^{VreI} no se produce en condiciones de limitación de hierro sino de fosfato inorgánico (Pi) (Faure et al., 2013). Curiosamente, los genes del regulón de σ^{VreI} también se expresan en bajo Pi a pesar de que el represor de σ^{VreI}, el factor anti-σ VreR, también se sintetiza en esta condición (Faure et al., 2013). El estudio presentado en el **capítulo 2** se realizó para entender cómo se modula la actividad de σ^{VreI} en condiciones de limitación de Pi. Nuestros análisis han determinado que aunque σ^{VreI} presenta cierta actividad basal en condiciones de bajo Pi, la máxima actividad de este factor σ sólo se produce tras la eliminación del factor anti-σ VreR, lo que sugiere que una señal adicional es necesaria para producir la activación de σ^{VreI}. Además, hemos demostrado que la actividad de σ^{VreI} está modulada no solo por VreR sino también por el factor de transcripción PhoB, y que la presencia de este regulador transcripcional es necesaria para que σ^{VreI} pueda unirse a la región promotora de los genes e iniciar la transcripción génica. Mediante mutagénesis dirigida se han determinado los sitios de unión de PhoB y σ^{VreI} al promotor de los genes del regulón de σ^{VreI}. Limitación de Pi es una situación fisiológica con la que frecuentemente se encuentran los patógenos en el entorno del hospedador y que en *P. aeruginosa* se sabe que induce un fenotipo virulento. En el **capítulo 3** hemos analizado la contribución de σ^{VreI} y VreR a este fenotipo. Mediante ensayos de virulencia utilizando embriones de pez cebra (*Danio rerio*) y células epiteliales del tracto pulmonar humano como hospedadores de *P. aeruginosa* demostramos que la mutación *vreI* reduce significativamente la virulencia y la citotoxicidad de este patógeno. Inesperadamente, la mutación del factor anti-σ *vreR* también reduce la virulencia de *P. aeruginosa*. Mediante análisis de transcriptómica por RNA-seq hemos determinado que VreR modula la expresión génica no solo a través de σ^{VreI} sino también de manera independiente a este factor σ, lo que podría explicar la disminución de virulencia observada en su ausencia. Además, hemos demostrado que σ^{VreI} se activa *in vivo* durante el proceso infeccioso.(Llamas et al., 2009)

Por otro lado, en el **capítulo 4** hemos identificado un sistema CSS de *P. aeruginosa* no caracterizado previamente y que hemos denominado Hxu que responde a la presencia de hemo. Dado que la mayor parte del hierro en mamíferos está acomplejado a esta molécula del hospedador representa una fuente importante de hierro para patógenos. Junto a los sistemas Phu y Has previamente identificados (Ochsner et al., 2000), Hxu representa el tercer sistema de hemo de *P. aeruginosa* y esta bacteria el único patógeno en el que se han descrito tres sistemas de hemo hasta el momento. Hemos determinado que estos tres sistemas de hemo no son redundantes ya que funcionan en respuesta a distintas concentraciones de hemo y tienen conexiones que les permite funcionar de manera más eficiente. Además, mostramos que los sistemas Has y Hxu son sistemas CSS que responden a complejos hemo-hemóforos y a hemo libre, respectivamente, y hemos identificado el mecanismo molecular responsable de su activación. Proponemos que a través de estos sistemas *P. aeruginosa* utiliza el hemo no sólo como fuente de hierro sino también como una molécula de señal para detectar al hospedador.

Finalmente, en el **capítulo 5** hemos analizado el mecanismo molecular que produce la activación de sistemas CSS de *Pseudomonas,* especialmente en lo referente a la compleja cascada proteolítica que procesa el factor anti-σ. Mediante análisis *in silico* y mutagénesis al azar por transposición hemos identificado cuatro nuevas proteasas que, junto con la proteasa RseP, participan directa o indirectamente en la activación de factores σ^{ECF} de *P. aeruginosa*. Esto incluye dos proteasas que actúan en el periplasma Prc y CtpA, y dos que actúan en el citosol PepA y FtsH. CtpA y Prc modulan la estabilidad del dominio periplásmico del factor CSS anti-σ y ajustan la respuesta del sistema CSS a la

señal inductora. La proteasa citosólica PepA parece funcionar como un inhibidor del factor CSS σ^{ECF} ya que la ausencia de esta proteasa incrementa tanto la actividad como la estabilidad del factor σ. FtsH parece activar la función del factor σ^{ECF} aunque el papel concreto de esta proteasa en la cascada de señalización no ha sido determinado todavía. Además, nuestros ensayos de virulencia muestran que mutantes en las proteasas *rseP* y *ctpA* están atenuados en virulencia mientras que la mutación *prc* aumenta la patogenicidad de *P. aeruginosa* probablemente al aumentar la producción de vesículas de membrana externa con las que este patógeno libera factores de virulencia en células del hospedador (Schwechheimer and Kuehn, 2015).

En resumen, en esta Tesis doctoral hemos identificado nuevas e importantes características de la señalización mediada por factores σ^{ECF} en *P. aeruginosa*. Los resultados presentados en esta Tesis han avanzado considerable nuestro conocimiento de cómo este patógeno de humanos es capaz de detectar y responder al hospedador. Dado que la inhibición de los sistemas de señalización bacterianos se ha propuesto como una estrategia prometedora para combatir patógenos, el trabajo presentado en esta Tesis proporciona nuevas perspectivas que podrían ser utilizadas para lograr este objetivo.

Chapter 1

General introduction

Bacterial kingdom has colonized the whole Earth surface. Bacteria can live in almost all locations from thermal waters to salty ecosystems, and they can freely inhabit soil and water. Bacteria can also live in symbiosis with plants and animals or infect both organisms. In fact, numerous bacterial species are able to colonize many of these different environments. There is a clear correlation between the size of a bacterial genome and the ability of a bacterium to colonize several environments. Bacterial genomes have a "core" of genes that form the backbone of genetic information, which is highly conserved among bacteria (Rodriguez-Valera and Ussery, 2012; Polz et al., 2013; Hutchison et al., 2016). Moreover, they have a flexible gene pool that is strain-specific and allows the adaptation to specific environmental conditions (Dobrindt and Hacker, 2001; Medini et al., 2005; Wiedenbeck and Cohan, 2011). Changes in the genome size occur by both loss and acquisition of genes and this is known as genomic plasticity. The loss of genetic material is known as genome evolution by reduction and usually occurs in obligated intracellular pathogens and parasites that live in stable environments (Cole et al., 2001). This event produces the removal of genes that are not necessary in these constant environments reducing in this way the energy cost needed for maintaining these genes (Cole et al., 2001; Moran, 2002). On the contrary, genome acquisition takes place in bacteria living in complex environments and in contact with several other species. Transfer of foreign DNA is knows as lateral or horizontal acquisition and occurs via three different ways: 1) by conjugation, which usually occurs through the type 4 secretion system and allows the transfer of DNA between two bacteria; 2) by transduction, which is performed by bacteriophages that transfer DNA from a bacterium to another; and 3) by transformation of naked DNA through transposon, plasmids, cosmids, integrative and conjugative elements, or pathogenic islands (Gyles and Boerlin, 2014). Bacteria also suffer genetic events that modify the original size of the genome, like point mutations or homologous recombination that produce gene deletions and duplications or inversion of genes (Dobrindt and Hacker, 2001). Large genomes usually reflect a higher metabolic versatility of the bacterium, which allows the colonization of more environments. However, both core and flexible pool genes are not expressed constantly and bacteria with large genomes also contain complex regulatory networks that regulate gene expression (Martinez-Antonio and Collado-Vides, 2003). In fact, laterally transferred genes usually co-transfer with their own regulators (Price et al., 2008). Bacteria are able to signal intracellular and extracellular cues that influence gene expression and permits the adaptation to the environment. Due to the highly cost of protein expression and the complications that a protein can generates in a non-suitable circumstance, genes are tightly regulated by several regulatory checkpoints that comprise from gene transcription to protein action.

A. Regulation of gene expression in bacteria

Gene expression is the process by which the information contained in a gene is used in the synthesis of a functional product. Several steps required in this process are regulated in bacteria including transcription, translation and post-translational modifications (Fig. 1).

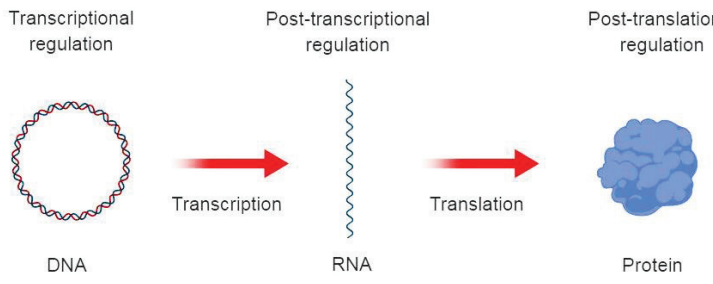

Fig. 1. Central dogma of molecular biology. Schematic representation of the steps from DNA to protein synthesis and their regulation mechanisms.

A1. Regulation of gene transcription

A1.1. Sigma (σ) factors

Regulation of gene expression in bacteria occurs primarily at the level of transcription initiation by modifying the affinity of the RNA polymerase (RNAP) for the DNA (Ishihama, 2000). The bacterial RNAP comprises a five-subunit core enzyme (RNAPc; subunit composition $\alpha 2\beta\beta'\omega$) and a dissociable σ subunit (Fig. 2). The σ subunit contains most promoter recognition determinants and is the subunit that directs the RNAP to the promoter region of the genes to be expressed. Therefore, this subunit confers specificity to the RNAP (Murakami and Darst, 2003). All bacteria contain a primary σ factor (i.e. σ^{70} or σ^{A}) that controls expression of essential housekeeping genes. In addition, most bacteria contain alternative σ factors that recognize alternative promoter sequences and activate expression of functions required only under specific circumstances (Ishihama, 2000). By modulating the use of standard and alternative σ factors, bacteria are able to adequately regulate general cell functions as well as specific functions. Indeed, the versatility and adaptability of bacteria is to a large degree reflected by the number of alternative σ factors they produce (Staron et al., 2009). The σ subunit is required not only for promoter recognition but also for transcription initiation. This subunit directs the RNAP catalytic core to the transcription start site and initiates the melting of the DNA double strand, which is the first step in the formation of the transcription bubble (Vassylyev et al., 2002). Once the elongation of the mRNA starts, the σ factor is released from the RNAP (Paget and Helmann, 2003).

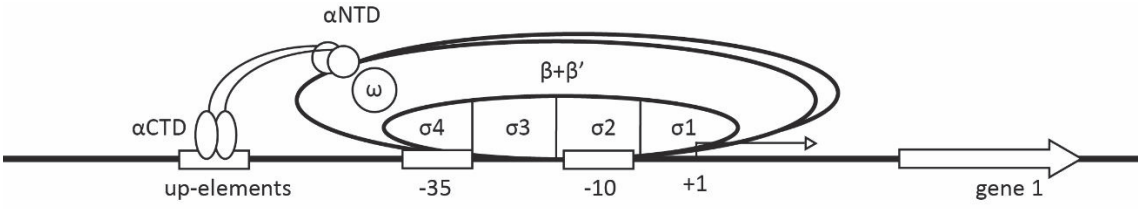

Fig. 2. Schematic representation of the bacterial RNA polymerase (RNAP). The scheme shows the interactions of the RNAP with the DNA. The thick black line represents the DNA and the rectangular boxes the σ^{70} conserved promoter sequences. The transcription start point is indicated by +1. Adapted from (Browning and Busby, 2004).

Bacteria contain two structurally different families of σ factors, the σ^{70} and σ^{54} families (Österberg et al., 2011), being the σ^{70} family the most extended group. In fact, all bacteria identified to date contain a primary σ factor of the σ^{70} family (Österberg et al., 2011). The working mechanism and promoter recognition by σ^{70} and σ^{54} factors differs. σ^{54} needs ATP-dependent enhancers for DNA melting and recognizes conserved promoter sequences located at the -24 and -12 bp upstream the transcription start point (+1) (Bush and Dixon, 2012). Genes under control of σ^{54} are required for RNA modification, chemotaxis, electron transport, flagellation or when a particular carbon or nitrogen source is available (Buck et al., 2000; Zhang and Buck, 2015). These σ factors are not present in GC-rich Gram-positive bacteria and cyanobacteria, and it is unusual to find two different forms of σ^{54} in the same organism (Buck et al., 2000). On the contrary, σ factors from the σ^{70} family do not need enhancers or ATP to function and they recognize promoter sequences located at the -35 and -10 bp upstream the +1

(Fig. 2). A bacterium can contain several members of the σ^{70} family. For example, *Escherichia coli* produces one σ^{54} and six σ^{70} factor members, including the primary σ^{70} factor and five alternative σ factors (named σ^{28}, σ^{32}, σ^{38}, σ^{E} and σ^{FecI}) (Ishihama, 2000; Paget, 2015). The σ^{70} family has been classified in four different subgroups according to their function and structure (Fig. 3) (Helmann, 2002; Paget and Helmann, 2003; Bastiaansen et al., 2012; Paget, 2015).

- Group 1 contains the primary σ factors, which are proteins of 40-70 KDa (i.e. *E. coli* σ^{70}) that are produced constantly along the time and have a high affinity for the RNAPc (Paget and Helmann, 2003). These σ factors are highly conserved and essential because trigger the transcription of genes required for general bacterial functions. Primary σ factors recognize promoter sequences matching the consensus TTGACA at -35 and TATAAT at -10 (Helmann, 2002; Feklistov et al., 2014). The conservation of the -35 and -10 sequences is related with the strength of the promoter and thus promoters close to the consensus sequences have higher affinity for the σ^{70}-loaded RNAP (Rhodius and Mutalik, 2010). This permits differences in the expression levels of genes regulated by the primary σ factor. Moreover, most strength promoters contains UP elements located upstream the -35 sequence where the C-terminal domain of the α subunit of the RNAP binds increasing the stability of the complex and thus transcription (Fig. 2) (Estrem et al., 1998). Structurally, Group 1 σ factors are divided in four different domains (Fig. 3). The σ^{1} domain is negatively charged, which avoids the binding of the free σ factor to the DNA (Dombroski et al., 1993; Schwartz et al., 2008). The σ^{2} domain is divided in several regions: the highly conserved 1.2 region is required for the formation of the DNA open complex and stabilization of the σ^{2} domain (Wilson and Dombroski, 1997; Haugen et al., 2008). Next to 1.2, there is a nonconserved region (NCR) that is variable in size and structure among the Group 1 σ factors. The regions 2.1 and 2.2 recognize the RNAPc and establish connections between the σ factor and the RNAP (Chan et al., 1996). The region 2.2 is the most conserved within the members of the σ^{70} family (Helmann and Chamberlin, 1988; Paget, 2015). Regions 2.3 and 2.4 make base specific interaction with the -10 promoter sequence of the non-template DNA strand (Harley and Reynolds, 1987; Voskuil et al., 1995). The σ^{3} domain is a compact three-helix bundle mainly implicated in the interaction with the RNAPc. Moreover, it interacts with the major groove of the DNA in promoters containing an extended -10 sequence (Fig. 3) (Paget, 2015). The 3.2 region acts as a linker to the σ^{4} domain and interacts with the template DNA strand facilitating the transcription initiation. The σ^{4} domain is divided in the 4.1 region, which is in charge of the interaction with transcription factors, and the 4.2 region, which is the region that recognizes and interacts with the -35 promoter sequence (Potvin et al., 2008; Feklistov et al., 2014; Llamas et al., 2014; Paget, 2015).
- The Group 2 of σ factors are closely related in function and structure to Group 1 with the difference that they do not contain the σ^{1} domain and are not essential (Fig. 3) (Paget, 2015). These σ factors trigger transcription of genes required for adaptation to stress conditions and nutrient limitation, which often occurs in the stationary phase of growth. The most studied Group 2 σ factor is *E. coli* σ^{S}, which is produced in the stationary phase of growth and induces expression of more than 500 genes (Paget, 2015).
- The σ factors of the Group 3 are more diverse in structure and function although they usually contain the σ^{2}, σ^{3} and σ^{4} domains (Fig. 3). This group is divided in four subgroups that differ phylogenetically and functionally, and are named according to the function they regulate: flagellum biosynthesis, heat shock response, general stress or sporulation

(Paget and Helmann, 2003). Group 3 σ factors recognized a more diverse range of -35 and -10 promoter sequences, which also vary within the group (Paget, 2015).

- The Group 4 contains the so-called extracytoplasmic function sigma (σ^{ECF}) factors because they were originally discovered as σ factors produced and activated in response to extracytosolic signals that triggered expression of cell envelope functions (i.e. secretion, iron transport or the response to periplasmic stress) (Lonetto et al., 1994). However, the genomic era has shown that this view is oversimplified and there are several members of the family that sense intracellular signals and govern responses that primarily influence the cytoplasm (Sineva et al., 2017). This is the biggest and more diverse group of the σ^{70} family and has been classified in 56 major and 32 minor phylogenetical subgroups based on sequence similarity and genomic contex (Staron et al., 2009; Huang et al., 2015; Sineva et al., 2017). The number of σ^{ECF} factors in bacteria can vary from zero in symbionts and obligate pathogens (e.g. *Chlamydiae*) to 115 in the aquatic bacterium *Gemmata obscuriglobus* (Jogler et al., 2012). Presence of a high amount of σ^{ECF} factors is related to a high capacity of adaptation to a wide range of niches and unstable environments (Österberg et al., 2011). Structurally, σ^{ECF} factors are the smallest σ factors of the family. They only contain domains σ^2 and σ^4 (Fig. 3), which thus seems to be the minimal requirement for a σ factor to work. In this group, the σ^3 domain has almost completely disappeared and only remains a non-structured linker that connects the σ^2 and σ^4 domains (Paget, 2015). The 2.4 region that recognizes the -10 promoter sequence is highly variable among σ^{ECF} factors, which suggests that the DNA binding specificity of this group is mainly determined by the -10 region (Staron et al., 2009; Paget, 2015). This is also supported by the presence of a conserved AAC sequence in the -35 region of most σ^{ECF} factors-dependent promoters (Lane and Darst, 2006), which further suggests that the specificity resides mainly in the -10 sequence. Due to the absence of the σ^3 domain, σ^{ECF} factors do not recognize extended -10 sequences (Campagne et al., 2015).

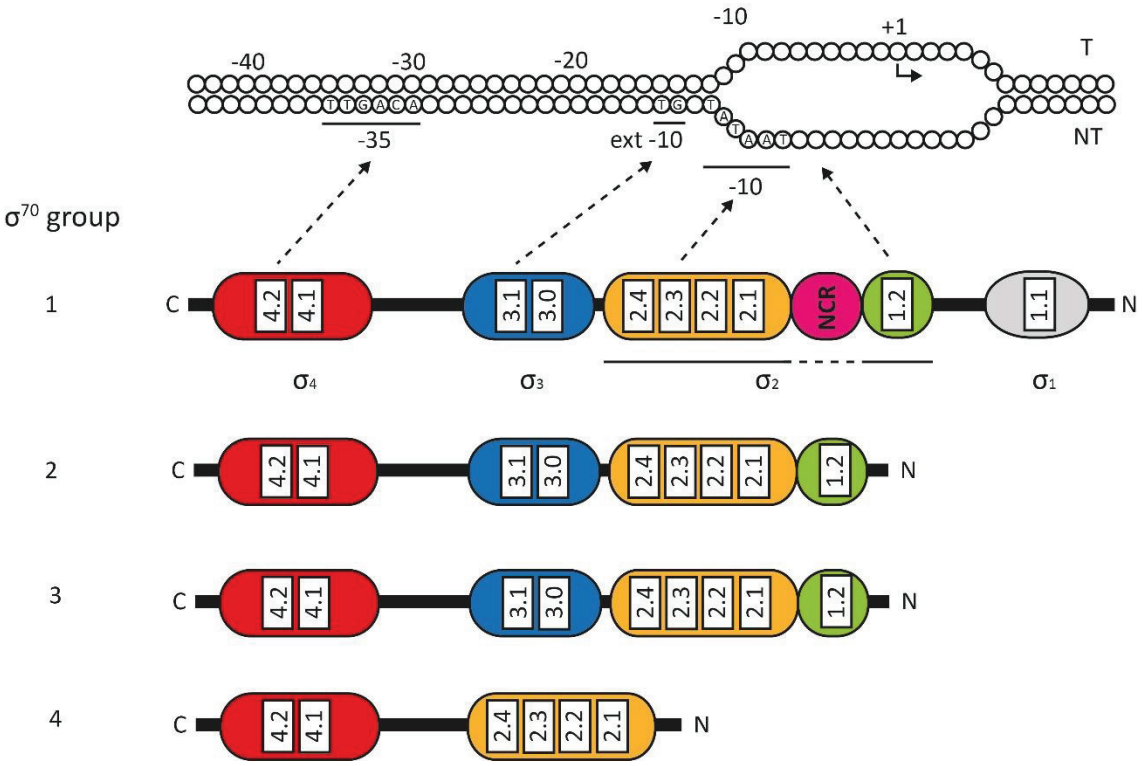

Fig. 3. Domain organization of the different groups of the σ^{70} factor family. The template (T) and non-template (NT) DNA strands, and the *E. coli* σ^{70} consensus promoter sequence are represented above the σ factors. Homologous σ domains are shown in the same color. Dash arrows indicate the interaction of the σ regions with the DNA. The non-conserved region (NCR) present in Group 1 σ^{70} factors is shown in pink. Adapted from (Paget, 2015).

Presence of high amount of σ factors in a bacterium raised the question of how they compete for binding to the RNAP, which is usually saturated with the primary σ factor (Ishihama, 2000; Maeda et al., 2000; Mauri and Klumpp, 2014). *E. coli* has ~4000 genes; however, during growth in rich medium the number of RNAP has been estimated in ~2000 molecules per cell (Ishihama, 2000). Therefore, alternative σ factors must overcome the number of RNAP molecules in the cell to be able to compete with the primary σ factor for the RNAP. There are inhibitory proteins that impede the binding of the primary σ factor to the RNAPc, as for example the alarmone (p)ppGpp (guanosine penta or tetraphosphate) (Potrykus and Cashel, 2008). ppGpp reduces the amount of the σ^{70}-RNAP complex but it does not reduce the amount of σ^{70} molecules *per se* and high levels of ppGpp usually correlate with expression of genes controlled by alternative σ factors (Hernandez and Cashel, 1995). A similar mechanism is also triggered by the DksA protein, a member of a family of regulators that binds to the RNAPc producing structural changes that impedes its interaction with the σ^{70} factor (Rutherford et al., 2009). Another protein, Rsd binds free σ^{70} impeding its interaction with the RNAPc and in vitro it is able to remove σ^{70} from the RNAP (Ilag et al., 2004; Westblade et al., 2004). Other proteins, as *E. coli* Crl, binds to alternative σ factors (i.e. σ^{38}) increasing its affinity for the RNAPc (Typas et al., 2007).

A1.2. Transcription factors (TFs)

Transcription factors (TFs) are sequence-specific DNA binding proteins that bind to a DNA region known as operator that is located between the -250 and +20 positions relative to the transcription start site (+1) (Collado-Vides et al., 1991). These proteins also modulate the

RNAP promoter recognition by either enhancing or inhibiting such recognition and therefore gene transcription (Browning and Busby, 2004). Then, the promoter recognition by the RNAP is modulated first by the substitution of the σ subunit and secondly by the interaction with TFs (Ishihama, 2000). TFs determine when a gene is expressed or repressed generally in response to intracellular or environmental signals (Martinez-Antonio et al., 2006). As occurs with alternative σ factors, TFs are also more abundant in bacteria with larger genomes, reflecting that it is necessary a greater number of regulatory elements to regulate expression of a greater number of genes (Lane and Martin, 2010). Most TFs are specific and control the transcription of few genes, usually one or two, while some of them are global regulators that control expression of several gene clusters (Martinez-Antonio and Collado-Vides, 2003). For example in *E. coli*, which contains 300 potential TFs, seven of them are general regulators responsible of the regulation of 50% of the genome (Browning and Busby, 2004).

TFs can work as activators or repressors, or have this dual-role (Perez-Rueda and Collado-Vides, 2000). Most activators directly interact with the RNAP and they have been classified in three different classes [reviewed in (Browning and Busby, 2004)]. Class I activators bind upstream the -35 sequences and interact with the C-terminal domain of the α subunit of the RNAP (Fig. 4A). Class II activation is based on the overlap of the TF binding site with the -35 promoter sequence element which produces the interaction of the TF with the σ subunit of the RNAP (Fig. 4A). Activators of the class III binds at or very near to the -35 and -10 promoter elements, even between them, and produces a conformational change in the DNA that expose these elements and allow their recognition by the RNAP (Fig. 4A). TFs acting as repressors reduce the efficiency of the transcription initiation process and have been also classified in three groups (Browning and Busby, 2004). Repressors of the class I sit at the promoter region of the gene and block the binding of the RNAP to the DNA (Fig. 4B). The class II repression is mediated by TFs that bind to promoter-distal sites inducing conformational changes in the DNA that hides the promoter to the RNAP (Fig. 4B). Class III repression is performed by anti-activators that bind to both the DNA and an activator inhibiting the activator (Fig. 4B).

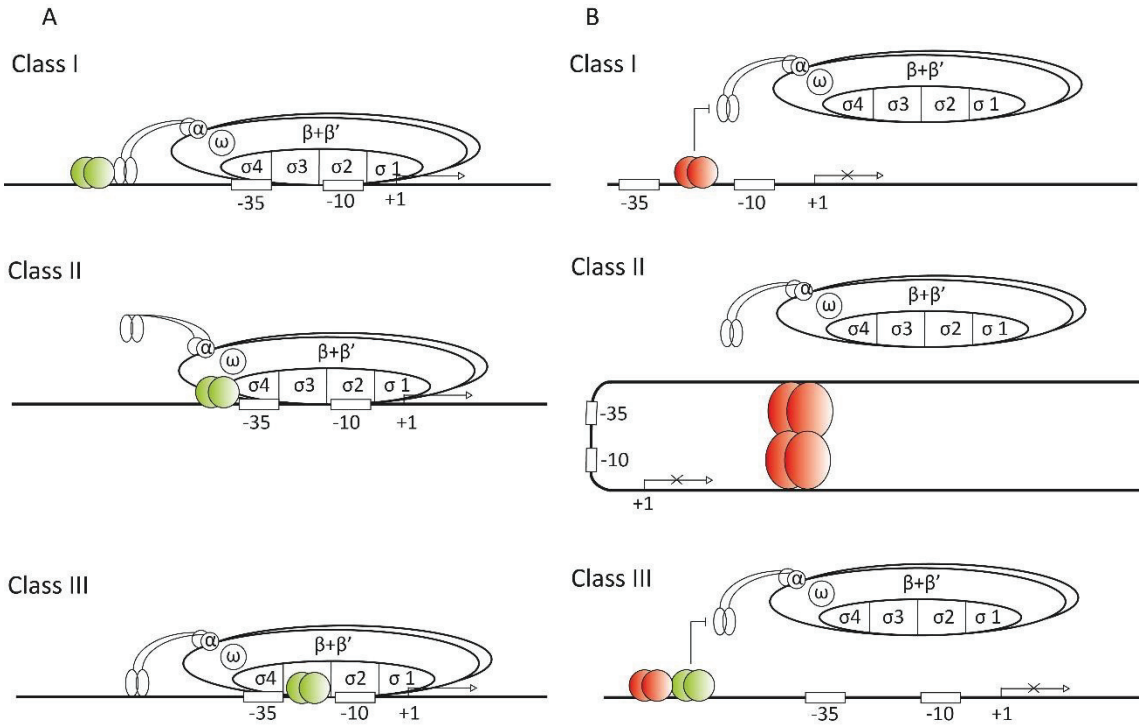

Fig. 4. Mode of action of transcription factor activators and repressors. Schematic representation of the gene transcription initiation by the bacterial RNAP and its interaction with TFs. TFs that function as activators are shown in green and repressors are shown in red. The black line represents the DNA, the rectangles the σ^{70} -35 and -10 promoter sequences and +1 the transcription start point. The three different classes of TF-mediated activation **(A)** and repression **(B)** are shown. Adapted from (Browning and Busby, 2004).

TFs can integrate several signals to modulate gene expression, which often occurs through the interaction with others TFs leading to complex regulatory networks (Browning and Busby, 2004). Complex networks often involve a repressor and an activator, or co-dependence of more than one activator (Fig. 5), and in few cases two repressors can modulate the activity of a promoter (Browning and Busby, 2004). A mechanism of integrated activation is performed by two TFs that interact independently with the RNAP and the DNA in different positions, as for example a class I and a class II activator (Fig. 5A). The interaction of two TFs that performs a co-operative binding to the DNA being unable to bind independently is another example (Fig. 5B). Other integration mechanisms are based on the repression of an activator. The appearance of a second activator breaks the inhibition of the primary activator allowing gene expression (Fig. 5C).

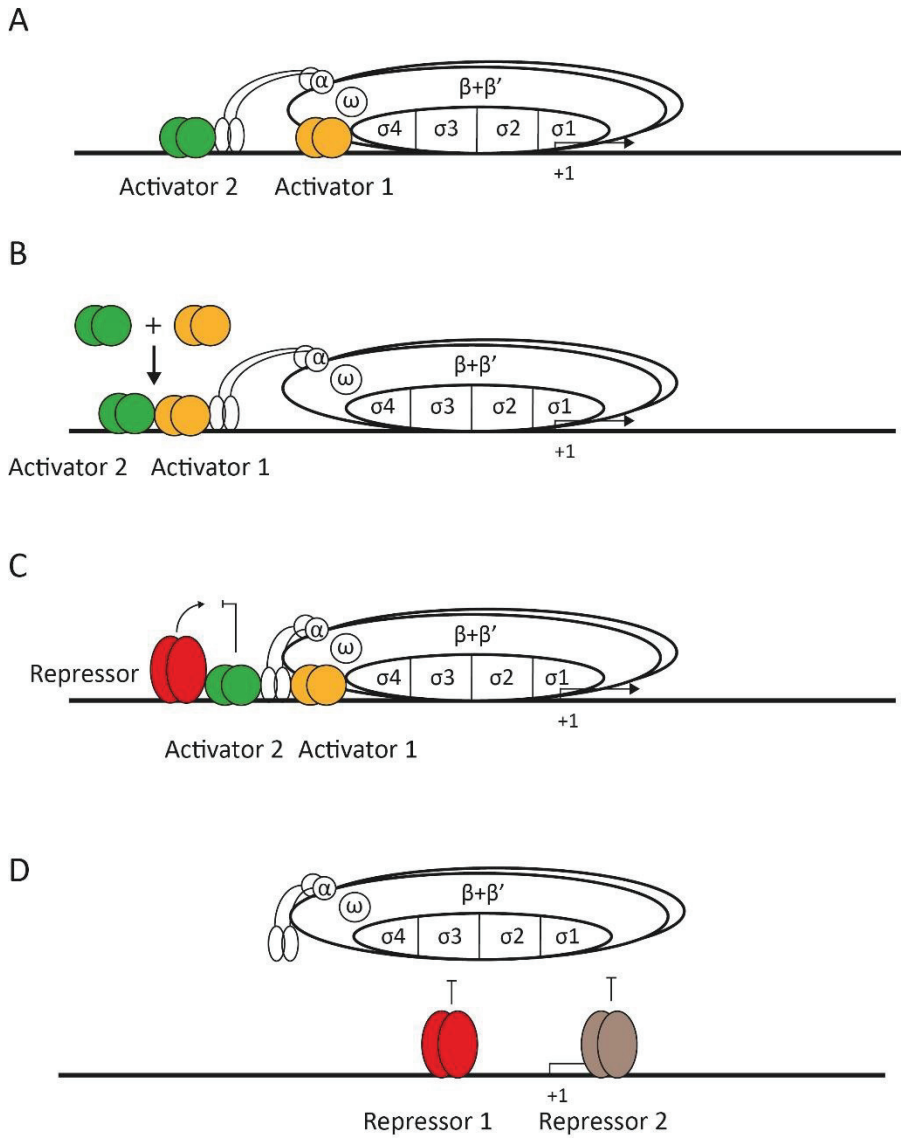

Fig. 5. Integration of signals by transcription factors. (A) Two TF activators that bind independently to the DNA and RNAP but both are needed for transcription. **(B)** Cooperation between two TFs that are not able to bind independently to the DNA and the RNAP. **(C)** Binding of the TF activator 2 (green) remove the inhibition exerted by the repressor (red) on the activator 1 (yellow) allowing gene transcription. **(D)** Two TFs repressors are inhibiting the gene transcription. Two signals are needed for removing the TFs and permits transcription. Adapted from (Browning and Busby, 2004).

A2. Post-transcriptional regulation of gene expression

Transcriptional regulation mediated by σ factors and TFs are the main checkpoints in gene regulation; however, there are several post-transcriptional regulations in the way towards an active protein. Post-transcriptional regulation permits a faster production of proteins, which is necessary in several situations for example under stress conditions or during the infectious process of pathogens (Cain et al., 2014). Post-transcriptional regulation affects primarily at mRNA translation and there are different mechanisms that perform this control, as described below.

A2.1. RNA thermometers

Temperatures changes deeply affect the efficiency of all process in bacteria because alter membranes fluidity, proteins folding, and the secondary structures of the RNA (Chursov et al., 2013). Bacteria have to react instantly to these setbacks and to do it they contain RNA thermometers (TRNAs) that respond to temperature in a immediately way [reviewed in (Kortmann and Narberhaus, 2012)] (Fig. 6A). TRNAs create secondary structures over the ribosome binding site (RBS) of mRNAs obstructing the translation by the ribosome. Changes in the temperature affect the secondary structure of the TRNAs, which expose the mRNA's RBS allowing translation. This type of regulation has been described in mRNAs that codify for cold shock, heat shock and virulence proteins, and permits a faster production of these proteins due to the presence in the cell of already transcribed mRNAs.

A2.2. Riboswitches

Riboswitches are encoded as part of the mRNA and thus work as *cis* regulatory elements [reviewed in (Sherwood and Henkin, 2016)]. These mRNA sequences are able to bind physiological signals that produce structural changes in the mRNA and the switch of the expression "on/off" (Fig. 6B). The ligand can be the product of a biosynthetic pathway that negatively regulates its own route as happens with the regulation by *S*-adenosyl-methionine of its metabolizing pathway (Epshtein et al., 2003). Acquired elements as iron are also common signals for riboswitches, which can repress the translation of mRNAs involved in its acquisition or induce the expression of proteins required to eliminate this toxic metal (Dann et al., 2007).

A2.3. Small RNAs (sRNAs)

Small RNAs (sRNAs) are 50-500 nucleotides molecules that interact with mRNAs inhibiting their translation [reviewed in (Gottesman and Storz, 2011; Storz et al., 2011)]. There are two types of sRNAs, true antisense sRNAs and limited complementary sRNAs. The first ones are transcribed in the opposite direction of the gene they control and thus share extensive complementary sequences with their cognate mRNA. This type of regulation often occurs in the expression of potentially toxic proteins. In contrast, sRNAs of the second group are encoded in different locations than the mRNAs they control. They usually bind to the RBS although other binding sites has been also described (Fig. 6C). In Gram-negative bacteria, the binding of limited complementary sRNAs to the mRNA is enhanced by the Hfq chaperone (Valentin-Hansen et al., 2004).

A2.4. RNA binding proteins (RBP)

Another post-transcriptional regulatory mechanism is based on proteins that interact with the mRNA and modulate its translation [reviewed in (Van Assche et al., 2015)]. RNA binding proteins (RBPs) directly compete with the ribosome for binding to the RBS or change the secondary structure of the mRNA near this region (Fig. 6D). In this case, the RBP produces the decay in the translation of the mRNA. RBPs can also act as platforms for assisting in the interaction of other molecules (as sRNAs or other proteins), which affect the stability or the translation efficiency of the mRNA. They predominantly compete with the ribosomes for the RBS. One of the most studied RBP is the carbon storage regulator A (CsrA) (Timmermans and Van Melderen, 2010). CsrA regulates mRNA stability and translation. It usually produces the decay of the mRNA by binding nearby or overlapping the Shine-Dalgarno sequence. It participates in multiple unrelated pathways such as central carbon metabolism, virulence, biofilm formation, motility or quorum sensing. The chaperone Hfq is another well studied RBP. As mentioned before, this protein binds sRNA and mRNA and induces their pairing under *in vitro* and *in vivo* conditions, enhancing the action of the sRNAs (Updegrove et al., 2016). Hfq binds to

A/U-rich single-stranded region of the sRNA; however, due to the low conservation of sRNAs a prediction-binding site is unreliable (Storz et al., 2011).

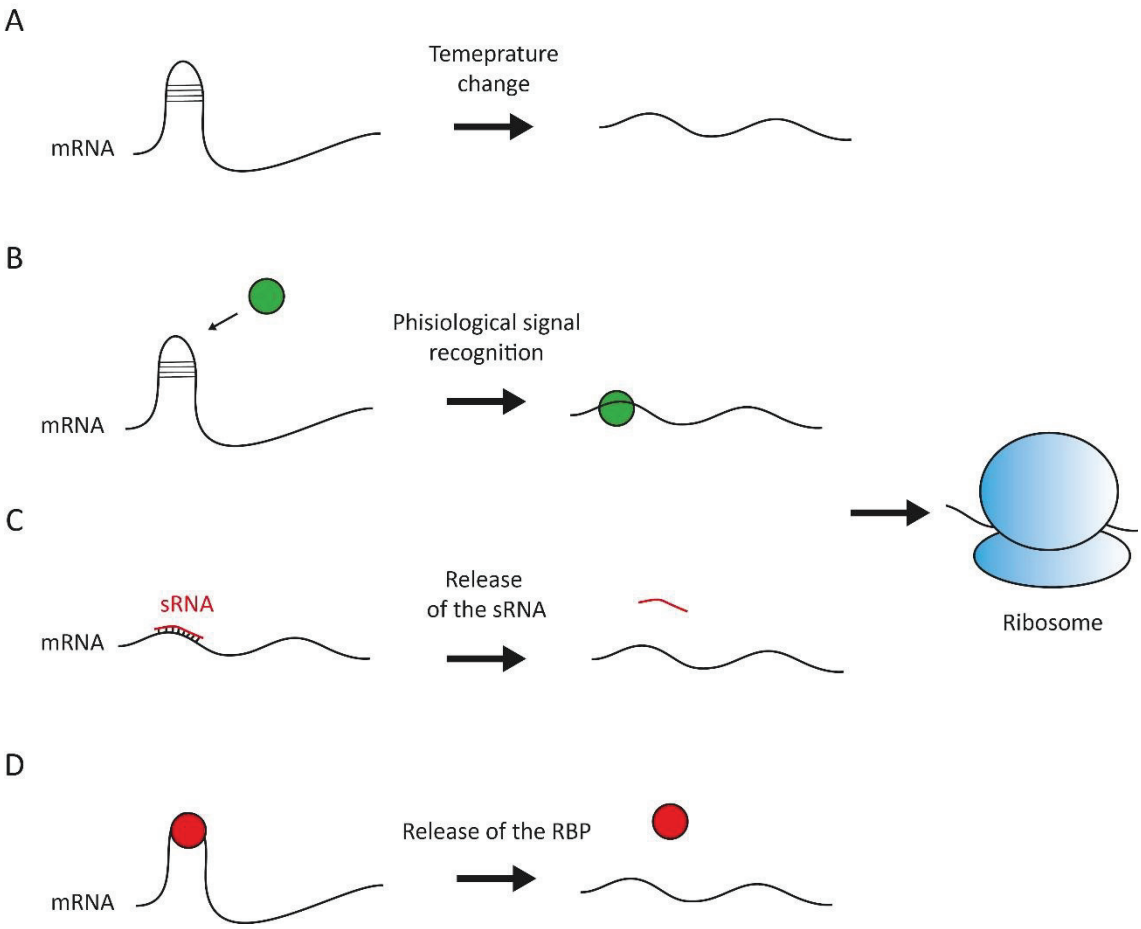

Fig. 6. Post-transcriptional regulations. (A) RNA thermometer. Temperature change eliminates the secondary structures and permits the translation by the ribosome. **(B)** Riboswitches recognize physiological signals (green) that induce structural changes allowing translation by the ribosome. **(C)** sRNA (red) binds to mRNA inhibiting its translation. sRNA is released and the mRNA can be translated. **(D)** RNA binding protein (RBP) (red) keeps the mRNA inaccessible to the ribosome. When RBP is released, translation occurs.

A3. Post-translational modifications (PTMs)

When an mRNA is translated, the generated protein does not usually have the freedom of acting immediately. There are post-translational modifications (PTMs) that can keep a protein inactive or can change its function. Although it was originally thought that PTMs was a specific process of eukaryotes, multiple PTMs have been described in bacteria in the last 50 years (Cain et al., 2014). PTMs have multiple effects in the function, stability and location of the proteins, and modulate their correct function in the right moment. Like post-transcriptional modifications, PTMs also permit a faster response to changes in the environment. There are several types of PTMs, which are briefly described below.

A3.1. Phosphorylation

Phosphate addition is one of the most common PTM in bacteria and occurs through the activity of protein kinases. These proteins transfer a phosphoryl group to another protein, which

usually results in the activation of the phosphorylated protein. In eukaryotes, protein phosphorylation takes place primarily in Ser, Thr and Tyr residues (Cohen, 2000). Although phosphorylation of these residues have been also identified in several bacterial species, the most common mechanism of protein phosphorylation in bacteria involves His and Asp residues (Cain et al., 2014), as occurs during the activation of two-component signaling systems (described in section B2. Two-component systems). Addition of phosphate to other aminoacids like Cys or Arg has been also described in bacteria (Macek et al., 2007). Phosphorylation is a reverse event. The removal of the phosphate group is performed by phosphatases that work in the opposite way than kinases. Protein phosphorylation and dephosphorylation work as an on/off protein switcher (Stock et al., 2000). This PTM is crucial for the modulation of a wide range of process from carbon, protein and nucleotide metabolisms to cell cycle, division, chemotaxis, signaling and virulence (Cozzone et al., 2004; Cain et al., 2014).

A3.2. Acetylation

Protein acetylation is the addition of acetyl groups in ε-amines of Lys residues located in side chains or in α-amines of N-termini residues (Cain et al., 2014). This process usually turns off the activity of the protein (Hu et al., 2010). Acetylation is carried out by a large family of enzymes known as acetyltransferases that mediate the transfer of the acetyl group from a donor (e.g. acetyl coenzyme A) to the acceptor protein (Fig. 7). The process is reversible by the action of deacetylates that remove acetyl groups and thus function as an 'on/off' switcher. Acetylation influences the activity of dozens of proteins that participate in a broad range of functions as motility, chemotaxis or stress response (Barak and Eisenbach, 2001; Ma and Wood, 2011; Cain et al., 2014) .

A3.3. Methylation

Protein methylation is widely distributed in bacteria and is one of the most versatile PTM due to its low energy cost, distribution and the multiple functions that it can alter (Murn and Shi, 2017). This PTM was originally discovered in *Salmonella typhimurium* as the process that modulates the activity of flagellar proteins (Ambler and Rees, 1959). Both N- and O-methylation occur in bacteria. N-methylation involves the addition of one, two or three methyl groups to the terminal amine of a aminoacid and can thus be performed over Lys, Arg and Gln residues, although Lys is the most methylated aminoacid (Lanouette et al., 2014). O-methylation takes place in the carboxylated side chain of Glu residues (Cain et al., 2014). Protein methylation usually inhibits the function of the protein in a reversible way via the function of methylesterases. There are several methyltransferases in bacteria that produce protein methylation. Protein methylation modulates important bacterial functions such as motility, adherence to surfaces, or immune evasion during infection (Paik et al., 2007).

A3.4. Glycosylation

Protein glycosylation is the most abundant protein modification in the nature and occurs by the covalently link of glycans to the protein (Nothaft and Szymanski, 2010). N-glycosylation involves the attachment of a glycan to the amide nitrogen of an Asn residue and O-glycosylation the binding of a glycan to the hydroxyl oxygen of Ser or Thr residues (Nothaft and Szymanski, 2010). N-glycosylation has been deeply studied in *Campylobacter jejuni* in which the process is mediated by the proteins encoded within the *pgl* (protein glycosylation) gene cluster (Nothaft et al., 2012). This bacterium has more than 100 N-glycosylated proteins and, among other functions, this modification is needed for the correct attachment of *C. jejuni* to epithelial cells

and colonization during infection (Karlyshev et al., 2004). Several pathogens like *Neisseria meningitides* or *Neisseria gonorrhoeae* suffers O-glycosylation in proteins of the type IV pili (Abu-Qarn et al., 2008). Absence of the glycosylaton machinery in these species significantly reduces bacterial motility and colonization, and therefore virulence. O-glycosylation needs a different pathway than the N-glycosylation. Glycans used for O-glycosylation are rare, and for that reason, they become a good target for combating the infection of these bacteria (Abu-Qarn et al., 2008).

A3.5. Lipidation

Lipidation is the process by which proteins are bound to lipids. This reversible modification permits the attachment of hydrophilic proteins to hydrophobic surfaces (e.g. bacterial outer membrane) (Okuda and Tokuda, 2009). Lipidation occurs in Cys residues present in a conserved motif known as lipobox (LB) (Wilson and Bernstein, 2016). The process takes place in the cytoplasmic membrane by the sequential action of three enzymes: a prolipoprotein diacylglycerol transferase (Lgt), a prolipoprotein signal peptidase (Lsp) and an apolipoprotein N-acyltransferase (Lnt) (Fig. 8) (Buddelmeijer, 2015). These three steps produce a mature *N*-acyl-*S*-diacylglyceryl-cysteine linked protein. Lipoproteins play a key role in important bacterial functions as cell wall homeostasis, nutrient uptake, signal transduction, conjugation, antibiotic resistance, transport and folding of membrane proteins, and virulence (Kovacs-Simon et al., 2011; Buddelmeijer, 2015). In fact, the Lnt and Lgt lipidation enzymes are essential in Gram-negative bacteria (Sutcliffe et al., 2012; Cain et al., 2014).

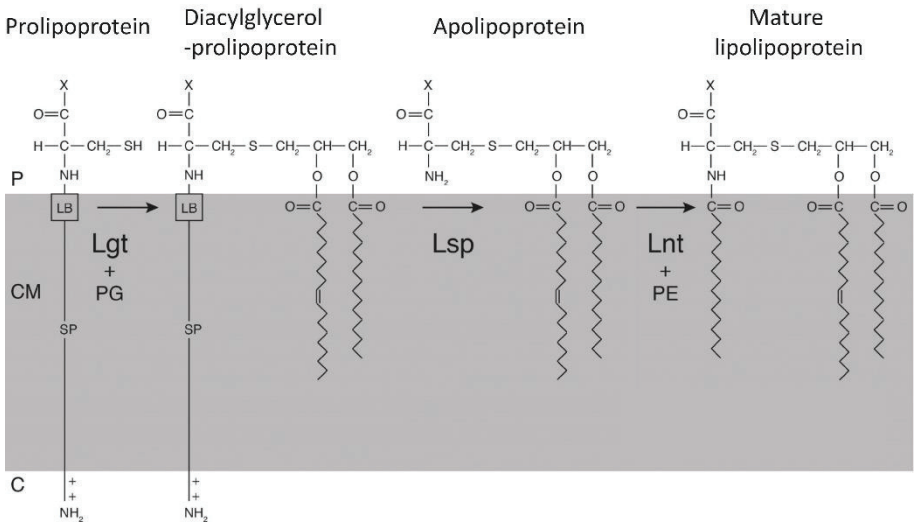

Fig. 8. Protein lipidation. Lgt attaches a diacylglycerol from phosphatidylglycerol (PG) to the sulfhydryl group of the Cys. Then Lsp cuts the signal peptide of the protein and produces the liberation of the α-amino group of the diacylglyceryl-cysteine. Finally, Lnt produces an N-acylation of the diacylglyceryl-cysteine using phosphatidylethanolamine (PE) as an acyl donor, generating a mature lipoprotein. C= cytoplasm, CM=cytoplasmic membrane, P=periplasm, LB=lipobox. Obtained from (Buddelmeijer, 2015).

A3.6. Pupylation

Pupylation is a PTM based on the addition of the "prokaryotic ubiquitin-like protein" (Pup) [reviewed in (Burns and Darwin, 2010; Cain et al., 2014)]. Pup is a peptide that works as a tag for sending proteins to degradation having thus the same function that ubiquitin in eukaryotes (Komander and Rape, 2012). Pupylation is mediated by the proteins PafA and Dop, which prepare and add the Pup to the target protein. In an irreversible manner, the pupylated

protein is directed to a eukaryotic-like proteasome, a compartment that contains proteolytic active sites, where the protein is degraded (Tanaka, 2013). The proteasome is only present in bacteria from the order of Actinomycetales (Murata et al., 2009). However, in other bacteria such as *E. coli* the proteasome is substituted by a Thr protease called heat-shock locus gene V (Murata et al., 2009). Before the tagged protein is degraded, pupylation is reversible. Dop also mediates the depupylation of the Pup tag rescuing the protein from degradation (Burns et al., 2010; Delley et al., 2012).

A3.7. Proteolysis

Proteolysis was firstly defined as an unspecific process for amino acids turnover. However, besides a general housekeeping proteolysis that degrades misfolded or damaged proteins in an unspecific manner, regulated proteolysis also occur in bacteria and eukaryotes (Gur et al., 2011). Regulated proteolysis processes selected substrates in response to specific signals in order to activate or to generate a new protein function (Henkel et al., 1993; Burkard et al., 2005; Berzigotti et al., 2012). When the action of a protein is needed immediately, transcription and translation are long standing processes, while protein cleavage produces a faster response. Proteolysis is a ubiquitous process and there is no other cellular mechanism implicated in the number of biological process than is proteolysis. DNA replication, cell cycle progress, cell proliferation or cell death are some of the biological process where proteolysis is needed (Turk, 2006). Proteolysis is an irreversible PTM that is generally performed by proteases and peptidases, which, depending on the active site residue or ion that carries out catalysis, are classified in serine, glutamic, threonine, aspartic, asparagine, cysteine or metallo proteases (Rawlings et al., 2012). Proteases can either act randomly producing the degradation of the protein, as occurs with proteases involved in amino acids recycling, or hydrolyze specific peptide bounds.

Regulated proteolysis has also solved the problem of how a signal can be transduced from one cell compartment to another across the cell membrane. In the field of cell signaling a new conceptual finding has emerged: the realization that transmembrane proteins can be cleaved within the plane of the membrane to liberate cytosolic fragment that modify gene transcription. This regulatory paradigm, known as regulated intramembrane proteolysis (RIP) (Fig. 9), governs transmembrane signaling responses ranging from the cell envelope stress response of several bacteria to the SREBP (sterol regulatory element-binding proteins) pathway of humans, or the production of the amyloid β peptide that is suspected to cause Alzheimer's disease (Brown et al., 2000). Typically, the RIP mechanism cleaves a transmembrane regulatory protein on either side of the membrane, resulting in release of the regulatory domain with a distinct biological function (Brown et al., 2000). These systems are most commonly found in compartmental membranes in eukaryotes and the cytoplasmic membrane of prokaryotes. The signal is generally sensed on the lumen or extracytoplasmic side of the membrane and the regulatory protein is released into the cytoplasm (Brown et al., 2000; Ehrmann and Clausen, 2004)

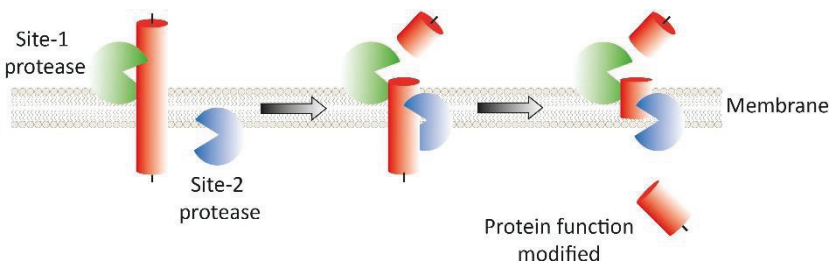

Fig. 9. Schematic representation of regulated intramembrane proteolysis (RIP). The intramembrane protein is processed by a site-1 and site-2 proteases. Site-2 protease usually recognizes substrate size. This allows its action only when the site-1 has cut before. The proteolytic process modifies the function of the protein.

B. Bacterial signal transduction mechanism

Changes in gene expression in bacteria often occur as a response to external and internal cues. Cell signaling is the process that allows bacteria to recognize signals and transduce the information to produce a response. Bacteria contain several mechanisms of cell signaling to detect both intracellular and extracellular signals, including one- and two-component regulatory systems, second messengers, chemotaxis, quorum sensing and extracytoplasmic function σ factors. The complexity of the bacterial cell signaling pathways has increased along the time and the interaction between different signaling pathways has allowed bacteria to integrate multiple signals to generate proper responses (Soyer et al., 2006; Ng and Bassler, 2009; Capra and Laub, 2012; Rowland and Deeds, 2014).

B1. One-component systems

One-component systems are the simplest structures for sensing cues in bacteria. These regulatory systems are broadly and highly present, and seems to be the primary signaling mechanism in bacteria and archaea (Ulrich et al., 2005). One-component systems are composed by a single cytoplasmic protein that carries out both recognition and response to the signal (Ulrich et al., 2005). These functions reside in two different domains of the protein: the signal is recognized by the ligand binding domain, also known as input domain, and the response is performed by the output domain (Fig. 10A) (Ulrich et al., 2005). One-component proteins have a wide diversity of input domains that allow the detection of multiple different signals (Ulrich et al., 2005). As output domain, around 84% of the one-component proteins have a helix-turn-helix (HTH) domain, which binds to the DNA activating or repressing gene transcription (Ulrich et al., 2005). However, a minority of these proteins contain other type of output domains including enzymatic domains that regulate cyclic nucleotides production and protein phosphorylation (Ulrich et al., 2005). The cytosolic location of the one-component proteins reduces the type of external signals that can access this bacterial compartment to be recognized by the sensor domain. For this reason, one-component systems only recognize signals produced in the cytosol or small molecules able to diffuse through the membrane, like light or gases (Ulrich et al., 2005). Upon signal recognition, the output domain undergoes a conformational change that induces the response usually by exposing the HTH domain of the protein allowing its binding to the DNA (Cashin et al., 2006). One-component proteins are usually not modified by phosphorylation or covalent modifications (like other signaling systems) .

A highly present and well-studied group of one-component systems is the AraC family (Gallegos et al., 1997; Egan, 2002; Ulrich et al., 2005). In *E. coli*, AraC regulates the transcription of enzymes responsible for utilizing L-arabinose as carbon and energy source (Schleif, 2003). The N-terminal input domain of this protein recognize L-arabinose and the C-terminal output domain binds to the DNA (Bustos and Schleif, 1993). AraC works as a homodimer. In absence of L-arabinose, AraC binds to two far regions of the *araBAD* promoter forming a repression loop in the DNA that inhibits transcription (Bustos and Schleif, 1993). In this case, AraC works as a class II inhibitor (Fig.4B). When L-arabinose is sensed, the AraC dimer binds to sites located next to the promoter (overlapping 4 bp with the -35 sequence) opening the repression loop and

inducing transcription (Bustos and Schleif, 1993; Gallegos et al., 1997). Another one-component system highly conserved in prokaryote, eukaryote and archaea is the LysR type transcriptional regulator (LTTR) family, which can work as activators or repressors (Maddocks and Oyston, 2008). The activators are usually composed by a HTH domain at the C-terminus and a co-factor binding domain at the N-terminal part, while the repressors are structured in the other way around (Perez-Rueda and Collado-Vides, 2000). LTTRs can bind to DNA in several positions, modifying the DNA topology and enhancing or repressing gene transcription (Maddocks and Oyston, 2008).

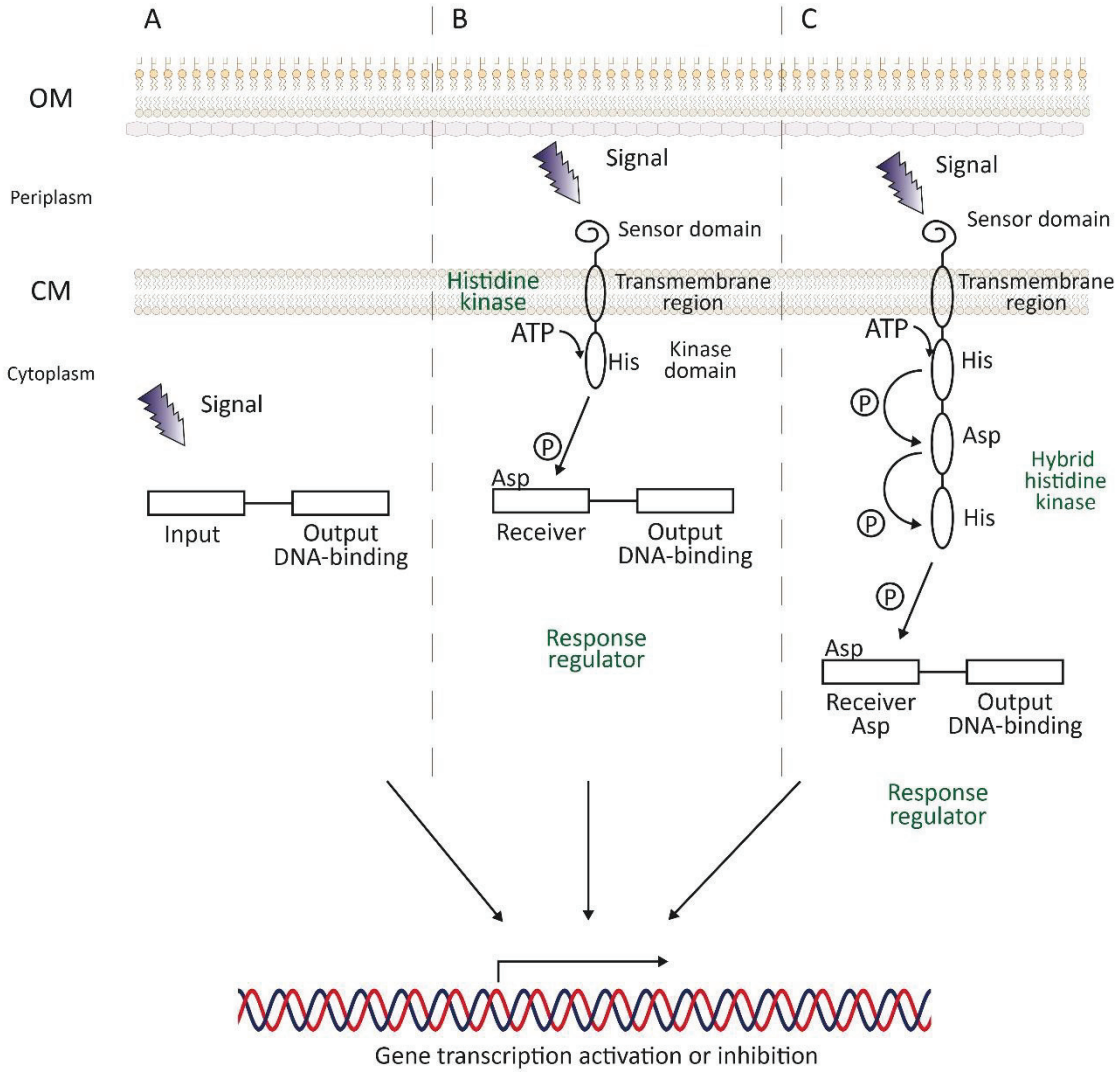

Fig. 10. Signal transduction by one- and two-component systems. (A) Schematic representation of one-component system composed by two domains. An input that sense signals and the output able to bind to DNA. **(B)** Representation of a two-component system. Once the signal is recognized by the input of the histidine kinase, it transfers the phosphate to the receiver domain of the response regulator, which turns active and is able to bind to the DNA. **(C)** Representation of a hybrid two-component system. The hybrid histidine kinase suffers several phosphorelays until the final phosphorylation of the RR that then can bind to DNA. Adapted from (Stock et al., 2000; Cashin et al., 2006).

B2. Two-component systems (TCSs)

The TCS signaling pathway was discovered in 1986 by Nixon and collaborators that found strong similarities between the pair of proteins NtrB and NtrC from *Bradyrhizobium* sp. with paired proteins of other organisms as *E. coli, Kliebsella pneumonie* or *Agrobacterium tumefaciens* (Nixon et al., 1986). They coined the term two-component system to name these pairs. In the same year, Ninfa and Magasanik discovered that activation of this signaling system involved a phosphorelay cascade (Ninfa and Magasanik, 1986). Since them, hundreds of bacterial TCSs have been identified and characterized (Salazar and Laub, 2015). In fact, TCSs are one of the main pathways for sensing environmental cues in prokaryotes, being also present in eukaryotes (Wolanin et al., 2002). These signaling systems respond to a variety of environmental changes such as pH variation, nutrient starvation, osmolarity, redox state, bacterial cell density or antibiotics, and modulate expression of genes required for bacterial adaptation to these changes (Stock et al., 2000). TCSs are therefore highly present in species able to live in multiple environments or in changing circumstances (Wuichet et al., 2010). TCSs are normally composed by two proteins: a histidine kinase (HK) that recognizes a specific stimuli and phosphorylates a response regulator (RR) that mediates the response (Fig. 10B) (Stock et al., 2000). There are several evidences suggesting that two-component systems (TCSs) have evolved from one-component systems, including the higher level of complexity of TCSs, the lower repertoire of TCSs than of one from one-component and the fact that TCSs are less distributed in prokaryotes than one-component systems (Ulrich et al., 2005). In fact, many species of archaea do not have TCSs but plenty of one-component systems, and it is believed that one-component systems were already present in the Last Universal Common Ancestor whereas TCSs were horizontally transferred later to some archaea (Wuichet et al., 2010). Likely, the evolutionary pressure as a result of the limitation in the (environmental) signals that can be recognized by one-component systems produced the separation of these systems in two proteins generating the TCSs.

TCSs recognize signals in the periplasm, within the membrane or in the cytoplasm (Mascher et al., 2006). TCSs that recognize periplasmic cues have HKs that are usually anchored to the cytoplasmic membrane through one or two membrane-spanning helices and work as homodimers (Surette et al., 1996; Mascher et al., 2006). Three different domains are found in these proteins including a variable sensor periplasmic domain, a transmembrane region, and a highly conserved cytosolic core kinase domain (Mascher et al., 2006). Once the signal is recognized by the sensor domain, a conformational change occurs that is transmitted through the membrane spanning helices to the core kinase domain. This allows the binding of ATP by the kinase domain, which produces the autophosphorylation of the kinase domain of its coupled monomer in a conserved His residue (Stock et al., 2000). Upon autophosphorylation, a phosphate transference reaction from the HK to its cognate RR occurs. Most RRs are TFs that possess two main domains: a conserved N-terminal regulatory domain and a variable C-terminal effector domain that generally binds to DNA (Stock et al., 2000). The regulator domain receives the phosphate group from the His of the HK in an Asp residue. This produces a conformational change in the RR that promotes its binding to the DNA. The RR can act as a transcription activator or repressor, and it is common that the RR induces the transcription of the genes encoding the TCS (Groisman, 2016). The half-life of RR-P can range from seconds to hours. The activity of the RR is turned off by phosphatases that hydrolyze the phosphate from the RR-P (Stock et al., 2000). Many RRs have autophosphatase activity that decreases the lifetime of the phosphorylation (Zapf et al., 1998; Stock et al., 2000). Moreover, numerous HKs also possess phosphatase activity, being able to dephosphorylate its cognate RR. Important variations from this archetypal model of TCS have evolved over time. The most outstanding modification is the existence of kinases with numerous phosphodonor sites (Fig. 10C) (Stock et al., 2000). They contain several His conserved residues and several domains with conserved Asp residues that transfer the

phosphate up to the Asp of the RR. This type of HKs are called hybrid HKs due to the presence of Asp residues, which are typical of RRs. The succession of several phosphorelay steps facilitates an accurate control of the activation of the pathway and reduces the occurrence of non-specific cross-talk because of the presence of several regulatory checkpoints (Stock et al., 2000). Moreover, there are phosphatases that act in specific phosphorelay sites being an extra level of regulation of the pathway (Grossman, 1995). Finally, multiple phosphorelay steps provide more communication spots for interaction with other signaling pathways (Appleby et al., 1996). These hybrid proteins are common in eukaryotes but are also present in prokaryotes (Trach and Hoch, 1993; Ogino et al., 1998; Rodrigue et al., 2000). Furthermore, TCSs pathways are sometimes formed by more than two proteins. There are convergent TCSs pathways in which several different HKs produce the phosphorylation of a single RR. Alternatively, a HK can phosphorylate several different RRs creating a divergent pathway (Groisman, 2016).

Although TCSs have been longer studied, the specific signal activating these pathways has been rarely identified and for most of them remain unknown. Example of TCSs with known signals include the CitA/CitB and DcuS/DcuR TCSs of *E. coli* also present in several other bacteria (Mascher et al., 2006). CitA senses citrate and DcuS citrate and C_4-dicarboxylate under anaerobic conditions, and their cognate RR, CitB and DcuR, respectively, induce the expression of enzymes and carriers for the processing of these molecules (Mascher et al., 2006; Liu et al., 2018). The PhoQ/PhoP from enterobacteria senses host-associated cues such as extracellular magnesium limitation, low pH, or the presence of cationic antimicrobial peptides, and regulates magnesium homeostasis, cell envelope composition, stress resistance and virulence [reviewed in (Groisman, 2001; Dalebroux and Miller, 2014)]. Recently, it has been discovered that this TCS is also able to recognize osmotic upshift by changes in the length of the transmembrane domain of the PhoQ HK, which produces the activation of the system (Yuan et al., 2017). These length changes are due to variations in the cell membrane thickness and lateral pressure produced by the osmotic shift. The PmrA/PmrB TCS is the major regulator of lipopolysaccharides (LPSs) modification [reviewed in (Chen and Groisman, 2013)], which are important for resistance to bactericidal agents and evasion of host immune defences. PmrB senses periplasmic Fe^{3+}, Al^{3+} and mildly acidic pH, and produces the phosphorylation of PmrA. The pair KdpD/KdpE is activated by three different stimuli: K^+ concentration, osmolarity and ATP concentration. They are sensed by KdpD that phosphorylates KdpE, which promotes transcription of the Kdp-ATPase potassium ion (K^+) pump and of diverse virulence loci [reviewed in (Freeman et al., 2013)]. The PhoR/PhoB TCS is the master regulator of phosphate metabolism in bacteria and is activated under inorganic phosphate (Pi) starvation conditions [reviewed in (Lamarche et al., 2008; Hsieh and Wanner, 2010)]. The HK PhoR senses Pi starvation through the associated Pst-PhoU ABC transport system (Fig. 11). This produces the activation of the phosphorylation and activation of the DNA binding protein PhoB. PhoB activates the expression of the *pst-phoU* operon and the machinery needed for Pi homeostasis. Moreover, PhoB enhances expression of cell surface components as phospholipids or exopolysaccharides, and participates in biofilm formation, virulence, toxin production, tolerance to acidity and resistance to antimicrobial compounds (Monds et al., 2001; Monds et al., 2007; Long et al., 2008; Zaborina et al., 2008; Zaborin et al., 2009; Santos-Beneit, 2015).

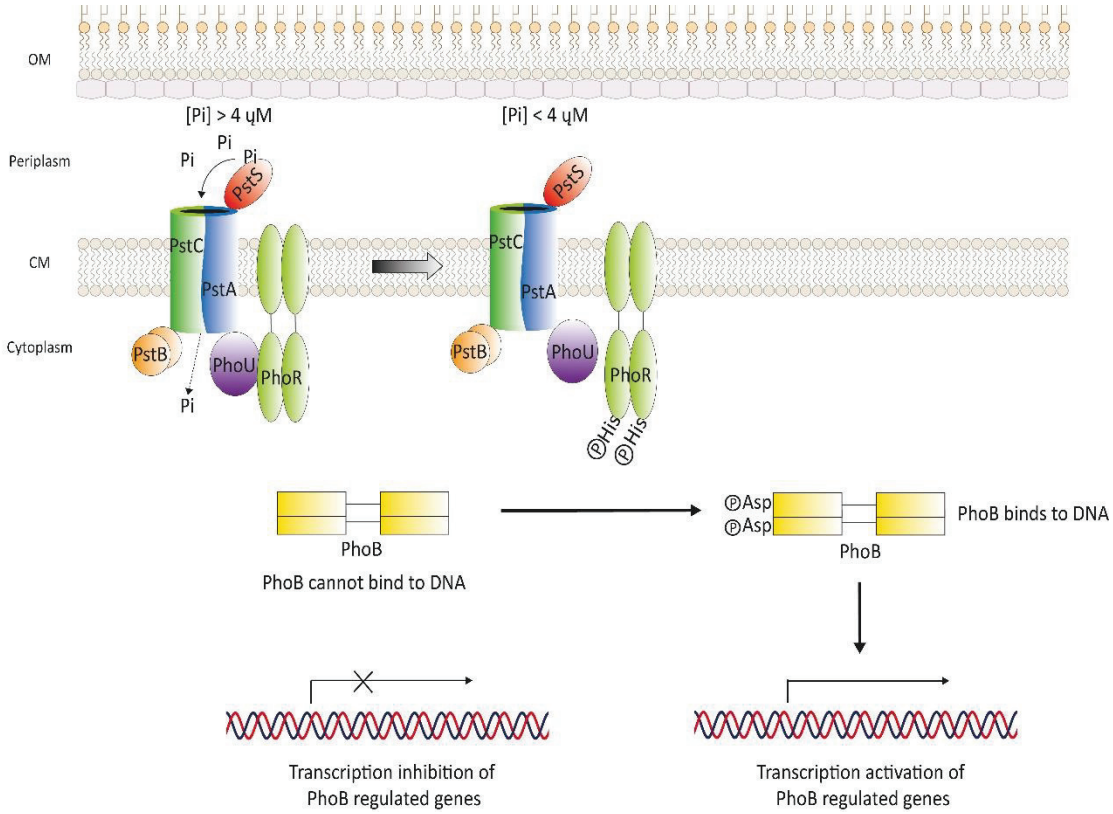

Fig. 11. Activation of the PhoR/PhoB two component system. Schematic representation of the Pst-PhoU ABC transporter and the TCS PhoR/PhoB. Pi starvation activates PhoB. In Pi sufficient conditions, Pi binds to the periplasmic binding protein PstS and is transported into the cytosol through the PstC/PstA channel in a process that requires the energy released by the ATP-dependent permease PstB. In this situation, the chaperone-like protein PhoU prevents PhoB phosphorylation (Lamarche et al., 2008). When the Pi concentration in the periplasm is less than 4 µM, the PhoU blockage disappears and PhoR autophosphorylates and transfers the phosphate to PhoB. Then PhoB-P binds to the DNA modifying gene transcription. Apated from (Stock et al., 2000; Cashin et al., 2006; Lamarche et al., 2008).

Besides the signals that target activation of TCSs, there are several unknown features about the working mechanism of these signaling systems. How the signal is transduced from the sensing to the kinase domain of the HK through the transmembrane domain or how phosphorylation provokes conformational changes in the RR are some of the open questions that require further research.

B3. Second messengers

A second messenger is a molecule that is synthesized in the cell after signaling of an external or internal cue. Second messengers induce the activity of an effector protein that generally produces changes in gene expression. This signaling mechanism has been described in both eukaryotes and prokaryotes, and is also present in archaea. Bacteria have evolved several second messengers being the three most studied the nucleotide-derived ppGpp, c-di-GMP and c-di-AMP. However, second messenger signalling is a recent field of study and the complexity of this signalling mechanism is currently under study.

B3.1. Guanosine penta- and tetraphosphate [(p)ppGpp]

The alarmones (p)ppGpp are important second messengers that orchestrate pleiotropic adaptations of bacteria and plant chloroplasts in response to starvation and stress. Severe nutrients limitation produces multiple changes in the bacterial metabolism, i.e. DNA synthesis stops, RNA synthesis is inhibited to preserve aminoacids and several non-essential reactions are relegated to a second place. The bacterium only focusses in the production of factors crucial to manage stress, and to induce glycolysis and amino acids synthesis. This tense situation is also called stringent response and (p)ppGp are involved in such response [reviewed in (Dalebroux and Swanson, 2012; Steinchen and Bange, 2016)]. The ppGpp pool of a bacteria cell is modulated by the enzymes that synthesizes and degrades this nucleotide. There are monofunctional synthetasas such as RelA that uses ATP and GTP for generating (p)ppGpp (Fig. 12A) (Dalebroux and Swanson, 2012). There are also bifunctional enzymes able to produce and degrade (p)ppGpp such as the synthetase-hydrolases SpoT and Rel (Fig. 12A) (Dalebroux and Swanson, 2012). Bacteria regulate the presence and functionality of these proteins to control (p)ppGpp production. ppGpp performs most of its biological functions by direct modulation of the RNAP and does not work alone but in complex with the protein DksA, which amplifies its impact in the cell (Fig. 12A) (Dalebroux and Swanson, 2012). Binding of the DksA-ppGpp complex to the RNAPc impedes the binding of the primary sigma factor σ^{70} to the RNAPc. This allows the interaction of alternative σ factors with RNAPc that produce the expression of alternative genes needed for the stringent response (Österberg et al., 2011). Apart from altering the RNAP σ composition, ppGpp produces the transcription of non-coding regulatory RNAs that regulates, together with the carbon storage regulator CsrA, the stability of mRNAs and inhibition of translation (Edwards et al., 2011). This regulation influences biofilm formation, motility or protein secretion (Dalebroux and Swanson, 2012). Furthermore, ppGpp controls the activity of numerous transcription factors that promotes transcription of virulence genes (Libby et al., 1994; Ellison and Miller, 2006; Zhao et al., 2008).

B3.2. Cyclic-di-GMP (c-di-GMP)

c-di-GMP is the most studied bacterial second messenger [reviewed in (Römling et al., 2013)]. This nucleotide usually acts as a monomer or dimmer, although bigger functional oligomers has also been detected (Gentner et al., 2012). Synthesis of c-di-GMP is carried out by diguanylate cyclases and degradation by specific phosphodiesterases, which are responsible of the pool of c-di-GMP in the cell (Fig. 12B) (Tal et al., 1998). These enzymes usually have three PAS-GGDEF-EAL domains (Römling et al., 2013), which are usually found in signalling and response regulator proteins. Both cyclases and esterases recognize specific signals that induce the synthesis or the degradation of c-di-GMP (Tal et al., 1998; Henry and Crosson, 2011). The c-di-GMP nucleotide is recognized by several structurally different cytosolic sensors (Römling et al., 2013). For instance by the PilZ domain, which is the domain with higher affinity for c-di-GMP discovered to date (Valentini and Filloux, 2016). Cytosolic sensors with PilZ domain have another domain that performs the output response. Several outputs domains have been described including type 2 glycosyltransferases domains, methyl-accepting protein domains, DNA binding domains and adenylate/guanylate cyclases domains (Römling et al., 2013). c-di-GMP control important bacterial functions including cell differentiation virulence, bacterial movement (swimming, swarming or twitching), the transition from motile to sessile lifestyle, or biofilm formation (Römling et al., 2013). However, this is only a small sample of the wide range functions that this second messenger governs.

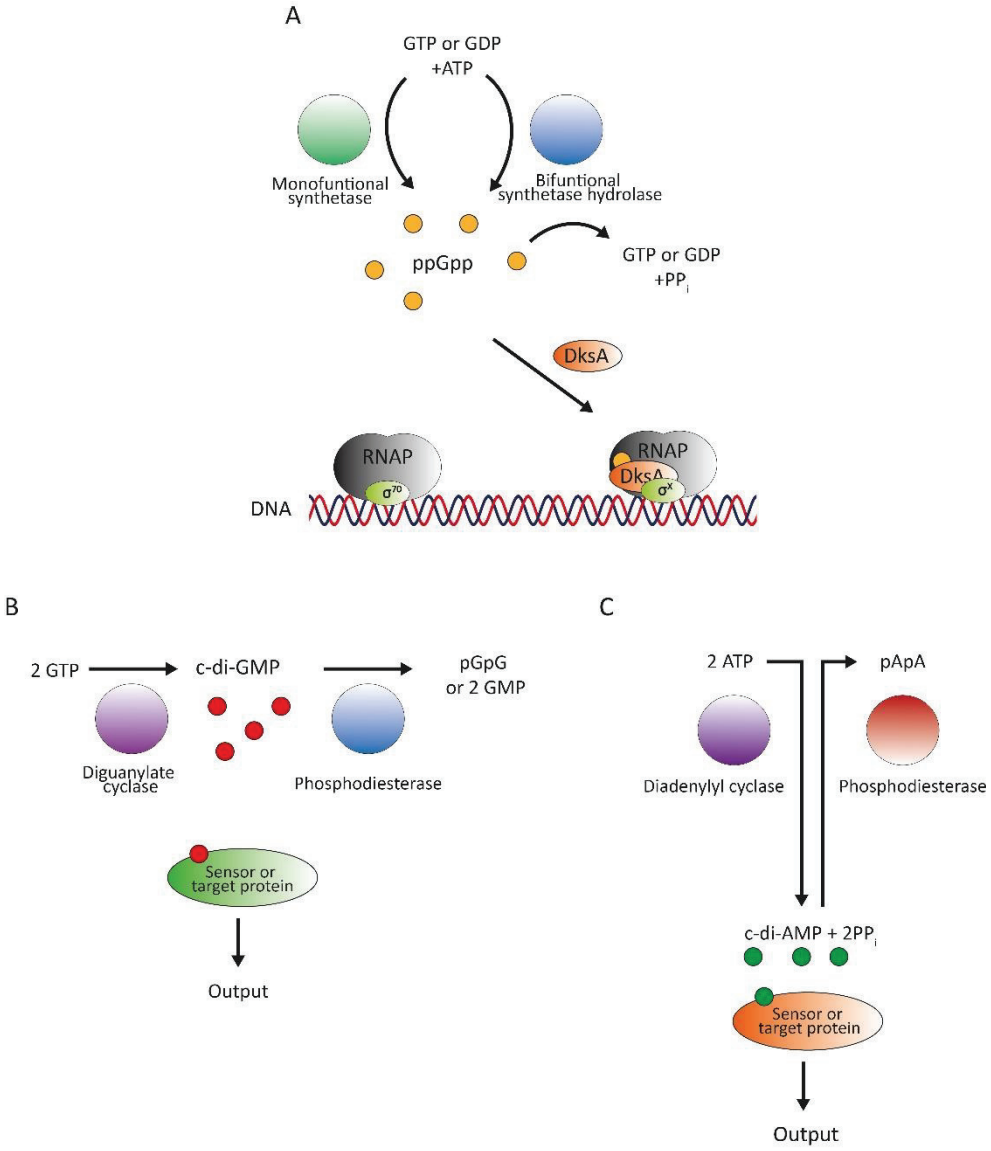

Fig. 12. Signalling by bacterial second messengers. (A) Schematic representation of the synthesis and degradation of ppGpp (yellow). ppGpp interacts with DksA (in orange) and inhibit the binding of the primary σ^{70} factor to the RNAPc, which allows the binding of alternative σ factors a. **(B)** Representation of the c-di-GMP (red) synthesis by diguanilate cyclases and degradation by phosphodiesterases. c-di-GMP interacts with cytosolic sensors and together generate a response. **(C)** Representation of the c-di-AMP (green) synthesisby diadenylyl cyclases and degradation by phosphodiesterases. C-di-AMP is recognized by cytosolic sensors and together produce a response. Adapted from (Dalebroux and Swanson, 2012; Corrigan and Grundling, 2013; Valentini and Filloux, 2016).

B3.3. Cyclic-di-AMP (c-di-AMP)

c-di-AMP was discovered in 2010 when Woodward and collaborators found c-di-AMP secreted by *Listeria monocytogenes* during intracellular infection (Woodward et al., 2010). C-di-AMP is produced from two molecules of ATP by diadenylyl cyclases (DAC) and degraded to pApA by phosphodiesterases (PDE) (Fig. 12C) [reviewed in (Corrigan and Grundling, 2013)]. Environmental signals are recognized directly or indirectly by DACs and PDEs, which then modulate the c-di-AMP pool in the cell. c-di-AMP binds and modify the activity of multiple proteins that control several cellular pathways. c-di-AMP is sensed by DNA binding proteins such as transcription factors of the tetracycline resistance family regulator (Zhang et al., 2013). c-di-

AMP is also able to regulate the activity of sensor histidine kinases as KdpD from *S. auerus*. Moreover, c-di-AMP is released from *L. monocytogenes* cells during infection and it is sensed by the host inducing the innate immune type I interferon response (Corrigan and Grundling, 2013).

B4. Quorum sensing (QS)

Bacteria can "talk" with each other and coordinate a specific response. This bacterial communication was discovered in 1970 by Nealson and collaborators when they observed that the fish symbiont *Vibrio fischeri* responded to high cell density by producing luminescence (Nealson et al., 1970). The term Quorum sensing (QS) that describes the bacterial ability to detect and to respond to cell population density was coined later by Fuqua and collaborators (Fuqua et al., 1994). QS enables bacteria to restrict expression of specific genes to high cell densities at which the resulting phenotypes will be most beneficial. This signaling mechanism is used for many bacteria species, both Gram-negative and Gram-positive. In Gram-negative bacteria QS is performed by autoinducers, which usually are homoserine lactones (HSLs) while Gram-positive bacteria use peptides of 5-17 aminoacids as autoinducers (Monnet et al., 2016; Papenfort and Bassler, 2016). HSLs are produced by HSL synthases that use S-adenosylmethionine (SAM) as substrate (Papenfort and Bassler, 2016). HSLs diffuse freely through the membrane to the extracellular medium and when the cell density is high and the concentration of HSLs is above the threshold, this molecule is recognized by a cytoplasmic or cytoplasmic membrane sensor that in response modifies gene expression (Fig. 13) (Papenfort and Bassler, 2016). The peptides produced by Gram-positive are not freely diffusible through the membrane and need to be actively transported to enter into the bacterial cytosol (Rutherford and Bassler, 2012). These peptides have two ways of being recognized by the cell. An extracellular pathway where a sensor domain of two-component histidine kinases in the inner membrane of the bacteria detects the peptide and activates a response regulator (Rutherford and Bassler, 2012). The second route is known as the intracellular pathway where a peptide transport system permits the entrance of the oligopeptide that then activates transcriptional factors.

QS induces expression of numerous genes that in some cases can achieve the 10% of the genome, as happens in *P. aeruginosa*. This large regulatory architecture is implicated in phenotypic modification as biofilm formation that usually implicates a sessile lifestyle, bacteriocins production, acid tolerance, antibiotics production, motility or stress response (Waters and Bassler, 2005; Reading and Sperandio, 2006; Williams et al., 2007).

Several bacteria produce molecules to interfere with QS signals used by other species with which they share the niche [reviewed in (Zhang and Dong, 2004)]. This includes the secretion of HSL degradation enzymes that silences the autoinducer or the production of triclosan, which inhibits a metabolic step in the HSL production (Zhang and Dong, 2004). Another interference mechanism is the synthesis of HSLs analogues like halogenated furanones that bind to the QS sensor and produce its degradation inhibiting the communication (Manefield et al., 2002). This latter mechanism is also used by infected host to prevent QS communication of pathogens (Manefield et al., 2002).

Besides for intraspecie communication, QS is also used by bacteria to communicate with other species with which maintain a symbiotic, competence or host-pathogen relations [reviewed in (Federle and Bassler, 2003; Lowery et al., 2008; Ryan and Dow, 2008)]. A SAM derived molecule present in Gram-negative and -positive for interspecies communication termed AI-2 (Federle and Bassler, 2003; Lowery et al., 2008), is sensed by the soluble periplasmic protein LuxP (Bassler et al., 1994). AI-2 varies in different species; however, it seems that they

are able to be recognized by several of them (Schauder and Bassler, 2001). The AI-2 interspecies communication has been related to bioluminescence production, expression of virulent factors and biofilm formation. Apart from AI-2 there are other interspecies signals as 2-heptyl-3-hydroxy-4-quinolone and related molecules that are exchanged through the 2-alkyl-4-quinolone (4Q) system from *P. aeruginosa*. This system is implicated in the growth inhibition of cohabit species (Lowery et al., 2008).

QS has been extensively studied in *P. aeruginosa* due to its main role in biofilm formation and virulence. *P. aeruginosa* contains three QS systems named LasR/LasI, RhlR/RhlI and PQS (Miller and Bassler, 2001; Williams and Camara, 2009; Rutherford and Bassler, 2012). LasI and RhlI synthesize two different HSLs that diffuse to the extracellular medium and are internally recognized by the LasR and RhlR sensors, respectively that bind to DNA an regulate gene transcription (Rutherford 2012). The third *P. aeruginosa* QS system does not involve a HSL but the 2-heptyl-3-hydroxy-4-quinolone (PQS from Pseudomonas Quinolone Signal), which is recognized by the cytosolic sensor PqsR, that binds to DNA and regulates gene transcription (Pesci et al., 1999). PQS also connects the Las and Rhl systems. There is a hierarchy in the three QS systems in *P. aeruginosa* regulation, which permits their synchronic activation. The Las system is at the top level. When LasR is activated, it induces transcription of the *rhlR* and *rhlI* genes. Moreover, LasR induce expression of the PQS system. PqsR also induce transcription of *rhlR* and *rhlI*. In addition, RhlR inhibits the expression of the PQS system (Papenfort and Bassler, 2016). The *P. aeruginosa* QS systems induce transcription of genes encoding elastases, proteases, pyocyanin, siderophores or biofilm formation (Schuster et al., 2003; Schuster and Greenberg, 2007), which in turn induces *P. aeruginosa* virulence (Smith and Iglewski, 2003).

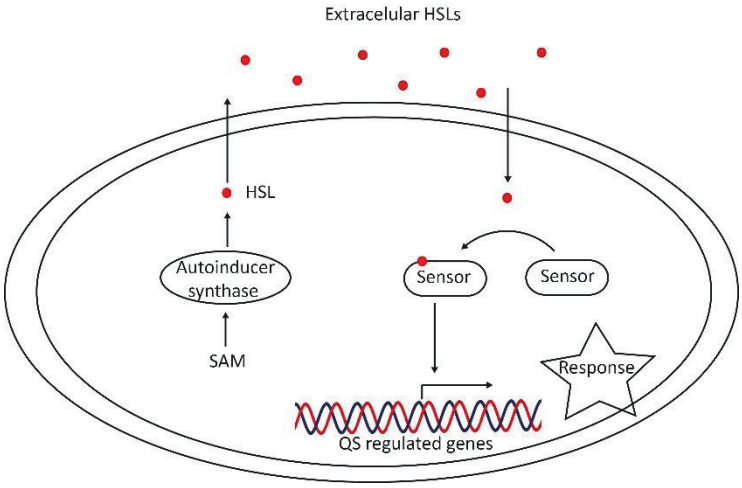

Fig. 13. Schematic representation of the Quorum sensing signaling. The autoinducer synthase generates the autoinducer HSL, which is released in the extracellular medium. Highconcentration of HSL generated in high cell density is recognized by the sensor, a DNA binding protein that modifies gene expression. Adapted from (Waters and Bassler, 2005).

B5. Chemosensory pathways

Chemosensory pathways were first identified as pathways that mediate chemotaxis, *i.e* the flagellum-mediated directed movement in response to a signal gradient (Parkinson et al., 2015). Chemotaxis allows bacteria to access compounds that serve as carbon and nitrogen-sources for growth (*i.e*. organic acids, sugars), compounds that serve as electron acceptors (*i.e*. oxygen) and compounds that inform the bacterium on a particular habitat (*i.e*. plant hormones, histamine) (Matilla and Krell, 2018). Chemotaxis also allows bacteria to escape from hazardous compounds. . A chemosensory pathway is composed by core and auxiliary proteins (Fig. 14). The

central element of a chemosensory pathway is the ternary complex formed by a chemoreceptor, the histidine kinase CheA and the coupling protein CheW (Parkinson et al., 2015). Binding of the signal to the ligand binding domain of a chemoreceptor induces a molecular stimulus that modulates activity of CheA that in turn alters the transphosphorylation activity to CheY (Fig. 14). Chemoeffectors can be recognized directly or in complex with a ligand binding protein (Matilla and Krell, 2018). Upon phosphorylation, CheY-P is released and binds to the flagellar motor and modifies the direction of the flagella rotation generating tumbling and subsequent change in direction (Fig. 14) (Porter et al., 2011). Pathway sensitivity is modulated by the methylation and demethylation of the receptor, which is mediated by the CheR methyltransferase or the CheB methylesterase, respectively (Fig. 14). (Wuichet and Zhulin, 2010). The end of the signaling is promoted by the CheZ phosphatase that dephosphorylates CheY-P.

Chemosensory pathways not only modulate flagellar rotation but also carry out alternative cellular functions, such as modulating second messenger levels or produce movement associated with twitching motility (Whitchurch et al., 2004; Hickman et al., 2005). This is for example the case of the *P. aeruginosa* Wsp pathway, which induces second messenger c-di-GMP production via a chemosensory cascade that phosphorylates and activates the diguanylyl cyclase WspR (Fig. 14) (section B3.2.) (Porter et al., 2011). Another chemosensory pathway of this bacterium, the Chp pathway, modulates twitching motility-via the phosphorylation and activation of the CheY-like response regulators PilG and PilH that controls the extension and retraction of the type IV pili (Fig. 14) (Bertrand et al., 2010). In *Myxococcus xanthus*, a chemosensory pathway modulates the sporulation process (Fig. 14) (Porter et al., 2011).

Chemosensory pathways are present in half of the prokaryotic genomes, mainly in bacteria with complex lifestyle able to colonize different niches (Wuichet and Zhulin, 2010). Although chemotaxis increases the ability of bacteria to find suitable niches to live, it is also an energetic costly process, which explains why this signaling process has not always been selected in evolution (Wuichet and Zhulin, 2010). Chemosensory pathways and chemotaxis are important for both beneficial and deleterious bacteria-plant interactions. For example, the plant growth promoting rhizobacterium *Pseudomonas putida,* which is able to colonize plant roots protecting the plant, is directed to the roots through chemosensory detection of root exudates and subsequent chemotaxis (Espinosa-Urgel et al., 2002). Phytopathogens use chemotaxis as a motility method for attraction towards specific niches suitable for infection and thus plant pathogens possess high number of chemoreceptors (Matilla and Krell, 2018). Around half of the animal pathogens possess chemosensory pathways and within this group, approximately 17 chemoreceptors are present in their genomes (Lacal et al., 2010). Interestingly, the majority of the gastrointestinal pathogens have chemosensory signaling genes that allow them to sense the non-friendly environment of the gastrointestinal tract (Matilla and Krell, 2018).

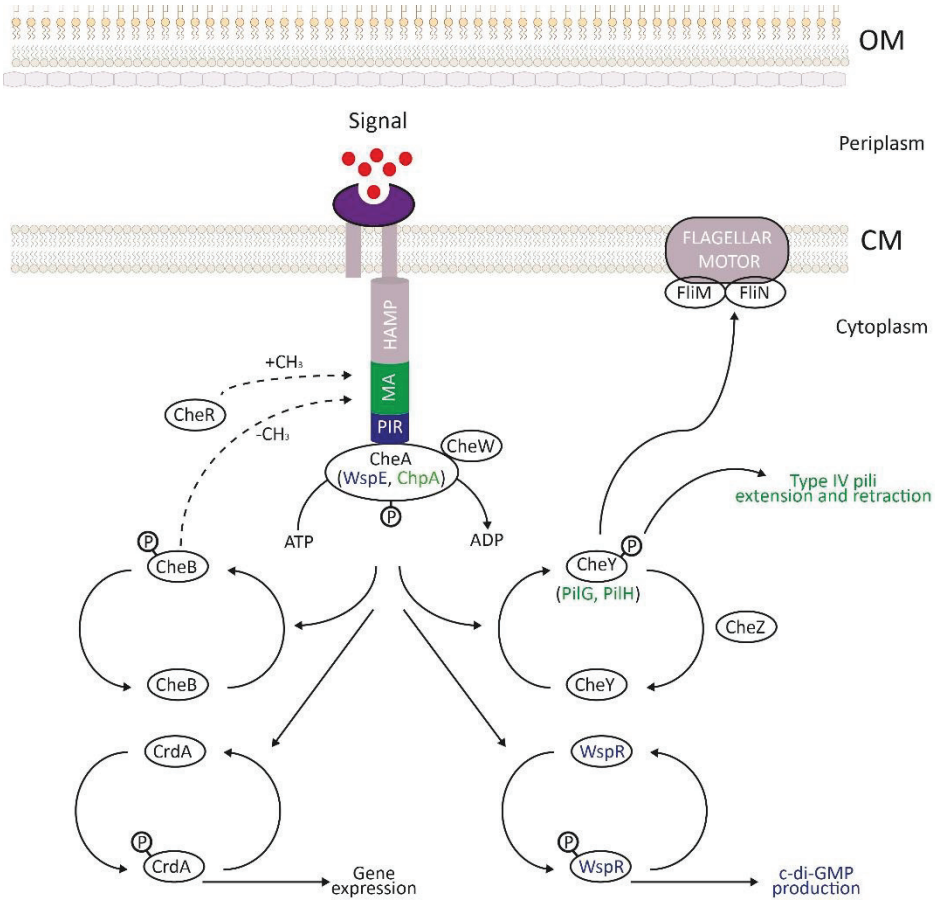

Fig. 14. Schematic representation of a chemosensory pathway. The signal is recognized by the receptor that promotes the autophosphorylation of CheA. CheA is able to phosphorylate two target proteins, CheB and CheY. CheY regulates the flagellar machinery and CheB participates in the adaptation of the chemosensory pathway together with CheR by de/methylation of the receptor. The CheA homolog WspE phosphorylates the diguanylyl cyclase WspR that increases the c-di-GMP concentration. The CheA homolog ChpA phosphorylates the CheY –like PilG and PilH regulating bacterial twitching through type IV pili. In M. xhantus, a CheA homolog phosphorylates the CrdA transcription factor that induces gene expression for sporulation. Adapted from (Matilla and Krell, 2018).

B6. σ^{ECF}-mediated signaling

The σ^{ECF} factor subfamily constitutes one of the major signal transduction systems in bacteria. Phylogenetic analyses have defined 56 major and 32 minor σ^{ECF} factor subgroups (Staron et al., 2009; Huang et al., 2015), which can be found in the Microbial Signal Transduction (MiST2) database (http://mistdb.com/). Most bacteria possess multiple σ^{ECF} factors, the number of which correlates with large genome size and a complex lifestyle. Expression and/or activation of σ^{ECF} factors are tightly regulated and only occur in response to specific signals. The post-translational control of σ^{ECF} factors is usually carried out by an anti-σ factor, which interacts with the σ^{ECF} factor in the absence of the inducing signal avoiding the binding of the σ^{ECF} factor to the RNAPc (Paget, 2015)

B6.1. The anti-σ factor

Similar to the HK/RR pair of two-component systems, the σ^{ECF}-dependent signaling pathway usually involves the σ^{ECF} and its cognate anti-σ factor, and the genes encoding these proteins normally co-transcribe, which maintains the stoichiometry and avoid uncoupled units (Österberg et al., 2011). Anti-σ factors share little primary sequence homology but share a

structural motif of about 80-90 aminoacids termed the anti-σ domain (ASD), which is the domain that binds the σ^{ECF} factor occluding the RNAPc binding determinants (Campbell et al., 2003; Campbell et al., 2007). Bioinformatic analyses have identified ASD-like domains in genes co-transcribed with σ^{ECF} factors for approximately 50% of the different σ^{ECF} subgroups (Campbell et al., 2007; Staron et al., 2009; Huang et al., 2015). Most of these proteins are single-pass transmembrane proteins with the ASD in the N-terminal cytosolic domain and a sensor domain in the C-terminal periplasmic domain. σ^{ECF} factors coupled to transmembrane anti-σ factors usually respond to signals sensed in the periplasm or in the outer membrane. The periplasmic domains of anti-σ factors vary considerably among different σ^{ECF} subgroups suggesting that they have diverged to recognize different signals (Staron et al., 2009; Huang et al., 2015). Over 30% of anti-σ factors with an ASD contain a Zn^{2+}-binding motif within the ASD known as ZAS domain (from zinc-dependent anti-sigma) (Campbell et al., 2007; Staron et al., 2009; Huang et al., 2015). These anti-σ factors are usually cytosolic proteins that sense redox changes and cytosolic stress.

Co-crystal structures have been solved for four σ^{ECF} factors from different subgroups bound to their cognate anti-σ factor, three cytoplasmic membrane anti-σ factors (RseA, RskA and RsiW) and a cytoplasmic anti-σ factor (ChrR) (Fig. 15) (Sineva et al., 2017)). The ASD fold is highly conserved among them, but, interestingly, the way in which each anti-σ factor binds to the σ^{ECF} factor varies considerably (Fig. 15). RseA, ChrR, and RskA bind between the σ^2 and σ^4 domains of the σ^{ECF} factor and dock on the cognate σ factor in different manners, while RsiW wraps the σ^{ECF} factor occluding the promoter-binding surface of the σ^4 domain (Fig. 15). Therefore, although the conformation of the ASD is highly conserved among anti-σ factors, the mechanism of inhibition of σ factor activity seems to be unique.

In addition to the ASD family, approximately 25% of the σ^{ECF} subgroups are co-transcribed with a second class of transmembrane anti-σ factor that contain a short (less than 50 aminoacids) N-terminal cytoplasmic domain that may act as an inhibitor of the σ factor (Staron et al., 2009; Huang et al., 2015). CnrY from *Alcaligenes eurotrophas*, belongs to this class of anti-σ factors and co-crystal structure showed that CnrY wraps across the σ^{ECF} factor blocking the sites where the β subunit of the RNAPc binds, which is analogous to the way of inhibition performed by the ASD anti-σ factor RsiW (Fig. 15) (Sineva et al., 2017).

A predicted anti-σ factor has not been identified in 20% of the σ^{ECF} subgroups (Staron et al., 2009; Huang et al., 2015). Some of these σ^{ECF} factors are primarily regulated at the level of expression (Sineva et al., 2017). In other subgroups, the σ^{ECF} factor itself contains additional domains that could be regulatory (Staron et al., 2009; Huang et al., 2015). The details about how these regulatory domain senses signals and controls RNAPc interaction remain to be elucidated.

Interestingly, some ASD transmembrane anti-σ factors do not only have anti-σ but also pro-σ activity and are required for σ^{ECF} activity in inducing conditions (Mettrick and Lamont, 2009). The pro-σ activity seems to reside within the ASD cytosolic domain because expression of this domain alone induces σ^{ECF} activity independently of the presence of the inducing signal (Mettrick and Lamont, 2009; Bastiaansen et al., 2015a). It has been proposed that the N-tail can protect the σ^{ECF} from degradation and that this domain may be bound to the σ^{ECF}-RNAP holoenzyme during the transcription process (Braun et al., 2006), although this has not been demonstrated yet.

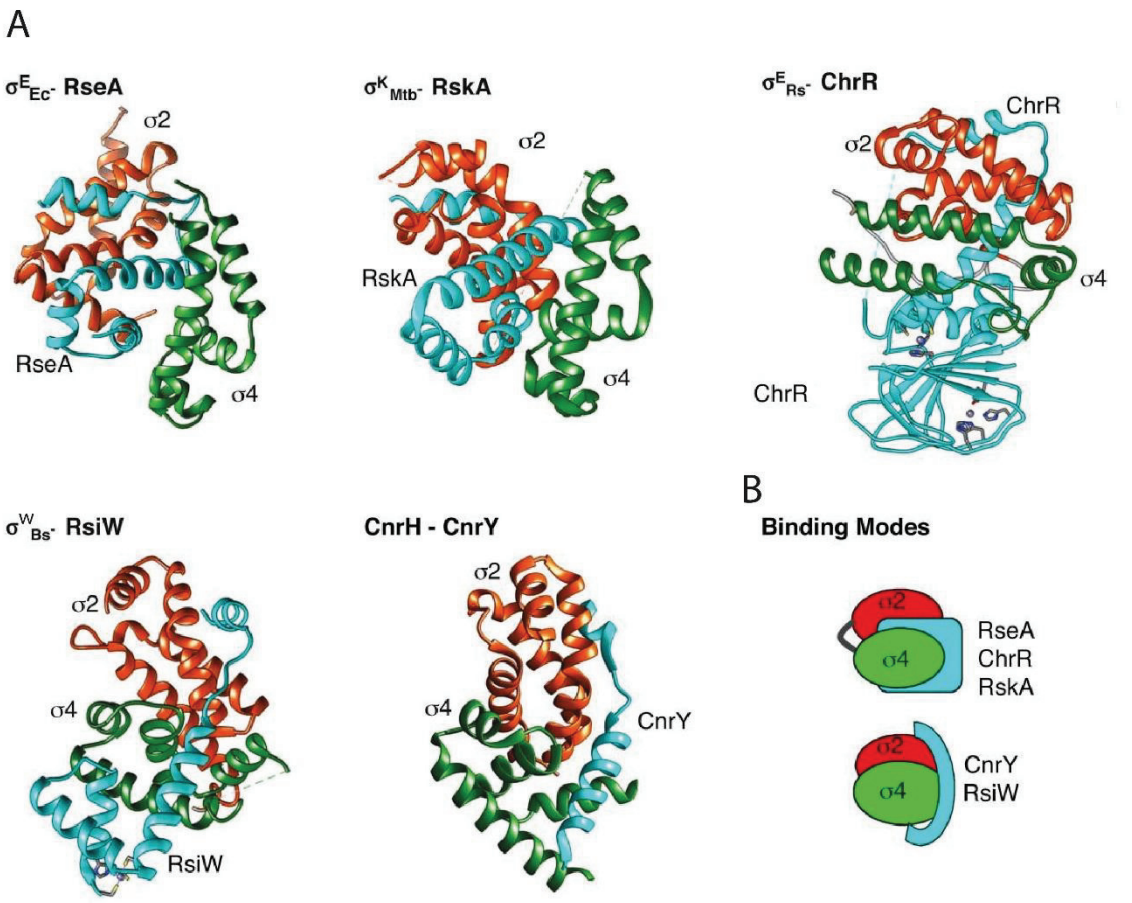

Fig. 15. Different ways of σ^{ECF}/anti-σ interaction. (A) Crystal structures of σ^{ECF}/anti-σ factors. Domains σ^2 and σ^4 of the σ^{ECF} are shown in red and green, respectively. The ASD of the anti-σ factors are shown in turquoise. RseA, ChrR and RskA bind between the σ^2 and σ^4 domains of the σ^{ECF} factor, RskA binds to σK blocking its DNA and RNAP binding sites. RseA and ChrR only block the RNAP binding site. The RsiW and CnrY anti-σ factors wrap the σ^{ECF} factor occluding the promoter-binding surface of the σ^4 domain. In the case of RsiW and CnrY, they bind to σ^W and CnrH respectively, wrapping it and keeping the DNA binding site of σ^2 domain blocked by the σ^4 domain. **(B)** Schematic representation of the two modes of σ^{ECF}/anti-σ interaction that inhibits the association of the σ^{ECF} factor with the RNAPc. Obtained from (Sineva et al., 2017).

B6.2. Signal sensing and σ^{ECF} factor activation

Most σ^{ECF} factors, except those that are only regulated at the level of transcription, are produced in an inactive state, either with an anti-σ factor or with a *cis*-acting inhibitory domain. This inhibitory interaction is disrupted in response to a signal, which is sensed directly by the anti-σ factor or via more complex signal transduction cascades. This produces the release and activation of the σ^{ECF} factor that can then associate with the RNAPc and trigger the transcription of responsive genes. Several major classes of activation mechanisms have been identified, including conformational changes by oxidation of cysteine residues, partner switching, regulated intramembrane proteolysis and cell-surface signaling.

a) Oxidation of cysteine residues - Redox sensing.

This mechanism of activation has been described for σ^{ECF} factors controlled by cytosolic anti-σ factors with ZAS domains. This domain binds Zn^{2+} and contains two cysteine residues that are required for the interaction with the σ^{ECF} (Fig. 16A). The σ^{ECF}/anti-σ factor interaction is

abrogated under cytoplasmic stress conditions in response to reactive electrophiles (i.e. diamides) or reactive oxygen species that produce the oxidation of the cysteine residues and the concomitant release of the Zn^{2+} ion and the σ^{ECF} factor (Fig. 16A). The best studied examples within this group include σ^{L}/RslA from *Mycobacterium tuberculosis* and σ^{R}/RsrA of *Streptomyces coelicolor* (Li et al., 2002; Zdanowski et al., 2006; Thakur et al., 2010). Variations to this scheme have been also described, as for example the ChrR anti-σ factor from *R. sphaeroides* that binds two Zn^{2+} ions in different part of the protein (one in the N-terminal part and the other in the C-terminal part) (Greenwell et al., 2011). The *B. subtillis* RsiW anti-σ factor has ZAS motif but curiously does not respond to oxidative stress (Sineva et al., 2017).

b) *Partner switching.*

Activation of σ^{ECF} and other alternative σ factors by partner switching systems (PSS) has been described in multiple organisms, but are highly abundant in Gram-positive bacteria (Bouillet et al., 2018). σ factor activation through this mechanism involves the participation of four main actors: the σ factor itself, an anti-σ factor with kinase activity, an anti-σ factor antagonist (also referred to as anti-anti-σ factor) and a PP2C-type phosphatase (Fig. 16B) (Bouillet et al., 2018). Although PSS always comprises these four components, the domain organization of the partners is highly diverse. While the anti-σ antagonist usually remains as a one-domain protein, the anti-σ factor can be associated with receiver domain of typical response regulator, PP2C-type phosphatase domain or domains of unknown function, while the phosphatase often associates with a signaling domain (Bouillet et al., 2018). Moreover, activation of the PPS can be controlled by other sensing modules that acts upstream of the PSS, like two component systems or complex pathways as the stressosome or chemosensory systems (Bouillet et al., 2018). In uninduced conditions, the anti-σ factor is bound to the σ factor and the anti-σ factor antagonist is phosphorylated through the kinase activity of the anti-σ factor. When the specific signal arises, the PP2C-type phosphatase gets active and dephosphorylates the anti-σ factor antagonist. The unphosphorylated anti-σ factor antagonist interacts with the anti-σ factor, which has higher affinity for this protein than for the σ factor and this produces the release and activation of the σ factor. In the Gram-positive bacteria *B. subtillis*, σ^{F} is regulated by this system. In this case, the anti-σ factor antagonist is SpoIIAA, the anti-σ factor is SpoIIAB and the phosphatase is known as SpoIIE. It is activated during the first stage of sporulation being crucial in this process. Other examples of σ factors regulated by PSS are σ^{B} from Bacillales or σ^{S} from *Shewanella oneidensis*. σ factors regulated by PSS are involved in virulence, biofilm formation, exoprotein production or stress response.

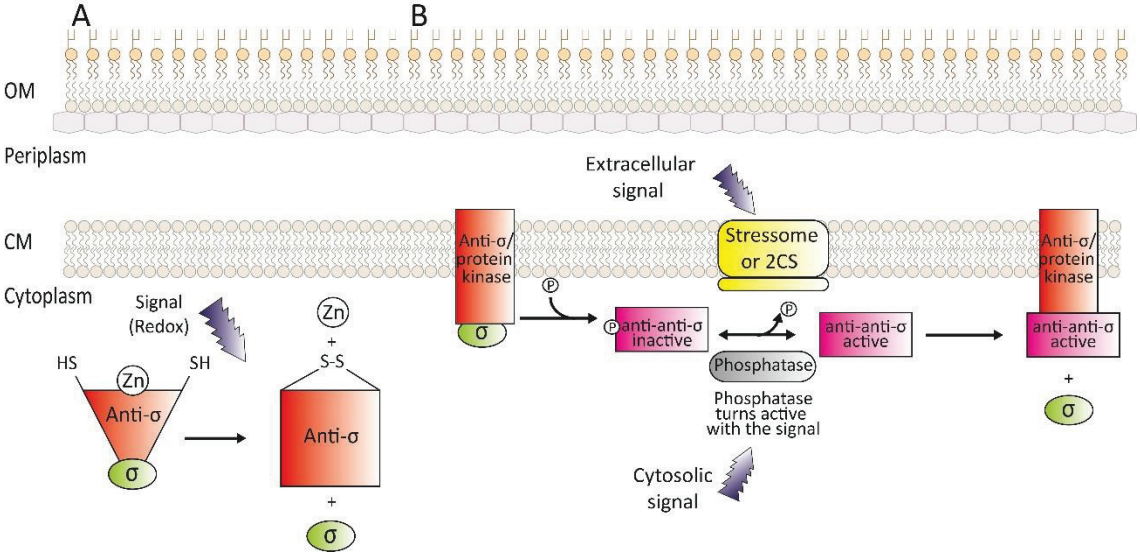

Fig. 16. σ^{ECF} factors activation by conformational changes. (A) Redox sensing by an anti-σ factor. The reduced anti-σ factor (red triangle) keeps the σ^{ECF} (green) inactive. The anti-σ factor senses redox signals that produce the oxidation of its cysteine groups that produces the release of the Zn^{2+} from the anti-σ (red square) and the σ^{ECF}, which turns active. **(B)** Partner switching. The anti-σ factor/ protein kinase (red rectangle) keeps the σ^{ECF} inactive. Moreover, the kinase domain phosphorylates the anti-anti-σ factor (pink rectangle) that remains inactive. The presence of the signal produce the dephosphorylation of the anti-anti-σ factor by a phosphatase (grey oval). The signal can be previously sensed by a stressome or a TCS (yellow). Active anti-anti-σ factor competes with the σ^{ECF} for the interaction with the anti-σ factor that produces the release of the σ^{ECF} and its activation. Adapted from (Mascher, 2013; Sineva et al., 2017; Bouillet et al., 2018).

c) *Regulated proteolysis*.

Regulated proteolysis of the anti-σ factors is one of the best-understood mechanisms of σ^{ECF} factor activation particularly for ASD transmembrane anti-σ factors that respond to signals sensed in the periplasm or the cell envelope. By this mechanism, the anti-σ factor is completely degraded in response to the signal by the sequential action of two periplasmic proteases (Fig. 9) followed by cytoplasmic proteases (Barchinger and Ades, 2013). The signal triggers the cleavage of the first protease in the periplasmic domain of the anti-σ factor, a process that allows the second protease to cleave the transmembrane region (Fig. 9). This releases the ASD cytosolic domain of the anti-σ factor bound to the σ^{ECF} factor in the cytoplasm, a fragment that is degraded by cytoplasmic proteases freeing the σ^{ECF} factor (Barchinger and Ades, 2013). This layout of the proteolytic cascade is conserved in diverse bacteria; however, some details differ, as for example how the signal that triggers the first proteolytic step is detected.

The *E. coli* σ^{E} and its homologue the *P. aeruginosa* σ^{AlgT} are the best studied examples of σ^{ECF} factors activated by regulated proteolysis of its cognate anti-σ factor (RseA and MucA, respectively) (Fig. 17). Activation occurs in response to periplasmic stresses that disrupt the outer membrane and these σ factors trigger expression of functions to relieve the bacterium from the stress. Two signals, unfolded outer membrane porins (OMPs) and intermediates in LPS biosynthesis trigger the proteolytic cascade responsible for degradation of RseA/MucA (Fig. 17) (Cezairliyan and Sauer, 2009; Lima et al., 2013). *B. subtilis* σ^{V} is also activated by regulated proteolysis of their cognate transmembrane anti-σ factors RsiV but in this case degradation of RsiV is triggered by binding of lysozyme to RsiV, which makes RsiV accessible to the first protease (Hastie et al., 2016).

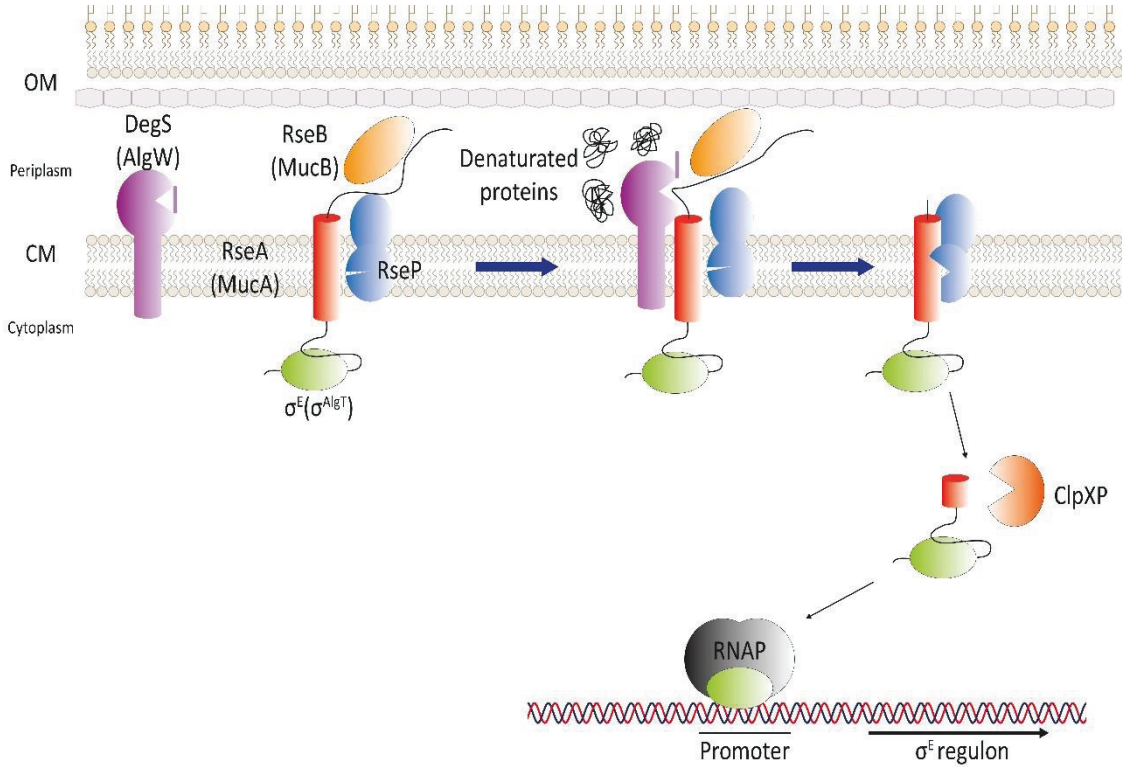

Fig. 17. Activation of *E. coli* σ^E and *P. aeruginosa* σ^{AlgT} by RIP. Activation of σ^E (σ^{AlgT}) by cell membrane stress due to accumulation of unfolded proteins (black). Unfolded proteins bind to the protease DegS (AlgW) activating it. RseA (MucA) has a proteolytic inhibitor called RseB (MucB) that is removed by an unknown mechanism. Then DegS (AlgW) produces the site-1 cleavage over RseA (MucA). This generates the substrate of the site-2 protease called RseP. RseP cuts RseA (MucA) in the intramembrane region releasing σ^E (σ^{AlgT}). The N-tail is degraded by ClpXP, then σ^E (σ^{AlgT}) is able to bind to the RNAPc and induce gene expression. Adapted from (Heinrich and Wiegert, 2009).

d) *Cell-surface signaling (CSS) systems.*

Several σ^{ECF}/anti-σ signaling pathways of Gram-negative bacteria have evolved to sense extracellular signals that do not necessarily have to enter into the cell to produce a response. This occurs via the functional, and often also genomic, association of the σ^{ECF}/anti-σ pair with a surface-exposed receptor located at the bacterial outer membrane. Together, these three proteins form a signal transfer system known as cell-surface signaling (CSS). The firsts CSS systems identified were the *E. coli* Fec and the *P. putida* Pup systems (Koster et al., 1994; Härle et al., 1995). Later, successive CSS systems have been studied in multiple Gram-negative bacteria, especially in *Pseudomonas* (Llamas et al., 2014). The CSS receptor belongs to a subfamily of the outer membrane TonB-dependent receptor (TBDR) family (Koebnik, 2005). These receptors are composed by 22 antiparallel β strands that form a β barrel that is inserted in the outer membrane. This β barrel forms a pore of 35-40 Å that is occluded by a globular plug domain that acts as a cork avoiding the entrance of unspecific molecules (Noinaj et al., 2010) (Fig. 18). In addition, CSS receptors have an N-terminal extension of about 80-90 amino acids located in the periplasm and known as the signaling domain (Koebnik, 2005; Noinaj et al., 2010) (Fig. 18). The C-terminal β barrel domain allow the passage of the CSS inducing signal while the N-terminal domain initiates the signaling cascade that leads to the activation of the CSS σ^{ECF} factor (Llamas et al., 2014). Transport requires the energy provided by the TonB-ExbB-ExbD system (Noinaj et al., 2010), hereby the name of TonB-dependent receptors.

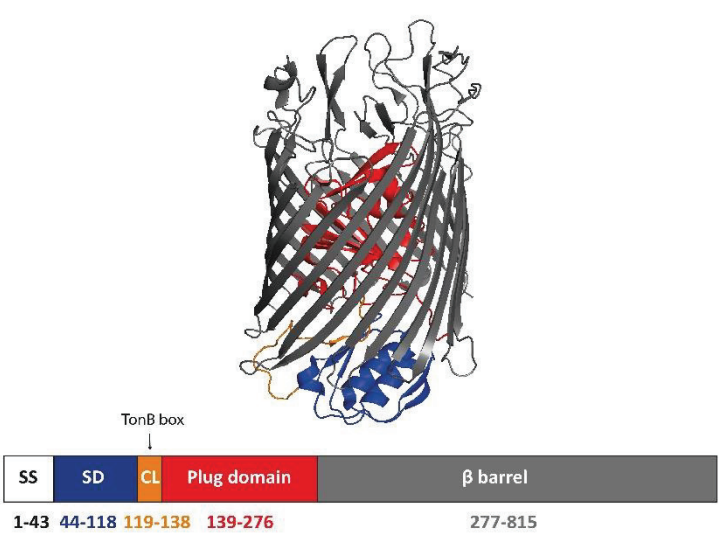

Fig. 18. Structure of the CSS TBDR FecA from *E.coli*. Representation of the mature tertiary structure of FecA receptor. The C-terminal β barrel domain formed by 22 anti-parallel β strands is shown in gray. The plug domain that blocks the β barrel pore is shown in red. The periplasmic connecting loop (orange) contains a 6 amino acids partially conserved known as TonB box where the TonB protein interacts providing the energy required for Fe-citrate transport. The signaling domain is shown in blue. Adapted from (Brillet et al., 2007).

Signaling occurs via the periplasmic interaction of the signaling domain of the CSS receptor with the C-terminal periplasmic domain of a transmembrane CSS anti-σ factor (Fig. 18) (Enz et al., 2003b). Recent analyses have shown that this interaction induces the regulated proteolysis of the CSS anti-σ factor (Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2015b; Bastiaansen et al., 2017). Remarkably, the first processing step of most CSS anti-σ factors occurs in the absence of the inducing signal by an enzyme-independent chemical reaction known as N-O acyl shift (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b) (Fig. 19). This produces the scission of the protein in two functional N- and C- domains that interact with each other in the periplasm and are both needed for proper anti-σ factor function (Bastiaansen et al., 2015a). The biological significance of this process is at present unknown since blocking this cleavage diminishes but does not inhibit the function of the protein and functional CSS anti-σ factors that do not undergo this processing step has been also identified (Bastiaansen et al., 2015a). In response to the inducing signal, both domains of the CSS anti-σ factors are proteolytically processed. The C-domain of the anti-σ seems to be degraded while the N-domain is subjected to RIP (see section B6.2.). Two proteases involved in the regulated proteolysis of CSS anti-σ factors has been identified, the membrane-anchored periplasmic carboxy-terminal protease Prc and the transmembrane protease RseP (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). RseP mediates the site-2 cleavage of the RIP of CSS anti-σ factors (Fig. 20), but the role of the Prc protease is still not clear (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). Prc produces the site-1 RIP cleavage of a unique *Pseudomonas* CSS σ^{ECF}/anti-σ hybrid protein (Bastiaansen et al., 2014). However, while involved, Prc is not the site-1 protease for classical (and most common) CSS anti-σ factors that are not fused to σ^{ECF} domains (Bastiaansen et al., 2015a). In these pathways, Prc seems to be involved in maintaining the levels of the anti-σ factor at a minimum in non-inducing conditions. Analysis of the CSS proteolytic cascade and that of the function of the Prc protease has been the subject of **chapter 5** of this Thesis.

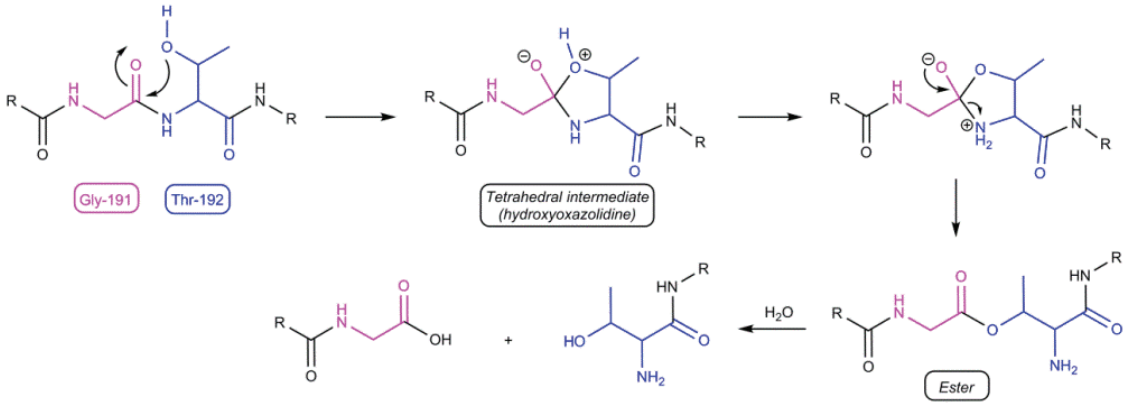

Fig. 19. Scheme of peptide bond hydrolysis initiated by an N-O acyl shift in the FoxR anti-σ factor from *P. aeruginosa*. The scheme of the autoproteolytic process of the FoxR anti-σ factor protein from th P.aeruginosa Fox CSS system, which undergoes self-cleavage between residues Gly-191 and Thr-192. The cores of the residues involved in the acyl shift have been coloured for clarity. The two R labels indicate the remainders of the protein chain. Obtained from (Bastiaansen et al., 2015b).

Proteolysis of CSS anti-σ factor results in the liberation and activation of the CSS σ^{ECF} factor that is then free to interact with the RNAPc and promote transcription of target genes. Several (although not all) CSS σ^{ECF} factors require the cytosolic ASD of its cognate anti-σ factor to function (Fig. 20B) (Llamas et al., 2014), and this fragment can be detected in the bacterial cell upon signal sensing (Bastiaansen et al., 2015a). The CSS σ^{ECF} regulon include genes to response to the CSS inducing signal, including the one encoding the CSS receptor itself (Llamas et al., 2014). This increases the amount of the receptor in the outer membrane and therefore the capacity of the bacterium to respond to and to transport (when required) the CSS signal.

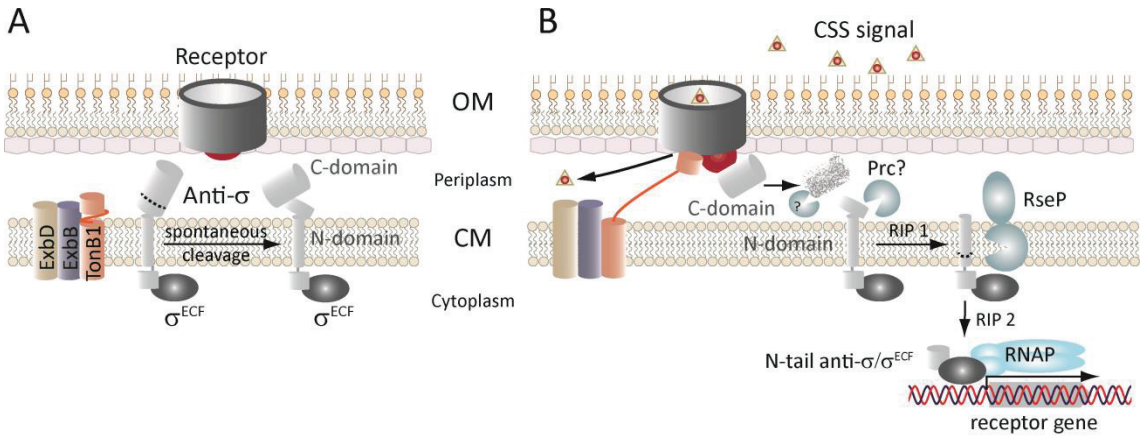

Fig. 20. Model of the complex proteolytic cascade controlling CSS activation based on studies on *P. aeruginosa*. (A) The receptor, anti-σ factor and σ^{ECF} of the CSS system are shown. Prior to signal recognition, the anti-σ factor undergoes a spontaneous cleavage that occurs by a protease-independent reaction in a conserved Gly-Thr (GT) cleavage site located in the periplasmic domain of the anti-σ factor. This cleavage produces two functional N- and C-domains that interact with each other in the periplasm and are both required for proper anti-σ factor function. **(B)** Signal recognition by the receptor produces the interaction of the signaling domain of the receptor (in red) with the C-domain of the anti-σ factor. This event triggers the degradation of the anti-σ factor C-domain by a still unknown mechanism, and the targeted proteolysis of the N-domain via RIP by the action of (at least) the Prc and RseP proteases. RseP

cleaves in the transmembrane domain of the anti-σ factor and produces the release of the σ^{ECF} into the cytoplasm likely bound to the N-tail domain of the anti-σ factor that can either be degraded or remain bound to the σ^{ECF} in the σ^{ECF}-RNAP complex (in case anti-σ factors with pro-σ activity). σ^{ECF} activates transcription of the CSS receptor gene and other genes involved in the response to the CSS signal. The TonB-ExbBD complex provides the energy required for the transport of the inducing signal to the CSS receptors that next to a function in signal transduction also have a function in the uptake of the inducing signal. OM, outer membrane; CM, cytoplasmic membrane; RNAP, RNA polymerase. Adapted from (Bastiaansen et al., 2014) with the findings from (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b).

B6.3. Function of σ^{ECF} factors

Among others, σ^{ECF} factors control bacterial functions as important as cytosolic and periplasmic stress responses, sporulation, iron uptake, bacterial competition and virulence. However, because σ^{ECF} signaling is a relative recent field of research and many of these regulatory proteins have not been studied yet, additional function are expected.

a) Stress responses.

Almost all bacteria are exposed during their life to any type of stress that has to be managed to survive. σ^{ECF} factors induce expression of genes that permits the response to handle with the stress. Bacteria try to maintain a reducing cytoplasm that permits to minimize deleterious action of reactive oxygen species. In the actinomycetes *S. coelicolor* the pair σ^{R}/RsrA regulates disulphide stress in the cytosol (Paget et al., 2001). Upon activation, σ^{R} induces the transcription of genes able to modulates the cellular thiol-disulphide status such as thioredoxin and glutaredoxin (Ritz and Beckwith, 2001; Li et al., 2003). Bacteria often suffer envelope stress since the cell envelope is the most exposed element to the hostilities of the environment. Most bacteria contain a specific σ^{ECF} factor that respond to this type of stress, which are homologues to the *E. coli* σ^{E} factor (Fig. 17). The σ^{E} regulon contains genes that encode proteases and chaperones to reduce the incorrect folding of outer membrane proteins that occurs under envelope stress (Rhodius et al., 2006; Rhodius and Mutalik, 2010). This regulon also includes genes that modulate the biosynthesis of phospholipids, lipopolysaccharides, fatty acids or lipoproteins (Hayden and Ades, 2008). There are σ^{E} homologs in numerous bacteria such as *S. typhimurium*, *Pseudomonas* spp or *Yersinia enterocolitica*. They control the response to cold shock, oxidative stress, osmotic stress, entrance to stationary phase (Rowley et al., 2006). In the Gram-positive *B. subtillis*, σ^{W} also plays a main role in envelope stress response by triggering expression of 60-90 genes implicated in resistance to antimicrobial agents by the expression of cytosolic enzymes that degrade them. σ^{W} also defends the cell by modifying the membrane against antibiotics and bacteriocins through the expression of the *fabHa-fabF* fatty acid biosynthesis operon (Helmann, 2016).

b) Sporulation.

Stressful situations like nutrient starvation can produce the bacterial sporulation in some species. In some organisms such as *B. subtillis*, this process is mainly mediated by two σ^{ECF} factors cascades initiated by σ^{H} and σ^{A} [reviewed in (Kroos, 2007)]. σ^{H} induces transcription of σ^{F} in the early smaller forespore. σ^{F} induces expression of several genes that participate in the formation of the small forespore and engulfment. Moreover, σ^{F} transcribes σ^{G} that is needed in the expression of the genes in the late small forespore. σ^{A} induces transcription of σ^{E}, which produces the expression of the numerous sporulation genes for engulfment and formation of the spore cortex. σ^{E} also induces expression of σ^{K}, which produces the expression in the late phase of mother cell (Kroos, 2007).

c) Iron uptake.

Iron is essential for bacterial survival because is used as a cofactor in multiple metabolic reaction such as DNA synthesis, the tricarboxylic acid cycle or being part of catalases and cytochromes (Andrews et al., 2003). Iron is mostly present in the environment in the oxidized ferric form (Fe^{3+}) that is hardly accessible to bacteria. In reduced and anaerobic environments, the soluble ferrous form (Fe^{2+}) is the most abundant. However, Fe^{2+} activates the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-$) that leads to the formation of hydroxyl radicals, which highly toxic for bacteria (Wandersman and Delepelaire, 2004). Therefore, maintenance of iron homeostasis is crucial for bacterial survival.

To fulfill their requirements for iron, bacteria produce and secrete low molecular weight iron chelating compounds called siderophores that bind ferric iron in the extracellular medium with high affinity (Wandersman and Delepelaire, 2004). Upon Fe^{3+} chelation, the Fe^{3+}-siderophore complex is recaptured by the bacterium through a specific outer membrane TBDR (Noinaj et al., 2010). Several bacteria are also able to transport and use siderophores produced by other bacteria or fungi (referred to as xenosiderophores) as source of iron (Ratledge and Dover, 2000), which provides an advantage in competing environments. And several pathogens use iron carriers from the host (i.e. transferrin, lactoferrin, heme or hemoglobin) to get iron (Wandersman and Delepelaire, 2004). A subgroup of σ^{ECF} factors known as iron starvation (IS) σ^{ECF} factors (Leoni et al., 2000) plays a key role in the regulation of the uptake of these different iron carriers. IS σ^{ECF} factors are usually activated by CSS cascades involving a CSS receptor and a CSS anti-σ factor (Fig. 20). Expression of these three proteins is often regulated by the ferric uptake regulator (Fur), which in iron rich environments binds Fe^{2+} and the DNA promoter region inhibiting transcription and in iron limiting conditions allows gene expression (Escolar et al., 1999). However, although produced under iron starvation, the IS σ^{ECF} factor is not active because an inhibitory interaction with its co-transcribed anti-σ factor occurs in this situation and the CSS receptor is only basally expressed (Fig. 20A) (Llamas et al., 2014). Only when the inducing iron carrier is present in the medium the CSS pathways is activated, which increases the production of the CSS receptor (Fig. 20B) (Llamas et al., 2014). In this way, the bacterium control both the import of iron and the production of the large outer membrane receptor needed for the transport of the iron carrier, which is an energetically costly process that only occurs when the iron carrier is present (Fig. 20B).

d) Bacterial competition.

Bacteria usually cohabit with other bacterial species in fierce competence for nutrients and resources. Bacteria produce bacteriocins that are cytotoxic secreted proteins able to kill bacteria of the same or closely related species through a range of mechanisms such as degradation of cellular DNA or RNA, inhibition of peptidoglycan synthesis or formation of pores in the cytoplasmic membrane. Bacteriocins secretion provides colonization advantages to the immune producing strains. *P. aeruginosa* produces a type of bacteriocins known as pyocins that can kill other pseudomonads (Michel-Briand and Baysse, 2002; Parret and De Mot, 2002), and contains a σ^{ECF} factor that triggers expression of these toxins (Llamas et al., 2008). This σ^{ECF} factor is located next to a CSS receptor gene that is likely involved in xenosiderophore uptake and it has been proposed that the bacterium uses this system to 'steal' siderophores from other bacteria simultaneously killing them (Llamas et al., 2008). The finding that a CSS can influence bacteriocin production suggests that bacteria not only use CSS to enhance its ability to acquire exogenous iron carriers, but also to modulate its killing activity on competing strains.

e) Virulence.

σ^{ECF} factors are important signaling proteins for bacterial pathogens during infection. A primary role of these proteins in virulence is related with their function in the regulation of iron

uptake. Iron withholding by the host becomes the first line of defense against a bacterial infection(Cassat and Skaar, 2013; Parrow et al., 2013). The low accessibility to iron due to the sequestration of iron by host molecules drastically reduces the chances of a pathogen to produce a successful infection. In fact, diseases like hemochromatosis or thalassemia that affects the ability of the host to produce iron withholding make the patient more accessible to pathogens (Bullen et al., 1991). To counteract the host iron sequestration, pathogens produce siderophores, which are considered important bacterial virulence determinants, as for example pyoverdine from *P. aeruginosa* (Lamont et al., 2002), mycobactin from *Mycobacterium tuberculosis* (Quadri et al., 1998) or anthrabactin and anthrachelin from *Bacillus anthracis* (Cendrowski et al., 2004) (Ratledge and Dover, 2000). Moreover, several pathogens are able to use the host molecules heme and haemoglobin, which represents the biggest source of iron in mammals, to obtain iron (Runyen-Janecky, 2013; Choby and Skaar, 2016). More successfully pathogens can use transferrin, lactoferrin, haptoglobin or other iron source for survival. Recognition and uptake of these host iron carriers often occurs through σ^{ECF}-mediated signaling.

Besides the regulation of iron uptake during infection, several σ^{ECF} factors have a key role in virulence by sensing and responding to host cues, which allow bacterial survival. This is the case of the σ^{E} factor of several pathogens (i.e. *Salmonella enterica* sv. Typhimurium, *Y. enterocolitica*, *Vibrio cholera*, *Haemophilus influenza* or *M. tuberculosis*), which responds to the oxidative stress encountered inside macrophages and promotes transcription of *htrA*, a gene required for oxidative stress resistance and bacterial survival within macrophages (Kazmierczak et al., 2005; Rowley et al., 2006; Fontan et al., 2008). Moreover, several σ^{ECF} factors trigger the transcription of virulent determinants as for example σ^{SigP} from *Porphyromonas gingivalis*, which regulates the type 9 secretion system (Nakayama, 2015; Kadowaki et al., 2016) or σ^{HrpL} factor that regulates the virulent type III secretion system (T3SS) in the plant pathogens *Erwinia amylovora*, *Pseudomonas syringae* and other phytopathogens (Mole et al., 2007; Cunnac et al., 2009; McNally et al., 2012). In the case of the *M. tuberculosis*, one of the σ^{ECF} factors related to virulence is σ^{C}, which is in charge of transcription of the *senX3-regX3* TCS that induces transcription of more than 50 genes involved in *Mycobacterium* virulence (Parish et al., 2003; Sun et al., 2004). In the human pathogen *P. aeruginosa*, signaling through σ^{ECF} factors is highly important during the infectious process as is described below. The importance of the σ^{ECF}-mediated signaling in bacterial pathogenicity and the absence of σ^{ECF}-like proteins in mammals makes them potential targets for drug development.

C. Signaling through σ^{ECF} factors and CSS systems in *Pseudomonas*

Bacteria of the genus *Pseudomonas* are widely distributed in the environment being found in soil and water and as a pathogen of both plants and animals, including humans (Palleroni, 2010). The genus includes bacteria that live in the plant rizosphere as *P. putida* and *Pseudomonas fluorescens*. They are plant growth promoters and they are also implicated in the root protection against pathogens working as a biocontrol agent (Espinosa-Urgel et al., 2002; Wu et al., 2011; Bernal et al., 2017). Interestingly, members of the same genus can be plant pathogens. This is the case of *Pseudomonas syringae* pathovars that can infect nearly every cultivated or wild plant (Mansfield et al., 2012). The opportunistic pathogen *P. aeruginosa* is highly versatile being able to live in numerous niches. *P. aeruginosa* is an opportunistic pathogen that infects the lungs of cystic fibrosis patient producing pneumonia. *P. aeruginosa* also generates bacteremia in immune system depressed patients as cancer, HIV, or severe burn

patients. Its infection usually starts with an acute infection that can develop to a chronic infection.

In general, *Pseudomonas* bacteria have large number of regulatory genes in comparison to the size of their genomes [e.g. 10% of the *P. aeruginosa* genome are gene regulators (Stover et al., 2000)]. This includes a high number of σ^{ECF} factors (Table 1) (Llamas et al., 2014).

Table 1. σ^{ECF} factors and CSS systems in *P. aeruginosa* and *P. putida* [adapted from (Llamas et al., 2014)].

***P. aeruginosa* PAO1**[a]			***P. putida* KT2440**[a]			
σ^{ECF} factor	**anti-σ factor**	**Receptor**	**σ^{ECF} factor**	**anti-σ factor**	**Receptor**	**Function**[b]**.**
PA0149	PA0150	PA0151	PP4608	PP4607	PP4606	Metal uptake (Llamas et al., 2008)
PA0472 (σ^{FiuI})	PA0471 (FiuR)	PA0470 (FiuA)	PP0352	PP0351	PP0350	Ferrichrome-mediated iron uptake (Llamas and Bitter, 2006)
PA0675 (σ^{VreI})	PA0676 (VreR)	PA0674 (VreA)	-	-	-	Virulence (Llamas et al., 2009) **(chapter 2 & 3)**
PA0762 (σ^{AlgT})	PA0763 (MucA)	-	PP1427	PP1428		(Mathee et al., 1997)
PA1300	PA1301	PA1302	-	-	-	Heme sensing **(chapter 4)**
PA1351	-	-	PP4553	-	-	Unknown
PA1363	PA1364	PA1365	-	-	-	Siderophore-mediated iron uptake (putative)
PA1776 (σ^{SigX})	-	-	PP2088	-	-	Virulence and biofilm (Gicquel et al., 2013; Blanka et al., 2014)
PA1912 (σ^{FemI})	PA1911 (FemR)	PA1910 (FemA)	PP3577 PP3086	PP3576 PP3086	PP3575 PP3084	Mycobactin-mediated iron uptake (Llamas et al., 2008)
PA2050	PA2051	PA2057/ PA2070	-	-	-	Metal uptake (Llamas et al., 2008)
PA2093	PA2094	PA2089/ PA2090	-	-	-	Siderophore-mediated iron uptake (Llamas et al., 2008)
PA2387 (σ^{FpvI})	PA2388 (FpvR)	PA2398 (FpvA)	PP4208	PP3555	-	Pyoverdine-mediated iron uptake (Beare et al., 2003)
PA2426 (σ^{PvdS})	PA2388 (FpvR)	PA2398 (FpvA)	PP4244 (σ^{PfrI})	-	-	Pyoverdine synthesis and virulence (Lamont et al., 2002)
PA2468 (σ^{FoxI})	PA2467 (FoxR)	PA2466 (FoxA)	PP0162	PP0161	PP0160	Ferrioxamine-mediated iron uptake (Llamas and Bitter, 2006)
PA2896 (σ^{SbrI})	PA2895 (SbrR)	-	-	-	-	Swarming motily and biofilm formation (McGuffie et al., 2015)
PA3285	-	-	-	-	-	Unknown
PA3410 (σ^{HasI})	PA3409 (HasS)	PA3408 (HasR)	-	-	-	Heme uptake (Ochsner et al., 2000) **(chapter 4)**
PA3899 (σ^{FecI})	PA3900 (FecR)	PA3901 (FecA)	PP4611	PP4612	PP4613	Citrate-mediated iron uptake (Banin et al., 2005)

P. aeruginosa PAO1[a]			*P. putida* KT2440[a]			
σ^{ECF} factor	anti-σ factor	Receptor	σ^{ECF} factor	anti-σ factor	Receptor	Function[b].
PA4896	PA4895	PA4897	-	-	-	Siderophore-mediated iron uptake, pyocins expression (Llamas et al., 2008)
-	-	-	PP0667	PP0668	PP0669	Siderophore-mediated iron uptake (putative)
-	-	-	PP0704	PP0703	-	Unknown
-	-	-	PP0865	PP0866	PP0867	Unknown
-	-	-	PP0994	-	-	Unknown
-	-	-	PP1008	PP1007	PP1006	Heme uptake (putative)
-	-	-	PP2192 (IutY)	PP2192 (IutY)	PP2193 (IutA)	Aerobactin-mediated iron uptake (Bastiaansen et al., 2014)
-	-	-	PP2888	PP2889	-	Unknown
-	-	-	PP3006	PP3005	-	Unknown

[a]ID numbers refers to the *Pseudomonas* Genome Database annotations (www.pseudomonas.com).
[b]The reference for analyzed systems is given.

Signaling through σ^{ECF} factors in *Pseudomonas* has been mainly studied in *P. aeruginosa*. In this bacterium, the most predominant function of σ^{ECF} factors is the regulation of iron or other metal uptake and thirteen out of the nineteen σ^{ECF} factors of *P. aeruginosa* are known or predicted to perform this function (Table 1) (Llamas et al., 2014). All these σ^{ECF} factors belong to the iron starvation (IS) group and are functionally and genomically associated with anti-σ factors and CSS receptors (Table 1). Activation of these σ^{ECF} factors usually occurs in response to iron carriers via a CSS cascade (Fig. 20) (Llamas et al., 2014). Most *P. aeruginosa* CSS systems resemble the archetypal *E. coli* iron-citrate Fec CSS system (Braun et al., 2006), including the Fox, Fiu and Fem systems that respond to the xenosiderophores ferrioxamine, ferrichrome and mycobactin, respectively (Fig. 21A) (Table 1) (Llamas et al., 2006; Llamas et al., 2008). However, important variations to this model have been identified in *Pseudomonas*. This includes the *P. aeruginosa* Fpv CSS system, which was the first CSS system analyzed in this bacterium. In contrast to archetypal CSS systems (Fig. 21A), the Fpv system controls the activity of not one but two σ^{ECF} factors (σ^{PvdS} and σ^{FpvI}) (Fig. 21B) and responds not to an exogenous signal such as xenosiderophores or iron-citrate but to a signal produced by the bacterium itself, the siderophore pyoverdine (Lamont et al., 2002; Beare et al., 2003). Moreover, *P. aeruginosa* contains CSS systems formed by, instead of one, two CSS receptors (Table 1) (Llamas et al., 2008) (Fig. 21C), which suggests that they can respond to different inducing signals. And in *P. putida* a unique CSS system formed by a hybrid σ^{ECF}/anti-σ protein (the IutY protein, Table 1) that contains both functions in a single polypeptide was recently identified (Fig. 21D) (Bastiaansen et al., 2014; Bastiaansen et al., 2017). The *P. putida* IutY hybrid protein is functional and responds to the xenosiderophores aerobactin via a signaling cascade initiated by the IutA CSS receptor (Bastiaansen et al., 2014). Signaling produces the targeted proteolysis of IutY, which separates the σ^{ECF} N-domain from the anti-σ C-domain (Bastiaansen et al., 2014; Bastiaansen et al., 2017). Identification and analysis of this system has established that CSS σ^{ECF} activation always requires the targeted proteolysis of the cognate anti-σ factor in response to the inducing signal (Fig. 20) (Llamas et al., 2014), changing the initial model that assumed that activation occurred via conformational changes of the CSS proteins.

Uncharacterized CSS systems of *P. aeruginosa* includes the Has and PA1300-PA1302 systems, which were earlier suggested to control heme uptake (Table 1) (Llamas et al., 2008; Cornelis, 2010). In case of the Has system, this suggestion was based on analyses of the HasAp hemophore and its co-transcribed CSS receptor HasR that showed that these proteins are involved in heme quelation and transport of the heme-hemophore complex, respectively (Ochsner et al., 2000; Smith and Wilks, 2015). However, the role of the *P. aeruginosa* HasR and that of the σ^{HasI}/HasR pair in signaling has not been analyzed and the prediction is based on the well characterized Has CSS system of *Serratia marcescens* (Biville et al., 2004). Regarding the PA1300-PA1302, *in silico* studies suggested some degree of similarity of the putative PA1302 receptor with heme receptors (Cornelis, 2010), although experimental analyses have not been performed yet. Both signaling systems have been analyzed in **chapter 4**.

IS σ^{ECF} and CSS systems are important for *P. aeruginosa* virulence, not only to supply iron to the bacterium during infection but also to trigger expression of virulence determinants. This is for example the case of σ^{PvdS} which induces expression of the virulence factors PrpL endoprotease and exotoxin A (Lamont et al., 2002). The PrpL endoprotease hydrolyses several host molecules like casein, lactoferrin, elastin or decorin, which produces subproducts that can be used by *P. aeruginosa* (Wilderman et al., 2001). The exotoxin A is a type II (T2SS) secreted *P. aeruginosa* toxin able to enter into host cells by binding to the KDEL receptors of the Golgi apparatus inducing apoptosis by activation of Caspase-8 and degradation of Mcl-1 protein that unleashes apoptosis (Hessler and Kreitman, 1997; Bleves et al., 2010; Michalska and Wolf, 2015). Prpl and Exotoxin A virulence have been studied using animal models showing their main role in virulence of *P. aeruginosa* (Hirakata et al., 1993; Wilderman et al., 2001). Both proteins are induced by σ^{PvdS} that as expected is also crucial for *P. aeruginosa* virulence. Other *P. aeruginosa* σ^{ECF} factor triggering expression of virulent determinants is σ^{Vrel} (Table 1). Among other genes, this σ factor promotes expression of a Type II secretion system (T2SS) involved in the secretion of a low molecular weight alkaline phosphatase and a two-partner secretion (TPS) system potentially involved in cell adhesion and pathogen dissemination (Llamas et al., 2009). In accordance, constitutive production of σ^{Vrel} increases *P. aeruginosa* virulence (Llamas et al., 2009). This σ^{ECF} factor, which is produced by the *vreAIR* operon that also encodes a CCS receptor-like protein (VreA) and an anti-σ factor (VreR) (Table 1), shares similarities with iron starvation σ^{ECF} factors but also important differences. This includes the nature of the receptor protein, which contains the signalling domain characteristic of CSS receptor but lacks the β barrel domain and seems to be located in the periplasm instead of in the outer membrane (Fig. 21E) (Llamas et al., 2009). This suggests that this receptor functions only in signalling but not in the uptake of the signal molecule. Moreover, recent analyses have shown that σ^{Vrel} is not expressed under iron but under inorganic phosphate (Pi) starvation, showing that its function is not related with iron (Faure et al., 2013). Intriguingly, the genes of the σ^{Vrel} regulon are also expressed in low Pi despite the fact that the σ^{Vrel} repressor, the anti-σ factor VreR, is also produced in this condition (Faure et al., 2013). The study presented in **chapter 2** was conducted to elucidate how the σ^{Vrel} activity is modulated in Pi starvation conditions. Pi starvation, a situation often encounters by pathogens in the host environment, has been longer known to induce a virulent phenotype in *P. aeruginosa* (Zaborin et al., 2009; Bains et al., 2012; Zaborin et al., 2012), and in **chapter 3** we have determined the contribution of σ^{Vrel} to this phenotype. Furthermore, presence of antibodies against σ^{Vrel}-regulated proteins in the serum of *P. aeruginosa*-infected patients and *in vivo* transcriptomics analyses suggested that σ^{Vrel} is activated *in vivo* during infection (Frisk et al., 2004; Chugani and Greenberg, 2007; Llamas et al., 2009), which has been analysed in **chapter 3**.

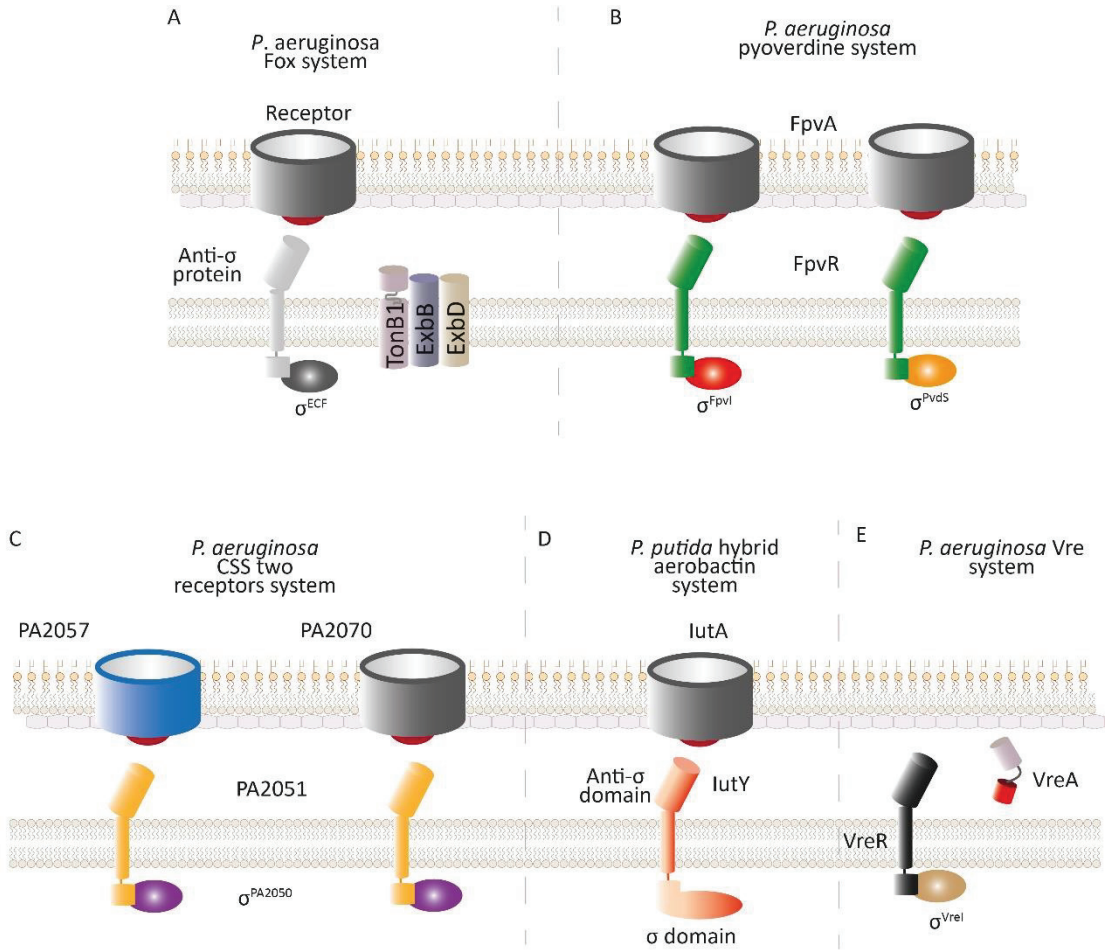

Fig. 21. Architectural variations of *Pseudomonas* CSS systems. (A) The archetypal CSS system represented by the *P. aeruginosa* Fox system is a three protein system formed by a CSS receptor (FoxA) that contains a β barrel domain inserted in the OM (dark grey) and a signaling domain in the periplasm (red), a transmembrane anti-σ factor (FoxR) (light grey) and a σ^{ECF} factor (σ^{FoxI}) (black). The TonB system provides the energy required for transport of the CSS inducing signal through the β barrel of the receptor. **(B)** The *P. aeruginosa* pyoverdine CSS system. The CSS receptor contains a β barrel domain (dark grey). The anti-σ factor FpvR (green) is able to bind two σ^{ECF} factors, σ^{FpvI} (red) and σ^{PvdS} (yellow). **(C)** The *P. aeruginosa* PA2050 CSS system. Two CSS receptors containing a β barrel domain (blue and dark gray) are able to induce activation of the σ^{PA2050} (purple) by inducing the process of PA2051 anti-σ factor (yellow). **(D)** The *P. putida* IutA/IutY hybrid CSS system. A TonB dependent receptor (gray) and a hybrid σ/anti-σ protein (orange) compose this CSS system. **(E)** The *P. aeruginosa* Vre system. A receptor-like protein is located in the periplasm (red and white). The VreR anti-σ factor (black) keeps σ^{VreI} (brown) inactive.

Pseudomonas species have a RpoE-like group σ^{ECF} factors (Fig. 17) (Staron et al., 2009). Some members of this group are σ^{AlgT}, σ^{SigX} or σ^{SrbI} from *P. aeruginosa* (Ramsey and Wozniak, 2005; Hoiby et al., 2010; Duchesne et al., 2013; McGuffie et al., 2015). They are activated in stress situations like presence of cell wall inhibitor antibiotics, hydrogen peroxide, unfolded proteins or osmotic stress (Chevalier et al., 2018). They respond in different ways for counteracting these stressful circumstances. *P. aeruginosa* can change from acute infection to a sessile lifestyle inducing a chronic infection, usually diagnosed in cystic fibrosis patients (Govan and Deretic, 1996). Bacteria usually loss the flagella and the fimbriae and secrete exopolimeric substances such as alginate. Alginate production is regulated by σ^{AlgT}, which produces the transcription of the alginate biosynthetic operon. Biofilms permits bacteria to live in

microcolonies in a sessile lifestyle and increase their capability of survival in presence of antibiotics and the oxidative stress generated by macrophages and neutrophils attack (Yu et al., 1996; Ramsey and Wozniak, 2005; Folkesson et al., 2012). Moreover, σ^{AlgT} downregulates the T3SS, during its virulence (Wu et al., 2004). Alginate overproduction strains have been found in most CF patients, for this reason, mucoid shift in *P. aeruginosa* is being deeply studied (Ciofu et al., 2012; Gaspar et al., 2013). Samples from patients have been analyzed and showed that most of *the P. aeruginosa* strains, which infect CF patients, have the σ^{AlgT} anti-σ factor MucA mutated (Boucher et al., 1997; Folkesson et al., 2012). This implicates the non-regulated action of σ^{AlgT}. In the case of σ^{SigX}, it shares 49% of similarity to σ^{W} from *B. subtillis* (Potvin et al., 2008). σ^{SigX} regulates expression of ion-gated channels, lipid A deacetylase, a glucose porin or the outer membrane porin known as OprF (Duchesne et al., 2013). As a general comment, σ^{SigX} participates in the outer membrane protein composition that is crucial for handling with cell envelope stress. σ^{SigX} also participates in virulence through the regulation of the assembly and expression of some effectors of the T3SS (Gicquel et al., 2013; Blanka et al., 2014). The new discovered σ^{SrbI} (McGuffie et al., 2015) regulates the expression of about 20 genes. One of these genes is *muiA* that is in charge of inhibition of alginate production and also inhibits swarming (Withers et al., 2014; McGuffie et al., 2015).

The plant pathogen *P. syringae* pv. tomato DC3000 has a σ^{ECF} factor known as HrpL that is implicated in virulence. HrpL regulates the T3SS. Concretely, HrpL regulates expression of multiple effectors injected through the T3SS. Moreover, HrpL regulates the expression of other genes that have a role in pathogenesis such as the coronatine biosynthesis genes (Ferreira et al., 2006; Zheng et al., 2012).

D. Targeting signalling pathways to develop new antimicrobial compounds

Bacterial infections have been one of the most important reasons of death along history. Until the discovery of antibiotics there were no efficient treatments against bacterial pathogens. The antibiotic era has permit the treatment of bacterial infections in a highly efficiently way reducing the human mortality drastically. However, the insensible use of antibiotics due to their abuse and the wrong use by reducing the time of treatment have generated the appearance of multiple antibiotic resistance pathogens. Therefore, the discovery of new antimicrobials and of new targets is needed to combat multi-resistant bacterial pathogens. A classical approach for drug development is to interfere with essential prokaryotic genes and functions (Hopkins, 2008). The selective pressure generated by the antibiotics that targets essential pathways is very high and usually leads to resistance. Targeting non-essential bacterial functions, as for example virulence, represents a promising strategy for the development of new antimicrobial compounds because this would reduce the selective pressure and thus the appearance of resistances. By reducing the pathogen virulence, the host immune system would be able to clear the infection. Because central to the infectious process is the ability of a pathogen to sense and respond to the host, inhibition of bacterial signalling cascades represents a promising target for antimicrobial development.

Both TCS and QS signalling systems have been proposed as promising targets for drug development (Kalia and Purohit, 2011; Tiwari et al., 2017). Besides controlling several virulence functions in bacteria, TCS also control some of the mechanisms that leads to antibiotic resistance as for example production of the efflux pumps used for bacteria to pump out antibiotics (Pages and Amaral, 2009; Sivaneson et al., 2011). Bacterial cell-cell communication is also critical for bacteria to know when a proper cell density for infection has been reached. The process of

interference or destruction of QS signals is known as “signal interference” or “quorum quenching” (Soukarieh et al., 2018), and enzymes able to degrade QS autoinducers or analogues to autoinducers that block sensing are promising molecules to block QS communication (Raina et al., 2009). Chemosensory pathways has been also proposed a potential targets for antimicrobial development because its essential role in the initial steps of infection (Matilla and Krell, 2018). Finally, because of the broad and important virulence-related functions controlled by σ^{ECF} factors, targeting these signalling pathways also represents a promising strategy to block the ability of bacteria to survive and colonize the host. This Thesis provides novel insights that potentially can be exploited for this purpose.

Objectives

OBJECTIVES

The main objective of this Thesis is to unravel the unknown features of the σ^{ECF}-mediated signalling in the human pathogen *P. aeruginosa,* by identifying σ^{ECF} factors that respond to host molecules and elucidating the molecular mechanism that triggers σ^{ECF} factors activation. The four main specific objectives of the Thesis are:

1. Elucidation of the mechanism that produce the expression and activation of the *P. aeruginosa* virulence regulator σ^{Vrel} in inorganic phosphate (Pi) starvation conditions. .
2. Determination of the *in vivo* conditions that triggers *P. aeruginosa* σ^{Vrel} activation.
3. Identification of *P. aeruginosa* σ^{ECF} factors that respond to host iron carriers (i.e. heme).
4. Role of the Prc protease and identification of new proteases involved in *Pseudomonas* σ^{ECF} factor activation.

Chapter 2

The activity of the *Pseudomonas aeruginosa* virulence regulator σ^{VreI} is modulated by the anti-σ factor VreR and the transcription factor PhoB

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ABSTRACT

Gene regulation in bacteria is primarily controlled at the level of transcription initiation by modifying the affinity of the RNA polymerase (RNAP) for the promoter. This control often occurs through the substitution of the RNAP sigma (σ) subunit. Next to the primary σ factor, most bacteria contain a variable number of alternative σ factors of which the extracytoplasmic function group (σ^{ECF}) is predominant. *Pseudomonas aeruginosa* contains nineteen σ^{ECF}, including the virulence regulator σ^{VreI}. σ^{VreI} is encoded by the *vreAIR* operon, which also encodes a receptor-like protein (VreA) and an anti-σ factor (VreR). These three proteins form a signal transduction pathway known as PUMA3, which controls expression of *P. aeruginosa* virulence functions. Expression of the *vreAIR* operon occurs under inorganic phosphate (Pi) limitation and requires the PhoB transcription factor. Intriguingly, the genes of the σ^{VreI} regulon are also expressed in low Pi despite the fact that the σ^{VreI} repressor, the anti-σ factor VreR, is also produced in this condition. Here we show that although σ^{VreI} is partially active under Pi starvation, maximal transcription of the σ^{VreI} regulon genes requires the removal of VreR. This strongly suggests that an extra signal, probably host-derived, is required *in vivo* for full σ^{VreI} activation. Furthermore, we demonstrate that the activity of σ^{VreI} is modulated not only by VreR but also by the transcription factor PhoB. Presence of this regulator is an absolute requirement for σ^{VreI} to complex the DNA and initiate transcription of the PUMA3 regulon. The potential DNA binding sites of these two proteins, which include a *pho box* and –10 and –35 elements, are proposed.

INTRODUCTION

Regulation of gene expression allows bacteria to adapt rapidly to alterations in their environment. This regulation occurs primarily at the level of transcription initiation by modifying promoter recognition of the RNA polymerase (RNAP) holoenzyme. The RNAP holoenzyme of bacteria comprises a five-subunit core enzyme (RNAPc; subunit composition $\alpha 2\beta\beta'\omega$) and a dissociable sigma (σ) subunit (Murakami and Darst, 2003). The σ factor contains most promoter recognition determinants and confers promoter specificity to the RNAP. All bacteria contain a primary σ factor (i.e. σ^{70}) that recognizes similar target promoter sequences and controls expression of genes required for general functions. Promoter recognition by σ^{70} is often modulated by transcription factors that either enhance or inhibit such recognition and therefore gene transcription (Ishihama, 2000; Martinez-Antonio et al., 2006). In addition, most bacteria contain several alternative σ factors that recognize alternative promoter sequences and activate expression of functions required only under specific circumstances (Ishihama, 2000). Therefore, the promoter recognition of the RNAP is modulated first by substitution of the σ subunit and secondly by the interaction with transcription factors.

The largest and most diverse group of bacterial alternative σ factors is the Group IV, which consists of the so-called extracytoplasmic function (ECF) σ factors (σ^{ECF}). These σ factors control expression of important bacterial functions such as stress responses, iron uptake and pathogenicity (Lonetto et al., 1994; Helmann, 2002; Bastiaansen et al., 2012; Mascher, 2013). Both expression and activation of σ^{ECF} are tightly regulated processes that usually occur in response to environmental signals. The post-translational control of σ^{ECF} is carried out by anti-σ factors that bind to and sequester the σ^{ECF}, which is only released and activated in the presence of an inducing signal. The functional unit of the σ^{ECF}-dependent signalling is therefore formed by the σ^{ECF} and its cognate anti-σ factor, and the genes encoding these two proteins are normally co-transcribed. This signal transduction cascade resembles that of the two-component systems in which a membrane bound histidine kinase controls the activity of a transcription factor (known as response regulator) that also mediates a cellular response through differential expression of target genes (Stock et al., 2000). However, whereas activation of two-components system involves phosphotransfer reactions, liberation and activation of the σ^{ECF} in response to the inducing signal requires the targeted proteolysis of the anti-σ factor (Qiu et al., 2007; Ades, 2008; Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a).

A high number of σ^{ECF} in a bacterial genome usually reflects the diversity of the bacterial living environment (Staron et al., 2009). The human opportunistic pathogen *Pseudomonas aeruginosa*, which thrives in diverse habitats ranging from soil to the human airways, encodes nineteen σ^{ECF} (Visca et al., 2002; Llamas et al., 2008; Llamas et al., 2014). Most *P. aeruginosa* σ^{ECF} belong to the iron-starvation group (Leoni et al., 2000) and initiate transcription of iron uptake functions. Expression of these σ factors is usually regulated by iron through the ferric-uptake regulator (Fur) repressor, and their function is normally activated by an iron carrier (i.e. siderophore) via a regulatory pathway known as cell-surface signalling (CSS) (Llamas et al., 2014). Apart from the σ^{ECF}/anti-σ factor pair, the CSS cascade also involves an outer membrane receptor of the TonB-dependent family (Llamas et al., 2014). CSS receptors usually have a dual function: transduce the presence of the signal to the anti-σ factor which activates the σ^{ECF} in the cytosol, and mediate the uptake of the inducing signal (i.e. siderophore) (Llamas et al., 2014). Moreover, *P. aeruginosa* contains two CSS σ factors that control expression of virulence genes (Llamas et al., 2014). This includes σ^{PvdS}, which responds to *P. aeruginosa*'s own siderophore pyoverdine and regulates the production of exotoxin A (*toxA*) and PrpL endoprotease (*prpL*)

(Lamont et al., 2002). The second example is σ^{VreI}, which regulates expression of several potential virulence factors, including secreted proteins and secretion systems (Fig. 1A), and induces *P. aeruginosa* virulence (Llamas et al., 2009). σ^{VreI} is encoded by the second gene of the *vreAIR* operon, which also encodes a CSS-like receptor (VreA) and an anti-σ factor (VreR) (Llamas et al., 2009). These three proteins form the PUMA3 CSS system (Llamas et al., 2009; Llamas et al., 2014). This system has a number of features that differentiate it from most CSS systems. First, the CSS receptor VreA lacks the C-terminal β barrel domain typical of TonB-dependent receptors and seems to be located in the periplasm instead of in the outer membrane (Llamas et al., 2009). This suggests that this protein is only involved in signal transduction and not in the uptake of the signal molecule. In addition, expression of the *vreAIR* operon is not regulated by iron and Fur but by phosphate (Pi) and the PhoB transcription factor (Faure et al., 2013). In *P. aeruginosa*, the level of Pi in the environment is sensed by the phosphate-specific ABC transport Pst system, which under Pi starvation conditions mediates Pi transport and activates the PhoR-PhoB two-component system (Lamarche et al., 2008). Upon activation, the PhoR histidine kinase promotes phosphorylation of its cognate DNA-binding response regulator PhoB. Phosphorylated PhoB controls the expression of a large set of genes by binding as a dimer to a *pho box*, a 22-bp specific DNA sequence in the promoter region of the PhoB regulon genes (Blanco et al., 2002). Interestingly, the genes belonging to the PUMA3 regulon are also expressed in response to Pi starvation in a σ^{VreI}-dependent manner (Faure et al., 2013). This is an intriguing observation since in this condition the genes encoding both σ^{VreI} and its cognate repressor, the VreR anti-σ factor, are expressed. This study was conducted to elucidate how the activity of σ^{VreI} is modulated in Pi starvation conditions.

RESULTS

Effect of the PUMA3 proteins on the expression of the vreAIR gene cluster and the σ^{VreI}-regulated genes under Pi starvation.

To assess the expression of the PUMA3 genes, we used *lacZ* transcriptional fusions to three PUMA3 promoters: P_{vreA}, P_{pdtA} and P_{phdA} (Fig. 1A). P_{vreA} is the promoter of the *vreAIR* operon, P_{pdtA} transcribes solely the *pdtA* gene, and P_{phdA} transcribes the *phdA*, *pdtB*, *exbB2D2*, *tonB4* and PA0696-PA0701 genes (Fig. 1A) (Faure et al., 2013). Activity of these three promoters was tested by β-galactosidase assay in the *P. aeruginosa* PAO1 wild-type strain and in the PUMA3 deletion mutants ΔvreA, ΔvreI and ΔvreR upon growth in low and high Pi conditions. A ΔphoB mutant was also included in the assay. The P_{vreA} was active in low Pi in a PhoB-dependent manner (Fig. 1B), as reported previously (Faure et al., 2013). In the ΔvreA, ΔvreI and ΔvreR mutants this promoter reached wild-type levels (Fig. 1B), showing that the PUMA3 CSS system is not involved in the regulation of its own expression. The PUMA3-regulated promoters P_{pdtA} and P_{phdA} were also active in low Pi and both the PhoB and σ^{VreI} proteins were required for such activation (Fig. 1B). However, the activity of these promoters in the ΔvreA mutant reached wild-type levels (Fig. 1B), which indicates that the VreA receptor is not involved in the expression of the PUMA3 regulon under Pi starvation. Expression from P_{pdtA} and P_{phdA} correlates with σ^{VreI} production, which occurs in the wild-type PAO1 and ΔvreA strains upon growth in low Pi but not in high Pi and does not occur in the ΔphoB mutant (Fig. 1C). This confirms that PhoB is required for σ^{VreI} production. Interestingly, in the ΔvreR mutant the activity of P_{pdtA} and P_{phdA} in low Pi was considerably higher than in the PAO1 wild-type strain (5.3- and 3.7-fold higher, respectively) (Fig. 1B). This indicates that full activation of σ^{VreI} requires the removal of VreR, which verifies

the anti-σ role of this protein. In accordance, the amount of σ^VreI in the ΔvreR mutant was considerably higher than in the PAO1 wild-type strain (Fig. 1C, - Pi). This supports previous results showing that an HA-tagged version of the σ^VreI protein is more stable in the absence of the anti-σ factor VreR (Llamas et al., 2009), and suggests that VreR promotes σ^VreI degradation. The high *pdtA* and *phdA* expression observed in the ΔvreR mutant was PhoB- and σ^VreI-dependent since activity of the *lacZ* fusions was completely abolished in a ΔphoB ΔvreR double mutant (Fig. 1B) that lacks PhoB and in which σ^VreI is not produced (Fig. 1C). Activity of the three promoters in the *phoB* mutants could be complemented by providing the *phoB* gene *in trans* (Fig. S1). Complementation was only partial (~35-55% of the activity in wild-type conditions) since overproduction of PhoB from plasmid slightly diminished promoter activities, as observed in the PAO1 wild-type strain (Fig. S1).

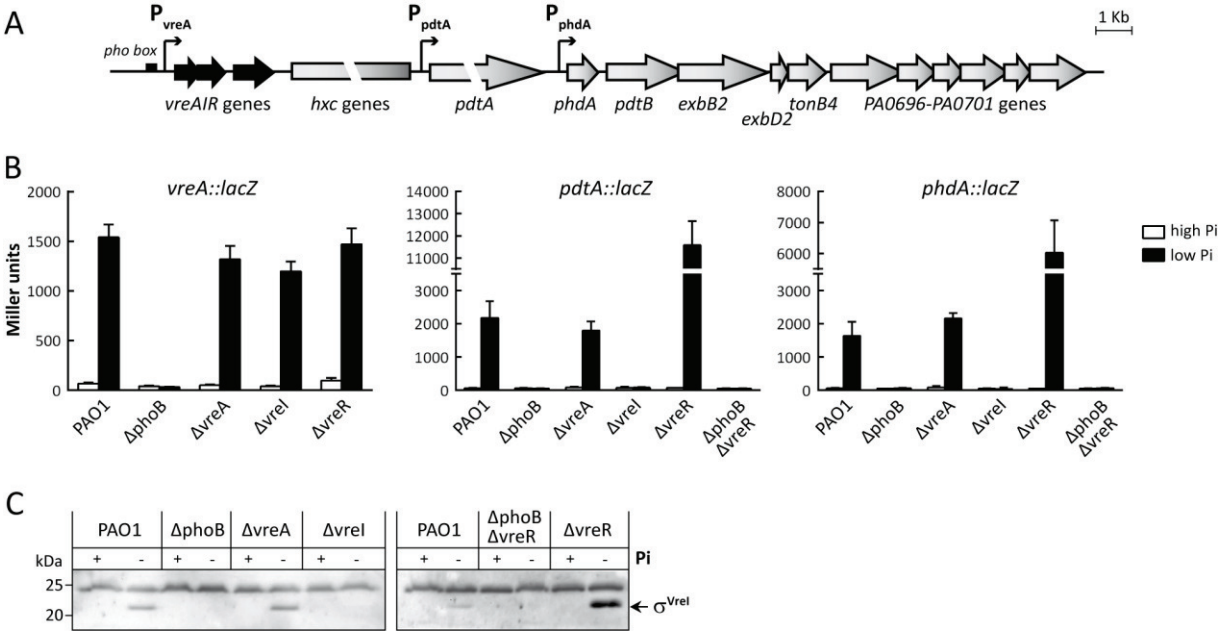

Figure 1. Genetic organization and expression of the PUMA3 regulon. (A) Transcriptional organization of the *vreAIR* locus encoding the PUMA3 CSS system (black) and the downstream PUMA3-regulated genes (grey). The big arrows represent the different genes, their relative sizes, and their transcriptional orientation. Below each arrow, the name of the gene, gene cluster or the PA number (http://www.pseudomonas.com) is indicated. Small arrows represent the promoters identified within the PUMA3 system (Llamas et al., 2009; Faure et al., 2013). The *pho box* present in the *vreA* promoter (Faure et al., 2013) is also indicated. **(B)** β-galactosidase activity of the *P. aeruginosa* PAO1 wild-type strain and the indicated mutants bearing the pMP220-derivated plasmids containing the indicated *lacZ* fusion (Table 2) after 18h of growth in high or low Pi conditions. **(C)** Detection of σ^VreI in *P. aeruginosa* PAO1 wild-type strain and the indicated mutants. Proteins were detected by Western-blot using a polyclonal anti-VreI antibody. The positions of the molecular size marker (in kDa) and of the σ^VreI protein are shown.

Role of the N-terminus of VreR in the regulation of σ^VreI activity.

To further analyse the role of VreR in the regulation of σ^VreI activity, we decided to focus on the N-terminal cytosolic tail (N-tail) of VreR. This anti-σ factor fragment (about 80-90 amino acids in length) is known to bind the σ^ECF (Campbell et al., 2007). Although it was originally described as the domain that keeps the σ^ECF sequestered and inactive in absence of the inducing signal, recent data have shown that the N-tail of some anti-σ factors has pro-sigma activity and

is required for σ^{ECF} functionality (Mettrick and Lamont, 2009; Bastiaansen et al., 2015a). To analyse the effect of the N-tail of VreR on σ^{VreI} activity, fragments of VreR of different lengths were cloned in the pMMB67EH plasmid under the control of an IPTG-inducible P*tac* promoter (Table S1). This includes VreR43 that contains the first 43 amino acids of the VreR protein, which constitutes only half of the cytosolic N-tail; VreR86 (amino acids 1-86), which contains the entire cytoplasmic portion of VreR (the N-tail); and VreR110 (amino acids 1-110), which contains the N-tail, the transmembrane domain and 4 periplasmic residues of VreR (Fig. 2A). Activity of σ^{VreI} in the presence of these protein fragments was tested in the ΔvreR mutant bearing the σ^{VreI}-dependent *phdA*::*lacZ* transcriptional fusion upon growth in high and low Pi conditions. Expression of a full-length VreR protein in the mutant restored σ^{VreI} activity to wild-type levels (Fig. 2B), showing that *in trans* production of VreR is able to complement the ΔvreR mutation. Amounts of σ^{VreI} in this strain were considerably lower than in the not complemented strain (Fig. 2C), confirming previous observations indicating that the presence of VreR promotes σ^{VreI} degradation (Fig. 1C and Llamas et al., 2009). Expression of the VreR43 fragment did not however affect σ^{VreI} activity (Fig. 2B) or stability (Fig. 2C), which were similar to those obtained in the not complemented ΔvreR mutant (Fig. 2B). This suggests that the VreR43 fragment, which contains only half of the VreR N-tail, is unable to bind σ^{VreI}. In contrast, production of the VreR86 fragment containing the complete cytosolic N-tail of VreR significantly reduced expression from the *phdA* promoter, suggesting that this fragment interacts with σ^{VreI} and inhibits its activity (Fig. 2B). Expression of VreR110 also reduced σ^{VreI} activity, but to a lesser extent than VreR86 (Fig. 2B), likely because the presence of the transmembrane domain in VreR110 hinders the binding of the N-tail to σ^{VreI}. Interestingly, whereas expression of VreR110 results in a less stable σ^{VreI} protein when compared with the not complemented ΔvreR mutant, expression of VreR86 results in higher amounts of σ^{VreI} (Fig. 2C). However, as described before, the σ factor is less active upon expression of VreR86 (Fig. 2B), which implies that, in contrast to the full-length VreR and the VreR110 proteins, the VreR86-mediated inhibition of σ^{VreI} activity does not involve σ^{VreI} degradation. In accordance with the reported structures of other σ^{ECF}/anti-σ pairs (Campbell et al., 2007), it is likely that the VreR86 fragment inhibits σ^{VreI} by binding to it and occluding its RNAPc binding determinants. All together these results show that overproduction of the N-tail of VreR inhibits σ^{VreI} activity, likely by interacting with this σ factor, and that therefore VreR does not contain pro-sigma activity.

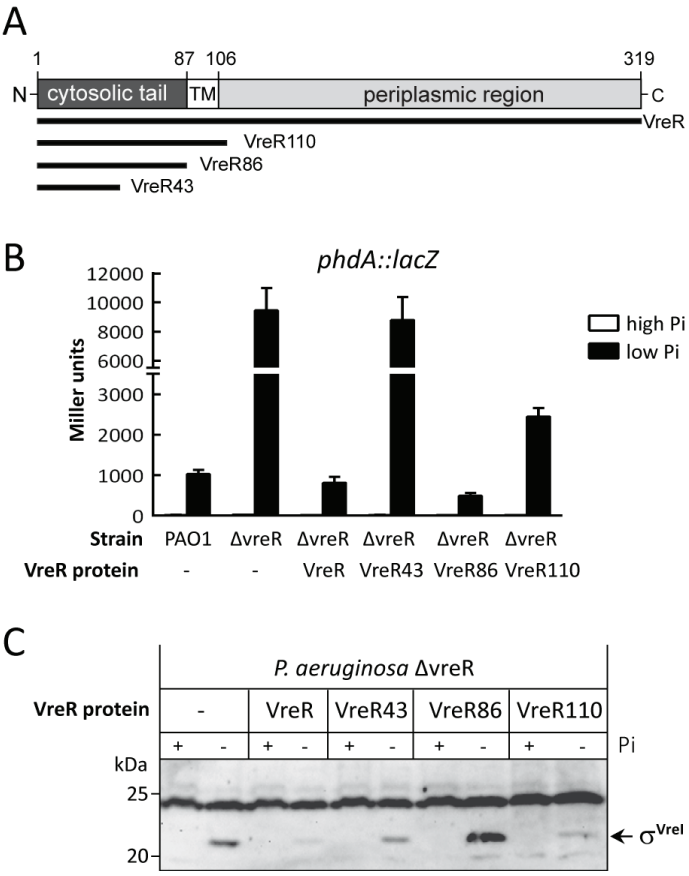

Figure 2. Effect of the N-tail of VreR on σ^VreI activity. (A) Schematic representation of the *P. aeruginosa* VreR protein. The VreR protein has been drawn to scale, and the cytosolic, transmembrane (TM), and periplasmic regions of the protein are shown. Numbers indicate amino acid positions. The produced VreR fragments are shown below the scheme. **(B)** β-galactosidase activity of the indicated *P. aeruginosa* strains bearing the transcriptional fusion *phdA*::*lacZ* and the pMMB67EH (-), pMMB-VreR, pMMB-VreR43, pMMB-VreR86 or pMMB-VreR110 plasmid expressing the indicated VreR fragment from the IPTG-inducible promoter P*tac* (Table 2). Strains were grown in high or low Pi in the presence of 1 mM IPTG. **(C)** Detection of σ^VreI in *P. aeruginosa* ΔvreR mutant upon expression of the indicated VreR fragment in high (+) or low (-) Pi and 1 mM IPTG. Proteins were detected by Western-blot using a polyclonal anti-VreI antibody. The positions of the molecular size marker (in kDa) and the σ^VreI protein are shown.

Effect of σ^VreI overproduction on expression of σ^VreI-regulated genes.

Several reports have shown that overproduction of σ^ECF, including σ^VreI, allows expression of their target genes in absence of the inducing signal (Koster et al., 1994; Pradel and Locht, 2001; Llamas et al., 2006; Llamas et al., 2008; Llamas et al., 2009; Faure et al., 2013). To study the effect of σ^VreI overproduction in the different *P. aeruginosa phoB* and PUMA3 mutants we used the pMUM3 plasmid, which contains the *vreI* gene expressed from a IPTG-inducible promoter (Llamas et al., 2009) (Table S1). Activity of both P_{pdtA} and P_{phdA} was null in high Pi when expression of *vreI* from pMUM3 was not induced by IPTG (Fig. 3A). Upon IPTG induction, a significant increase in activity was observed in all strains tested, including the two *phoB* mutants (Fig. 3A). This effect was considerably stronger in low Pi conditions (Fig. 3A). The fact that there is promoter activity in high Pi and in the *phoB* mutants when *vreI* expression is induced by IPTG indicates that overproduction of σ^VreI can bypass the low Pi and PhoB requirements for P_{pdtA} and P_{phdA} activity, as observed previously (Llamas et al., 2009; Faure et al., 2013). In fact, σ^VreI is present in extremely high amounts when its expression from pMUM3 is induced by IPTG (Fig. 3B). Activity of P_{pdtA} and P_{phdA} in low Pi without IPTG was similar to that observed in low Pi in absence of the pMUM3 plasmid: Maximal in the ΔvreR single mutant and null in both *phoB* mutants (Compare Fig. 3A and Fig. 1B). Moreover, the ΔvreI mutant could be complemented with pMUM3 in this condition (Fig. 3A, low Pi -IPTG), which suggests that σ^VreI is also produced from the plasmid in absence of IPTG. This was confirmed by Western-blot (Fig. 3B). Interestingly, the activity of P_{pdtA} and P_{phdA} in the complemented ΔvreI mutant was considerably higher than that obtained in the PAO1 wild-type strain in low Pi without IPTG (3.9- and 3-fold higher,

respectively) and similar to that of the ΔvreR mutant (Fig. 3A). Since the absence of the VreR anti-σ factor results in maximal σ^{VreI} activity (Fig. 1B), the observed phenotype could be due either to a polar effect of the *vreI* mutation on the expression of the downstream *vreR* gene or to the instability of the VreR protein in absence of σ^{VreI}. To check these two possibilities, VreR production/stability was analysed by Western-blot using an anti-VreR antibody that detects the chromosomally produced protein, and VreR stability was assayed using an anti-HAtag antibody that detects a C-terminally HA-tagged VreR protein constitutively produced from plasmid. This analysis showed that VreR is not produced in the ΔvreI mutant (Fig. 3C, left panel), and that the stability of the protein is not affected in absence of *vreI* since even higher amount of the VreR-HA protein were detected in the ΔvreI mutant (Fig. 3C, right panel). These results indicate that the *vreI* mutation exerts a polar effect on the expression of *vreR*. Therefore, both production of σ^{VreI} from pMUM3 in absence of IPTG and the lack of VreR explain the high promoter activity observed in the complemented ΔvreI mutant. Importantly, P_{pdtA} and P_{phdA} are not active in strains bearing the pMUM3 plasmid in absence of IPTG in high Pi—a condition in which PhoB is not active (Lamarche et al., 2008)—and in the two *phoB* mutants, despite the fact that σ^{VreI} is being produced and present in sufficient amount to target transcription (Fig. 3A and 3B). This strongly suggests that PhoB is not only required for expression of the *vreI* gene but also to enhance the σ^{VreI}-mediated expression of the PUMA3 regulon genes.

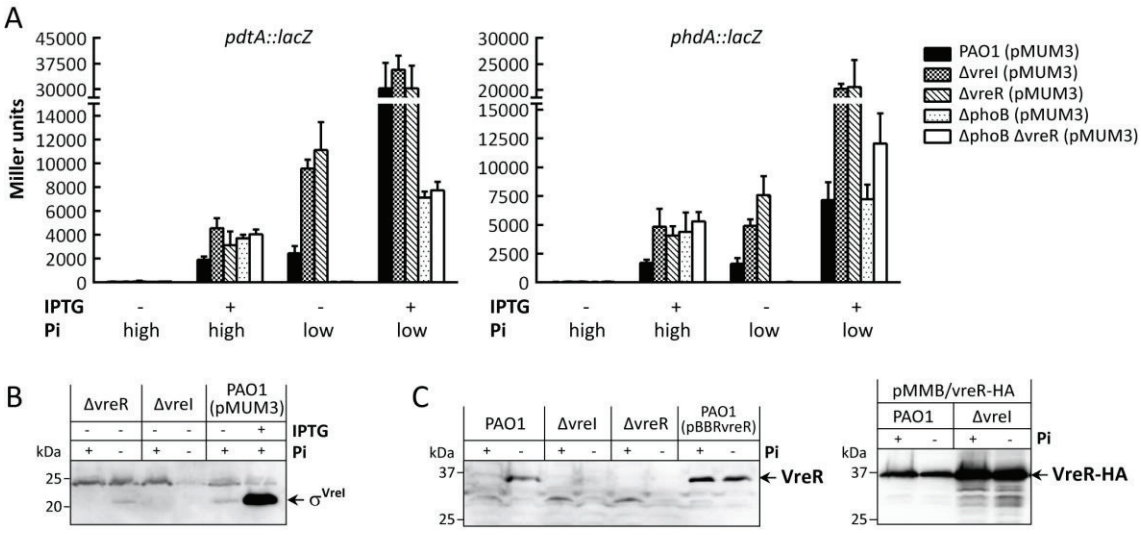

Figure 3. Effect of σ^{VreI} overproduction in the expression of the PUMA3 regulon. The indicated *P. aeruginosa* strains were grown 18h under high (+) or low Pi (-) conditions without (-) or with (+) 1 mM IPTG. **(A)** β-galactosidase activity of *P. aeruginosa* strains bearing the indicated *lacZ* fusion and the pMUM3 plasmid expressing the *vreI* gene from the IPTG-inducible promoter P*tac* (Llamas et al., 2009) (Table 2). **(B)** Detection of σ^{VreI} by Western-blot using a polyclonal anti-VreI antibody. The positions of the molecular size marker (in kDa) and the σ^{VreI} protein are shown. **(C)** Detection of VreR by Western-blot using a polyclonal anti-VreR antibody (left panel) or a monoclonal anti-HA antibody (right panel). The production of the VreR-HA protein from plasmid (Table 2) was induced with 1 mM IPTG. The positions of the molecular size marker (in kDa) and the VreR proteins are shown.

Defining the promoter region of σ^{VreI}-regulated genes.

In order to study the effect of the PhoB transcriptional regulator on the expression from P_{pdtA} and P_{phdA}, we decided to first define these promoter regions by locating the transcription initiation point of the *pdtA* and *phdA* genes (Fig. 1A). Transcription start sites were mapped by

5' RACE (Invitrogen) using RNA from the *P. aeruginosa* PAO1 wild-type strain or the ΔvreR mutant after growth in low Pi medium to induce maximal *pdtA* and *phdA* expression (Fig. 1B). This strategy located the transcriptional start site of *pdtA* at an adenine residue situated 53-bp upstream the *pdtA* translational start codon and that of *phdA* at a thymine residue situated 198-bp upstream the *phdA* translational start codon (Fig. 4A). In order to confirm these results and to rule out the possibility of the presence of other transcription initiation points not identified by 5' RACE, we carried out primer extension analyses. Total RNA isolated from *P. aeruginosa* PAO1 cells was annealed to a 5'-labeled oligonucleotide complementary to either the *pdtA* or the *phdA* gene (Table S2). A single cDNA product was obtained for each gene when the RNA was isolated from *P. aeruginosa* PAO1 or ΔvreR cells grown in low Pi, the amount of these products being considerably higher in the ΔvreR mutant (Fig. 4B). In fact, the *pdtA* cDNA product could be detected only in the ΔvreR mutant (Fig. 4B). This confirms the maximal *lacZ* activity of the transcriptional fusions observed in ΔvreR (Fig. 1B). The sizes of the cDNA products (73-bp for *pdtA* and 178-bp for *phdA*) corresponded with the transcription initiation points identified by 5' RACE. These bands were absent when total RNA was isolated from *P. aeruginosa* cells grown in high Pi or in the ΔvreI and *phoB* mutants (Fig. 4B), confirming that expression of these genes occurs under Pi starvation in a σ^{VreI}- and PhoB-dependent manner (Fig. 1B). An alignment of the DNA regions upstream the +1 site of *pdtA* and *phdA* genes allowed us to identify highly conserved DNA sequences centred within the −10 and −35 regions (Fig. 4A). These sequences did not exhibit similarity to the consensus sequence recognized by σ^{70} (TATAAT at −10 and TTGACA at −35), and could therefore be an alternative promoter sequence recognized by the RNAP loaded with σ^{VreI}. Interestingly, a putative *pho box* was detected in both promoter regions. PhoB binds DNA as a dimer and recognizes a 22-bp region with two 7-bp direct repeats followed by an A/T-rich region of 4-bp (Blanco et al., 2002), a sequence that was present in P_{pdtA} and P_{phdA} (Fig. 4A). The presence of a *pho box* further suggests the direct involvement of the PhoB regulator in the expression from these promoters.

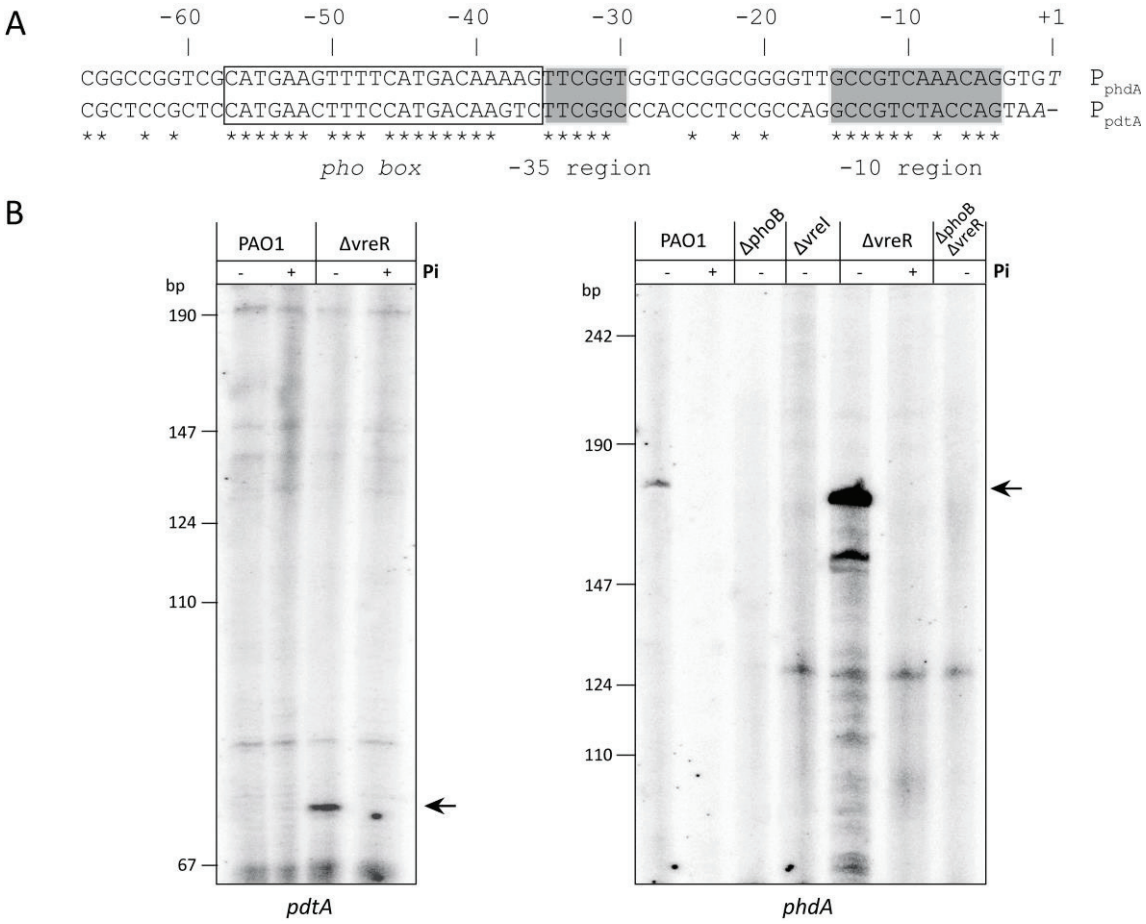

Figure 4. Determination of the transcription initiation points of the *pdtA* and *phdA* genes. (A) Identification of the +1 site by 5' RACE and *pdtA* and *phdA* promoter analysis. The *P. aeruginosa* PAO1 genomic sequence corresponding to the region upstream of the *pdtA* and *phdA* gene is shown. Nucleotides in italic represent the proposed +1 site. The identified promoter elements (*pho*, −35 and −10 boxes) are indicated. Identical nucleotide residues in both promoter regions are marked with a star. **(B)** Primer extension analysis of *pdtA* and *phdA* mRNA. *P. aeruginosa* PAO1 cells and the indicated mutants were grown in low or high Pi medium, and samples were taken in stationary phase for total RNA isolation. The autoradiogram shows the cDNA products obtained after reverse transcription of 12 µg of total RNA with the 5'-end-labeled PA0690R or PA0691R (Table S2) oligonucleotides hybridizing with the *pdtA* or the *phdA* mRNA, respectively.

Contribution of the −10 and −35 regions and the pho box to the activity of the pdtA promoter.

To determine the contribution of the identified −10, −35 and *pho box* regions to the activity of the *pdtA* promoter, we made several constructs in which these sequences were disrupted by single or multiple substitutions (S), by insertions (I), or by deletions (Δ) (Table 2). These constructs were then fused to the *lacZ* reporter gene and transferred to the *P. aeruginosa* PAO1 wild-type strain and the ΔvreR mutant to test their activity upon growth in Pi starvation conditions. Activity of all constructs in high Pi conditions was null in both strains (data not shown), indicating that none of the mutations resulted in a constitutively active promoter. Importantly, the effect of the mutations in the promoter activity upon growth in Pi starvation was very similar when tested in the PAO1 and in the ΔvreR mutant (Table 2) in which σ^{VreI} activity is maximal, indicating that their activity depends on σ^{VreI}. Single and multiple mutations in the −10 region showed that changes in the nucleotides **–**5 to **–**11 had a dramatic effect on promoter

activity, which was completely abolished (Table 2). However, mutation of the −3 and −4 nucleotides had little effect (70% of the activity retained); substitution of the −12, −13 and −14 nucleotides reduced, but did not abolish the activity (30-55% of the activity retained); and mutation of only the −13 and −14 nucleotides had no effect on promoter activity (Table 2). Substitutions within the region between the –10 and –35 boxes did not affect promoter activity (Table 2; S-19 and S-24), whereas changing the size of this region by either inserting or deleting one nucleotide did significantly affect expression (Table 2; I-22 and Δ-22). Within the –35 region, substitution of the nucleotides –30 to –34 and that of the –33 and –34 considerably reduced *pdtA* promoter activity (Table 2). In contrast, changing the –29 and –30 GC nucleotides into TA resulted in a more active promoter (Table 2). The contribution of the identified *pho box* to the *pdtA* promoter activity was also analysed. Complete disruption of the *pho box* (S pho box) or disruption of only one of the two 7-bp direct repeat sequences (S-40 to -45 or S-50 to -56) completely abolished promoter activity (Table 2). This indicates that intact –10, –35 and *pho* boxes are required for *pdtA* expression.

Table 1. Mutagenesis of the *pdtA* promoter and activity[a]

Promoter	Sequence[b]	% activity in low Pi compared to WT promoter[c]	
		PAO1 (wild-type)	ΔvreR mutant
P_{pdtA}	−50 −30 −10 +1		
WT promoter	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**100** (2408 ± 433)	**100** (17585 ± 3706)
S−3−4	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCCTTAA	**70.2** (1691 ± 311)	**71.3** (12542 ± 2971)
S−5−6−7−8	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCGGGTTAGTAA	**7.9** (190 ± 21)	**0.8** (148 ± 36)
S−5−7−9	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCCCCGAGTAA	**3.7** (88 ± 5)	**17.5** (3072 ± 965)
S−5−8	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCGACAAGTAA	**7.1** (172 ± 29)	**0.9** (153 ± 31)
S−6	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTATCAGTAA	**7.8** (187 ± 11)	**0.9** (157.5 ± 25)
S−7	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTTCCAGTAA	**8.3** (200 ± 17)	**0.9** (162 ± 20)
S−7−8	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCCCCAGTAA	**7.6** (182 ± 17)	**0.9** (161 ± 28)
S−9−10−11−12	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCGCGTTACCAGTAA	**8.6** (207 ± 28)	**0.7** (118 ± 11)
S−9−10−11	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCACATACCAGTAA	**1.0** (24 ± 6)	**0.7** (122 ± 46)
S−9−10−12	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCTGGGTACCAGTAA	**7.3** (178 ± 15)	**0.7** (119 ± 19)
S−9−10	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGGTTACCAGTAA	**7.2** (174 ± 25)	**0.7** (120 ± 20)
S−10	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGGCTACCAGTAA	**7.5** (180 ± 24)	**0.6** (109 ± 22)
S−11	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCCGTCTACCAGTAA	**7.0** (169 ± 15)	**0.7** (123 ± 33)
S−9 to −14	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGTAATGATACCAGTAA	**1.7** (41 ± 4)	**0.3** (49 ± 16)
S−12−13−14	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGTAAGTCTACCAGTAA	**28.7** (692 ± 93)	**57.2** (10,054 ± 2281)
S−13−14	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGTACGTCTACCAGTAA	**103** (2487 ± 148)	**117** (20,574 ± 2964)
S−19	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCTCCAGGCCGTCTACCAGTAA	**116** (2789 ± 348)	**114** (20,051 ± 979)
S−24	CATGAACTTTCCATGACAAGTCTTCGGCCCACACTCCGCCAGGCCGTCTACCAGTAA	**97** (2329 ± 357)	**121** (21,348 ± 3169)
I−22	CATGAACTTTCCATGACAAGTCTTCGGCCCACCCTTCCGCCAGGCCGTCTACCAGTAA	**13.3** (320 ± 35)	**27.1** (4772 ± 1039)
Δ−22	CATGAACTTTCCATGACAAGTCTTCGGCCCACCC-CCGCCAGGCCGTCTACCAGTAA	**51.4** (1237 ± 283)	**27.1** (4763 ± 969)
S−30 to −34	CATGAACTTTCCATGACAAGTCGGATTCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**19.0** (458 ± 183)	**4.0** (710 ± 169)
S−29−30	CATGAACTTTCCATGACAAGTCTTCGTACCACCCTCCGCCAGGCCGTCTACCAGTAA	**142.7** (3436 ± 381)	**124** (21,716 ± 3920)
S−33−34	CATGAACTTTCCATGACAAGTCGCCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**7.2** (172 ± 23)	**0.7** (121 ± 22)
S−35 to −40	CATGAACTTTCCATGAACCTGATTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**37.3** (898 ± 75)	**74.1** (13,023 ± 1582)
S−40 to −45	CATGAACTTTCTGGTGTAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**3.5** (85 ± 16)	**4.4** (771 ± 262)
S−50 to −56	ACCACCATTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**3.6** (86 ± 25)	**3.2** (557 ± 160)
S pho box	TTTTTTTCTCTTTTTTTCCTTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA	**1.7** (41 ± 9)	**0.8** (136 ± 58)

[a] The promoter activity was measured by β-galactosidase assay

[b] The +1 site is in italic, the −10 and −35 regions are shaded, and the *pho box* is underlined

[c] Miller units and standard deviation from three biological repetitions are shown between brackets

The PhoB transcription factor binds to the vreA*,* pdtA *and* phdA *promoters.*

The results obtained here with the mutational analysis of the *pho box* of the *pdtA* promoter (Table 2) and those obtained previously with a similar analysis of the *pho box* of the *vreA* promoter (Faure et al., 2013), suggest that PhoB directly binds to these promoter regions. To confirm this, we performed electrophoretic mobility shift assays (EMSA) using a fixed amount of fluorescein-labelled dsDNA probes obtained by annealing oligonucleotides that contain the promoter region of the *vreA*, *pdtA* or *phdA* genes (Table S3). Addition of increasing concentrations of purified and phosphorylated PhoB protein to the DNA fragments resulted in a slower complex that at higher protein concentration became the predominant (Fig. 5A), showing that PhoB indeed binds the *vreA*, *pdtA* and *phdA* promoters. Since non-isotopic DNA labelling can alter the affinity and/or stoichiometry of the protein-DNA interaction, we also performed the EMSA using ^{32}P-labelled dsDNA (Fig. 5). In this condition, two retarded DNA bands were observed (Fig. 5B, *pdtA* promoter). Since PhoB is known to bind to DNA as a dimer of which each monomer contacts one direct repeat of the *pho box* (Makino et al., 1996; Blanco et al., 2002), it is possible that these bands are the result of PhoB binding first as a monomer (complex I) and at higher concentrations as a dimer, generating the second retardation band (complex II). Two DNA retardation bands were also observed when the *pstC* promoter, which is known to contain a *pho box* (Nikata et al., 1996; Jensen et al., 2006), was used as a positive control (Fig. S2). No band shifts were however detected when the *fiuA* promoter, which is not regulated by low Pi (Llamas et al., 2006) and does not have a *pho box*, was used as DNA probe (Fig. S2). This confirms the specific binding of the purified PhoB protein to promoters containing a *pho box*. Moreover, addition of increasing amounts of an unlabelled competitor dsDNA to the EMSA reactions resulted in the complete disappearance of the second retardation band and in a considerably increase in the amount of free DNA (Fig. S2). Although the complex I was still formed (probably because the amounts of unlabelled DNA did not reach the level needed for complex I to disappear), this indicates that there is competition between the DNAs and therefore that the retardation bands are the specific result of PhoB-DNA complexes formation. Interestingly, when a *pdtA* promoter containing mutations in the first direct repeat of the *pho box* was used as DNA probe, the binding of PhoB was considerably impaired and a higher concentration of the protein was needed for the formation of the PhoB-DNA complex (Fig. 5C). Mutation of the two direct repeats of the *pho box* completely abolished PhoB binding (Fig. 5C), which suggests that this mutated region contains the PhoB binding site. Altogether, our results show that PhoB binds to the promoter region of the *vreAIR* operon and, importantly, to that of the σ^{VreI}-dependent promoters *pdtA* and *phdA*.

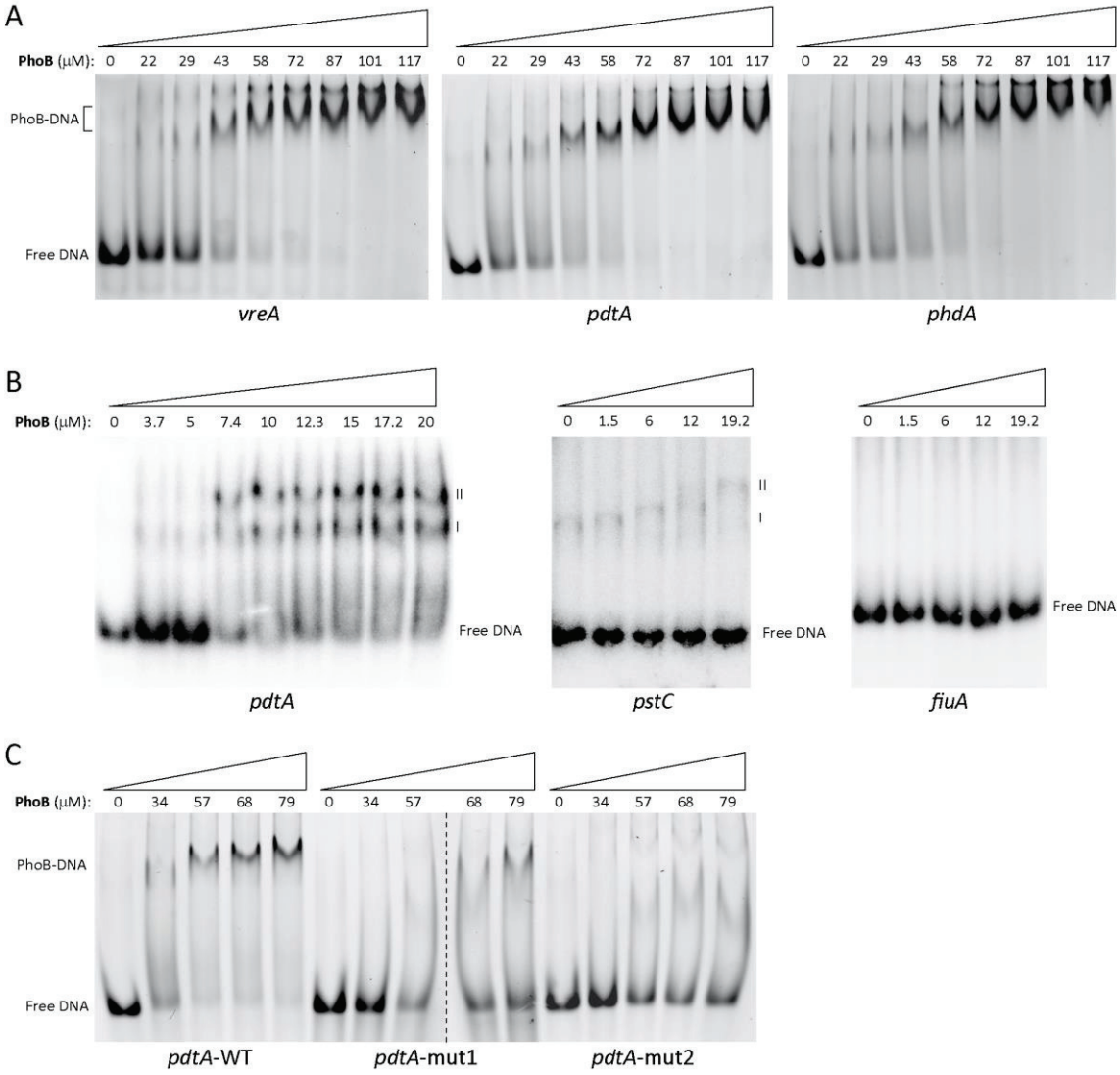

Figure 5. Binding of PhoB to the *vreA*, *pdtA* and *phdA* promoter regions. EMSA gels using fluorescein-labelled **(A and C)** or ^{32}P-labelled **(B)** dsDNA probes (Table S3) containing the indicated *P. aeruginosa* promoter and increasing amounts of phosphorylated PhoB protein. Upper numbers indicate the concentration of PhoB used in the assay (in μM). In A and B wild-type (WT) promoter sequences were used as DNA probes. In C *pdtA* promoters with mutations in the first direct repeat (*pdtA*-mut1) or in both direct repeats (*pdtA*-mut2) of the *pho box* were used. The position of the free DNA and of the PhoB-DNA complexes (I and II) are indicated.

PhoB is required for the binding of σ^VreI to the pdtA promoter.

Next, we assayed the binding of σ^VreI to the *pdtA* promoter by EMSA. Several attempts using the σ^VreI protein alone or reconstituted σ^VreI-RNAP holoenzyme did not result in DNA retardation bands (data not shown), suggesting that σ^VreI alone could not bind to this promoter region. Therefore, we tested the binding of σ^VreI to PhoB-DNA complexes. Addition of increasing amounts of σ^VreI in EMSA reactions containing the PhoB protein (in a concentration that results in the formation of the complex II) and the *pdtA* promoter resulted in the appearance of a new retarded DNA band (Fig. 6). This change in mobility likely reflects the formation of a σ^VreI-PhoB-DNA complex, which was not formed in absence of σ^VreI or PhoB (Fig. 6). This indicates that σ^VreI cannot interact with the promoter region of *pdtA* and *phdA* genes in the absence of PhoB, which

is in agreement with the PhoB-requirement for expression of these genes even in conditions in which σ^VreI is present (Fig. 3A, low Pi -IPTG). The σ^VreI-PhoB-DNA complex was not formed when the *vreA* promoter was used as DNA probe (Fig. 6), in agreement with σ^VreI not being involved in the expression from this promoter (Fig. 1B). This confirms the specific binding of the σ factor to σ^VreI-dependent promoters and shows the requirement of PhoB for this binding to occur.

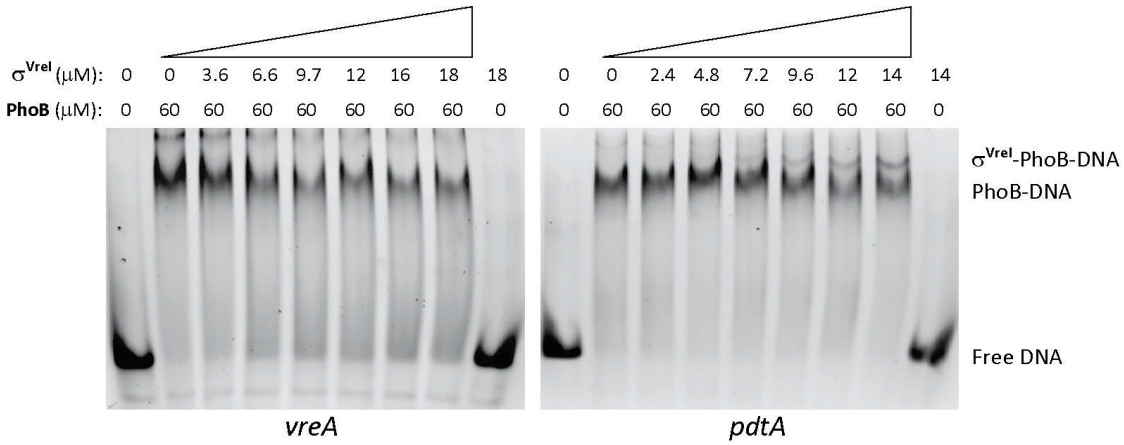

Figure 6. Binding of σ^VreI to the *pdtA* promoter. EMSA gels using fluorescein-labelled dsDNA probes containing the *P. aeruginosa vreA* or *pdtA* promoter (Table S3). Increasing amounts of σ^VreI were added to a preformed PhoB-DNA complex. Upper numbers indicate the concentration of phosphorylated PhoB and σ^VreI proteins used in the assay (in μM). The position of the free DNA and of the PhoB-DNA and σ^VreI-PhoB-DNA complexes are indicated.

DISCUSSION

The PUMA3 system of *P. aeruginosa* is an unusual CSS cascade in various functional and architectural aspects. Importantly, this signal transduction system is directly involved in the regulation of virulence and, unlike most *P. aeruginosa* CSS systems, does not control iron uptake (Llamas et al., 2009; Llamas et al., 2014). The architectural variations of the system mainly concern the VreA receptor-like component, which is smaller than regular CSS receptors and seems to function only in signalling and not in the transport of the signal molecule (Llamas et al., 2009). In addition, the genetic organization of the *vreAIR* genes encoding the PUMA3 system is different than that of most CSS pathways. While CSS σ^ECF are generally co-transcribed with their cognate anti-σ factor and the receptor gene is located in a separate transcriptional unit (Koebnik, 2005; Llamas et al., 2014), the *vreAIR* genes form an operon (Fig. 1A). In *P. aeruginosa* expression of most σ^ECF/anti-σ operons is controlled by iron through the Fur regulator, which allows production of these proteins in iron depleted conditions (Llamas et al., 2014). In contrast, expression of the *vreAIR* operon is targeted by Pi starvation and requires the phosphate regulator PhoB. A *pho box* is present in the *vreA* promoter region (Faure et al., 2013), and direct binding of this transcription factor to this promoter region has been demonstrated in this work (Fig. 5). The *vreAIR* gene products, including σ^VreI, are not involved in the expression from the *vreA* promoter, and in accordance, σ^VreI does not bind to this promoter (Fig. 6). This indicates that another σ factor, likely the *P. aeruginosa* primary σ factor σ^70, targets transcription of the *vreAIR* operon under Pi starvation and in a PhoB-dependent manner.

Interestingly, the genes belonging to the PUMA3 regulon are expressed in response to Pi starvation in a σ^VreI-dependent manner, despite the fact that in this condition the σ^VreI repressor VreR is also produced. A specific inducing signal is typically required to relieve the anti-σ-

mediated inhibition of σ^{ECF} activity. In presence of such a signal, the anti-σ factor protein is removed by regulated proteolysis allowing the σ^{ECF}-mediated transcription (Qiu et al., 2007; Ades, 2008; Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a). We show here that deletion of the *vreR* anti-σ factor gene is required for maximal σ^{VreI} activity in low Pi (Fig. 1B). This suggests that an additional stimulus not present in Pi starvation is needed to remove VreR and produce full σ^{VreI} activation. This hypothesis is supported by the fact that the VreA receptor, which by analogy with most CSS systems likely initiates the PUMA3 signalling cascade (Llamas et al., 2014), is not required for the transcription of the PUMA3 regulon genes in low Pi. Therefore, the detected σ^{VreI} activity in Pi starvation seems not to be the result of actual signalling through the PUMA3 CSS system, but represents 'leaky' activity of σ^{VreI}. Previous studies have shown that σ^{VreI}-regulated genes are induced upon contact of *P. aeruginosa* with human airway epithelial cells (Frisk et al., 2004; Chugani and Greenberg, 2007). Moreover, antibodies against the PdtA and PA0697 proteins of the PUMA3 regulon (Fig. 1A) have been detected in the serum of patients infected with *P. aeruginosa* (Llamas et al., 2009). This suggests that the molecule targeting PUMA3 signalling could be host-derived, and our current work aims at identifying such a signal.

The fact that VreR removal produces maximal activation of σ^{VreI} indicates that this anti-σ factor only has anti-σ function. Two divergent classes of CSS anti-σ factors have been reported, mere anti-σ factors and anti-σ factors with pro-sigma activity (also called sigma factor regulators) (Mettrick and Lamont, 2009; Llamas and Bitter, 2010; Llamas et al., 2014). Proteins within the first group only contain anti-σ activity and inhibit activity of their cognate σ^{ECF} in absence of the CSS inducing signal. Deletion of these proteins results in signal-independent transcription of the σ^{ECF}-regulated genes (Mettrick and Lamont, 2009). In contrast, deletion of anti-σ factors of the second group does not result in activation of its cognate σ^{ECF} since these anti-σ factors are required for σ^{ECF} activity. The pro-sigma activity of these proteins seems to reside within the short cytosolic N-terminal region (N-tail), since the expression of this domain alone induces σ^{ECF} activity independently of the presence of the signal (Ochs et al., 1995; Cuív et al., 2006; Mettrick and Lamont, 2009). Recently, we have shown that the N-tail of anti-σ factors is indeed produced *in vivo* in response to the inducing signal and that the transmembrane protease RseP is responsible for this process (Bastiaansen et al., 2015a). Although still not experimentally determined, it has been proposed that the N-tail can protect the σ^{ECF} from degradation and that this domain may be bound to the σ^{ECF}-RNAP holoenzyme during the transcription process (Mahren and Braun, 2003). However, this does not seem to be the case for the N-tail of VreR since this protein fragment does not enhance σ^{VreI} activity. In fact, overexpression of the N-tail of VreR inhibits the activity of σ^{VreI} (Fig. 2). This indicates that VreR does not contain pro-sigma activity, which is in accordance with the higher σ^{VreI} activity detected in the ΔvreR mutant. The role of VreR as a mere anti-σ factor is further supported by the fact that σ^{VreI} is more stable in absence of VreR. Our results suggest that VreR employs at least two mechanisms to inhibit σ^{VreI} activity: binding to the σ factor likely shielding the binding determinants of σ^{VreI} for the RNAPc, and promotion of σ^{VreI} degradation. The N-tail of VreR (aminoacids 1-86) seems to be sufficient to prevent binding of σ^{VreI} to the RNAPc, but this fragment alone does not promote σ^{VreI} degradation (Fig. 2) and the complete protein seems to be required for this. Another *P. aeruginosa* CSS anti-σ factor, FvpR, has also been reported to induce degradation of its cognate σ^{ECF} (Spencer et al., 2008), although the mechanism behind this process is still unknown. These observations further indicate that *in vivo* and upon sensing the PUMA3 inducing signal, VreR needs to be completely removed in order for σ^{VreI} to reach maximal activity.

Importantly, we show in this work that activity of σ^{VreI} is also modulated by a transcription factor, the phosphate regulator PhoB. This is an important finding since, while modulation of primary σ factors activity by trans-acting factors has been extensively reported, such modulation of σ^{ECF} has not been extensively investigated yet. As demonstrated in this study, PhoB is not only required for σ^{VreI} production but also for the binding of σ^{VreI} to the promoter region of its target genes. In fact, the two proteins bind to the promoter of the σ^{VreI}-regulated genes and, in accordance, expression of these genes does not occur unless both proteins are present and active in the cell. Only extremely high levels of σ^{VreI}, which we obtained by overexpressing the *vreI* gene from an IPTG-inducible promoter, can bypass the PhoB requirement for the transcription of the σ^{VreI} target genes. However, these σ^{VreI} levels are not likely to be ever reached *in vivo*. As mentioned before, it is expected that upon sensing the PUMA3 inducing signal VreR is proteolytically degraded and σ^{VreI} released. Thus, the maximal σ^{VreI} amount expected *in vivo* upon induction of the PUMA3 cascade likely resembles the level obtained in the ΔvreR mutant, which is considerably lower than that obtained when production of σ^{VreI} from plasmid was induced with IPTG (Fig. 3B). Therefore, both PhoB and σ^{VreI} are needed to target transcription of the PUMA3 regulon genes *in vivo*. The potential DNA binding sites for PhoB and σ^{VreI} in the promoter regions of PUMA3 regulon genes have been identified. A conserved *pho box* (Blanco et al., 2002) containing two 7-bp direct repeats is located upstream of the –35 region of both the *pdtA* and *phdA* promoters. Mutation of this region, either one of the direct repeats or the entire box, completely abrogates gene expression (Table 2), and, when the two direct repeats are mutated, also the binding of PhoB (Fig. 5C). Based on these results, we propose that this region within the *pdtA* and *pdhA* promoters is the PhoB binding site. Although in *E. coli* the *pho box* is usually located near the σ^{70} –10 promoter region substituting the –35 region (Makino et al., 1986; Blanco et al., 2002), this does not seem to be the case for σ^{VreI}-dependent promoters. Downstream of the *pho box*, highly identical sequences centred within the –35 and –10 positions have been identified in the *pdtA* and *phdA* promoters (Fig. 4A). Members of the σ^{70} family are known to recognize promoter sequences located at positions −35 and −10 from the transcriptional start point and regions 4.2 and 2.4, respectively, of the primary σ^{70} protein are involved in such recognition (Brooks and Buchanan, 2008). σ^{ECF} are the smallest σ factors of the σ^{70} family and lack two of the four conserved domains of primary σ factors (domains 1 and 3) (Lonetto et al., 1994; Bastiaansen et al., 2012). However, promoter recognition by σ^{ECF} involves the same σ factor regions (Enz et al., 2003a; Wilson and Lamont, 2006). Interestingly, region 2.4, which recognizes the −10 promoter element, shows most variation within the σ^{ECF} subfamily, which likely reflects differences in promoter binding specificity (Lonetto et al., 1994). This suggests that promoter specificity of σ^{ECF} is predominantly determined by the −10 promoter element and the region 2.4 of the σ^{ECF}. In agreement, single mutations within the −10 promoter sequence of *pdtA* (nucleotides −5 to −11) completely abrogated gene expression, in both the wild-type strain and the ΔvreR mutant, which strongly indicates that this region is essential for the σ^{VreI}-mediated transcription of this gene. Although σ^{ECF} usually share a high degree of similarity in their −35 promoter element (Enz et al., 2003a), which has a conserved AA motif that is important for DNA geometry and thus for σ^{ECF}-DNA interaction (Lane and Darst, 2006), this is not the case for the σ^{VreI}-dependent promoters. The absence of this motif in the −35 region could impair the binding of σ^{VreI} to the DNA, which would be facilitated by the binding of the PhoB protein to the *pho box*. Our results strongly suggest a model in which PhoB recruits σ^{VreI} to the promoter region to trigger transcription, which is similar to the mechanism employs by PhoB with the primary σ^{70} factor (Makino et al., 1996; Blanco et al., 2011). Although studies focused on the structure of the PhoB-σ^{VreI}-DNA complex are required to fully understand the process, it is likely that the PhoB-σ^{VreI} interaction involves, as shown for σ^{70} (Blanco et al., 2011), the region

4 of σ^{VreI}, which is the region that contacts the −35 sequence and is potentially the closest to PhoB in the complex.

In summary, our results show that the activity of the *P. aeruginosa* σ^{VreI} in Pi starvation is modulated by both the anti-σ factor VreR and the transcription factor PhoB. Pi starvation is an important environmental cue that induces transcription of the so-called *pho* regulon, which in *P. aeruginosa* includes multiple potential virulence factors (Lamarche et al., 2008). It is therefore not surprising that Pi starvation enhances *P. aeruginosa* lethality in mice and nematodes, while providing excess phosphate protects from killing (Long et al., 2008; Zaborina et al., 2008; Zaborin et al., 2009; Zaborin et al., 2012). Overexpression of σ^{VreI} has been demonstrated to increase *P. aeruginosa* lethality in zebrafish embryos (Llamas et al., 2009) and preliminary results from our group indicate that Pi starvation enhances the virulence of this bacterium in this infection model (data not shown). Moreover, there are several indications that the *P. aeruginosa pho* regulon is induced *in vivo* during infection (Frisk et al., 2004; Datta et al., 2007; Long et al., 2008). Since the PUMA3 CSS system is produced under Pi starvation and the currently unknown inducing signal is likely host-derived, it will be of interest to determine the contribution of σ^{VreI} and the PUMA3 regulon proteins to the low Pi-induced virulence of *P. aeruginosa*.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table S1. Bacteria were grown in liquid LB (Sambrook et al., 1989) or in 0.3% (w/v) proteose peptone (DIFCO) containing 100 mM HEPES, 20 mM NH_4Cl, 20 mM KCl, 3.2 mM $MgCl_2$, and 0.4% (w/v) glucose (pH 7.2), without (low Pi) or with 10 mM KH_2PO_4 (high Pi), on a rotatory shaker at 37ºC and 200 rpm. When required, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to induce full expression from the pMMB67EH P*tac* promoter. Antibiotics were used at the following final concentrations (μg ml^{-1}): ampicillin (Ap), 100; kanamycin (Km), 50; piperacillin (Pip), 25; streptomycin (Sm), 100; tetracycline (Tc), 20.

Plasmids construction and molecular biology. Plasmids used are described in Table S1 and primers listed in Table S2. PCR amplifications were performed using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) or Expand High Fidelity DNA polymerase (Roche). Nucleotide substitutions or deletions in the *pdtA* and *phdA* promoters were generated by whole plasmid PCR site-directed mutagenesis (Fisher and Pei, 1997) with a pair of complementary mutagenic primers using the pTOPO-Pr0690 and pTOPO-Pr0691b plasmids (Table S1), respectively, as templates. After mutagenesis, the promoters were subcloned in the pMP220 plasmid as EcoRI-XbaI (P_{pdtA}) or BglII-KpnI (P_{phdA}) restriction fragments. All constructs were confirmed by DNA sequencing and transferred to *P. aeruginosa* by electroporation (Choi et al., 2006). Construction of null mutants was performed by allelic exchange using the suicide vector pKNG101 as described before (Bastiaansen et al., 2014). Southern blot analyses to confirm the chromosomal gene deletion were performed as described (Llamas et al., 2000).

Enzyme assay. β-galactosidase activities in soluble cell extracts were determined using *o*-nitrophenyl-b-D-galactopyranoside (ONPG) (Sigma-Aldrich) as described before (Llamas et al., 2006). Each condition was tested in duplicate in at least three biologically independent experiments and the data given are the average with error bars representing standard deviation (SD). Activity is expressed in Miller units.

Production of α-VreI and α-VreR antibodies. To obtain relatively pure protein recombinant, VreI and VreR were expressed as an insoluble protein in *E. coli* TOP10F' using an aggregation tag. Inclusion bodies were isolated as followed: bacterial cells were resuspended in 5 ml solution buffer (50 mM Tris-Hcl, 25% sucrose, 1 mM NaEDTA, 10 mM DTT, 0.4 mg/ml lysozyme, 20 µg/ml DNAse I and 2 mM $MgCl_2$). Following sonication, 5 ml lysis buffer was added (50 mM Tris-HCl, 1% Triton X-100, 1% Na deoxycholate, 100 mM NaCl, 10 mM DTT) and the suspension was incubated on ice for 1 h. After a snap freezing and thawing cycle the total amount of NaEDTA and $MgCl_2$ was increased to 15 mM and 6 mM, respectively. Inclusion bodies were pelleted at 11.000 x *g* for 20 min at 4 °C and washed with a buffer containing 50 mM Tris-HCl, 1% Triton X-100, 100 mM NaCl, 1 mM NaEDTA and 1 mM DTT. Following sonication to obtain a homogenous suspension and another centrifugation step, washing was performed in the same buffer omitting Triton X-100. Subsequently, inclusion bodies were boiled in SDS-PAGE sample buffer. Proteins were analysed by SDS-PAGE containing 12% (w/v) acrylamide and the VreI and VreR proteins were excised from the gel following an imidazole-zinc staining. The proteins were electroeluted out of the gel and purified VreI and VreR were sent to Innovagen (Sweden) for antibody production. Rabbits were immunized at day 0 and subsequently given boosters at days 14, 28, 49, and 70. At day 84 rabbits were sacrificed and serum was isolated. Prior Western-blot, serum was concentrated using 30K centrifugal filter units (Millipore) at 4000 rpm for 15 min.

SDS-PAGE and Western-blot. Bacteria were grown until late log phase and pelleted by centrifugation. Samples were normalized according to the OD_{660} of the culture, solubilized in Laemmli buffer and heated for 10 min at 95°C. Proteins were separated by SDS-PAGE containing 12 or 15% (w/v) acrylamide and electrotransferred to nitrocellulose membranes. Ponceau S staining was performed as a loading control. Immunodetection was realized using polyclonal antibodies directed against the σ^{VreI} or the VreR proteins, or a monoclonal antibody directed against the influenza hemagglutinin epitope (HA.11, Covance). The second antibody, either the horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) or the horseradish peroxidase-conjugated rabbit anti-mouse (DAKO), was detected using the SuperSignal® West Femto Chemiluminescent Substrate (Thermo Scientific). Blots were scanned and analysed using the Quantity One version 4.6.7 (Bio-Rad).

RNA preparation. *P. aeruginosa* cells were grown until late exponential phase in low or high phosphate medium, or in LB supplemented with 1 mM IPTG. Total bacterial RNA was isolated by the hot phenol method using the TRI® Reagent protocol (Ambion) as described before (Llamas et al., 2008). RNA quantity and quality was assessed by UV absorption at 260 nm in a ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

5' RACE. The transcription start points were determined using the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen). RNA isolated from *P. aeruginosa* PAO1 or ΔvreR cells grown in low Pi was used as the template for 5' RACE analysis. The primers used in this analysis are shown in Table S2. The 5' RACE reactions were performed as recommended by the manufacturer and analysed by agarose gel electrophoresis to assess purity and product size. Single cDNA bands were obtained for the reactions and, upon purification, were sequenced using a nested gene-specific primer to locate the 5' end of the transcript. The sequencing results of the 5' RACE product were aligned with the *P. aeruginosa* PAO1 genome sequence.

Primer extension analysis. Primer extension analyses were done basically as described by Marques et al. (Marques et al., 1994) using 12 µg of total RNA in each reaction. About 10^5 cpm

of [γ-^{32}P]-labelled 5'-end oligonucleotides was used as primers in extension reactions (Table S2). The cDNA products obtained after the reverse transcriptase reaction were separated and analysed in urea-polyacrylamide sequencing gels. Visualization of the gels was performed using the Fujifilm imaging plate BAS-MS 2040.

PhoB and σ^{VreI} protein purification. His-tagged PhoB and σ^{VreI} proteins were produced in *E. coli* BL21 from the pET-phoB and pET-vreI plasmids, respectively, and purified by affinity chromatography. Cells were grown overnight at 18ºC in LB supplemented with 0.1 mM IPTG and harvested by centrifugation. The pellet was resuspended in 30 ml of buffer A (20 mM Tris-HCl, 0.1 mM EDTA, 300 mM NaCl, 5% glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol; pH 7.25) supplemented with 1x Complete protease inhibitor cocktail (Roche) and broken by repeated French Press passages at 1000 psi. Following centrifugation at 20.000 *x g* for 1 h the soluble fraction was passed through a 0.22 µm filter (Millipore) and loaded onto a 5 ml HisTrapHP chelating column (GE Healthcare) previously equilibrated in buffer A. PhoB and σ^{VreI} were eluted with a 10 mM to 500 mM imidazole gradient in buffer A and dialyzed against buffer B (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT).

Electrophoretic mobility shift assays. Two different EMSA methods were used in this work, the classic method using radioactive labelled DNA (Rojas et al., 2003) and a new method using fluorescein labelled DNA (Blanco et al., 2011). In both methods, phosphorylated PhoB protein was used. The protein was phosphorylated in 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT and 9 mM acetylphosphate reaction buffer at 37ºC for 60 min as previously described (McCleary, 1996). For the radioactive method, dsDNA probe containing the promoter region of the *P. aeruginosa pdtA* gene was obtained by annealing non-labelled complementary oligonucleotides (Table S3). The *pstC* and *fiuA* promoter regions were amplified by PCR using genomic DNA from *P. aeruginosa* PAO1. These DNA fragments were then end-labelled with [γ-^{32}P] deoxy-adenosinetriphosphate (ATP) using the T4 polynucleotide kinase. A 10 µl sample containing 0.002 pmols of labeled DNA (1.5×10^4 cpm) was incubated with increasing concentrations of phosphorylated PhoB and/or σ^{VreI} proteins for 20 min in binding buffer (12 mM Tris-HCl, pH 7, 23.6 mM NaCl, 0.12 M magnesium acetate, 0.24 mM EDTA, 0.24 mM DTT, 1.2% [v/v] glycerol and 2.3 mM acetylphosphate) containing 20 µg/ml of polyd(IC) and 200 µg/ml of bovine serum albumin (BSA). DNA-protein complexes were resolved by electrophoresis on 4% (w/v) non-denaturing polyacrylamide gels in Tris/Glycine buffer (512 mM Tris, 58.6 mM glycine). For the second method, a 5' end fluorescein-labelled oligonucleotide was annealed with the complementary strand (Table S3) to obtain dsDNA. In a final volume of 10 µl EMSA samples contained 0.025 nmols of fluorescein-labelled dsDNA and variable amounts of purified PhoB and/or σ^{VreI} proteins were incubated in the same binding buffer described above containing polyd(IC) but not BSA during 20 min at 37ºC. In the competition experiment, increasing amounts of an unlabelled competitor dsDNA was added to the EMSA reaction. Samples were loaded onto 8% non-denaturing polyacrylamide gels prepared in Tris/Glycine buffer and run at 50 V at room temperature. The fluorescence signal was detected on a conventional UV transilluminator and pictures were taken with the gel-recoding apparatus Minilumi bio-imaging system (Bio-Imaging Systems Ltd).

Computer-assisted analyses. Sequence analyses of the *Pseudomonas* genomes were performed at http://www.pseudomonas.com (Winsor et al., 2011) and sequence alignments with ClustalW (Thompson et al., 1994).

SUPLEMTENTAL INFORMATION

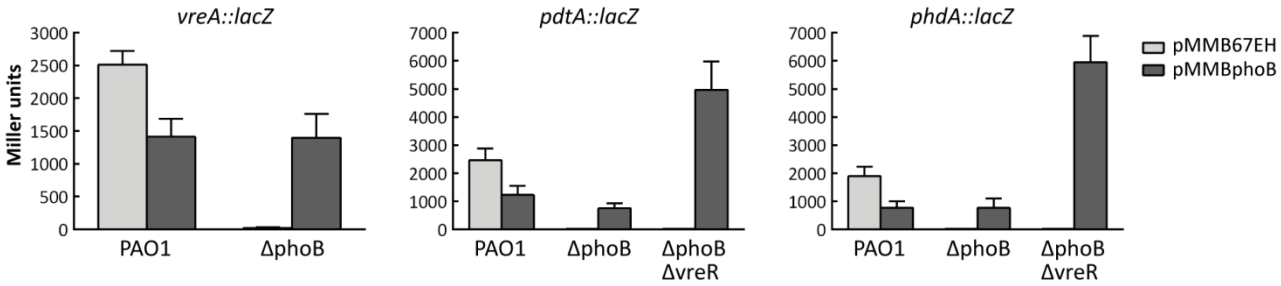

Figure S1. Complementation of the *P. aeruginosa phoB* mutants. β-galactosidase activity of the indicated *P. aeruginosa* strains bearing the *vreA*, *pdtA* or *phdA lacZ* transcriptional fusion and the pMMB67EH (empty) or pMMBphoB (containing the *phoB* gene) plasmids upon growth in Pi starvation conditions.

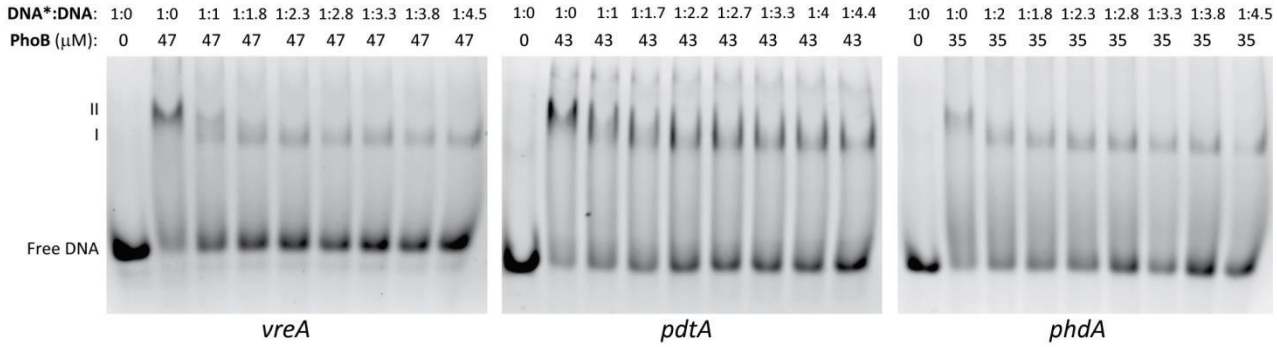

Figure S2. Competition EMSA reactions with unlabelled probes. EMSA gels using fluorescein-labelled dsDNA probes and increasing amounts of an unlabelled competitor (Table S3) containing the indicated *P. aeruginosa* promoter. Upper numbers indicate the ratio of labelled-DNA (*) and unlabelled-DNA, and the concentration of phosphorylated PhoB protein used in the assay (in μM). The position of the free DNA and of the PhoB-DNA complexes (I and II) are indicated.

Table S1. Bacterial strains and plasmids used in this study[a]

Strains or plasmid	Relevant characteristics	Reference
E. coli		
BL21 (DE3)	F⁻ *lon ompT hsdS* (r_B^- m_B^-) *gal dcm* λ(DE3)	(Jeong et al., 2009)
CC118λ*pir*	Δ(*ara-leu*) *araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1*, lysogenized with λ*pir*; Rif^R	(Herrero et al., 1990)
DH5α	*supE44 Δ*(*lacZYA-argF*)*U169* ϕ80 *lacZ*ΔM15 *hsdR17* (r_K^- m_K^+) *recA1 endA1 gyrA96 thi1 relA1*; Nal^R	(Hanahan, 1983)
P. aeruginosa		
PAO1	Wild-type strain	(Jacobs et al., 2003)

ΔphoB	Markerless PAO1 null mutant in the *phoB* (PA5360) gene	(Faure et al., 2013)
ΔvreA	Markerless PAO1 null mutant in the *vreA* (PA0674) gene	This study
ΔvreI	Markerless PAO1 null mutant in the *vreI* (PA0675) gene	(Faure et al., 2013)
ΔvreR	Markerless PAO1 null mutant in the *vreR* (PA0676) gene	This study
ΔphoB ΔvreR	Markerless PAO1 double null mutant in the *phoB* and *vreR* genes	This study
Plasmids		
pBBR1MCS-5	Broad-host range plasmid, *ori*TRK2; Gm^R	(Kovach et al., 1995a)
pBBRvreR	pBBR1MCS-5 carrying in KpnI-HindIII a 0.96-Kb PCR fragment containing the entire *P. aeruginosa vreR* (PA0676) gene; Gm^R	This study
pCR2.1-TOPO	TA cloning vector for the direct ligation of PCR products; Ap^R, Km^R	Invitrogen
pTOPO-Pr0690	pCR2.1-TOPO carrying the *P. aeruginosa pdtA* (PA0690) promoter amplified by PCR from the pMP0690 plasmid; Ap^R, Km^R	This study
pTOPO-Pr0691b	pCR2.1-TOPO carrying the *P. aeruginosa phdA* (PA0691) promoter amplified by PCR from the pMP0691b plasmid; Ap^R, Km^R	This study
pET28b(+)	Translation vector for cloning and expressing recombinant proteins in *E. coli*. Contains a 6xHis fusion tag; Km^R	Novagen
pET-phoB	pET28b(+) carrying in NdeI-BamHI a 0.69-Kb PCR fragment containing the *P. aeruginosa phoB* (PA5360) gene downstream a 6xHis tag; Km^R	This study
pET-vreI	pET28b(+) carrying in NdeI-BamHI a 0.56-Kb PCR fragment containing the *P. aeruginosa vreI* (PA0675) gene downstream a 6xHis tag; Km^R	This study
pKNG101	Gene replacement suicide vector, *ori*R6K, *oriT*RK2, *sacB;* Sm^R	(Kaniga et al., 1991)
pKΔvreA	pKNG101 carrying in XbaI-BamHI a 2.7-Kb PCR fragment containing the regions up- and downstream the *P. aeruginosa vreA* (PA0674) gene; Sm^R	This study

[a] Ap^R, Cm^R, Nal^R, Rif^R, Sm^R and Tc^R, resistance to ampicillin, chloramphenicol, nalidixic acid, rifampicin, streptomycin and tetracycline, respectively.

Table S2. Sequence of the primers used in this study

Amplified (or deleted) gene and promoter region	Plasmid/assay	Name	Sequence (5' → 3')a
vreA (PA0674)	pKΔvreA	PA0673F-X	CAT**TCTAGA**CAACCTGCTGACCAAC GAG
		ΔPUMA3R-E	CCA**GAATTC**ATTCCAGTCCGAACTA CCC
		vreIF-E	GAT**GAATTC**TGACGGAGGGAGTGG GAGGG
		C-VreRR-B	AAT**GGATCC**TCAGCCGAGCAGCACC ACC
vreI (PA0675)	pET-vreI	VreIF-Nd	ACA**CATATG**AGCGATTCGCGGCAA AGC
		PA0675R-B	TAT**GGATCC**CCTGCTTATGCTTATGA CGG
vreR (PA0676)	pKΔvreR	pUCMA3F-X	TCA**TCTAGA**GTCCACTCGTCCG
		ΔvreRR-E	ACT**GAATTC**GACTGTGCTGTACGGA CAC
		ΔvreRF-E	AAA**GAATTC**GGGGTGGTGCTGCTC GGC
		PA0678R-B	TTC**GGATCC**ATCTTCGTCGGGCACT CTG
	pMMB-VreR and pBBRvreR	PA0676F_4-Kp	GAAT**GGTACC**ATGACAGCCTCAGAC TCCGC
		PA0676R_960-H	ACAC**AAGCTT**TCAGCCGAGCAGCAC CACC
	pMMB-VreR43	PA0676F_4-Kp	GAAT**GGTACC**ATGACAGCCTCAGAC TCCGC
		PA0676R_43-H	ACA**AAGCTT**ACGCACACCACTGGCG GAAGG
	pMMB-VreR86	PA0676F_4-Kp	GAAT**GGTACC**ATGACAGCCTCAGAC TCCGC
		PA0676R_85-H	TTA**AAGCTT**AGCGACGACCGAAAC GCCTC
	pMMB-VreR110	PA0676F_4-Kp	GAAT**GGTACC**ATGACAGCCTCAGAC TCCGC
		PA0676R_110-H	TTA**AAGCTT**AGCCATGGTCGAGCAG CGACG
	pMMB/VreR-HA	PA0676Fa-E	AAA**GAATTC**ATGACAGCCTCAGACT CCGCCGCC

		CHA-PA0676-X	TTT**TCTAGA**TTAGCACGCGTAGTCC GGCACGTCGTACGGGTAGCCGAGC AGCACCACCCCGCCCGG
pdtA (PA0690)	pMP0690	PR0690F-E	TTA**GAATTC**TCATGAGCGCCTTCATC ACTGGTA
		PR0690R-X	TAA**TCTAGA**AACGCTGCAACTGCTG GTTGA
	pTOPO-Pr0690	PR0690 F-E	TTA**GAATTC**TCATGAGCGCCTTCATC ACTGGTA
		MCS220R	ATCAACGGTGGTATATCC
	5' RACE	GSP1B-0690	GAACATCCTGCGGGAGATTC
		GSP2-0690	TGCTGGTTGACCCGCTGCTGCT
	Primer extension	PA0690R	GACGGCGAACGGGACGGCA
phdA (PA0691)	pTOPO-Pr0691b	PR0691F2Bg	TAA**AGATCT**GATCGCCGCGCTGTTC CCCGAG
		MCS220R	ATCAACGGTGGTATATCC
	5' RACE	GSP1-0691	CCGACGAACCAGCGATACAG
		GSP2-0691	CGCCCCCTGCCAATCTTCCTG
	Primer extension	PA0691R	CGTATTCACCGGAGTGACCA
phoB (PA5360)	pET-phoB	PhoBF-Nd	TAG**CATATG**GTTGGCAAGACAATCC TC
		PhoBR-B	AAC**GGATCC**TCAGCTCTTGGTGGAG AAACG
	pMMB-phoB	1147F-E	AGAG**GAATTC**AACCTGTTGAGCATA GCTC
		1148R-H	AGAG**AAGCTT**TCAGCTCTTGGTGGA GAAAC

[a] The sequences of the restriction sites are indicated in bold

Table S3. Oligonucleotides used in the EMSA reactions

Name	Sequence (5' → 3')[a]
Flu-PvreAF	[Flc]ACCGCAGGTACCGTCACACCACAGTCACACAGTGCCATCAGGATGTCCCTCCTGGGTGATGGCCATCTGG
PvreAF	ACCGCAGGTACCGTCACACCACAGTCACACAGTGCCATCAGGATGTCCCTCCTGGGTGATGGCCATCTGG
PvreAR	CCAGATGGCCATCACCCAGGAGGGACATCCTGATGGCACTGTGTGACTGTGGTGTGACGGTACCTGCGGT
Flu-PpdtAF	[Flc]CGCTCCGCTCCATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA
PpdtAF	CGCTCCGCTCCATGAACTTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA
PpdtAR	TTACTGGTAGACGGCCTGGCGGAGGGTGGGCCGAAGACTTGTCATGGAAAGTTCATGGAGCGGAGCG
Flu-PpdtAmutF1	[Flc]CGCTCCGCTC**ACCACCA**TTTCCATGACAAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA
PpdtAmutR1	TTACTGGTAGACGGCCTGGCGGAGGGTGGGCCGAAGACTTGTCATGGAAATGGTGGTGAGCGGAGCG
Flu-PpdtAmutF2	[Flc]CGCTCCGCTC**ACCACCA**TTTC**TGGTGT**AAGTCTTCGGCCCACCCTCCGCCAGGCCGTCTACCAGTAA
PpdtAmutR2	TTACTGGTAGACGGCCTGGCGGAGGGTGGGCCGAAGACTTACACCAGAAATGGTGGTGAGCGGAGCG
Flu-PphdAF	[Flc]CGGCCGGTCGCATGAAGTTTTCATGACAAAAGTTCGGTGGTGCGGCGGGGTTGCCGTCAAACAGGTGT
PphdAF	CGGCCGGTCGCATGAAGTTTTCATGACAAAAGTTCGGTGGTGCGGCGGGGTTGCCGTCAAACAGGTGT
PphdAR	ACACCTGTTTGACGGCAACCCCGCCGCACCACCGAACTTTTGTCATGAAAACTTCATGCGACCGGCCG

[a]Flc indicates fluorescein. The *pho box* is underlined and nucleotide substitutions relative to the wild-type sequence are in bold.

Chapter 3

The alternative sigma factor σ^{Vrel} is active during infection and contributes to phosphate starvation-induced virulence of *Pseudomonas aeruginosa*

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ABSTRACT

Extracytoplasmic function sigma (σ^{ECF}) factors are the largest and most diverse group within the σ^{70} family of σ factors. These proteins bind to the RNA polymerase (RNAP) and direct it to the promoter of target genes initiating transcription. σ^{ECF} factors are normally produced with an anti-σ factor that inhibits the binding of the σ^{ECF} to the RNAP. This inhibitory interaction is suppressed in response to a specific inducing signal allowing transcription of signal responsive genes. Signaling mediated by σ^{ECF}/anti-σ factor pairs is a fundamental mechanism for bacterial colonization of the environment and the host. The human pathogen *Pseudomonas aeruginosa* contains nineteen σ^{ECF} factors, including σ^{Vrel} that promotes transcription of virulence determinants. σ^{Vrel} is produced together with the anti-σ factor VreR under phosphate (Pi) starvation, a physiological condition often encountered in the host that increases *P. aeruginosa* pathogenicity. σ^{Vrel} is basally active under Pi limitation but evidence indicates that an additional signal is required for complete σ^{Vrel} activation. Using zebrafish embryos and the A549 cell line as *P. aeruginosa* hosts, we demonstrate in this work the activation of σ^{Vrel} *in vivo* during infection and its contribution to the Pi starvation-induced virulence of this pathogen.

INTRODUCTION

The human pathogen *Pseudomonas aeruginosa* causes a wide array of life-threatening acute and chronic infections, particularly in immunocompromised and cystic fibrosis (CF) patients (Lyczak et al., 2000; Sadikot et al., 2005; Gellatly and Hancock, 2013). This bacterium is moreover one of the leading causes of nosocomial infections affecting hospitalised patients (Buhl et al., 2015). Treatment of *P. aeruginosa* infections can be particularly challenging due to the natural resistance of this bacterium to antibiotics and to its ability to acquire new resistances (Lister et al., 2009; Breidenstein et al., 2011). *P. aeruginosa* infections are thus a serious threat to human health worldwide and the World Health Organization has declared this bacterium as the second priority pathogen for research and development of new antibiotics(WHO, 2017). To achieve this aim, a further knowledge of the *P. aeruginosa* pathogenicity is essential.

P. aeruginosa is able to colonize several different environments within the host, which is facilitated by its metabolic versatility and the multiple virulence factors this bacterium contains (Lyczak et al., 2000; Bleves et al., 2010; Klockgether and Tummler, 2017). Moreover, this bacterium produces many global regulators and signalling systems that form complex regulatory networks responsible of phenotypic adaptation in changing environments and virulence (Jimenez et al., 2012; Balasubramanian et al., 2013). Gene expression in bacteria is regulated primarily at the level of transcription initiation by modifying the affinity of the RNA polymerase (RNAP) for the DNA. The first step of such modification occurs through the substitution of the sigma (σ) subunit of the RNAP, which is the subunit that contains most promoter recognition determinants and thus confers promoter specificity to the RNAP (Ishihama, 2000). The second step is orchestrated by transcriptional regulators that enhance or repress RNAP activity and thus gene transcription. *P. aeruginosa* contains a plethora of these regulatory proteins. Besides a primary σ factor (i.e. σ^{70}) that promotes transcription of general functions, *P. aeruginosa* produces several alternative σ factors that promote expression of genes that are necessary only under specific conditions. Of those, the σ^{ECF} form the most numerous group and are among the most important signaling proteins in *P. aeruginosa* (Llamas et al., 2014; Chevalier et al., 2018). Activity of most *P. aeruginosa* σ^{ECF} is controlled through an inhibitory interaction with a co-transcribed anti-σ that prevents the binding of the σ^{ECF} to the RNAP in the absence of the inducing stimulus. Therefore, the functional unit of the *P. aeruginosa* σ^{ECF}-mediated signaling pathway is normally formed by the σ^{ECF}/anti-σ factor pair and σ^{ECF} activation in response to the stimulus requires the targeted proteolysis of the anti-σ factor (Qiu et al., 2007; Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a).

P. aeruginosa σ^{ECF} factors control important processes required during infection such as iron acquisition, cell envelope stress response, production of the exopolysaccharide alginate, and synthesis of virulence factors (Llamas et al., 2014; Chevalier et al., 2018). Most *P. aeruginosa* σ^{ECF} respond to and stimulate the acquisition of iron quelating compounds, including siderophores, heme/hemophore and iron-citrate (Llamas et al., 2014; Chevalier et al., 2018). These σ^{ECF} are produced together with an anti-σ factor under iron starvation and respond to the extracellular presence of the iron quelating compound by a signal transduction cascade known as cell-surface signalling (CSS) that also involves a TonB-dependent outer membrane receptor (TBDR) (Llamas et al., 2014). CSS receptors have a dual function both in signal transduction, which is mediated by the N-terminal signalling domain of the receptor that interacts with the anti-σ factor, and in the transport of the inducing signal that occurs through the C-terminal β barrel domain (Fig. S1A) (Llamas et al., 2014). Because iron is involved in essential cellular processes ranging from DNA replication to important metabolic reactions, acquisition of this metal is key for bacterial survival and thus for the development of a successful infection (Becker

and Skaar, 2014). Apart from their role in virulence through control of iron acquisition during infection, several *P. aeruginosa* iron starvation σ^{ECF} also stimulate the transcription of virulence determinants, like for example σ^{PvdS} that promotes production of exotoxin A, the PrpL and AprA secreted proteases, and several type III secretion system (T3SS) toxins (Llamas et al., 2014; Chevalier et al., 2018). Otherwise, the *P. aeruginosa* σ^{AlgT}, σ^{SigX} and σ^{SbrI} factors are activated in response to cell envelope stress and stimulate expression of genes involved in peptidoglycan and membrane homeostasis to maintain the integrity of the cell envelope (Chevalier et al., 2018). Moreover, these σ factors affect the production of several virulence determinants including secretion systems and secreted proteases (Chevalier et al., 2018). Especially σ^{AlgT} plays a key role in *P. aeruginosa* virulence by expressing, among others, the genes required for production of the exopolysaccharide alginate (Wood and Ohman, 2012), which is responsible of the *P. aeruginosa* mucoid phenotype that leads to the establishment of chronic infection and produces airways inflammation (Lyczak et al., 2002). Activation of these stress responsive σ factors is controlled by an anti-σ factor but not by a TBDR (Chevalier et al., 2018).

The *P. aeruginosa* σ^{Vrel}, which is encoded within the *vreAIR* operon together with a receptor-like protein (VreA) and an anti-σ factor (VreR) (Fig. 1), also controls *P. aeruginosa* virulence (Llamas et al., 2009). This σ factor shows analogy with iron starvation σ^{ECF} but also important differences, including the nature of the receptor-like component VreA (Fig. S1A) (Llamas et al., 2009; Llamas et al., 2014). VreA contains the N-terminal signaling domain that interacts with the anti-σ factor in the CSS signal transduction cascade, but does not contain the C-terminal β barrel that mediates the transport of the CSS inducing signal (Fig. S1A) (Llamas et al., 2009). Moreover, σ^{Vrel} is not produced under iron limitation but under phosphate (Pi) starvation, and its activity is modulated not only by the VreR anti-σ factor but also by the phosphate transcriptional regulator PhoB, which recruits the σ^{Vrel}-RNAP complex to the promoter region of the σ^{Vrel} regulon genes (Fig. S1B) (Quesada et al., 2016). Our earlier analyses showed that σ^{Vrel} stimulates expression of *P. aeruginosa* virulent determinants, including secretion systems and secreted proteins (Fig. 1), and that constitutive activation of this σ factor increases *P. aeruginosa* virulence (Llamas et al., 2009). Antibodies directed against the secreted proteins of the σ^{Vrel} regulon (i.e. PdtA, Fig. 1) are detected in the serum of most *P. aeruginosa* infected patients (Llamas et al., 2009), which suggests that this regulon is transcribed *in vivo* during infection and thus that σ^{Vrel} is active in this condition. This is also suggested by reports showing that interaction of *P. aeruginosa* with host cells induces the expression of several σ^{Vrel} regulon genes (Frisk et al., 2004; Chugani and Greenberg, 2007). However, direct evidence showing the activation of σ^{Vrel} *in vivo* during infection has not been reported yet. Expression of the *vreAIR* operon occurs under Pi starvation (Quesada et al., 2016), a condition often encountered by pathogens in the host environment that is known to induce a virulent phenotype in *P. aeruginosa* (Zaborin et al., 2009). A basal activity of σ^{Vrel} is observed under Pi limitation; however, maximal activity of this σ factor requires the removal of the VreR anti-σ factor, which does not occur under Pi starvation (Quesada et al., 2016). Because activation of *P. aeruginosa* σ^{ECF} factors in response to the inducing signal occurs through the regulated proteolysis of the anti-σ factor (Qiu et al., 2007; Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a), another signal is expected to be required for complete σ^{Vrel} activation. Therefore, we hypothesized that σ^{Vrel} is activated during infection by a host-derived condition and contributes to the Pi starvation-induced virulence of *P. aeruginosa*, which we show in this work.

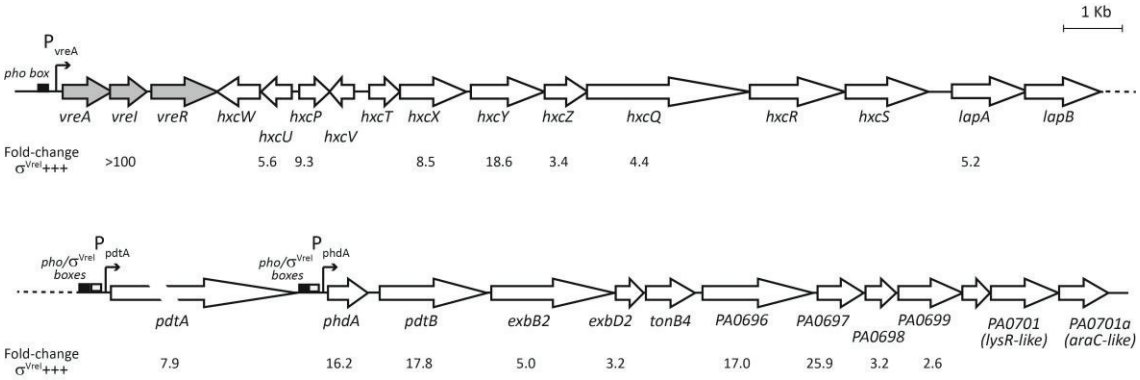

Fig. 1. Genetic organization of the σ^Vrel regulon. Transcriptional organization of the *vreAIR* locus (grey) and the downstream σ^Vrel-regulated genes (white). Big arrows represent the different genes, their relative sizes, and their transcriptional orientation, with the name of the gene or the PA number (http://www.pseudomonas.com/) indicated below the arrow. The promoters and regulatory boxes identified within this locus are indicated (Ball et al., 2002; Faure et al., 2013; Quesada et al., 2016). Numbers indicate the fold-change in the expression of the gene in cells overproducing σ^Vrel as determined earlier by microarray (Llamas et al., 2009). The *hxc* genes encode for a type II secretion system involved in the secretion of the low molecular weight alkaline phosphatase LapA (Ball et al., 2002). *pdtA* and *pdtB* encode a functional two-partner secretion (TPS) system involved in *P. aeruginosa* virulence in the *C. elegans* model (Faure et al., 2014). *phdA* encodes a homologue of the prevent-host-death (Phd) protein family and is required for biofilm formation and eDNA release (Petrova et al., 2011). *exbB2-exbD2-tonB4* encode a still uncharacterized putative TonB system. The function of the PA0696-PA0700 gene products is still unknown. PA0701 encode for a putative LysR-like transcriptional regulator and PA0701a, which is not annotated in the PAO1 genome but it is in the *P. aeruginosa* PA14 genome, encodes for a putative AraC-like transcriptional regulator.

RESULTS

σ^Vrel and VreR are required for P. aeruginosa *virulence in zebrafish embryos*

The virulence of *P. aeruginosa* was assayed using zebrafish (*Danio rerio*) embryos as host. *P. aeruginosa* is able to lethally infect zebrafish embryos when the amount of cells injected exceeds the phagocytic capacity of the embryo (Anne Clatworthy et al., 2009; Llamas et al., 2009; Llamas and van der Sar, 2014). Since the expression of the *vreAIR* operon is induced under phosphate (Pi) starvation (Faure et al., 2013; Quesada et al., 2016), we first determined the effect of the Pi concentration in *P. aeruginosa* virulence. One-day old embryos were injected into the blood stream to generate a systemic infection with *P. aeruginosa* cells previously grown either in low or high Pi conditions and embryo survival was monitored during five days. Survival was considerable lower upon injection of PAO1 wild-type cells grown in low than in high Pi conditions ($P<0.001$) (Fig. 2A), which indicates that Pi starvation induces a *P. aeruginosa* virulent phenotype. The contribution of σ^Vrel to this phenotype was determined using a null Δvrel mutant. The virulence of this mutant was significantly lower than that of the PAO1 strain ($P<0.05$) (Fig. 2B), which suggests that σ^Vrel contributes to the low Pi-induced virulence of *P. aeruginosa*. Unexpectedly, a ΔvreR anti-σ factor mutant in which σ^Vrel is highly active(Quesada et al., 2016) also presented attenuated virulence ($P<0.001$) (Fig. 2B). This prompts that VreR may have more functions in *P. aeruginosa* than just controlling σ^Vrel activity. In contrast, the absence of the receptor-like protein VreA did not have any effect on *P. aeruginosa* virulence ($P=0.35$) (Fig. 2B), which suggests that this protein is not involved in the σ^Vrel/VreR-mediated virulence of this bacterium.

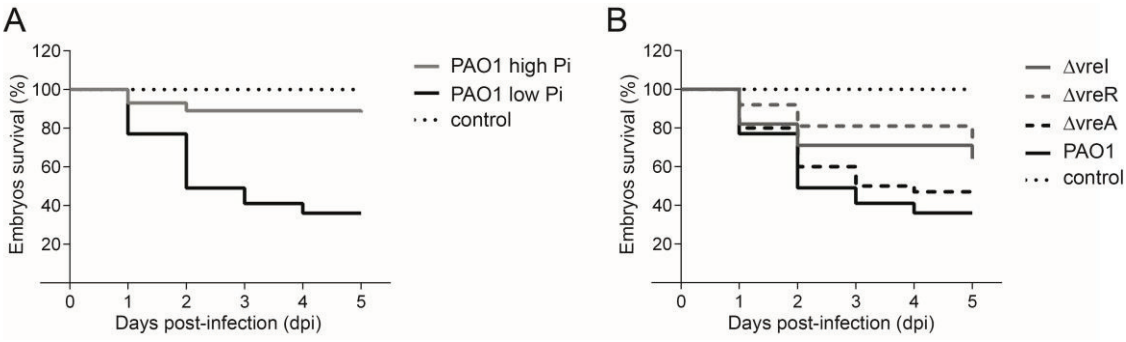

Fig. 2. Klapan-Meier survival curves of zebrafish embryos upon infection with *P. aeruginosa*. One day-old embryos were injected with ~1000 colony forming units (CFU) of the *P. aeruginosa* PAO1 wild-type strain grown either in Pi-restricted or Pi-sufficient conditions **(A)** or with the indicated PAO1 isogenic mutant grown under Pi starvation **(B)**. Uninfected control (non-injected) is shown. Data represents the mean of four biologically independent replicates (N=4) with 20 embryos/group in each replicate.

σ^VreI and VreR are required for P. aeruginosa *cytotoxicity.*

P. aeruginosa often invades the respiratory tract of the human being. Therefore, we used the A549 human respiratory epithelial cell line as *P. aeruginosa* host. First we assayed the cytotoxicity of *P. aeruginosa* toward the eukaryotic cells by determining A549 cell viability after co-incubation with the bacteria. Similar to the results obtained in the zebrafish embryos, we observed that the growth of *P. aeruginosa* under Pi starvation increases the bacterial cytotoxicity as the eukaryotic cells were more damaged by bacteria grown in low than in high Pi medium (Fig. 3A). Both σ^VreI and VreR contribute to this phenotype since mutants lacking these proteins were significantly less efficient in damaging the A549 cells than the PAO1 wild-type strain (Fig. 3A).

We also measured internalization of *P. aeruginosa* into A549 cells. Although considered an extracellular pathogen, *P. aeruginosa* is able to enter into non-phagocytic host cells such as epithelial cells (Chi et al., 1991; Engel and Eran, 2011). The internalization efficiency of a given strain depends on several factors including the T3SS profile (Wolfgang et al., 2003; Wareham and Curtis, 2007). Strains that are more efficient in internalizing are less cytotoxic while less invasive strains kill the eukaryotic cells more rapidly. Therefore, there is an inverse correlation between internalization and cytotoxicity, and thus between internalization and acute virulence (Fleiszig et al., 1996). Upon *P. aeruginosa* infection of A549 cells, we observed a ~3-fold increase in the internalization capacity of the ΔvreI and ΔvreR mutants with respect to the PAO1 wild-type strain (Fig. 3B). This is consistent with the lower cytotoxicity displayed by these mutants (Fig. 3A) and with the impaired capacity of these mutants to produce a systemic infection in zebrafish embryos (Fig. 2B). All together these results suggest that σ^VreI and VreR are required during *P. aeruginosa* acute infections.

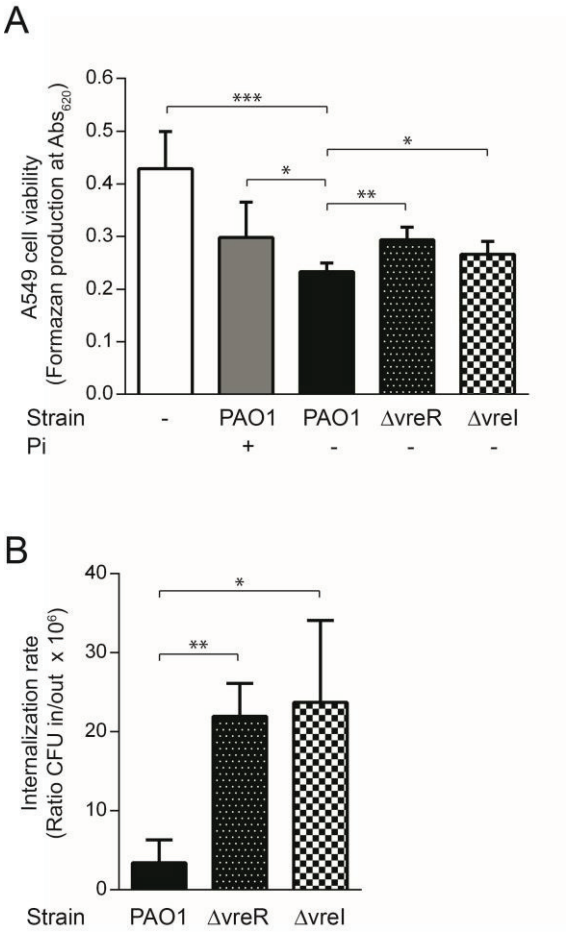

Fig. 3. *P. aeruginosa* infections in the A549 cell line. (A) A549 cell viability. The *P. aeruginosa* PAO1 wild-type strain and the indicated isogenic mutant were grown in Pi-restricted (-) or Pi-sufficient (+) conditions prior to infection. Formazan production upon addition of the MTT tretazolium salt was determined spectrophotometrically at 620 nm. Uninfected cells (white bar) were used as control. (**B**) *P. aeruginosa* internalization into A549 cells. A549 cells were infected with the indicated *P. aeruginosa* strain previously grown in in Pi-restricted conditions. Internalization is reported as the ratio between bacteria CFU inside (in) the A549 cells and CFU in the culture supernatant (out). In both panels data are means ± SD from three biological replicates (N=3). P-values were calculated by unpaired two-tailed *t*-test as described in Materials and Methods and brackets indicate the comparison to which the P-value applies.

σ^Vrel is activated in vivo *during infection.*

To analyze whether σ^Vrel is activated *in vivo*, we used zebrafish embryos and A549 cells as hosts, and a σ^Vrel-dependent fluorescent construct in which the promoter of the σ^Vrel regulated gene *pdtA* (Fig. 1) was cloned in front of a red fluorescent protein (*rfp)* gene (Table S1). *P. aeruginosa* strains were grown in high Pi conditions to avoid σ^Vrel expression and activation prior to infection (Quesada et al., 2016). Bacteria were injected in the hindbrain region of the zebrafish embryos (Fig. 4A) to produce an infection that remains initially localized, which facilitates fluorescence measurements. At 0 hours post-infection (hpi), red fluorescence was undetectable, indicating that there was no transcription from the σ^Vrel-dependent *pdtA* promoter and thus that σ^Vrel is not active (Fig. 4B, red channel). At this early infection point there was no neutrophil recruitment in the hindbrain because of the absence of a developed infection (Fig. 4B, green channel). However, at 12 hpi embryos injected with both the PAO1 wild-type strain and the Δvrel mutant were clearly infected as is evident from the recruitment of neutrophils in the head of the embryos (Fig. 4B, green channel) and the formation of necrotic

tissue within this area (not shown). Importantly, at this time point red fluorescence was observed in the embryos infected with the wild-type but not with the ΔvreI mutant (Fig. 4B, red channel). This shows that *pdtA* expression occurs during infection in a σ^VreI/VreR-dependent manner and therefore suggests that this σ factor is expressed and active in these conditions.

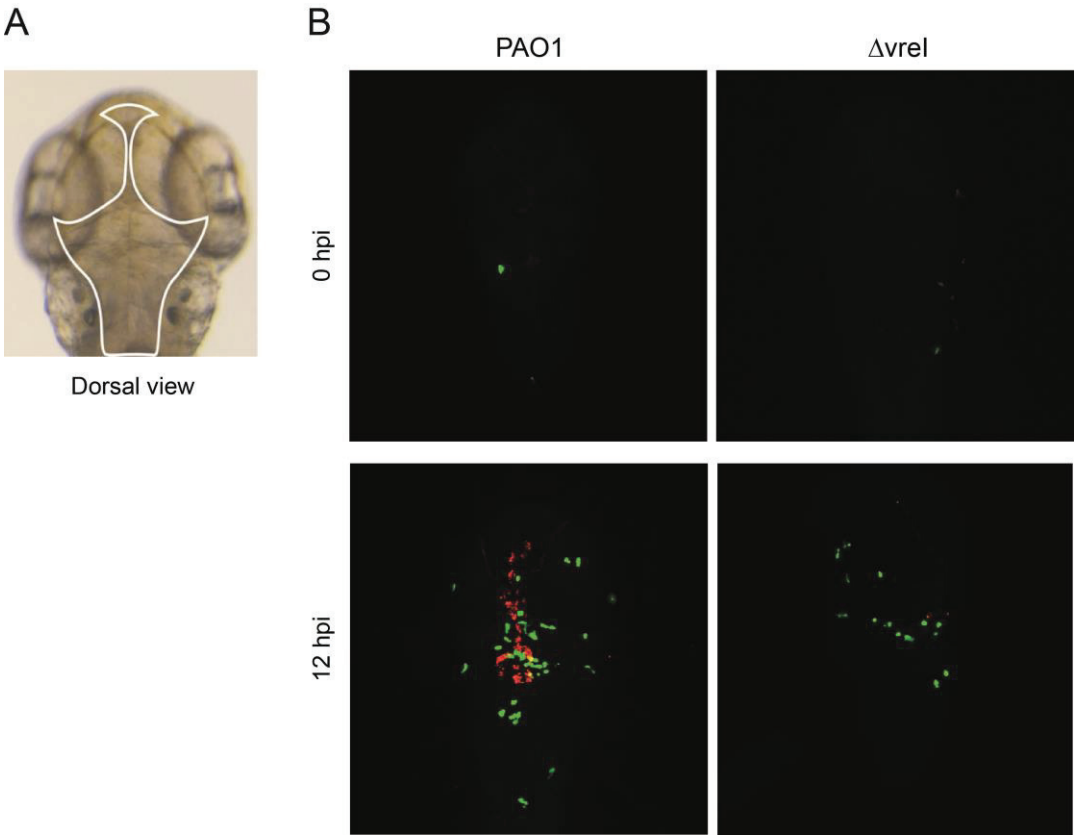

Fig. 4. σ^VreI activation during *P. aeruginosa* infection in zebrafish embryos. (A) Dorsal view of the head of a two-days old zebrafish embryo. The hindbrain region were *P. aeruginosa* was injected is highlighted. (**B**) Confocal images of the head of two-days old embryos injected in the hindbrain region with 2000 CFUs of the *P. aeruginosa* PAO1 wild-type strain or its isogenic ΔvreI mutant bearing the σ^VreI-dependent *pdtA::rfp* transcriptional fusion (pMP0690mCherry plasmid, Table S1) (red channel) at 0 and 12 hpi. *P. aeruginosa* was grown in high Pi medium prior injection. Neutrophils expressing constitutively a green fluorescent protein (GFP) are also visualized (green channel).

We also analyzed σ^VreI activation upon interaction of *P. aeruginosa* with the A549 human lung epithelial cell line. Green fluorescence-labelled *P. aeruginosa* strains bearing the σ^VreI-dependent *pdtA::rfp* transcriptional fusion were inoculated in A549 cultures. As control, bacteria were also inoculated in cell-free cultures. At 0 hpi, red fluorescence was undetectable (Fig. 5A). However, at 10 hpi red fluorescence was observed in the PAO1 wild-type cells and was considerably higher in the A549-containing cultures than in the A549-free cultures ($P<0.01$) (Fig. 5A and 5B). In contrast, fluorescence remained undetectable in the ΔvreI mutant independently of the presence or absence of the A549 epithelial cells (Fig. 5A). Together this suggests that the expression of *pdtA* is induced by the presence of the human cells and that this induction depends on the σ^VreI-VreR signaling cascade. To confirm this result, we measured *pdtA* expression by qRT-PCR after PAO1 inoculation in A549-free and A549-containing cultures. The cycle threshold (ct) value of a total of eight biologically independent co-incubations was determined (Fig. 6). In

A549-containing cultures the expression of *pdtA* increased between 3- and 40-fold compared to the cell-free cultures (Fig. 6). This confirms that expression of *pdtA* is induced upon contact of *P. aeruginosa* with the eukaryotic cell. Altogether, these results show that σ^VreI is active during infection, and suggests the presence of a host-derived signal triggering its activation.

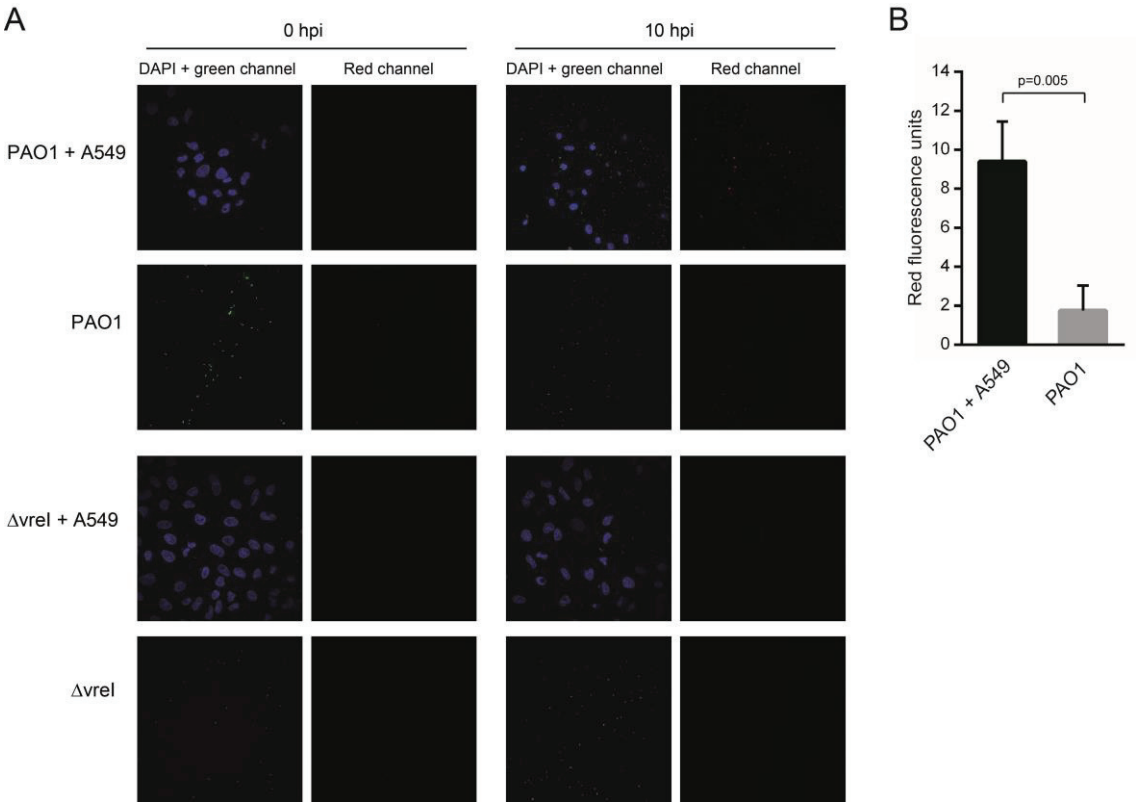

Fig. 5. Activation of σ^VreI upon interaction of *P. aeruginosa* with A549 eukaryotic cells. (A) Confocal images of *P. aeruginosa*-A549 co-cultures at 0 and 10 hpi. Green-fluorescent labelled (pBBRmEos3.1 plasmid, Table S1) *P. aeruginosa* PAO1 wild-type strain and its isogenic ΔvreI mutant containing the σ^VreI-dependent *pdtA::rfp* transcriptional fusion (pMP0690mCherry plasmid, Table S1) (red channel) were grown in high Pi medium and inoculated in A549-containing and A549-free cultures. A549 cells DNA was stained with DAPI (blue channel). Images are representative of three independent experiments (N=3). **(B)** Quantification of the red fluorescence intensity observed in (A) was performed as described in Materials and Methods, and the total corrected cellular fluorescence (TCCF) is given. Data are means ± SD from three biological replicates (N=3). P-value was calculated by unpaired two-tailed *t*-test.

VreR influences P. aeruginosa *gene expression also in a σ^VreI-independent manner*

The phenotype of the ΔvreR mutant in which reduced instead of increased virulence was observed prompted us to analyse gene expression in this mutant by RNA-seq. We compared the transcriptome of the *P. aeruginosa* PAO1 wild-type strain with that of the ΔvreR mutant upon bacterial growth in Pi starvation, a condition known to induce *vreR* expression (Quesada et al., 2016). A total of forty-six transcripts were more abundant in the ΔvreR mutant than in the PAO1 wild-type strain, and nine were less abundant (including the *vreR* transcript) (Table 1). Increased expression of some of these genes in the ΔvreR mutant was confirmed by qRT-PCR (Fig. S2). Of the forty-six upregulated genes, nineteen are located immediately downstream to the *vreAIR* locus and most of them belong to the σ^VreI regulon previously identified by microarray of cells overproducing σ^VreI (Fig. 1). Other upregulated transcripts belong to genes located in different loci widespread in the *P. aeruginosa* PAO1 genome (Table 1). These include genes encoding

metabolic and energy obtaining functions, transport, and several regulators of gene expression (Table 1).

Table 1. Differentially expressed *P. aeruginosa* genes in ΔvreR versus PAO1

ORF	Gene name	Function and reference[a]	Fold-change[b]
PA0141		Polyphosphate kinase 2 (PPK2) (utilizes poly P to make GTP, which is needed for the synthesis of alginate) (Ishige et al., 2002)	2.3
PA0200		Unknown function	5.9
PA0677	*hxcW*	Hxc T2SS (Ball et al., 2002)	19.1
PA0680	*hxcV*	Hxc T2SS (Ball et al., 2002)	7.6
PA0681	*hxcT*	Hxc T2SS (Ball et al., 2002)	15.9
PA0682	*hxcX*	Hxc T2SS (Ball et al., 2002)	17.7
PA0683	*hxcY*	Hxc T2SS (Ball et al., 2002)	25.8
PA0685	*hxcQ*	Hxc T2SS (Ball et al., 2002)	13.5
PA0688	*lapA*	Low-molecular-weight alkaline phosphatase A, secreted by the Hxc T2SS (Ball et al., 2002)	54.6
PA0690	*pdtA*	TPS partner A, large secreted exoprotein (Faure et al., 2014)	
PA0691	*phdA*	Prevent-host-death protein A, involved in biofilm formation (Petrova et al., 2011)	17.8
PA0692	*pdtB*	TPS partner B, outer membrane protein involved in *pdtA* secretion (Faure et al., 2014)	25.4
PA0693	*exbB2*	ExbB proton channel	19.9
PA0694	*exbD2*	ExbD protein family	22.0
PA0695	*tonB4*	TonB energy protein	26.6
PA0696		Unknown function, putative outer membrane porin	18.7
PA0697		Unknown function, structural homology with channel-forming colicins	31.5
PA0698		Putative sensory transduction regulator of the YbjN protein family	30.0
PA0699		Probable peptidyl-prolyl cis-trans isomerase, PpiC-type	24.1
PA0701		Probable LysR-type transcriptional regulator	15.9
PA0701a		Probable AraC-type transcriptional regulator	4.1
PA1196	*ddaR*	σ^{54} dependent transcriptional regulator, regulates methylarginine degradation (Lundgren et al., 2017)	2.3
PA1414		Unknown function	3.7

PA1429		Probable cation-transporting P-type ATPase	2.9
PA1546	*hemN*	Oxygen-independent coproporphyrinogen-III oxidase (heme biosynthesis) (Rompf et al., 1998)	4.6
PA1556	*ccoO2*	Cytochrome c oxidase, cbb3-type, CcoO subunit (energy metabolism, electron transport)	5.3
PA1673		Probable bacteriohemerythrin (non-heme diiron oxygen transport proteins)	5.4
PA1746		Unknown function. Orthologue to Appr-1-p (ADP-ribose-1"-monophosphate) processing protein	3.5
PA2753		Unknown function	2.8
PA2819.1		tRNA-Gly	
PA3278		Unknown function	2.4
PA3305.1	*phrS*	Non-coding RNA involved in quorum sensing regulation (Sonnleitner et al., 2011)	6.9
PA3337	*rfaD*	ADP-L-glycero-D-manno-heptose-6-epimerase	5.2
PA3458		Probable transcriptional regulator of the MarR family	2.3
PA3880		Unknown function	4.1
PA4067	*oprG*	Outer membrane porin, transport of cations and small aminoacids (Kucharska et al., 2015)	7.4
PA4159	*fepB*	Ferrienterobactin-binding periplasmic protein (Chu et al., 2014)	2.7
PA4280.5		16S ribosomal RNA	
PA4348		Unknown function, contains a metallo-beta-lactamase domain	8.3
PA4358	*feoB*	Fe^{2+} transporter (Seyedmohammad et al., 2016)	5.7
PA4359	*feoA*	Fe^{2+} transporter, probable activator of FeoB (Seyedmohammad et al., 2016)	11.9
PA4577		Unknown function, probable TraR/DksA family transcriptional regulator	3.0
PA4610		Unknown function, probable copper export protein	2.4
PA5027	*dadA*	Universal stress (UspA)-like protein	5.1
PA5427	*adhA*	Alcohol dehydrogenase	3.9
PA5475		Unknown function, N-acetyltransferase domain	7.0
PA0676	*vreR*	Anti-σ factor (Quesada et al., 2016)	-887.0
PA0878		Unknown function	-2.5

PA2356	*msuD*	Methanesulfonate sulfonatase (sulfur metabolism)(Kertesz et al., 1999)	-3.5
PA2357	*msuE*	NADH-dependent FMN reductase (sulfur metabolism)(Kertesz et al., 1999)	-4.1
PA3510		Unknown function	-2.2
PA4022	*hdhA*	Hydrazone dehydrogenase (Taniyama et al., 2012)	-2.0
PA4280.2		23S ribosomal RNA	-14.8
PA4690.2		23S ribosomal RNA	-4.2
PA4690.5		16S ribosomal RNA	-4.2

[a]T2SS, type II secretion system; TPS, two partner secretion

[b]Minus indicates that expression of the gene is downregulated in ΔvreR

We then wondered whether the increased expression of these genes in the ΔvreR mutant was due to σ^{VreI}, which is highly abundant and active in this mutant (Quesada et al., 2016). Therefore, we compared the relative expression of these genes in the ΔvreR mutant with that of the ΔvreI mutant in which *vreR* is also not produced (Quesada et al., 2016) (referred as a ΔvreI *vreR* mutant in Fig. 7). A total of twenty-five genes were selected, eight known to belong to the σ^{VreI} regulon (Fig. 1), which, as expected, were expressed considerably less in the ΔvreI *vreR* mutant than in ΔvreR (Fig. 7). This shows that the high expression of these genes in the ΔvreR mutant directly depends on σ^{VreI}. The relative expression of eight genes (PA0141, *ccoO2*, PA1673, PA1746, PA3880, PA4348, *dadA* and *adhA*) was similar in both mutants (Fig. 7) which indicates that their expression is not affected by the absence of σ^{VreI} and therefore that this σ factor is not involved in their transcription. The expression of nine other genes (PA0200, *ddaR*, PA1414, *hemN*, *phrS*, *rfaD*, *oprG*, *feoA* and PA5475) was higher in the ΔvreI *vreR* mutant that in ΔvreR (Fig. 7). This suggests that σ^{VreI} not only does not mediate the transcription of these genes but impairs their expression when it is present and active in the cell.

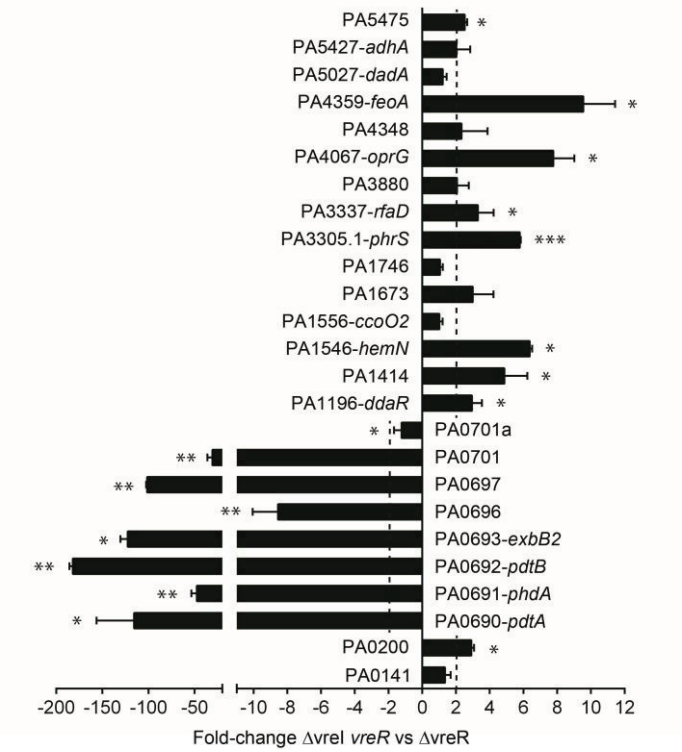

Fig. 7. Differential gene expression in the *P. aeruginosa* ΔvreR and ΔvreI *vreR* mutants. mRNA levels of the indicated genes were obtained by qRT-PCR upon growth of the *P. aeruginosa* mutants in low Pi medium. The $2^{-\Delta\Delta CT}$ method was used to determine the fold-change range in gene expression in ΔvreI *vreR* versus ΔvreR. Data are means ± SD from three biological replicates (N=3) each one including three technical replicates. P-values were calculated by one-sample *t*-test to a hypothetical value of 1 as described in Materials and Methods.

DISCUSSION

Pi starvation has been described as a signal of host weakness for pathogens. During surgical operations and also in severe burn patients the reabsorption of Pi from the kidneys is reduced and exudative losses are higher than normal producing hypophosphatemia, which can increase upon treatments with bisphosphonates or antivirals (Berger et al., 1997; Cohen et al., 2004; Datta et al., 2007). During respiratory alkalosis induced by sepsis or mechanical respiration, a redistribution of the Pi into cells occurs and this reduces the extracellular content of Pi (Bugg and Jones, 1998). Increased concentration of circulating catecholamines, which occurs in CF patients (Elborn et al., 1993), has been also associated with hypophosphatemia (Bugg and Jones, 1998). Evolution has benefited pathogens that are able to recognize this marker and react virulently (Lamarche et al., 2008), taking advantage of the lower activity of the host immune system in this condition (Marinella, 2003). *P. aeruginosa* recognizes low Pi environments through the phosphate-specific ABC transport Pst system, which under Pi starvation mediates Pi transport and activates the PhoR-PhoB two-component system (Lamarche et al., 2008). Upon activation, the PhoR histidine kinase promotes phosphorylation of the DNA-binding response regulator PhoB, which induces transcription of the so-called *pho* regulon that in *P. aeruginosa* includes multiple virulence factors (Lamarche et al., 2008). It is therefore not surprising that Pi starvation enhances *P. aeruginosa* lethality in zebrafish embryos (Fig. 2A), as also observed in mice and nematodes (Long et al., 2008; Zaborina et al., 2008; Zaborin et al., 2009; Zaborin et al., 2012), while providing excess of Pi reduces virulence (Fig.

2A). The *pho* regulon includes the *vreAIR* operon and the σ^{VreI} regulon (Fig. 1 and Fig. S1B)(Quesada et al., 2016), and we have shown here that VreR and σ^{VreI} are required for the low Pi-induced *P. aeruginosa* virulence (Fig. 2B and 3). The observed reduced virulence of the *vreI* mutant is in line with previous results showing that overproduction of this σ factor considerably increases *P. aeruginosa* pathogenicity (Llamas et al., 2009). Surprisingly, the absence of the anti-σ factor VreR resulted in a similar phenotype (Fig. 2B), despite the fact that σ^{VreI} is considerably active in absence of VreR (Quesada et al., 2016). Our transcriptomic analyses have revealed that VreR influences gene expression not only in a σ^{VreI}-dependent but also in a σ^{VreI}-independent manner. This includes several functions that could affect *P. aeruginosa* virulence, and several regulators of gene expression (Table 1). How VreR modulates gene expression independently of σ^{VreI} is at present unknown but likely involves some of these regulatory proteins. In contrast, the third component of the operon, VreA, does not seem to play a role in the low Pi-induced virulence of *P. aeruginosa*. The function of this CSS-like receptor in the signalling cascade, if any, is at present unknown. VreA has unique domain architecture, with an N-domain that resembles the signaling domain of CSS receptors and a C-domain that resembles the periplasmic domain of TonB proteins (Fig. S1A) (Llamas et al., 2009). The TonB protein interacts with outer membrane proteins, including TBDR to which TonB delivers the energy required for substrate passage (Noinaj et al., 2010; Klebba, 2016). Based on this prediction we initially proposed two different working mechanisms for VreA and the σ^{VreI} signaling cascade (Llamas et al., 2009; Llamas et al., 2014). In the first model, VreA would interact with an outer membrane protein through its C-domain being thus the nexus that connects the receptor of the system with the VreR anti-σ factor. However, pull-down assays we carried out have not resulted in the identification of such receptor. Alternatively, the C-domain of VreA could interact with the N-domain, which contains a potential TonB box (Fig. S1A) (Llamas et al., 2009). This would prevent the interaction of VreA with the anti-σ factor VreR in the absence of the inducing signal whereas signaling would allow it by disrupting the N-VreA/C-VreA interplay. However, VreA does not seem to be involved in σ^{VreI} activation either *in vitro* (Quesada et al., 2016) or *in vivo* (Fig. 2B), which suggests that the σ^{VreI}-VreR signaling cascade does not require this protein.

Furthermore, we have shown in this work that σ^{VreI} activation occurs *in vivo* during infection. *pdtA* expression, which *in vitro* completely depends on the σ^{VreI}/VreR signaling cascade and the PhoB regulator (Fig. S1B) (Quesada et al., 2016), occurs during *P. aeruginosa* infections in zebrafish embryos and upon interaction with human epithelial cells, and in these *in vivo* situations expression of *pdtA* also completely depends on σ^{VreI}. The activation of σ^{VreI} *in vivo* is in accordance with the increased expression of the *vreAIR* operon and the σ^{VreI} regulon (including *pdtA*) in mouse models of acute and chronic infections (Turner et al., 2014), and with the presence of antibodies against the PdtA protein in the serum of *P. aeruginosa* infected patients (Llamas et al., 2009). The host signal that activates σ^{VreI} is at present unknown. This σ factor seems to be active both in bacterial cells that are and cells that are not directly in contact with the A549 eukaryotic cell (Fig. 5A, PAO1), which suggests that the host-derived signal could be a molecule released by the host cell into the culture medium. Alternatively, the activating signal could be produced by *P. aeruginosa* itself in response to the host environment. An example of such a mechanism is the activation of σ^{PvdS} in *P. aeruginosa*, which in response to the iron starvation conditions encountered in the host (Cassat and Skaar, 2013; Parrow et al., 2013) produces pyoverdine that in turn increases σ^{PvdS} activation (Lamont et al., 2002; Beare et al., 2003). Another example is the activation of the *P. aeruginosa* σ^{AlgT} factor in response to the oxidative stress generated by the oxygen radicals produced by leucocytes or in response to the elevated temperatures that are often produced in infected hosts (Mathee et al., 1999; Rowley et al., 2006). Both situations lead to cell envelope stress and the accumulation of misfolded

proteins in the bacterial periplasm, which trigger σ^{AlgT} activation (Wood and Ohman, 2009). Further research will be conducted to clarify how activation of σ^{VreI} in response to the host occurs.

METHODS

Bacterial strains and growth conditions. Strains used are listed in Table S1. Bacteria were routinely grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37 ºC in a rotary shaker at 200 rpm. For differential expression of the *vreAIR* operon low and high phosphate media were used (Quesada et al., 2016). When necessary, antibiotics (Sigma-Aldrich) were used at the following final concentrations (μg ml^{-1}): ampicillin (Ap), 100; gentamicin (Gm), 20; hygromycin B (Hg), 100; nalidixic (Nal), 10; and tetracycline (Tc), 20.

Plasmids construction and molecular biology. Plasmids used are described in Table S1 and primers listed in Table S2. PCR amplifications were performed using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes). All constructs were confirmed by DNA sequencing and transferred to *P. aeruginosa* by electroporation (Choi et al., 2006).

Zebrafish maintenance, embryo care and infection procedure. Transparent adult *casper* mutant zebrafish *(mitfa $^{w2/w2}$;roy $^{a9/a9}$)* and adult labelled *Tg(mpx:GFP)^{i114} casper* zebrafish producing green neutrophils *(Renshaw et al., 2006; White et al., 2008)* were conserved at 26 °C in aerated 5 L tanks with a 10/14 h dark/light cycle. Zebrafish embryos were collected during the first hour post-fertilization (hpf) and kept at 28 °C in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl{\cdot}2H_2O$, 0.33 mM $MgCl_2{\cdot}7H_2O$) supplemented with 0.3 mg/L methylene blue. Prior to infection, 1 or 2 days post-fertilization (dpf) embryos were mechanically dechorionated and anaesthetized in 0.02 % (*w/v*) buffered 3-aminobenzoic acid methyl ester (pH 7.0) (Tricaine, Sigma-Aldrich). Zebrafish embryos were individually infected by microinjection with 1 nl of *P. aeruginosa* either in the hindbrain ventricle (localized infection) or in the caudal vein (systemic infection) as described elsewhere (Llamas and van der Sar, 2014). All procedures involving zebrafish embryos were according to local animal welfare regulations.

Virulence assay in infected zebrafish embryos. Zebrafish embryos were injected in the caudal vein with ~1000 CFU of exponentially grown *P. aeruginosa* cells in low or high phosphate conditions previously resuspendend in phosphate-free physiological salt containing 0.5% (w/v) of phenol red. After infection, embryos were kept in 12-well plates containing 60 μg/mL of Sea salts (Sigma-Aldrich) at 32 ºC with 20 individually injected embryos in each group per well. Embryo survival was determined by monitoring live and dead embryos at fixed time points during five days. Three biologically independent experiments were performed and the data given are the average. P-values were calculated by log-rank (mantel-Cox) test.

Confocal fluorescence imaging of zebrafish embryos. For confocal imaging, zebrafish embryos were injected in the hindbrain ventricle with ~2000 CFU of *P. aeruginosa* cells containing a σ^{VreI}-dependent red fluorescence transcriptional fusion. At 0 and 12 hpi, embryos were fixated overnight in 4 % (v/v) paraformaldehyde in phosphate buffered saline (PBS). Fixated embryos were embedded in 1.5 % (w/v) low-melting-point agarose using an open uncoated 8-well microscopy μ-slide (Ibidi®). Confocal images were generated with a Leica TCS SP8 Confocal Microscope. Leica Application Suite X and ImageJ software was used to process the confocal images, specifically for brightness/contrast enhancements as well as for creating merged images.

Cytotoxicity assay in A549 human lung epithelial cells. *P. aeruginosa* cytotoxicity on A549 cells was assayed using a colorimetric assay that detects the number of metabolically active eukaryotic cells able to cleave the MTT tetrazolium salt (Sigma-Aldrich) to the insoluble formazan dye. The A549 cell line (ATCC® CCL-185™) was maintained in DMEM medium supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco) in a 5% CO_2 incubator at 37 ºC. One day prior infection, the A549 cells were placed in 96-well plates at a concentration of 4 x 10^4 cells/well and cultured in phosphate-free DMEM medium (Gibco) with 5 % (v/v) FBS. In this condition, cell mitosis does almost not occur. Late exponentially grown *P. aeruginosa* strains in low or high phosphate conditions were then inoculated at a multiplicity of infection (MOI) of 20. At 3 hpi, 30 µl of a 5 mg/ml MTT solution in PBS was added to the wells and the plates were incubated for 2 h. The culture medium was then removed and 100 µl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan. Production of formazan, which directly correlates to the number of viable cells, was quantified using a scanning multi-well spectrophotometer (Infinite® 200 PRO Tecan) at 620 nm.

Confocal fluorescence imaging of A549 cells. For confocal imaging, 2 x 10^5 A549 cells were seeded in 24-well plates containing 11 mm round glass coverslips and phosphate-free DMEM medium with 5 % (v/v) FBS one day prior infection. Infections with green-labelled *P. aeruginosa* cells containing a σ^{Vrel}-dependent red fluorescence transcriptional fusion were performed at a MOI of 10. At 0 and 10 hpi, the cells were fixated with 4% (v/v) paraformaldehyde in PBS. Samples were washed with PBS and coverslips were mounted on glass slides containing the Fluoroshield mounting medium with DAPI (Sigma-Aldrich) to retain fluorescence and stain the A549 cells DNA. Confocal images were generated with a Nikon A1R confocal scanning laser microscope. NIS-Elements and ImageJ software were used to process the confocal images. Total corrected cellular fluorescence (TCCF) was calculated using the following equation: TCCF = integrated density – (area of selected cell × mean fluorescence of background readings), as described before(Burgess et al., 2010; McCloy et al., 2014).

Internalization assay. To enumerate bacteria internalized into A549 cells, a polymyxin B protection assay was performed with *P. aeruginosa* strains grown at late exponential phase in low phosphate conditions. A549 cells were cultured in 24-well plates at a concentration of 2 x 10^5 cells/well in phosphate-free DMEM medium supplemented with 5% (v/v) FBS. Bacterial infections were performed at a MOI of 10. At 6 hpi, the culture supernatants were collected and serial dilutions plated on LB for bacterial counting. Fresh DMEM medium containing 20 µg/ml polymyxin B was added to the infection wells and incubated during 45 min to kill extracellular bacteria. Afterward, the antibiotic-containing medium was removed and the cells were lysed with 1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS during 10 min. Serial dilution of the lysed cells were plated on LB for bacterial counting. Internalization is reported as the ratio between the CFU after the lysis of the A549 cells and CFU in the culture supernatant.

RNA isolation. Total RNA was extracted by the hot phenol method using the TRI® Reagent protocol (Ambion) as described elsewhere (Llamas et al., 2008) and subjected to two DNase I treatments with the Turbo DNA-free kit (Ambion) and RNaseOUT (Invitrogen). RNA quality, including purity, integrity and yield, was assessed by electrophoresis of 1 µg of total RNA and by UV absorption at 260 nm in a ND-1000 spectrophotometer (NanoDrop Technologies, USA).

Quantitative RT-PCR analyses. A549 cells cultured in 6-well plates in phosphate-free DMEM medium with 5% (v/v) FBS were infected with exponentially grown *P. aeruginosa* cells in high phosphate conditions at a MOI of 10. At 3 hpi, the culture supernatants were spin down at 1600

x gand 4ºC and total RNA was isolated. cDNA from eight biologically independent replicates was produced in triplicate by reverse transcription reactions of 0.5-1 µg RNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers as primers according to the protocol supplied. Real-time PCR amplification were carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 optical system software (version 2.1.97.1001) in 11.5 µl reaction mixture containing 6.25 µl of iQ SYBR green Supermix (Bio-Rad), 0.4 µM of each primer (Table S2) and 1 µl of the template cDNA (diluted 1000-fold when measuring the 16S rRNA reference gene). Thermal cycling conditions were the following: one cycle at 95°C for 10 min and then 40 cycles at 95°C for 15 s, 65°C for 30 s, and 72°C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. Melting curve analyses were performed by gradually heating the PCR mixture from 55 to 95°C at a rate of 0.5°C per 10 s for 80 cycles. The relative expression of the genes was normalized to that of 16S rRNA and the results were analyzed by means of the comparative cycle threshold (ΔΔct) method (Pfaffl, 2001).

RNA-seq analysis. *P. aeruginosa* PAO1 and its isogenic ΔvreR mutant were grown until late exponential phase in low Pi medium and total RNA was isolated. A mixture of the three isolations was used for RNA-seq analyses, which were performed at Era7 Bioinformatics (Granada, Spain). First, the rRNA from 4 µg of total RNA was removed using the Ribo-Zero rRNA removal kit (Illumina) following the manufactures' recommendations. Sequencing libraries were prepared with the NEBNext Ultra directional RNA library Prep kit (New England Biolabs). RNA was fragmented at 94 °C for 7.5 min and first strand cDNA synthesis was performed at 42 °C during 50 min using the adaptor and primers recommended by the manufacturer (NEBNext Multiplex Oligos, Illumina). Samples were then processed on the Illumina NextSeq500 Sequencer in one run with a read length of 2 × 75 bp. The pipeline recommended in the tool Cufflinks 2.2.1 (Trapnell et al., 2013) was used to analyze the data and obtain the set of differentially expressed genes following these steps: 1) Raw reads quality analysis with the tool FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc); 2) Reads mapping to the *P. aeruginosa* PAO1 reference genome (NCBI reference sequence NC_002516.2) with the tool Bowtie integrated in the Tophat suite (Trapnell et al., 2009); and 3) analysis of differences in gene expression with Cufflinks 2.2.1 (Trapnell et al., 2013). All the samples passed the quality analysis. Some tests failed in some of the samples, particularly the tests related to overrepresented sequences (corresponding to overexpressed genes in this case, e.g. *pdtA*, Table 1). A q-value ≤ 0.05 and a fold-change ≥ 2 were set as threshold of significance in order for a transcript to be considered differentially abundant. RNA-seq data set have been deposited in NCBI's Gene Expression Omnibus (GEO) database (Edgar et al., 2002) under accession number GSE122253 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122253). RNA-seq results were confirmed by qRT-PCR using RNA from three biological replicates obtained from *P. aeruginosa* cells grown in the same conditions that for the RNA-seq assay and primers listed in Table S2.

Other bioinformatic analyses. Statistical analyses are based on *t*-test in which two conditions are compared independently. P-values from raw data were calculated by independent two-tailed *t*-test and from ratio data to the control by one-sample *t*-test using GRAPHPAD PRISM version 5.01 for Windows and are represented in the graphs by *, P<0.05; **, P<0.01; and ***, P<0.001.

SUPPLEMENTAL INFORMATION

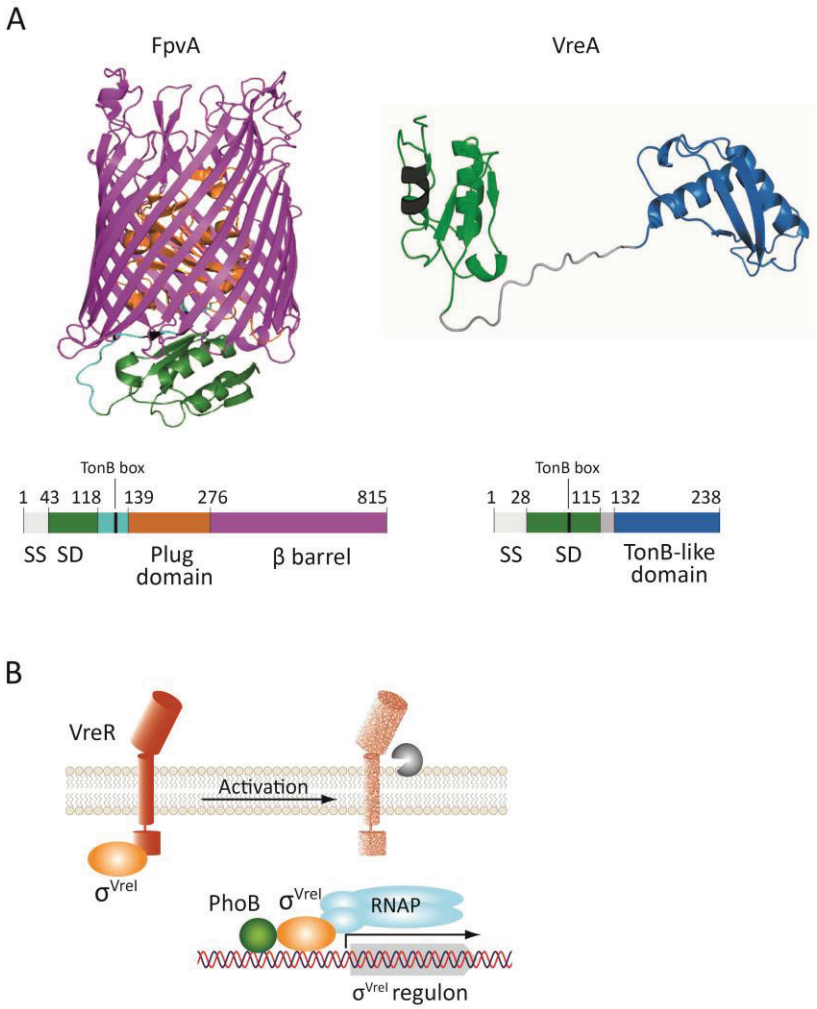

Fig. S1. The *P. aeruginosa* VreA-VreR-σ^VreI system. (A) Structural model of the *P. aeruginosa* FpvA CSS receptor and VreA. The periplasmic signalling domains (SD) of the proteins is coloured in green and the potential TonB boxes are indicated in black. The C-terminal β barrel domain of FpvA (purple) forms a pore in the outer membrane that is occluded by a plug domain (orange). VreA lacks this β barrel domain and its C-terminal domain (blue) has structural homology with the periplasmic domain of the TonB protein. The N- and C-domains of VreA are connected by a connecting loop (grey). Images adapted from (Greenwald et al., 2009; Llamas et al., 2009) **(B)** Modulation of the σ^VreI activity by VreR and PhoB. Activation of σ^VreI requires its release from the VreR anti-σ factor, which likely occurs by the proteolytic degradation of VreR in response to the inducing signal. Upon activation, σ^VreI binds to the RNAPc and the transcription factor PhoB recruits the σ^VreI-RNAP complex to the promoter region of target genes to trigger transcription.

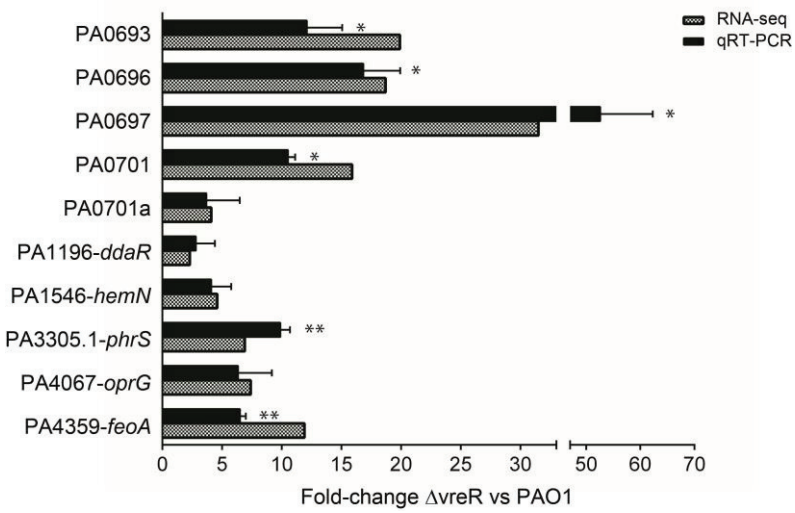

Fig. S2. Comparison between the RNA-seq and qRT-PCR measurements of the differential gene expression in *P. aeruginosa* ΔvreR and PAO1. mRNA levels of the indicated genes were obtained by RNA-seq (Table 1) or qRT-PCR upon growth of the *P. aeruginosa* strains in low Pi medium. The $2^{-\Delta\Delta CT}$ method was used to determine the fold-change range in gene expression in ΔvreR vs PAO1 by qRT-PCR and these data are means ± SD from three biological replicates (N=3) each one including three technical replicates. P-values were calculated by one-sample *t*-test to a hypothetical value of 1 as described in Materials and Methods.

Table S1. Bacterial strains and plasmids used in this study[a]

Strain	**Characteristics**	**Reference**
E. coli		
DH5α	*supE44 Δ(lacZYA-argF)U169* ϕ80 *lacZ*ΔM15 *hsdR17* (r_K^- m_K^+) *recA1 endA1 gyrA96 thi1 relA1*; Nal^R	(Hanahan, 1983)
P. aeruginosa		
PAO1	Wild-type strain	(Jacobs et al., 2003)
ΔvreA	Markerless PAO1 null mutant in the *vreA* (PA0674) gene	(Quesada et al., 2016)
ΔvreI	Markerless PAO1 null mutant in the *vreI* (PA0675) gene; the mutation affects the expression of the downstream *vreR* gene	(Faure et al., 2013; Quesada et al., 2016)
ΔvreR	Markerless PAO1 null mutant in the *vreR* (PA0676) gene	(Quesada et al., 2016)
Plasmid		
pMP0690	Plasmid containing a *P. aeruginosa pdtA::lacZ* transcriptional fusion; Tc^R	(Quesada et al., 2016)
pRS-mCherry	pRSET-B plasmid (Invitrogen) containing a mCherry dsRed with an improved Shine/Dalgarno (SD) sequence to optimize for gene expression in bacteria; Ap^R	(Shaner et al., 2004; Llamas et al., 2009)

pME6031	Broad-host-range plasmid, pVS1 replicon; Tc^R	(Heeb et al., 2000)
pME-mCherry	pME6031 carrying in HindIII a 0.74-Kb from the pRS-mCherry plasmid containing the mCherry dsRed gene; Tc^R	This study
pMP0690mCherry	pMP0690 carrying in XbaI a 0.53-Kb PCR fragment containing the mCherry dsRed gene in between and in the same orientation that the *pdtA* promoter and the *lacZ* gene (*pdtA*::*mCherry* transcriptional fusion); Tc^R	This study
pBBR-PoprF	pBBR1MCS-5 carrying the promoter region of the *P. aeruginosa* PAO1 *oprF* gene; Gm^R	(Llamas et al., 2009)
pSMT3-hsp60-mEos3.1	Source of the mEos3.1green fluorescent protein (*gfp*) gene; Hg^R	(Boot et al., 2017)
pBBR-mEos3.1	pBBR-PoprF carrying in EcoRI-XbaI a 1.0-Kb PCR fragment containing the mEos3.1 *gfp* gene; Gm^R	This study

[a] Ap^R, Gm^R, Hg^R, Nal^R and Tc^R, resistance to ampicillin, gentamycin, hygromycin, nalidixic acid and tetracycline, respectively.

Table S2. Primers used in the qRT-PCR analyses

***P. aeruginosa* gene**	**Primer name**	**Primer sequence (5'→ 3')**
PA0141	PA0141F	ACTATCC CTATCAC ACGCGG
	PA0141R	GTGTTCCATGAAGCGCTTGA
PA0200	PA0200F	ATTTCCTCTCCATCCCAGCC
	PA0200R	TCCTCCGTTCCTTCGACTTC
PA0690-*pdtA*	PA0690F2	AGCAGCGGGTCAACCAGCAGTT
	PA0690R	ATCCTGCGGGAGATTCAGCG
PA0691-*pdhA*	PA0691F	GCAGGAACTTGATCAGCGCCTA
	GSP2-0691	CGCCCCCTGCCAATCTTCCTG
PA0692-*tpsB*	PA0692F	CTGGCTGTCGCATTGTCGTG
	PA0692R	CGGTTGTCCAGCACGGTGTT
PA0693-*exbB2*	PA0693F	TGCGGCGATCAACGACAGTC
	GSP2-0693	GGCGGCGACGAAGCGGATGT
PA0696	PA0696F	CTGGGGATGGGCGTCATGCT
	GSP2-0696	CCAGCAGGCGGATCAGGTTG
PA0697	PA0697F1	AGTTGCGCAGCACCACCCAGC
	PA0697R1	CCTGTTGCTGGGCCGAGAG
PA0701	PA0701F	ACGCGACGACCGTGCCTATT
	PA0701R	ATGAAACAGCCGCAACGGG
PA0701a	701-1F	ATTGGCAGGGCGAGGTCT
	701-1R	GTCTCCGCCAGCAGCAACT
PA1196-*ddaR*	PA1196F	GAGGAACCGACGATTTCCAC
	PA1196R	TGTAGTAGACGAAGCGACCG
PA1414	PA1414F	ACGCCGGTCTACTCTTTCCT
	PA1414R	GGTTTTGCAGCCAGAGTTTC
PA1546-*hemN*	PA1546F	GCGTTACACCTCCTATCCGA
	PA1546R	GTAGTAGCAGATGTTGGCGC
PA1556-*ccoO2*	PA1556F	TGTTCTTCCAGGACGTGACC
	PA1556R	AGCCTTCGCGGATATAGATG
	PA1673F	ATCAACACAAACGCATCGTC

PA1673	PA1673R	AGTCCACCAGCTCCTCGAT
PA1746	PA1746F	GTCGATGGTTCGCGACTAC
	PA1746R	GACGATGCACTGGGTCTTG
PA3305.1-*phrS*	PA3305.1F	CTCATGGTCGCTTTCTTCGG
	PA3305.1R	CTTGCGTGCTCTGTGTATCC
PA3337-*rfaD*	PA3337F	GGCGAGACCGACATCATC
	PA3337R	CGTTCTGGTCCAGGTAGTCG
PA3880	PA3880F	GCTGCATTGCGTGAAAGG
	PA3880R	GCCGTAGCGCTTCTTCAG
PA4067-*oprG*	PA4067F	CGCTGCGGATATTCAAGGAC
	PA4067R	TCCAGCTTGATGTCGGAACT
PA4348	PA4348F	CCAACCAGTTCCTGATCGTC
	PA4348R	AAGCGTTTCGACAATTCCAG
PA4359-*feoA*	PA4359F	CATTGCAACCGTCCCGTTC
	PA4359R	AGAAGCCCCATGGAGAACAG
PA5027	PA5027F	ATGATCCGCCACCTCCTG
	PA5027R	TCGTACACGTGCAGCAGACT
PA5304-*dadA*	PA5304F	AGTGCGTATTACCTGGCCC
	PA5304R	TTTCCAGCAGCCACTTCATG
PA5427	PA5427F	CCTGCCACAGACCATGAAAG
	PA5427R	GAGGCTTCGATCTTCACCAG
PA5475	PA5475F	CGACCATTGGGTCGAGTC
	PA5475R	GTTGATGAACTGGCGTTCG
RNAr 16S	13-RNAr 16S	AAAGCCTGATCCAGCCAT
	14-RNAr 16S	GAAATTCCACCACCCTCTACC

Chapter 4

Pseudomonas aeruginosa possesses three distinct systems for sensing and using the host molecule heme

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ABSTRACT

Because iron is essential for bacterial survival, iron acquisition is key for successful infections. Pathogens have developed several strategies to obtain iron during infection, including the use of iron-containing molecules from the host. Heme accounts for the vast majority of the iron pool in vertebrates and thus represents an important source of iron for pathogens. In this work we analyze the response of the human pathogen *Pseudomonas aeruginosa* to heme. Using a proteomic approach, we have identified a previously uncharacterized system, which we name Hxu, that together with the known Has and Phu systems, is used to respond to heme. This finding makes *P. aeruginosa* the only pathogen in which three heme systems have been reported to date. We show that the *P. aeruginosa* Has and Hxu systems are functional signal transduction pathways of the cell-surface signaling class. By genetics and molecular approaches we have identified the mechanism triggering the activation of these signaling systems. The Has system senses and responds to heme-hemophore complexes, whereas Hxu responds to free heme. Both signaling cascades involve an outer membrane receptor that upon sensing heme in the extracellular medium produces the activation of an σ^{ECF} factor in the cytosol. In this way, *P. aeruginosa* uses heme not only as a source of iron but also as a signal molecule to detect the host. Blocking heme sensing and utilization has been proposed as a promising strategy to fight *P. aeruginosa* and our work provides new insights that could be exploited to achieve this aim.

INTRODUCCIÓN

Iron is essential for the survival of virtually all organisms. This metal is involved in a wide range of cellular processes ranging from DNA replication to important metabolic reactions. For most pathogens, iron acquisition is a key factor in colonization and infection (Becker and Skaar, 2014). Vertebrates have evolved several mechanisms to reduce the availability of iron as a strategy to combat pathogens, a process referred to as 'nutritional immunity'. The vast majority of iron in vertebrates is complexed within the porphyrin ring of heme in hemoproteins, such as hemoglobin, or intracellularly sequestered by the iron storage protein ferritin. Extracellular iron is, at physiological pH, present in the oxidized form Fe^{3+}, which is insoluble and therefore not available for pathogens. Moreover, most extracellular iron of vertebrates is bound to the serum protein transferrin for transport through the body, or to lactoferrin, a glycoprotein of the transferrin family found in milk, tears, and the mucosa of nasal passages (Cassat and Skaar, 2013; Parrow et al., 2013). Increased synthesis of these iron-scavenging proteins, or of haptoglobin and hemopexin that conjugate free hemoglobin and heme, are some of the strategies used by the host to reduce the amount of iron available for invading microorganisms (Cassat and Skaar, 2013; Parrow et al., 2013). In response, microorganisms synthetize and secrete siderophores, high affinity iron chelating compounds able to scavenge minute quantities of free iron from the environment and the host. Several pathogens also produce hemophores that bind host heme with high affinity, and most successful pathogens can also directly use the host-iron complexes transferrin, lactoferrin and hemoglobin as sources of iron (Wandersman and Delepelaire, 2004). The battle for iron is an important process in the host-pathogen interaction, with evolution shaping host iron-scavenging proteins into variants that are less well recognized by the pathogen, and the bacterium facing this process by counterselecting receptors able to recognize the altered host proteins (Armitage and Drakesmith, 2014; Barber and Elde, 2014).

Pseudomonas aeruginosa is a Gram-negative free-living bacterium commonly found in soil and water. This bacterium is increasingly recognized as an emerging human pathogen of high clinical relevance because it causes a wide array of life-threatening acute and chronic infections, particularly in immunocompromised and cystic fibrosis (CF) patients (Sadikot et al., 2005). This bacterium is moreover one of the leading causes of nosocomial infections affecting hospitalized patients (Rosenthal et al., 2016). *P. aeruginosa* infections are often difficult to treat due to its natural resistance to antibiotics and to its capacity to acquire such resistance (Lister et al., 2009; Breidenstein et al., 2011). *P. aeruginosa* contains a large repertoire of virulence determinants and a complex regulatory network that allows the bacterium to colonize many different host environments (Lyczak et al., 2000; Balasubramanian et al., 2013; Moradali et al., 2017). This is also driven by the ability of this pathogen to scavenge iron during infection. The different strategies used by *P. aeruginosa* to acquire iron include: 1) the production of two siderophores, pyoverdine and pyochelin; 2) the uptake of siderophores produced by other microorganisms (referred as xenosiderophores) with which *P. aeruginosa* shares the niche; 3) the utilization of heme from host hemoproteins; and 4) the uptake of Fe^{2+} (Cornelis and Dingemans, 2013). The bacterium uses these different mechanisms of iron acquisition depending on the type of infection it causes (Cornelis and Dingemans, 2013). Pyochelin, which has lower affinity for iron than pyoverdine, is the first siderophore synthesized by *P. aeruginosa*, which switches to pyoverdine when the iron availability becomes more limited (Dumas et al., 2013). Pyoverdine is able to scavenge iron from the host protein transferrin and is essential for *P. aeruginosa* to cause acute infections (Takase et al., 2000; Imperi et al., 2013). In hypoxic environments, such as the CF lungs and biofilms, free iron is mainly found in the reduced Fe^{2+} form (Hunter et al., 2013), which can be acquired by *P. aeruginosa* through the Feo system

(Cartron et al., 2006). In these chronic infections, there is a long term decline in pyoverdine production, although co-occurrence of pyoverdine producers and non-producers within CF patients occurs (Marvig et al., 2014; Andersen et al., 2015). Loss of pyoverdine production correlates with increased synthesis of heme receptors (Marvig et al., 2014; Nguyen et al., 2014). Heme from hemoglobin becomes available as an iron source after lysis of erythrocytes and globin degradation and *P. aeruginosa* secretes several factors that facilitate this process (Ochsner et al., 2000). Two systems involved in heme acquisition have been identified in *P. aeruginosa*, the Phu (from *Pseudomonas* heme uptake) and the Has (from heme assimilation system) (Ochsner et al., 2000) (Fig. 1A and 1B). In both systems the transport of heme through the highly impermeable outer membrane of *P. aeruginosa* occurs through specialized receptors of the TonB-dependent receptor (TBDR) family in an energy-dependent process (Noinaj et al., 2010) (Fig. 1C). The Phu system mediates the direct acquisition of free heme through the PhuR receptor, whereas the Has system involves the secretion of a heme-binding protein, the hemophore HasAp, and the HasR receptor that extracts the heme from the hemophore and transports it into the periplasm (Fig. 1C). Heme is internalized into the cytoplasm through the PhuSTUV system and degraded by the heme oxygenase HemO, which results in the release of iron (O'Neill et al., 2012) (Fig. 1C). Expression of both systems occurs in iron restricted and not in iron sufficient conditions, a control exerted by the Fur repressor protein that in high iron conditions binds to the *hasR* and *phu* promoter regions inhibiting gene transcription (Ochsner et al., 2000). Although it was initially proposed that the Has system was the high-affinity transport system for heme (Letoffe et al., 1998; Ochsner et al., 2000), a recent study proposes that the PhuR receptor plays a major role in heme acquisition whereas the primary role of HasR is to sense the presence of heme (Smith and Wilks, 2015). HasR belongs to a special subfamily of TBDR that are not only involved in uptake but also in sensing and signaling. These receptors contain an N-terminal extension of about 90-100 amino acids referred to as the signaling domain that is responsible of the signal transduction function (Koebnik, 2005; Noinaj et al., 2010) (Fig. 1C, red ball of the HasR receptor), and are functionally, and normally also genomically, associated with extracytoplasmic function sigma factor (σ^{ECF}) and anti-σ factor pairs (Llamas et al., 2014) (Fig. 1B). These three proteins form a signal transduction pathway known as cell-surface signaling (CSS) (Llamas et al., 2014). In response to the presence of the cognate CSS inducing signal in the extracellular medium, the CSS receptor initiates a cascade that produces the targeted proteolysis of the CSS anti-σ factor and the release and activation of the CSS σ^{ECF} in the cytosol (Draper et al., 2011; Bastiaansen et al., 2014; Llamas et al., 2014; Bastiaansen et al., 2015a). Upon activation, the σ^{ECF} binds to the core enzyme of the RNA polymerase (RNAPc) activating the transcription of the genes required to respond to the signal. One of the genes usually stimulated by CSS σ^{ECF} is the one encoding the CSS receptor (Llamas et al., 2008; Llamas et al., 2014). This positive feedback mechanism increases the amount of the receptor in the outer membrane and, therefore, the capacity of the bacterium to sense and respond to the CSS signal. Although not experimentally analyzed yet, by analogy with the well-studied hemophore-mediated CSS pathway of *Serratia marcescens* (Rossi et al., 2003; Biville et al., 2004) we and others have proposed that the *P. aeruginosa* HasR receptor together with the σ^{HasI}/HasS proteins forms a CSS involved in the detection and uptake of heme (Llamas et al., 2014; Smith and Wilks, 2015). The purpose of this work was to analyze the response of *P. aeruginosa* to the presence of heme, which is important to get new insights to block *P. aeruginosa* iron acquisition during infection and, therefore, virulence.

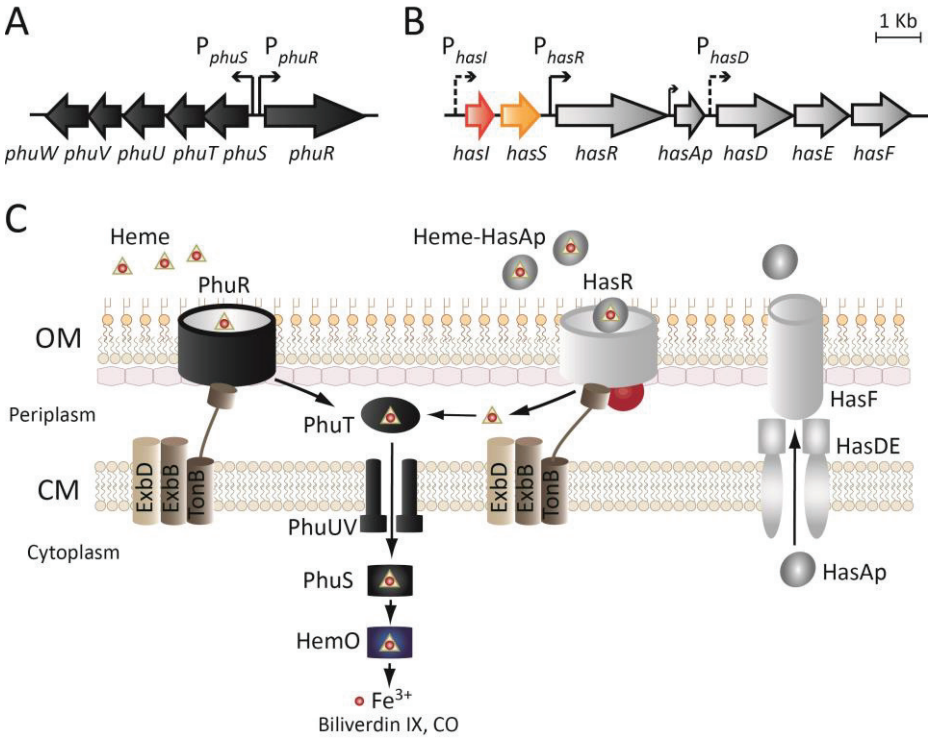


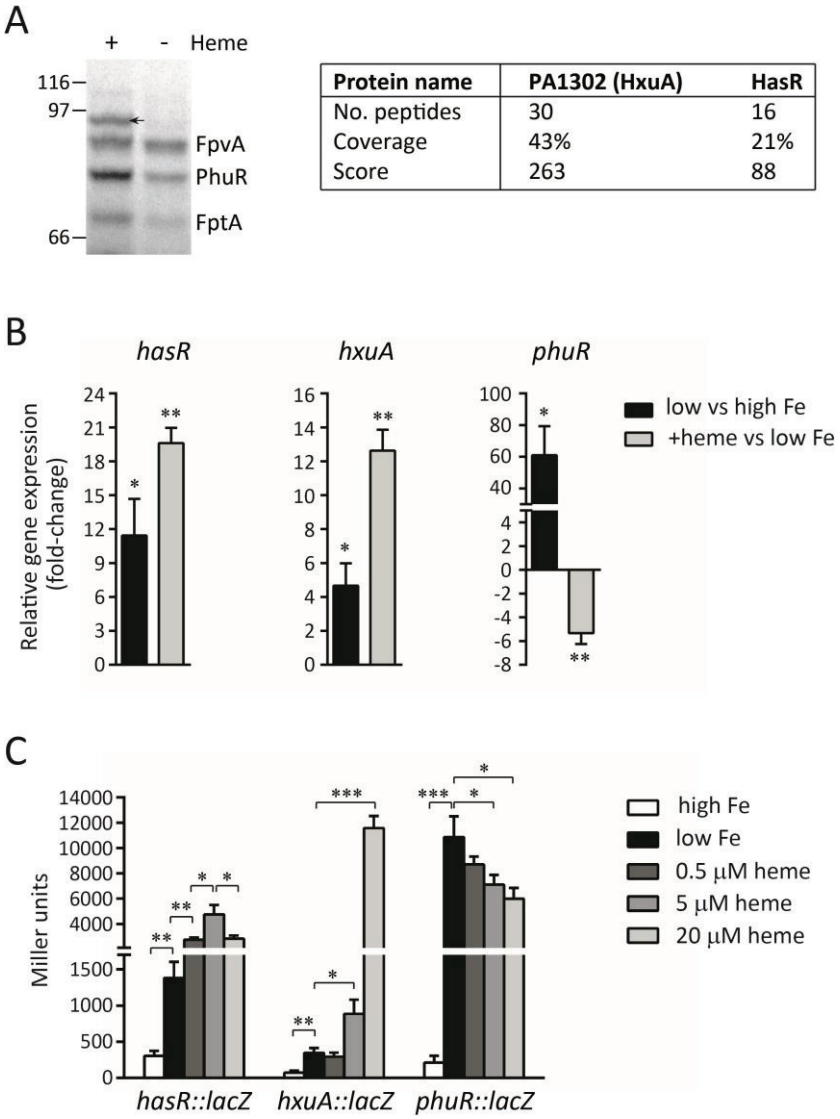
Fig. 1. Genetic organization and schematic representation of the *P. aeruginosa* Phu and Has heme utilization systems. (A, B) Block arrows represent the different genes and their transcriptional orientation, and small arrows identified (solid line) or proposed (dashed line) promoters. **(A, C)** The Phu system is encoded by two divergently transcribed operons (Ochsner et al., 2000). PhuR transports free heme to the periplasm in a process that requires the energy provided by the ExbBD-TonB complex (Noinaj et al., 2010). In the periplasm the heme binding protein PhuT delivers the heme to the PhuUV ABC transporter that translocates it into the cytoplasm where heme is sequestered by the heme-trafficking protein PhuS. PhuS forms a complex with the heme oxygenase HemO that degrades heme to biliverdin IX and CO releasing the ferric ion (O'Neill et al., 2012). **(B, C)** The Has system is arranged in three potential operons, with the first encoding a σ/anti-σ factor pair (σ^{HasI}/HasS) not previously characterized. The second operon encodes the HasR receptor together with the hemophore HasAp. Presence of a second weak promoter has been proposed within this operon (Ochsner et al., 2000). The last operon encodes a potential type I secretion system that, by homology with the *S. marcescens* HasD-HasE-HasF exporter system, is likely involved in the secretion of HasAp (Ochsner et al., 2000). HasAp binds and delivers heme to HasR, which, in an energy-requiring process extracts and translocates the heme into the periplasm (Smith et al., 2015), which is then internalized in the cytoplasm by the components of the Phu system (Smith and Wilks, 2015). The N-terminal extension of HasR potentially involved in signaling is shown in red.

RESULTS

P. aeruginosa *responds to the presence of heme in the extracellular medium.*

We focused on the outer membrane composition of *P. aeruginosa* to analyze the response of the bacterium to heme. Cell envelope preparations enriched by outer membrane proteins of *P. aeruginosa* cells grown in low iron without or with heme were analyzed by SDS-PAGE. Three main protein bands were observed in the low iron control sample (Fig. 2A), which, as identified by proteomic in previous work (Llamas et al., 2006), likely consist of the pyoverdine receptor FpvA, the heme receptor PhuR and the pyochelin receptor FptA, respectively (Fig. 2A). A

differentially expressed protein band appeared in cells grown in heme (Fig. 2A). Proteomic analyses showed that this band contained the HasR receptor and also the uncharacterized TBDR PA1302 (Fig. 2A). In fact, PA1302 was the major protein within this sample as judged by the higher number of tryptic peptides that matched this protein as compared with those matching HasR (Fig. 2A). PA1302 presents the N-terminal extension typical of TBDR involved in CSS and it is genomically associated with an σ^{ECF}/anti-σ factor pair (PA1300 and PA1301, respectively). Protein sequence analysis and classification using InterPro revealed that this protein belongs to the TonB-dependent heme/hemoglobin receptor family (IPR011276), of which the *Haemophilus influenzae* HxuC heme receptor is the best characterized (Zambolin et al., 2016). Therefore, we re-named PA1302 as HxuA, and the associated σ^{ECF}/anti-σ factor proteins as σ^{Hxul} and HxuR, respectively, following the nomenclature adopted for the components of CSS systems in *Pseudomonas* (Llamas et al., 2014).



Protein name	PA1302 (HxuA)	HasR
No. peptides	30	16
Coverage	43%	21%
Score	263	88

Fig. 2. Response of *P. aeruginosa* to heme. (A) SDS-PAGE and proteomic analyses of *P. aeruginosa* PAO1 outer membrane proteins after growth in iron-restricted conditions without (-) or with (+) heme. The arrow indicates the position of the protein band that was excised and subjected to proteomic analysis by MALDI-TOF. The results of the proteomics analyses are shown and include the number of peptides matching each protein, coverage (%) of the protein, and the score expressed as the -10 log(*P*) where P is

the probability that the observed match is a random event. Protein scores shown are significant (P<0.05). The positions of the receptors FpvA, PhuR and FptA, identified by proteomics in a previous work (Llamas et al., 2006), are indicated on the right and those of the molecular size markers on the left. SDS-PAGE is representative of two biological replicates (N=2). **(B)** Analysis of the expression of the heme receptors by qRT-PCR. *P. aeruginosa* PAO1 wild-type strain was grown under iron-restricted conditions (low Fe) or iron-restricted medium supplemented with $FeCl_3$ (high Fe) or heme (+heme). qRT-PCR on total RNA was performed with primers hybridizing the indicated gene (Table S2). Data are means ± SD from three biological replicates (N=3) each one including three technical replicates. P-values were calculated by one-sample *t*-test to a hypothetical value of 1 as described in Materials and Methods. (**C)** β-galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain after growth under iron-restricted conditions (low Fe) or iron-restricted medium supplemented with $FeCl_3$ (high Fe) or the indicated amount of heme. Data are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods and brackets indicate the comparison to which the P-value applies.

Quantitative real-time PCR (qRT-PCR) analyses to measure expression of the identified receptor genes showed that *hasR* and *hxuA* mRNA levels were higher in low than in high iron (12- and 4.5-fold, respectively) conditions and increased when heme was added to the low iron medium (20- and 13-fold, respectively) (Fig. 2B). Expression of the third heme receptor gene *phuR* was also higher in low than in high iron conditions but decreased upon addition of heme (Fig. 2B). This effect is likely due to the supply of iron that *P. aeruginosa* receives through heme, and shows that, in contrast to *hasR* and *hxuA* that both require iron limitation and heme to be fully expressed, iron limitation alone is enough for full expression of *phuR*. This is in agreement with PhuR being detected in the outer membrane of *P. aeruginosa* cells grown solely in low iron (Llamas et al., 2006), but not HasR and HxuA (Fig. 2A).

To know if the heme receptors were differentially produced depending on the amount of heme available, we measured their expression in different concentrations of heme using transcriptional fusions in which the promoter-containing region upstream the receptor gene was fused to *lacZ* (S1 and S2 Tables). *phuR* promoter activity was maximal in low iron and addition of heme resulted in a concentration-dependent decrease of its activity (Fig. 2C), in accordance with the previous observation indicating that the supply of iron diminishes the mRNA levels of this receptor (Fig. 2B). In contrast, *hasR* promoter activity was induced by all heme concentrations tested, although maximal induction was observed at 5 μM (Fig. 2C). At this heme concentration the activity of the *hxuA* promoter was only moderately induced and higher amounts of heme (20 μM) were required to fully induce the expression from this promoter (Fig. 2C). These results suggest that *P. aeruginosa* adapts the production of its three heme receptors to the amount of heme it encounters.

The P. aeruginosa *PhuR and HasR receptors play a role in heme utilization.*

The contribution of the identified heme receptors to the utilization of heme by *P. aeruginosa* was assayed by constructing *phuR, hasR,* and *hxuA* single, double, and triple mutants and measuring their growth in low iron medium either or not supplemented with heme. All strains grew poorly in low iron conditions but grew at a similar rate as the PAO1 wild-type strain when iron ($FeCl_3$) was added to the medium (Fig. 3A). Addition of 20 μM of heme to the low iron medium allowed the growth of the PAO1 wild-type strain, whereas addition of lower amounts of heme (0.5 and 5 μM) did not (Fig. 3B). The growth of the single phuR-Tn and ΔhasR mutants upon supplementation of heme was delayed, especially that of the ΔhasR mutant (Fig. 3C). However, the ΔhxuA mutant grew in heme at similar rate as the wild-type strain (Fig. 3C). This indicates that PhuR and especially HasR play a larger role in heme utilization than HxuA.

Surprisingly, the growth of double and triple receptor mutants in heme, although impaired, was less affected than that of the *hasR* single mutant (Fig. 3C). This could be due to a switch in the predominant use of heme by *P. aeruginosa* to another source of iron when two or more heme receptors are lacking. In fact, production of the *P. aeruginosa* siderophore pyoverdine was higher in the heme receptor mutants than in the wild-type strain, especially in those mutants that were more impaired for heme utilization (i.e. *hasR* and *phuR* mutants) (S1 Fig.). This indicates that *P. aeruginosa* adapts to a situation in which utilization of heme is less efficient by using other sources of iron.

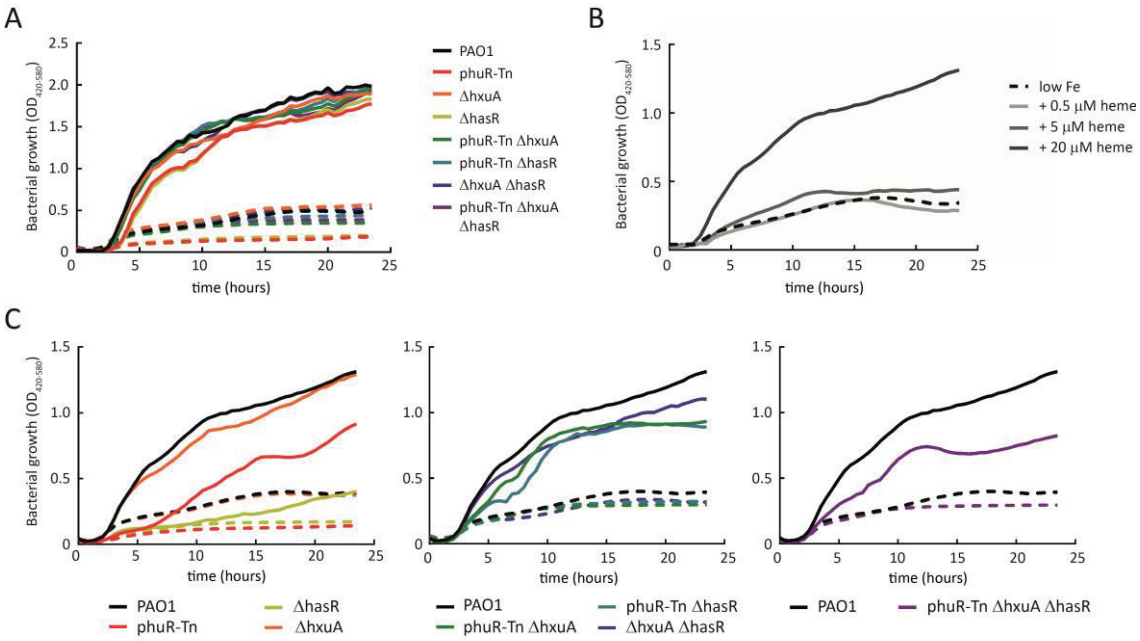


Fig. 3. Growth of *P. aeruginosa* heme receptor mutants. (A) Growth of *P. aeruginosa* PAO1 wild-type strain and the indicated mutants in low iron (dashed line) or low iron supplemented with $FeCl_3$ (solid line). **(B)** Growth of *P. aeruginosa* PAO1 wild-type strain under iron-restricted conditions (low Fe) or iron-restricted medium with the indicated amount of heme. **(C)** Growth of *P. aeruginosa* PAO1 wild-type strain and the indicated single, double and triple heme receptor mutant under iron-restricted conditions (dashed line) or iron-restricted medium supplemented with the indicated amount of heme (solid line). In all panels data are means from three biological replicates (N=3) with SD below 0.18 in all cases (error bars are not included to allow a better visualization of the data).

P. aeruginosa *compensates for a lack of HasR by increasing HxuA production.*

Next, we wondered whether *P. aeruginosa* compensates a lack of one of the three heme receptors by increasing the amounts of the others. Thus, we assayed *hasR*, *hxuA* and *phuR* promoter activity in the single, double and triple receptor mutants (Fig. 4). Because the mutants contain a transposon (Tn) that confers tetracycline (Tc) resistance, a gentamycin (Gm) cassette was cloned in the pMP220-derived plasmids used to measure promoter activity (S1 Table). For an unknown reason, presence of the Gm cassette reduced the *lacZ* activity of the original plasmids (without Gm, Fig. 2C) but had no effect on the overall response of the promoters to low iron or heme (Fig. 4, PAO1 strain). *hasR* expression was unaffected in the phuR-Tn mutant, only slightly reduced in the ΔhxuA mutant and totally abolished in the ΔhasR mutant (Fig. 4A). Complementation of the ΔhasR mutation with a copy of the *hasR-hasAp* operon (Fig. 1B) restored *hasR* expression but not with a copy of the *hasR* gene alone (S2A Fig.). This suggests that the *hasR* deletion affects the expression of its downstream *hasAp* gene and thus the

production of the HasAp hemophore, which seems to be necessary for *hasR* expression. Providing the *hasAp* gene alone to ΔhasR did not restore *hasR* expression (S2A Fig.), which indicates that the HasR receptor is also required for its own expression. Besides, our results also indicate that the lack of PhuR or HxuA is not compensated by increased synthesis of HasR (Fig. 4A). In agreement with this last observation, *hasR* expression was also reduced and not increased in the double mutants, especially the heme-induced expression of this receptor (Fig. 4A). This was more pronounced in the phuR-Tn ΔhasR double and in the triple mutant where *hasR* expression was almost abolished (Fig. 4A).

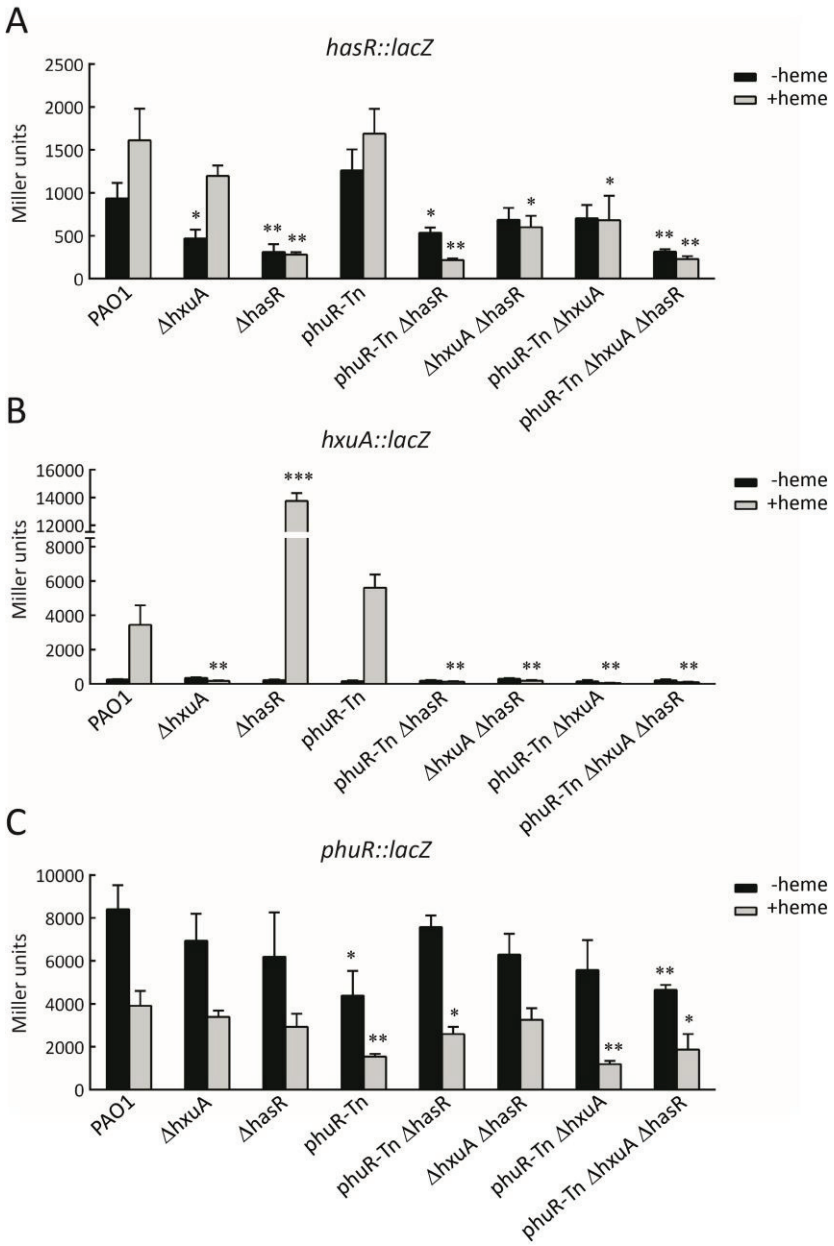


Fig. 4. Expression of the *hasR*, *hxuA* and *phuR* receptors in the absence of one, two or the three heme receptors. β-galactosidase activity of the *hasR* **(A)**, *hxuA* **(B)** or *phuR* **(C)** *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type and the indicated mutants. Strains were grown under iron-restricted conditions without (-) or with (+) heme. Data are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained in the mutant with that of the PAO1 wild-type strain in the same growth condition.

Regarding *hxuA*, expression of this receptor in heme-containing medium was completely null in the ΔhxuA mutant, unaffected in the phuR-Tn mutant and considerably higher (4-fold) in the ΔhasR mutant (Fig. 4B). Complementation of the ΔhxuA and ΔhasR mutations by providing a wild-type copy of the *hxuA* gene or the *hasR-hasAp* operon, respectively, restored the expression of *hxuA* in these mutants to wild-type levels (S2B Fig.). Increased expression of the *hxuA* gene in the ΔhasR mutant correlates with more HxuA receptor protein detected in the outer membrane of this mutant than in the wild-type strain (S3 Fig.). This indicates that *P. aeruginosa* compensates the lack of HasR by increasing the synthesis of HxuA. These results also show that HxuA is essential for its own heme-induced expression. In accordance, *hxuA* expression did not occur in any of the mutants containing the ΔhxuA deletion (Fig. 4B). However, *hxuA* expression was also totally abolished in a phuR-Tn ΔhasR double mutant, despite the presence of an intact HxuA receptor. This suggests that both presence of HxuA and of either the PhuR or the HasR receptor are necessary for *hxuA* expression.

Finally, expression of the PhuR receptor was only affected in the mutants containing the phuR-Tn mutation (Fig. 4C), showing that the loss of HasR or HxuA is not compensated by increasing the synthesis of PhuR and that expression of *phuR* does not depend on HasR and/or HxuA.

The hemophore HasAp is essential for expression of the P. aeruginosa *hasR receptor gene.*

The complementation assay of the ΔhasR mutant suggested that not only the HasR receptor but also the hemophore HasAp is needed for *hasR* expression (S2A Fig.). In accordance, expression of *hasR* was completely null in a hasAp-Tn mutant (Fig. 5A) but it was restored by providing a copy of the wild-type *hasAp* gene to the mutant (S2C Fig.). This phenotype was observed both in absence and presence of heme, which shows that both the low iron- and the heme-induced expression of *hasR* depend on the production of the hemophore HasAp. In contrast, *hxuA* and *phuR* expression was only slightly lower in the mutant (Fig. 5A), which indicates that the hemophore plays a minor role in the production of these receptors. These results suggest a role for HasAp as a signal molecule able to trigger the synthesis of HasR. This was confirmed using the supernatant of *P. aeruginosa* iron-restricted cultures which contain the secreted hemophore (Letoffe et al., 1998). Addition of the supernatant of PAO1 cultures (hemophore-containing supernatant) to the *hasAp*-deficient strain induced *hasR* expression but not that of the hasAp-Tn mutant (hemophore-free supernatant) (Fig. 5B). Interestingly, the supernatant of both PAO1 iron-restricted cultures without heme (apo-HasAp) and with heme (holo-HasAp) were able to induce *hasR* expression (Fig. 5B). This indicates that the HasAp hemophore is able to trigger *hasR* expression even in the absence of external heme. Hemophore production in the ΔhasR mutant was as affected as in the hasAp-Tn mutant according to the *hasR* expression obtained upon addition of ΔhasR culture supernatant (Fig. 5B). This confirms that the downstream *hasAp* gene is not expressed in the ΔhasR mutant, as suggested by the complementation assay (S2A Fig.). In contrast, HasAp production was unaffected in the ΔhxuA and phuR-Tn receptor mutants (Fig. 5B), which is in line with the wild-type *hasR-hasAp* promoter activity measured in these mutants (Fig. 4A).

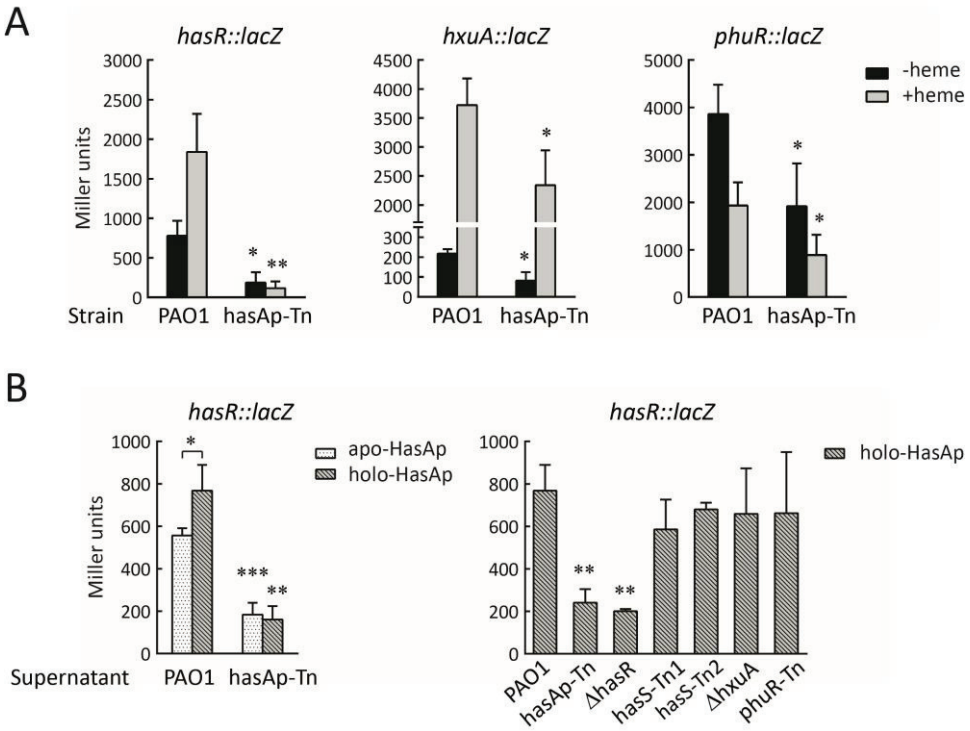


Fig. 5. Expression of the *P. aeruginosa* heme receptors in the absence of hemophore. (A) β-galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain or the hasAp-Tn hemophore mutant after growth under iron-restricted conditions without (-) or with (+) heme. **(B)** β-galactosidase activity of *hasR*::*lacZ* promoter fusion in the *P. aeruginosa* hasAp-Tn hemophore mutant after growth under iron-restricted conditions and the supernatant of the indicated strain cultured in the absence (apo-HasAp) or the presence (holo-HasAp) of external heme. In both panels data are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained in the mutant with that of the PAO1 wild-type strain in the same growth condition or by comparing different growth conditions of a same strain (indicated by brackets in this case).

The HasR and HxuA receptors are part of CSS regulatory pathways.

Both HasR and HxuA, but not PhuR, contain an N-terminal extension typical of TBDR involved in CSS pathways (Fig. 6A) and are genomically associated with σ^{ECF}/anti-σ factor pairs (Llamas et al., 2014) (Fig. 1B). Therefore, we hypothesized that these receptors promote their own expression in response to the inducing signal (i.e. heme) through the activation of their cognate σ^{ECF}. To test this, we measured *hxuA* and *hasR* promoter activity in mutants in each one of the three components of the potential CSS systems. Both promoter activities were null in the CSS receptor mutant (hxuA-Tn and hasR-Tn, respectively) and in the σ^{ECF} mutant (hxuI-Tn and hasI-Tn, respectively) (Fig. 6B), which indicates that these proteins are essential in the signaling pathway that triggers *hxuA* and *hasR* expression. Complementation of the mutants with a wild-type copy of the mutated gene restored expression (Figs. S2A, S2B and S2D). The hasR-Tn mutation could be complemented with the *hasR-hasAp* operon but not with the *hasR* gene alone (S2A Fig.), likely because the Tn insertion affects the expression of the downstream *hasAp* gene (as also observed in the ΔhasR mutant). Constitutive production of σ^{HxuI} and σ^{HasI} resulted in constitutive expression of *hxuA* and *hasR*, respectively (S2D Fig.). Thus, overproduction of σ^{HxuI} and σ^{HasI} can bypass the need of low iron and heme for signaling, as occurs with other σ^{ECF} factors (Llamas et al., 2008; Bastiaansen et al., 2014; Quesada et al., 2016). All these results are in line

with HxuA and HasR being the sensing protein, and σ^{HxuI} and σ^{HasI} the response protein of a CSS pathway (Llamas et al., 2014).

Regarding the CSS anti-σ factor component, mutation of *hxuR* increased *hxuA* promoter activity both in absence and presence of heme (Fig. 6B, hxuR-Tn mutant). Providing a copy of the *hxuR* gene to this mutant restored the wild-type activity (S2E Fig.). Lack of HxuR is thus enough to have expression of *hxuA*, likely because σ^{HxuI} is free in this situation and can promote *hxuA* transcription even when the signaling system has not been activated by heme. In contrast, overproduction of HxuR completely abolished *hxuA* expression (S2E Fig., PAO1 strain). These phenotypes are in line with the anti-σ factor role of this protein. However, unlike *hxuR*, mutation of the *hasS* anti-σ factor did not enhance but impair the low iron-induced expression of *hasR*, which was lower than in the PAO1 wild-type strain (although not null), and did not affect the heme-induced expression of this receptor (Fig. 6B). This effect was observed in both a *hasS* mutant that contains the Tn insertion in the *hasI-hasS* intergenic region (hasS-Tn1) and in a mutant where the Tn is inserted in the periplasmic domain of the protein (hasS-Tn2) (S1 Table and Fig. 7A). A possible effect of the Tn insertion on the expression of the downstream *hasR-hasAp* genes was discarded because the supernatant of both mutants induced *hasR* expression in a *hasAp*-deficient strain (Fig. 5B, *hasS*-Tn1 and -Tn2), which indicates that production of the HasAp hemophore is not affected.

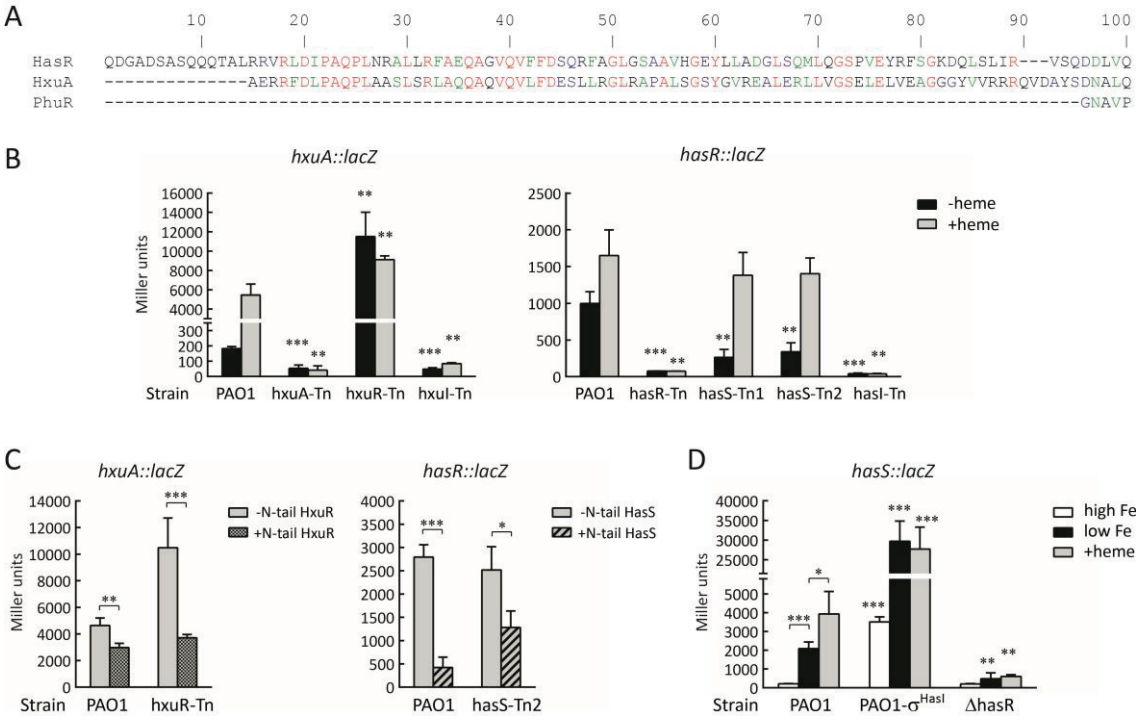


Fig. 6. Analysis of the Has and Hxu CSS pathways. (A) N-terminal extension of mature *P. aeruginosa* HasR and HxuA proteins compared to mature PhuR protein. The numbering of amino acid residues is indicated. Colors indicate identical (red), strongly similar (green) and weakly similar (blue) residues. **(B)** β-galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type and the indicated transposon mutant. Strains were grown under iron-restricted conditions without (-) or with (+) heme. **(C)** β-galactosidase activity of the indicated *lacZ* promoter fusion gene in the PAO1 wild-type and the indicated transposon mutant bearing the empty pMMB67EH plasmid (- N-tail) or the pMMB67EH-derived plasmid expressing the indicated N-tail (+ N-tail) (Table S1). Strains were grown under iron-restricted conditions with IPTG and heme. **(D)** β-galactosidase activity of the *hasS*::*lacZ* promoter fusion in the *P. aeruginosa* wild-type strain bearing the pMMB67EH empty plasmid (PAO1) or the pMMB-hasI

plasmid (PAO1-σ^{HasI}), and in the ΔhasR receptor mutant. Strains were grown under iron-restricted conditions with IPTG and in the absence (low Fe) or presence of $FeCl_3$ (high Fe) or heme (+heme). Data in all panels are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained in the mutant or in cells overproducing σ^{HasI} with that of the PAO1 wild-type strain in the same growth condition, or by comparing different growth conditions of a same strain (indicated by brackets in this case).

Two types of CSS anti-σ factor proteins have been identified, those that function as mere anti-σ factors and those that, next to the anti-σ function, also contain pro-σ activity (Mettrick and Lamont, 2009; Bastiaansen et al., 2015a). The pro-σ activity lies within the cytosolic N-terminal domain (N-tail) of the anti-σ factor protein (Fig. 7A), which has been proposed to be bound to the σ^{ECF} in the σ^{ECF}-RNAP complex (Mahren and Braun, 2003; Llamas et al., 2014). Complete deletion of the first class of anti-σ factors results in constitutive σ^{ECF} activity whereas that of the second class results in a lack of σ^{ECF} activity. The position of the Tn insertion in the hxuR-Tn and hasS-Tn1 mutants (S1 Table) produces the complete absence of the corresponding anti-σ factor protein, including the N-tail domain. According to the phenotype of these mutants (Fig. 6B), HxuR seems to function as a mere anti-σ factor but HasS could also have pro-σ activity. To investigate this, the N-tail of these proteins was overexpressed, because overproduction of this domain has been shown to promote σ^{ECF} inhibition in the case of mere anti-σ factors and activation in the case of anti-σ factors with pro-σ activity (Mettrick and Lamont, 2009). *hxuA* promoter activity was significantly lower in PAO1 wild-type cells overexpressing the N-tail of HxuR, an effect that was more pronounced in cells lacking the HxuR anti-σ factor (Fig. 6C). This confirms that the N-tail of HxuR only contains anti-σ activity. Similarly, overexpression of the N-tail of HasS protein resulted in reduced *hasR* expression (Fig. 6C), indicating that HasS also solely contains anti-σ activity.

hasS *is expressed from its own promoter in a* σ^{HasI}*-dependent manner.*

The phenotype of the *hasS* mutants in which decreased instead of increased σ^{HasI} activity was observed (Fig. 6B) prompted us to investigate whether *hasS* is expressed differently than other anti-σ factors. Most *P. aeruginosa* CSS σ^{ECF}/anti-σ factor genes overlap and are thus co-transcribed from a single transcriptional unit (Llamas et al., 2014), as observed for the *hxuI* and *hxuR* genes that overlap 4 bp (S4 Fig.). However, *hasI* and *hasS* are separated by 64 bp and RT-PCR analyses showed that these genes are not co-transcribed (S4 Fig.). Thus, we constructed a *lacZ* transcriptional fusion of this intergenic region to analyze the presence of an active promoter. Activity of this fusion was null in iron sufficient conditions and was ~10-fold induced in iron deficient conditions (Fig. 6D). This indicates that the *hasI-hasS* intergenic region contains an active promoter for transcription of *hasS* that is regulated by iron. Addition of heme to the low iron medium resulted in increased promoter activity (~2-fold) (Fig. 6D). This pattern of activity resembles that of the *hasR* promoter (Figs. 2C and 6B), suggesting a role of the Has CSS system and σ^{HasI} also in the expression of *hasS*. In agreement, *hasS* promoter activity was extremely high in cells in which σ^{HasI} was constitutively produced, even in iron sufficient conditions, and was totally abolished in a *hasR* mutant (Fig. 6D). Because constitutive production of CSS σ^{ECF} is enough to produce expression of target genes (Llamas et al., 2008; Bastiaansen et al., 2014; Quesada et al., 2016), these results indicate that transcription of *hasS* is initiated by σ^{HasI} upon activation of this σ factor by HasR and the CSS pathway.

The HxuR and HasS anti-σ factors are subject to proteolysis.

Activation of CSS pathways requires the targeted proteolysis of the CSS anti-σ factor (Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). CSS anti-σ factors are usually cytoplasmic membrane proteins that contain a short cytosolic N-domain that binds the σ^{ECF}, a large periplasmic C-domain that receives the CSS signal from the receptor, and a transmembrane domain that connects both regions (Fig. 7A). To determine if proteolysis of the anti-σ factor is also responsible for Has and Hxu CSS activation, processing of N-terminally HA-tagged versions of HxuR and HasS was analyzed by Western-blot. A predominant protein band of ~21-22 kDa was obtained for both proteins in heme-free and heme-containing medium (Fig. 7B, PAO1 strain). This fragment, which is considerably smaller than the size of the full protein (~35-36 kDa), likely corresponds to the N-domain that is produced after the spontaneous cleavage of CSS anti-σ factors. We have recently determined that most CSS anti-σ factors undergo self-cleavage immediately upon production (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b). This spontaneous cleavage occurs in a conserved Gly-Thr (GT) cleavage site located in the periplasmic domain of CSS anti-σ factors by a protease-independent chemical reaction known as N-O/S acyl rearrangement (Bastiaansen et al., 2015b). This cleavage produces two functional N- and C- anti-σ factor domains (Fig. 7A) that interact with each other in the periplasm and are required for proper CSS function (Bastiaansen et al., 2015a). Both HasS and HxuR proteins contain GT residues in the cleavage site (Fig. 7C), and it is therefore not surprising that these proteins undergo the spontaneous cleavage.

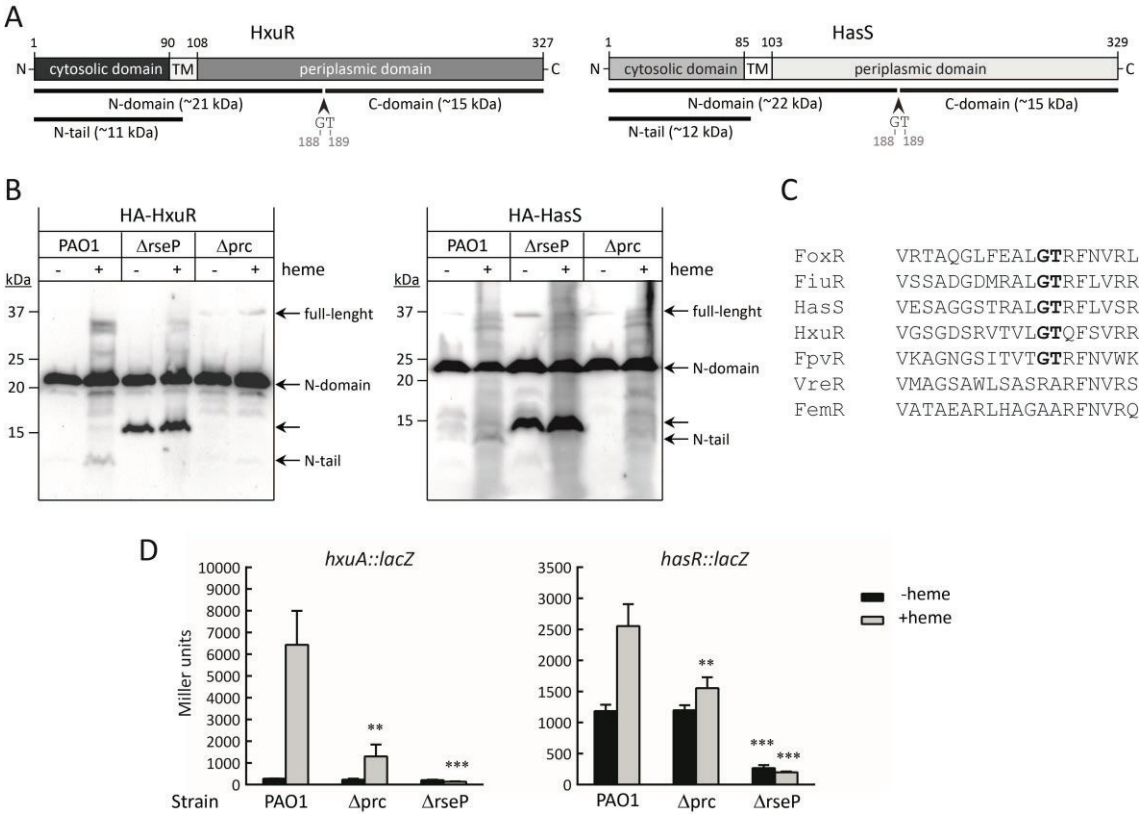


Fig. 7. Processing of the HasS and HxuR anti-σ factors and role of the Prc and RseP proteases. (A) Schematic representation of the HxuR and HasS proteins. The proteins have been drawn to scale, and the cytosolic, transmembrane (TM), and periplasmic domains are shown. Numbers indicate amino acid positions. The spontaneous cleavage site between Gly^{188} and Thr^{189} is indicated as well as the respective N- and C-domains generated from this cleavage and their molecular masses. The estimated size of the N-tail is also indicated. **(B)** *P. aeruginosa* PAO1 wild-type strain and their isogenic Δprc and ΔrseP mutants

expressing an N-terminally HA-tagged HasS or HxuR protein were grown to late log-phase under iron-restricted conditions with IPTG and in the absence (-) or presence (+) of heme. Proteins were immunoblotted for HA. Position of the protein fragments and the molecular size marker (in kDa) is indicated. Presence of the HA-tag adds ~1 kDa to the molar mass of the protein fragments. Blots are representative of four biological replicates (N=4). **(C)** Alignment of the region of the indicated *P. aeruginosa* anti-σ factor proteins that contains the conserved Gly and Thr residues (GT) (in bold) where the spontaneous cleavage of these proteins occurs. **(D)** Activity of the Has and Hxu CSS systems in the Δprc and ΔrseP protease mutants. β-galactosidase activities were determined after bacterial growth in iron-restricted conditions without (-) or with (+)heme. Data are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained in the mutant with that of the PAO1 wild-type strain in the same growth condition.

In response to heme, the N-domains of HxuR and HasS were further processed producing protein subfragments of about 12-13 kDa (Fig. 7B, PAO1), which is the expected size of the cytosolic tail (N-tail) of the proteins (Fig. 7A). Two proteases, RseP and Prc have been identified to be involved in CSS anti-σ factor processing in response to the CSS signal (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). RseP cuts within the transmembrane (TM) domain of CSS anti-σ factors generating the N-tail subfragment (Bastiaansen et al., 2015a). This cleavage allows the release and activation of the σ^{ECF} factor and it is therefore indispensable for CSS activity (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). In accordance with this model, the N-tails of HxuR and HasS were not detected in a ΔrseP mutant (Fig. 7B). Instead, larger HxuR and HasS protein fragments were accumulated (Fig. 7B). These fragments likely are the RseP substrates and their accumulation in the mutant indicates that the N-domains of HxuR and HasS undergo another cleavage prior the RseP cleavage. In agreement with RseP being essential for CSS σ^{ECF} activation, expression of *hxuA* and *hasR* was completely abolished in the ΔrseP mutant in both absence and presence of heme (Fig. 7D). The HxuR and HasS protein fragments that accumulated in the *rseP* mutant where absent in a Δprc mutant (Fig. 7B). This suggests that Prc is responsible of the proteolytic event that generates these RseP substrates or acts upstream of it, thus being essential for this event to occur. Activity of the Hxu and Has CSS systems in the Δprc mutant was considerably lower than in the wild-type strain, especially that of the Hxu system, although not null (Fig. 7D). In fact, small amounts of the HxuR and HasS N-tail subfragments, usually indicative of CSS activation (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b), could still be detected in the Δprc mutant (Fig. 7B). This indicates that the Hxu and Has systems can be partially activated in a Prc-independent manner.

Transcription of phuR*,* phuS *and* hemO *is not promoted by* σ^{HasI} *or* σ^{HxuI}*.*

We next wondered whether expression of the heme utilization genes *phuR*, *phuSTUVW* and *hemO* (Fig. 1A and 1C) depends on the Has and/or Hxu CSS pathways. Therefore, we tested the ability of the CSS response proteins σ^{HasI} and σ^{HxuI} to activate expression from the promoter of these genes. However, constitutive production of σ^{HasI} or σ^{HxuI} did not induce the activity of *phuR*, *phuS* and *hemO* promoters while it did induce the activity of the σ^{HasI}-dependent promoter *hasR* and that of the σ^{HxuI}-dependent promoter *hxuA* (Fig. 8). This indicates that the transcription of *phuR*, *phuS* and *hemO* is not mediated by these σ factors and thus that their expression does not depend on the Has and Hxu signaling pathways. In accordance, the *phuR*, *phuS* and *hemO* promoter activities did not increase in response to the Has and Hxu CSS inducing signal heme (Fig. 8). As previously observed for *phuR* (Fig. 2B and 2C), expression of *phuS* and *hemO* seem to be controlled solely by the intracellular concentration of iron and thus occurs in low but not in high iron conditions and diminishes in presence of the iron-supplier heme (Fig. 8).

Besides, analysis of *hasR* expression in cells producing σ^{HxuI} and that of *hxuA* in cells producing σ^{HasI} showed that there is no cross-talk between the Has and Hxu signaling systems at the σ factor level because σ^{HasI} did not induce *hxuA* promoter activity and σ^{HxuI} did not affect that of *hasR* (Fig. 8). In contrast, a considerably reduction in *hxuA* expression was observed in cells overproducing σ^{HasI} (Fig. 8). This suggests that high amounts of σ^{HasI} impair the σ^{HxuI}-mediated transcription of *hxuA* likely due to the competition of these σ factors for binding to the RNAPc.

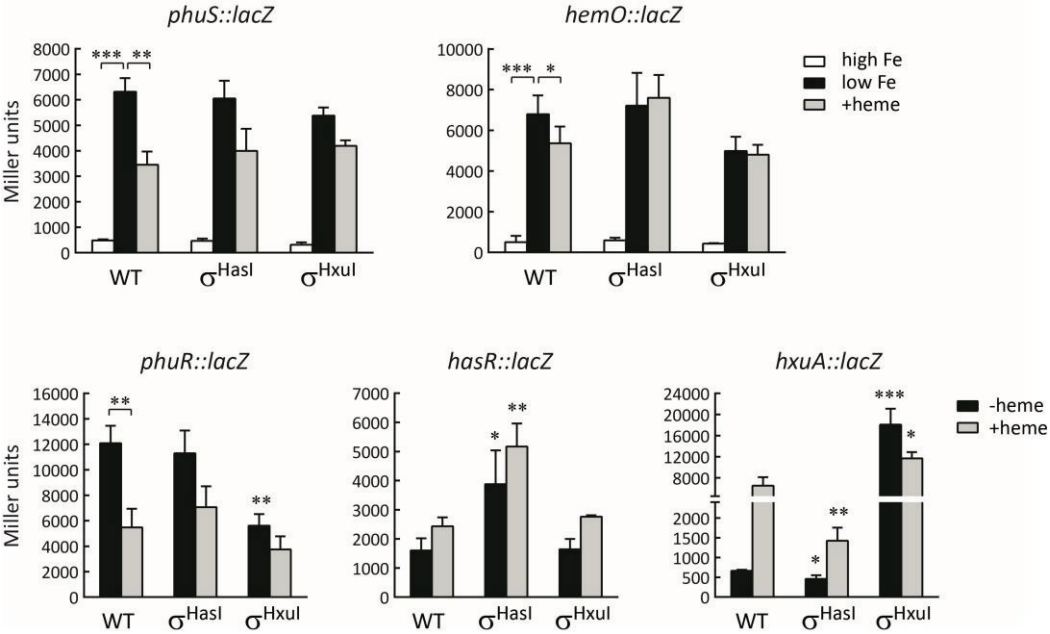


Fig. 8. Expression of heme genes in cells overexpressing σ^{HasI} or σ^{HxuI}. β-galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain bearing the pMMB67EH empty plasmid (WT), the pMMB-hasI plasmid (σ^{HasI}), or the pMMB-hxuI plasmid (σ^{HxuI}). Strains were grown in IPTG-containing iron-restricted medium (low Fe or -heme) supplemented with $FeCl_3$ (high Fe) or heme (+heme). Data are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by comparing the value obtained in the cells overexpressing the σ factor with that of the PAO1 wild-type strain in the same growth condition, or by comparing different growth conditions of a same strain (indicated by brackets in this case).

DISCUSSION

Acquisition of iron by pathogens is key for a successful infection (Ratledge and Dover, 2000; Sheldon and Heinrichs, 2015). Because heme accounts for the vast majority of the iron pool in mammalian hosts (Parrow et al., 2013), the capacity of a pathogen to use this host molecule as source of iron represents an important advantage. In the human pathogen *P. aeruginosa*, the Phu and Has systems discovered by Ochsner and coworkers in 2000 (Ochsner et al., 2000) (Fig. 1) have since been considered the only heme utilization systems (Smith and Wilks, 2015; Huang and Wilks, 2017). However, we report here the identification of a third heme system in this bacterium, the Hxu system, which makes *P. aeruginosa* the only pathogen in which three heme systems have been identified. The three systems involve a receptor of the TBDR family. These outer membrane proteins have an N-terminal plug domain that is sequestered inside a C-terminal 22-stranded β barrel domain in the absence of ligand and is ejected from the β barrel upon ligand binding, thereby allowing its transport (Noinaj et al., 2010). Most bacterial heme receptors bind heme through two His residues located within the plug domain and the FRAP/PNPNL extracellular loop of the protein, respectively(Bracken et al., 1999; Krieg et al.,

2009). The *P. aeruginosa* HasR receptor presents this bis-His coordination (His^{221} and His^{624}) but not the PhuR receptor that binds heme through His^{124} and Tyr^{519} (Smith et al., 2015) (S5 Fig.). The HxuA receptor contains a conserved His residue within the plug domain (His^{223}) but not within the extracellular loop where a Tyr residue is present instead (Tyr^{596}) (S5 Fig.). It has been proposed that the emergence of the His-Tyr coordination in heme receptors provides an advantage in scavenging and acquiring heme over the bis-His coordination (Smith et al., 2015). These authors suggest that this is in line with PhuR being the main heme utilization receptor of *P. aeruginosa*, a suggestion based primarily on the impaired heme utilization phenotype they observed in a *phuR* mutant but not in a *hasR* mutant (Smith and Wilks, 2015). However, our results are not in line with this observation because the growth of both *hasR* and *phuR* mutants in heme is considerably impaired (Fig. 3C), which is in accordance with the results originally reported by Ochsner and coworkers (Ochsner et al., 2000). Moreover, HxuA, which seems to coordinate heme through His-Tyr residues (S5 Fig.) does not appear to play a predominant role in heme acquisition (Fig. 3C). Our results suggest that *P. aeruginosa* adapts the production of its three heme receptors to the amount of heme encountered. In low iron conditions PhuR is fully and HasR partially produced, making the bacterium ready to use heme if this source of iron becomes available. Production of PhuR goes down when iron is being supplied as heme and that of HasR when high amounts of heme are encountered. This correlates with increased synthesis of HxuA, which would allow *P. aeruginosa* to continue using heme to obtain iron and/or to sense heme and, therefore, the host.

We have shown that the *P. aeruginosa* HasR and HxuA receptors are involved in sensing and signaling and that, together with an σ^{ECF}/anti-σ pair, form part of functional signal transduction pathways of the CSS class. Our results are in accordance with a model in which sensing of free heme by HxuA or the hemophore HasAp by HasR, induces a signaling cascade that promotes the activation of σ^{HxuI} and σ^{HasI} in the cytosol, which then initiates transcription of *hxuA* and *hasR*, respectively (Fig. 9). Most *has* and *hxu* gene promoter regions (except that of *hasS*) contain a Fur binding-site (S6 Fig.), which suggests that the regulation exerted by iron occurs through the Fur repressor protein that inhibits gene transcription when high intracellular amounts of iron are encountered and allows it when the iron levels are low (Escolar et al., 1999). The anti-σ factor *hxuR* is co-transcribed with *hxuI* (S4 Fig.) and therefore also expressed under iron starvation, which produces the inhibition of σ^{HxuI} (Fig. 9A). The basal amount of HxuA produced under iron starvation allows heme sensing when heme becomes available, which then results in σ^{HxuI} activation and maximal HxuA production (Fig. 9B). Activation of the Has system is slightly different because this signaling pathway is already active under iron starvation. This activation depends completely on the hemophore HasAp (Fig. 5A), which is co-transcribed with *hasR* under iron starvation (Ochsner et al., 2000) (Fig. 1B). This suggests that HasAp is able to activate the Has CSS pathway even in the absence of external heme. In fact, the supernatant of heme-free low iron cultures of *P. aeruginosa* induces *hasR* expression (Fig. 5B, apo-HasAp), which further supports that the hemophore produced and secreted by *P. aeruginosa* in absence of external heme can signal through the Has system. In *S. marcescens* a HasA hemophore protein mutant impaired in heme binding is unable to signal (Rossi et al., 2003). However, some degree of signaling is observed with purified apo-HasA, probably due to the traces of heme that the purified protein contains (Rossi et al., 2003). A similar situation could occur in *P. aeruginosa* because this bacterium can synthesize heme (Kawasaki et al., 1997), and thus activation of the Has system in absence of external heme could occur through HasAp molecules loaded with endogenous heme. Further research will clarify this matter.

Activation of the Has and Hxu signaling pathways requires the targeted proteolysis of the anti-σ factor component HasS and HxuR, respectively. The first proteolytic event takes place in the absence of heme (Fig. 9A). Presence of GT residues in the cleavage site of HasS and HxuR suggests that this step occurs by a protease-independent mechanism known as N-O acyl rearrangement, as we demonstrated for the *P. aeruginosa* FoxR anti-σ factor (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b). This spontaneous cleavage produces a HasS and HxuR N-domain that by analogy with FoxR(Bastiaansen et al., 2015a) likely interacts with its corresponding C-domain in the periplasm (Fig. 9A). However, contrary to what other authors have proposed (Huang and Wilks, 2017), this proteolytic step is not responsible for the liberation and activation of either σ^{HasI}, or σ^{HxuI}, because it takes place in situations in which σ^{HasI} and σ^{HxuI} are inactive (e.g. absence of heme or in the ΔrseP mutant) (Fig. 7). Activation of these σ factors involves the targeted proteolysis of, at least, the N-domain of its cognate anti-σ factor (Fig. 9B). Proteolysis of CSS anti-σ factors in response to the inducing signal occurs through regulated intramembrane proteolysis (RIP) , a conserved mechanism in which a transmembrane protein is subjected to several processing steps in order to liberate and activate a cytosolic effector (Brown et al., 2000). The RIP of CSS anti-σ factors always involves the site-2 metalloprotease RseP, which cuts within the transmembrane domain of the protein generating the cytosolic N-tail (Draper et al., 2011; Bastiaansen et al., 2014; Llamas et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). Production of the N-tail coincides with CSS σ^{ECF} activation, likely due to the release of the σ factor in the cytosol (Fig. 9B). In accordance with being activated by RIP of their cognate anti-σ factor, σ^{HasI} and σ^{HxuI} activities are null in the ΔrseP mutant (Fig. 7D). RseP substrate recognition and cleavage occurs through the size-filtering function of the two PDZ domains of RseP rather than by the recognition of a specific sequence/motif (Hizukuri et al., 2014), and the RseP substrate is normally generated by a site-1 protease (Brown et al., 2000). The first evidence that a site-1 cleavage of CSS anti-σ factors takes place is the accumulation of a slightly larger fragment than the RseP product in an *rseP* mutant (Bastiaansen et al., 2014; Bastiaansen et al., 2015a), which we have also observed for HasS and HxuR (Fig. 7B). The periplasmic protease Prc is required for the generation of the RseP substrate of the HasS and HxuR proteins (Fig. 7B). However, whether or not Prc cuts directly on these anti-σ factors needs to be investigated. A difference of the Has CSS pathway with respect to the Hxu system is the functioning and expression of the anti-σ factor component. Whereas HxuR is totally dispensable for σ^{HxuI} activity, σ^{HasI} activity seems to require HasS (Fig. 6B), although no pro-σ activity has been observed for any of these anti-σ factors (Fig. 6C). Besides, *hasS* transcription is promoted by σ^{HasI}. This is not a common feature of CSS anti-σ factors which are normally co-transcribed with their cognate σ^{ECF} gene by the σ^{70}-RNAP holoenzyme (Llamas et al., 2014). This dependency on σ^{HasI} for *hasS* transcription also happens in *S. marcescens* and it has been proposed that this mechanism allows fine tuning to the heme-hemophore signal (Biville et al., 2004). In this way σ^{HasI}, which does not promote its own expression (Biville et al., 2004), autoregulates its activity.

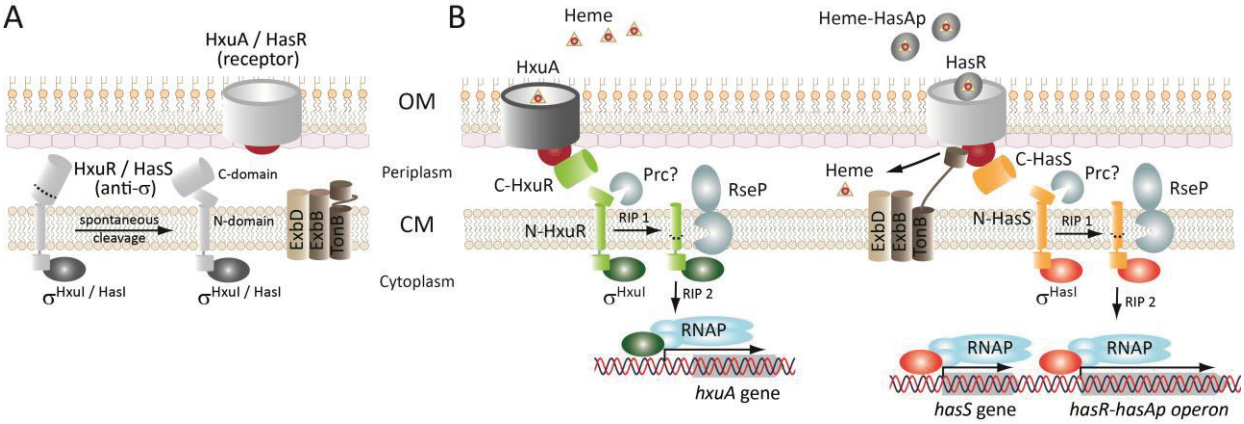


Fig. 9. Schematic models of the *P. aeruginosa* Has and Hxu CSS systems. (A) The three proteins of the Hxu and Has CSS systems (receptor, anti-σ factor and σ^{ECF}) are shown. Immediately upon production and in the absence of heme, the HxuR and HasS anti-σ factors undergo a spontaneous cleavage that results in the generation of two protein domains. By analogy with other anti-σ factor (Bastiaansen et al., 2015a), it is likely that these domains interact with each other in the periplasm. The N-domain of the anti-σ factor binds to and keeps sequester the σ^{ECF}. **(B)** Detection of free heme by HxuA or the heme-hemophore (HasAp) complex by HasR generates a signal that is transmitted by the signaling domain of these receptors (in red). This triggers the regulated intramembrane proteolysis (RIP) of the N-domain of HxuR and HasS by the action of, at least, the Prc and RseP proteases. Liberation of σ^{HxuI} results in *hxuA* gene transcription and that of σ^{HasI} in *hasS* and *hasR-hasAp* transcription. Uptake of heme through HasR occurs in an energy-dependent process involving the ExbBD-TonB complex (Smith et al., 2015). OM, outer membrane; CM, cytoplasmic membrane. Figure adapted from (Llamas et al., 2014) with the results from this work.

Liberation and activation of σ^{HasI} and σ^{HxuI} result in the transcription of target genes by the σ^{HasI}- or σ^{HxuI}-loaded RNAP holoenzyme (Fig. 9B). The regulons of these σ factors have not been identified yet, but we have shown here that the σ^{HasI} regulon includes the *hasR* and *hasS* genes, and the σ^{HxuI} regulon the *hxuA* gene, and that none of them include the *phuR*, *phuSTUVW* and *hemO* genes (Figs. 1 and 8), as proposed for σ^{HasI} previously (Smith and Wilks, 2015). Actually, these authors observed a considerably higher expression of *phuS* in a *hasR* receptor mutant (Smith and Wilks, 2015). Because activation of CSS σ^{ECF} does not occur in CSS receptor mutants (Llamas et al., 2014), increased expression of *phuS* in a *hasR* mutant is the opposite of what would be expected if σ^{HasI} would indeed be involved in the transcription of this gene. In fact, the σ^{HasI}-transcribed genes *hasR* and *hasS* are not expressed in the *hasR* mutant (Figs. 6B and 6D). σ^{HasI} and σ^{HxuI} are activated under the same condition, i.e. presence of external heme, and they thus likely compete for binding to the RNAPc in this situation. We have observed increased production of the HxuA receptor upon heme induction when the Has CSS pathway, and therefore σ^{HasI}, is inactive (i.e. in the ΔhasR mutant) (Figs. 4B and S3). This can be due to the formation of more σ^{HxuI}-RNAP complexes in conditions in which σ^{HasI} is not active, which results in increased *hxuA* transcription. The opposite effect is not observed, which suggests that σ^{HasI} binds more strongly to the RNAPc than σ^{HxuI}. Indeed, overproduction of σ^{HasI} considerably reduces the σ^{HxuI}-mediated expression of *hxuA* whereas overeproduction of σ^{HxuI} does not affect *hasR* expression (Fig. 8). Because HasR and HxuA are both involved in heme sensing, this mechanism of increasing HxuA production when HasR is lacking would ensure the ability of *P. aeruginosa* to continue recognizing this host molecule. Identification of the regulons of σ^{HasI} and σ^{HxuI} is an important topic that we will undertake in the near future to understand how *P. aeruginosa* uses heme not only as a source of iron but also to respond to the host.

In summary, we report here that the human pathogen *P. aeruginosa* contains three heme systems. These systems are highly conserved among *P. aeruginosa* clinical isolates (S1 Dataset), which highlights the importance of this host molecule during infection. In accordance, expression of most *P. aeruginosa* heme genes is considerably induced in murine models of acute and chronic infections (Turner et al., 2014; Damron et al., 2016). Because a mutant lacking the HasR heme receptor has strongly attenuated virulence (Damron et al., 2016), blocking heme sensing and acquisition may be a promising strategy to combat *P. aeruginosa* infections, and our work has revealed important new information that could be used to achieve this aim.

METHODS

Bacterial strains. Strains used in this study are listed in S1 Table. *P. aeruginosa* PAO1 wild-type strain and all the transposon insertion mutants used were from the comprehensive *P. aeruginosa* transposon mutant library at the University of Washington Genome Center (Jacobs et al., 2003). The locations of the mutations were confirmed by PCR with primers flanking the insertion sites. Construction of null mutants was performed by allelic exchange using the suicide vector pKNG101 as described before (Bastiaansen et al., 2014). Southern blot analyses to confirm the transposon insertion or the chromosomal gene deletion were performed as described (Llamas et al., 2000). Bacteria were routinely grown in liquid LB (Sambrook et al., 1989) on a rotatory shaker at 37ºC and 200 rpm. For low iron conditions, cells were cultured in CAS medium (Llamas et al., 2006) containing 400 µM of 2,2'-bipyridyl. The low iron medium was supplemented with 20 µM of heme (Sigma-Aldrich) (other amounts used are indicated) or 100 µM of $FeCl_3$ (high iron medium). The concentration of heme in the stock solutions was determined by a pyridine hemochromagen assay (Barr and Guo, 2015). When required, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to induce full expression from the pMMB67EH P*tac* promoter. Antibiotics were used at the following final concentrations (µg/ml): ampicillin (Ap), 100; gentamycin (Gm), 10; piperacillin (Pip), 25; streptomycin (Sm), 100; tetracycline (Tc), 20.

Plasmid construction and molecular biology. Plasmids used are described in S1 Table and primers listed in S2 Table. PCR amplifications were performed using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) or Expand High Fidelity DNA polymerase (Roche). All constructs were confirmed by DNA sequencing and transferred to *P. aeruginosa* by electroporation (Choi et al., 2006).

Outer membrane proteins isolation and proteomics. *P. aeruginosa* PAO1 was cultured overnight in 1 liter of low iron medium without or with 20 µM heme at 37 ºC. Cells were harvested and centrifuged at 10.000 × g for 30 min. Pellets were frozen at -80 ºC during 15 min and then resuspended in 30 ml of buffer A (20 mM Tris, 0.1 mM EDTA, 300 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, pH 7.35) supplemented with 1x Complete protease inhibitor cocktail (Roche) and benzonase (Merck). Cells were lysed by repeated French Press passages at 1.000 psi. Cell lysates were centrifuged at 10.000 × g for 20 min to remove unbroken cells. Supernatants were then centrifuged at 68.000 × g in an ultracentrifuge (Model ULTRA-100, Thermo Scientific) during 1 h 30 min. Outer membrane proteins from pellets were extracted with 15 ml of buffer A containing 1% (v/v) Triton X-100 at room temperature for 4 h. Proteins were then concentrated in an ultrafiltration device (Amicon Ultra, Millipore) by centrifugation at 8.000 × g and separated by SDS-PAGE containing 7.5% (w/v) acrylamide. Proteins were

visualized by Coomasie blue staining and bands of interest excised for proteomic analyses at the Proteomics facility of the University Complutense of Madrid (Spain). Proteins were digested with trypsin and the resulting peptides analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) to obtain the peptide fingerprint. Proteins ambiguously identified by matching experimental masses with those of theoretical digests of bacterial proteins were subjected to MS/MS sequencing analyses. The identification of proteins by peptide mass footprints were carried out with MASCOT version 2.3 (Matrix Science) in NCBI database without and with taxonomic restriction to bacteria. A Mascot's MOWSE (for molecular weight search) score >89 and a sequence coverage ≥16% was used as cut off for protein identification. The identified proteins exceeded the score that the mascot's MOWSE algorithm estimated to be statistically significant with a P-value < 0.05.

Enzyme assay. β-galactosidase activities in soluble cell extracts were determined using *o*-nitrophenyl-b-D-galactopyranoside (ONPG) (Sigma-Aldrich) as described before (Llamas et al., 2006). Each condition was tested in duplicate in at least three biologically independent experiments and the data given are the average of the three biologically independent experiments with error bars representing standard deviation (SD). Activity is expressed in Miller units.

Real-time PCR analyses. Total RNA from late exponentially grown *P. aeruginosa* PAO1 cells in low iron medium or low iron medium supplemented with 200 μM $FeCl_3$ (high iron) or 20 μM heme was extracted by the hot phenol method using the TRI® Reagent protocol (Ambion) as described elsewhere (Llamas et al., 2008). The isolated RNA was subjected to two DNase I treatments with the Turbo DNA-free kit (Ambion) and RNaseOUT (Invitrogen) to eliminate contaminated DNA. RNA quality, including purity, integrity and yield, was assessed by electrophoresis of 1 μg of total RNA and by UV absorption at 260 nm in a ND-1000 spectrophotometer (NanoDrop Technologies, USA). RT-PCR to assess the co-transcription of the *hasI-hasS* and *hxuI-huxR* genes was performed on total RNA using the Titan One-Tube RT-PCR system (Roche) in accordance with the manufacturer's recommendations and primers that amplify the *hasIS* or *hxuIR* intergenic regions (Table S2). For each reaction, 50 ng of total RNA from three biologically independent PAO1 cultures was used. Thermal cycling conditions were the following: one cycle at 42°C for 30 min, one cycle at 95ºC for 2 min, and then 35 cycles at 95°C for 30 sec, 58°C for 30 s and 68°C for 30 s, with a final cycle at 68ºC during 10 min. DNA contamination of the RNA samples was ruled out by performing a PCR with Taq DNA polymerase (Roche). A positive control using *P. aeruginosa* genomic DNA was included. For quantitative Real-time PCR (qRT-PCR), cDNA was produced in triplicate by reverse transcription reactions of 0.5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers as primers according to the protocol supplied. Amplifications were carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 optical system software (version 2.1.97.1001) in 11,5 μl reaction mixture containing 6,25 μl of iQ SYBR green Supermix (Bio-Rad), 0.4 μM of each primer (Table S2) and 1 μl of the template cDNA (diluted 1000-fold when measuring the 16S rRNA reference gene). Thermal cycling conditions were the following: one cycle at 95°C for 5 min and then 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. Melting curve analyses were performed by gradually heating the PCR mixture from 55 to 95°C at a rate of 0.5°C per 10 s for 80 cycles. The relative expression of the genes was normalized to that of 16S rRNA and the results were analyzed by means of the comparative cycle threshold (ΔΔct) method

(Pfaffl, 2001). Three biologically independent experiments were performed each one including three technical replicates and the data given are the average.

Growth curves. *P. aeruginosa* strains were cultured overnight in LB medium. Cultures were then diluted and inoculated in a Honeycomb® plate (Oy Growth Curves AB Ltd) containing 200 µl of CAS medium with 800 µM of 2,2'-bipyridyl (low Fe medium) supplemented or not with 200 µM $FeCl_3$ or 20 µM heme. Plates were placed in a Bioscreen C Microbiology Reader™ and incubated at 37 ºC with shaking during 24 h. Turbidity measurement were performed every 30 min using a wideband filter (420-580 nm). Three technical repeats were performed in three biologically independent experiments and the data given are the average.

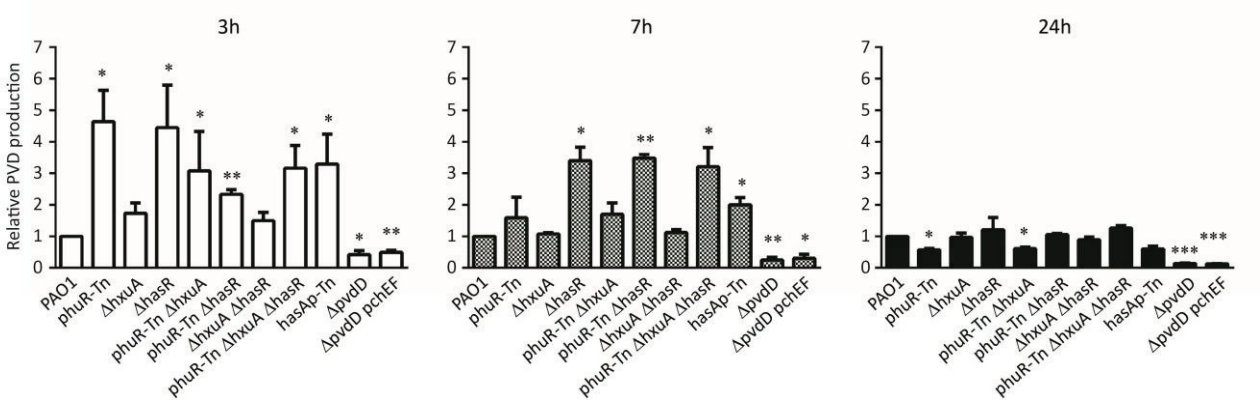
Preparation of hemophore containing supernatants. Hemophore containing supernatants were obtained from iron-restricted cultures of *P. aeruginosa* PAO1 and hemophore free supernatants from *P. aeruginosa* hasAp-Tn cultures in the absence (apo-HasAp) or presence (holo-HasAp) of 20 µM heme (Sigma-Aldrich). Supernatants were filtered through a 0.2 µm filter (GE Healthcare) and added in 1:1 proportion to iron restricted cultures. Presence of the hemophore in the supernatants was analysed by measuring the activity of the *hasR* promoter (pMPhasRGm plasmid) in the *P. aeruginosa* hasAp-Tn hemophore mutant. At least three biologically independent experiments were performed by using supernatants from three different cultures on a given strain.

Pyoverdine production. Overnight cultures of *P. aeruginosa* strains in LB medium were diluted and inoculated in CAS medium containing 800 µM of 2,2'-bipyridyl supplemented with 20 µM heme. The production of pyoverdine was measured spectrophotometrically as described before (Wilderman et al., 2001). Briefly, 1 mL of the culture at the indicated growth time was centrifuged and the supernatant filtered through a 0.22 µm pore size filter. The absorbance at 380 nm was measured and normalized for differences in cell density with the OD at 660 nm of the cultures. Values were normalized to that obtained with the PAO1 wild-type strain. Data given are the average of three biologically independent experiments with error bars representing standard deviation (SD).

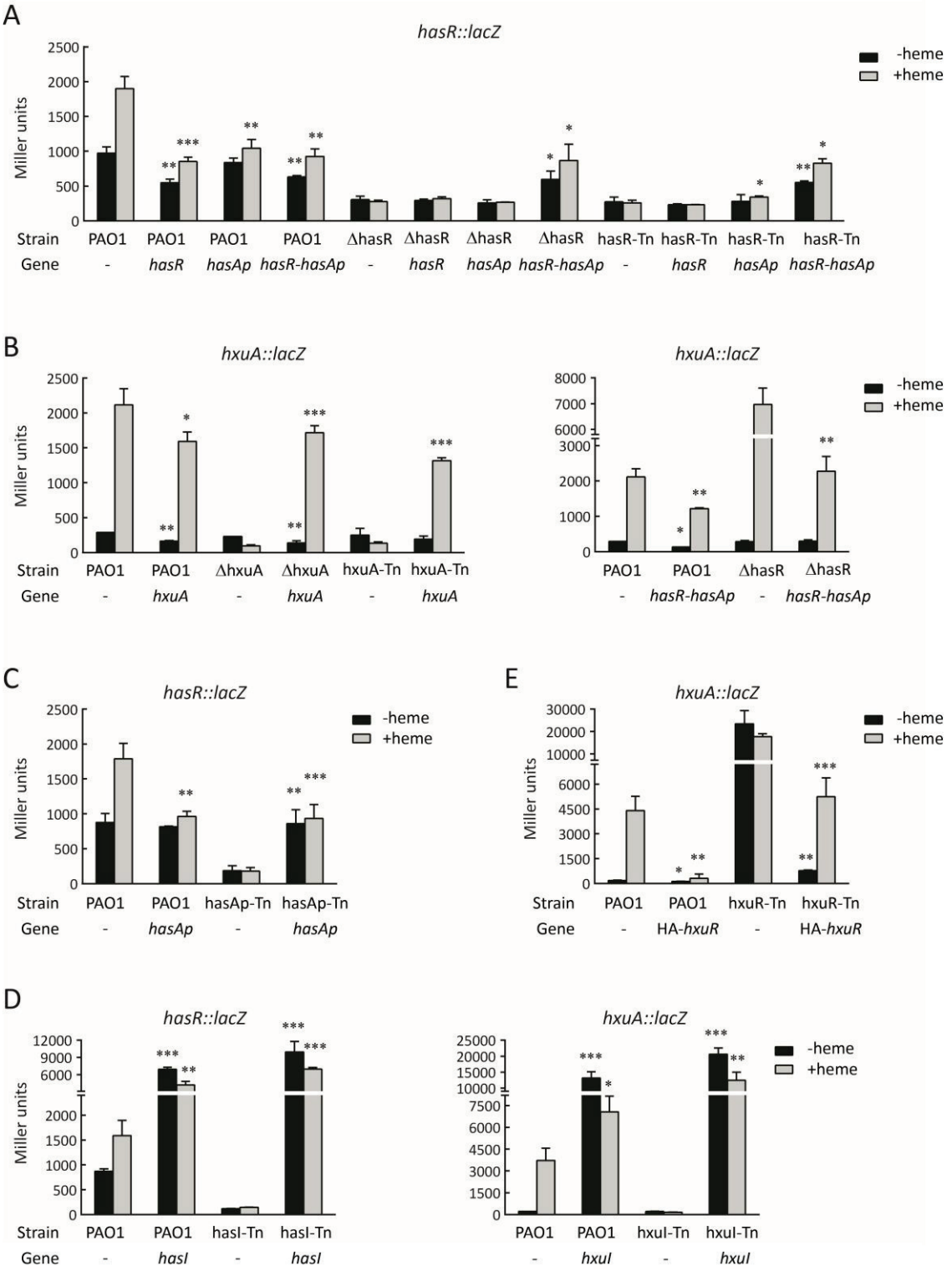
SDS-PAGE and Western-blot. *P. aeruginosa* was grown in iron-limited medium containing 1 mM IPTG and without or with 20 µM heme until late log phase. Cells were pelleted by centrifugation and heated for 10 min at 95 °C following solubilization in SDS-PAGE sample buffer. Sample normalization was done according to the OD_{660} of the bacterial culture. Proteins were separated by SDS-PAGE containing 12 or 15% (w/v) acrylamide and electrotransferred to nitrocellulose membranes (Millipore). Ponceau S (Serva) staining was performed as a loading control. HA-tagged proteins were detected using a monoclonal antibody directed against the influenza hemagglutinin epitope (HA.11, Covance) as previously described (Bastiaansen et al., 2014).

Computer-assisted analysis. Protein sequence analysis and classification were performed using InterPro (Finn et al., 2017) and alignments with ClustalW (Thompson et al., 1994). BLASTn and DIAMOND BLASTp analyses of heme gene promoter regions and proteins were performed at http://www.pseudomonas.com/ (Winsor et al., 2016) using a total of 93 *P. aeruginosa* complete genome sequences (S1 Dataset). Statistical analyses are based on *t*-test in which two conditions are compared independently. P-values from raw data (i.e. miller units from β-galactosidase assays) were calculated by independent two-tailed *t*-test and from ratio data to the control (i.e. qRT-PCR and pyoverdine production) by one-sample *t*-test using GRAPHPAD PRISM version 5.01 for Windows and are represented in the graphs by *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

SUPPLEMENTAL INFORMATION

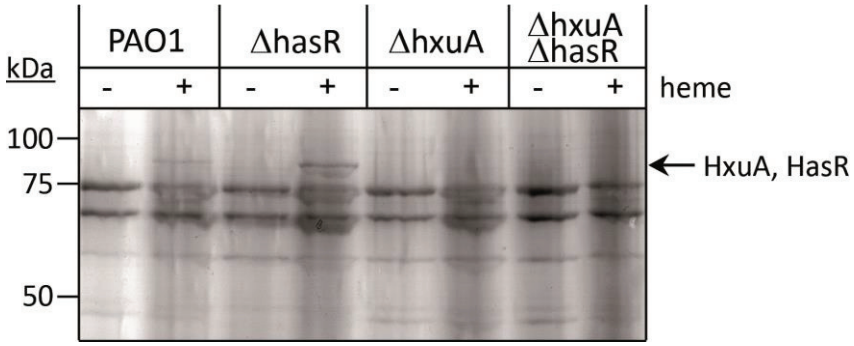


S1 Fig. Production of pyoverdine by *P. aeruginosa* heme receptor mutants. *P. aeruginosa* strains were grown in low iron medium with heme. The production of pyoverdine (PVD) relative to the production in the PAO1 wild-type strain after 3, 7 and 24 hours of growth of each mutant is shown Mutants deficient in pyoverdine (*pvdD*) production were used as control. Data are means ± SD from three biological replicates (N=3). P-values were calculated by one-sample *t*-test to a hypothetical value of 1 as described in Materials and Methods.

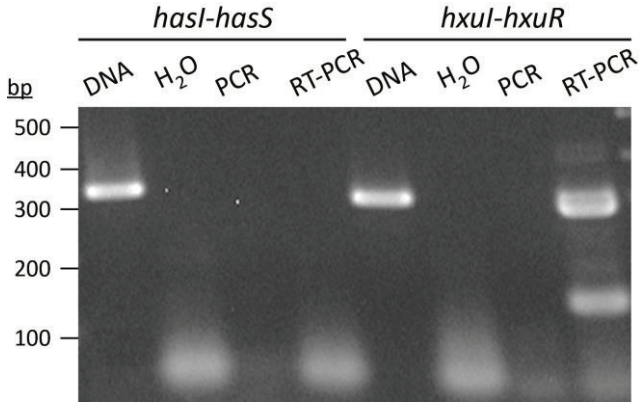


S2 Fig. Complementation of key heme mutant phenotypes. Complementation of **(A, B)** the HasR and HxuA receptor mutants**, (C)** the hemophore hasAp-Tn mutant, **(D)** the hxuI-Tn and hasI-Tn σ factor mutants, and **(E)** the hxuR-Tn anti-σ factor mutant. β-galactosidase activity of the indicated *lacZ* promoter fusion gene in the *P. aeruginosa* PAO1 wild-type strain and the indicated mutant bearing the empty (-) or the pMMB67EH-derived plasmid (Table S1) expressing the indicated gene. Bacteria were grown in iron-restricted conditions without (-) or with (+) heme. The expression of the *hasAp* and HA-*hxuR* genes from the pMMB67EH Ptac promoter was induced by adding IPTG to the cultures. Data are means ± SD from three biological replicates (N=3). P-values were calculated as described in Materials and Methods by

comparing the value obtained in the strain bearing the empty plasmid (-) with that of the same strain expressing the indicated gene in the same growth condition.



S3 Fig. Outer membrane composition of *P. aeruginosa hxuA* and *hasR* receptor mutants. SDS-PAGE of *P. aeruginosa* PAO1 outer membrane proteins after growth in iron-restricted conditions without (-) or with (+) heme. The position of the band corresponding to a mixture of the HasR and HxuA receptor proteins is indicated on the right and that of the molecular size marker on the left. SDS-PAGE is representative of two biological replicates (N=2).



S4 Fig. Analysis of the co-transcription of the *has* and *hxu* σ^{ECF}/anti-σ genes by RT-PCR. Total RNA was isolated from the *P. aeruginosa* PAO1 wild-type strain grown under iron-restricted conditions supplemented with heme. Reactions were performed with primers amplifying the *hasI-hasS* or *hxuI-hxuR* intergenic regions (Table S2, expected sizes 327 and 312 bp, respectively). Positive controls using *P. aeruginosa* genomic DNA and negative controls containing either H_2O instead of RNA or Taq polymerase (PCR) instead of the reverse transcriptase (RT-PCR) were included. Next to the expected cDNA product, an unspecific product was also obtained as result of the *hxuI-hxuR* RT-PCR. Data show is representative of two biological replicates (N=2).

Receptor

HasR	602	FATYGKG**WRPP**AVTESLITGRP**H**GGGAENMY**PNPFL**SPERSKA	644
PhuR	503	YGQYAQG**FRTP**TAKAL**Y**GRFENLQAGYHIE**PNPNL**KPEKSQSF	545
HxuA	580	FADYTET**WRAP**VIDEQ**Y**EVQNSSTIGG**SSRDL**DAERIHAIRGG	622

S5 Fig. Sequence alignment of the heme-coordination extracellular loop of *P. aeruginosa* heme receptors. The conserved FRAP/PNPNL loop required for heme uptake (Krieg et al., 2009) is shown in bold and the His or Tyr coordination residues within this loop are grey boxed.

S6 Fig. Predicted Fur boxes in the promoter region of the *P. aeruginosa* heme utilization genes. Predicted Fur binding sites are grey boxed with residues matching the consensus shown in bold. The translational-start sites are indicated with arrows. The intergenic region between the divergent *phuR* and *phuS* genes contains a strong and a weak Fur box (Ochsner et al., 2000).

Table S1. Bacterial strains and plasmids used in this study[a]

Strain	Characteristics	Reference
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1*, lysogenized with λ*pir*; Rif^R	(Herrero et al., 1990)
DH5α	*supE44* Δ(*lacZYA-argF*)*U169* ϕ80 *lacZ*ΔM15 *hsdR17* (r_K^- m_K^+) *recA1 endA1 gyrA96 thi1 relA1*; Nal^R	(Hanahan, 1983)
P. aeruginosa		
PAO1	Wild-type strain	(Jacobs et al., 2003)
hasAp-Tn	PAO1 carrying a ISphoA/hah transposon in the *hasAp* (PA3407) gene (insertion in codon 79, ID: PW6748); Tc^R	(Jacobs et al., 2003)
hasR-Tn	PAO1 carrying a ISlacZ/hah transposon in the *hasR* (PA3408) gene (insertion in codon 354, ID: PW6750); Tc^R	(Jacobs et al., 2003)
hasS-Tn1	PAO1 carrying a ISlacZ/hah transposon at the -10 position upstream the start codon of the *hasS* (PA3409) gene (ID: PW6754); Tc^R	(Jacobs et al., 2003)
hasS-Tn2	PAO1 carrying a ISphoA/hah transposon in the *hasS* (PA3409) gene (insertion in codon 155, ID: PW6751); Tc^R	(Jacobs et al., 2003)
hasI-Tn	PAO1 carrying a ISphoA/hah transposon in the *hasI* (PA3410) gene (insertion in codon 137, ID: PW6755); Tc^R	(Jacobs et al., 2003)
hxuA-Tn	PAO1 carrying a ISlacZ/hah transposon in the *hxuA* (PA1302) gene (insertion in codon 200, ID: PW3356); Tc^R	(Jacobs et al., 2003)
hxuR-Tn	PAO1 carrying a ISphoA/hah transposon in the *hxuR* (PA1301) gene (insertion in codon 8, ID: PW3355); Tc^R	(Jacobs et al., 2003)
hxuI-Tn	PAO1 carrying a ISlacZ/hah transposon in the *hxuI* (PA1300) gene (insertion in codon 147, ID: PW3353); Tc^R	(Jacobs et al., 2003)
phuR-Tn	PAO1 carrying a ISphoA/hah transposon in the *phuR* (PA4710) gene (insertion in codon 417, ID: PW8935); Tc^R	(Jacobs et al., 2003)
ΔhasR	Markerless PAO1 null mutant in the *hasR* (PA3408) gene	This study
ΔhxuA	Markerless PAO1 null mutant in the *hxuA* (PA1302) gene	This study
phuR-Tn ΔhasR	phuR-Tn with the *hasR* markerless mutation (double mutant); Tc^R	This study
phuR-TnΔhxuA	phuR-Tn with the *hxuA* markerless mutation (double mutant); Tc^R	This study
ΔhxuA ΔhasR	ΔhxuA with the *hasR* markerless mutation (double mutant)	This study

phuR-Tn ΔhxuA ΔhasR	phuR-Tn ΔhxuA with the *hasR* markerless mutation (triple mutant); Tc^R	This study
ΔpvdD	Allelic unmarked mutant deficient in pyoverdine (*pvdD*) production	(Ghysels et al., 2004)
ΔpvdD pchEF	Allelic unmarked mutant deficient in pyoverdine (*pvdD*) and pyochelin (*pchEF*) production	(Ghysels et al., 2004)
Plasmid		
pBSL141	Source of the Gm cassette; Ap^R, Gm^R	(Alexeyev et al., 1995)
pKNG101	Gene replacement suicide vector, *ori*R6K, *oriT*RK2, *sacB;* Sm^R	(Kaniga et al., 1991)
pKΔhasR	pKNG101 carrying in XbaI-BamHI a 2.4-Kb PCR fragment containing the regions up- and downstream the *P. aeruginosa hasR* (PA3408) gene; Sm^R	This study
pKΔhxuA	pKNG101 carrying in XbaI-BamHI a 2.5-Kb PCR fragment containing the regions up- and downstream the *P. aeruginosa hxuA* (PA1302) gene; Sm^R	This study
pMMB67EH	IncQ broad-host range plasmid, $lacI^q$; Ap^R	(Furste et al., 1986)
pMMB/hasI	pMMB67EH carrying a 0.62-Kb EcoRI-XbaI PCR fragment expressing the *P. aeruginosa hasI* (PA3408) gene; Ap^R	This study
pMMB/hxuI	pMMB67EH carrying a 0.76-Kb EcoRI-XbaI PCR fragment expressing the *P. aeruginosa hxuI* (PA1300) gene; Ap^R	This study
pMMB/HA-hxuR	pMMB67EH carrying a 1.1-Kb XbaI-HindIII PCR fragment containing an N-terminally HA-tagged *P. aeruginosa hxuR* (PA1301) gene; Ap^R	This study
pMMB/HA-hasS	pMMB67EH carrying a 1.3-Kb EcoRI-XbaI PCR fragment containing an N-terminally HA-tagged *P. aeruginosa hasS* (PA3409) gene; Ap^R	This study
pMMB/N-tail HasS	pMMB67EH carrying a 0.48-Kb EcoRI-XbaI PCR fragment expressing the N-tail of the *P. aeruginosa* HasS (PA3409) protein; Ap^R	This study
pMMB/N-tail HxuR	pMMB67EH carrying a 0.42-Kb XbaI-HindIII PCR fragment expressing the N-tail of the *P. aeruginosa* HxuR (PA1301) protein; Ap^R	This study
pMMB/hasAp	pMMB67EH carrying a 0.84-Kb EcoRI-XbaI PCR fragment containing the *P. aeruginosa* hasAp (PA3407) gene; Ap^R	This study
pMMB/hasR	pMMB67EH carrying a 3-Kb EcoRI-XbaI PCR fragment containing the *P. aeruginosa hasR* (PA3408) gene; Ap^R	This study
pMMB/hasR-hasAp	pMMB67EH carrying a 3.7-Kb EcoRI-XbaI PCR fragment containing the *P. aeruginosa hasR-hasAp* (PA3408-PA3407) genes; Ap^R	This study
pMMB/hxuA	pMMB67EH carrying a 2.8-Kb EcoRI-XbaI PCR fragment containing the *P. aeruginosa hxuA* (PA1302) gene; Ap^R	This study
pMP220	IncP broad-host-range *lacZ* fusion vector; Tc^R	(Spaink et al., 1987)
pMPhasR	pMP220 carrying a 387-bp EcoRI-XbaI PCR fragment containing the *P. aeruginosa hasR* (PA3408) promoter region upstream the *lacZ* gene; Tc^R	This study
pMPhasRGm	pMPhasR carrying in BglII-EcoRI, and in the opposite direction to the *lacZ* gene, a 0.97-kb BamHI-EcoRI fragment of pBSL141 containing a Gm cassette; Gm^R, Tc^R	This study

pMPhxuA	pMP220 carrying a 331-bp EcoRI-XbaI PCR fragment containing the *P. aeruginosa hxuA* (PA1302) promoter region upstream the *lacZ* gene; Tc^R	This study
pMPhxuAGm	pMPhxuA carrying in BglII-EcoRI, and in the opposite direction to the *lacZ* gene, a 0.97-kb BamHI-EcoRI fragment of pBSL141 containing a Gm cassette; Gm^R, Tc^R	This study
pMPhemO	pMP220 carrying a 401-bp BglII-KpnI PCR fragment containing the *P. aeruginosa hemO* (PA0672) promoter region upstream the *lacZ* gene; Tc^R	This study
pMPphuR	pMP220 carrying a 430-bp EcoRI-XbaI PCR fragment containing the *P. aeruginosa phuR* (PA4710) promoter region upstream the *lacZ* gene; Tc^R	This study
pMPphuRGm	pMPphuR carrying in BglII-EcoRI, and in the opposite direction to the *lacZ* gene, a 0.97-kb BamHI-EcoRI fragment of pBSL141 containing a Gm cassette; Gm^R, Tc^R	This study
pMPphuS	pMP220 carrying a 243-bp EcoRI-XbaI PCR fragment containing the *P. aeruginosa phuR* (PA4709) promoter region upstream the *lacZ* gene; Tc^R	This study

[a] Ap^R, Gm^R, Nal^R, Sm^R and Tc^R, resistance to ampicillin, gentamycin, nalidixic acid, streptomycin and tetracycline, respectively.

Table S2. Sequence of the primers used in this study

***P. aeruginosa* gene (or promoter region)**	**Plasmid**	**Primer name**	**Primer sequence (5'→ 3')[a]**
hemO (PA0672)	pMPhemO	PrhemOF-Bg	AAT**AGATCT**CCGCCGGGTAGCAGTGCGTC
		PrhemOR-Kp	ATT**GGTACC**TTTGCCGAGTGGATTCAGGG
hxuI (PA1300)	pMMB/hxuI	hxuI2F-E	AAA**GAATTC**AGGAACGCATTTCCCAGTC
		hxuI2R-X	AAA**TCTAGA**TCATCCTTCGCCTCCCTGGC
	hxuI-hxuR RT-PCR	RThxuIF	AGCATGGTGGAAAAGCACAT
		RThxuIR	GCTCGGGAATATCGTCCA
hxuR (PA1301)	pMMB/HA-hxuR	HA-hxuRF-X	AAA**TCTAGA**ATGTACCCGTACGACGTGCCGG ACTACGCGAAGCCGCTGGAACGACTGG
		ΔhxuAR-H	CAC**AAGCTT**AGGTCATGCTGGAACTCTCC
	pMMB/N-tail HxuR	N-tail hxuRF-X	AAA**TCTAGA**CGTTCGTCCTGCACAAGTT
		N-tail hxuRR-H	AAA**AAGCTT**TCAGAAGAACGCCGCCGCCAG
hxuA (PA1302)	pKΔhxuA	hxuRF-X	AAA**TCTAGA**GGAAAAGCACATCGCCAGCG
		ΔhxuAR-H	CAC**AAGCTT**AGGTCATGCTGGAACTCTCC
		ΔhxuAF-H	TTC**AAGCTT**ACAGCCAGGGACGCAACGC
		PA1303R-B	ATT**GGATCC**GCTTCGTCTTTGCCATCGCC
	pMPhxuA	PrPA1302F-E	AAA**GAATTC**CCGACGATTTTCTCCTCGC
		PrPA1302R-X	AAA**TCTAGA**GGCAGGTCGAAGCGTCGTTC
	pMMB/hxuA	PrPA1302F-E	AAA**GAATTC**CCGACGATTTTCTCCTCGC
		hxuAR-X	AAA**TCTAGA**TCAGAAGAACTGGGTGACGC
	qRT-PCR	qhxuAF	GTGTTCATCGAACCGGAAAT
		qhxuAR	TCGTTGGAGTGATAGCCGTA
hasAp (PA3407)	pMMB/hasAp	hasApF-E	AAA**GAATTC**GTGAGCGATGAACTGACC
		hasApR-X	AAA**TCTAGA**TCAGGCCGCCAGGGCCAGGT
		hasSF-X	AAC**TCTAGA**CAGCAGATCCGCCAGCAGGC

hasR (PA3408)	pKΔhasR	ΔhasRR-E	AAA**GAATTC**ATCCACGGCTCGAAAATGCG
		hasApF-E	AAA**GAATTC**GTGAGCGATGAACTGACC
		hasApR-B	AAA**GGATCC**GCCGAGCAGCAGGTAGAGG
	pMPhasR	PrhasRF-E	ATA**GAATTC**GGCATTTGCTGGTGGCTCCC
		PrhasRR-X	AAA**TCTAGA**CGCACGGCACTCCATCCACG
	pMMB/hasR	PrhasRF-E	ATA**GAATTC**GGCATTTGCTGGTGGCTCCC
		hasRR-X	AAA**TCTAGA**TCAGAACTGGTATTCGAGGG
	pMMB/hasR-hasAp	PrhasRF-E	ATA**GAATTC**GGCATTTGCTGGTGGCTCCC
		hasApR-X	AAA**TCTAGA**TCAGGCCGCCAGGGCCAGGT
	qRT-PCR	qhasRF	CGGTGGAATACCGCTTCTC
		qhasRR	CGTCTGGTAGACCCAGTCGT
hasS (PA3409)	pMMB/HA-hasS	HA-hasSF-E	AAA**GAATTC**ATGTACCCGTACGACGTGCCGG ACTACGCGAACAGCCCCCAGGAACAACAGC
		PrhasRR-X	AAA**TCTAGA**CGCACGGCACTCCATCCACG
	pMMB/N-tail HasS	N-tail hasSF-E	AAA**GAATCC**CGTGAGATCTTCCGTCTCAA
		N-tail hasSR-X	AAA**TCTAGA**TCACGCGGCCCAGCCGCG
hasI (PA3410)	pMMB/hasI	hasIF-E	AAA**GAATTC**CTGAGCCCGGCATCTCACAA
		hasIR-X	AAA**TCTAGA**GAACCGGCAAGACTCGGTAA
	hasI-hasS RT-PCR	RThasIF	CCTGGAAATCTCCGATAGCTC
		RTHasSR	ATCCCAGCTGTTTCCAGGTC
phuS (PA4709)	pMPphuS	PrphuS2F-E	AAA**GAATTC**CTGCCAGAAAAACGCTAATG
		PrphuS2R-X	AAA**TCTAGA**CTTCGCTGACCTGCAACAG
phuR (PA4710)	pMPphuR	PrPAphuRF-E	TAT**GAATTC**GCTCGCCTTCGCTGACCTGC
		PrPAphuRR-X	TAT**TCTAGA**CGGGTGGCGGTGATGGTGG
	qRT-PCR	qphuRF	GATCACCGGCTACAACATCC
		qphuRR	TTTACGATGTCCGGATCGAC

[a] The sequences of the restriction sites are indicated in bold and the annealing region is underlined

Chapter 5

Identification of new proteases involved in the control of cell-surface signaling activity and *Pseudomonas aeruginosa* virulence

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ABSTRACT

In the human pathogen *Pseudomonas aeruginosa*, extracytoplasmic function sigma (σ^{ECF}) factors control important biological functions required for colonization of different environments and the host. Most *P. aeruginosa* σ^{ECF} factors are associated with a transmembrane anti-σ factor that binds to, and prevents the binding of the σ^{ECF} to the RNA polymerase, thus impairing gene transcription. Only when a specific signal arises, the σ^{ECF} factor is released and initiates transcription of the signal responsive genes. Most σ^{ECF}/anti-σ factor pairs of *P. aeruginosa* are associated with a surface-exposed outer membrane receptor forming a signal transduction system known as cell-surface signaling (CSS). Activation of CSS σ^{ECF} has recently been shown to require the regulated and sequential proteolysis of the anti-σ factor, but many features of this complex proteolytic cascade have not been elucidated yet. Next to the previously identified proteases RseP and Prc, we here identify three other proteases that modulate CSS activity, namely the periplasmic carboxyl-terminal processing protease CtpA, the cytoplasmic membrane-anchored protease subunit HflK, and the cytosolic leucine aminopeptidase PepA (also called PhpA). Using the archetypal ferrioxamine-induced Fox CSS system of *P. aeruginosa*, we show that Prc and CtpA control CSS activation by modulating the levels of the FoxR anti-σ factor C-domain. CtpA works upstream Prc in the proteolytic cascade and its function seems to be prevention of the Prc-mediated proteolysis of C-FoxR. HflK, which itself is not a protease but a negative regulator of the FtsH protease, likely impedes the proteolysis of the N-domain of the FoxR anti-σ factor. Finally, PepA modulates the level of the σ^{FoxI} factor thus controlling the last step in the activation of the CSS system. Furthermore, using zebrafish embryos and the A549 cell line as *P. aeruginosa* hosts we show that mutants in the *rseP* and *ctpA* proteases are attenuated in virulence while the *prc* mutation increases virulence likely by increasing the production of *P. aeruginosa* outer membrane vesicles.

INTRODUCTION

Cell-surface signaling (CSS) is a signal transfer system of Gram-negative bacteria that allow bacteria to adapt to environmental changes through the detection of specific extracellular signals and the modification of gene expression. These three-protein systems are composed by an outer membrane receptor, an anti-σ factor and an extracytoplasmic function σ factor (σ^{ECF}) (Llamas et al., 2014) (Fig. 1). The CSS receptor belongs to the TonB-dependent receptor family and usually is involved in both the transport of the CSS inducing signal and signaling. Transport occurs through the large C-terminal domain of the protein which forms a β barrel in the outer membrane and needs the energy provided by the TonB-ExbBD system (Noinaj et al., 2010). Signaling occurs via a small N-terminal domain located in the periplasm of the bacteria which interact with the CSS anti-σ factor (Enz et al., 2003b) (Fig. 1). CSS anti-σ factors usually are cytoplasmic membrane-anchored proteins that contain a large periplasmic C-domain and a short cytosolic N-domain connected by a transmembrane segment (Llamas et al., 2014). They bind and keep the σ^{ECF} factor sequestered in the absence of the CSS signal. σ^{ECF} factors belong to the σ^{70} family of bacterial σ factors and it is the largest and most diverse group (Lonetto et al., 1994; Staron et al., 2009; Bastiaansen et al., 2012). The σ factor is the subunit of the RNA polymerase (RNAP) that directs it to the promoter initiating gene transcription and thereby is the subunit that confers promoter specificity to the RNAP (Ishihama, 2000). Shifts in the σ component of the RNAP represents thus the first level in the regulation of bacterial gene transcription (Ishihama, 2000).

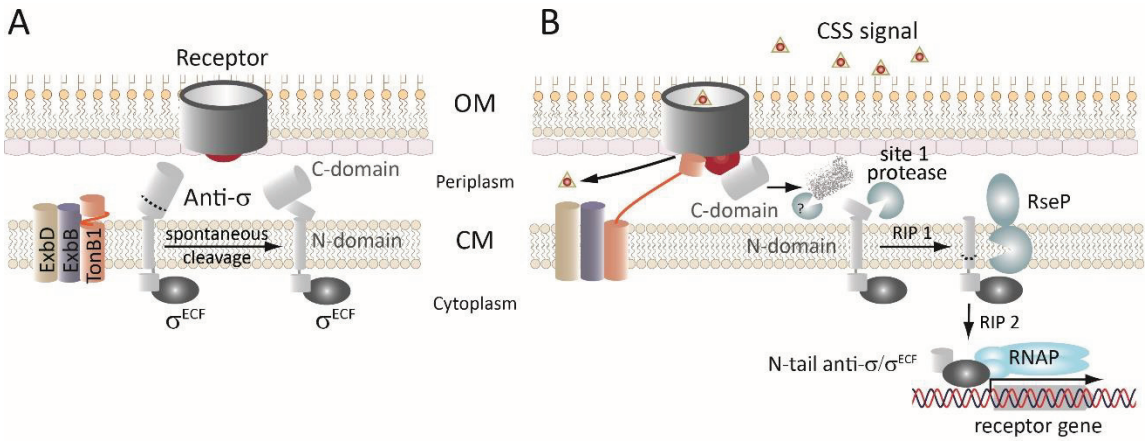

Fig. 1. Model of the complex proteolytic cascade controlling CSS activation based on studies in *P. aeruginosa*. (A) The receptor, anti-σ factor and σ^{ECF} of the CSS system are shown. Prior to signal recognition, the anti-σ factor undergoes a spontaneous cleavage that occurs by a protease-independent reaction in a conserved Gly-Thr (GT) cleavage site located in the periplasmic domain of the anti-σ factor. This cleavage produces two functional N- and C-domains that interact with each other in the periplasm and are both required for proper anti-σ factor function. **(B)** Signal recognition by the receptor produces the interaction of the signaling domain of the receptor (in red) with the C-domain of the anti-σ factor. This event triggers the degradation of the anti-σ factor C-domain by a still unknown mechanism, and the targeted proteolysis of the N-domain via regulated intramembrane proteolysis (RIP) by the action of (at least) two proteases. The site 2 protease RseP cleaves in the transmembrane domain of the anti-σ factor and produces the release of the σ^{ECF} into the cytoplasm likely bound to the N-tail domain of the anti-σ factor that can either be degraded or remain bound to the σ^{ECF} in the σ^{ECF}-RNAP complex (in case anti-σ factors with pro-σ activity). σ^{ECF} activates transcription of the CSS receptor gene and other genes involved in the response to the CSS signal. The TonB-ExbBD complex provides the energy required for the transport

of the inducing signal to the CSS receptors that next to a function in signal transduction also have a function in the uptake of the inducing signal. OM, outer membrane; CM, cytoplasmic membrane; RNAP, RNA polymerase. Adapted from (Llamas et al., 2014)with the findings from (Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2015b; Bastiaansen et al., 2017).

CSS is extensively present in *Pseudomonas* bacteria, especially in *P. aeruginosa*, *P. putida* and *P. protegens* that contain between 13-17 of these systems (Llamas et al., 2014). Signaling systems increas the bacterial ability to detect and survive in many different environments, a characteristic of *Pseudomonas* bacteria (Spiers et al., 2000; Silby et al., 2011). In this genus, CSS is mainly involved in the regulation of iron uptake by sensing and responding to iron-chelating compounds like siderophores, iron-citrate or hemophores, but also in theregulation of bacterial competition and virulence (Llamas et al., 2014). The analysis of *Pseudomonas* CSS systems has revealed that CSS activation in response to the inducing signal requires the targeted proteolysis of the CSS anti-σ factor (Fig. 1B). This occurs through regulated intramembrane proteolysis (RIP), a conserved mechanism in which a transmembrane protein is subjected to several proteolytic steps in order to liberate and activate a cytosolic effector (Brown et al., 2000). The RIP of CSS anti-σ factors always involves the site-2 metalloprotease RseP, which cuts within the transmembrane domain of the protein liberating the CSS σ^{ECF} factor in the cytosol (Fig. 1B) (Draper et al., 2011; Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). RseP substrate recognition and cleavage occurs through size-filtering rather than by the recognition of a specific sequence/motif (Hizukuri et al., 2014), and the RseP substrate is normally produced by a site-1 protease (Brown et al., 2000). Evidence that a site-1 cleavage of CSS anti-σ factors occurs is the accumulation of a slightly larger fragment than the RseP product in *rseP* mutants (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). The carboxyl-terminal processing serine protease Prc is required for the site-1 cleavage of a unique *P. putida* CSS protein, the IutY protein, that contains an σ^{ECF} and an anti-σ factor domain in a single protein separated by transmembrane domain (Bastiaansen et al., 2014). In accordance, Prc is essential for activation of the σ^{IutY} domain in response to the inducing signal (i.e. the siderophore aerobactin) and overproduction of Prc leads to IutY processing and σ^{IutY} activation in absence of the signal (Bastiaansen et al., 2014). However, the role of Prc in the activation of archetypal CSS systems in which the σ^{ECF} and anti-σ factors are two different proteins is still not completely clear. Prc is required for activation of these systems (Bastiaansen et al., 2014), but whether or not Prc directly cleaves these anti-σ factors has not been determined yet. Moreover, the potential protease that mediates the site-1 cleavage of these anti-σ factors and produces the RseP substrate has also not been identified. This study was conducted to shed light on these unknown features of CSS activation and has resulted in the identification of three new proteases involved in the activation of this signaling cascade.

RESULTS

The proteolytic activities of Prc and RseP are required for CSS activation.

To further analyse the role of the Prc protease in the activation of CSS systems, we constructed a proteolytically inactive *P. putida* Prc protein in which the active site protease residue Ser-485 was changed to alanine. As control, the active site residue His-23 of the *P. putida* RseP protease (Koide et al., 2007) was changed to alanine. Then we assayed the activity of the *P. putida* Fiu and Fox CSS pathways, which are activated by the siderophores ferrichrome and

ferrioxamine, respectively (Bastiaansen et al., 2014), in Δprc and ΔrseP mutants complemented with either a wild-type Prc or RseP protein or with the Prc-S485A or RseP-H23A active site mutants. CSS activity was measured using *fiuA::lacZ* and *foxA::lacZ* transcriptional fusions, of which expression completely depends on the activation of their respective CSS pathways (Bastiaansen et al., 2014). Activity of both pathways was completely abolished in the ΔrseP and considerably reduced in the Δprc mutant (Fig. 2), as shown previously (Bastiaansen et al., 2014). Complementation of ΔrseP with the wild-type RseP protein was able to restore the activity of both systems in the presence of the inducing siderophore, while the proteolytically inactivated version RseP-H23A could not (Fig. 2). This further confirms that the proteolytic activity of RseP is required for activation of these CSS pathways, as expected from the protease that cleavages the transmembrane domain of CSS anti-σ factors producing the liberation and activation of the CSS σ^{ECF} (Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). Regarding Prc, production of the wild-type protein in the Δprc mutant restored the ferrioxamine- and the ferrichrome-mediated transcription of *foxA* and *fiuA*, respectively (Fig. 2), and resulted in a small but significant increase of expression in absence of the siderophores (Fig. 2). Interestingly, the Prc-S485A active site mutant was not only unable to complement the Δprc mutation, but also exhibited a dominant negative effect by blocking the residual *foxA* and *fiuA* promoter activity observed in the Δprc background in presence of the inducing siderophore (Fig. 2). Moreover, in contrast to wild-type Prc, this mutant protein did not cause any signal-independent activity of the *foxA* and *fiuA* promoters (Fig. 2). These results confirm the importance of the proteolytic action of both the Prc and RseP proteases in the activation of CSS pathways.

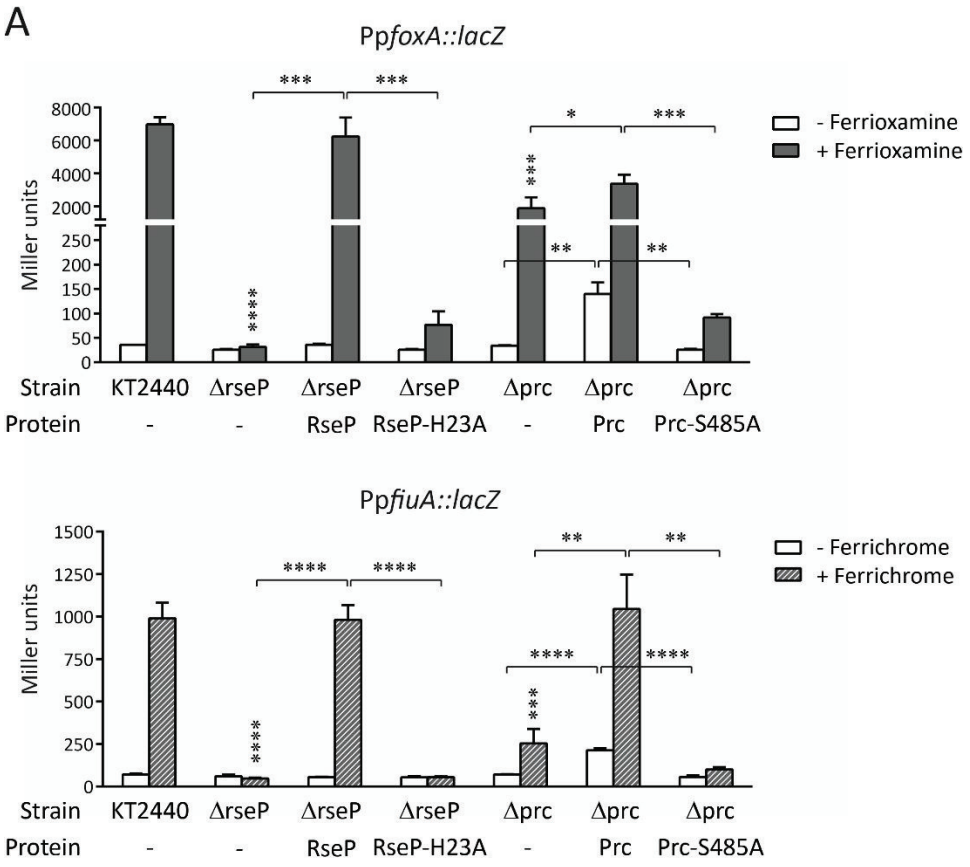

Fig. 2. Role of the proteolytic activity of the Prc and Rsep proteases in CSS activation. β-galactosidase activity of the *P. putida foxA::* or *fiuA::lacZ* fusion genes in the KT2440 wild-type

strain and its isogenic ΔrseP and Δprc mutants bearing a pBBR1MCS-5 derivative plasmid expressing the indicated protein (-, empty plasmid). Strains were grown under iron-restricted conditions without (-) or with (+) the indicated siderophore. Data are means ± SD from three biological replicates (N=3). P-values were calculated by two-tailed *t*-test and brackets indicate the comparison to which the P-value applies. The P-value in the ΔrseP and Δprc mutants corresponds to the comparison with the KT2440 wild-type strain.

The Prc-homologue CtpA is involved in CSS activation in P. aeruginosa.

As shown before [Fig. 2 and (Bastiaansen et al., 2014)], when Prc is lacking in *Pseudomonas* there is still some CSS activity in response to the signal. This residual activity could be due to the function of another protease in the absence of Prc. *Pseudomonas* species produces a second carboxyl-terminal processing protease known as CtpA, a soluble periplasmic serine protease that in *P. aeruginosa* has been involved in the activation of the SbrI σ^{ECF} factor (Seo and Darwin, 2013; McGuffie et al., 2015). Therefore, we introduced a *ctpA* deletion (ΔctpA) in *P. aeruginosa* and assayed CSS activity using CSS σ^{ECF}-dependent *lacZ* fusions. Unexpectly, lack of CtpA considerably increased the response of the *P. aeruginosa* Fox and Fiu CSS systems to the presence of their cognate inducing siderophore (Fig. 3A). Activity of these systems in the ΔctpA mutant was however not affected in the absence of the siderophore (Fig. 3B), which indicates that the absence of CtpA does not lead to constitutive activation of the CSS pathways. Production of CtpA from a low-copy number plasmid did not affect the activity of the Fox system in the PAO1 wild-type strain but it was able to complement the *P. aeruginosa* ΔctpA mutation (Fig. 3B), which further indicates that CtpA is required for CSS activation. Mutation of *ctpA* in *P. putida* also produced an increase in the activation of the Fiu CSS system of this bacterium, although it did not affect the activity of the Fox system (Fig. S1). Even so, CtpA overproduction led to a decrease of the *P. putida* Fox system activity (Fig. S1). These results suggest that the CtpA protease fine-tunes the response to the CSS signal. However, CtpA does not seem to compensate for the lack of Prc as initially hypothesized since the *ctpA* mutation results in increased instead of decreased CSS activity. In fact, introduction of the *ctpA* deletion in the Δprc mutant did not alter the phenotype observed in the single Δprc mutant (Fig. 3A). The lack of the ΔctpA phenotype in the Δprc ΔctpA double mutant suggests that CtpA works upstream of Prc in the modulation of the CSS activity.

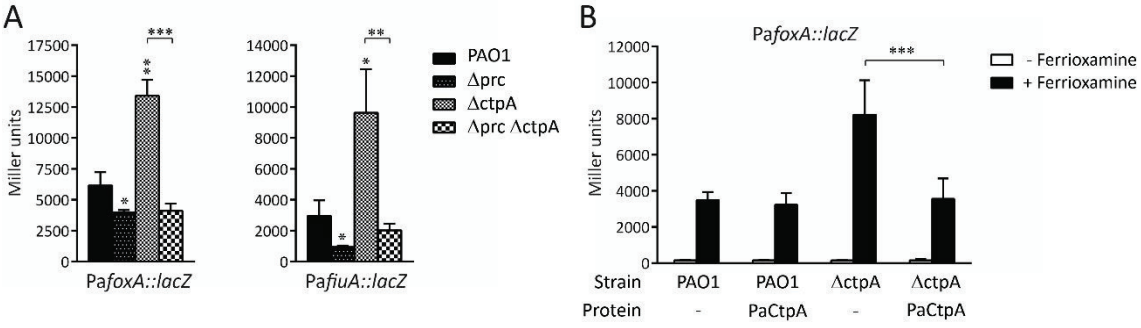

Fig. 3. Activity of the *P. aeruginosa* Fox and Fiu CSS systems in *ctpA* mutants. β-galactosidase activity of the indicated *lacZ* fusion gene in the *P. aeruginosa* PAO1 wild-type strain and the indicated mutant. **(A)** Strains were grown under iron-restricted conditions with ferrioxamine (Pa*foxA::lacZ*) or ferrichrome (Pa*fiuA::lacZ*). **(B)** In addition to the *lacZ* fusion, strains bear the pBBR1MCS-5 empty (-) or the pBBR-PaCtpA plasmid (Table S1) and were grown in iron-restricted conditions without (-) or with (+) ferrioxamine. In both panels data are means ± SD from three biological replicates (N=3). P-values were

calculated by two-tailed *t*-test by comparing the value obtained in the mutant with that of the PAO1 wild-type strain in the same growth condition. Comparisons between other strains are indicated by brackets.

We next focused on the identification of the CSS substrate of the *P. aeruginosa* CtpA protease. We first considered the CSS anti-σ factor and analysed the *P. aeruginosa* FoxR (PaFoxR) anti-σ factor. To detect PaFoxR, we used N- and C-terminally HA-tagged protein variants. We have determined before that PaFoxR undergoes a spontaneous cleavage immediately upon production that leads to a ~22-23 kDa N-domain and a ~15 kDa C-domain (Fig. 1A) (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b). Both domains were detected by Western-blot (Fig. 4A and 4B, PAO1 strain). In response to ferrioxamine, the N-domain is proteolytically processed by RIP through the action of an unknown site-1 protease and the RseP site-2 protease (Fig. 1B) (Bastiaansen et al., 2015a). RseP cuts within the transmembrane domain of N-FoxR and generates the FoxR cytosolic domain, which we called N-tail (Fig. 1B), a domain that was detectable upon ferrioxamine induction (Fig. 4A). Lack of CtpA did not affect the RIP of N-PaFoxR and the same N-FoxR subproducts were obtained in the wild-type and ΔctpA strains (Fig. 4A). However, the amount of the PaFoxR N-domain, and especially that of the N-tail, was significantly higher in the ΔctpA mutant (Fig. 4A). Higher amounts of the N-tail usually correlates with increased CSS activity likely because this process produces the liberation and activation of the σ^{FoxI} factor (Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2015b), as also observed in the ΔctpA mutant (Fig. 3). Introduction of the Δprc mutation in the ΔctpA mutant diminished the amount of the FoxR N-tail, which was similar to that obtained in the single Δprc mutant (Fig. 4A), in agreement with the lower CSS activity observed in the Δprc ΔctpA double mutant (Fig. 3A). Interestingly, the *ctpA* mutation had a considerable effect on the C-FoxR domain which was hardly detected in this mutant, especially in presence of ferrioxamine (Fig. 4B). The amount of C-PaFoxR in the Δprc ΔctpA double mutant was similar to that in the wild-type strain (Fig. 4B) confirming that the *prc* mutation abolished the effect of the *ctpA* mutation. Together these results suggest that the FoxR anti-σ factor is not the substrate of CtpA although the absence of this protease considerably affects the stability of both domains of this protein.

Because the absence of CtpA increases CSS activity in a Prc-dependent manner, we wondered whether Prc itself could be the substrate of CtpA. The level of the Prc protease slightly increased in the wild-type strain in presence of ferrioxamine (Fig. 4C, PAO1). However, this increase was not observed in the ΔctpA mutant (Fig. 4C). This suggests that Prc is more stable in conditions in which the Fox CSS system is active and that the CtpA protease stabilizes Prc in this condition.

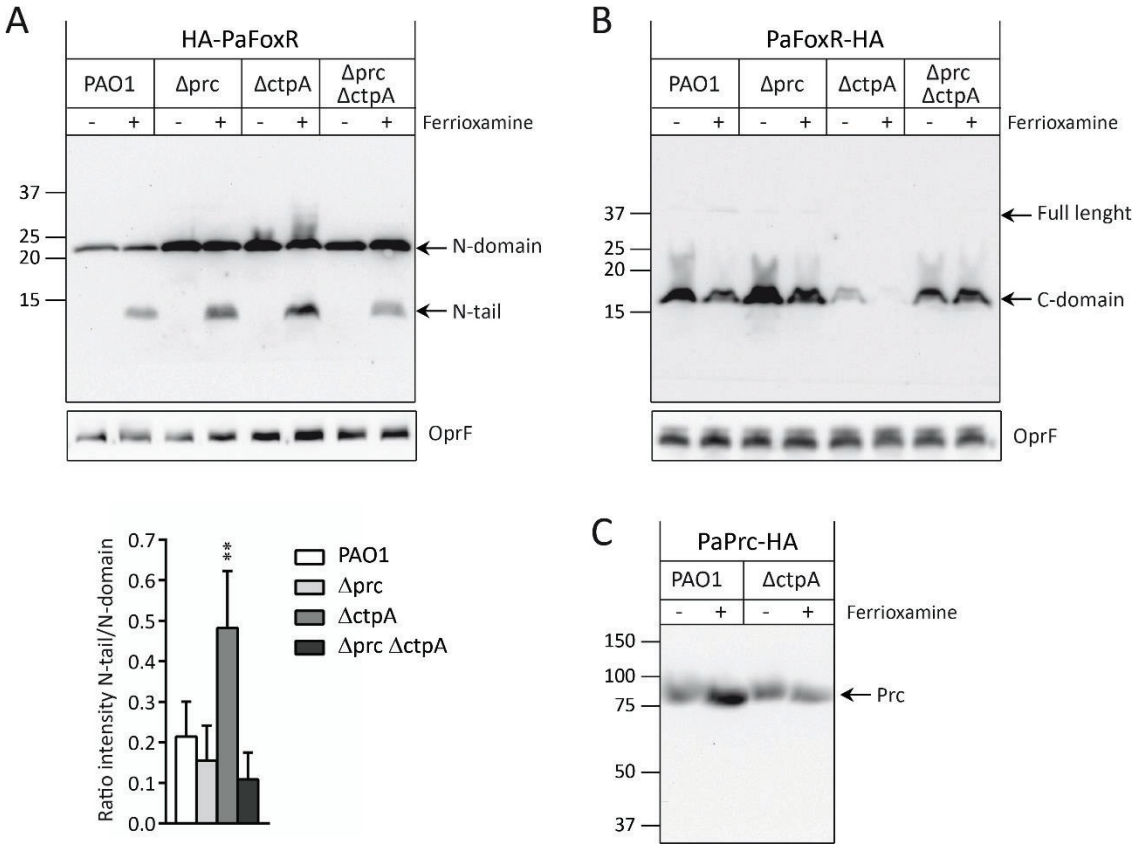

Fig. 4. Role of the CtpA protease in processing *P. aeruginosa* CSS components. In all panels, the *P. aeruginosa* PAO1 wild-type strain and the indicated isogenic mutants were grown to late log-phase under iron-restricted conditions and in the absence (-) or presence (+) of ferrioxamine. In **(A)** Strains express an N-terminally HA-tagged *P. aeruginosa* FoxR protein (HA-PaFoxR), in **(B)** a C-terminally HA-tagged *P. aeruginosa* FoxR protein and in **(C)** a C-terminally HA-tagged Prc protein. Proteins were immunoblotted for HA using a monoclonal antibody. Position of the protein fragments and the molecular size marker (in kDa) is indicated. Presence of the HA-tag adds ~1 kDa to the molar mass of the protein fragments. Blots are representative of at least three biological replicates (N=3). Detection of the OprF protein was used in **(A)** and **(B)** as loading control. The graph in **(A)** shows the ratio between the intensity of the N-tail and the N-domain protein bands, and data are means ± SD from three biological replicates (N=3). P-values were calculated by two-tailed *t*-test by comparing the value obtained in the mutant with that of the PAO1 wild-type strain.

Lack of RseP, Prc and CtpA proteases affects P. aeruginosa *virulence*

The CtpA protease has been shown to be required for *P. aeruginosa* virulence in a mouse model of acute infection (Seo and Darwin, 2013), but the role of RseP and Prc in virulence is unknown. To analyze the pathogenicity of *P. aeruginosa* we used zebrafish (*Danio rerio*) embryos, which are lethally infected by this pathogen when the amount of bacterial cells injected exceeds the phagocytic capacity of the embryo (Clatworthy et al., 2009; Llamas et al., 2009; Llamas and van der Sar, 2014). Wild type and mutant bacteria were injected in the bloodstream of one-day old embryos to generate a systemic infection and embryo survival was monitored during five days (Fig. 5A). The ΔctpA mutant showed reduced virulence in zebrafish embryos ($P<0.0001$) (Fig. 5A), in accordance with the reduced virulence previously observed in mice (Seo and Darwin, 2013). The survival of the embryos injected with the ΔrseP mutant was similar to that of the control group injected only with phosphate-free physiological salt showing that this mutant was completely attenuated for virulence ($P< 0.0001$) (Fig. 5A). In contrast, the

prc mutation leads to a strain that was considerably more virulent than the PAO1 wild-type strain (P<0.0001) (Fig. 5A).

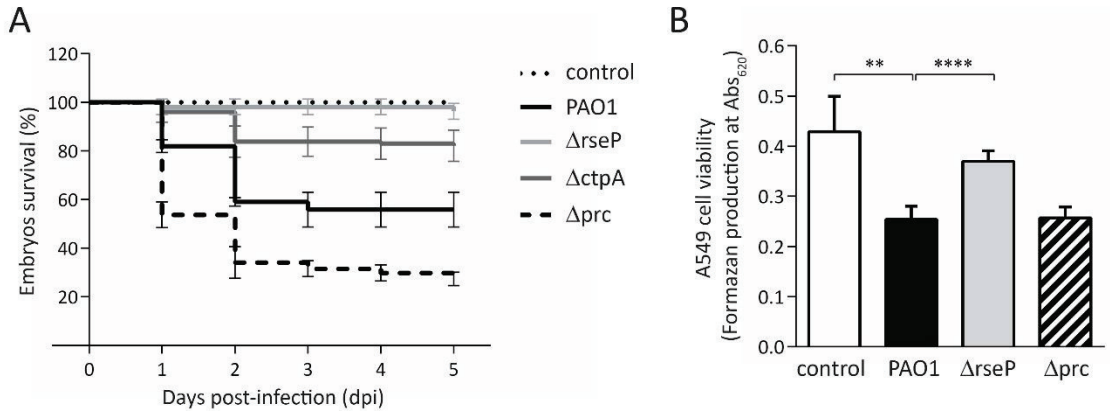

Fig. 5. *P. aeruginosa* infections in zebrafish embryos and in the A549 cell line. (A) Klapan-Meier zebrafish embryo survival curves upon infection with *P. aeruginosa*. One day-old embryos were injected with ~1000 CFU of the *P. aeruginosa* PAO1 wild-type strain or with the indicated isogenic mutant. Uninfected control (non-injected) is shown. Data are means ± SD of five biologically independent replicates (N=5) with 20 embryos/group in each replicate. **(B)** A549 cell viability. The *P. aeruginosa* PAO1 wild-type strain and the indicated isogenic mutant were co-incubated with the eukaryotic cells. Formazan production upon addition of the MTT tretazolium salt was determined spectrophotometrically at 620 nm. Uninfected cells (white bar) were used as control. Data are means ± SD from five biological replicates (N=5). P-values were calculated by unpaired two-tailed *t*-test and brackets indicate the comparison to which the P-value applies.

We also used the A549 human respiratory epithelial cell line as host since *P. aeruginosa* often colonizes the human respiratory tract. The cytotoxicity of *P. aeruginosa* toward the eukaryotic cells was determined by measuring A549 cell viability after co-incubation with the bacteria. The ΔrseP mutant was less efficient in damaging the A549 cells than the PAO1 wild-type strain, while the cytotoxicity of the Δprc mutant was similar to that of the wild-type strain (Fig. 5B). In accordance, time-lapse imaging showed that the A549 cells detached, which is the first indication of cell death, after co-incubation with the wild-type strain but not with the ΔrseP mutant (Video 1). In fact, the percentage of detached cells was considerably lower following infection with the mutant (P<0.001) (Fig. S2). All together, these results indicate that the *rseP* mutation leads to a considerably less virulent *P. aeruginosa* strain.

Prc modulates production of P. aeruginosa *outer membrane vesicles (OMVs).*

An unexpected result from the virulence assays was the hypervirulent phenotype of the Δprc mutant (Fig. 5). In *E. coli*, the Prc protease (also known as Tsp) modulates the activity of the murein DD-endopeptidase MepS, a hydrolase that cleaves peptidoglycan cross-links for insertion of new material (Singh et al., 2015). The absence of Prc increases MepS levels, and uncontrolled activity of MepS increases the formation of outer membrane vesicles (OMVs) (Schwechheimer et al., 2015). Because secretion of OMVs is used by *P. aeruginosa* to deliver multiple virulence factors directly into the host cell cytoplasm (Bomberger et al., 2009; Koeppen et al., 2016), we hypothesized that the hypervirulent phenotype of the Δprc mutant (Fig. 5A) could be related with higher OMVs production. SDS-PAGE and transmission electron microscopy (TEM) analyses showed that OMVs production was indeed considerably higher in the Δprc

mutant than in the PAO1 wild-type strain, a phenotype that could be complemented by providing the *prc* gene to the mutant in *trans* (Fig. 6A and 6B). This indicates that the absence of Prc raises OMVs secretion, which would indeed increase *P. aeruginosa* virulence.

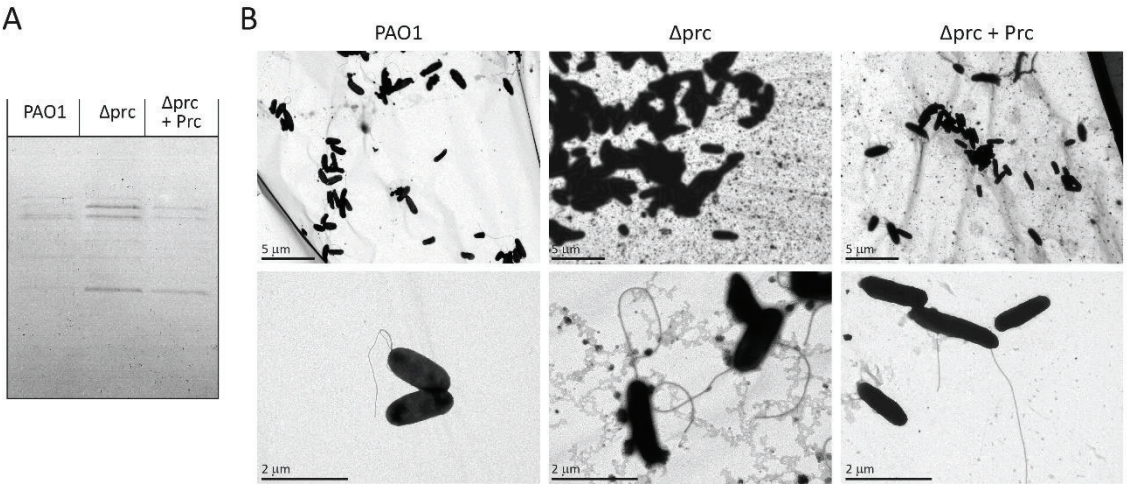

Fig. 6. *P. aeruginosa* outer membrane vesicles (OMVs) production and role of the Prc protease. *P. aeruginosa* PAO1 wild-type strain, the isogenic Δprc mutant, and the Δprc mutant bearing the pBBR/PAPrc plasmid (Table S1) (Δprc + Prc) were grown to exponential phase in low phosphate medium. **(A)** Outer membrane proteins were isolated and visualized by Coomasie staining. Molecular size marker (in KDa) is indicated. SDS-PAGE is representative of three biological replicates (N=3). **(B)** Strains were negatively stained with phosphotungstic acid. TEM images were taken in several fields at 6.000 x and 25.000 x. Images are representative of three biological replicates (N=3).

The HflK and PepA proteases affect CSS activation.

To identify other proteases involved in CSS activation, we performed a random mutagenesis in *P. putida* and a withe/blue screening of mutants bearing the *foxA*::*lacZ* fusion to select clones in which the Fox CSS activity was affected. Amongst others, we obtained clones with insertions in the PP0980 and PP4892 locus that are annotated in the Pseudomonas genome database as the cytosolic leucine aminopeptidase PepA (also termed PhpA) and the cytoplasmic membrane-anchored protease subunit HflK, respectively (Fig. S3). The effect of these mutations on *P. aeruginosa* CSS activity was assayed using *pepA* and *hflK* transposon mutants (Table S1). Although originally selected in *P. putida* as a mutation that reduced Fox activity (Fig. S3), in *P. aeruginosa* the *pepA* mutation led to an increase in Fox and Fiu CSS activity in response to the inducing siderophore (ferrioxamine and ferrichrome, respectively). This difference between both species can be due to the position of the Tn insertion in the corresponding *pepA* gene, at the beginning in the *P. aeruginosa* mutant (in codon 33 of a 495 aa protein) and almost at the end in *P. putida* mutant (in codon 412 of a 497 aa protein) (Table S1). Mutation of *hflK* in *P. aeruginosa* decreased Fox and Fiu CSS activity (Fig. 7A), as also observed in *P. putida* (Fig. S3). Moreover, a slight but significant increase of Fox activity was observed in the *P. aeruginosa pepA-Tn* mutant in the absence of the inducing signal (Fig. 7A). Providing the *pepA* gene in *trans* to this mutant diminished the Fox activity (Fig. 7B). Activity in the complemented strain was lower than in the wild-type strain, likely due to the negative effect that PepA production from plasmid has on Fox activity, as observed in the PAO1 wild-type strain (Fig. 7B). In contrast, the *hflK* mutation could not be complemented because production of HflK from plasmid

considerably decreases CSS activity in both the PAO1 wild-type strain and the hflK-Tn mutant (Fig. 7B).

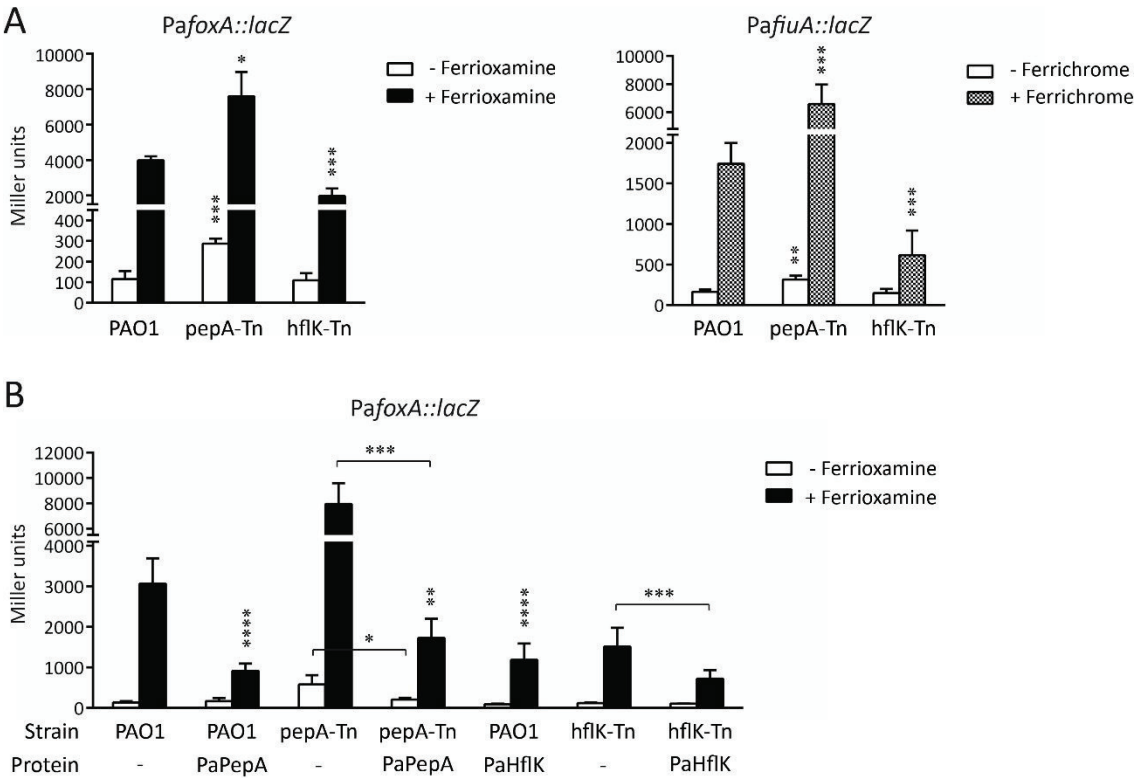

Fig. 7. CSS activity in *P. aeruginosa pepA* and *hflK* mutants. β-galactosidase activity of the indicated *lacZ* fusion gene in the *P. aeruginosa* PAO1 wild-type strains and the indicated isogenic mutant. **(A)** Strains were grown under iron-restricted conditions with ferrioxamine (*foxA::lacZ*) or ferrichrome (*fiuA::lacZ*). **(B)** In addition to the *lacZ* fusion, strains bear the empty (-) or the pBBR1MCS-5-derived plasmid (Table S1) expressing the indicated *P. aeruginosa* (Pa) gene and were grown in iron-restricted conditions without (-) or with (+) ferrioxamine. In both panels data are means ± SD from three biological replicates (N=3). P-values were calculated by two-tailed *t*-test by comparing the value obtained in the mutant or the complemented strain with that of the wild-type strain in the same growth condition. Comparisons between other strains are indicated by brackets.

Because PepA and HflK perform their protease activities in the cytosol (Ito and Akiyama, 2005; Reijns et al., 2005), we assayed the effect of these protease mutations on the stability of the cytosolic factor σ^{FoxI} of *P. aeruginosa*. We detected the σ factor by Western-blot using an N-terminally HA-tagged version constitutively produced from a low-copy number plasmid (Table S1). Introduction of the HA-tag did not affect the activity of σ^{FoxI} (Fig. S4). Lack of PepA resulted in increased stability of σ^{FoxI} (Fig. 8C), which correlates with the higher activity of the σ^{FoxI}-dependent *foxA::lacZ* fusion observed in pepA-Tn (Fig. 7). In contrast, lack of *hflK* did not affect σ^{FoxI} levels (Fig. 8C). This suggests that the higher activity of the Fox system observed in the PepA-Tn mutant could be due to the increased stability of the σ^{FoxI} factor protein.

We also assayed *P. aeruginosa* FoxR protein stability in these strains to determine if these mutations affected the stability of the cytosolic N-tail domain of this anti-σ factor, which is known to be required for σ^{FoxI} activity (Fig. 1B) (Mettrick and Lamont, 2009; Llamas et al., 2014). *pepA* and *hflK* mutations did not affect the RIP of the PaFoxR N-domain in response to ferrioxamine (Fig. 8A). However, these mutations did affect the stability of the protein and

increased amounts of the N-tail subfragment were observed in pepA-Tn and less amounts in hflK-Tn (Fig. 8A). The level of the N-tail in the mutants correlates with the observed CSS activity, higher in pepA-Tn and lower in hflK-Tn (Fig. 7A).

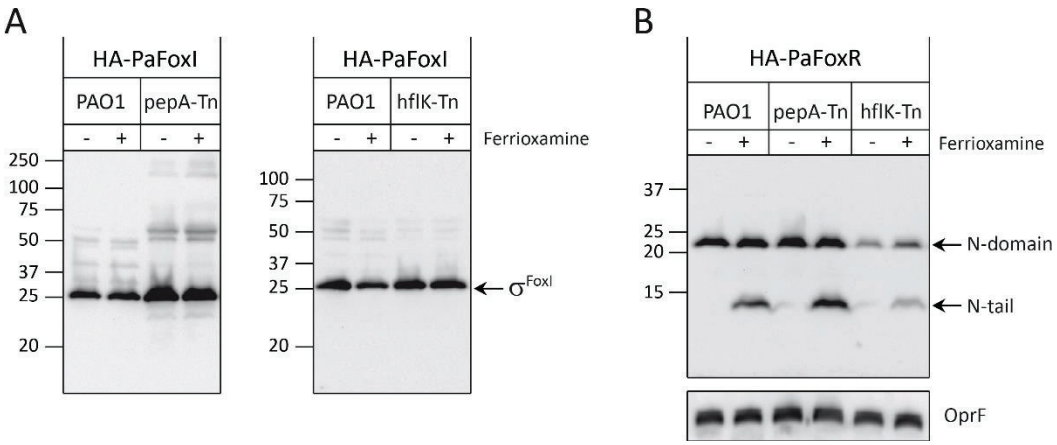

Fig. 8. Role of the PepA and HflK proteases in *P. aeruginosa* σ^{FoxI} and FoxR stability. *P. aeruginosa* PAO1 wild-type strain and the indicated isogenic mutant expressing an a N-terminally HA-tagged σ^{FoxI} **(A)** or FoxR **(B)** protein were grown to late log-phase under iron-restricted conditions with IPTG and in the absence (-) or presence (+) of ferrioxamine. Proteins were immunoblotted for HA. Position of the protein fragments and the molecular size marker (in kDa) is indicated. Presence of the HA-tag adds ~1 kDa to the molar mass of the protein fragments. Blots are representative of at least three biological replicates (N=3). Detection of the OprF protein was used as loading control.

DISCUSSION

In recent years, it has become clear that activation of CSS pathways occurs through the regulated proteolysis of the CSS anti-σ factor, a process that results in the release and activation of the CSS σ^{ECF} in the cytosol (Fig. 1B) (Bastiaansen et al., 2014; Bastiaansen et al., 2015a; Bastiaansen et al., 2017). The carboxy-terminal processing serine protease Prc was, together with the cytoplasmic membrane anchored protease RseP, the first protease playing a role in this process identified. Our analyses on an unusual CSS pathway of *P. putida* formed by a hybrid σ^{ECF}/anti-σ factor protein, the IutY protein, revealed that Prc is required to eliminate the anti-σ C-domain thereby allowing RseP to cut into the transmembrane domain of IutY and the release of an active σ^{IutY} domain (Bastiaansen et al., 2014). RseP also cuts the transmembrane segment of archetypal CSS anti-σ factors that are not fused to σ^{ECF} domains, and, in accordance, inactivation of the proteolytic function of RseP completely blocks activation of these CSS systems (Fig. 2). The role of Prc in the proteolytic cascade of archetypal CSS systems was less clear (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). We show in this work that a proteolytically inactive version of the Prc protease (Prc-S485A) completely inhibits CSS activation (Fig. 2). This is different from what is observed in cells lacking Prc altogether in which some degree of CSS activation still occurs (Fig. 2 and 3A, Δprc mutant), and confirms that the protease activity of Prc is required for activation of the CSS pathway. The dominant negative effect exerted by the Prc-S485A protein is likely the result of tight binding but not cleavage of this protein to its substrate, probably the anti-σ factor, which avoids other proteolytic events to happen. Remarkably, overproduction of Prc resulted in low but significant signal-independent activation of *P. putida*

CSS systems (Fig. 2). The fact that Prc seems to be active under non-inducing conditions (Bastiaansen et al., 2017) suggests that the function of Prc in absence of the signal is blocked by another element. This role could be performed by the second carboxy-terminal protease that *Pseudomonas* bacteria contain, the CtpA protease, since lack of this protein increases CSS activation in a Prc-dependent manner (Fig. 3A). Western-blot analyses of the *P. aeruginosa* FoxR (PaFoxR) anti-σ factor, which undergoes spontaneous cleavage immediately upon production that separates the protein in an N- and a C-domain (Fig. 1A) (Bastiaansen et al., 2015a; Bastiaansen et al., 2015b), indicate that both Prc and CtpA modulate the levels of the PaFoxR C-domain. Lack of CtpA reduces while that of Prc increases the stability of this domain (Fig. 4B). This suggests that Prc degrades the C-PaFoxR domain while CtpA prevents this degradation to occur. CtpA could perform this function by interacting directly with C-PaFoxR protecting it from degradation or by inhibiting Prc function. Reduced instead of increased Prc protein level are observed in absence of CtpA (Fig. 4C), which indicates that CtpA does not degrade Prc. Still, CtpA could act on another element required for Prc function. In *E. coli*, Prc (also named Tsp) forms a complex with the outer membrane lipoprotein NlpI, which enhances the stability and activity of Prc (Su et al., 2017). This seems to be a common characteristic for the family of carboxy-terminal serine proteases since in *P. aeruginosa* the CtpA protease has been recently found to also form a complex with a lipoprotein (LbcA) (Srivastava et al., 2018). Several substrates of the *P. aeruginosa* CtpA protease are outer membrane lipoproteins (Srivastava et al., 2018), and this could include a still unidentified lipoprotein that stabilizes and works with *P. aeruginosa* Prc. Further research is needed to test this hypothesis.

Mutation of *ctpA* and *prc* does not prevent the regulated intramembrane proteolysis (RIP) that the N-domain of *P. aeruginosa* FoxR undergoes upon signal recognition (Fig. 1B and 4A). Therefore, none of these proteases seems to be the site-1 protease that initiates the RIP pathway of N-PaFoxR in response to ferrioxamine, a cleavage that is required to generate the substrate of the site-2 protease RseP (Fig. 1B) (Bastiaansen et al., 2014; Bastiaansen et al., 2015b). However, absence of CtpA increases the amount of the PaFoxR RIP product, the N-tail domain (Fig. 4A). This effect is likely due to the lack of the C-PaFoxR domain in the *ctpA* mutant (Fig. 4B) since it has been shown that this domain protects the N-domain from the RIP cascade (Bastiaansen et al., 2015a). In fact, when the C-PaFoxR domain is absent the activity of the Fox CSS pathway increases (Bastiaansen et al., 2015a), as also observed in the ΔctpA mutant (Fig. 3A). Together, these results indicate that CtpA fine-tunes the activity of the *P. aeruginosa* Fox CSS pathway by preventing degradation and thus maintaining the correct levels of the C-FoxR anti-σ factor domain, which in turn prevent the RIP pathway of the FoxR N-FoxR domain.

In attempt to identify the protease that works upstream of RseP in the RIP pathway of FoxR (Fig. 1B), we performed a random mutagenesis screen that resulted in the identification of the cytoplasmic proteases PepA (also called PhpA in *Pseudomonas*) and HflK. The *pepA* mutation increases activation of the *P. aeruginosa* Fox CSS system (Fig.7), likely by increasing the stability of the σ^{FoxI} factor and that of the FoxR N-tail domain (Fig. 8). PepA is a leucine aminopeptidase present in multiple organisms that participates in amino acids recycling and in degradation of abnormal proteins (Colloms, 2012). Moreover, PepA can also bind to DNA and modify gene expression (Reijns et al., 2005; Nguyen Le Minh et al., 2016). Loss of the PepA aminopeptidase activity in *P. aeruginosa* results in increased transcription of the alginate biosynthetic genes (*i.e. algD*) (Woolwine et al., 2001), which is performed by the σ^{ECF} factor σ^{AlgT} (also known as σ^{22}) (Wood and Ohman, 2012). This resembles the situation described in this work as we also observed increased expression of the σ^{FoxI}-dependent gene *foxA* in the *pepA* mutant (Fig. 7). Thus, it is tempting to suggest that the PepA protease has a direct role on the modulation of the

σ^{ECF} factor activity in *P. aeruginosa*, likely by maintaining the correct σ^{ECF} factor level. Future research will clarify the function of PepA in σ^{ECF} factor activity.

Regarding HflK, mutation of this protease subunit decreases Fox CSS activity in *Pseudomonas* (Fig. 7 and Fig. S3). The function of this protein in *Pseudomonas* has not yet been characterized. However, in *E. coli*, HflK is known to form a membrane-anchored complex with HflC that negatively regulates the activity of the FtsH protease (Ito and Akiyama, 2005; Bittner et al., 2017). FtsH is one of the five AAA^+ proteases of *E. coli* and the only membrane anchored protease of this group, although its Zn^{2+} metalloprotease domain is located in the cytosol (Ito and Akiyama, 2005). FtsH degrades a set of short-lived proteins, as for example the alternative heat-shock σ factor of *E. coli* (σ^{32}), enabling cellular regulation at the level of protein stability. FtsH also degrades unassembled membrane proteins by dislocating membrane protein substrates out of the membrane (Ito and Akiyama, 2005). The HflKC complex negatively modulates FtsH-mediated proteolysis of membrane proteins (Ito and Akiyama, 2005). It has been proposed that HflKC prevents the entry of membrane protein substrates in the active site of FtsH, thus indirectly decreasing the capability of FtsH to degrade these substrates (Kihara et al., 1998). Mutation of the HflK protein in *P. aeruginosa* does not affect the stability of the σ^{FoxI} factor but reduces the levels of the N-domain of the FoxR anti-σ factor (Fig. 8). This produces lower levels of the FoxR N-tail in the cell, and because this anti-σ domain is required for σ^{FoxI} activity (Fig. 1B), this is likely the reason for the lower CSS activity observed in the *hflK* mutant. Thus, it is possible that HflK modulates the activity of the Fox CSS pathway by preventing the FtsH-mediated degradation of the N-domain of the FoxR anti-σ factor. Members of the AAA^+ protease group as ClpP are known to contribute to the degradation of anti-σ factors in *P. aeruginosa* and other bacterial species (Flynn et al., 2004; Zellmeier et al., 2006; Bishop et al., 2017). Further research is needed to determine the role of HflK and FtsH on CSS anti-σ factor degradation. Alternatively, HflK and FtsH could affect CSS activation in a more indirect way because one of the substrates of the FtsH protease is the ExbD protein (Arends et al., 2016), which together with ExbB and TonB form the energizing machinery of CSS receptors (Fig. 1) (Noinaj et al., 2010). This possibility will be also assessed in future research.

σ^{ECF}-mediated signaling controls important virulence functions in *P. aeruginosa*, including iron acquisition during infection, the response to the oxidative and cell envelope stress produced by components of the immune system of the host, synthesis of the exopolysaccharide alginate responsible of the virulent mucoid phenotype of *P. aeruginosa*, biofilm formation, and production of several virulent determinants (i.e. toxins, exoproteases, secretion systems and secreted proteins) (Hershberger et al., 1995; Llamas et al., 2009; Llamas et al., 2014; McGuffie et al., 2015; Chevalier et al., 2018). Therefore, we analyzed the role of some of the proteases involved in σ^{ECF} factor activation in *P. aeruginosa* virulence. Importantly, a mutant in the RseP protease, which is required for activation of all σ^{ECF} factors of *P. aeruginosa* associated with transmembrane anti-σ factors (at least 16 out of 19) (Llamas et al., 2014; Chevalier et al., 2018) is completely attenuated for virulence and shows significantly reduced cytotoxicity toward host cells. Although we cannot rule out the possibility that this effect may be due to the role of RseP in processing other transmembrane proteins (Akiyama et al., 2004), we believe that the inability of the *rseP* mutant to carry out σ^{ECF}-signaling also contributes to this phenotype. Furthermore, mutation of the CtpA protease reduced *P. aeruginosa* virulence in zebrafish embryos, as previously observed by other authors in mice (Seo and Darwin, 2013). This phenotype is likely due to the impaired functioning of the type 3 secretion system (T3SS), which injects toxic effector into eukaryotic cells, and the reduced cytotoxicity of the *ctpA* mutant (Seo and Darwin, 2013). Surprisingly, mutation of the Prc protease produces a *P. aeruginosa* hypervirulent strain.

To our knowledge, this phenotype has not been reported yet for any bacteria. On the contrary, the *prc* mutation decreases the ability of a pathogenic *E. coli* strain to cause bacteremia and increases its sensitivity to complement-mediated serum killing (Wang et al., 2012). The higher virulence of the *P. aeruginosa prc* mutant could be related to the increased production of outer membrane vesicles (OMVs) observed in this mutant. *P. aeruginosa* OMVs are known to transport virulence factors to host cells and to promote inflammatory response (Bomberger et al., 2009; Ellis and Kuehn, 2010; Lee et al., 2016). Further analyses will confirm this relation.

In summary, we report here the identification of three new proteases that directly or indirectly participate in the activation of *P. aeruginosa* σ^{ECF} factors shedding more light on the complex proteolytic pathway that controls this process.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table S1. *P. aeruginosa* PAO1 wild-type strain and the transposon insertion mutants were obtained from the comprehensive *P. aeruginosa* transposon mutant library at the University of Washington Genome Center (Jacobs et al., 2003). *P. putida* KT2440 and the *P. putida* ctpA-Tn miniTn5-Km transposon mutant were derived from the *P. putida* transposon mutant library at the EEZ-CSIC (Molina-Henares et al., 2010). The locations of the mutations were confirmed by PCR with primers flanking the insertion sites. Construction of null mutants was performed by allelic exchange using the suicide vector pKNG101 as described before (Bastiaansen et al., 2014). Southern blot analyses to confirm the transposon insertion or the chromosomal gene deletion were performed as described (Llamas et al., 2000). Bacteria were routinely grown in liquid LB (Sambrook J 1989) on a rotatory shaker at 37 ºC and 200 rpm. For low iron conditions, cells were cultured in CAS medium (Llamas et al., 2006) containing 400 µM (for *P. aeruginosa*) or 200 µM (for *P. putida*) of 2,2'-bipyridyl. For induction experiments the low iron media was supplemented with 1 µM of ferrioxamine B (Sigma-Aldrich) or 40 µM of iron-free ferrichrome (Santa Cruz Biotechnology). For phosphate starvation conditions, *P. aeruginosa* was cultured in 0.3 % (w/v) proteose peptone (DIFCO) as described elsewhere (Quesada et al., 2016). When required, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to induce full expression from the pMMB67EH P*tac* promoter. Antibiotics were used at the following final concentrations (µg/ml): ampicillin (Ap), 100; chloramphenicol (Cm), 30; kanamycin (Km), 100; nalidixic acid (Nal), 25; piperacillin (Pip), 25; streptomycin (Sm), 100; tetracycline (Tc), 20.

Plasmid construction and molecular biology. Plasmids used are described in Table S1 and primers listed in Table S2. PCR amplifications were performed using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) or Expand High Fidelity DNA polymerase (Roche). All constructs were confirmed by DNA sequencing and transferred to *P. aeruginosa* by electroporation (Choi et al., 2006).

β-galactosidase activity assay. β-galactosidase activities in soluble cell extracts were determined using *o*-nitrophenyl-b-D-galactopyranoside (ONPG) (Sigma-Aldrich) as described before (Llamas et al., 2006). Each condition was tested in duplicate in at least three biologically independent experiments and the data given are the average of the three biologically independent experiments with error bars representing standard deviation (SD). Activity is expressed in Miller units.

Transposon mutagenesis and screening. A *P. putida* mini-Tn5 transposon mutant bank was created as described before (Molina-Henares et al., 2010) by triparental mating using the KT2440 wild-type strain as recipient, *E. coli* CC118λpir (pUT-Km) as donor, and *E. coli* HB101 (pRK600) as helper. Approximately 31.000 colonies were collected and the pull electroporated with the pMP-PPfoxA plasmid. Clones were plated in CAS medium containing 200µM 2,2'-bipyridyl, 1 µM ferrioxamine, 0.56 mg/ml 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-gal) (Panreac), and the antibiotics Tc and Km. White colonies were selected and the Fox CSS activity assayed by β-galactosidase. Transposon locations were determined by arbitrary PCR using the TNEXT2, ARB1A and CEK62B primers (Table S2) in the first PCR round and the TNINT and ARB2A primers (Table S2) in the second PCR round, and sequencing.

SDS-PAGE and Western-blot. *P. aeruginosa* was grown in iron-limited medium containing 1 mM IPTG (when necessary) and without or with 1 µM ferrioxamine. Cells were pelleted by centrifugation and heated for 10 min at 95 °C following solubilization in SDS-PAGE sample buffer (Laemmli, 1970). Sample normalization was done according to the OD_{660} of the bacterial culture. Proteins were separated by SDS-PAGE containing 15 % or 12,5 % (w/v) acrylamide and electrotransferred to nitrocellulose membranes (Millipore). Ponceau S staining was performed as a loading control prior to immunodetection. HA-tagged proteins were detected using a monoclonal antibody directed against the influenza hemagglutinin epitope (HA.11, Covance) as previously described (Bastiaansen et al., 2014) or the MA4-4 monoclonal antibody against the *P. aeruginosa* OprF protein (Rawling et al., 1995).

Outer membrane vesicles (OMVs) isolation and detection. *P. aeruginosa* OMVs were isolated following the protocol described before (Balsalobre et al., 2006) with some modifications. Briefly, overnight LB cultures of *P. aeruginosa* were diluted at 0.05 OD_{600} in low phosphate medium. At OD_{600} of ~1, the cultures were collected and centrifuged at 6.000 x g, 4 ºC. Approximately 10 ml of the supernatants were filtered through a 0.45 µm pore size filter (Millipore) to remove non-pelleted cells and the OMVs were pelleted by ultracentrifugation at 150.000 x g, 180 min, 4 °C. After carefully removal of the supernatant, the pellet was resuspended in PBS containing 15 % (v/v) glycerol in a volume proportional to the final OD_{600} of the culture and the amount of supernatant ultracentrifuged (e.g. 10 ml of supernatant of a 1.2 OD_{600} culture was resuspended in 120 µl of PBS-glycerol). 5 µl of the suspension was plated to verify that it was free of bacteria. OMV proteins (40 µl) were separated by SDS-PAGE containing 15 % (w/v) acrylamide and visualized by Coomassie staining.

Transmission electron microscopy (TEM). TEM samples were prepared following the method described before (Remuzgo-Martinez et al., 2015). Overnight cultures of *P. aeruginosa* in phosphate starvation conditions were centrifuged at 0.6 x g and the pellets were washed four times in PBS. Bacteria cells were placed in 100 mesh copper grids containing the support carbon-coated Formvar film (Electron Microscopy Sciences) and air dried. Cells were negatively stained, with 1 % (w/v) phosphotungstic acid in distilled water, for 20 sec and examined with a JEOL JEM-1011 transmission electron microscope equipped with an ORIUS SC 1000 CCD camera (GATAN).

Zebrafish maintenance, embryo care and infection procedure. Transparent adult *casper* mutant zebrafish *(mitfa* $^{w2/w2}$*;roy* $^{a9/a9}$*) (Renshaw et al., 2006; White et al., 2008)* were conserved at 26 °C in aerated 5 L tanks with a 10/14 h dark/light cycle. Zebrafish embryos were collected during the first hour post-fertilization (hpf) and kept at 28 °C in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl{\cdot}2H_2O$, 0.33 mM $MgCl_2{\cdot}7H_2O$) supplemented with 0.3 mg/L methylene blue. Prior to infection, 1 day post-fertilization (dpf) embryos were mechanically dechorionated

and anaesthetized in 0.02 % (*w/v*) buffered 3-aminobenzoic acid methyl ester (pH 7.0) (Tricaine, Sigma-Aldrich). Zebrafish embryos were individually infected by microinjection with 1 nl of *P. aeruginosa* in the caudal vein (systemic infection) as described elsewhere (Llamas and van der Sar, 2014). All procedures involving zebrafish embryos were according to local animal welfare regulations.

Virulence assay in infected zebrafish embryos. Zebrafish embryos were injected in the caudal vein with ~1000 CFU of exponentially grown *P. aeruginosa* cells in low phosphate conditions previously suspended in phosphate-free physiological salt containing 0.5 % (w/v) of phenol red. After infection, embryos were kept in 12-well plates containing 60 µg/mL of Sea salts (Sigma-Aldrich) at 32 ºC with 20 individually injected embryos in each group per well. Embryo survival was determined by monitoring live and dead embryos at fixed time points during five days. Five biologically independent experiments were performed and the data given are the average. P-values were calculated by log-rank (mantel-Cox) test.

Cytotoxicity assay in A549 human lung epithelial cells. *P. aeruginosa* cytotoxicity on A549 cells was assayed using a colorimetric assay that detects the number of metabolically active eukaryotic cells able to cleave the MTT tetrazolium salt (Sigma-Aldrich) to the insoluble formazan dye. The A549 cell line (ATCC® CCL-185™) was maintained in DMEM medium supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco) in a 5 % CO_2 incubator at 37 ºC. One day prior infection, the A549 cells were placed in 96-well plates at a concentration of 4 x 10^4 cells/well and cultured in phosphate-free DMEM medium (Gibco) with 5 % (v/v) FBS. In this condition, cell mitosis does almost not occur. Late exponentially grown *P. aeruginosa* strains in low or high phosphate conditions were then inoculated at a multiplicity of infection (MOI) of 20. At 3 hpi, 30 µl of a 5 mg/ml MTT solution in PBS was added to the wells and the plates were incubated for 2 h. The culture medium was then removed and 100 µl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan. Production of formazan, which directly correlates to the number of viable cells, was quantified using a scanning multi-well spectrophotometer (Infinite® 200 PRO Tecan) at 620 nm.

Time-lapse imaging assay. Time-lapse microscopy was performed on a Nikon Eclipse Ti-E microscope (Nikon), equipped with a PlanFluor 20–40 × 0.6NA objective (Nikon) and a CO_2 incubator. A549 cells were seeded in coated 4-well µ-slides (Ibidi, Martinsried, Germany) in the same conditions as in the cytotoxic assays. Late exponentially grown *P. aeruginosa* strains in low phosphate conditions were then inoculated at a multiplicity of infection (MOI) of 20. Images were collected from 0 to 240 min post-infection every 2 min with an ORCA- R2 CCD camera (Hamamatsu) powered by Nis Elements 3.2 software. Videos where edited with ImageJ.

Computer-assisted analysis. Images processing and band intensity measurements were performed using the ImageJ software. Statistical analyses are based on *t*-test in which two conditions are compared independently. P-values from raw data were calculated by independent two-tailed *t*-test and Kaplan-Meyer survival curves by Log-Rank (Mantel-Cox) using GRAPHPAD PRISM version 5.01 for Windows and are represented in the graphs by ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001; and ****, P<0.0001.

SUPPLEMENTAL INFORMATION

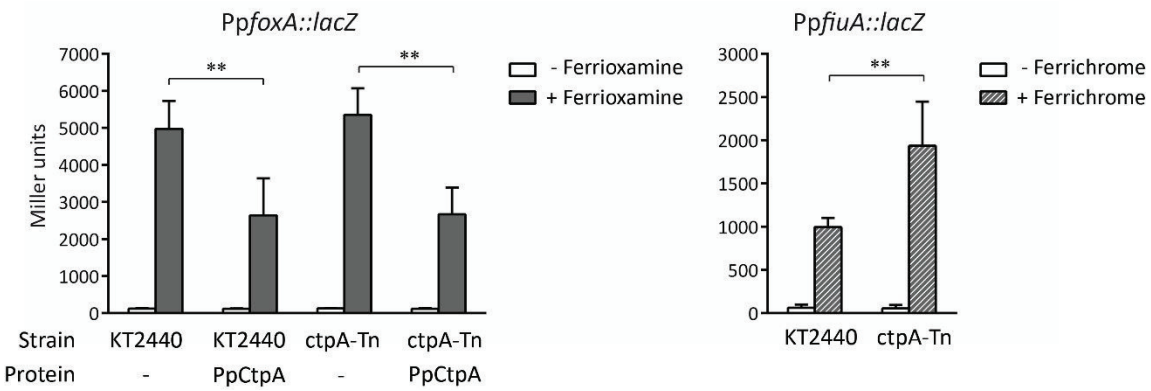

Fig. S1. CSS activity in the *P. putida* ctpA-Tn mutant. β-galactosidase activity of the indicated *lacZ* fusion gene in the *P. putida* KT2440 wild-type strain and its isogenic ctpA-Tn mutant bearing the pBBR1MCS-5 empty (-) or pBBR-PpCtpA (Table S1) plasmid. Strains were grown under iron-restricted conditions without (-) or with (+) the cognate inducing siderophore. Data are means ± SD from three biological replicates (N=3). P-values were calculated by two-tailed *t*-test by comparing the value obtained in the mutant with that of the PAO1 wild-type strain in the same growth condition. Comparisons between other strains are indicated by brackets.

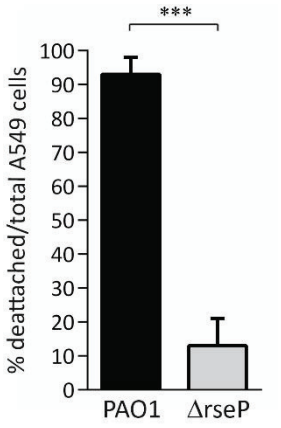

Fig. S2. Percentage of detached A549 cells infected with *P. aeruginosa*. The percentage of detached cells following infection with the indicated *P. aeruginosa* strain was obtained from the time-lapse imaging assay at 2.5 hours post-infection (hpi). Data are means ± SD from three biological replicates (N=3). P-value was calculated by two-tailed *t*-test.

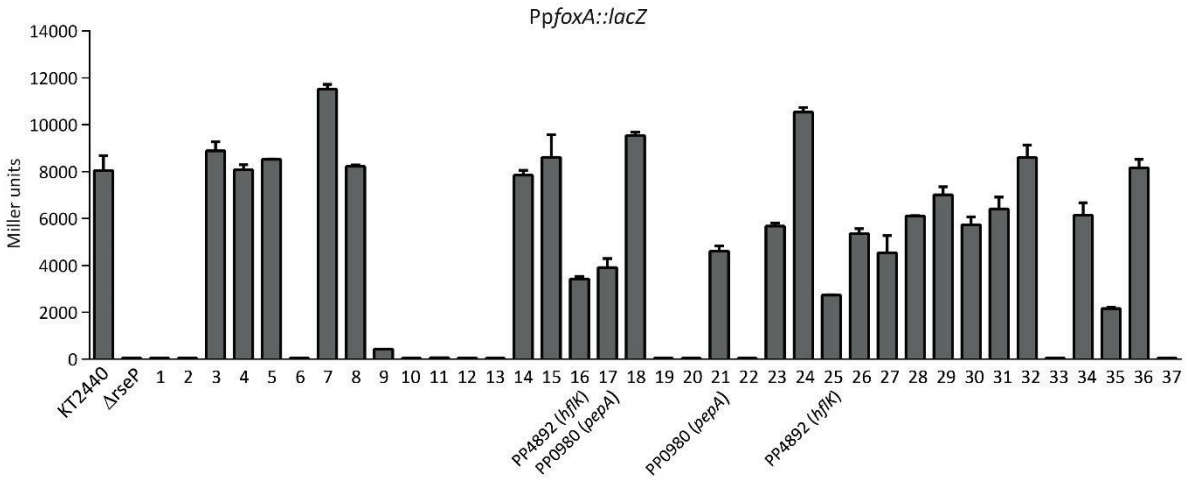

Fig. S3. Analysis of the Fox CSS activity in *P. putida* transposon mutants. β-galactosidase activity of the clones selected from the random mutagenesis screen in *P. putida* KT2440 bearing the *foxA::lacZ* fusion.

Strains were grown to late log-phase under iron-restricted conditions in presence of ferrioxamine. Data are means ± SD from two biological replicate (N=2).

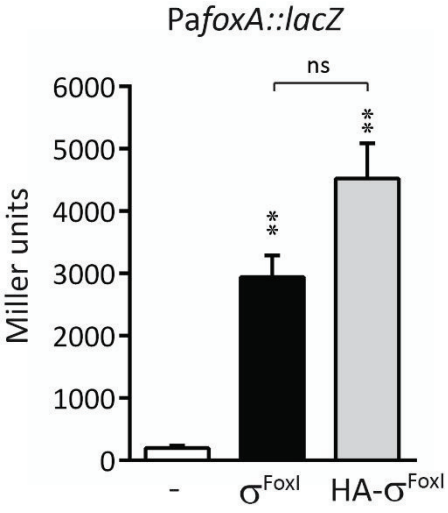

Fig. S4. Activity of an N-terminally HA-tagged *P. aeruginosa* σ^{FoxI} factor. β-galactosidase activity of *P. aeruginosa* PAO1 wild-type strain bearing the *foxA::lacZ* fusion and the pMMB67EH empty plasmid (-) or the pMMB67EH-derived plasmid expressing the indicated protein (Table S1). Strains were grown in LB with 1 mM IPTG to induce expression from the pMMB67EH Ptac promoter. Data are means ± SD from two biological replicates (N=2). P-values were calculated by two-tailed *t*-test by comparing the value obtained in cells overexpressing σ^{FoxI} with that of the strain bearing the empty plasmid. Comparisons between other strains are indicated by brackets.

Video 1. Time-lapse imaging of *P. aeruginosa* infections in A549 cells. Infections with the *P. aeruginosa* PAO1 wild-type strain are shown on the left and with the ΔrseP mutant on the right. Video was recorded from 0 to 240 min post-infection and pictures were taken every 2 min. The scale bar indicates 50 µm.

Discussion

Signal transduction cascades mediate the sensing and processing of stimuli. These molecular circuits detect, amplify, and integrate molecular signals to generate cell responses. This process is essential for adaptation to changing circumstances. In recent years it has become clear that extracytroplasmic function sigma (σ^{ECF}) factors are the third fundamental mechanism of bacterial signal transduction (Helmann, 2002; Staron et al., 2009). *Pseudomonas* bacteria contain a plethora of σ^{ECF} factors (Martinez-Bueno et al., 2002; Potvin et al., 2008; Llamas et al., 2014), which highlights the important of this signaling mechanism in the adaptation of these bacteria to the environment and the host. In this Thesis, we have studied σ^{ECF}-mediated signaling in the human pathogen *P. aeruginosa.* We have focused on σ^{ECF} factors that allow *P. aeruginosa* to sense and respond to the host, enabling a successful infection (**chapter 2, 3 and 4**). Moreover, we have discovered new features of the molecular mechanism responsible of *Pseudomonas* σ^{ECF} factors activation (**chapter 5**). We have identified components of the σ^{ECF}-mediated signaling that are essential for *P. aeruginosa* virulence and could thus be new targets for the development of antibacterial drugs, as proposed and discussed below.

Role of σ^{ECF} factors in P. aeruginosa *virulence.*

Most *P. aeruginosa* σ^{ECF} factors promote expression of iron uptake machineries in response to specific iron carriers. These σ^{ECF} factors are known as iron-starvation σ factors (Leoni et al., 2000), and are usually expressed in an operon with an anti-σ under conditions of iron limitation (Llamas et al., 2014). Iron participates in multiple metabolic reactions such as electron transport or DNA and amino acids synthesis, acquisition of this metal is thus crucial for pathogens to be able to produce an infection (Ratledge and Dover, 2000; Wandersman and Delepelaire, 2004). The optimal iron concentration for growth of most bacteria is much higher than the concentration that is freely accessible in the mammalian host (Andrews et al., 2003). Moreover, the ferrous ion (Fe^{2+}) is potentially toxic because its ability to catalyze the production of reactive oxygen and nitrogen species. The potential toxicity of iron is managed by both pathogens and the host by tightly controlled systems dedicated to balancing cellular and whole iron acquisition, storage and utilization (Graf et al., 1984). *P. aeruginosa* σ^{ECF} factors play a key role in this process. They control the production of several outer membrane receptors needed for the uptake of different iron carriers (Table 1, chapter 1). This regulation prevents the synthesis of these large outer membrane proteins, which is energetically expensive, when they are not necessary and avoids an excess of iron going into the cell.

Besides the regulation of iron uptake, several iron starvation σ^{ECF} factors of *P. aeruginosa* also promote expression of virulence functions. The best studied is σ^{PvdS}, which promotes transcription of the biosynthetic locus of the siderophores pyoverdine, as well as of the virulent factors exotoxin A and endoprotease PrpL (Lamont et al., 2002). Activation of this σ factor occurs in response to the Fe-pyoverdine complex in the extracellular medium through the Fpv CSS pathway. This σ^{ECF} factor system plays a major role in *P. aeruginosa* acute virulence (Minandri et al., 2016). However, during chronic infections, study of chronic lung infection patients and using chronic infection animal models showed a reduced pyoverdine production (Smith et al., 2006; Turner et al., 2014) indicating a relegated role of pyoverdine in this infection stage.

Heme is the most abundant iron source in mammals (Ganz and Nemeth, 2006). We have shown in this Thesis that *P. aeruginosa* contains two iron-starvation σ^{ECF} factors, σ^{HasI} and σ^{HxuI}, which likely promote heme acquisition (chapter 4). Although previously proposed (Cornelis et al., 2009; Llamas et al., 2014), the involvement of σ^{HxuI} σ factor in heme sensing and uptake have not been experimentally demonstrated before our study. The role of σ^{HxuI} in promoting heme

acquisition, although likely, is however less clear because a direct transport of heme through the HxuA receptor has not been shown yet. The existence of three heme utilization systems in *P. aeruginosa* that are not redundant permits sensing of a wide range of heme concentrations suggesting a predominant use of heme as source of iron during infection. In fact, transcription of heme genes increases in mice models of infection (Turner et al., 2014; Damron et al., 2016). The genes that regulates the heme degradation once it is inside the bacteria are also crucial for a proper infection. The heme oxygenases HemO, which degrades the heme in the cytoplasm, is needed for the virulence of *P. aeruginosa* (Hom et al., 2013). Moreover, heme signaling produces the activation of the σ^{HasI} and σ^{HxuI} that induce expression of genes. Their operons include their cognate receptors (as usual in the σ^{FecI}-like σ^{ECF} factors) but probably also regulates more genes. Identification of these regulons is important to determine whether these σ factors triggers, as σ^{PvdS}, the expression of *P. aeruginosa* virulent determinants (Beare et al., 2003). The virulence of *P. aeruginosa hasI* and *hxuI* σ factor knockouts has not been analyzed yet, but it has been shown that mutation of the *hasR* receptor diminishes the bacterial burden in mice lungs after 16 hours of acute infection (Damron et al., 2016). However, a *P. aeruginosa hasR phuR* mutant is as virulent as the wild-type strain after 14 days of infection (Minandri et al., 2016), which suggests that the mutant is able to recover from the initial bacterial burden diminution that produces the *hasR* mutation. This suggests that the HasR receptor is required in the initial step of the infection process, being less important when the pathogen has colonized the host. Alternatively, the fact that the virulence of the *hasR phuR* double mutant is not affected can be also due to the presence of the HxuA receptor. The existence of compensation in the activity of the heme systems could explain this result.

Another *P. aeruginosa* σ^{ECF} factor initially classified in the iron-starvation group that triggers expression of virulence functions is σ^{VreI} (Llamas et al., 2009). Our analyses have however shown that this classification is incorrect because σ^{VreI} is not expressed in conditions of iron limitation and only responds in inorganic phosphate limitation in coordination with the PhoR/PhoB two-component system [(Faure et al., 2013) and chapter 3]. Microarray analyses initially determined that σ^{VreI} promotes expression of virulent determinants (Llamas et al., 2009). However, the regulation of σ^{VreI} independent genes, in the absence of the anti-σ factor VreR shows a more complex regulated network. Although not analyzed yet, this regulation could be performed by the transcription factors which expression is modulated by σ^{VreI} (Table 1, chapter 3). Alternatively, VreR could control the activity of not only σ^{VreI} but also another *P. aeruginosa* σ factor. An anti-σ factor controlling the activity of two σ factors has been already described in *P. aeruginosa* (i.e. FpvR) (Beare et al., 2003) and other bacteria (e.g. the *B. subtillis* SpoIIAB anti-σ factor) (Kellner et al., 1996). There are two σ^{ECF} factors in *P. aeruginosa* without a putative anti-σ factor, PA3285 and PA1351 (Table 1, chapter 1). They should be the first candidates to be analyzed. This new framework with the possibility of two σ^{ECF} factors controlled by VreR could explain the σ^{VreI}-independent modulation of gene expression exerted by VreR.

P. aeruginosa has more σ^{ECF} factors involved in virulence like σ^{SigX} or σ^{AlgT}. These two σ^{ECF} factors, as σ^{VreI}, are not iron-starvation σ factors. σ^{SigX} is induced membrane stress conditions (Bouffartigues et al., 2012). σ^{SigX}, apart from expressing *oprF*, participates in the assembly of the T3SS and regulates some *exs* genes. A *P. aeruginosa* strain lacking σ^{SigX} is not able to use the T3SS correctly being its virulence highly reduced (Gicquel et al., 2013; Blanka et al., 2014). σ^{AlgT} produces the transcription of the alginate biosynthetic operon. Production of this exopolysaccharide causes the biofilm formation that permits the transition from motile to a sessile lifestyle. Biofilms increase resistance to antibiotics and impedes a proper defense from the host immune system (deeply described in chapter 1).

A major portion of the *P. aeruginosa* virulence is regulated by σ^{ECF} factors. In the acute infection, σ^{PvdS}, σ^{SigX} and the here described σ^{Vrel} manage most of the main virulence mechanisms. During chronic infection, σ^{AlgT} has a crucial role in the alginate production and biofilm formation. Furthermore, the necessity of iron during infection is mainly handle by σ^{Fpvl} and probably by σ^{Hasl} and σ^{Hxul}.

Activation of σ^{ECF} factors by host signals

Interaction with the host gives pathogens the information about the circumstances that surround them. These interactions let bacteria known whether they are located in a favorable or hostile environment (i.e. lack of nutrients or presence of hazardous molecules). During infection, the host is also able to recognize the pathogen (i.e. via Toll-like receptors), which triggers an immune response to eradicate the pathogen (Akira et al., 2006). Therefore, pathogens need to coordinate the virulent attack to prevent alarming the immune system too early. Host recognition is crucial to achieve the host invasion. One of the most important signals that bacteria use to recognize the host is the temperature change, which is recognized by RNA thermometers. Variation in temperature produces conformational changes in the secondary structures of the RNA thermometer, usually exposing the mRNA RBS inducing its transcription. Mammalian host temperature is around 37 ºC. Pathogens have virulent genes that are already transcribed that are RNA thermometers, which only permit translation in increased temperatures, like host temperatures, releasing their pathogenicity (Narberhaus, 2010; Kortmann and Narberhaus, 2012). Bacteria also have receptors for recognizing components of the host immune system. The enterohemorragic *E. coli* O157:H7 is able to recognize the mammalian norepinephrine and epinephrine hormones, which are released by the host during stress. They are sensed by the HK QseC that phosphorylates the response regulator QseB, which together with the QS induce transcription of virulent genes as the T3SS or the flagella regulon (Sperandio et al., 2003; Clarke et al., 2006). Antimicrobial peptides and lysozyme from the host, are used by pathogens for activating gene expression through σ^{ECF} factors (Bishop and Finlay, 2006; Hastie et al., 2014). σ^{E} from *M. tuberculosis* induces activation of virulence genes in response to oxidative stress, which is usually generated by the action of leukocytes (Fontan et al., 2008). *P. aeruginosa* also have mechanisms for recognizing the host infection. The OprF outer membrane protein from *P. aeruginosa* is able to recognize interferon-γ from the host and induce the expression of PA-I lectin through QS activation. PA-I lectin participates in cell adherence to the host (Wu et al., 2005). Dynorphin is an opioid expressed in the lungs of the host that induces the *P. aeruginosa* QS system by upregulating the transcription of the *pqsABCDE* operon that produces the increase of the biofilm formation and virulence (Zaborina et al., 2007). In this Thesis, we have studied several σ^{ECF} factors that are regulated by host signals.

In the case of the σ^{Vrel} factor, a probable second signal (apart from low phosphate) fully activates it (chapter 2 and 3). The probably existence of two signals for activating σ^{Vrel} (low Pi and host-derived molecule), tightly regulates the system. When a mechanism has a complex regulation is probably due to the highly cost or the consequences of its activation. Induction of virulence in a wrong time point can produce the total annihilation of the pathogen by the host immune system or a waste of energy whether the expressed proteins are not needed in that circumstance. When gene expression is integrated by several cues, one of them is usually a global metabolic signal (low Pi) and the other signal produces a concrete response (host-derived molecule) (Browning and Busby, 2004). Similar signaling have most of the σ^{ECF} factors that regulates iron uptake. A first metabolic signal is the absence of iron. In this case, the Fur negative regulation disappears. Then the siderophore quelated with iron is recognized, which acts as a

second signal that produces a concrete response (Llamas et al., 2014). The host-derived signal would show the bacteria that is infecting a host. The host could be assumed as a source of elements and nutrients for the bacteria (Abu Kwaik and Bumann, 2013; Santic and Abu Kwaik, 2013). Because Pi participates in multiple reactions in bacterial metabolism, a low Pi environment must be corrected. The host could be used by bacteria as a source of Pi as happens with multiple nutrients (Abu Kwaik and Bumann, 2013). Expression of the virulent genes of the σ^{Vrel} regulon increases the virulence of *P. aeruginosa* that kills more host cells. The cell lysis permits an easier access to the nutrients for the bacterial development. Apart from the already cited signals, more compounds are released from the host cells that could act as signal. The epithelial lung cells release enzymes such as lysozyme or secretory phospholipase A_2, iron binding proteins, cationic nuclear proteins like histones, and antimicrobial peptides (Bals et al., 1998). They could act as a signal for activation of σ^{Vrel}. Future experiments exposing these compounds to *P. aeruginosa* would reveal which signal is in charge of σ^{Vrel} activation.

Another host-derived molecule used by the pathogen is heme. Pathogens are able to use them as signaling molecules for gene expression. *P. aeruginosa* has evolved (at least) two heme signaling systems (Has and Hxu). They permit *P. aeruginosa* to sense heme with high efficiency in different heme concentrations that permit to cope a wide range of situations. Heme signaling induce the activation of σ^{Hasl} and σ^{Hxul}. In both cases, two signals are activating the σ^{ECF} factors. First, the physiological signal of iron starvation induce the expression of the system (Fur regulated). Second, the recognition of the host-derived signal heme is sensed and activates the σ^{ECF} factors. Most iron present in mammalian host is forming heme as part of hemoglobin. The ability of *P. aeruginosa* to heme recognition and uptake (at least by the Has system) permits the acquisition of the heme needed for a correct development of the infection. *P. aeruginosa* is not the only organism that activates σ^{ECF} factors using heme as a host-derived signal. *H. influenze* has a system homolog to the Hxu system from *P. aeruginosa*, that also senses heme (Cope et al., 1995). *S. marcescens* possess a Has heme acquisition system homolog to the Has system from *P. aeruginosa* (Ghigo et al., 1997).

Molecular mechanism responsible of CSS activation

Activity of most σ^{ECF} factors are usually controlled by its cognate anti-σ factor. Moreover, several σ/anti-σ pairs have an outer membrane receptor associated, in charge of sensing signals. The three proteins form a CSS system. When the correct signal appears, the receptor has to transmit the activation from the outer membrane to the cytosol, where the σ^{ECF} is located. Signal transduction across membranes is always a challenge due to the impossibility of signals to freely diffuse through them. In previous works, our group demonstrated that RIP of the anti-σ factor takes place transducing the signal through the inner membrane (Bastiaansen et al., 2014; Bastiaansen et al., 2015a). Along organisms, RIP is usually performed by two proteases. A first proteolytic event takes place in one side of the membrane (site-1 proteolysis), a second one (site-2 proteolysis) happens in the intramembrane region. Prc and RseP (site-2) proteases play a main role in *P. putida* and *P. aeruginosa* anti-σ factors RIP events (Bastiaansen et al., 2014). However, only in the IutA/IutY *P. putida* system Prc has been described directly processing the anti-σ factor as a site-1 protease. In the other CSS systems, we determined that Prc needs its proteolytic activity but is not clear which substrate is processing. In this scenario, another protease should be acting in site-1. In our search of the site-1 protease, we proposed the carboxy-terminal protease CtpA as a candidate, however; we concluded that it is participating in the CSS system activation but not as a site-1 protease (chapter 5). What is sure is that the site-1 proteolysis takes place in the absence of Prc and RseP keeps working as site-2 protease.

It is not clear how the RIP is initiated. In the σ^E/RseA pair from *E. coli*, the protease DegS has a domain called PDZ that keeps the proteolytic activity inactive. Several stresses generate unfolding of outer membrane porins. The C-terminal part of these unfolded porins is recognized by the PDZ domain of DegS, which produces a conformational change activating the protease function (Walsh et al., 2003). The site-2 protease RseP keeps inhibited until the action of DegS. RseA has a Gln rich inhibitory domain that due to its chemical nature impedes the action of RseP (Kanehara et al., 2003). DegS removes this region in the site-1 proteolysis permitting the action of RseP. Moreover, RseP has a periplasmic PDZ domain that in this case cannot inhibits the intramembrane active site of its protein due to its location but, it is thought that the PDZ domain could be interacting with the Gln rich region impeding the action of the active site (Ades, 2004). In the CSS systems, the receptor plays a role in the activation of the cascade. Apart from its role as signal receptor, it transduces the signal recognition to the anti-σ factor, through its signaling domain. Several evidences (preliminary data from the group) show the *in vitro* interaction between the receptor FoxA and the anti-σ factor FoxR, as was observed between FecA and FecR from *E. coli* (Enz et al., 2003b). This interaction takes place in the absence of the signal. We hypothesize that the signaling domain of the receptor protects the anti-σ factor from RIP. The recognition of the external signal produces a conformational change in the receptor. Apart from the conformational change that permits the entrance of the signal into the cell, the signaling domain also suffers a conformational change. In the case of the FpvA receptor (pyoverdine system from *P. aeruginosa*) the signaling domain changes its position drastically (Brillet et al., 2007; Schalk et al., 2012). This movement could expose the anti-σ factor to the action of proteases that initiate the RIP cascade.

Focusing on the cytoplasmic part of the anti-σ factor, two different groups have been organized in relation to its activity. The mere anti-σ factors have the ability to inhibit the σ factor activation. The second group of anti-σ factors have two functions. First, they inhibit the σ factor but when the RIP takes place, their N-terminal part is needed for the function of the σ factors. They are known as anti-σ factor regulators (Mettrick and Lamont, 2009; Llamas et al., 2014). Up to date, the reason why some σ^{ECF} factors need the anti-σ factor N-tail is not clear. In the case of the FpvR N-tail, it is degraded by the ClpXP protease complex. However, in the case of the FecR from *E. coli* or *P. aeruginosa* the N-tail is needed for the correct function of σ^{FecI} (Ochs et al., 1995; Mettrick and Lamont, 2009). These groups have several differences that explain these two functions. Anti-σ factors are structurally similar to each other but with different amino acids sequences where the specific affinity to a concrete σ^{ECF} factor resides (Campbell et al., 2007). Interestingly, the interacting region of the σ^{ECF} factor to the anti-σ factor varies from one to other (described in chapter 1). This variability of interactions could expose or not the N-tail to proteases like the ClpXP protease complex. Even differences in where the RseP protease produces the site-2 cleavage could affect the sensitivity to protease degradation. Apart from the variations in the anti-σ factors, the key of the existence of two anti-σ factor groups could be lying on the σ^{ECF} factor. The N-tail probably stabilizes the σ^{ECF} factor (Mettrick and Lamont, 2009). Moreover, the number of σ^{ECF} molecules probably plays a role, because the over expression *in trans* of σ^{FoxI} in a ΔfoxR mutant bypasses the absence of the N-tail (Mettrick and Lamont, 2009). Besides, increased affinity to the RNAPc has been observed when purified σ^{FecI} is exposed to RNAPc in presence of the FecR N-tail (Mahren and Braun, 2003). Even the complex N-tail-σ^{FecI}-RNAPc has been purified, showing that probably the N-tail is part of the transcription machinery (Mahren and Braun, 2003). In conclusion, we hypothesize that the σ^{ECF} factors could vary in stability, amount and affinity to RNAPc, and some of them need the N-tail of its cognate anti-σ factor for its function. Solving the structure of several sigma factors, their cognate anti-σ factors

and interaction crystal structures would give more light about the double function of the σ factor regulators.

Targets for developing new antimicrobial drugs

The WHO (WHO, 2017) has listed *P. aeruginosa* as the second most important pathogen that needs new antibiotics development due to its high capacity of treatment resistance. To achieve this aim, a deeper knowledge of this bacterium is needed. In this Thesis, we studied several protein pathways needed for virulence and iron uptake during infection. We also found possible targets for new antibiotics development. The wrong use of antibiotics and the high selective pressure that they generate have permitted the appearance of resistant strains that are difficult to treat. Even the combination of several antibiotics is not enough for killing resistant bacteria. The worldwide alarm has been activated for finding out new antibiotics able to treat antibiotics-resistant infections. A novel drug strategy has been proposed. The innovative wave is moving forward a new drug era where antibiotics reduce the virulence of the pathogen facilitating the clearing of the infection through the immune system. Whether bacteria have a reduced virulence, the immune system could clear the infection more efficiently. The selective pressure is low in this new type of antibiotics, reducing the possible resistant strains. The use of molecules that can block the activity of σ^{ECF} factors is a promising strategy for treating bacterial infections. Due to the σ^{ECF} factors function in virulence and their role in stress response, they are good targets for drug development. Moreover, the unlikely similarity to human molecules permits the development of drugs that do not act non-specifically generating side effects (Bashyam and Hasnain, 2004). An interesting source of possible drug compounds are the bacteriophages. The AsiA protein from the bacteriophage T4 binds to the *E.coli* σ^{70} factor inhibiting its binding to the RNAPc that then is bound to σ factors from the bacteriophage (Orsini et al., 1993). Another example is the use of flucytosine in *P. aeruginosa*. Flucytosine inhibits the transcription of *pvdS* impairing the production of pyoverdine, PrpL protease and exotoxin A with the associated reduction in virulence (Imperi et al., 2013). The use of σ factors as a target for drugs development has been already proposed and we support this idea with new data explaining the relevant role of σ^{ECF} factors in virulence and critical process as iron uptake.

Apart from σ^{ECF} factors, another important proposed are proteases (Turk, 2006). The absolutely reduced virulence observed in the *P. aeruginosa* ΔrseP mutant (Chapter 5) makes RseP as a perfect candidate for drug development. Proteases participate in multiple processes. They participate in stress response, sporulation, cell division and cell differentiation (Koide et al., 2008). Absence of RseP probably not only affects the RIP of anti-σ factors, being more processes affected by its impaired function. RseP is a suitable candidate as a target for drug development because it is not essential for its survival, and this permits a low selective pressure reducing the appearance of resistant strains. One of the main problems of drug development is to overcome the presence of the membranes that impedes the action of the drugs inside bacteria. The use of CSS receptors as an entrance door for the drug has been proposed. Siderophores bound to a drug molecule can enter to the bacterium through the CSS receptors as a Trojan horse (Mollmann et al., 2009; Gorska et al., 2014). Once the drug is inside the cell it can blocks its target impairing the correct function of the bacteria.

Concluding remarks

In this work, we have studied some of the interactions that happen between the host and the opportunistic pathogen *P. aeruginosa*. During infection, the iron availability is an issue that bacteria have to overcome for a successful infection. Several mechanisms have been

developed for bacterial iron uptake. We have discovered a new CSS system that is in charge of sensing heme (chapter 4). Being heme a major source of iron, an efficient uptake would probably give an advantage for achieving an infection. Heme is not the only host-derived signal able to induce gene expression in *P. aeruginosa*. There are more interactions with the host. The pair σ^{VreI}/VreR plays a major role in the low Pi dependent virulence (chapter 3). Low Pi partially activates σ^{VreI} (chapter 2), a common situation in immune comprised patients. We have discovered that the full activation of σ^{VreI} probably depends on a host-derived signal secreted from the host. However, a complete analysis of the molecules released by the host cell is needed for the recognition of the σ^{VreI} activating signal. Finally, we have investigated the signal transduction of the CSS systems. We focused on the RIP that produces the anti-σ factor cleavage and the release of the σ^{ECF} factor. We found three new proteases, which directly or indirectly participate in the σ^{ECF} factors regulation (chapter 5). Future studies of the substrates of these proteases will give more information about how the CSS systems activation works.

Conclusions/Conclusiones

CONCLUSIONS

1. Under inorganic phosphate (Pi) starvation, the transcriptional regulator PhoB binds to the promoter region of the *P. aeruginosa vreAIR* operon, which encodes a σ^{ECF} factor (σ^{VreI}), an anti-σ factor (VreR) and an outer membrane-associated protein (VreA), and promotes its expression.

2. The release and activation of the *P. aeruginosa* σ^{VreI} factor in response to the inducing signal requires the removal of the anti-σ factor VreR. After having been released into the cytosol, the σ^{VreI} factor is recruited by the transcription factor PhoB in the promoter region of the σ^{VreI} regulon genes, which contains a *pho* box and −10 and −35 sequences for the binding of these regulatory proteins that in turn promotes expression of these genes.

3. The virulence of *P. aeruginosa* on zebrafish embryos and its cytotoxicity towards A549 human lung epithelial cells increases under Pi starvation conditions. The σ^{VreI} and VreR factors, but not the VreA protein, are required for the low Pi-induced virulence of *P. aeruginosa*. σ^{VreI} is active *in vivo* during infection, which produces the expression of several virulence factors that increases the pathogenicity of this bacterium.

4. The VreR anti-σ factor influences gene expression not only through the activation of σ^{VreI} but also independently of this σ factor. VreR modulates the expression of several regulators of gene expression.

5. *P. aeruginosa* contains three systems for acquisition and detection of heme namely Phu, Has and Hxu. The *P. aeruginosa* Has and Hxu systems are cell-surface signaling (CSS) systems that respond to the presence of heme-hemophore complexes and free heme, respectively. Both signalling cascades involve an outer membrane receptor (HasR and HxuA, respectively) that upon sensing heme in the extracellular medium produces the activation of a σ^{ECF} factor (σ^{HasI} and σ^{HxuI}, respectively) in the cytosol. The *P. aeruginosa* σ^{HasI} factor promotes expression of the *hasR-hasAp* operon encoding the HasR receptor and the HasAp hemophore, respectively, whereas the σ^{HxuI} factor triggers expression of the *hxuA* receptor gene. *P. aeruginosa* is therefore able to use heme as a signal molecule to induce expression of response genes.

6. Activation of the σ^{HasI} and σ^{HxuI} factors requires the targeted proteolysis of their cognate anti-σ factors HasS and HxuR, respectively. The first proteolytic step of these anti-σ factors occurs in absence of heme by a spontaneous cleavage in the periplasmic domain of these proteins that separates them into an N-terminal and a C-terminal domain. In response to heme, the N-domain is further processed by a process involving the Prc and RseP proteases. This leads to the activation of the σ^{HasI} and σ^{HxuI} factors in the cytosol and the expression of the genes regulated by these factors σ. HasS and HxuR function only as anti-σ factors and do not have pro-σ activity.

7. The *P. aeruginosa* σ^{ECF} *hxuI* gene is co-transcribed with its anti-σ factor gene *hxuR* but the *hasI* and *hasR* genes are not, in contrast to the situation found with most σ^{ECF}/anti-σ factor pairs of *P. aeruginosa* that allows to maintain the stoichiometry of the system and avoids the presence of active σ units in the absence of inducing signal. However, σ^{HasI} stimulates the transcription of its anti-σ factor *hasS*, thereby self-regulatingits own activity.

8. The proteolytic activities of the RseP and Prc proteases are required for the activation of the *Pseudomonas* Fox and Fiu CSS pathways.

9. The periplasmic carboxy-terminal processing protease CtpA, the cytoplasmic membrane protease subunit HflK and the cytosolic protease PepA are involved directly or indirectly in the activation of the *P. aeruginosa* Fox CSS pathway. The absence of CtpA decreases the amount of the C-domain of the FoxR anti-σ factor which is the domain that interacts with and protects the N-terminal domain of FoxR from the subsequent proteolytic process. Reduced C-FoxR level thus increases the proteolysis of the N-FoxR domain, which in turn increases σ^{FoxI} factor activation and expression of the *foxA* gene. The lack of PepA increases the stability of the σ^{FoxI} factor, which results which results in higher *foxA* gene expression. The absence of HflK reduces CSS activity and *foxA* expression by a still unknown mechanism.

10. The absence of the RseP and CtpA proteases reduces the virulence of *P. aeruginosa* on zebrafish embryos and human A549 cells, whereas that of Prc increases the virulence of this pathogen. Lack of Prc increases the production of *P. aeruginosa* outer membrane vesicles, which are used by this pathogen to deliver virulence factors directly into the cytoplasm of host cells. This increased production of outer membrane vesicles may underlie the increased virulence observed in the absence of Prc.

CONCLUSIONES

1. En condiciones de limitación de fosfato inorgánico (Pi), el regulador transcripcional PhoB se une a la región promotora del operón *vreAIR* de *P. aeruginosa,* que codifica un factor σ^{ECF} (σ^{VreI}), un factor anti-σ (VreR) y una proteína periplásmica asociada a la membrana externa (VreA), e induce su expresión.

2. La liberación y activación del factor σ^{VreI} de *P. aeruginosa* en respuesta a la señal inductora requiere la eliminación del factor anti-σ VreR. Tras ser liberado en el citosol, el factor σ^{VreI} es reclutado por el factor transcripcional PhoB en la región promotora de los genes del regulón de σ^{VreI} que contiene una caja *pho* y secuencias −10 y −35 a las que se unen estas proteínas reguladoras promoviendo la expresión de esos genes.

3. La virulencia de *P. aeruginosa* sobre embriones de pez cebra y su citotoxicidad hacia células humanas epiteliales de pulmón A549 aumenta en condiciones de limitación de Pi. Los factores σ^{VreI} y VreR, pero no la proteína VreA, son necesarios para que se induzca la virulencia de *P. aeruginosa* en estas condiciones. σ^{VreI} está activo *in vivo* durante el proceso infeccioso lo que produce la expresión de varios factores de virulencia y el aumento en la patogenicidad de esta bacteria.

4. El factor anti-σ VreR modula la expresión de genes no sólo a través de la activación de σ^{VreI} sino también de forma independiente a este factor σ. VreR modula la expresión de varios reguladores transcripcionales.

5. *P. aeruginosa* contiene tres sistemas para la adquisición y detección de hemo, los sistemas Phu, Has y Hxu. Los sistemas Has y Hxu de *P. aeruginosa* son sistemas de señalización de la superficie celular (CSS) que responden a la presencia del complejo hemo-hemóforo y a hemo libre, respectivamente. En ambas cascadas de señalización participa un receptor de membrana externa (HasR y HxuA, respectivamente) que tras detectar hemo en el medio extracelular producen la activación de un factor σ^{ECF} (σ^{HasI} y σ^{HxuI}, respectivamente) en el citosol. El factor σ^{HasI} de *P. aeruginosa* induce la expresión del operon *hasR-hasAP* que codifica el receptor HasR y el hemóforo HasAp, mientras que σ^{HxuI} produce la expresión del receptor *hxuA*. *P. aeruginosa* es por tanto capaz de utilizar hemo como una molécula señal para inducir la expresión de genes respuesta.

6. La activación de σ^{HasI} y σ^{HxuI} requiere la proteólisis de los factores anti-σ a los que están asociados, HasS y HxuR, respectivamente. El primer paso de la proteólisis de estos factores anti-σ se produce en ausencia de hemo mediante una escisión espontánea en el dominio periplásmico de estas proteínas que las separa en un dominio N-terminal y un dominio C-terminal. En respuesta a hemo, el domino N-terminal es procesado en un proceso en el que intervienen las proteasas Prc y RseP. Esto produce la activación de los factores σ^{HasI} y σ^{HxuI} en el citosol y la expresión de los genes regulados por estos factores σ. HasS y HxuR funcionan sólo como factores anti-σ y no tienen actividad pro-σ.

7. El gen del factor σ *hxuI* de *P. aeruginosa* se co-transcribe con su factor anti-σ *hxuR* pero los genes *hasI* y *hxuI* no, al contrario de lo que ocurre con la mayoría de los pares σ^{ECF}/anti-σ de *P. aeruginosa* para mantener la estequiometria y evitar la presencia de unidades σ activas en ausencia de señal inductora. Sin embargo, σ^{HasI} está a cargo de la transcripción de su factor anti-σ *hasS* autorregulando de esta manera su propia actividad.

8. Las actividades proteolíticas de las proteasas RseP y Prc son necesarias para la activación de los sistemas CSS Fox y Fiu de *P. putida*.

9. La proteasa periplásmica carboxy-terminal CtpA, la subunidad proteica HflK y la proteasa citosólica PepA están involucradas directa o indirectamente en la activación del sistema CSS Fox de *P. aeruginosa*. La ausencia de CtpA reduce la cantidad del domino C-terminal del factor anti-σ FoxR que es el dominio que interacciona y protege al dominio N-terminal de FoxR de un proceso proteolítico posterior. Como consecuencia de una menor cantidad de C-FoxR se incrementa la proteólisis del dominio N-FoxR, lo que activa el factor σ^{FoxI} y produce una mayor expresión del gen *foxA*. La ausencia de PepA incrementa la estabilidad del factor σ^{FoxI}, lo que resulta en un aumento de la expresión de *foxA*. La ausencia de HflK reduce la actividad CSS y la expresión de *foxA* mediante un mecanismo aún desconocido.

10. La ausencia de las proteasas RseP y CtpA reduce la virulencia de *P. aeruginosa* sobre embriones del pez cebra y células humanas A549, mientras que la de Prc aumenta la virulencia de este patógeno. La falta de Prc eleva la producción de vesículas de la membrana externa de *P. aeruginosa* las cuales son utilizadas por el patógeno para introducir factores de virulencia directamente en el citoplasma de células del hospedador. Este aumento en la producción de vesículas de la membrana externa podría ser la causa de la mayor virulencia observada en ausencia de Prc.

General Methods

This section describes the materials and methods commonly used in this Thesis. However, each chapter has its own section describing the specific materials and methods used.

Bacterial strains and growth conditions. Wild-type bacterial strains used in this study are listed in Table 1. Bacteria were routinely grown in liquid LB (Sambrook et al., 1989) on a rotatory shaker at 37 or 30 °C and 200 rpm. For low iron conditions, cells were cultured in CAS medium [3.18 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 50 mM PIPES, pH 6.8, containing 1 mM K_2HPO_4, 1% (wt/vol) casaminoacids, and 1% (wt/vol) glycerol] (Llamas et al., 2006) supplemented with 400 µM of 2,2'-bipyridyl for *P. aeruginosa* or 200 µM for *P. putida*. CAS medium was complemented with 1 µM of ferrioxamine B (Sigma-Aldrich), 40 µM of ferrichrome (Santa Cruz Biotechnology), 20 µM (other amounts used are indicated) of heme (Sigma-Aldrich), or 100-200 µM of $FeCl_3$ (high iron medium). For low phosphate condition, cells were cultured in 0.3% (w/v) proteose peptone (DIFCO) containing 100 mM HEPES, 20 mM NH_4Cl, 20 mM KCl, 3.2mM $MgCl_2$, and 0.4% (w/v) glucose (pH 7.2), without (low Pi) or with 10 mM KH_2PO_4 (high Pi). When necessary, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to induce full expression from the pMMB67EH Ptac promoter. Antibiotics were used at the final concentrations (µg ml^{-1}): ampicillin (Ap), 100; gentamicin (Gm), 20; kanamycin (Km), 50; nalidixic acid (Nal), 10; piperacillin (Pip), 25; rifampicin (Rif) 10; streptomycin (Sm), 100; tetracycline (Tc), 20.

Table 1. Bacterial strains used in this Thesis.

Bacterial Strains	Characteristics	References
Escherichia coli		
BL21 (DE3)	F^- *lon ompT hsdS* (r_B^- m_B^-) *gal dcm* λ(DE3)	(Jeong et al., 2009)
CC118λpir	Δ(*ara-leu*) *araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1, lysogenized with λpir*; Rif^R	(Herrero et al., 1990)
DH5α	*supE44* Δ*(lacZYA-argF)U169* ϕ80 *lac*ZDM15 *hsdR17* (r_K^- m_K^+) *recA1 endA1 gyrA96 thi1 relA1*; Nal^R	(Hanahan, 1983)
Pseudomonas aeruginosa		
PAO1	Wild-type strain	(Jacobs et al., 2003)
Pseudomonas putida		
KT2440	*hsdR1*, wild-type strain; Rif^R	(Franklin et al., 1981)

Plasmids construction and molecular biology. PCR amplifications were performed using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) or Expand High Fidelity DNA polymerase (Roche). PCR amplifications and plasmids where digested and ligated using Roche and New England Biolabs enzymes. General vectors and primers used in this Thesis are listed in Table 2. All constructs were confirmed by DNA sequencing and transferred to *P. aeruginosa* and *P. putida* by electroporation (Choi et al., 2006). Electroporation was performed from miniprep of the plasmid of interest obtained by a miniprep extraction kit (Quiagen). Container strain was grown to late exponential phase and 1 ml was washed two times with sucrose 300 mM to remove salts. Approximately 100 µg of the plasmid was added to the cells and electroporated using a 2 mm gap cuvette and an electroporator (BioRad) as the manufacturer describes.

Table 2. Vectors and primers used in this Thesis.

Vectors		
pBBR1MCS-5	Broad-host range plasmid, *ori*TRK2; Gm^R	(Kovach et al., 1995b)
pCR2.1-TOPO	TA cloning vector for direct ligation of PCR products; Ap^R, Km^R	Invitrogen
pET28b(+)	Translation vector for cloning and expressing recombinant proteins in *E. coli*. Contains a 6xHis fusion tag; Km^R	Novagen
pKNG101	Gene replacement suicide vector, *ori*R6K, *ori*TRK2, *sacB*; Sm^R	(Kaniga et al., 1991)
pMMB67EH	IncQ broad-host range plasmid, *lacI*^{q}; Ap^R	(Furste et al., 1986)
pMP220	IncP broad-host-range *lacZ* fusion vector; Tc^R	(Spaink et al., 1987)
General primers	**5'→3'**	**Characteristics**
Hah-138	CGGGTGCAGTAATATCGCCCT	Binds to the IS*PhoA*/hah transposon
LacZ-148	GGGTAACGCCAGGGTTTTCC	Binds to IS*lacZ*/hah transposon
M13F	CAGGAAACAGCTATGACCATG	Binds upstream the multiple cloning site (mcs) of pCR2.1-TOPO
M13R	CGCCAGGGTTTTCCCAGTCACGAC	Binds downstream the mcs of pCR2.1-TOPO
MMB5´(3)	TGGTATGGCTGTGCAGGTCGTAAA	Binds upstream the mcs of pMMB67EH
MMB673'	TGTTTTATCAGACCGCTTC	Binds downstream the mcs of pMMB67EH
MCS220F3	TAACGCGGGCCTCCCAT	Binds upstream the mcs of pMP220
MCS220R	ATCAACGGTGGTATATCC	Binds downstream the mcs of pMP220
pBBR1-F	TTAGCTCACTCATTAGGCACCCC	Binds upstream the mcs of pBBR1MCS-5
pBBR1-R	GGTAACGCCAGGGTTTTCCC	Binds downstream the mcs of pBBR1MCS-5
pKNG5'	TCTACTGTACGATACACTTCCGC	Binds upstream the mcs of pKNG101
pKNG3'	CAGCGACACTGAATACGGGG	Binds downstream the mcs of pKNG101
T7	TAATACGACTCACTATAGGG	Binds upstream the mcs of pET28b(+)
T7T	GCTAGTTATTGCTCAGCGG	Binds downstream the mcs pET28b(+)

Gene deletion by pKNG101 suicide vector. *P. aeruginosa* null mutants were constructed by allelic exchange using the suicide vector pKNG101 (Kaniga et al., 1991) following the protocol described previously with some variations (Bastiaansen et al., 2014). Regions upstream and downstream the gene to be mutated were cloned in the pKNG101 vector plasmid and

transformed in *E. coli* CC118λpir. The *P. aeruginosa* recipient strain, the *E. coli* CC118λpir bearing a pKNG101-derived plasmid (donor strain), and the *E. coli* HB101 (pRK600) helper strain were grown overnight (o/n) in LB at 37 °C. Strains were washed twice with PBS (phosphate buffered saline). The helper and donor strain were mixed together and spotted on top of a 2 cm diameter with 0.2 µm of pore filter (Millipore) placed on solid LB plates, while the recipient strain was incubated at 42 °C to inhibit the function of the extracellular DNA degradation machinery. After 2 h of incubation, the recipient strain was spotted with the helper and donor strains and the mix incubated o/n at 37 °C. Transconjugants were selected in minimal M9 medium (Sambrook et al., 1989) with 0.3% (w/v) citrate as the sole carbon source and Sm 2000 µg/ml. Controls containing the single strains were also plated in the same medium. The first recombination event was confirmed by colony PCR. Selected transconjugants were streaked on LB agar plates without NaCl supplemented with 20-25% (w/v) sucrose. Plates were incubated at RT for 48-72 h. This allows the production of the second recombination step required for plasmid excision and obtainment of mutant colonies. PCR and/or southern-blot were performed for mutant confirmation (Llamas et al., 2000).

β-galactosidase activity assay. β-galactosidase activities in soluble cell extracts were determined as described before (Llamas et al., 2006). Briefly, bacteria were grown until late log phase and pelleted by centrifugation. Samples were resuspended in Z Buffer (60 mM NA_2HPO_4, 40 mM NAH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.27 % (v/v) β-mercaptoethanol). 100 µl sample were diluted in 500 µl of Z buffer and treated with 20 µl of chloroform and 20 µl SDS 0.05 % (w/v). Then, samples were vortexed and incubated at 30 °C for 10 min. Reaction was triggered with 100 µl of o-nitrophenyl-b-D-galactopyranoside (ONPG) 4 mg/ml (Sigma-Aldrich) and stopped with 260 µl of Na_2CO_3 1 M. Reactions were measured at 550 and 420 nm and cell density at 660 nm using a Shimadzu UV-266 spectrophotometer. Activity was expressed in Miller units (Miller, 1972). Each condition was tested in duplicate in at least three biologically independent experiments and the data given are the average with error bars representing standard deviation (SD).

SDS-PAGE and Western-Blot. Bacteria were grown until late log phase and pelleted by centrifugation. Samples were normalized according to the OD_{660} of the culture, solubilized in Laemmli buffer (Laemmli, 1970) and heated for 10 min at 95 °C. Proteins were separated by SDS-PAGE containing 8, 12 or 15% (w/v) acrylamide and electrotransferred to nitrocellulose membranes. Ponceau S staining was performed as a loading control. Immunodetection was performed using polyclonal antibodies directed against a particular protein or a monoclonal antibody directed against the influenza hemagglutinin epitope (HA.11, Covance). The second antibody, either the horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) or the horseradish peroxidase-conjugated rabbit anti-mouse (DAKO), was detected using the SuperSignal® West Femto Chemiluminescent Substrate (Thermo Scientific). Blots were scanned and analyzed using the Quantity One version 4.6.7 (Bio-Rad).

Zebrafish maintenance, embryo care and infection procedure. Transparent adult casper mutant zebrafish (*mitfa*$^{w2/w2}$;*roy*$^{a9/a9}$) and adult labelled *Tg(mpx:GFP)*^{i114} casper zebrafish producing green neutrophils (Renshaw et al., 2006; White et al., 2008) were conserved at 26 °C in aerated 5 L tanks with a 10/14 h dark/light cycle. Zebrafish embryos were collected during the first hour post-fertilization (hpf) and kept at 28 °C in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl{\cdot}2H_2O$, 0.33 mM $MgCl_2{\cdot}7H_2O$) supplemented with 0.3 mg/L methylene blue. Prior to infection, 1 or 2 days post-fertilization embryos were mechanically dechorionated and

anaesthetized in 0.02 % (w/v) buffered 3-aminobenzoic acid methyl ester (pH 7.0) (Tricaine, Sigma-Aldrich). Prior to infection, 1 or 2 days post-fertilization (dpf) embryos were mechanically dechorionated and anaesthetized in 0.02 % (*w/v*) buffered 3-aminobenzoic acid methyl ester (pH 7.0) (Tricaine, Sigma-Aldrich). Zebrafish embryos were individually infected by microinjection with 1 nl of *P. aeruginosa* either in the hindbrain ventricle (localized infection) or in the caudal vein (systemic infection) as described elsewhere (Llamas and van der Sar, 2014). All procedures involving zebrafish embryos were according to local animal welfare regulations.

Human epithelial lung cell maintenance. A549 cell line (ATCC® CCL-185™) was maintained in DMEM medium supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco) in a 5% CO_2 incubator at 37 °C. One day prior infection, the A549 cells were placed in well plates at the indicated concentrations and cultured in phosphate-free DMEM medium (Gibco) with 5 % (v/v) FBS.

RNA isolation. Total RNA was extracted by the hot phenol method using the TRI® Reagent protocol (Ambion) as described elsewhere (Llamas et al., 2008) and subjected to two DNase I treatments with the Turbo DNA-free kit (Ambion) and RNaseOUT (Invitrogen). RNA was isolated by phenol-chloroform extraction and precipitation with 2.5 × volume 100% (v/v) ethanol and 0.1 × volume 3 M sodium acetate pH 4.8. RNA quality, including purity, integrity and yield, was assessed by electrophoresis, in a 2% (w/v) agarose gel, of 1 µg of total RNA and by UV absorption at 260 nm in a ND-1000 spectrophotometer (NanoDrop Technologies, USA).

Quantitative RT-PCR analyses. cDNA from at least three biologically independent replicates was produced in triplicate by reverse transcription reactions of 0.5-1 µg RNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers as primers according to the protocol supplied. Real-time PCR amplification were carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 optical system software (version 2.1.97.1001) in 11.5 µl reaction mixture containing 6.25 µl of iQ SYBR green Supermix (Bio-Rad), 0.4 µM of each primer and 1 µl of the template cDNA (diluted 1000-fold when measuring the 16S rRNA reference gene). Thermal cycling conditions are indicated in each chapter. A single fluorescence measurement per cycle was performed according to the manufacturers' recommendations. Melting curve analyses were performed by gradually heating the PCR mixture from 55 to 95 °C at a rate of 0.5 °C per 10 s for 80 cycles. The relative expression of the genes was normalized to that of 16S rRNA and the results were analyzed by means of the comparative cycle threshold (ΔΔct) method (Pfaffl, 2001).

Computer-Assisted Analyses. BLASTn and DIAMOND BLASTp analyses of the *Pseudomonas* genomes were performed at http://www.pseudomonas.com (Winsor et al., 2011). Other BLASTp analyses were performed at the NCBI website (Boratyn et al., 2013) and sequence alignments with ClustalW (Thompson et al., 1994). Statistical analyses are based on t-test in which two conditions were compared independently. P-values from raw data (were calculated by independent two-tailed t-test and from ratio data to the control by one-sample t- test. Statistics for zebrafish survival curves were analyzed by Log-Rank (Mantel-Cox). The analysis were performed using GRAPHPAD PRISM version 5.01 for Windows and are represented in the graphs by *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

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