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Papel de las proteínas reguladoras de la familia Rsm en la señalización mediada por diguanilato cíclico y el modo de vida multicelular de *Pseudomonas putida* KT2440

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Papel de las proteínas reguladoras de la familia Rsm en la señalización mediada por diguanilato cíclico y el modo de vida multicelular de *Pseudomonas putida* KT2440

Memoria que presenta el Licenciado en Bioquímica

Oscar Huertas Rosales

para aspirar al Título de Doctor

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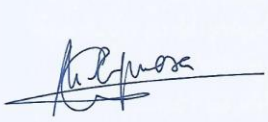

Fdo.: Manuel Espinosa Urgel

Fdo.: María Isabel Ramos González

Doctorando

Fdo.: Oscar Huertas Rosales

Al contemplar la Naturaleza
No perdáis nunca de vista
ni el conjunto ni el detalle
que en su vastedad magnífica
nada está dentro ni fuera;
y por rara maravilla
anverso y reverso son
en ella una cosa misma.
De este modo, ciertamente,
aprenderéis en seguida
este sagrado secreto
que miles de voces publican.

Johann Wolfgang von Goethe – "Epirrema"

No reconocer como verdadero sino lo evidente, dividir cada dificultad en cuantas porciones sea preciso para mejor atacarlas, comenzar el análisis por el examen de los objetos más simples y más fáciles de ser comprendidos, para remontarse gradualmente al conocimiento de los más complejos…

René Descartes - "Discurso del método"

La ciencia, sin divulgación, es la mitad de la ciencia.

Parafraseando a ***Luís Buñuel*** *(La realidad, sin imaginación, es la mitad de la realidad)*

A mi madre

A mi padre y mi hermano

Al amor de mi vida, Elena

A mi hijo Mario

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pero en gran medida por los aspectos personales. Solo cuando se tiene miedo, se puede ser valiente. Por otro lado, ha sido quizá la época más interesante e intensa de mí vida hasta ahora y con estas palabras quiero volver la cabeza atrás para comprobar que sin todos vosotros no habría sido posible.

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Vamos con las casas y escudos:

Casa Proteína. En su escudo figura un inyector de ITC sobre una estructura tridimensional de un receptor de membrana. Sus habitantes han sido una ayuda constante durante este periodo. Gracias Cristina, David, Álvaro y Miguel. Pero sobre todo gracias a Andrés, Dr. Andrés a estas alturas, el mexicano menos mexicano que he conocido nunca. Compañero de fatigas siempre dispuesto a ayudarme cuando he tenido cualquier duda sobre proteínas. Espero haber correspondido en la ayuda y te deseo la mayor de las suertes. Y mi pequeña Gnoma Miriam, Dra. Miriam. Gracias, gracias y mil veces gracias. No recuerdo una sola vez en

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Casa Sigma. En el blasón figura un western-blot con dos pipetas cruzadas. Es sin duda el laboratorio más internacional. Dr. Josemi, siempre trabajando, siempre dispuesto. Gracias por ayudarme en tantas y tantas cosas. Todas las rt-PCR de la tesis te las dedico a ti. Gracias Joaco, Cristina, Alicia, Carlijn y Kity. Lo he pasado genial con vosotros.

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Casa Anaerobia. Su escudo tiene una mancha de chapapote sobre la que hay bacterias "regordotas" zampando. La casa más fértil de todas, sin duda. Gracias Patri por todas las veces que amablemente me has ayudado y asesorado, eres un sol. Pacheco, ole tu gracia, ole tus formas, ole tu acento y ole tú, que eres un tío grande. La vida nos da pataillas, pero la vamos saltando y regateando de buena manera. Sophie, el solete italiano que ha iluminado los días más oscuros. Gracias por estar ahí siempre guapa. Suerte con todo, que seguro que a partir de ahora todo va mejor. Felicidades por ese doctorado que ya se acaba y por la nueva maternidad.

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A partir de ahora… LANIAKEA

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List of Abbreviations

aa	Amino acid
AHL	N-Acilhomoserine lactone
Alg	Alginate
Ap	Ampicillin
Bcs	Cellulose

bp	Base pair
cDNA	Complementay Deoxyribonucleic Acid
c-di-GMP	Bis-(3´-5´)-cyclic dimeric guanosine monophosphate or 3´,5´-Cyclic diguanylic acid
Cm	Chloramphenicol
CSLM	Confocal scanning laser microscopy
Csr	Carbon Storage Regulator
DGC	Diguanilate cyclase
DNA	Deoxyribonucleic acid
EPS	Exopolysacharides
E. coli	*Escherichia coli*
g	*Grams*
gac	Global Activator of secondary metabolism
GGDEF	Motif Gly-Gly-Asp-Glu-Phe
GFP	Green Fluorescent Protein
h	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
Km	Kanamycin
LB	Luria-Bertani media
M	Molar
MCS	Multicloning Site
mg	Milligram

min	Minute
ml	Milliliter
mm	Millimeter
mM	miliMolar
Mob+	A vector containing MOB genes, also called Dtr for DNA-transfer replication
mRNA	Messenger Ribonucleic Acid
nm	nanometer
O.D.	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase Chain Reaction
P. fluorescens	*Pseudomonas fluorescens*
pOHR Rosales	Plasmid constructed by Oscar Huertas
P. putida	*Pseudomonas putida*
QS	Quorum Sensing
RBS	Ribosome binding site
Rif	Rifampicin
RNA	Ribonucleic Acid
rpm	Revolutions per minute
Rsm	Repressor of Secondary Metabolism
RT-PCR reaction	Reverse transcription polymerase chain
s	Seconds
Sm	Streptomycin

sRNA	small Ribonucleic Acid
Tc	Tetracycline
TCS	Two-component system
WT	Wild Type
µl	Microliter
ΔX	Mutant in X gen
ºC	Celsius Degrees

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RESULTS

Chapter 1. Self-Regulation and Interplay of Rsm Family Proteins Modulate the Lifestyles of *Pseudomonas putida*

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Resumen

Los organismos procariotas son la forma de vida autónoma más exitosa que existe en la tierra tanto por número de individuos como por la diversidad de especies. Esto es una consecuencia de la capacidad de

adaptación de estos microorganismos a condiciones cambiantes, que está relacionada con una gran versatilidad metabólica y la posibilidad de adoptar diferentes estilos de vida.

En el medio ambiente, las bacterias se encuentran predominantemente formando comunidades multicelulares adheridas a las superficies más variopintas y formando estructuras de diverso tipo como capas, esferas, flóculos, lodos o las denominadas biopelículas o biofilms (Wingender *et al.*, 1999). Las biopelículas son probablemente la forma de vida más antigua en la tierra, como revela el registro fósil (Hall-Stoodley *et al.*, 2004). Estas comunidades son habitualmente multiespecíficas, aunque alguna especie pueda ser la mayoritaria. Sin embargo, para su estudio en el laboratorio se suele simplificar el sistema a modelos monoespecíficos. De esta forma se ha conseguido profundizar en el conocimiento y los mecanismos moleculares que gobiernan la dinámica de las biopelículas.

Los biofilms están constituidos por conjuntos tridimensionales de células bacterianas ancladas a superficies bióticas o abióticas rodeados por una matriz compleja producida por las propias bacterias. Esta matriz extracelular se compone de exopolisacáridos, proteínas, lípidos y ADN extracelular (Hall-Stoodley *et al.*, 2004). En algunas de estas estructuras, las bacterias pueden llegar a representar solo el 10% del peso seco, mientras que la matriz constituiría el 90% restante. Estas estructuras complejas, robustas y a la vez flexibles proporcionan protección a las bacterias que lo forman frente a condiciones adversas como la exposición a luz ultravioleta, la desecación, el exceso de salinidad (Flemming, 1993), la toxicidad por metales (Teitzael y Parsek, 2003), así como los depredadores y la respuesta inmune del hospedador o el estrés oxidativo, éstos últimos cuando los biofilms se forman dentro o sobre otros organismos (Costerton *et al.*, 1999; Flemming y Wingender, 2010; Römling y Balsalobre, 2012).

La formación de una biopelícula es un proceso que se regula de una forma muy fina y que responde a señales medioambientales. La red de regulación que conecta el estímulo con los cambios que se producen en la expresión génica es amplia, compleja y aunque no se conoce en su totalidad tiene lugar a distintos niveles (Colley *et al.,* 2016; Valentini and Filloux, 2016). Esta red regulatoria ayuda a coordinar la síntesis y degradación de ciertos componentes bacterianos y es además necesaria para adaptar el metabolismo de la célula a la situación compleja que representa vivir en biopelículas (Fazli *et al.*, 2014; Mhatre *et al.*, 2014).

Existen diferentes niveles de regulación relacionados con la dinámica de formación y dispersión de las biopelículas. Señales ambientales como la presencia o ausencia de nutrientes y la biodisponibilidad de hierro estimulan el anclaje de las células a una superficie (O'Toole and Kolter, 1998; Yu *et al.,* 2016). Aunque no se conoce completamente la naturaleza de estas señales y puede además variar de unas estirpes bacterianas a otras, lo que parece claro es la implicación de un nivel de regulación representado por los sistemas de dos componentes en la regulación de la formación del biofilm. En un sistema de dos componentes típicamente interviene un sensor con actividad histidina quinasa y un regulador de respuesta que se une a DNA modulando la transcripción de un conjunto de genes. Estos sistemas constituyen los mecanismos mejor estudiados que tienen las bacterias para detectar cambios externos (ej. en cuanto a la presencia de nutrientes, pH, temperatura, osmolaridad o tóxicos). Un sistema de dos componentes relevante en este trabajo de Tesis es el sistema GacS/GacA. Sin embargo hay reguladores de respuesta que en lugar de funcionar como reguladores transcripcionales presentan dominios con actividad enzimática como es el caso de los dominios GGDEF y EAL/HD-GYP responsables de la síntesis y degradación, respectivamente, del segundo mensajero c-di-GMP. Este segundo mensajero presente en eubacterias,

auque no en arqueas, tiene un papel crucial en la adopción de un estilo de vida sésil frente a planctónico.

GacS se activa (autofosforila) tras el reconocimiento por una señal exógena desconocida y transforsforila al regulador de respuesta GacA. A pesar del efecto global que ejerce GacS/GacA, el número de dianas a las que se une directamente GacA parece ser muy limitado, reduciéndose casi exclusivamente a los RNA pequeños (sRNAs) rsmX, rsmY y rsmZ (Kay *et al.*, 2005).

Otro agente de regulación lo constituyen las señales bióticas producidas por la población bacteriana. En la evolución de una microcolonia hacia una biopelícula madura participan las señales de quorum sensing (QS) (Zhang and Dong, 2004), que permiten sensar la densidad de población y ajustar así la expresión de un conjunto de genes de forma coordinada en la población.

Esta Tesis ha versado sobre el estudio de la formación y regulación de biopelículas bacterianas usando como modelo *Pseudomonas putida* KT2440. Esta bacteria coloniza de forma eficiente la rizosfera de plantas de interés agrícola, como el maíz, y de interés básico como Arabidopsis. Esta cepa puede promover el crecimiento vegetal y también activa la resistencia sistémica inducida frente a patógenos (Matilla *et al.*, 2010). Con ella se han llevado a cabo estudios transcriptómicos que resultaron pioneros para identificar genes de expresión preferencial en rizosfera (Matilla *et al.,* 2007). Uno de estos genes, *rup4959*, codifica un regulador de respuesta con actividad diguanilado ciclasa, el único con dominios GGDEF y EAL presente en KT2440 (Matilla *et al.*, 2011), cuya regulación ha constituido uno de los objetos de estudio en esta Tesis Doctoral. Durante el curso de la misma, el gen se ha redenominado como *cfcR*, siendo ésta la nomenclatura usada en este trabajo.

En el capítulo 1, "**Self-Regulation and Interplay of Rsm Family Proteins Modulate the Lifestyle of *Pseudomonas* putida**", se describe el papel regulador de las tres proteínas de la familia

de los reguladores post-transcripcionales de la familia CsrA/RsmA presentes en *P. putida* KT2440. A través de la construcción de mutantes simples, dobles y triple, así como la sobreexpresión de los genes por separado y bajo la influencia de un promotor constitutivo, se ha investigado el papel regulador de estas proteínas (RsmA, RsmE y RsmI) en el estilo de vida de la bacteria. Se ha podido observar que un mutante triple presenta menor movilidad tanto de tipo "swimming" como de tipo "swarming" además de una cinética alterada de formación de biopelículas, observándose una más temprana y mayor biomasa adherida a superficies y una dispersión también más temprana del biofilm. Por el contrario, la sobreexpresión tanto de RsmE como de RsmI provocó una disminución en la capacidad de adhesión. El análisis de la expresión de los diferentes exopolisacáridos y adhesinas en los fondos mutantes reveló que estas alteraciones bien podrían deberse a una alteración en la composición de la matrix extracelular así como en los tiempos en que tenía lugar su síntesis. Por último, el estudio de la expresión de las propias proteínas Rsm nos indicó la existencia de mecanismos de autoregulación. Las numerosas herramientas generadas en este trabajo (mutantes, construcciones para sobreexpresión y fusiones reporteras) nos permitirá seguir profundizando en la disección de la regulación post-transcripcional en *P. putida* y otras bacterias relacionadas.

En el capítulo 2, "**The *Pseudomonas putida* CsrA/RsmA homologues negatively affect c-di-GMP pools and biofilm formation through the GGDEF/EAL response regulator CfcR",** se profundiza en el estudio de la regulación de la expresión de *cfcR*. Previo a este trabajo de Tesis se conocía que la expresión de este gen está regulado a nivel transcripcional por RpoS y ANR (Matilla *et al.*, 2011) y FleQ (Ramos-González *et al.,* 2016) y que además su funcionalidad como diguanilato ciclasa require del multisensor híbrido con actividad histidina kinasa CfcA (Ramos-González et al., 2016). En este capítulo se describe un nivel adicional de regulación sobre *cfcR* que opera a nivel post-

transcipcional a través de las proteínas RsmA, RsmE y RsmI. Se ha demostrado la unión directa de estas proteínas a un motivo conservado (5´CANGGANG3´) que solapa con el codón de inicio de la traducción de *cfcR*. La mutación de las proteínas Rsm causa una desrepresión de *cfcR* que va acompañada de un adelanto e incremento en los de niveles de c-di-GMP que no tiene lugar cuando se deleciona este gen, pudiéndose así establecer que el efecto de la cascada Gac/Rsm sobre el biofilm tiene lugar a través de CfcR en *P. putida*.

En el tercer capítulo "**Global analysis of the Rsm regulon in *Pseudomonas putida* KT2440"**, se ha realizado un estudio RIP-seq para determinar asociaciones RNA/proteínas Rsm en *P. putida*. Hasta 168 dianas para RsmI, 311 para RsmE y 102 para RsmA se han detectado como zonas de unión de estas proteínas al RNA, implicando a más de 400 genes, algunas regiones intergénicas y posibles sRNA, entre ellos los responsables de modular a las propias proteínas Rsm (rsmY y rsmZ). Se han identificado dianas expecíficas para cada una de las proteínas Rsm y otras que son compartidas por dos de ellas (95) o las tres (38). Hemos comprobado que tanto *cfcR* como elementos implicados en su regulación aparecen entre las dianas, como era esperable a partir de los resultaos del capítulo 2. También aparecen otros genes relacionados con la formación de biofilms cuya regulación se puede inferir a partir de los resultados de los otros dos capítulos (otras proteínas con dominios GGDEF/EAL, la adhesina LapA, y uno de los clúster de exopolisacáridos). Aunque el análisis de dianas realizado es preliminar, debido a las limitaciones temporales propias de una tesis, este trabajo ha permitido generar un largo listado de genes y nuevos sRNA cuyo estudio nos permitirá completar el mapa de la regulación asociada a proteínas Rsm en *P. putida* KT2440.

Esta Tesis Doctoral aporta información clave del proceso de formación de biopelículas por parte de *P. putida* KT2440 que ha permitido la propuesta de un modelo más completo acerca de la

regulación de la formación de biopelículas. Se demuestra por otro lado la conexión en *P. putida* entre el sistema Gac/Rsm y la formación de biopelículas a través de la regulación por diguanilato cíclico (una conexión que en *P. aeruginosa* se realiza a través de la proteína SadC, sin homólogos en *P. putida*). Además, abre el camino para analizar en detalle distintos aspectos de la red global de regulación asociada a las proteínas Rsm.

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I. GENERAL INTRODUCTION

1. Biofilm formation: a bacterial colonization and adaptation strategy

Prokaryotes are the most successful life form on earth, both from the point of view of number of individuals and considering the diversity of species. This is a consequence of their ability to colonize very diverse and changing environments, even in extreme conditions. The majority of prokaryotes do not live as pure cultures of dispersed single cells but form polymicrobial aggregates such as mats, flocs, sludge or biofilms (Wingender *et al.*, 1999). Biofilms are probably the most ancient multicellular life forms on earth, as evidenced by fossil records (Hall-Stoodley *et al.*, 2004). Biofilms are convoluted three-dimensional bacterial cell assemblages anchored to biotic or abiotic solid surfaces and surrounded by a complex self-produced matrix consisting on exopolysaccharides (EPS), proteins, lipids and extracellular DNA (Flemming and Wingender, 2010). In biofilms, the microorganisms often account for less than 10% of the dry mass, while the matrix in which the cells are embedded can account for over 90%. The structural components of the biofilm matrix give rise to a robust structure with high tensile strength that aids in the protection of enclosed bacterial populations from adverse conditions. These can include environmental hazards such as UV exposure, desiccation, salinity (Flemming, 1993) and metal toxicity (Teitzel and Parsek, 2003), predation, or host responses like oxidative stress and immune defense mechanisms (Costerton *et al.*, 1999; Flemming and Wingender, 2010; Römling and Balsalobre, 2012).

2. The developmental cycle of a biofilm

Five stages have been proposed to take place during the formation of biofilms (Figure 1). Initially, free-swimming bacteria move to reach a surface and establish first contact, which is considered to be stochastic and usually driven by flagellar motility and influenced by hydrodynamic forces (Beloin *et al.*, 2008; Petrova and Sauer, 2012). A weak and transient initial attachment to the surface ① is mediated by several components of the bacterial envelope including flagella and pili (Karatan

and Watnick, 2009) that allow bacteria to overcome repulsive hydrodynamic forces and scan the surface properties (Stecher *et al.*, 2004; Jin *et al.*, 2011). Environmental conditions and the ability of bacteria to make firm contact with the surface determine whether cells will detach from the surface or remain and initiate the transition from reversible to permanent attachment ② (Belin *et al.*, 2008). Once bacteria are firmly attached to the surface, they show an aggregative behavior resulting from cell-to-cell interactions that have not been fully characterized yet but possibly throw the participation of LapF proteins (in pink in Fig. 1) (Martínez-Gil *et al.*, 2010). Metabolic changes along with cell division are also associated to the formation of a microcolony ③. Bacteria forming microcolonies then produce the extracellular matrix containing EPSs, proteins and nucleic acids characteristic of mature biofilms ④. The microenvironments within a biofilm give rise to subpopulations of bacteria with different gene expression in response to the local nutritional status and oxygen availability (Domka *et al.,* 2007). Overgrowth of the biofilm and some of these environmental changes eventually lead to the release of some bacterial cells from the biofilm, either passively through shearing forces or detaching actively from the biofilm ⑤. These planktonic cells can then colonize new niches (Lasa, 2006; Harmsen *et al.*, 2010; Sauer *et al.,* 2004; McDougald *et al.,* 2011).

The structural elements required for each stage have been characterized in detail in several microorganisms. In different bacteria, surface proteins mediate the transition from reversible to permanent attachment. In *Pseudomonas fluorescens* and *Pseudomonas putida*, for example, the large adhesin LapA mediates cell-to-surface interactions and is essential for irreversible attachment (Hinsa *et al.*, 2003; Hinsa and O´Toole, 2006; Ivanov *et al.,* 2012; Martínez-Gil *et al.,* 2010). Similar adhesins have also been described in *Salmonella*, *Staphylococcus* and *Enterococcus* (Toledo-Arana *et al.*, 2001; Cucarella *et al.*, 2001; Latasa *et al.,* 2005). In other bacterial species, EPS are required in early stages.

Studies on the initial steps of biofilm formation at a single cell level in *Pseudomonas aeruginosa* have revealed that in this bacterium, the species-specific polysaccharide Psl is required for irreversible attachment (Römling *et al.*, 2013). The bacterial cell leaves a trail of extracellular polysaccharide as it moves along during surface scanning using type IV pili for reversible attachment. The cells have more affinity to Psl trails and deposit additional Psl, thus conditioning a particular area where bacteria preferentially adhere to start microcolony formation.

Cell-to-cell interactions leading to microcolony formation require appendages like curli or type IV pili, EPS, and/or extracellular proteins. In *P. putida*, a second large extracellular protein, LapF, has been shown to be implicated in microcolony development (Martínez-Gil *et al.*, 2010). Finally, EPS production provides the structural framework for the bacterial community in mature biofilms. EPSs are also believed to interact with other matrix components. In *P. aeruginosa*, the extracellular polysaccharides Psl, Pel and alginate, the large secreted adhesin CdrA, and extracellular DNA constitute the structural scaffold in mature biofilms (Friedman and Kolter, 2004; Branda *et al.*, 2005). In addition, EPSs are able to absorb organic compounds allowing the recruitment of nutrients within the biofilm (Wolfaardt *et al.*, 1998) and they may also act as a physical barrier against antimicrobial compounds (Nichols *et al.,* 1988). In the case of *Salmonella typhimurium* and *Escherichia coli*, the EPS cellulose is a crucial component of the extracellular matrix (Zogaj *et al.*, 2001; Solano *et al.*, 2002). The ability of different bacteria to produce extracellular cellulose has been recognized for a long time (Ross *et al.*, 1991), and was already recorded in the mid-1940's as the main component of pellicles formed by *Gluconacetobacter xylinus* (formerly *Acetobacter xylinus*) at the air-liquid interface when growing under static conditions (Hestrin *et al.*, 1947; Schramm and Hestrin, 1954). Studies of bacterial multicellular behaviors over the past decade have shown that many other species are capable of producing

cellulose during biofilm development. However, many species can produce more than one EPS type, each with different roles depending on the environmental conditions and colonized surface.

3. Regulation of biofilm formation

Biofilm formation is a highly regulated process which responds to different environmental signals. The regulatory network that connects these stimuli with changes in gene expression is large and complex and not totally understood. This regulatory network allows the coordinated synthesis of the different components that are needed in each step, and the adjustment of bacterial metabolism to the biofilm lifestyle.

There are several levels of regulation involved in biofilm development. Environmental signals such as nutrient or iron availability stimulate bacterial attachment. Although the nature of these signals is not completely known and may vary from one species to another, several two-component signaling systems (TCSs) have been described to participate in biofilm formation and regulate the transcriptional profile of the community These TCSs, which consist of a sensor histidine kinase and a response regulator, are the predominant signaling mechanism in bacteria and allow them to sense a wide variety of external conditions including nutrients, temperature, pH, osmolarity and toxic substances. TCSs have been studied in *P. aeruginosa* biofilms (Petrova and Sauer, 2009), where several TCSs have been shown to control different stages of the biofilm development process (Figure 1). The most relevant for the objectives of this Thesis is the GacS/GacA system, which will be discussed in following sections.

A second level of regulation can come from signals produced by the bacterial populations. Intracellular communication via quorum sensing (QS) has been described to participate in the development from microcolonies to mature biofilm (Zhang and Dong, 2004). QS is based on the production of small diffusible molecules that bacteria release into

their environment, the extracellular accumulation of which is directly proportional to the population density of the producing organisms. These signaling molecules can be detected by the bacterial cells, in this way individual cells can sense the local density of bacteria and this allows the population as a whole to initiate a concerted action once a minimal threshold stimulatory concentration ("quorum") has been reached.

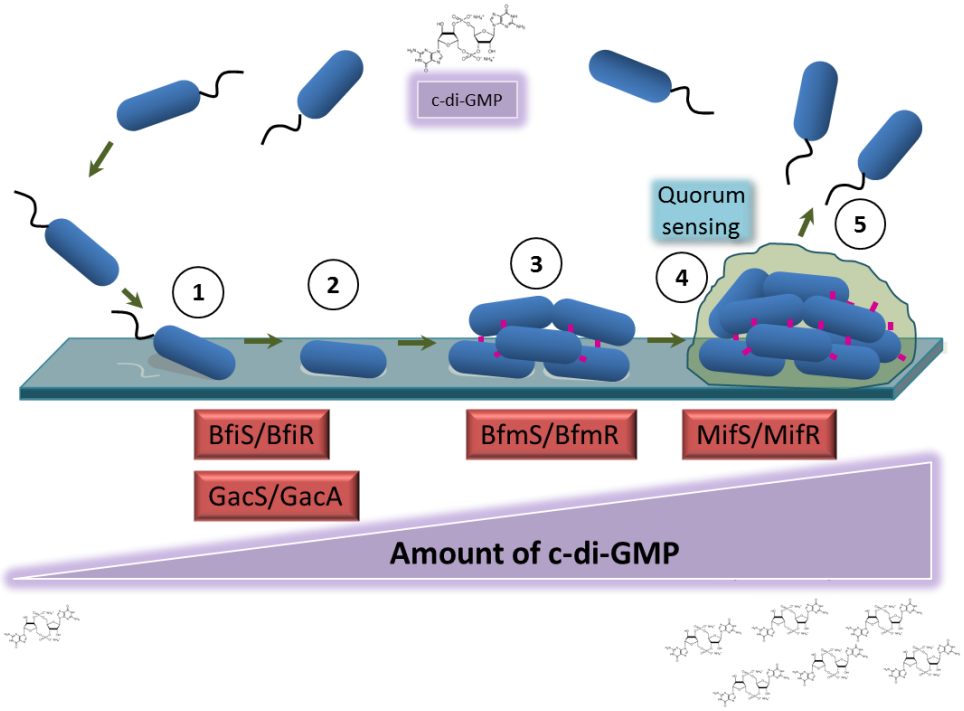

Figure 1. Scheme of biofilm formation process on abiotic surface. The stages are detailed in the text. Elements involved in biofilm life cycle regulation including two component regulatory systems of *P. aeruginosa* are indicated in red box. Adapted from (Lasa, 2006; Römling and Amikam, 2006; Petrova and Sauer, 2009; Mikkelsen *et al*., 2011; Martínez-Gil *et al*., 2014). BfiSR (biofilm initiation), BfmSR (biofilm maturation) and MifSR (microcolony formation).

It is therefore not surprising that QS is important in such a densely populated environment as a biofilm. Additional control levels include transcriptional and post-transcriptional regulators, connected by a key molecule acting as intracellular second messenger: bis-(3´-5´)-cyclic dimeric guanosine monophosphate (c-di-GMP).

4. Bis-(3´-5´)-cyclic dimeric guanosine monophosphate (c-di-GMP) signaling

Cyclic nucleotides, such as cAMP and cGMP, are recognized as important signaling molecules in both prokaryotes and eukaryotes, with diverse functions as secondary messengers. For example, cAMP is known to relieve glucose catabolite repression in bacteria by complex mechanisms that include also other global regulators (Botsford and Harman, 1992; Notley-McRobb *et al.*, 1997). Whereas prokaryotes do not seem to take advantage of cGMP as a signaling molecule, bacterial pathogens can interfere with the cGMP signaling of their eukaryotic host cells (Uzzau and Fasano, 2000).

Bacteria make extensive use of another cyclic guanosine compound, cyclic dimeric-guanosine-monophosphate (c-di-GMP). C-di-GMP was first described as an allosteric activator absolutely required for the functionality of the cellulose synthase BcsA. C-di-GMP is widely found in bacteria but not in archaea (Galperin, 2004) and has been established as a universal bacterial second messenger (Paul *et al.*, 2004; Simm *et al.*, 2004). The cellular levels of c-di-GMP are regulated by the opposing activities of GGDEF and EAL/HD-GYP domain proteins, named after conserved amino acid motifs observed in proteins involved in the biosynthesis and degradation of the second messenger, respectively.

4.1 C-di-GMP turnover

C-di-GMP is synthesized from two guanosine triphosphate (GTP) molecules by the diguanylate cyclase (DGC) activity encoded in GGDEF domains with the release of two inorganic phosphorus molecules. GGDEF domain proteins function as homodimers, where each monomer contributes one bound GTP molecule to form a phosphodiester bond. Two divalent metal ions (Mg^{+2} or Mn^{+2}) are required for this bond

formation between the GTP´s molecules to form pppGpG (Ross *et al.*, 1987; Wassmann *et al.*, 2007).

Five amino acids upstream the GGDEF motif, the "I site" (Inhibitory site) is located. It has an allosteric inhibitory function when c-di-GMP or structural homologues bind. The first crystal structure of a GGDEF domain, corresponding to the PleD protein, revealed that c-di-GMP can bind to this "I site", defined by an RxxD motif (Paul *et al.*, 2004; Christen *et al.*, 2006).

The degradation of c-di-GMP into pG3´p5´G or two GMP molecules is mediated by the phosphodiesterase activity (PDE) encoded in EAL/HD-GYP domains respectively (Ross *et al.*, 1987; Bobrov *et al.*, 2005; Ryan *et al.*, 2006). The majority of EAL domain proteins characterized so far exist as dimers (Tarutina *et al.*, 2006; Rao *et al.,* 2008). Some studies have indicated the existence of at least two classes of EAL domains, one with c-di-GMP-specific PDE activity and a second class that seems to lack this catalytic activity. The first class includes, for example, the *E. coli* EAL domain proteins YahA and DOS (Schmidt *et al.*, 2005), YhjH from *S. enterica* serovar *Typhimurium* and VieA from *V. cholera* (Simm *et al.*, 2004; Tischler and Camilli, 2004). On the other hand, proteins that contain GGDEF/EAL domains but only show DGC activity have been described to participate in cellulose biosynthesis in the fruit-degrading bacterium *G. xylinus* (Tal *et al.* 1998) or c-di-GMP-dependent biofilm formation in *S. typhimurium* (García *et al.*, 2004). In *P. putida*, CfcR (previously Rup4959) (only shows DGC activity, despite the presence of a predicted EAL domain (Matilla *et al.*, 2011).

Rao and colleagues have categorized the EAL domains in three classes, correlating sequence conservation with the proposed enzymatic activity. EAL domains belonging to class 1 possess conserved catalytic residues and a conserved loop 6. Class 1 EALs function as a PDEs (Schmidt *et al.*, 2005; Christen *et al.,* 2005; Tamayo *et al.,* 2005; Weber *et al.*, 2006; Simm *et al.*, 2007; Rao *et al.,* 2008). Class 2 EAL domains

contain conserved catalytic residues and a degenerated loop 6 (e.g. YkuI and DGC2). These EAL domains are most likely catalytically inactive, but the potential to be activated by terminal signaling domains cannot be excluded. Class 3 EAL domains lack one or more of the catalytic residues, have a degenerated loop 6, and are predicted to be catalytically inactive. The proteins CsrD and YcgF from *E. coli* and YdiC from *Salmonella Typhimurium* harbor EAL domains which belong to this class (Suzuki *et al.*, 2006; Newell *et al.,* 2009; Simm *et al.*, 2009; Tschowri *et al.*, 2009). The case of CfcR from *P. putida* seems to be different, having the domain but not certains residues in the domain. It´s not clear if the EAL domain of CfcR is active or not in certain conditions (Matilla *et al.*, 2011)

Proteins containing GGDEF/EAL domains are broadly distributed among bacterial species. Also, the genomes of some plants and lower eukaryotes such as sea anemones, hydra and *Dictyostelium* appear to encode GGDEF/EAL domain proteins (Römling, 2012; Römling *et al.*, 2013). Actually, it has been shown that the social amoeba *Dictyostelium* synthesizes c-di-GMP (Chen and Schaap, 2012). The number of these domain proteins encoded in a single genome varies from none to several dozen (Galperin, 2004). The apparent redundancy that could be inferred from the abundance of genes coding proteins involved in c-di-GMP metabolism in one single bacterial genome has led to the notion of a complex signaling regulation of c-di-GMP by spatial patterns and temporal activity of such proteins (Römling, 2005; Ryjenkov *et al.,* 2006).

4.2 C-di-GMP receptors

The binding of c-di-GMP to protein targets alters the structure of these effectors and their functionality (Hengge, 2009). C-di-GMP binding proteins identified so far include:

- Proteins containing PilZ domains (Amikam and Galperin, 2006), such as BcsA, the cellulose synthase in *G. xylinus, E. coli, S. typhimurium*

and other bacteria, YcgR in *E. coli* and *S. typhimurium* (Ryjenkov *et al.,* 2006), DgrA in *Caulobacter crecentus* (Christen *et al.*, 2007) and PlzCD in *V. cholera* (Newell *et al.*, 2009; Römling and Amikam, 2006; Pultz *et al.*, 2012).

- RxxD motif of enzymatically non-functional GGDEF domain proteins.

- Enzymatically inactive EAL domain proteins, such as FimX in *P. aeruginosa* (Qi *et al.*, 2011).

- FleQ of *P. aeruginosa* (Hickman and Harwood, 2008) and *P. putida* (Molina-Henares *et al.*, 2016), a member of the Ntrc/DctD family of transcriptional regulators.

- CsgD-like proteins such as VspT of *V. cholerae*.

- CRP/FNR-type transcriptional activators (Gomelsky, 2009; Leduc and Roberts, 2009; Fazli *et al.*, 2011).

4.3. Functions associated with c-di-GMP signaling: Motility vs. sessility

Best documented roles of c-di-GMP is the regulation of the transitions from motility to sessility and from acute infection to chronic infection (Römling and Balsalobre, 2012; Römling and Amikam, 2006). However, c-di-GMP can modulate other processes. For example, survival and transmission of the obligate intracellular pathogen *Borrelia burgdorferi* in insect and mammalian hosts (He *et al.*, 2011), heterocyst formation in *Cyanobacteria*, multicellular development and antibiotic production in *Streptomyces* (Neunuebel and Golden, 2008), long-term nutritional stress survival and lipid metabolism and transport in *Mycobacteria* (Bharati *et al.*, 2012; Li and He, 2012) have been reported to be controlled by c-di-GMP signaling as recently reviewed (Römling *et al.*, 2013).

The transition from motility to sessility and promotion of biofilm formation is by far the most studied role of c-di-GMP (Simm *et al.*, 2004;

Méndez-Ortiz *et al.*, 2006; Weber *et al.*, 2006; Römling, 2012). High intracellular levels of the second messenger correlate with reduced motility and enhanced biofilm formation, and vice-versa. C-di-GMP has been shown to regulate several kinds of multicellular behaviors including pellicle formation at the air liquid interface, adhesion to abiotic surfaces and biofilm formation in continuous flow systems.

The role of c-di-GMP in cellulose biosynthesis in *E. coli, S. typhimurium* and *G. xylinus* has already been mentioned. The second messenger binds the PilZ domain of BcsA, the cellulose synthase enzyme and functions as an allosteric activator (Ross *et al.*, 1987; Ryjenkov *et al.*, 2006). In different bacteria, c-di-GMP controls also the production of other exopolysaccharide components of the extracellular matrix. These include PAG (poly-β-1,6-*N*-acetylglucosamine) in *Yersinia pestis, E. coli and Pectinobacterium atrosepticum* (Kirillina *et al.,* 2004; Steiner *et al.*, 2013) and alginate, Pel and PsI in *P. aeruginosa* (Hickman *et al.*, 2005; Hickman and Harwood, 2008; Oglesby *et al.*, 2008). The production of other major constituents of some bacterial biofilms, such as proteinaceus fimbriae, can also be regulated by c-di-GMP. In *Klebsiella pneumoniae*, synthesis of type 3 fimbriae is regulated by MrkH, a transcriptional regulator that binds c-di-GMP (Langstraat *et al.*, 2001; Ong *et al.*, 2010). Similarly, c-di-GMP activates the expression of curli in *S. typhimurium* and *E. coli* and Cup fimbriae in *P. aeruginosa* (Kader *et al.*, 2006; Meissner *et al.*, 2007; Rao *et al.,* 2008) . In addition, c-di-GMP regulates type IV pili in *P. aeruginosa* and *X. axonopodis* (Huang *et al.*, 2003; Navarro *et al.,* 2009), as well as various non fimbrial adhesins such as LapA in *P. putida* and *P. fluorescens* (Newell *et al.*, 2009) and a β-helical adhesin and CdrA in *P. aeruginosa* (Ueda and Wood, 2009; Borlee *et al.,* 2010).

Paired with the synthesis of adhesins and matrix components, transition to sessility requires the inhibition of motility by c-di-GMP. In *Pseudomonas aeruginosa* PA14, c-di-GMP inversely controls biofilm

formation and surface swarming motility, with high levels of this dinucleotide signal stimulating biofilm formation and repressing swarming. *P. aeruginosa* encodes two stator complexes, MotAB and MotCD that participate in the function of its single polar flagellum. When c-di-GMP level is high, the MotAB stator can displace MotCD from the motor, thereby affecting motor function (Kuchma *et al.*, 2015).

In *S. typhimurium* and *E. coli* this process involves binding of the second messenger to the PilZ domain protein YcgR. This allows YcgR to interact with FliG and FliM, the subunits of the flagellar switch complex to induce (counterclockwise) CCW biased rotation and slow down the flagellar motor rotation. YcgR gradually reduces flagellar motor function by inactivating individual stator units in a brake-like fashion. YcgR brake mechanism involves electrostatic interaction between MotA and the rotor protein FliG. In adition, there exist networks of five c-di-GMP signaling proteins that work synergistically to adjust the concentration of c-di-GMP in *E. coli* cells and thereby fine-tunes bacterial velocity (Boehm *et al.*, 2010). C-di-GMP also regulates motility at the transcriptional level, for example in *V. cholerae*, where the transcriptional factor VpsT requires c-di-GMP for its activity to suppress flagellar gene expression (Krasteva *et al.*, 2010).

In *P. aeruginosa*, flagellar gene expression decreases as a biofilm matures (O´Toole and Kolter, 1998; Vallet *et al.,* 2001; Whiteley *et al.*, 2001; Sauer *et al.*, 2002; Klausen *et al.*, 2003; Wolfgang *et al.*, 2004). The transcriptional regulator FleQ is responsible for the coordination between flagellar and biofilm gene expression. When c-di-GMP levels are low, FleQ activates flagellar genes and represses *pel* operon expression (Arora *et al.*, 1997; Hickman and Harwood, 2008). However, when c-di-GMP levels rise in the cells, FleQ binds to c-di-GMP and activates rather than represses *pel* transcription (Hickman and Harwood, 2008; Baraquet *el al.*, 2012). The mechanism of the FleQ functional switch is due to

conformational changes caused by the interactions with both c-di-GMP and FleN, the antagonist of FleQ. Binding of FleQ to c-di-GMP does not inhibit flagellar gene expression significantly, and presumably other mechanisms are involved in the decrease in flagellar gene expression during biofilm formation (Baraquet *et al.*, 2012). Late in biofilm maturation, motility is re-activated to permit cell dispersal (Sauer *et al.*, 2002; Davies and Marques, 2009). A diffusible chemical signal, cis-2-decenoic acid, has been reported to trigger biofilm dispersion in *P. aeruginosa* and other bacteria (Davies and Marques, 2009), and to promote the reversion of persisters to metabolically active cells in biofilms (Marques *et al.*, 2014).

4.4. Regulation of c-di-GMP signaling

GGDEF/EAL domain proteins are often located at the C-terminus of membrane associated and sensory domains such as PAS/PAC, MASE and GAF. Proteins containing these sensory domains monitor cytoplasmic (intracellular) or periplasmic (extracellular) levels of their respective ligands and respond by altering the synthesis or hydrolysis of c-di-GMP. Thus intracellular levels of c-di-GMP may ultimately regulated in response to environmental signals. Although in many cases the signals have not been identified, several works have described and characterized sensor domain containing diguanylate cyclases and phosphodiesterases that sense oxygen, nitric oxyde and light (Chang *et al*, 2001; Barends *et al.*, 2009; Qi *et al.*, 2009; Tuckerman *et al.*, 2009; Wan *et al.*, 2009; Matilla *et al.,* 2011). Similarly, stand-alone sensory domains encoded by neighbouring genes may also interact with diguanylate cyclases and phosphodiesterases (Liu *et al.*, 2012; Plate and Marletta, 2012). An additional mechanism of regulating c-di-GMP levels relapses upon the carbon storage regulator CsrA. CsrA can interact with mRNA transcripts of c-di-GMP metabolizing proteins and structural proteins involved in c-di-GMP dependent processes (Jonas *et al.*, 2008 and 2010). In *E. coli*,

CsrA suppresses the expression of two diguanylate cyclases, YcdT and YcdH (Jonas *et al.*, 2008), which inhibit motility. The mode of action of CsrA and related proteins is detailed below.

5. The GacS/GacA system and the Gac/Rsm cascade

A widespread signal transduction pathway in Gram-negative bacteria is the so-called Gac/Rsm cascade, typically regulating at the post-transcriptional level the expression of extracellular products and carbon storage compounds. In animal- and plant-pathogenic bacteria, mutants defective in this pathway lose part or all of their virulence, whereas in plant-beneficial, root-colonizing species, such mutants have a reduced ability to suppress plant diseases that are caused by phytopathogenic fungi (Haas and Défago, 2005). In *E. coli*, the same pathway is important for the regulation of central carbon metabolism and storage (Romeo, 1998; Heeb and Haas, 2001; Babitzke and Romeo, 2007).

The GacA/GacS two-component system is conserved in pseudomonads. GacS (=LemA), "global activator of secondary metabolism", was first described in the plant pathogen *P. syringae* B728a as an essential factor for lesion manifestation on bean leaves, where inactivation of the *gacS* gene resulted in the loss of virulence (Hrabak and Willis, 1992; Hirano *et al.*, 1997). GacA, the cognate response regulator, was first identified as a global activator of antibiotic and cyanide production in *P. fluorescens* CHA0 (Laville *et al.*, 1992). GacA/GacS homologues have been identified in *E. coli* (BarA/UvrY), *Salmonella* (BarA/SirA), *Erwinia* (ExpS/ExpA) and *Vibrio* (VarS/VarA) as well as in several pseudomonads: *P. fluorescens, P. aeruginosa, P. syringae*, *P. aureofaciens* and *P. putida* (Laville *et al.*, 1992; Reimmann *et al.*, 1997; Hrabak and Willis, 1992; Chancey *et al.*, 1999; Heeb and Haas, 2001). GacA/GacS positively control the expression of genes involved in the production of a variety of secondary metabolites, extracellular products

and virulence factors in *P. aeruginosa* (Reimmann *et al.*, 1997; Pessi and Haas, 2001). QS molecules are also regulated by this system in some pseudomonads, as demonstrated by the production of C4-HSL in *P. aeruginosa* (Reimmann *et al.*, 1997).

GacS is activated by an as yet unknown signal, leading to auto-phosphorylation and then phosphoryl group transfer onto the response regulator GacA. Despite the global effect of GacA/GasS, the number of targets directly bound by GacA appears to be very limited. Rather, the mechanism by which this two component system controls the expression of target genes is via a post-transcriptional network involving RNA-binding proteins and the transcription of small, untranslated regulatory RNAs. Upon activation, the response regulator GacA promotes the transcription of one or several small, non-coding RNAs, depending on the bacterial species (Kulkarni *et al.*, 2006). In *P. protegens* CHA0, GacA controls the expression of three small RNAs termed RsmX, RsmY and RsmZ. In *E. coli* K-12, there are two functionally equivalent, UvrY-inducible RNAs, CsrB and CsrC. These small RNAs have high affinity for RNA binding proteins of the RsmA/CsrA family (Liu *et al.*, 1997; Heeb *et al.*, 2002; Valverde *et al.*, 2003; Weilbacher *et al.*, 2003; Kay *et al.*, 2005).

5.1 CsrA/RsmA family proteins

Proteins belonging to the CsrA/RsmA family are small (less than 60-amino acids) RNA-binding proteins that play key roles in the regulation of gene expression. Originally identified in *E. coli* and *Salmonella enterica* (Romeo, 1998; Altier *et al.*, 2000), now at least 200 putative CsrA homologues are known to be distributed among 150 different eubacteria , with several species encoding more than one homologue (e.g. *Coxiella burnetii, Legionella pneumophila, Pseudomonas fluorescens, Pseudomonas putida* and *Pirellula* sp.) (Bateman et al., 2004). CsrA controls gene expression post-

transcriptionally, by binding specifically and with high affinity to the 5-untranslated leader of target mRNAs, and affecting translation and RNA stability (Liu *et al.,* 1997; Wei *et al.*, 2001; Baker *et al.*, 2002; Dubey *et al.*, 2003; Wang *et al.*, 2005). In *E. coli*, CsrA represses glycogen biosynthesis, gluconeogenesis, peptide transport, and biofilm formation while activating glycolysis, acetate metabolism, and motility (Sabnis *et al.*, 1995; Wei *et al.*, 2001; Dubey *et al.*, 2003). Its target genes include *cstA*, *glgCAP*, *flhDC*, and *pgaABCD*.

CsrA is also known to be critical for the regulated expression of virulence factors in pathogens of both animals and plants. For example, *L. pneumophila* CsrA is essential for the repression of transmission phase genes during infection of amoebas and alveolar macrophages (Molofsky and Swanson, 2003); *Salmonella enterica* CsrA regulates pathogenicity island (SPI1) genes required for intestinal epithelial cell invasion (Altier *et al.*, 2000). In the opportunistic human pathogen *P. aeruginosa*, the CsrA homolog RsmA ("Repressor of secondary metabolism") negatively regulates the production of hydrogen cyanide, pyocyanin, LecA (PA-1L) lectin and N-acylhomoserine lactone (AHL) quorum sensing signal molecules (Pessi and Haas, 2001; Pessi *et al.*, 2001). *Erwinia carotovora* RsmA represses expression of extracellular enzymes needed for plant pathogenicity and development of soft rot disease as well as production of homoserine-lactone quorum-sensing signals (Cui *et al.*, 1995).

CsrA/RsmA proteins are sequestered by the small RNAs described above as targets of GacA (Baker *et al.,* 2002; Dubey *et al.,* 2003; Valverde *et al.*, 2003; Wang *et al.*, 2005). Thus, when RsmX/Y/Z or CsrB/C are induced and abundant, they exert the opposite influence on target genes, commonly relieving the repression effect of those RNA-binding proteins.

5.2 CsrA/RsmA proteins: structure and function

A major step in understanding CsrA structure was taken when three CsrA (RsmA) proteins were independently solved, all of which exhibited

the same overall topology (Gutiérrez *et al.,* 2005; Rife *et al*., 2005; Heeb *et al*., 2006). These studies proved CsrA to be a novel class of RNA-binding protein and confirmed previous work that suggested that CsrA forms a homodimer (Dubey *et al.,* 2003). They also highlighted the unusual way in which the dimer is formed; two interlocking CsrA monomers produce a hydrophobic core composed of 10 strands and two winglike-helices. CsrA dimer is a barrel-like structure stabilized noncovalently by an extensive network of hydrogen bonds from backbone amino and carboxyl groups (Fig. 2) (Heeb *et al.,* 2006). With the benefit of a CsrA three-dimensional structure it was possible to map protein regions essential for RNA binding and/or regulation of gene expression.

Two regions were identified in CsrA/RsmA proteins that were critical for regulation and RNA binding. They were located within the first (1, residues 2–7) and containing the last (5, residues 40–47) strands of CsrA. The 1 and 5 strands of opposite monomers lie adjacent and parallel to each other in the three-dimensional structure of this protein. Given the symmetry of the CsrA dimer, these findings imply that two distinct RNA binding surfaces or functional subdomains lie on opposite sides of the protein (Fig. 3) (Mercante *et al.,* 2006). The structure of CsrA permits simultaneous binding to two target sites within a transcript (bridging or dual-site binding).

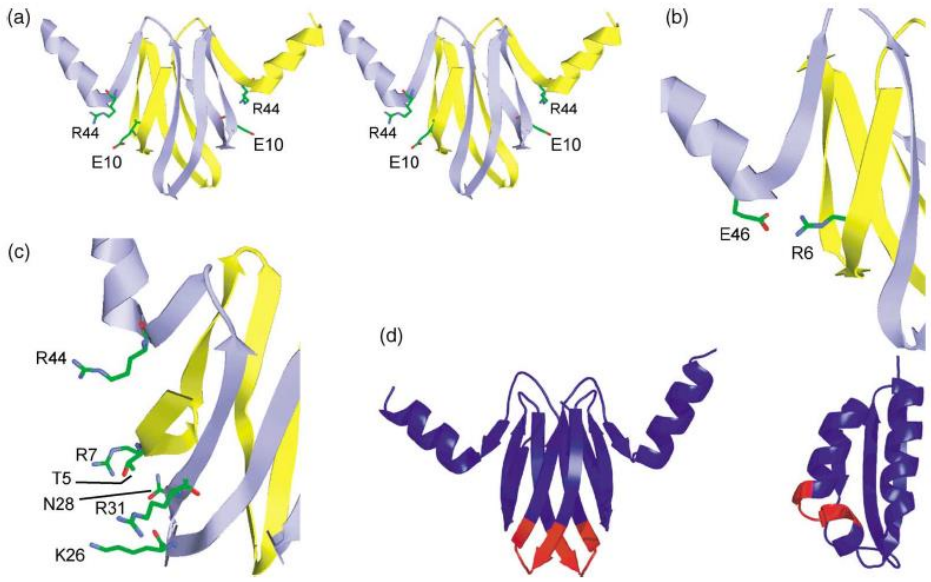

Figure 2. Structures of the *Y. enterocolitica* RNA binding protein RsmA. (a) Stereo view of the RsmA dimer composed of two five-stranded antiparallel b-sheets and two ahelices. The b-sheets form a barrel-like structure. The side-chains of two solvent-exposed residues, Glu10 and Arg44, examined in detail here are indicated. (b) Salt-bridges, important for structural maintenance of RsmA, form between R6 in the b1 sheets and E46 in the a-helices from two different subunits. (c) Charged, solvent-exposed, and highly conserved residues such as Arg44 may be important residues defining the RNA-binding site of RsmA/CsrA proteins. (d) Comparison of RsmA (right), the eukaryote protein Nova (left), and the domains having sequences similar to the KH RNA-binding domain (Figure extracted from Heeb *et al.*, 2006).

An intersite distance of ~18 nucleotides (nt) is described as optimal, although bridging occurred with an intersite distance of 10 to ≥63 nt. The close 10-nt spacing reduced the stability of dual-site binding, as competition for one site by a second CsrA dimer readily occurred (Mercante *et al.*, 2009). The finding that a symmetric CsrA dimer contains two functional subdomains on opposite sides of the protein may explain the longstanding observation that CsrB RNA, which contains 18 CsrA target sequences, binds to 18 CsrA subunits (nine functional dimers) to form a globular complex (Liu *et al.*, 1997).

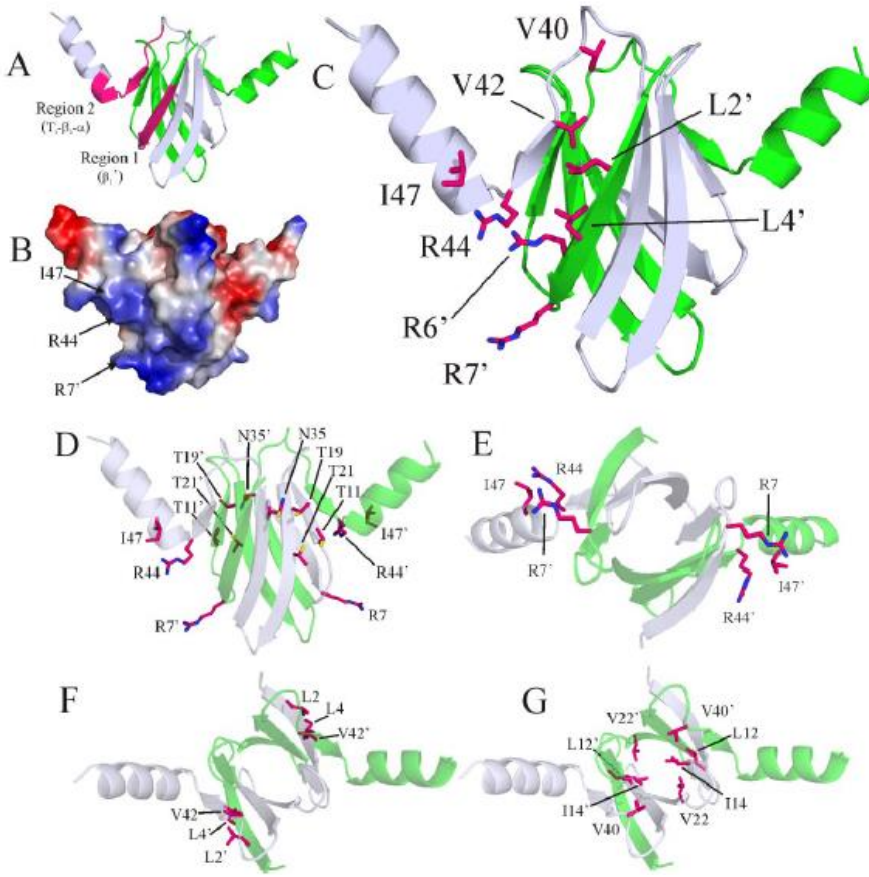

Figure 3. Critical residues of CsrA mapped onto the three-dimensional model of *Y. enterocolitica* CsrA. *A*, *ribbon diagram* of CsrA highlighting functional regions 1 and 2 (*red*) identified in the study of Mercante et al. 2006. The individual CsrA monomers are colored *white* and *green*. The second functional region found on the opposite side of the homodimer is not highlighted. *B*, identical view angle of CsrA, as shown in *A*, displaying electrostatic surface potential. Electropositive areas are represented in *blue*, whereas the electronegative areas are in *red*. *C*, CsrA displaying critical residues found in regions 1 (Leu2, Leu4, Arg6, and Arg7) and 2 (Val40, Val42, Arg44, and Ile47). The corresponding critical residues found on the opposite side of the protein are not illustrated.*D*, front view of a semitransparent CsrA displaying residues that are probably involved directly in RNA binding (Arg44, Ile47, and Arg7) and several residues found outside regions 1 and 2 (Thr11, Thr19, Thr21, and Asn35) that affect regulation. *E*, *bottom-up view* of the likely RNA binding residues listed in *D*. *F* and *G*, *top-down view* illustrating amino acids that compose a separate but adjacent hydrophobic pocket (Leu2, Leu4, and Val42) (*F)* and those that point inward and stabilize the CsrA hydrophobic core (Leu12, Ile14, Val22, and Val40) (*G*). (Taken from Mercante *et al.*, 2006)

5.3 CsrA/RNA interactions

Although their nucleotide sequence conservation is poor, small regulatory RNAs, whose length varies from 112 to approximately 345 nucleotides, have a conserved predicted secondary structure, suggesting they have analogous modes of action (Heurlier *et al.*, 2004). They retain a characteristic GGA motif located in the distal zone of stem-loops structures (Fig. 4).

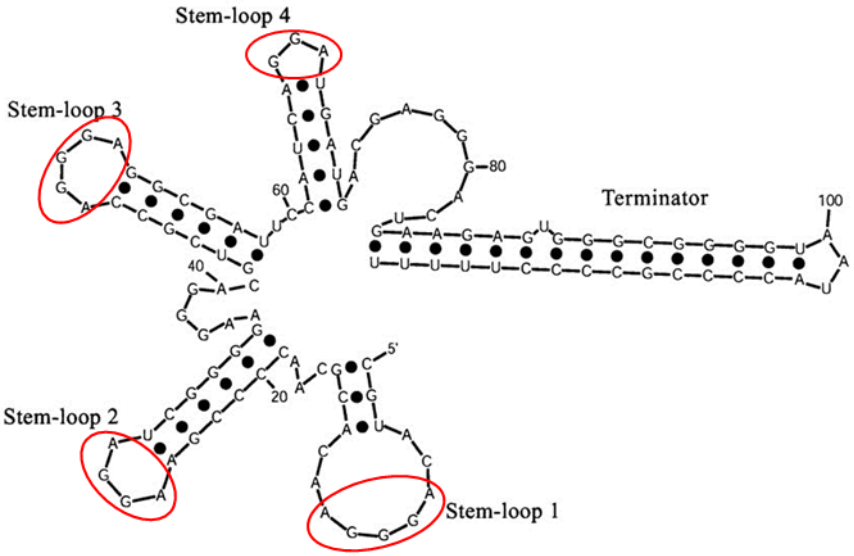

Figure 4. Predicted secondary structure of RsmZ. GGA motif located in the distal zone of stem-loops are circled in red (Adapted from Heurlier et al., 2004) of *P. protegenes* CHA0 at 30°C using MFOLD (Zuker *et al.*, 1999).

Repeated, unpaired GGA motifs in the small regulatory RNAs are assumed to be the hallmarks to be recognized by the RsmA/CsrA proteins. In the case of RsmY of pseudomonads, it has been verified experimentally that multiple GGA repeats are indeed essential for RsmA binding *in vivo* (Valverde *et al.*, 2004) and *in vitro* (Sorger-Domenigg *et al.*, 2007). However, a heptameric loop of CsrB carrying a single GGA

motif did not bind CsrA *in vitro* (Gutiérrez *et al.*, 2005), suggesting that the motif alone is insufficient to promote binding. Three target mRNAs of *E. coli*, i.e the untranslated 5´ leader sequences of the *glgC*, *pgaA* and *csrA* genes, have been investigated for CsrA binding in vitro, and from these studies an ANGGA consensus can be deduced as a conserved recognition element. In each of the three mRNAs, one ANGGA element overlaps the Shine-Dalgarno (SD) sequence. Thus, CsrA can block ribosome binding (Baker *et al.*, 2002; Dubey *et al.*, 2003; Wang *et al.*, 2005). CsrA could theoretically bridge two target sites (e.g. within two hairpin loops) (Mercante *et al.,* 2006). Subsequent studies provided experimental demonstration of the function of dual RNA-binding sites of CsrA in regulation. Using model RNA substrates, Mercante and colleagues (2009) showed that CsrA can simultaneously bind two target sites within a transcript.

The optimal binding of CsrA to some small RNA has been investigated with high-affinity RNA ligands containing a single CsrA binding site and the systematic evolution of ligands by exponential enrichment (SELEX) (Dubey *et al.*, 2005). That study revealed a consensus sequence (RUACARGGAUGU) where the ACA and GGA motifs were 100% conserved and the GU sequence was present in all but one of the experimental ligands. The majority of ligands contained GGA in the loop of short hairpins (Fig. 5) within the most stable predicted structure (Dubey *et al.* 2005).

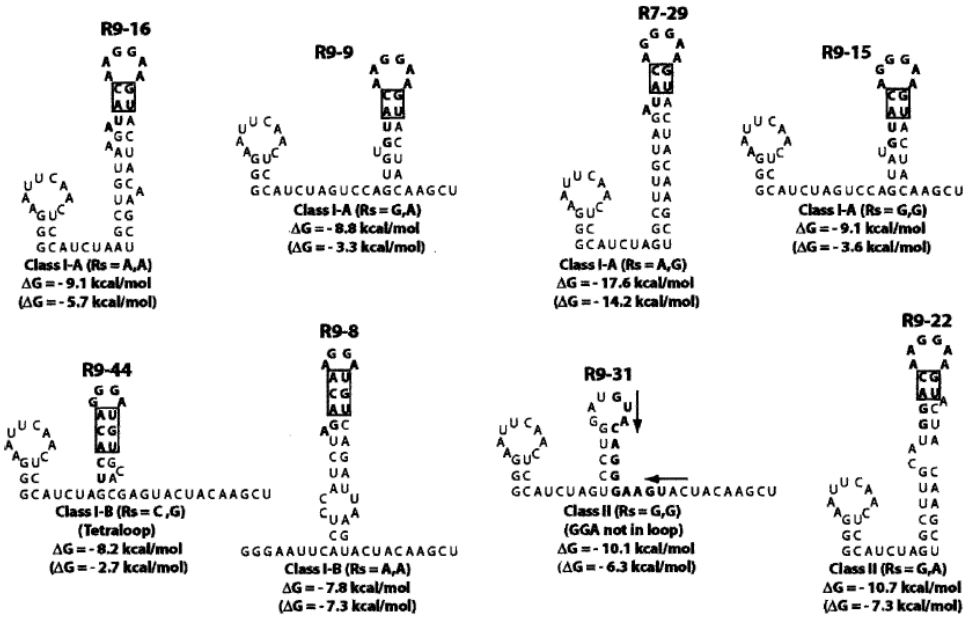

Figure 5. Predicted secondary structures of representative selected RNA ligands. The identity of the purines (R) in the SELEX-derived CsrA binding site consensus (RUACARGGAUGU) is indicated. For R9-44, one of these residues is a C. The apparent CsrA binding site for each transcript is shown in bold type, while the conserved residues predicted to be involved in base-pair formation are boxed. Arrows for R9-31 show a less stable alternative pairing arrangement in which the GGA motif would be present in the loop of a hairpin. The predicted free energy of each structure is shown, while the predicted free energy of the hairpin containing the CsrA binding site is in parentheses (Taken from Dubey *et al.,* 2005).

Since part of the consensus sequence RUACARGGAUGU is found in the stem, it was suggested that the hairpin structure partially melts after initial recognition, leading to additional base-specific contacts (Dubey *et al.*, 2005). The CsrA binding site for CsrC and CsrB (CAGGAUG) is slightly different from the SELEX-derived sequence, and not all natural CsrA binding sites contain a GGA motif. In CsrB, four are replaced with a GGG, while GGA is replaced with AGA in one of the *pgaA* binding sites (Wang *et al.*, 2005).

Several works have advanced in the study of RNA-protein complexes, their formation and the residues involved. The NMR solution

structure of RsmE from *P. protegens* CHA0 was determined as a complex with a target RNA containing the RBS of the *hcnA* gene (encoding hydrogen cyanide synthase subunit A).

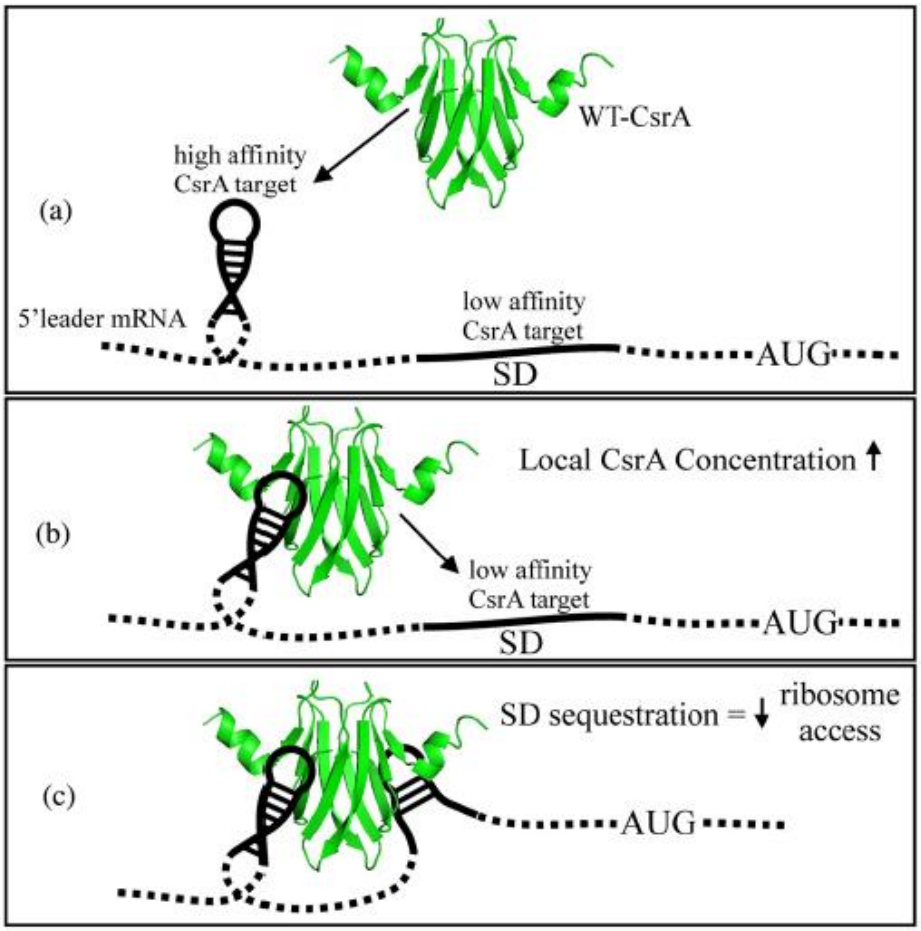

Figure 6. Model for CsrA regulation by binding to the 5′ leader of repressed transcripts. (a) Wild type WT-CsrA initially binds at a high affinity target site, commonly located within a hairpin structure that lies close to the SD. (b) After initial binding, the increased local concentration of CsrA allows the free RNA-binding surface of the protein to interact with the downstream low-affinity target site overlapping the SD sequence. (c) Binding of the low-affinity target site sequesters the SD sequence, thereby blocking ribosome loading and decreasing translational initiation (Taken from Mercante *el al.,* 2009).

Only the binding of RsmE to the 12 nt RNA sequence containing the RBS produced a stable stem/loop, whereas there was no evidence of free RNA forming a similar structure. The RsmE homodimer has two binding sites and makes optimal contact with a 5´-$^{A}/_{U}$CANGGANG$^{U}/_{A}$-3´ sequence. When bound to RsmE, the ANGGAN core folds into a loop structure, favoring the formation of a 3 bp stem (Schubert et al., 2007). It is interesting to note that the consensus sequence 5´-$^{A}/_{U}$CANGGANG$^{U}/_{A}$-3´ closely matches the ideal 5´-AAGGAGGU-3´ Shine Dalgarno (SD) sequence, which has been proposed as a low affinity target for the proteins of the RsmA/CsrA family (Fig. 6).

The study by Mercante *et al.*, 2009 also represented the first experimental demonstration of the function of dual RNA-binding sites of CsrA in regulation. As well as the wild type (WT), a heterodimer was used (HD), where one of the binding surfaces had an alanine mutation at the R44 site, previously shown to be required for biological function (Heeb *et al.,* 2006). CsrA binds to the 5‘-untranslated leader sequence of target transcripts and alters their translation and/or stability. The example used was the *glgCAP* 5‘-leader, which has four RNA binding sites, only two of which had been previously characterized. Compared to the WT-CsrA, the HD-CsrA had only a third of the affinity for a single target. The heterodimeric CrsA, was ~14 fold less effective at repression using a *glgC*’-‘*lacZ* reporter fusion. When a GGA site upstream the RNA target was deleted, the difference in the HD-CsrA was unchanged, but relative to the WT-CsrA regulation decreased by 7 folds.

6. *Pseudomonas putida* KT2440: biofilm regulation in a versatile environmental microorganism.

Pseudomonas putida KT2440, the organism used in this Doctoral Thesis, is considered a paradigm of metabolic versatility and has been extensively characterized at the physiological, genetic and biochemical levels (Timmis, 2002; Wu *et al.*, 2011). It is certified as safe for cloning

and expression of heterologous genes by the National Institutes of Health (NIH) of the United States (Register, 1982). This accreditation has allowed *P. putida* KT2440 to become a model organism for biodegradation and biotransformation of aromatic compounds studies, metals resistance, heterologous gene expression and interaction with plants (Dos Santos *et al.*, 2004; Scott *et al.*, 2013; Matilla *et al.*, 2010; Graf and Altenbuchner, 2014). *Pseudomonas putida* KT2440 is a derivative of *P. putida* mt-2, which was isolated from garden soil in Japan based on its ability to use 3-methylbenzoate (Nakazawa, 2002). Its genome encodes information for a large number of activities related to protection against toxic substances (dioxygenases, monooxigenases, oxidoreductases, ferredoxins, cytochromes, dehydrogenases, proton pumps, glutathione transferases, etc), enzymes and routes for degradation of aromatic compounds (Jiménez *et al.,* 2002). Its great metabolic versatility is consistent with the identification of at least 350 transport systems in the cytoplasmic membrane (12% of the entire genome sequence), many of them involved in the transport of aromatic compounds (Nelson *et al.*, 2002). The *in silico* metabolic networks of this bacterial strain have been reconstructed at the genomic scale (Nogales *et al.,* 2008; Puchalka *et al.*, 2008; Van Duuren *et al.*, 2013).

Previous work in our group and other laboratories has allowed the identification of structural elements involved in attachment of *P. putida* KT2440 to abiotic and plant surfaces, and in biofilm formation. The regulatory mechanisms controlling expression of some of these structural elements have started to be explored, and have led to a sequential model (Fig. 7) where first LapA (in red in figure 7) is required for cell-surface interactions and then LapF (in pink in figure 7) mediates cell-cell interactions (Martínez-Gil *et al.*, 2010). In a mature biofilm, both proteins, along with exopolysaccharides, form the extracellular matrix. *P. putida* has the potential to produce 4 different EPSs, namely cellulose, alginate and two species-specific ones (Pea and Peb). Pea is the most

relevant for biofilm formation under laboratory conditions (Espinosa-Urgel *et al.*, 2000; Yousef-Coronado *et al.*, 2008).

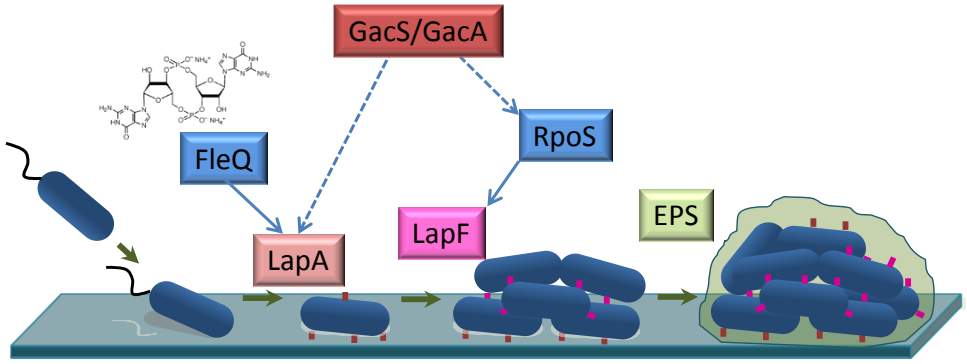

Figure 7. Structural and regulatory elements involved in biofilm formation in *P. putida* KT2440

Particularly relevant to the objectives of this work is the identification of the GacS/GacA system as a key modulator of expression of the large adhesins LapA and LapF (Martínez-Gil *et al.*, 2014), and the characterization of Rup4959, a unique response regulator with diguanylate cyclase activity. The gene coding for Rup4959 was found to be preferentially expressed in *P. putida* populations colonizing the rhizosphere of corn plants (Matilla *et al.*, 2007), as compared with laboratory culture conditions. The presence of this gene cloned in a multicopy plasmid causes an increase in the intracellular levels of c-di-GMP, which is associated to the appearance of a pleiotropic phenotype that includes increased biofilm formation, flocculation in liquid medium, crinkly colony morphology, and increased pellicle formation at the air-liquid interface in static cultures (Matilla *et al.*, 2011). These previous studies also explored the transcription pattern and regulation of *rup4959* (recently renamed as *cfcR*, the nomenclature used hereafter), and hypothesized the possible existence of post-transcriptional regulation mediated by Rsm proteins. This was based on the presence of two motifs

coincident with the consensus recognition site for RsmA in the untranslated leader sequence of *cfcR* mRNA (Fig. 8) and the inconsistency found between expression patterns based on promoter activity and mRNA levels under anaerobic conditions (Matilla *et al.*, 2011).

Figure 8. Features in the *cfcR* promoter sequence. Pseudopalindromic sequence likely being a rho independent transcriptional terminator for PP_4960 is indicated with arrowheads. Transcriptional initiation points are in bold in capital letters. Putative -35 sequence compatible with RpoS requirement are in bold. Shine Dalgarno (AGAGAA) and start codon (ATG) are shown in capital. Sequences compatible with the consensus of the global posttranscriptional regulator Rsm are underlined (Adapted from Thesis of Matilla, M.A., 2011)

The role of GacS/GacA in biofilm formation and the possible connection with c-di-GMP signaling via Rsm proteins and CfcR, was the starting point of this Thesis.

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II. AIM OF THE THESIS

Our Research group has been studying the process of colonization of plant surfaces by the plant growth-promoting rhizobacterium *Pseudomonas putida* KT2440, and how this process correlates with biofilm formation. While the structural elements involved in these processes have been studied in detail, the regulatory factors influencing their expression are less characterized. The aims of this Thesis are based on two findings:

- The identification of the GacS/GacA two component system as a relevant regulatory element for adhesins involved in biofilm formation in *P. putida* KT2440.
- The existence of potential targets for RsmA recognition in the leader sequence of *cfcR* mRNA, which encodes the only response regulator with diguanylate cyclase activity in this microorganism.

These evidences pointed to a relevant role of the Gac/Rsm cascade in the regulation of biofilm formation by *P. putida* KT2440, likely influencing the synthesis of c-di-GMP. Thus, we decided to address the following questions in the frame of this Thesis:

1. Characterization of the three Rsm proteins present in KT2440 and their role in biofilm formation.
2. Influence of the Rsm proteins on the expression of the response regulator with diguanylate cyclase activity CfcR, and their impact on intracellular levels of c-di-GMP.
3. Global analysis of genes potentially regulated at the post-transcriptional level by Rsm proteins.

III. MATERIAL AND METHODS

MATERIAL AND METHODS – Chapter 1

Bacterial strains and culture conditions. The strains and plasmids used in this work are listed in Table 1. *P. putida* KT2440 is a plasmid-free derivative of *P. putida* mt-2, which was isolated from a field planted with vegetables and whose genome is completely sequenced (Nakazawa, 2002, Nelson, *et al.*, 2002). Fluorescently labeled strains with a single chromosomal copy of mCherry were obtained by conjugation using the plasmid miniTn*7*Ptac-mChe (Tecon *et al.,* 2009) as detailed below. *E. coli* and *P. putida* strains were routinely grown at 37°C and 30°C, respectively, in LB medium (Lennox, 1955) under orbital shaking (200 rpm). M9 minimal medium (Sambrook *et al.*, 2001) was supplemented with trace elements (Yusef-Coronado *et al.,* 2008) and glucose or citrate at the concentrations indicated in each case. For pyoverdine quantification, King's B medium was used (King *et al.*, 1954). When appropriate, antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; streptomycin, 50 μg/ml (*E. coli*) or 100 μg/ml (*P. putida*); and tetracycline, 10 μg/ml or 20 μg/ml (as indicated). Cell growth was followed by measuring turbidity at 600 nm (optical density at 600 nm [OD600]) or 660 nm (OD660), except for experiments done in a BioScreen apparatus with a wide-band filter (450 to 580 nm).

DNA techniques. Preparation of chromosomal DNA, digestion with restriction enzymes, dephosphorylation, ligation, and electrophoresis were carried out using standard methods (Sambrook *et al.,* 2001; Ausubel *et al,*. 1987) and following the manufacturers' instructions (Roche and New England BioLabs). Plasmid DNA isolation and recovery of DNA fragments from agarose gels were done with Qiagen miniprep and gel extraction kits, respectively. The DIG DNA labeling and detection kit (Roche) was used for Southern blots, according to the manufacturer's instructions. Electrotransformation of freshly plated

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype/Relevant characteristics[a]	Reference/source
E. coli		
CC118λpir	Rif^r, *λpir*	Herrero *et al* 1990
DH5α	*supE*44 *lac*U169(Ø80*lacZ*ΔM15) *hsdR*17 ($r_k^-m_k^-$) *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Woodcock *et al* 1989
HB101	Host of helper plasmid pRK600	V. de Lorenzo
P. putida		
KT2440	Wild type; derivative of *P. putida* mt-2 cured of pWW0	Nakazawa, 2002
pvdS	Mutant in *pvdS*, defective in pyoverdine synthesis	Matilla *et al* 2007
ΔI	Null mutant derivative of KT2440 in PP_1746 (*rsmI*)	This study
ΔE	Null mutant derivative of KT2440 in PP_3832 (*rsmE*)	This study
ΔA	Null mutant derivative of KT2440 in PP_4472 (*rsmA*)	This study
ΔIE	Null mutant derivative of KT2440 in PP_1746 and PP_3832	This study
ΔEA	Null mutant derivative of KT2440 in PP_3832 and PP_4472	This study
ΔIA	Null mutant derivative of KT2440 in PP_1746 and PP_4472	This study
ΔIEA	Null mutant derivative of KT2440 in PP_1746, PP_3832 and PP_4472	This study
Plasmids		
pGEM®-T Easy	Ap^r, PCR cloning vector with β-galactosidase α-complementation	Promega
PCR2.1 TOPO	Km^r, PCR cloning vector with β-galactosidase α-complementation	Invitrogen
pKNG101	Sm^r, *oriR6K mobRK2 sacBR*	Kaniga *et al* 1991
pMP220-BamHI	Tc^r, pMP220 with deletion of a 238-bp BamHI fragment, removing the ribosome binding site and 52 codons of *cat* that precede '*lacZ*	Matilla *et al* 2011
pKR600	Cm^r, *mob tra*	V. De Lorenzo
pUC18Not	Ap^r, cloning vector, MCS of pUC18 flanked by NotI sites	Herrero *et al* 1990
pME6032	Tc^r, pVS1-p15A derivative; broad-host-range *lacI*^{q}-P_{tac} expression vector	Heeb *et al* 2002
pMMG1	Tc^r, transcripcional fusion *lapF::´lacZ* containing RBS and first codons	Martínez-Gil *et al* 2010
pMMGA	Tc^r, transcriptional fusion *lapA::´lacZ* containing RBS and first codons	Martínez-Gil *et al* 2014
pMIR125	Tc^r, transcriptional fusion *algD*::'*lacZ* containing RBS and first codons	Molina-Henares *et al* 2016
pMP-bcs	Tc^r, transcriptional fusion PP_2629::'*lacZ* containing RBS and first codons	Molina-Henares *et al* 2016
pMP-pea	Tc^r, transcriptional fusion PP_3132::'*lacZ* containing RBS and first codons	Molina-Henares *et al* 2016
pMP-peb	Tc^r, transcriptional fusion PP_1795::'*lacZ* containing RBS and first codons	Molina-Henares *et al* 2016
pOHR14	Ap^r, pUC18NotI derivative with 1.9 kb NotI fragment containing the *rsmA* null allele	This study
pOHR20	Sm^r, pKNG101 derivative for the *rsmA* null allele replacement with the 1.9 kb NotI fragment of pOHR14 cloned at the same site of pKNG101	This study
pOHR30	Ap^r, pUC18NotI derivative with 1.7 kb NotI fragment containing the *rsmI* null allele	This study
pOHR33	Sm^r, pKNG101 derivative for the *rsmI* null allele replacement with the 1.7 kb NotI fragment of pOHR30 cloned at the same site of pKNG101	This study
pOHR32	Ap^r, pUC18NotI derivative with 2 kb NotI fragment	This study

	containing the *rsmE* null allele	
pOHR34	Sm^r, pKNG101 derivative for the *rsmE* null allele replacement with the 2 kb NotI fragment of pOHR32 cloned at the same site of pKNG101	This study
pME6032-*rsmA*	Tc^r, pME6032 derivative for the ectopic expression of *rsmA* under the control of *lacI*^{q}-P$_{tac}$	This study
pME6032-*rsmE*	Tc^r, pME6032 derivative for the ectopic expression of *rsmE* under the control of *lacI*^{q}-P$_{tac}$	This study
pME6032-*rsmI*	Tc^r, pME6032 derivative for the ectopic expression of *rsmI* under the control of *lacI*^{q}-P$_{tac}$	This study
pOHR46	Tc^r, pMP220-BamHI derivative containing translational fusion RsmI-LacZ	This study
pOHR47	Tc^r, pMP220-BamHI derivative containing translational fusion RsmE-LacZ (with proximal promoter)	This study
pOHR48	Tc^r, pMP220-BamHI derivative containing translational fusion RsmE-LacZ (with proximal and distal promoters)	This study
pOHR52	Tc^r, pMP220-BamHI derivative containing translational fusion RsmA-LacZ	This study

[a]Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampin; Sm, streptomycin; Tc, tetracycline MCS, multicloning site; RBS, ribosome-binding site

Pseudomonas cells were performed as previously described (Cornish *et all*, 1998). For PCR amplifications, Expand High Fidelity polymerase (Roche) was used if amplicons were used in further cloning.

Conjugation. Overnight cultures (0.5 ml) of donor, helper, and recipient bacteria were mixed, centrifuged (12,000 rpm; 2 min), washed with 1 ml of fresh LB medium to remove antibiotic traces, and resuspended in 50 µl of LB medium. The mixture was spotted onto a 0.22-µm filter placed on an LB plate and incubated overnight at 30°C. The mating mixture was then suspended in 2 ml of M9 salts and plated in M9 medium with 15 mM sodium citrate to counterselect *E. coli* strains and with the corresponding antibiotics for transconjugant selection. For strains harboring miniTn*7*Ptac-mChe, the presence of a chromosomal copy of the transposon at an extragenic location near *glmS* was checked by PCR, as described previously (Kock *et al.*, 2001).

Generation of null mutants. The general strategy for the construction of null mutants consisted of replacement by homologous recombination of the wild-type allele with a null allele. Fragments (0.7 to 1 kb) of the regions flanking each *rsm* gene were amplified by PCR with oligonucleotides containing unique restriction sites (Table 2) and then

cloned into pGEM-T Easy or pCR2.1-TOPO. The absence of missense mutations in the PCR amplicons was confirmed by sequencing. The null allele was first cloned in pUC18Not (Herrero *et al.,* 1990) and subsequently subcloned in the NotI site of plasmid pKNG101 (Kaniga, 1991), which is unable to replicate in *Pseudomonas*. The derivative plasmids of pKNG101 containing the null mutations were mobilized from *E. coli* CC118 λ*pir* into *P. putida* KT2440 by conjugation as described above, using HB101(pRK600) as a helper. Merodiploid exconjugants were first selected in minimal medium with citrate and streptomycin and then incubated in LB medium supplied with 12% sucrose to obtain clones in which a second recombination event had removed the plasmid backbone. Sm-sensitive clones were repurified, and the presence of the null mutations was checked by PCR, followed by sequencing of the corresponding chromosomal region and Southern blotting. The nomenclature of the mutants in more than one locus indicates the order in which the null alleles were introduced.

Overexpression of Rsm proteins. Plasmid pME6032 (Heeb *et al,*. 2002) was used to overexpress RsmI, RsmE, and RsmA. The three genes were amplified from *P. putida* KT2440 chromosomal DNA by PCR using oligonucleotides PP1746HistagF and PP1746HistagR, PP_3832HistagF and PP_3832HistagR, and PP_4472HistagF and PP_4472HistagR, respectively (Table 2); digested by EcoRI and XhoI; and inserted into EcoRI/XhoI-cut pME6032 to obtain pOHR40, pOHR38, and pOHR37, respectively. The integrity of all the constructs was verified by sequencing to discard any mutation in the PCR amplicons.

Construction of Rsm-LacZ translational fusions. Translational fusions were generated by PCR amplification of a fragment covering the promoter regions plus initiation codons of *rsmI*, *rsmE*, and

rsmA designed to ensure in-frame cloning in pMP220-BamHI (Matilla *et al.,* 2011). The primers used are listed in Table 2. PCR amplicons of 222 bp (for RsmI-LacZ), 204 bp (for RsmE-LacZ with the proximal promoter), 340 bp (for RsmE-LacZ with the distal and proximal promoters), and 1,000 bp (RsmA-LacZ) containing the ribosome-binding sites, and the first 7 to 9 codons of each gene were cloned in pCR2.1-TOPO and sequenced to ensure the absence of mutations, followed by digestion with BamHI and subsequent cloning into the same site of pMP220-BamHI to yield pOHR46, pOHR47, pOHR48, and pOHR52, respectively.

RNA purification. Bacterial cells grown in liquid LB medium were harvested at the indicated times by centrifugation, immediately frozen with liquid nitrogen, and stored at -80°C. Alternatively, cells were collected from patches grown on LB plates for 24 or 48 h and resuspended in M9 salts prior to centrifugation and freezing. Total RNA from the mutants and the wild type was extracted by using TRI reagent (Ambion, Austin, TX, USA), as recommended by the manufacturer, except that Tripure isolation reagent was preheated at 70°C, followed by purification with an RNeasy purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA traces were then removed from RNA samples with RNase-free DNase I (Turbo RNA-free; Ambion), as specified by the supplier. The RNA concentration was determined with a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), its integrity was assessed by agarose gel electrophoresis, and the absence of any residual DNA was checked by PCR.

qRT-PCR. Expression analyses by quantitative real-time PCR (qRTPCR) were performed using iCycler Iq (Bio-Rad, Hercules, CA, USA) with total RNA preparations obtained from three independent cultures

(three biological replicates). Total RNA (1 μg) treated with Turbo DNA free (Ambion) was retrotranscribed to cDNA with Superscript II reverse transcriptase (Invitrogen) using random hexamers as primers. Template cDNAs from the experimental and reference samples were amplified in triplicate using the primers listed in Table 2. Each reaction mixture contained 2 μl of a dilution of the target cDNA (1:10 to 1:10,000) and 23 μl of Sybr Green mix (Molecular Probes). Samples were initially denatured by heating at 95°C for 10 min, followed by a 40-cycle amplification and quantification program (95°C for 15 s, 62°C for 30 s, and 72°C for 20 s) with a single fluorescence measurement per cycle. The PCR products were between 150 and 200 bp in length. To confirm the amplification of a single PCR product, a melting curve was obtained by slow heating from 60°C to 99.5°C at a rate of 0.5°C every 10 s for 80 cycles, with continuous fluorescence scanning. The results were normalized relative to those obtained for 16S rRNA. Quantification was based on the $2^{-\Delta\Delta CT}$ method (Livak *et al.,* 2001).

Transcription initiation site determination. The rapid amplification of cDNA ends (RACE) system version 2.0 (Invitrogen) was used to determine the 5´ ends of *rsm* transcripts. Total RNA was extracted from *P. putida* KT2440 cultures at OD600s of 0.8 and 2.8, and the RACE technique was carried out with the oligonucleotides listed in Table 2, following the manufacturer's instructions. Ten clones were sequenced for each transcript, and in all cases, 8 or more gave the same result in terms of identification of the +1 site.

TABLE 2 Primers used in this work

Primer name	Sequence (5´-3´) [a]	Comment
PP_4472UpF	TTGAGCTCCAGCATCACTACCCTGGGTC	*rsmA* null mutant construction
PP_4472UpR	TGGGATCCCATTCAGGGGTAACAGTCTTGG	
PP_4472DwF	TCGGATCCGAAGGATGAAGAGCCAAGCC	
PP_4472DwR	TCGGATCCGATTGTTGTGGATGGGAAAGC	
PP_3832UpF	GAATTCCGACCAGCACAAATACGGG	Null *rsmE* mutant construction
PP_3832UpR	TCTAGACTCCTTGGTGATGTATAAGTCCG	
PP_3832DwF	TCTAGAGAAGACACACACTGAGCGTCAC	
PP_3832DwR	AAGCTTGACATCATTGGGCCTGGC	
PP_1746UpF	GAATTCCCGATGTCAACGAAGCC	Null *rsmI* mutant construction
PP_1746UpR	TCTAGAGTTCCGATCCTCCTGCG	
PP_1746DwF	TCTAGAGCAGAGCAAGGCCTGAAG	
PP_1746DwR	AAGCTTCTGGCGTAGCGGCATTG	
PP_4472HistagF	GAATTCATGcatcatcatcatcatATGTTGATTCTGACT	189-bp EcoRI/XhoI fragment for *rsmA* ectopic expression
PP_4472HistaR	CGTCG CTCGAGCTATTATAAAGGCTTGGCTCTTCATCC	
PP_3832HistagF	GAATTCATGcatcatcatcatcatATGCTGATACTCACC	198-bp EcoRI/XhoI fragment for *rsmE* ectopic expression
PP_3832HistagR	CGTAAG CTCGAGCTATTATCAGTGTGTGTCTTCGTGTTTC	
PP_1746HistagF	GAATTCATGcatcatcatcatcatATGCTGGTAATAGG	180-bp EcoRI/XhoI fragment for *rsmI* ectopic expression
PP_1746HistagR	GCGC CTCGAGCTATTATCAGGCCTTGCTCTGC	
PP_4472GSPR1	GAATCAACATAGCTTTCTCCTTACGCA	+1 determination of *rsmA* by RACE-PCR
PP_4472GSPR2	TTGCCTTTGACGCCAA	
PP_3832GSPR1	AGTGTGTGTCTTCGTGTTTCTCC	+1 determination of *rsmE* by RACE-PCR
PP_3832GSPR2	TCAGTTAATAGGCACCTG	
PP_1746GSPR1	AGGCCTTGCTCTGCTTGGAC	+1 determination of *rsmI* by RACE-PCR
PP_1746GSPR2	GACCTGGAAAGCCAGCAAT	
PP_4472PromF	GGATCCGCTTCCAGGGCGTC	RsmA-LacZ fusion
PP_4472PromR	GGATCCCGACGAGTCAGAATCAA	
PP_3832ShortPromF	GGATCCCCGTTGACGGTTTGC	$RsmE_{P2}$-LacZ fusion (proximal promoter)
PP_3832ShortPromR	GGATCCCCAACCTTACGGGTG	
PP_3832LargePromF	GGATCCGGCCTTGCTGTTGTTTC	$RsmE_{P1P2}$-LacZ fusion (proximal + distal promoters)
PP_3832ShortPromR	GGATCCCCAACCTTACGGGTG	
PP_1746PromF	GGATCCGCGGCTGTATGACG	RsmI-LacZ fusion
PP_1746PromR	GGATCCCCTACTTCGCGCCCTA	
qRTAlgF	GCTTCCTCGAAGAGCTGAA	qRT_PCR; *alg* cluster (PP_1277; *algA*)[b]
qRTAlgR	CTCCATCACCGCATAGTCA	
qRTPebF	GCAATGTCTCCACAGGCAC	qRT_PCR; *peb* cluster (PP_1795)[b]
qRTPebR	TCATCTGATTGGCGACCAG	
qRTCelF	GTCGAGAGCAGCCAGCTTC	qRT_PCR; *bcs* cluster (PP_2629)[b]
qRTCelR	GCCTCATACAGTGCCAGCTC	
qRTPeaF	TGCTCAGCACGCCGACACG	qRT_PCR; *pea* cluster (PP_3132)[b]
qRTPeaR	GGTCTCGCTGTTCAGCA	
qRT16SF	AAAGCCTGATCCAGCCAT	qRT_PCR control 16S rRNA
qRT16SR	GAAATTCCACCACCCTCTACC	

[a]Restriction sites inserted in the primer for the cloning strategy are underlined; 5x Histidine tail is in lower case

[b]The specific loci given

Assays for β-galactosidase activity. Overnight cultures were diluted 1/100 in fresh LB medium supplemented with 10 μg/ml tetracycline and grown at 30°C for 1 h. Then, the cultures were diluted 1/10 in fresh LB medium and cultivated at 30°C for 1 more hour to allow the reduction of most of the remaining β-galactosidase accumulated in the overnight cultures. Finally, the cultures were diluted to an OD600 of 0.05 in fresh LB medium supplemented with 10 μg/ml tetracycline. The cells were allowed to grow at 30°C, and at the indicated time points, aliquots were taken and β-galactosidase activity was measured as described previously (Miller, 1972). Experiments were carried out in triplicate with two experimental replicates, and all the data represent averages and standard deviations.

Biofilm assays. Biofilm formation was analyzed in LB medium under static conditions using a microtiter plate assay described previously (O´Toole *et al.,* 1998). Alternatively, biofilm development was followed during growth in LB medium in borosilicate glass tubes incubated with orbital rotation at 40 rpm at 30°C. In both cases, the OD600 of the cultures was adjusted to 0.02 or 0.05, respectively, at the start of the experiment. At the indicated times, the liquid was removed and nonadherent cells were washed away by rinsing with distilled water. The biofilms were stained with 1% crystal violet (Sigma) for 15 min, followed by rinsing twice with distilled water. Photographs were taken, and the cell-associated dye was solubilized with 30% acetic acid and quantified by measuring the absorbance at 580 nm (*A*580). Assays were performed in triplicate. For confocal laser scanning microscopy (CLSM) analysis of biofilms, cultures were grown in LB medium diluted 1/10 in 24-well glass bottom plates (Greiner Bio-One, Germany). At the indicated times, the biofilms were visualized using a Zeiss LSM 510 Meta/AxioVert 200 confocal microscope, and three-dimensional reconstruction was performed with Imaris software (Bitplane).

Motility assays. Swimming motility was tested on LB plates containing 0.3% (wt/vol) agar. Cells from exponentially growing cultures (2 μl) were inoculated into the plates. Swimming halos were measured after 24 h of inoculation, and the area was calculated. Assays were performed three times with three replicates each. Surface motility assays were done as previously described (Matilla *et al.,* 2007) on plates containing 0.5% (wt/vol) agar.

Statistical methods. One-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test (set at a *P* value ≥0.05) or Student's *t* test for independent samples (*P* ≥0.05), was applied using the R program for all statistical analyses.

MATERIAL AND METHODS – Chapter 2

Bacterial strains, culture media and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 respectively. *Pseudomonas putida* KT2440 is a plasmid-free derivative of *P. putida* mt-2, which was isolated from a vegetable-planted field and whose genome is sequenced (Nakazawa, 2002, Nelson et al., 2002). *Pseudomonas putida* strains were grown at 30°C as indicated, in either Luria-Bertani (LB) medium (Bertani, 1951) or M9 defined medium (Sambrook et al., 1989) supplemented with 1 mM $MgSO_4$, 6 mg/L ammonium ferric citrate and trace metals as described previously (Yousef-Coronado et al., 2008). Glucose (27mM) or sodium citrate (15mM) was added as alternative carbon sources to defined M9-minimal medium. *Escherichia coli* strains were grown at 37°C in LB.

Table 1. Bacterial strains used in this work

Strain	Genotype/relevant characteristics	Reference or source
Escherichia coli		
CC118*λpir*	Rif^R, *λpir*, donor strain for pKNG101-derivative plasmids	Herrero *et al.*, 1990
DH5α	*supE*44 *lac*U169(Ø80*lacZ*ΔM15) *hsdR*17 ($r_k^-m_k^-$) *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Woodcock *et al.*, 1989
HB101 (pRK600)	Helper strain harboring a Cm^R *mob tra* plasmid	V. De Lorenzo
P. putida		
KT2440	Wild-type, prototroph, cured of pWWO derivative of *P. putida* mt-2	Nakazawa (2002)
ΔcfcR	Km^R, nonpolar null PP_4959 mutant	Matilla *et al.*, 2011
ΔrsmI	Null PP_1476 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmE	Null PP_3832 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmA	Null PP_4472 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmIE	Double null PP_1746/PP_3832 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmEA	Double null PP_3832/PP_4472 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmIA	Double null PP_1746/PP_4472 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmIEA	Triple null PP_1746/PP_3832/PP_4472 derivative of KT2440	Huertas-Rosales *et al.*, 2016
ΔrsmIEAcfcR	PP_4959 null derivative of *ΔrsmIEA*	This study

Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline

When appropriate, antibiotics were added to the medium at the following final concentrations (μgml^{-1}): ampicillin 100; kanamycin 25; streptomycin 50 (*E. coli*) or 100 (*P. putida*); gentamycin 50 or 100 as indicated; tetracycline 10, 20 or 200 as indicated. Cell growth was followed by measuring turbidity at 660 nm.

Table 2. Plasmids used in this work

Plasmid	Relevant characteristics	Reference or source
pBBR1-MCS5	Gm^r, *oriRK2 mobRK2*	Kovach *et al.*, 1995
pCdrA::*gfp*^{C}	Ap^r, Gm^R, FleQ dependent c-di-GMP biosensor	Rybtke *et al.*, 2012
pCR™2.1-TOPO	Km^R, PCR cloning vector with β-galactosidase α-complementation	Invitrogen
pGEM®-T	Ap^R, PCR cloning vector with β-galactosidase α-complementation	Promega
pKNG101	Sm^R, *oriR6K mobRK2 sacBR*	Kaniga *et al.*, 1991
pMP220	Tc^R, oriRK2, *'lacZ*, vector used for transcriptional fusions	Spaink *et al.*, 1987
pMP220-BamHI	Tc^R, derivative of pMP220 with a deletion of a 238-bp BamHI fragment, which removes the ribosome binding site and 52 codons of the *cat* gene that precede '*lacZ;* used as vector for translational fusions	Matilla *et al.*, 2011
pUC18Not	Ap^r, derivative of pUC18 with two NotI sites flanking the polylinker	Herrero *et al.*, 1990
pMAMV21	Tc^R, *rpoS'-'lacZ* translational fusion in pMP220-BamHI	Matilla *et al.*, 2011
pME6032	Tc^R, pVS1-p15A derivative. Broad host range *lacI*^{q}-P$_{tac}$ expression vector	Heeb *et al.*, 2002
pME6032-*rsmA*	Tc^R; pME6032 derivative for the ectopic expression of *rsmA* under the control of *lacI*^{q}-P$_{tac}$	Huertas-Rosales *et al.*, 2016
pME6032-*rsmE*	Tc^R; pME6032 derivative for the ectopic expression of *rsmE* under the control of *lacI*^{q}-P$_{tac}$	Huertas-Rosales *et al.*, 2016
pME6032-*rsmI*	Tc^R; pME6032 derivative for the ectopic expression of *rsmI* under the control of *lacI*^{q}-P$_{tac}$	Huertas-Rosales *et al.*, 2016
pMIR153	Km^R, pKNG101 derivative harboring *cfcR* inactivation	Matilla *et al.*, 2011
pMIR219	Tc^R, *cfcR'-'lacZ* translational fusion in pMP220-BamHI	This study
pMIR200	Tc^R, *cfcR'::'lacZ* transcriptional fusion in pMP220	This study
pMIR220	Tc^R, *cfcR'*$_{(bmod)}$-'*lacZ* translational fusion in pMP220-BamHI	This study

Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; MCS, multicloning site; Tc, tetracycline

DNA techniques. Digestion with restriction enzymes, dephosphorylation, ligation and electrophoresis were carried out using standard methods (Ausubel, *et al*, 1987; Sambrook, *et al.*, 1989) and following the manufacturers' instructions. Plasmid DNA isolation and recovery of DNA fragments from agarose gels were done using QIAGEN miniprep and gel extraction kits, respectively. Competent cells were prepared using calcium chloride and transformations were performed

using standard protocols. Electrotransformation of freshly plated *Pseudomonas* cells was performed as previously described (Sambrook, *et al.*, 1989). Polymerase chain reactions (PCR) were carried out using Taq DNA polymerase (Roche).

Triparental conjugations. Transfer of plasmids from *E. coli* to *P. putida* strains was performed by triparental matings using *E. coli* (pRK600) as a helper. For each strain, cells were collected from 0.5 ml of overnight LB cultures via centrifugation, then rinsed and suspended in 50 µl of fresh LB, and finally spotted on mating filter (0.25 µm pore diameter) on LB-agar plates. After overnight incubation at 30°C, cells were scraped off from the mating filter and suspended in 2 ml of M9 salts media and serial dilutions were plated on selective citrate-supplied M9 minimal medium with the appropriate antibiotics to select exconjugants and counterselect donor, helper, and recipient strains.

RNA purification. *Pseudomonas putida* bacterial cells were incubated at 30°C with orbital shaking (200 rpm) and samples harvested at indicated times by centrifugation, instantly frozen with liquid nitrogen and stored at -80°C. Total RNA was extracted using an RNA isolation kit (Macherey-Nagel) and following the manufacturer´s instructions. RNA samples were subsequently treated with RNase-free DNase I (Turbo DNA-free kit, Ambion) to remove DNA traces, as specified by the supplier. RNA concentration was determined using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), RNA integrity was assessed by agarose gel electrophoresis, and the absence of DNA was verified by PCR.

Quantitative Real-time PCR (qRT-PCR). Analysis by qRT-PCR was performed using total RNA preparations obtained from three independent cultures (three biological replicates) using iCycler Iq (Bio-

Rad, Hercules, CA, USA). DNA-free RNA samples (1 μg) were retrotranscribed to cDNA using Superscript II reverse transcriptase (Invitrogen) and random hexamers as primers. Template cDNA from the experimental and reference samples were amplified using the primers listed in table S1. Three experimental replicates were amplified. Each reaction contained 2 μl of a dilution of the target cDNA (1:10-1:10.000) and 23 μl SyBR Green mix (Molecular Probes). Samples were initially denatured by heating at 95°C for 10 min. A 40-cycle amplification and quantification program was then followed (95°C for 15s, 62°C for 30s, and 72°C for 20s) with a single fluorescence measurement per cycle according to manufacturer´s recommendations. PCR products were between 150 and 200 bp in length. To confirm the amplification of a single PCR product, a melting curve was obtained by slow heating from 60°C to 99.5°C at a rate of 0.5°C every 10s, for 80 cycles, with continuous fluorescence scanning. The results were analysed by means of the comparative critical threshold (ΔΔCt) method (Pfaffl, 2001) and normalized to those obtained for 16S rRNA.

Table 3. Primers used in this work.

Prime name	Sequence (5´→3´)[a,b,c]	Use
qRTRpoSF qRTRpoSR	GGCTCTCAGTAAAGAAGTGCCG GTGGCATCGAGCGCCCTG	qRT_PCR (*rpoS*, PP_1623)
qRTCfcRF qRTCfcRR	CTCGACGTTGTGCAACTGAC TCGTGTACGCGGTAGATCTG	qRT_PCR (*cfcR*, PP_4959)
qRT16SF qRT16SR	AAAGCCTGATCCAGCCAT GAAATTCCACCACCCTCTACC	qRT_PCR (16S rRNA)
4959KpnIF 4959rev2	GGTACCAGCGCTACCTGAAA GCATGCGTTATGTGACCGACTTCAA	P_{cfcR}*::'lacZ* transcriptional fusion in pMIR200
4959KpnIF ruprev	GGTACCAGCGCTACCTGAAA AGATCTGCATCCATGCTGCTTCTC	*cfcR'-'lacZ* translational fusion in pMIR219
4959KpnIF ruprev2	GGTACCAGCGCTACCTGAAA AGATCTAACATGCTGCTTCTCTGTTA TGTG	*cfcR*$_{(bmod)}$*'–'lacZ* translational fusion in pMIR220
ATTO700- labeled	AAAAAAAACCCCCCCCC	InfraRedDye-labeled DNA primer
PT7cfcRF PT7cfcRR	TTTTCTGCAGTAATACGACTCACTAT AGGATAGCGTTCTGATTGCATT *AAAAAAAACCCCCCCCC*GTCGTCTAC CACCAGAAGAA	DNA-CfcR(ab) template used for the synthesis of labeled RNA-CfcR(ab); hybridized with the labeled ATTO700 oligo
PT7cfcRF PT7cfcRR2	TTTTCTGCAGTAATACGACTCACTAT AGGATAGCGTTCTGATTGCATT GTCGTCTACCACCAGAAGAA	DNA-CfcR(ab) template used for the synthesis of RNA-CfcR(ab) specific unlabeled competitor (SUC)
PT7Loop1F PT7Loop1R	TTTTCTGCAGTAATACGACTCACTAT AGGATAGCGTTCTGATTGCATT *AAAAAAAACCCCCCCCC*GACTTCAAA ATACGGTTGGA3	DNA-CfcR(a) template used for the synthesis of labeled RNA-CfcR(a); hybridized with labeled ATTO700 oligo
PT7Loop1F PT7Loop1R2	TTTTCTGCAGTAATACGACTCACTAT AGGATAGCGTTCTGATTGCATT GACTTCAAAATACGGTTGGA	DNA-CfcR(a) template used for the synthesis of RNA-CfcR(a) specific unlabeled competitor (SUC)
PT7Loop2F PT7Loop2R	TTTTCTGCAGTAATACGACTCACTAT AGGTCCAACCGTATTTTGAAGTC *AAAAAAAACCCCCCCCC*GTCGTCTAC CACCAGAAGAA	DNA-CfcR(b) template used for the synthesis of labeled RNA-CfcR(b); hybridized with labeled ATTO700 oligo
PT7Loop2F PT7Loop2R 2	TTTTCTGCAGTAATACGACTCACTAT AGGTCCAACCGTATTTTGAAGTC GTCGTCTACCACCAGAAGAA	DNA-CfcR(b) template used for the synthesis of RNA-CfcR(b) specific unlabeled competitor (SUC)

[a] Restriction sites inserted in the primer for the cloning strategy are underlined

[b] T7 polymerase promoter is double underlined

[c] Italics indicate the sequence used to hybridize with labeled ATTO700

Generation of ΔIEAcfcR mutant by homologous recombination. The pMIR153 plasmid (a derivative of pKNG101) containing the inactivated *cfcR* allele (Matilla, *et al.*, 2011) was mobilized from *E. coli* CC118 *λpir* into *P. putida Δrsm*IEA (Huertas-Rosales, *et al.*, 2016) by conjugation using HB101 (pRK600) as a helper, as described

above. Merodiploid exconjugants were first selected in minimal medium with citrate and streptomycin and then incubated in LB medium supplied with 12% sucrose to obtain clones in which a second recombination event had removed the plasmid backbone. Sm-sensitive clones were re-isolated and the presence of the *cfcR* mutation was checked by PCR, followed by sequencing of the corresponding chromosomal region and Southern blotting.

Construction of *cfcR'-'lacZ* translational fusions and P_{cfcR}*::'lacZ* transcriptional fusion. Two translational fusions, one containing a native motif B and the other a modified motif B, were designed to ensure in-frame cloning to '*lacZ* in pMP220-BamHI (Table 2). PCR amplicons of 261 bp contained the *cfcR* promoter, both +1 sites previously determined experimentally (Matilla *et al.*, 2011), ribosome binding site (RBS) and the first 13 nucleotides of the gene. Primers used are listed in table S1. These amplicons were cloned into pCR2.1-TOPO to generate pMIR217 and pMIR218, respectively. The absence of mutations was assessed by sequencing. Subsequently, these plasmids were double digested with Acc65I/BglII and the resulting fragments were each cloned into Acc65I/BamHI sites of pMP220-BamHI to yield pMIR219 and pMIR220.

The transcriptional fusion expanded 246 bp that contained the *cfcR* promoter and both +1 sites of the gene. Primers used are listed in table S1. This amplicon had been cloned into pGEM®-T to generate pMIR199. The absence of mutations was assessed by sequencing. Subsequently, these plasmids were double digested with Acc65I/SphI and the resulting fragment was cloned in pMP220 to yield pMIR200. RBS and ATG for *'lacZ* in pMIR200 were those of *cat* gene in pMP220.

Assay for β-galactosidase activity. Specific β-galactosidase activity from bacterial suspensions growing in liquid cultures was

measured as described (Miller, 1972). An overnight culture of the strain of interest was diluted 1/100 in fresh LB medium supplied with the required antibiotics and grown at 30°C for 1 h. Then the cultures where diluted 1/2 in fresh LB medium and grown at 30°C for 1 more hour to better dilute out any remaining β-galactosidase that may have accumulated in overnight cultures. Finally cultures were diluted to an OD_{600nm} of 0.05 (time 0). Cells were then incubated at 30°C under orbital shaking (200 rpm). At the indicated time points, aliquots were measured for optical density (A_{600}) and β-galactosidase activity. Experiments were carried out in triplicate on two experimental replicates.

Production of Rsm proteins. The expression plasmid pME6032 (Heeb *et al.*, 2002) was used to express His-tagged Rsm proteins (His6-Rsm) in their natural host *P. putida* KT2440. Overnight cultures (10 mL) of KT2440 harboring plasmids for His6-Rsm expression, pME6032-*rsmA*, pME6032-*rsmE* and pME6032-*rsmI* (Huertas-Rosales et al., 2016) were used to inoculate LB rich medium (500 mL) containing the appropriate antibiotic. The later cultures were incubated at 30°C with shaking to reach an OD_{660nm} of 0.8. At this point, expression of His6-Rsm was induced by the addition of IPTG to a final concentration of 0.5 mM. After 6 h, bacterial cells were harvested by centrifugation and cell pellets stored at -80°C. His6-Rsm were purified using Ni-NTA Fast Start Kit (Qiagen). Purifications from cultures involving the empty vector plasmid pME6032 were performed and used as controls for any unspecific binding in EMSA. Because proteins were not purified to homogeneity, the level of purity observed in gels was taken into account in the final quantification. Bradford assay and spectrophotometry techniques were used for protein quantification.

RNA synthesis and fluorescence-based electrophoretic mobility shift assays (fEMSA). DNA-CfcR(ab), DNA-CfcR(a) and

DNA-Cfc(b) templates were generated by PCR using primers that incorporated a T7 promoter at the 5´end and a 17 nt tag at the 3´end (table S1). These PCR amplicons were then used for the synthesis of RNA probes using MAXIscript T7 kit (Life Technologies). RNAs molecules thus obtained and free of DNA could be detected by hybridization with an ATTO700-labeled primer as described by Ying and colleagues (2007). The concentration of this primer in the hybridization reaction was in excess (20-fold >RNA concentration) in order to maximize RNA detection. Purified His6-Rsm proteins at the indicated concentrations were incubated with 5 nM RNA probe in 1 x binding buffer (10 mM Tris-Cl pH [7.5], 10 mM $MgCl_2$, 100 mM KCl), 0.5 µg/µl total yeast tRNA (Life Technologies), 7.5% (vol/vol) glycerol, 0.2 units SUPERase In RNase Inhibitor (Life Technologies). Reactions with or without unlabelled competitor RNA (500 nM) were incubated for 30 min at 30°C, then bromophenol blue was added (0.01% wt/vol) and immediately the samples were subjected to electrophoresis at 4°C on 6% (wt/vol) native polyacrylamide TBE gel (47 mM Tris, 45 mM boric acid, 1 mM EDTA, pH [8.3]). Images were obtained using a 9201 Odyssey Imaging System (LI-COR Biosciences) with Image Studio V5.0 software.

Microtiter plate-based c-di-GMP reporter assays. Microtiter plate-based assays containing the pCdrA::*gfp*^{C} reporter strains were carried out as follows. LB-overnight cultures were diluted to an OD_{660nm} of 0.05 in 1/10 LB in the presence of 20 µg/mL Gm. Growth (OD_{660nm}) and fluorescence (excitation/reading filter 485/535 nm) were monitored in an Infinite 200 Tecan plate reader using Greiner 96 well plates (black flat bottom polystyrene wells). The assays were conducted in triplicate for 24 hours in static with a pulse of shaking just before the measures were registered every 30 minutes. Optical density values of LB and fluorescence values of KT2440 without reporter plasmid pCdrA::*gfp*^{c}

were subtracted from all readings (turbidity and fluorescence, respectively).

Biofilm assays. Time course biofilm formation was assayed by determining the ability of cells to grow adhered to the wells of sterile polystyrene microtiter plates (96 flat base multiwells) as previously described (O'Toole and Kolter, 1998) and monitored in a Tecan Sunrise plate reader. An overnight LB culture was diluted down to a final OD_{660nm} of 0.05 in fresh medium and dispensed at 200 µl/well. Inoculated plates were incubated under static conditions at 30°C for up to 24 h. In order to measure the degree of attachment, every 2 h culture was removed from selected wells, which were then rinsed with 200 µl of distilled water and processed with crystal violet, as detailed below. In addition, biofilm formation was measured in borosilicate tubes. Initially overnight LB cultures were diluted to an OD_{660} of 0.05 in fresh medium and 2 ml of this cell suspension was added to 16x150-mm borosilicate glass tubes, which were incubated in a tube rotator at 40 rpm for up to 24 h. At indicated points, non-adhered cells were removed and the biofilms rinsed with distilled water. Biofilms were stained by the addition of 200 µl of 1% crystal violet (Sigma) for 15 min followed by rinsing with distilled water. Photos were taken and the cell-associated dye was solubilized in 200 µl of acetic acid 30% (v/v) and quantified by measuring the OD_{590nm} of the resulting solution. Experiments were performed twice in triplicate.

Microscopy. Images were taken using Leica stereomicroscope M165FC. Excitation/Emission filter 480/510 nm was used for monitoring GFP fluorescence. Required exposure times varied as indicated.

Statistical methods. Student´s t-test for independent samples ($P<0.01$ or $P<0.05$) was applied as appropiate using "R" program for all statistical analyses.

MATERIAL AND METHODS – Chapter 3

Bacterial strains, culture media and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas putida* KT2440 is a plasmid-free derivative of *P. putida* mt-2, which was isolated from a vegetable-planted field (Nakazawa, 2002) and whose genome is sequenced (Nelson *et al.*, 2002). *Pseudomonas putida* strains were grown at 30ºC, in Luria-Bertani (LB) medium (Bertani, 1951) or M9 defined medium (Sambrook et al., 1989) supplemented with 1mM $MgSO_4$, 6 mg/L ammonium ferric citrate and trace metals as described previously (Yousef-Coronado *et al.*, 2008). Glucose (27mM) or sodium citrate (15mM) was added as alternative carbon sources to defined M9-minimal medium. *Escherichia coli* strains were grown at 37ºC in LB. When appropriate, antibiotics were added to the medium at the following final concentrations (μg/ml): ampicillin 100; kanamycin 25; streptomycin 50 (*E. coli*) or 100 (*P. putida*); gentamycin 50 or 100 as indicated; tetracycline 10, 20 or 200 as indicated. Cell growth was followed by measuring optical density at 660 nm.

DNA techniques. Digestion with restriction enzymes, dephosphorylation, ligation and electrophoresis were carried out using standard methods (Ausubel, 1987, Maniatis *et al.*, 1982) and following manufacturers' instructions. Plasmid DNA isolation and recovery of DNA fragments from agarose gels were done using QIAGEN miniprep and gel extraction kits, respectively. Competent cells were prepared using calcium chloride and transformations were performed using standard protocols (Maniatis *et al.*, 1982). Electrotransformation of freshly plated *Pseudomonas* cells was performed as previously described (Choi *et al.*, 2006).

Table 1. Bacterial strains and plasmid used in this work

Strain or plasmid	Genotype/relevant characteristics	Reference or source
E. coli		
CC118*λpir*	Rif^R, *λpir*, donor strain for pKNG101-derivative plasmids	Herrero *et al.*, 1990
DH5α	*supE*44 *lac*U169(Ø80*lacZ*ΔM15) *hsdR*17 ($r_k^-m_k^-$) *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Woodcock *et al.*, 1989
HB101 (pRK600)	Helper strain harboring a Cm^R *mob tra* plasmid	DeLorenzo
P. putida		
KT2440	Wild-type, prototroph, cured of pWWO derivative of *P. putida* mt-2	Nakazawa (2002)
ΔrsmIEA	Triple null PP_1746/PP_3832/PP_4472 derivative of KT2440	Huertas-Rosales *et al.*, 2016
pME6032	Tc^R, pVS1-p15A derivative. Broad host range *lacI*^{q}-P_{tac} expression vector	Heeb *et al.*, 2002
pOHR37	Tc^R, derivative of pME6032 for the expression of *rsmA* gene	Huertas-Rosales *et al.*, 2016
pOHR38	Tc^R, derivative of pME6032 derivative for the expression of *rsmE* gene	Huertas-Rosales *et al.*, 2016
pOHR40	Tc^R, derivative of pME6032 for the expression of *rsmI* gene	Huertas-Rosales *et al.*, 2016

Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; MCS, multicloning site; Tc, tetracycline

Triparental conjugations. Transfer of plasmids from *E. coli* to *P. putida* strains was performed by triparental matings using *E. coli* (pRK600) as a helper. For each strain, cells were collected from 0.5 ml of overnight LB cultures via centrifugation, then washed and suspended in 50 μl of fresh LB, and finally spotted on mating filters (0.25 μm pore diameter) on LB-agar plates. After overnight incubation at 30°C, cells were scraped off from the mating filter and suspended in 2 ml of M9 salts media and serial dilutions were plated on selective citrate-supplemented M9 minimal medium with the appropriate antibiotics to select exconjugants and counterselect donor, helper, and recipient strains.

Purification of total RNA and RNA from Rsm-RNA complexes. Plasmid pME6032 (Heeb *et al.*, 2002) was used to express His-tagged Rsm proteins in its natural host *P.* putida KT2440. Overnight

cultures (10 mL) harbouring His-tagged Rsm expression plasmids were used to inoculate LB rich medium (500 mL) containing Tc 100 µg/mL. These cultures were incubated at 30°C under shaking to reach an OD_{660nm} of 0.8. At this point, expression of His-tagged-Rsm proteins was induced for 6 h by the addition of IPTG to a final concentration of 0.5 mM. Three biological replicates were run in parallel. Alliquots of 1.5 ml were harvested by centrifugation, instantly frozen with liquid nitrogen and stored at -80°C for total RNA purification. Cells from the remaining cultures were also harvested and cell pellets stored at -80°C. His-tagged Rsm-RNA complex purification were isolated using Ni-NTA Fast Start Kit (Qiagen).

Total RNA and RNA from Rsm-RNA complex was extracted using RNA isolation kit (Macherey-Nagel) following the manufacturer´s instructions. RNA samples were subsequently treated with RNase-free DNase I (Turbo DNA-free kit, Ambion) to remove DNA traces, as specified by the supplier. Total RNA quality was assessed using Agilent RNA 6000 Nano Kit (Agilent Tecnologies, 5067-1511) in the Agilent 2100 Bioanalyzer. RNA concentration was measured using Qubit RNA BR assay kit (Life technologies, Q10210). 1µg of RNA was used for rRNA depletion using Ribo-Zero rRNA Removal Kit (Illumina, MRZMB126).

Generation of c-DNA libraries and sequencing. Strand-specific cDNA libraries were then generated using NEBNext Ultra Directional RNA Library Prep kit for Illumina (NEB, E7420S). Dual Index Primers Set 1 was used to generate bar-coded multiplex libraries (NEB, E7600S). Library QC was performed using bioanalyser HS kit (Agilent biotechnologies, 5067-4626). cDNA libraries were quantified using qPCR (Kapa Biosystems, KK4824). Libraries were pooled at desired concentrations, denatured and loaded for sequencing according to manufacturer's instructions. Sequencing was performed on the Illumina NextSeq500 sequencing platform to generate 2 x 75bp reads.

Bioinformatic analysis. Filtered reads were aligned to reference genome *P. putida* KT2440 [GenBank] with Bowtie v2 (Langmead et al., 2009). Alignment .sam file was analyzed by MACS v14 to detect reads enriched regions in the genome (Zhang *et al.*, 2008).

Enriched regions with a score $-10 \cdot \text{Log}_{10}(P\text{-value})$, ≥ 200 (for RsmA and RsmI) or ≥ 300 (for RsmE) and a fold enrichment e≥ 3 (for RsmA and RsmI) or ≥ 4 (for RsmE) were selected for further studies.

For further analysis only peaks present in the three technical replicates were considered.

InterPro and Gene Ontology semantic values were used to assign categories to genes close to enriched regions.

Statistical analysis. SigmaStat software package (Systat software) was used for all statistical analyses. The data were compared using Student´s t-test for independent samples ($P<0.05$ or $P<0.1$).

Biolog plates growth curves. We used the compounds present in the BIOLOG plates (Biolog Inc. Hayward, CA, USA; http://www.biolog.com/microID.html) to assess growth of the triple Δ*rsmIEA* mutant and the WT strain under different nitrogen and carbon sources. Starting from M9 with glucose as carbon source undershaking cultures, bacterial cells were cultured at 30ºC in M8 defined medium (Sambrook *et al.,* 1989) supplemented with 0.1 g/L NH_4Cl, 1mM $MgSO_4$, 6 mg/L ammonium ferric citrate and trace metals as described previously (Yousef-Coronado *et al.,* 2008). To study the effect of different sulfur and phosphate sources, the trace metals solution was prepared with $CuCl_2$ and $MgCl_2$ instead of $CuSO_4$ and $MgSO_4$.

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IV. RESULTS

Chapter 1

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Summary

In the plant-beneficial bacterium *Pseudomonas putida* KT2440, three genes have been identified that encode posttranscriptional regulators of the CsrA/RsmA family. Their regulatory roles in the motile and sessile lifestyles of *P. putida* have been investigated by generating single-, double-, and triple-null mutants and by overexpressing each protein (RsmA, RsmE, and RsmI) in different genetic backgrounds. The *rsm* triple mutant shows reduced swimming and swarming motilities and increased biofilm formation, whereas overexpression of RsmE or RsmI results in reduced bacterial attachment. However, biofilms formed on glass surfaces by the triple mutant are more labile than those of the wild-type strain and are easily detached from the surface, a phenomenon that is not observed on plastic surfaces. Analysis of the expression of adhesins and exopolysaccharides in the different genetic backgrounds suggests that the biofilm phenotypes are due to alterations in the composition of the extracellular matrix and in the timing of synthesis of its elements. We have also studied the expression patterns of Rsm proteins and obtained data that indicate the existence of autoregulation mechanisms.

Importance

Proteins of the CsrA/RsmA family function as global regulators in different bacteria. More than one of these proteins is present in certain species. In this study, all of the RsmA homologs in *P. putida* are characterized and globally taken into account to investigate their roles in controlling bacterial lifestyles and the regulatory interactions among them. The results offer new perspectives on how biofilm formation is modulated in this environmentally relevant bacterium.

Introduction

Proteins belonging to the CsrA/RsmA family are small (less than 7 kDa) RNA-binding proteins that play key roles in the regulation of gene expression in diverse Gram-negative and Gram-positive bacteria. CsrA (Carbon Storage Regulator) was first described in *Escherichia coli* (Romeo *et al.*, 1993, Romeo, 1998), where it plays a major role in controlling the intracellular carbon flux, acting as a negative regulator of glycogen metabolism and several enzymes involved in central carbohydrate metabolism (Yang *et al.*, 1996, Sabnis *et al.,* 1995). Members of the CsrA/RsmA family have subsequently been found to be important elements in global posttranscriptional regulation in many other bacterial genera. In the opportunistic human pathogen *Pseudomonas aeruginosa*, the CsrA homolog RsmA (repressor of secondary metabolism) negatively regulates the production of virulence determinants, such as hydrogen cyanide, pyocyanin, or LecA (PA-1L) lectin, as well as *N*-acylhomoserine lactone (AHL) quorum-sensing signal molecules (Pessi and Haas 2001; pessi *et al.*, 2001). In this bacterium, RsmA also represses the translation of the *psl* operon, responsible for the synthesis of one of the two main exopolysaccharides (EPSs) that contribute to the extracellular matrix of biofilms in nonmucoid strains of *P. aeruginosa* (Irie *et al.,* 2010). RsmA promotes the planktonic lifestyle of *P. aeruginosa*, functioning in opposition to the increase in the second messenger c-di-GMP, which leads to a sessile lifestyle, and some of the molecular elements connecting the two regulatory networks are being characterized (reference Colley *et al.,* 2016 and references therein).

RsmA homologs act posttranscriptionally, often by binding to mRNA at or near the ribosome-binding site, thus modulating translation (Liu *et al.,* 1995; Liu and Romeo, 1997). For example, in *Pseudomonas fluorescens*, RsmA represses the production of hydrogen cyanide during exponential growth by reducing the translation rate of *hcnA*, the gene coding for hydrogen cyanide synthase. A specific sequence near the

ribosome-binding site was shown to be required for RsmA activity to be evident on *hcnA*::*lacZ* translation (Blumer *et al.*, 1999). In *E. coli*, CsrA mediates posttranscriptional repression of glycogen biosynthesis by binding to the 5´ leader transcript of *glgC* and inhibiting its translation (Baker *et al.*, 2002). CsrA can also act directly or indirectly as a positive regulator of gene expression: in *E. coli*, it activates genes involved in glycolysis and the glyoxylate shunt (Pernestig *et al.*, 2003) and in flagellar motility. In the last case, binding of CsrA to a 5´ segment of *flhDC* mRNA stimulates its translation and extends its half-life (Wei *et al.*, 2001).

The effects of RsmA/CsrA are relieved by small regulatory RNA molecules that sequester multiple units of the proteins, thereby modulating their activity. Such antagonistic small RNAs include CsrB and CsrC in *E. coli* (Romeo *et al.*, 1993; Weilbacher *et al.,* 2003); RsmB in *Erwinia carotovora* (Liu *et al.,* 1998); and RsmX, RsmY, and RsmZ in *P. fluorescens* (Heeb *et al.*, 2002; Kay *et al.*, 2005). This kind of posttranscriptional control may facilitate rapid, potentially reversible regulation of diverse cellular functions.

The RsmA family proteins and their cognate small RNAs described so far in *Pseudomonas* are part of the GacS/GacA signal transduction pathway, which operates an important metabolic switch from primary to secondary metabolism in many Gram-negative bacteria and also affects enzyme synthesis and secretion (Blumer *el al.,* 1999; Heeb and Haas 2002; Hytiainen *et al.,* 2001). GacS is a sensor histidine kinase that responds to an as yet unidentified signal and phosphorylates the response regulator GacA, causing its activation. In *P. aeruginosa*, GacA positively regulates the quorum-sensing machinery and the expression of several virulence factors via a mechanism involving the participation of RsmA as a negative-control element (Pessi and Haas, 2001; Reimmann *et al.,* 1997). The main regulatory targets of GacA correspond to the small RNAs of the *rsmXrsmY-rsmZ* family that interact with RsmA (Lapouge *et al.*, 2008).

In this study, we analyzed the roles and expression of the three RsmA family proteins present in the plant-beneficial bacterium *Pseudomonas putida* KT2440. Our results indicate that these proteins have different effects on motility, biofilm formation, and dispersal of *P. putida* KT2440, altering the expression of adhesins and exopolysaccharides. These effects may depend on regulatory interactions between the three Rsm proteins.

Results

CsrA/RsmA family proteins and their cognate small RNAs (sRNAs) in *P. putida* KT2440. An *in silico* analysis of the *P. putida* KT2440 genome indicated that there are three genes in the bacterium encoding CsrA/RsmA family proteins (PP_1746, PP_3832, and PP_4472), which show 46%, 70%, and 75% amino acid identity with CsrA of *E. coli*, respectively. Alignment of these protein sequences with those of RsmA of *P. aeruginosa* PAO1 and the three homologs annotated in the genome of *P. fluorescens* F113 (Fig. 1) led to the following gene nomenclature in KT2440: PP_1746 corresponds to *rsmI*, PP_3832 to *rsmE*, and PP_4472 to *rsmA*. RsmA (62 amino acids) and RsmE (65 amino acids) of *P. putida* share 54% identical residues, while RsmI (59 amino acids) shows 43% identity with the other two proteins (Fig. 1). RsmI is also the most divergent in terms of conserved residues that interact with RNA (Fig. 1). None of the proteins is equivalent to RsmF/RsmN of *P. aeruginosa*, which are different in structure and sequence from other Rsm proteins, and no homolog of RsmN could be found in *P. putida* KT2440 based on sequence analysis. In *P. fluorescens*, three noncoding sRNAs interact with Rsm proteins: *rsmX*, *rsmY*, and *rsmZ* (Heeb et al., 2002, Kay et al., 2005). Previous work had allowed the identification of the sRNAs *rsmY* and *rsmZ* in *P. putida* DOT-T1E (Molina-Santiago *et al.,* 2015). Based on sequence homology analysis, we have identified the equivalent sRNAs in KT2440 in the intergenic regions

between the loci PP_0370 and PP_0371 (corresponding to *rsmY*) and between the loci PP_1624 and PP_1625 (corresponding to *rsmZ*). These sRNAs are 72% and 78% identical to their counterparts in *Pseudomonas protegens* Pf-5 (Fig.1). The presence or identity of *rsmX*, on the other hand, is less clear. Based on comparison with the *rsmX* sequences of *P. protegens* Pf-5 and *P. fluorescens* F113, a potential candidate could be located in the intergenic region between PP_0214 and PP_0215 (Fig. 1), but the percent identity was much lower than that of the other two sRNAs (55%).

Generation of *rsm* single, double, and triple mutants. We generated *rsmI*, *rsmE*, and *rsmA* null mutants by complete deletion of the open reading frames without introducing antibiotic resistance cassettes, as well as the three double-mutant combinations and the triple mutant (see Materials and Methods for details). For simplicity, here, these mutants are designated ΔI, ΔE, ΔA, ΔIE, ΔEA, ΔIA, and ΔIEA. The growth of all the mutants was analyzed in liquid cultures in rich and defined minimal media. No differences were observed in LB medium (not shown), whereas in minimal medium, the triple mutant showed an extended lag phase with respect to the wild type, regardless of the carbon source. The same delay was also observed in the ΔEA mutant growing in minimal medium with citrate as a carbon source and, although less pronounced, in the ΔA and ΔEA mutants in minimal medium with glucose as a carbon source (Fig. 2).

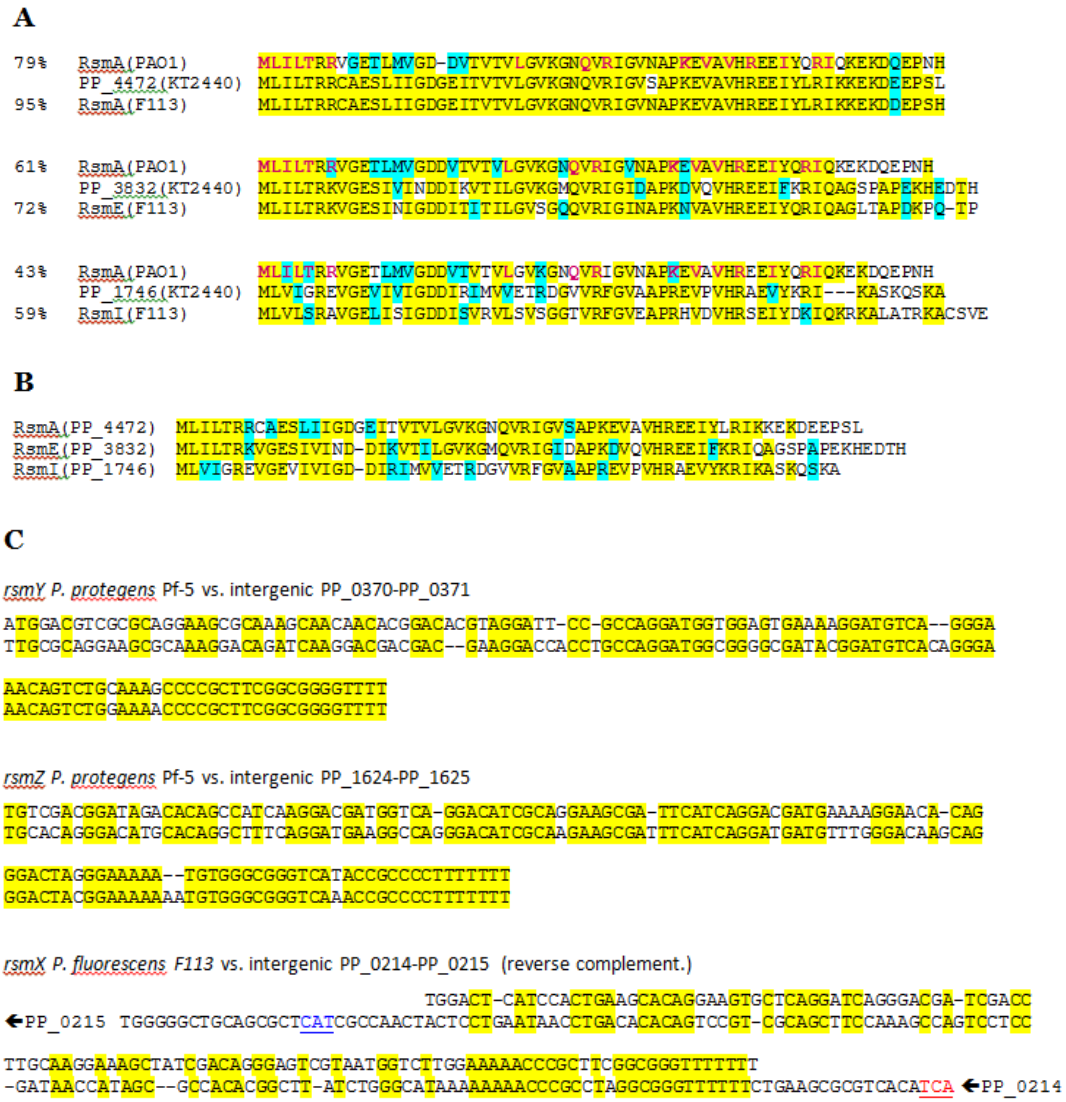

Figure 1. A) Alignment and percent identities of each Rsm protein of *P. putida* KT2440 with RsmA of *P. aeruginosa* and their respective homolog in *P. fluorescens* F113. **B)** Amino acid sequence comparison between the three Rsm family proteins of *Pseudomonas putida* KT2440. In both panels A and B, yellow and turquoise shading indicate identical residues and conservative changes, respectively. Residues involved in mRNA recognition are shown in magenta boldface (Schubert M. et al 2007). **C)** Identification of chromosomal regions encoding small RNAs *rsmY*, *rsmZ* and putative *rsmX* in *P. putida* KT2440, by comparison with the corresponding sequences of *P. protegens* Pf-5 and P. *fluorescens* F113. Identical nucleotides are shaded in yellow. Start and stop codons of PP_0215 and PP_0214 are underlined and shown in blue and red, respectively.

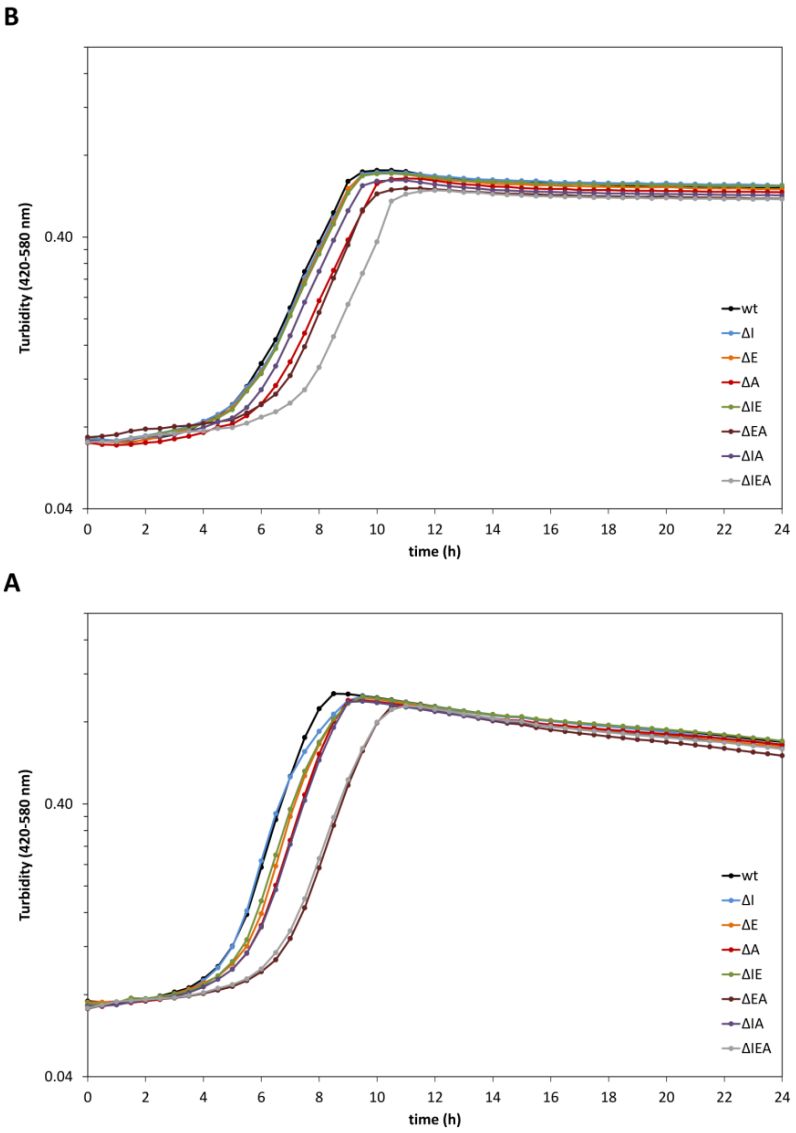

Figure 2. Growth of wild type KT2440 (KT WT) and *rsm* mutants in minimal medium with 10 mM citrate **A)** or 5mM glucose **B)** as carbon source. Cultures were grown overnight and inoculated in each medium at an initial OD_{660} of 0.05, in triplicate. Turbidity was monitored every 30 min. during growth at 30°C in a BioScreen and average growth curves of each strain are shown.

Specific *rsm* mutations alter the motility of *P. putida*. In *P. fluorescens* F113, it has been reported that mutants affected in the *gacS-gacA* regulatory system show hypermotility, and a regulatory cascade involving RsmE and RsmI has been proposed (Martínez-Granero *et al.,* 2012). This, and the fact that in different bacteria the main target of the response regulator GacA is the RsmA/*rsmX-rsmY-rsmZ* regulon and that CsrA modulates flagellar genes in *E. coli* (Wei *et al.,* 2001), led us to test the influence of *rsm* mutations on the motility of *P. putida* KT2440. As shown in Fig. 3A, the triple mutant and the ΔEA strain presented reduced swimming motility with respect to the wild type and the rest of the mutants in LB plates with 0.3% agar.

The effect of *rsm* mutations on surface motility was then analyzed on 0.5% agar plates, as previously described (Matilla *et al*., 2007). Whereas the wild type had nearly covered the surface of the plate after 24 h, none of the mutants showed movement at that time, with the exception of ΔI (Fig. 3B), although that mutant was unable to completely cover the plate surface. This type of motility requires pyoverdine-mediated iron acquisition in KT2440 (Matilla *et al.,* 2007). This prompted us to check if any of the mutants showed altered pyoverdine production that could correlate with the defect in swarming. Pyoverdine was measured in the supernatants of cultures grown overnight in King's B medium (Fig. 4). All the mutant strains showed a reduction in pyoverdine production with respect to the wild type that could be associated with the observed alteration in swarming motility. However, the difference in motility between ΔI and the remaining mutants cannot be explained simply in terms of pyoverdine production, which was not significantly different between them (Fig. 4).

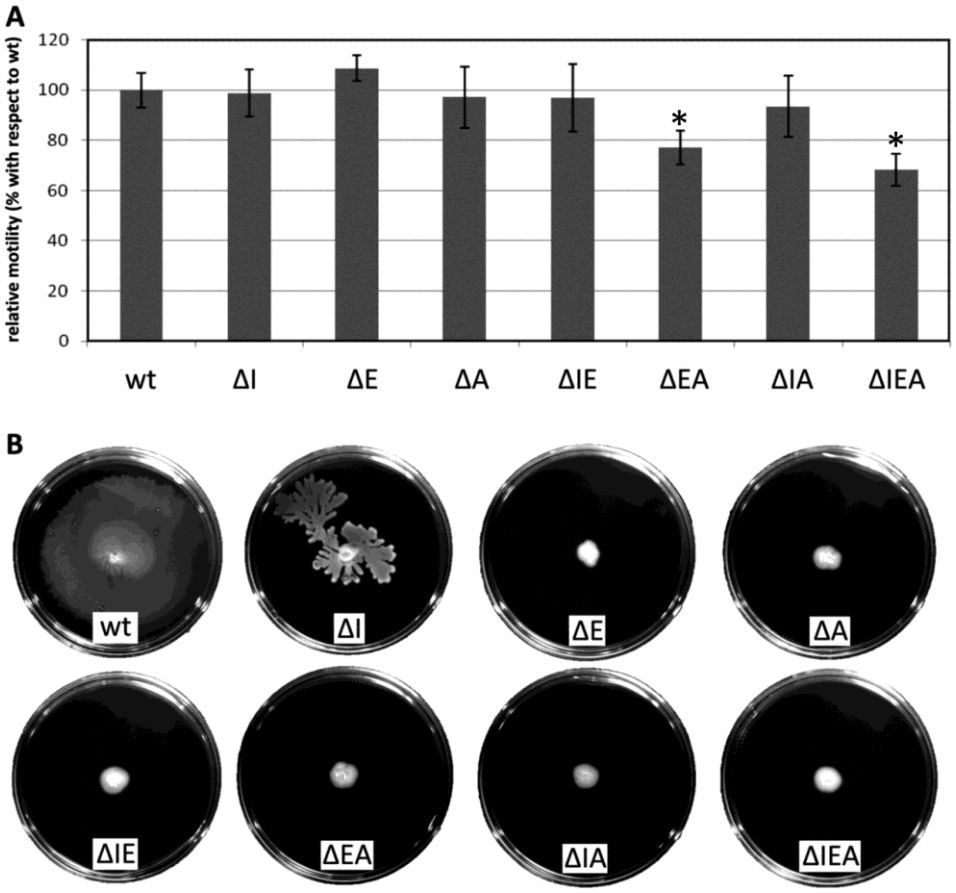

Fig. 3 Influence of *rsm* mutations on motility of *P. putida*. **A)** Swimming motility on LB plates with 0.3% agar. The graph indicates the areas covered by swimming halos after overnight growth. The data are the averages and standard deviations of 9 replicates. The asterisks indicate statistically significant differences (ANOVA; $P < 0.05$). **B)** Surface motility of KT2440 and the *rsm* single, double, and triple mutants. The images were taken after 48 h of growth and show a representative experiment out of three independent replicates.

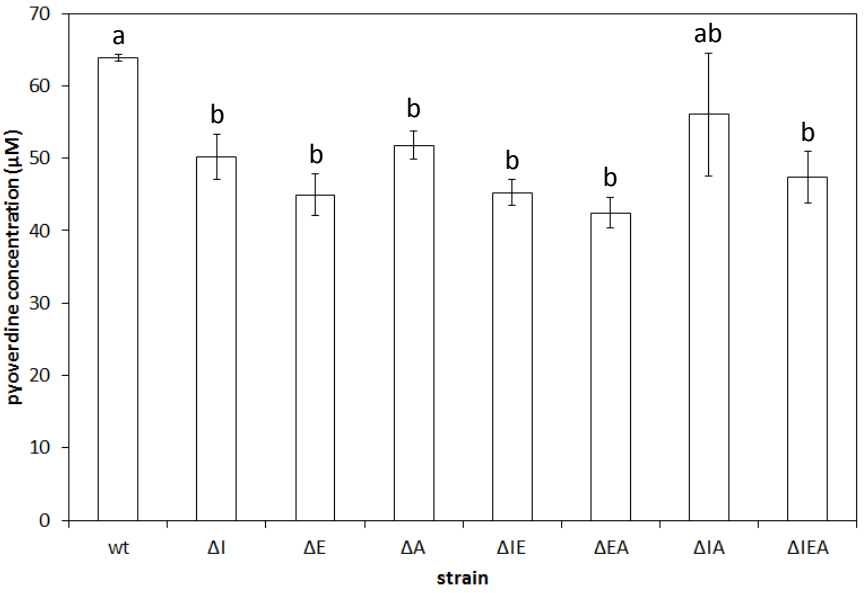

Figure 4. Pyoverdine concentration in supernatants of cultures grown overnight in King's B medium, calculated by measuring absorbance at 405 nm (molar extintion coefficient of pyoverdine = $1{,}9\times10^{-4}$ $M^{-1}\times cm^{-1}$). Data are averages and standard deviations of three biological replicas with three technical replicas each, normalized to $OD_{660} = 1$ to correct for potential growth differences between cultures. Different letters are indicative of statistically significant differences between datasets (1-way ANOVA, $P<0.05$).

Rsm proteins modulate biofilm development. Previous results have shown that *gacS* is involved in the regulation of biofilm formation in *P. putida* KT2440 (Martínez-Gil *et al.,* 2014). We therefore examined the effects of *rsm* mutations on surface attachment and biofilm development. Assays were first done with cultures grown statically in polystyrene multiwell plates, following the attachment/detachment dynamics over time by staining the surface-associated biomass with crystal violet. As shown in Fig. 5A, each of the *rsm* single mutants behaved differently in these assays. The ΔI mutant initiated attachment like the wild type but showed early detachment from the surface. The ΔE and ΔA strains, on the other hand, presented reduced attachment during the first hours, reaching attached biomass values similar to those of the wild type at later times. The double and triple mutants were then

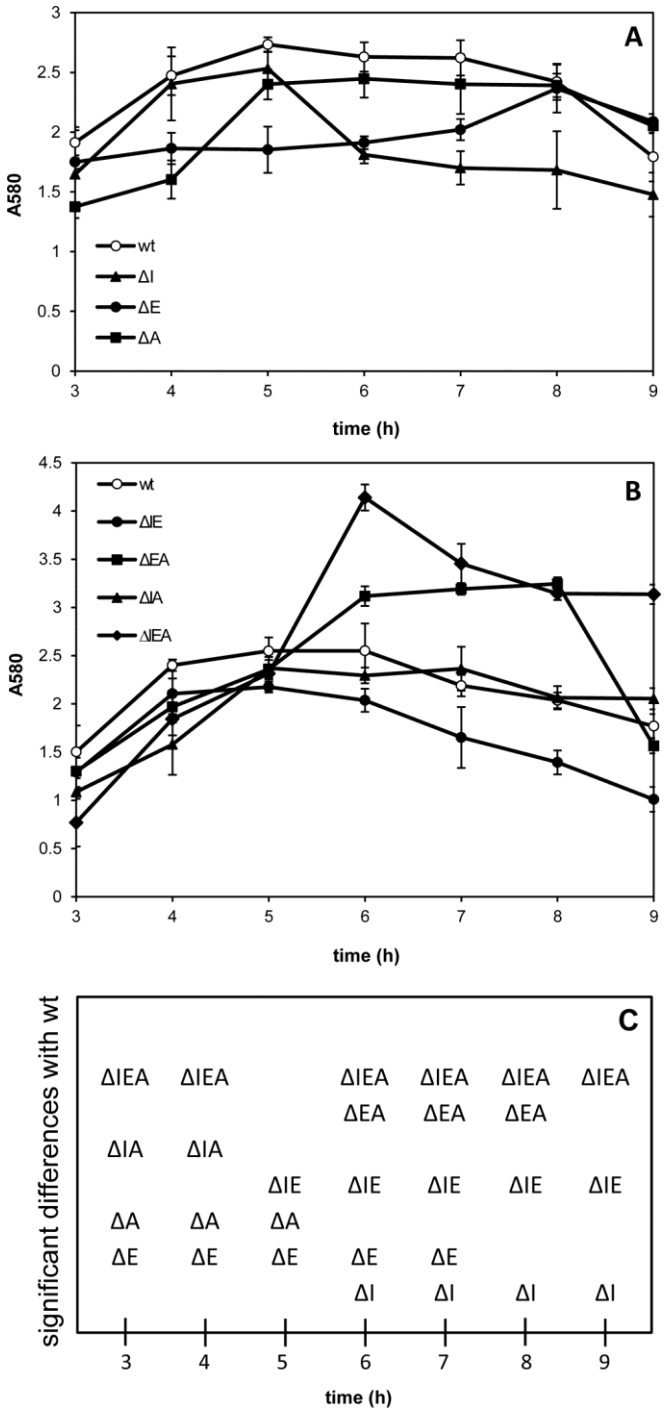

FIG 5 Biofilm formation by KT2440 and *rsm* mutant derivatives in polystyrene multiwell plates. Overnight cultures grown in LB medium were diluted to an OD660 of 1, and 5 µl was added to each well containing 200 µl of LB medium. Attachment was followed by removing the liquid from the wells at the indicated times and staining with crystal violet. The data correspond to the measurement of absorbance at 580 nm (*A*580) after solubilization of the dye and are the averages and standard deviations of three independent experiments, each with three replicates per strain. **A)** Wild type and single mutants. **B)** Wild type, double mutants, and triple mutant. **C)** Mutants showing significant differences from the wild type at a given time point (Student's *t* test; $P \geq 0.05$).

analyzed (Fig. 5B). In ΔEA and ΔIEA, the attached biomass was significantly above that of the wild type after 6 h, whereas ΔIA and ΔIE kept a kinetic similar to that of the wild type, although the values for the latter were slightly below those of the wild type throughout.

Experiments were also done in borosilicate glass tubes with cultures grown under orbital rotation. We first compared the wild type and the triple mutant over time (Fig. 6A). Observation of the tubes before staining with crystal violet indicated similar attachment dynamics at early time points, with the ΔIEA mutant forming a significantly denser biofilm than the wild type at later time points. However, at these times, the biofilm of the mutant proved to be more labile than that of the wild type, so that it was washed from the surface during staining with crystal violet. A follow-up of this phenomenon in the remaining mutants indicated that ΔEA behaved like the triple mutant and was washed from the surface during staining after 7 h of growth (Fig. 6B). The ΔA mutant was also partly removed at this time, whereas the remaining mutants showed this phenotype at later times, while the biofilm of the wild type remained stainable.

The development of biofilms of KT2440 and the triple mutant tagged with mCherry was also followed in microscopy-ready multiwell plates by CLSM during growth under static conditions. The results presented in Fig. 7 show that under these conditions, ΔIEA started colonizing the surface faster than the wild type and also detached earlier.

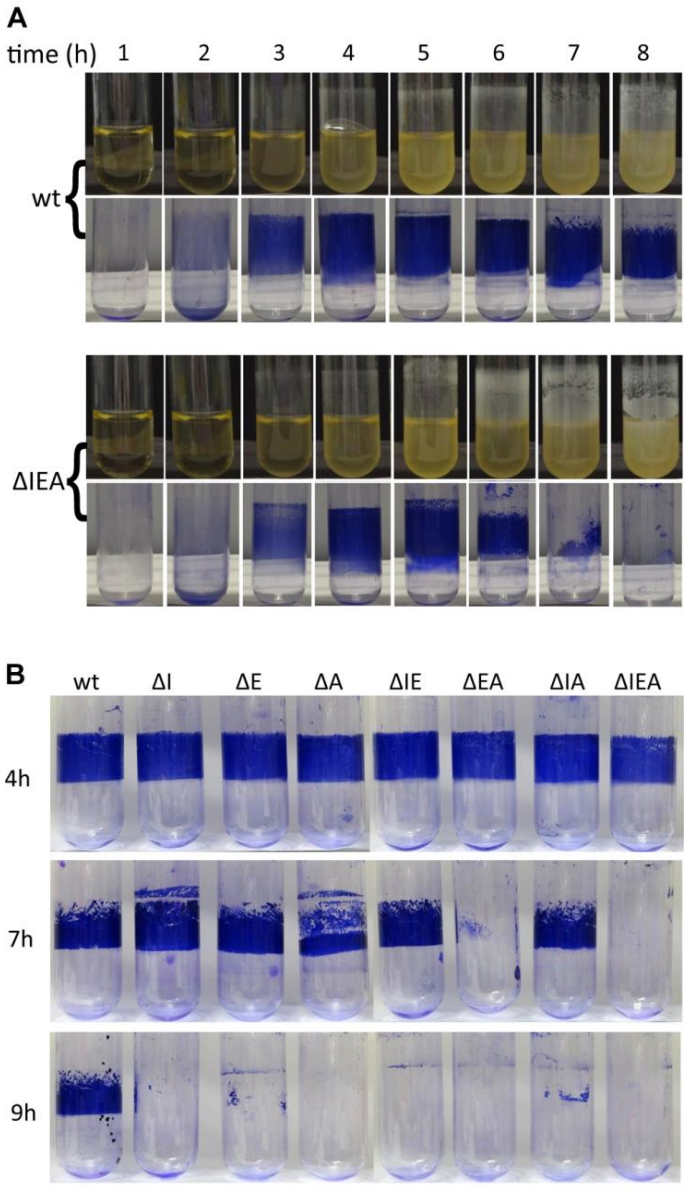

FIG 6 Kinetics of biofilm formation. **A)** Biofilm formation by KT2440 and the *rsm* triple mutant (ΔIEA) growing in LB medium in borosilicate glass tubes under orbital rotation. At the indicated times, tubes were removed and images (top) were taken before discarding planktonic cells and staining with crystal violet (bottom). **B)** Evaluation of the surface-attached biomass in KT2440 and the seven *rsm* mutants at different times of biofilm development. Growth conditions were as for panel A. Images from a representative experiment out of three replicates are shown (different experiments are represented in panels A and B).

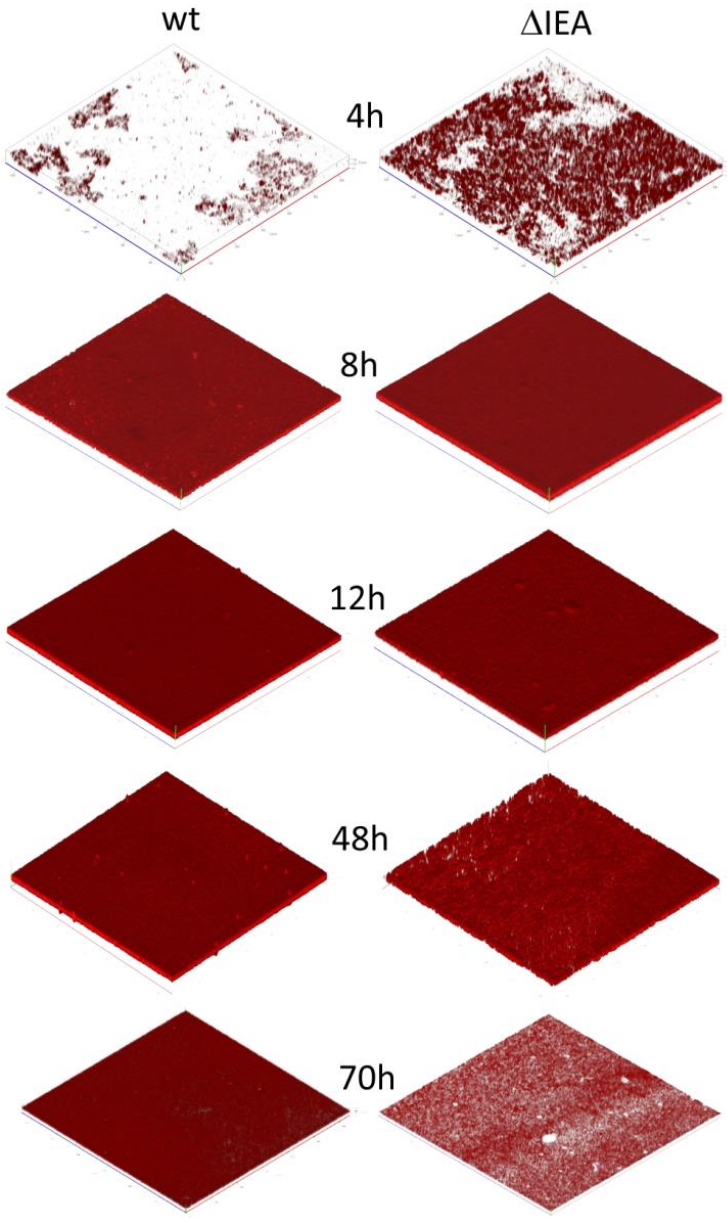

FIG 7 CLSM follow-up of biofilm formation by KT2440 and the *rsm* triple mutant, ΔIEA. Strains were tagged with miniTn*7*Ptac-mChe (*miniTn7:: mCherry*) in single copy at an intergenic location in the chromosome. Bacterial cells were grown in LB medium diluted 1:10 in microscopy-ready multiwell plates. Three-dimensional reconstructions, generated with Imaris, of representative fields are shown.

Overexpressing RsmE or RsmI, but not RsmA, causes reduced biofilm formation in KT2440. To further explore the role of Rsm proteins in biofilm formation, each protein was cloned independently in an expression vector that is stable in *Pseudomonas* (Heeb *et al.*, 2002) under the control of IPTG (isopropyl-β-D-thiogalactopyranoside). These constructs were introduced into the wild-type KT2440, and biofilm formation was analyzed during growth in LB medium in the presence or absence of IPTG. As shown in Fig. 8, overexpressing RsmE caused a reduction in biofilm formation. The increase in RsmI had a similar, although less pronounced, effect, whereas the increase in RsmA had no obvious influence on biofilm formation. Quantification of the attached biomass after solubilization of the dye revealed 70% and 50% reductions due to overexpression of RsmE and RsmI, respectively (Fig. 9).

To investigate the potential interplay between the different Rsm proteins, we also introduced each of the expression constructs in all the mutants and analyzed biofilm formation on borosilicate glass tubes (Fig. 8 and Fig. 9). As with the wild type, overexpression of RsmA had no significant effect regardless of the genetic background, whereas overexpression of RsmE resulted in decreased biofilm formation in all the strains, with small differences between them. Interestingly, the effect observed in the wild type when RsmI was overexpressed was nearly lost in the triple mutant. Analysis of the remaining mutants showed that the presence of intact *rsmA* was required for the reduced biofilm phenotype associated with RsmI overexpression. It should be noted that at the time at which this analysis was done (5 h after inoculation), the reduction in crystal violet staining was correlated with the visual observation of attached biomass, and it was not a consequence of the biofilm lability that can be observed at later times (shown in Fig. 6).

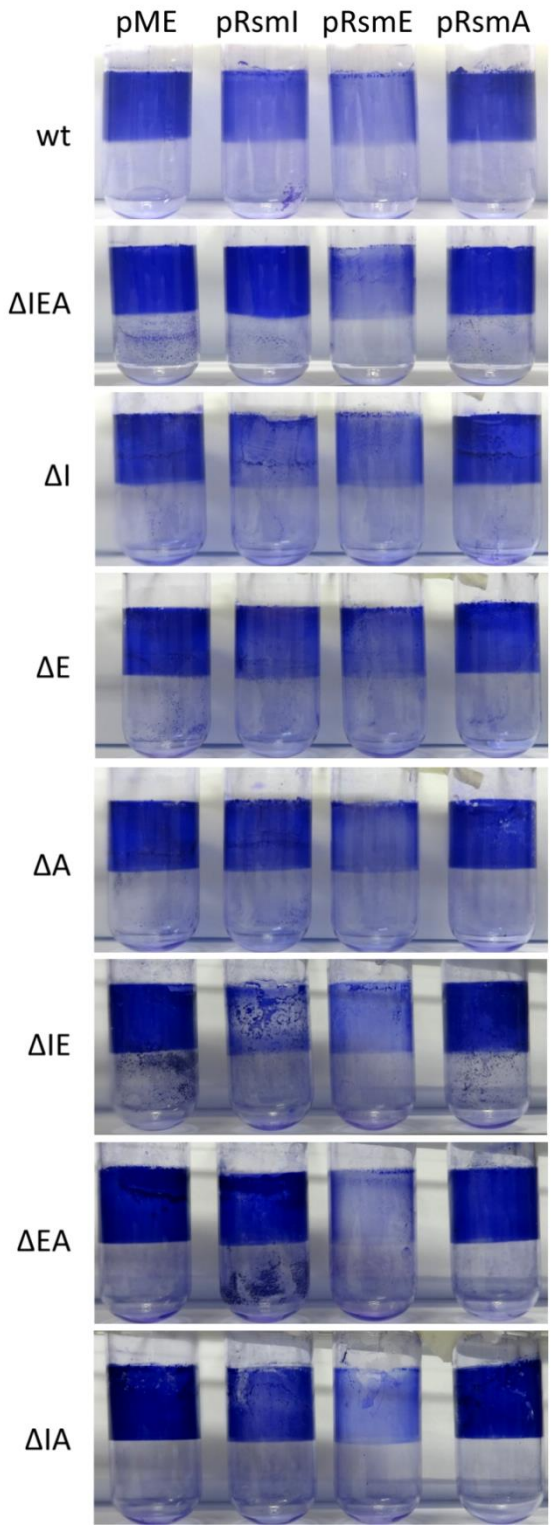

FIG 8 Effects of overexpressing each Rsm protein on biofilm formation by KT2440 and the seven *rsm* mutant derivatives. The genes *rsmI*, *rsmE*, and *rsmA* were cloned in the broad-host-range expression vector pME6032. The resulting constructs (pRsmI, pRsmE, and pRsmA), as well as the empty vector (pME) as a control, were introduced into all the strains. Experiments were done as for Fig. 3. The images show attached biomass stained with crystal violet after 5 h of growth in the presence of 0.1mMIPTG and 20 μg/ml tetracycline. wt, wild type.

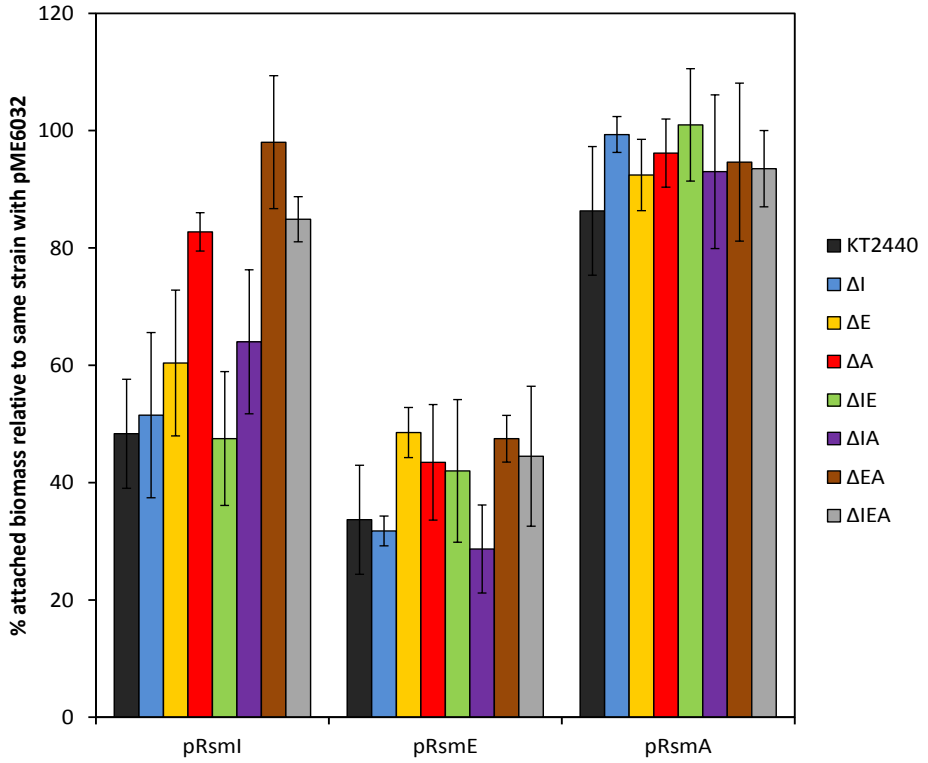

Figure 9. Quantitative analysis of the influence of overexpressing each Rsm protein on biofilm formation by KT2440 and *rsm* mutant backgrounds. Attached biomass (see Fig. 5) was quantified by measuring absorbance at 580 nm after crystal violet staining and solubilization of the dye with 30% acetic acid. Values are given in percentage relative to the same strain carrying the empty vector pME6032, which corresponds to a value of 100% (Averages and S.D. from three different experiments).

Influence of Rsm proteins on expression of structural elements of *P. putida* biofilms. The results described so far could suggest the possibility that Rsm proteins participate in biofilm formation by altering the surface characteristics of *P. putida* cells, which would explain why the triple mutant showed thicker but more labile biofilms on glass surfaces while remaining attached on plastic surfaces. This, and the fact that in *P. aeruginosa* RsmA modulates expression of the Psl

exopolysaccharide (Irie *et al.*, 2010), prompted us to investigate if any of the mutations caused changes in the expression of structural elements known to participate in the buildup of *P. putida* biofilms under different conditions, namely, the two large adhesins LapA and LapF and the exopolysaccharides Pea, Peb, cellulose (Bcs), and alginate (Alg).

To determine if *rsm* mutations had an influence on the mRNA levels of genes involved in exopolysaccharide biosynthesis, qRT-PCR was done with RNA extracted from the wild-type and ΔIEA strains grown on solid medium for 24 or 48 h, using primers that correspond to the loci PP_1277 (Alg), PP_1795 (Peb), PP_2629 (Bcs), and PP_3132 (Pea).

As shown in Fig. 10, there was a significant increase in the mRNAs corresponding to Peb (2.5-fold) and Bcs (5-fold) in the triple mutant with respect to the wild type, whereas no significant differences were observed for Pea (PP_3132) and Alg (PP_1277). Analysis of these differences in the single mutants after 48 h indicated that in the case of Peb, the lack of either RsmE or RsmA had an effect similar to that observed in the triple mutant, while RsmI did not appear to influence its expression (Fig. 10B). For Bcs, increased expression was observed in ΔE and ΔA, but in neither case did it reach the levels observed in ΔIEA, suggesting a cumulative effect of both proteins.

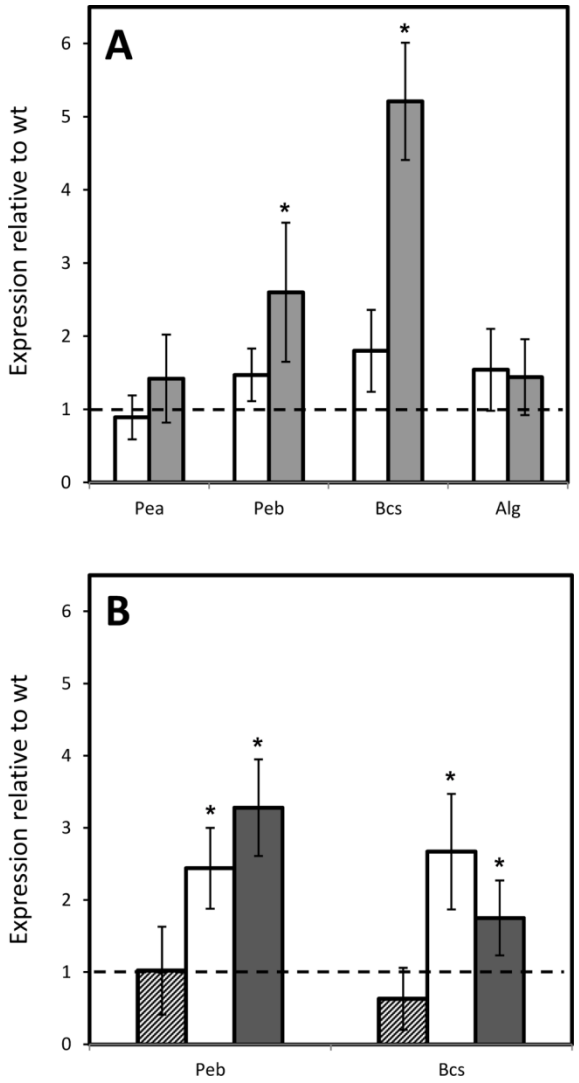

FIG 10 Influence of *rsm* mutations on mRNA levels of EPS-encoding genes in cultures grown in solid medium. **A)** mRNA levels of EPS genes in the *rsm* triple mutant relative to the wild type analyzed by qRT-PCR. RNA was isolated from samples grown on LB agar plates after 24 h (white bars) or 48 h (gray bars). A value of 1 (dashed line) indicates expression levels identical to those of the wild type. **B)** Relative expression of genes corresponding to cellulose (Bcs) and the specific EPS Peb in mutants ΔI (hatched bars), ΔE (gray bars), and ΔA (white bars) with respect to the wild type after 48 h of growth on LB agar plates. The data are the averages and standard deviations from three biological replicates with three technical replicates. The values significantly different from those of the wild type are indicated by asterisks (Student's *t* test; $P \geq 0.05$).

Since Rsm proteins may regulate translation with or without effects onmRNAtranscript levels, we decided to expand this analysis by checking the expression patterns of fusions of the first gene in each EPS cluster (including the promoter and first codons) with the reporter gene *lacZ* devoid of its own promoter, as described elsewhere (Molina-Henares *et al.,* 2016).

The results obtained comparing the wild types with the ΔIEA mutant are shown in Fig. 11. In all cases, the pattern of expression showed alterations at different times during growth in liquid medium, with an overall increase in activity in the mutant. In the case of the fusion corresponding to Pea, the differences were significant only between 4 and 8 h of growth, which could explain why qRT-PCR at 24 at 48 h did not reveal alterations in the ΔIEA mutant. Analysis of the fusions in all the mutant backgrounds indicated that in all cases except Peb, the combination of *rsmA* and *rsmE* mutations was responsible for most of the observed changes in the triple mutant (Fig. 11A, C, and D), whereas no single or double mutant had the same pattern as ΔIEA in the case of Peb (data not shown).

Next, expression of the two adhesins was followed during growth by measuring the β-galactosidase activity of *lapA*::´*lacZ* and *lapF*::´*lacZ* fusions carried on plasmids pMMGA and pMMG1, respectively (Martínez-Gil *et al*., 2010; Martínez-Gil *et al.,* 2014), in the wild type and all the *rsm* mutants. No great differences were observed for *lapA* in the different genetic backgrounds, except for a slight overall increase in ΔIEA (Fig. 11E) that was not significant. In contrast, expression of *lapF*::´*lacZ* was clearly influenced by the three Rsm proteins, and the triple mutant showed earlier induction and increased β-galactosidase activity with respect to the wild type (Fig. 11F).

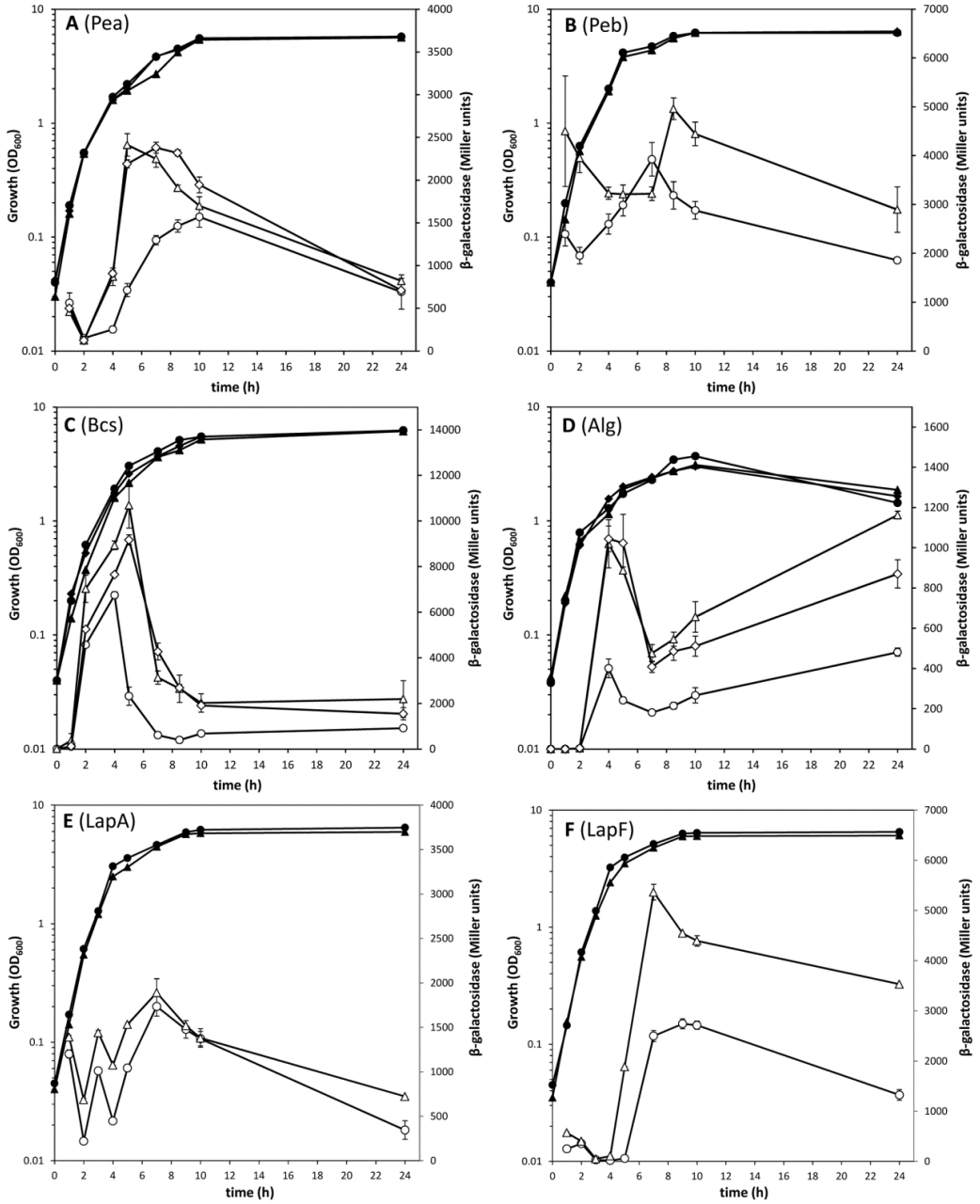

FIG 11 Influence of *rsm* mutations on expression of EPS- and adhesin-encoding genes during growth in liquid medium. Growth (solid symbols) and β-galactosidase activity (open symbols) of KT2440 (circles), ΔIEA (triangles), and ΔEA (diamonds) carrying reporter fusions corresponding to Pea (PP_3132::=*lacZ*) **A)**, Peb (PP_1795::=*lacZ*) **B)**, Bcs (PP_2629::=*lacZ*) **C)**, Alg (*algD*::=*lacZ*) **D)**, *lapA*::*lacZ* **E)**, and *lapF*::*lacZ* **F)** were followed over time. The data are averages and standard deviations from three biological

replicates with two technical repetitions. Statistically significant differences between the wild type and ΔIEA were detected from 4 to 8 h **A)**; at 2, 4, 8, 10, and 24 h **B)**; from 2 to 10 h **C)**; from 4 h onward **D)**; and from 5 h onward **F)** (Student's *t* test; $P \geq 0.05$). (D) D-Cycloserine (75 μg/ml) was added after 2 h of growth, since in *P. putida*, the *algD* promoter is silent in liquid medium in the absence of cell wall stress (M. I. Ramos-González, unpublished data).

Detailed analysis in each of the single mutants indicated that the absence of RsmA and RsmE had a cumulative influence on the overall increase in expression, while the lack of RsmI mostly contributed to the earlier peak of activation (Fig 12).

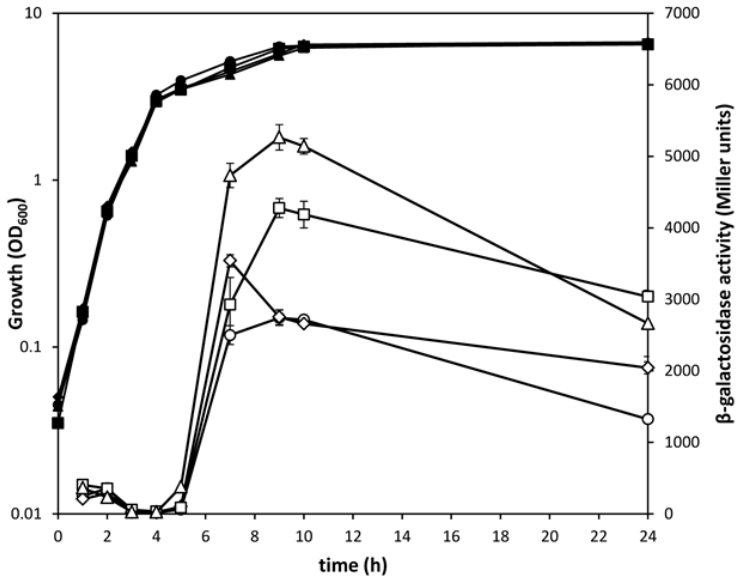

Figure 12. Influence of Rsm proteins on *lapF* expression. Growth (closed symbols) and β-galactosidase activity (open symbols) of KT2440 (circles) and mutants ΔI (diamonds) ΔE (squares) and ΔA (triangles), carrying the *lapF::lacZ* fusion in pMMG1. Data are averages and standard deviations from two biological replicas with two technical replicas.

Expression patterns of *rsm* genes. RACE was used to determine the transcription initiation sites of the three *rsm* genes. In *rsmI* and *rsmA*, the +1 site was located 63 and 245 bp upstream of the ATG, respectively, while in *rsmE*, two transcription initiation sites were identified at bases -53 (proximal +1 site) and -183 (distal +1 site) (Fig.

13). The same initiation sites were identified in transcripts from cultures in the exponential or stationary phase of growth.

>*rsmI*
[GCGGCTGTATGACGAGATGGCCGAAATGGCGGTGAAAATGCGGGCAAAAGCCCCGTCG
CGATGATTGGTCTGTAGGAGTAGTTACTCAAAAGTGTAAGAGGCTTCTGAATCGCAGT
GGAACTATTGAGTAGCTGAATGCCTACGGGGCAGGCTCCGGTTTTCCCTTTCGCAGGAG
GATCGGAACATGCTGGTAATAGGGCGCGAAGTAGGG]

>*rsmE*
[GGCCTTGCTGTTGTTTCTTCAGGCACTTGAGGAACAGGAGGACCATTGAGCTTGTCTAT
TCAGCCCATGATGCGAAGAAATTTCCCATTAGCCGCCAACCCCCCTATTCTTGCCAGCG
AGCACCTTGGCCGACAAA[CCGTTGACGGTTTGCTTTGGCACCTGCAACTGAAGACACG
CAACAGAATTTGTCTGTCGGGGAATTACTATCCGCCGAAAGCGTCTTTAATGAGAAGGA
AGAGGGACAAGCTCCCGCCGCCGCGGCAACTAGCCCAGCGGACTTATACATCACCAAGG
AGAACTACATGCTGATACTCACCCGTAAGGTTGGC]

>*rsmA*
[CTTCCAGGGCGTCGACGAGCACGGCAGCATCACTACCCTGGGTCGTGGTGGCTCTGAT
ACCACCGGCGTGGCCCTGGCGGCGGCGCTGAAGGCTGATGAGTGCCAGATCTACACTGA
TGTTGATGGCGTCTACACCACCGACCCGCGCGTCGTGCCACAGGCCCGTCGCCTGGAGA
AGATCACCTTCGAAGAGATGCTGGAAATGGCCAGCCTTGGTTCCAAGGTGCTGCAGATC
CGTTCGGTGGAGTTCGCCGGCAAGTACAACGTTCCGCTGCGCGTGCTGCACAGCTTCAA
GGAGGGTCCGGGTACCCTCATTACCATTGATGAAGAGGAATCCATGGAACAGCCGATCA
TTTCCGGTATCGCCTTCAACCGTGATGAAGCAAAGCTGACCATTCGCGGCGTGCCGGAT
ACCCCGGGCGTGGCCTTCAAGATCCTCGGCCCGATCAGCGCTTCGAACATCGAAGTCGA
CATGATCGTGCAGAACGTTGCGCACGATAACACCACCGACTTCACCTTCACCGTGCACCG
CAATGAGTACGAGAAGGCGCAGAGCGTGCTGGAAAACACCGCGCGCGAAATCGGTGCC
CGTGAAGTGATCGGCGACACCAAGATCGCCAAGGTCTCGATCGTCGGCGTCGGCATGCG
TTCGCACGCCGGTGTTGCCAGCTGCATGTTCGAAGCCCTGGCCAAGGAGAGCATCAACA
TCCAGATGATCTCCACTTCCGAGATCAAGGTTTCGGTGGTGCTCGAAGAGAAGTACCTG
GAGCTGGCCGTACGCGCGCTGCATACCGCGTTCGATCTGGATGCTCCTGCCCGACAGGG
CGAGTAAGGCGCTGCCTGGAAGGGCGCGGCTTGCCGCGCCCTTCGCGTTTCTGCTCGCC
GCGCAGGTCCTGTCCTGCCTTGTGCGGCGAGTAACCGGGGCCATGGTCGTCGTCCGCGA
CCCGGGCCCGATATCCAAGACTGTTACCCCTGAATGTTTTGCGTAAGGAGAAAGCTATG
TTGATTCTGACTCGTCGG]

Figure 13. Transcriptional start sites (highlighted in turquoise) identified in *rsmI, rsmE* and *rsmA* by RACE. The ribosomal binding sites are underlinded and start codons are highlighted yellow. Stop codons of upstream genes are shown in red. Fragments included in translational fusions with LacZ are indicated by purple square brackets. Sequences compatible with σ^{54}- and σ^{70}-dependent promoters in *rsmI* and *rsmE* are shaded in grey, with conserved bases in bold.

Based on this information, two translational fusions were constructed constructed with the reporter gene *lacZ* in plasmid pMP220-BamHI containing the *rsmI* and *rsmA* regions upstream of the +1 site (pOHR46 and pOHR52), respectively, and two with *rsmE*, a fragment containing the proximal +1 site closer to the ATG (pOHR47) and a larger one including both the proximal and distal transcription start sites (pOHR48). All the constructs included the ribosome-binding site and translation initiation codon. The constructs were introduced in KT2440, and β-galactosidase activity was followed during growth in LB medium. The activity of the RsmI-LacZ fusion was very low and was detected only when cultures had already reached the stationary phase (Fig. 14A). The results for RsmA-LacZ (Fig. 14B) showed a gradual increase in activity during exponential growth and the early stationary phase, followed by a decrease later. In the case of RsmE, both constructs showed an increase in expression in midexponential phase (Fig. 14C and D). The difference between the construct harboring the proximal promoter region (P2) and the construct with both promoter regions can be explained if the activity of the distal promoter (P1) is maintained longer than that of P2.

The observations made in different genetic backgrounds with respect to biofilm formation and expression of the extracellular matrix components suggested the existence of cross-regulation between *rsm* genes. This possibility was first explored by introducing the above-mentioned constructs in the triple mutant.

Analysis of β-galactosidase activity showed, in all cases, altered patterns and/or increased levels of expression with respect to the wild type (Fig. 14). The different fusions were then introduced in the single and double mutants. The results obtained with ΔE and ΔA indicated that both RsmE (Fig. 15A) and RsmA (Fig. 15B) have a clear negative effect on their own expression, with the self-repression effect of RsmA during the late stationary phase especially evident (Fig. 15B), while in the remaining

combinations of mutations and reporter fusions, the observed changes were minor or nonexistent (data not shown).

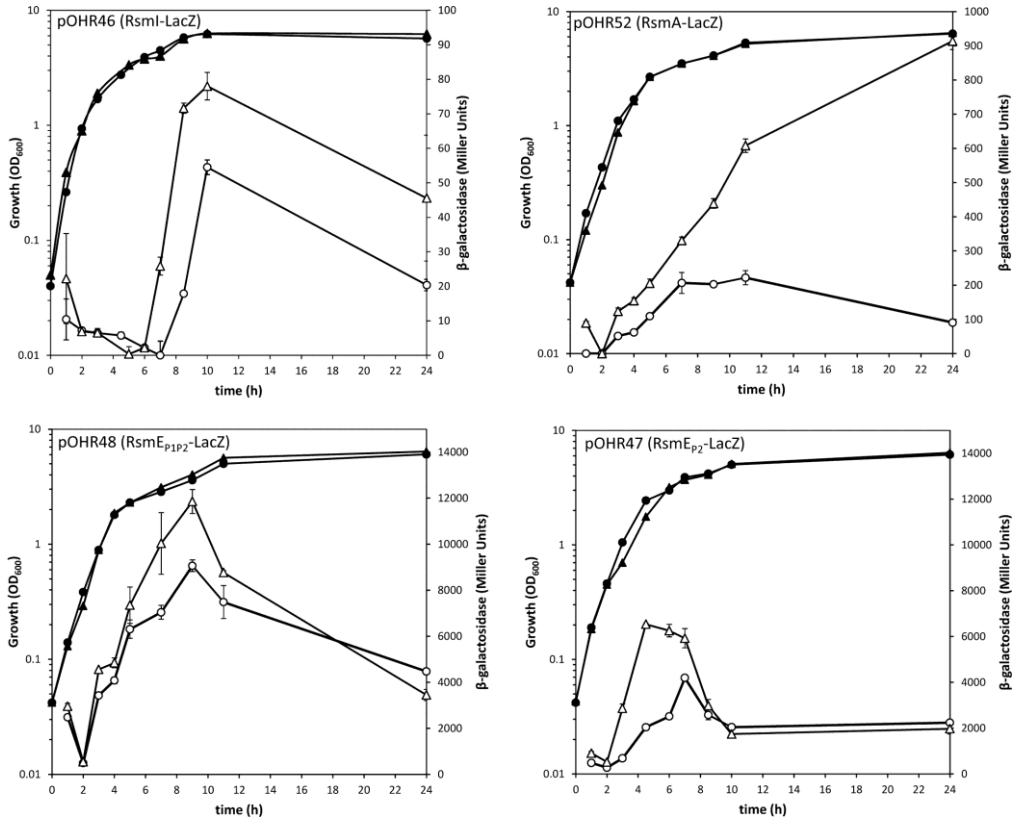

FIG 14 Expression patterns of Rsm proteins in the wild-type KT2440 and the *rsm* triple mutant ΔIEA. Growth (solid symbols) and β-galactosidase activity (open symbols) of KT2440 (circles) and βIEA (triangles) carrying the different Rsm-LacZ fusions are indicated in each panel. The RsmEP1P2-LacZ fusion contains both distal and proximal promoters, and the RmsEP2-LacZ fusion contains only the proximal promoter. The data are averages and standard deviations from three biological replicates with two technical repetitions. Statistically significant differences between the wild type and ΔIEA were detected from 7 h onward for pOHR46, from 7 h onward for pOHR52, at 7 and 8 h for pOHR48, and from 3 to 7 h for pOHR47 (Student's *t* test; $P \geq 0.05$).

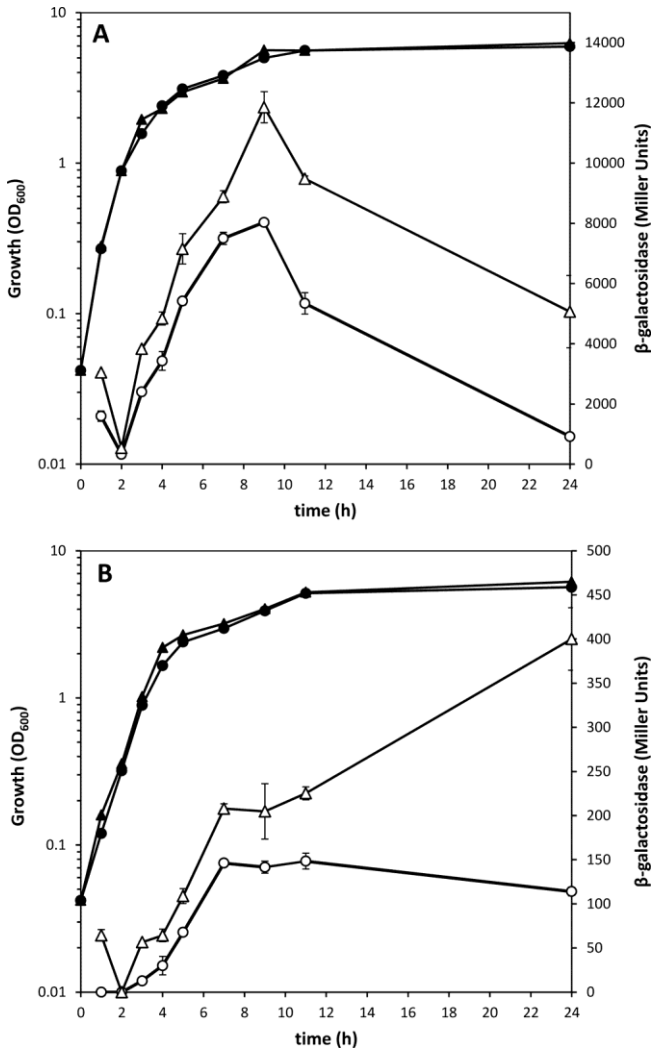

FIG 15 Self-repression exerted by Rsm proteins. Growth (solid symbols) and β-galactosidase activity (open symbols) of KT2440 (circles) and single mutants (triangles) carrying RsmEP1P2-LacZ (ΔE mutant) **A)** and RsmA-LacZ (ΔA mutant) **B)** fusions. The data are averages and standard deviations from three biological replicates with two technical repetitions. Statistically significant differences between the wild type and the mutant were detected from 3 h onward for RsmEP1P2-LacZ and from 7 h onward for RsmA-LacZ (Student's *t* test; $P \geq 0.05$).

Discussion

The regulatory cascade involving posttranscriptional regulators of the Rsm family and their cognate small RNAs has gained increasing relevance because, beyond its role in secondary metabolism, it is arising as a central element in bacterial gene expression regulation. RsmA homologs are present in diverse bacteria, from generalist species able to adapt to a variety of environments, like *P. putida*, to highly specialized bacteria with relatively few regulatory proteins, such as the human gastric pathogen *Helicobacter pylori*, where RsmA controls virulence and the stress response (Barnard *et al.*, 2004). It is noticeable that in the former, which has been the focus of this work, and in related species, such as *P. fluorescens* or *P. protegens*, there are three very similar Rsm proteins. Although this could imply the existence of regulatory redundancy or something like a "backup system," our data suggest that there is a combination of differentiated and cumulative effects attributable to these proteins. This is exemplified by the fact that RsmA and RsmE have roles in swimming motility that can be detected only when both are deleted, whereas the lack of RsmI has no detectable influence on swimming. On the other hand, swarming is completely abolished in all the mutants except ΔI. Other members of the RsmA/ CsrA family have been previously described as relevant elements for swarming motility in different bacteria, such as *P. aeruginosa* (Heurlier *et al.,* 2004), *Serratia marcescens* (Ang *et al.,* 2001), and *Proteus mirabilis* (Liaw *et al.,* 2003).

We have shown that Rsm proteins function as negative regulators in the process of biofilm development: the lack of all three proteins causes an increase in biofilm formation, but the robustness of the biofilm in terms of bacterial association with the surface is reduced, resulting in relatively easy and early detachment (Fig. 6 and 7). This effect is observed on glass surfaces, especially with ΔEA and ΔIEA, but it is not evident on plastic, where these strains remain attached to the surface. Under these

conditions the single ΔE and ΔA mutants' show delayed attachment relative to the wild type, whereas the ΔI mutant presents early detachment.

We have also analyzed the effect of overexpressing each Rsm protein in the wild type and in each mutant background. Our results pinpoint RsmE as the main element modulating bacterial attachment, with RsmI having a less pronounced effect that is dependent on the presence of an intact RsmA, which indicated that there is a regulatory interplay between Rsm proteins, so that they may act in a concerted way on their targets. Expression of the translational fusions constructed in the different genetic backgrounds indicated the existence of self-regulation in *rsmA* and *rsmE* and also revealed sequential activation of the different *rsm* genes. Thus, *rsmE* and *rsmA* would be the first to be expressed during growth in rich medium at 30°C, followed by *rsmI* in stationary phase. Expression of each gene also seems to be turned off sequentially: first *rsmA*, then *rsmE*, and finally *rsmI*. It is also worth mentioning the presence in *rsmE* of two distinct promoters with different expression dynamics. Although the expression sequence of *rsm* genes in the same bacterium had not been previously investigated, small RNAs are known to be sequentially expressed. In *P. fluorescens* (now *P. protegens*) CHA0, the expression patterns of the three sRNAs that interact with RsmA homologs have been described, with *rsmX* and *rsmY* showing a linear increase during growth while *rsmZ* is expressed at a later time (Kay *et al.*, 2005). However, a possible correlation with the expression patterns of Rsm proteins has not been studied. It may also be that different environmental conditions cause alterations in the progression of expression of the various elements. Detailed analysis of these aspects will be of great interest in terms of the responses of *P. putida* to different environmental conditions.

To have a clearer picture of the molecular basis for the role of Rsm proteins in biofilm formation, we have examined their influence on the expression of elements that are required for surface attachment (LapA), cell-cell interactions (LapF), and extracellular matrix composition (both adhesins and EPS). We have shown that the lack of Rsm proteins causes earlier and increased expression of LapF and also increases expression of cellulose and the strain-specific EPS Peb. Previous work has supported the notion that Pea (the other strain-specific EPS) and, to a minor extent, Peb are the exopolysaccharides with the main structural roles in *P. putida* biofilms grown under conditions similar to those used here, while alginate and cellulose would have a role in different environmental situations (Nielsen *et al.*, 2011; Nilsson *et al.*, 2011). Therefore, removing Rsm proteins (particularly RsmE and RsmA) likely promotes cell-cell interactions mediated by LapF, giving rise to thicker biofilms, and causes alterations in the balance and composition of the biofilm extracellular matrix. This probably also has consequences for characteristics such as hydrophobicity, which would explain the lability of the biofilms formed by the triple mutant, despite their increased biomass on glass surfaces, and the differences observed with plastic surfaces. In *P. aeruginosa*, RsmA negatively influences the expression of *psl*, one of its two species-specific EPS operons, involved in the architecture of biofilms (Irie *et al.*, 2010). Similarly, RsmA and RsmE influence the expression of one of the two *P. putida*-specific EPSs, Peb. However, the modulation of biofilm formation by the Rsm system appears to be far more complex in this bacterium, not only because of the existence of three proteins, but also because of their influence on additional elements involved in surface colonization that are absent in *P. aeruginosa* (LapF and cellulose).

The results obtained here provide evidence of the regulatory complexities associated with the adaptation of a versatile bacterium like *P. putida* KT2440 to different environmental conditions. The tools generated in this work (mutants, overexpression constructs, and reporter

fusions) and the knowledge gained will be of great importance for further dissection of the elements involved in posttranscriptional control of expression in *P. putida*.

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Chapter 2

The *Pseudomonas putida* CsrA/RsmA homologues negatively affect c-di-GMP pools and biofilm formation through the GGDEF/EAL response regulator CfcR

Summary

Expression of *cfcR,* encoding the only GGDEF/EAL response regulator in *Pseudomonas putida,* is transcriptionally regulated by RpoS, ANR and FleQ, and the functionality of CfcR as a diguanylate cyclase requires the multisensor CHASE3/GAF hybrid histidine kinase named CfcA. Here an additional level of *cfcR* control, operating post-transcriptionally via the RNA-binding proteins RsmA, RsmE and RsmI, is unraveled. Specific binding of the three proteins to an Rsm binding motif (5'CANGGANG3') encompassing the translational start codon of *cfcR* was confirmed. Although RsmA exhibited the highest binding affinity to the *cfcR* transcript, single deletions of *rsmA*, *rsmE* or *rsmI*, caused minor derepression in CfcR translation compared to a *ΔrsmIEA* triple mutant. RsmA also showed a negative impact on c-di-GMP levels in a double mutant *ΔrsmIE* through the control of *cfcR,* which is responsible for most of the free c-di-GMP during stationary phase in static conditions. In addition, a CfcR-dependent c-di-GMP boost was observed during this stage in *ΔrsmIEA* confirming the negative effect of Rsm proteins on CfcR translation and explaining the increased biofilm formation in this mutant compared to the wild type. Overall these results suggest that CfcR is a key player in biofilm formation regulation by the Rsm proteins in *P. putida*.

Importance

In this work we have unraveled that the influence on biofilm formation observed for Rsm proteins (Huertas-Rosales *et al.*, 2016) takes place through direct repression of *cfcR*, which is responsible for most of c-di-GMP free pool in *Pseudomonas putida* during the stationary phase of growth. It was known that this gene is tightly regulated at the transcriptional level and encodes an orphan response regulator with GGDEF/EAL domains, which requires an hybrid histidine kinase named CfcA to trigger diguanylate cyclase activity. The connection between the

Gac/Rsm pathway and the formation of biofilm through the control of c-di-GMP metabolism is just beginning to be understood. This is not an easy issue given the presence of several dozens of genes encoding GGDEF/EAL domains containing proteins in the genomes of pseudomonads. To our knowledge SadC is the only diguanylate cyclase involved in biofilm formation controlled by the Gac/Rsm pathway in *P. aeruginosa* but there is no orthologue of this gene in the genome of *P. putida*. On the other hand, CfcR/CfcA are absent of *P. aeruginosa,* but they are present in many plant-associated pseudomonads (either beneficial or pathogenic). Thus a comprehensive analysis of the functioning and regulation of this system is of the highest significance for expanding our knowledge on biofilm formation by these bacteria of environmental interest.

Introduction

Proteins belonging to the CsrA/RsmA (acronyms for carbon storage regulator and regulator of secondary metabolism) family are small sequence specific RNA-binding regulators that activate or repress gene expression by altering translation, RNA stability and/or transcript elongation (Romeo *et al.*, 2013). They are present in diverse Gram-negative and Gram-positive bacteria (Ulrich and Zhulin, 2010). CsrA was first described in *Escherichia coli* (Romeo *et al.*, 1993; Romeo, 1998), where it plays a major role in controlling the intracellular carbon flux, by negatively regulating glycogen metabolism and several enzymes involved in central carbohydrate metabolism (Sabnis *et al.*, 1995; Yang *et al.*, 1996). In *Pseudomonas protegens* CHA0 (previously *P. fluorescens*) the CsrA homologues, RsmA and RsmE, not only control metabolism but also the production of biocontrol-related traits (Reimmann *et al.*, 2005). Furthermore, CsrA/RsmA systems have been shown to play a key role in the control virulence through complex regulatory networks (Vakulskas *et al.*, 2015).

The activity of RsmA/CsrA has been shown to be modulated by small regulatory RNA molecules that can bind these regulators with high affinity titrating them out and preventing them from binding to their target mRNAs. These include the sRNAs RsmY, RsmW and RsmZ in *P. aeruginosa* (Kay *et al.*, 2006) and RsmX, RsmY, and RmsZ in *P. protegens* (Heeb *et al.*, 2002; Kay *et al.*, 2005). The RsmA family proteins and their cognate small RNAs are part of the GacS/GacA signal transduction pathway. It is known that titration of RsmA by the sRNAs increases bacterial attachment and biofilm formation, whereas excess of Rsm proteins promote the planktonic lifestyle of these bacteria; the later functions in opposition to that of the second messenger c-di-GMP, an increase of which leads to cellular aggregation (Römling *et al.*, 2013). Some of the molecular elements connecting the Rsm and c-di-GMP regulatory networks are being characterized (Colley *et al.*, 2016; Valentini and Filloux, 2016 and references therein).

GGDEF and EAL protein domains are responsible for the synthesis and hydrolysis of c-di-GMP through their role in diguanylate cyclase (DGC) and phosphodiesterase (PDE) activities respectively. These activities are key in controlling the turn-over of this second messenger in bacterial cells (Hengge, 2009; Römling and Simm, 2009). The gene *rup4959*, which encodes the unique response regulator containing both GGDEF and EAL domains in *P. putida* KT2440, was identified as being preferentially expressed in the corn rhizosphere (Matilla *et al.*, 2007). When overexpressed, *rup4959* increases the levels of free c-di-GMP in the bacterial cells and confers a pleiotropic phenotype that includes enhanced biofilm and pellicle formation capacity, cell aggregation and crinkle colony morphology. In order to trigger the DGC activity, the protein encoded by *rup4959* requires to be phosphorylated at the Asp65 in its REC domain (Matilla *et al.*, 2011) and also the multi-sensor (CHASE3/GAF) hybrid histidine kinase CfcA. Therefore we have recently renamed Rup4959 to CfcR (Ramos-González *et al.*, 2016).

Previous studies focused in the regulation of *cfcR* have highlighted that its transcription is entirely dependent on RpoS and positively modulated by ANR (Matilla *et al.*, 2011) and FleQ (Ramos-Gonzalez *et al.*, 2016). In addition, a post-transcriptional regulation of *cfcR* has been suggested. Two motifs which share conservation with the SELEX-derived consensus for CsrA/RsmA binding 5'RUACARGGAUGU3' (Dubey *et al.*, 2005) were identified in the *cfcR* mRNA (Matilla *et al.*, 2011). The first (motif A) overlap with a distal transcription initiation site of the gene and the second (motif B), showing higher similarity to the consensus, encompasses the translation start codon of *cfcR* (Fig. 1A).

The genome of *P. putida* encodes three CsrA/RsmA homologues named RsmA, RsmE and RsmI (Nelson *et al.*, 2002; Winsor *et al.*, 2016). Although RsmA and RsmE are more related to CsrA than RsmI (Huertas-Rosales *et al.*, 2016), we have found that RsmI still shares predictive secondary structures with CsrA/RsmA/RsmE. All seven possible mutant strains as result of the deletion of one, two or the three *rsm* genes have been generated in *P. putida* previously to this work. The *rsm* triple mutant showed increased biofilm formation, whereas overexpression of RsmE or RsmI resulted in reduced bacterial attachment (Huertas-Rosales *et al.*, 2016). This suggests that these Rsm proteins may exert a negative regulation upon diguanylate cyclases in this bacterium and that this effect may be mediated via the control of *cfcR* expression.

In this study we have analyzed the direct interaction between the three Rsm proteins of *P. putida* and specific motifs in the leader sequence and translation initiation of the *cfcR* mRNA and evaluated the role of these proteins on *cfcR* expression and the free pool of c-di-GMP. Our results indicate that the influence on biofilm formation observed for Rsm proteins takes place through direct repression of *cfcR,* which results in reduced levels of c-di-GMP in the stationary phase. Therefore, we show

that CfcR is a central player in Rsm-controlled biofilm formation in *P. putida*.

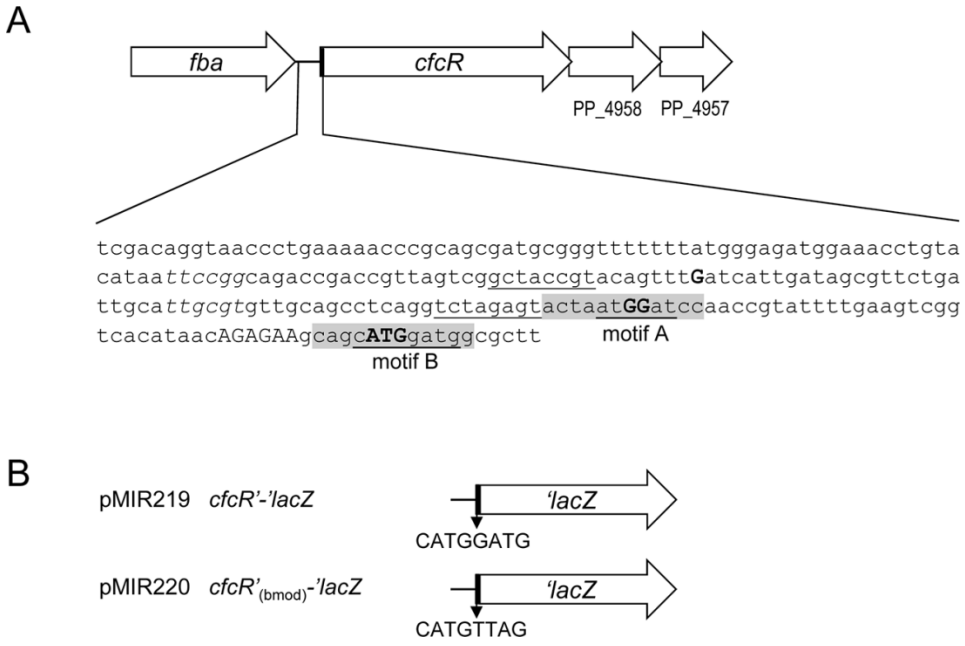

Fig. 1. Physical map and constructs involving *cfcR*. **A)** Features in the promoter, leader and coding sequences of *cfcR*. Transcription initiation points, previously determined experimentally, distal and proximal (double) are indicated in bold as capital "G"(Matilla *et al.*, 2011). Two sequences matching the SELEX-derived consensus for the global posttranscriptional regulator CsrA (RUACARGGAUGU) (Dubey *et al.*, 2005) are in grey boxes (motif A, overlapping the proximal transcription initiation points; and motif B, overlapping the start codon). Nucleotides in these boxes coinciding with the consensus are underlined. Putative -35 RpoS binding sequences are in italic. Predicted sequences for an extended -10 are underlined. Shine Dalgarno (AGAGAA) and start codon (ATG in bold) are indicated. Diagram not to scale. **B)** Translational fusions involving the *cfcR* gene. Plasmids pMIR219 and pMIR220 each contain a translational fusion to *'lacZ* in the vector pMP220-BamHI (Table 2). The predicted Rsm binding site CATGGATG (motif B) of pMIR219 was replaced by CATGTTAG in pMIR220.

Results

The Rsm proteins repress the expression of the response regulator CfcR and its transcriptional regulator RpoS. To investigate the influence of the Rsm proteins from *P. putida* KT2440 upon the expression of *cfcR* a translational fusion *cfcR'-'lacZ* was generated in pMIR219 (Fig. 1B) and β-galactosidase activity determined under optimal aeration conditions in the wild type strain and a battery of seven mutants hampered in production of one, two or the three Rsm proteins present in this bacterium. These mutants were generated in a previous work (Huertas-Rosales *et al.*, 2016). As expected for an RpoS-dependent gene (Matilla *et al.*, 2011) the expression of *cfcR* is initiated in the transition from the exponential to the stationary phase of growth in the wild type. In the triple *ΔrsmIEA* mutant, *cfcR* expression was activated earlier and enhanced at the onset of stationary phase, an increase that was maintained throughout this phase with levels of β-galactosidase activity around 1.5 times higher than those of the wild type (Fig. 2A).

The deletion of single *rsm* genes caused only minor incremental changes in *cfcR* expression as measured at the advanced stationary phase of growth (Fig. 3). In the double mutants *ΔrsmIA* and *ΔrsmEA* a slight increase in expression was also observed at the onset of stationary phase. In the latter strain this was maintained until further into the stationary phase; as such, the expression pattern of *cfcR* in the double mutant *ΔrsmEA* (Fig. 3), where only RsmI remains, is most similar to the triple mutant. In the double mutant *ΔrsmIE*, with only RsmA active, a slight decrease in expression was observed at earlier stages of growth. These results suggest a potential gradual relevance of RsmA, RsmE and finally RsmI in the control of *cfcR* expression under the experimental conditions tested.

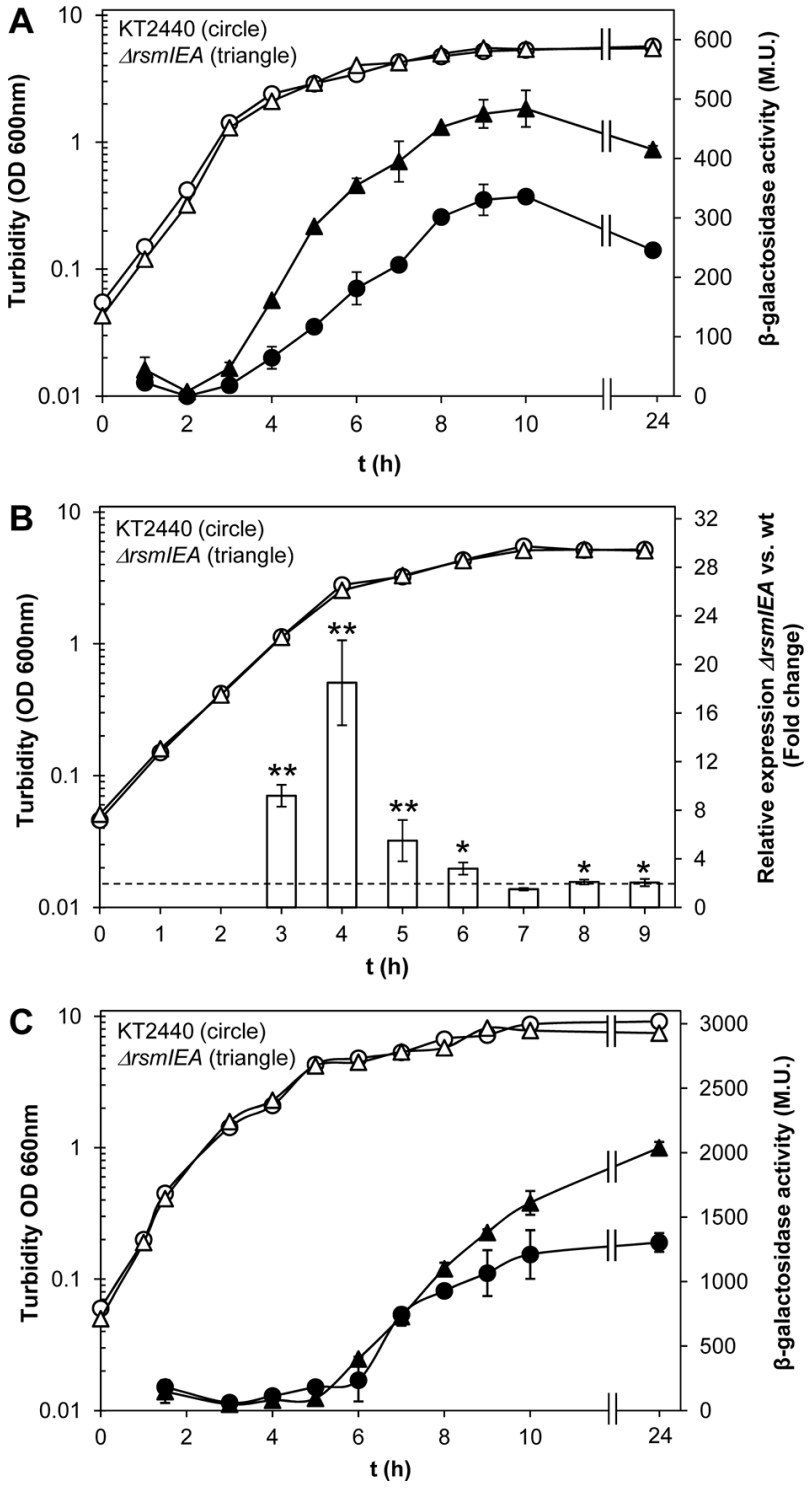
A
KT2440 (circle)
ΔrsmIEA (triangle)
Turbidity (OD 600nm)
β-galactosidase activity (M.U.)
t (h)
B
KT2440 (circle)
ΔrsmIEA (triangle)
Turbidity (OD 600nm)
Relative expression ΔrsmIEA vs. wt (Fold change)
t (h)
C
KT2440 (circle)
ΔrsmIEA (triangle)
Turbidity OD 660nm
β-galactosidase activity (M.U.)
t (h)

Fig. 2 (previous page). Expression of *cfcR* in the wild type and the triple mutant *ΔrsmIEA*. A, Activity of the translational fusion *cfcR'-'lacZ* in pMIR219. Cultures growing in LB supplied with Tc as described in the experimental procedures were analyzed for turbidity (hollow symbols) and β-galactosidase activities (solid symbols) at the indicated times. The experiment was performed in triplicate and for each biological replicate activities were assayed in triplicate. Average data and standard deviations are plotted from one representative experiment. Statistically significant differences between wild type and *ΔIEA* β-galactosidase activities were detected from 4 h onwards (Student's *t* test; $P < 0.05$). **B**, Time course of the relative quantities of CfcR-RNA in the triple mutant *ΔIEA* vs. the wild type strain *P. putida* KT2440. Growth curve of the strains in LB are plotted. Fold changes were based on mRNA measurements obtained with qRT-PCR. The experiment was carried out in triplicate with three experimental replicates. Average data and standard deviations are plotted. One or two asterisks indicate when results for the triple mutant *ΔIEA* are significantly different from wild type (Student's *t* test, $P<0.05$ and $P<0.01$, respectively). A fold change of 2 is indicated with a dotted line. **C**, Activity of the transcriptional fusion P_{cfcR}*::'lacZ* in pMIR200. Samples were analyzed for turbidity (hollow symbols) and β-galactosidase activities (solid symbols). Experiments and statistical analysis were performed as indicated above for panel A.

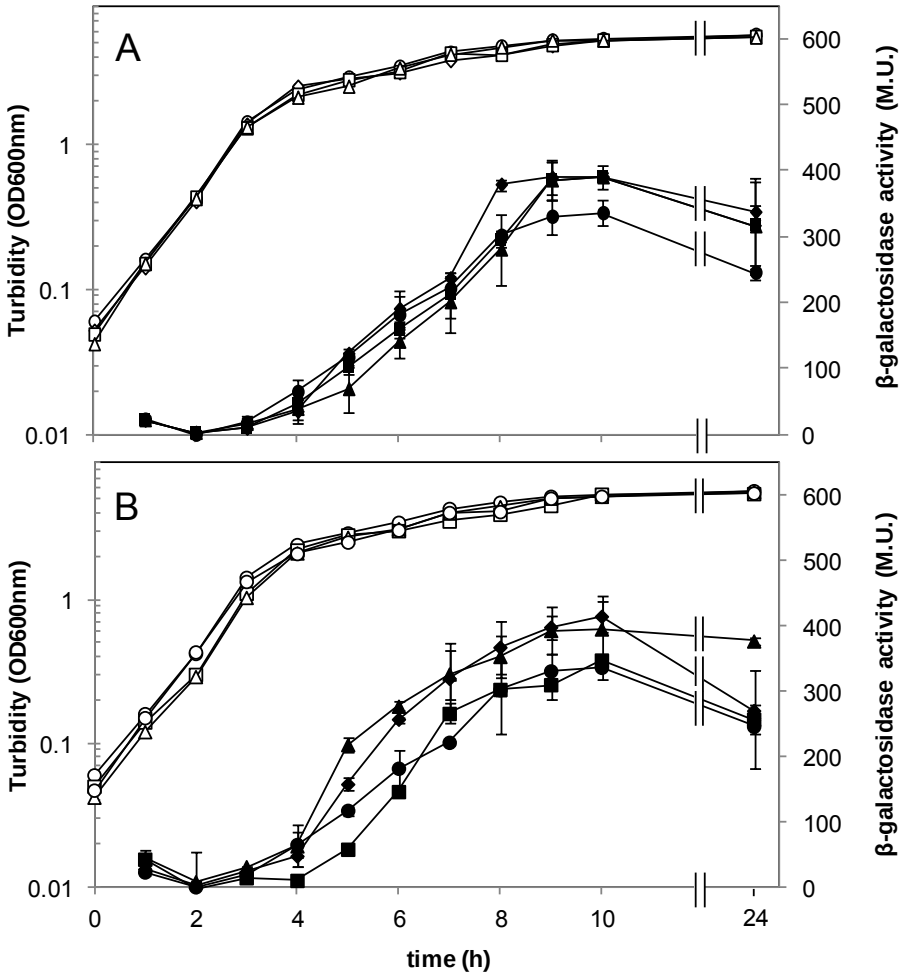

Fig. 3. Activity of the *cfcR'-'lacZ* fusion in single and double *rsm* mutants. LB cultures, prepared as described in the experimental procedures section, were analyzed for turbidity (hollow symbols) and β-galactosidase activity (solid symbols) at the indicated times. A, wild type KT2440 (circle), *rsmA* (triangle), *rsmE* (square) and *rsmI* (diamond); B, wild type KT2440 (circle), *rsmEA* (triangle), *rsmIE* (square) and *rsmIA* (diamond). Experiments were performed in triplicate. Average and standard deviations are plotted for one representative experiment using data obtained from activities measured in duplicate. Statistically significant differences in β-galactosidase values were detected between wild type and *ΔrsmI* (at 8 h); *ΔrsmEA* (at 5, 6 and 7, and 24 h); *ΔrsmIA* (at 5, 6 and 7 h); and *ΔrsmIE* (at 5 h) (Student's *t* test; $P < 0.05$). Non significant differences are not pointed out.

The relative expression of *cfcR* along the growth curve was also examined by RT-qPCR in the triple *ΔrsmIEA* mutant versus the wild type and the results showed higher levels of expression in the mutant. The increase in the relative values (fold change) of mRNA was particularly evident during the exponential phase and reached a peak of ~18-fold at the onset of stationary phase, after which it gradually decreased with time (Fig. 2B). Minor effects upon *cfcR* expression were observed in the single *ΔrsmE* and *ΔrsmA* mutants (Fig. 4), whereas transient increases were observed in the double *ΔrmsEA* mutant, where RsmI remained active, and to a lesser extent in the *ΔrsmIA* strain, where only RsmE remained active (Fig. 5).

In the double mutant *ΔrsmIE,* with an active RsmA, no differences were observed in the relative mRNA *cfcR* transcripts. Again these results indicated that although the individual loss of Rsm proteins did not have much impact on *cfcR* expression, when RsmA or RsmE remained as unique Rsm proteins they seem to still exert a major negative effect (RsmA causing more important repression than RsmE). Interestingly, the increase in *cfcR* expression took place at earlier stages of growth as the number of deleted *rsm* genes increased, which is in agreement with the progress in biofilm formation previously described for the triple mutant (Huertas-Rosales *et al.*, 2016).

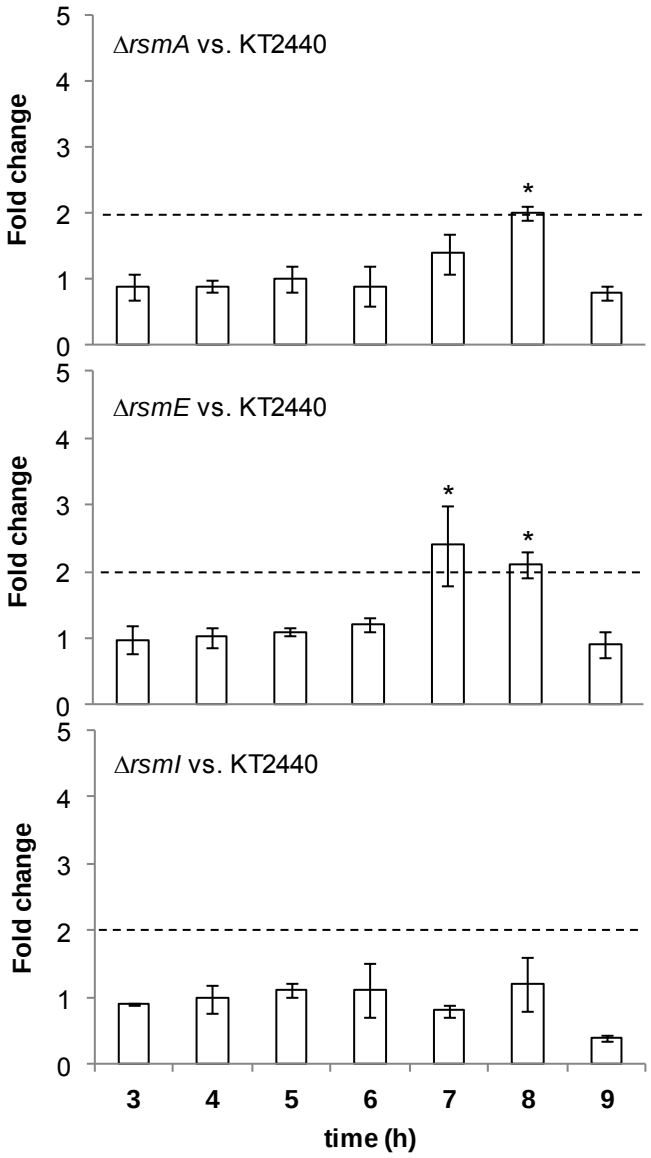

Fig. 4. Time course of the relative quantities of CfcR-RNA in the single *rsm* mutants versus the wild type *P. putida* KT2440 strain. Fold changes were calculated using qRT-PCR data. Averages and standard deviation of three biological replicates and three experimental replicates are plotted. Asterisks indicate when values for each mutant are significantly different from wild type values (Student's t test, $p<0.05$). Fold change of 2 is indicated with a dotted line.

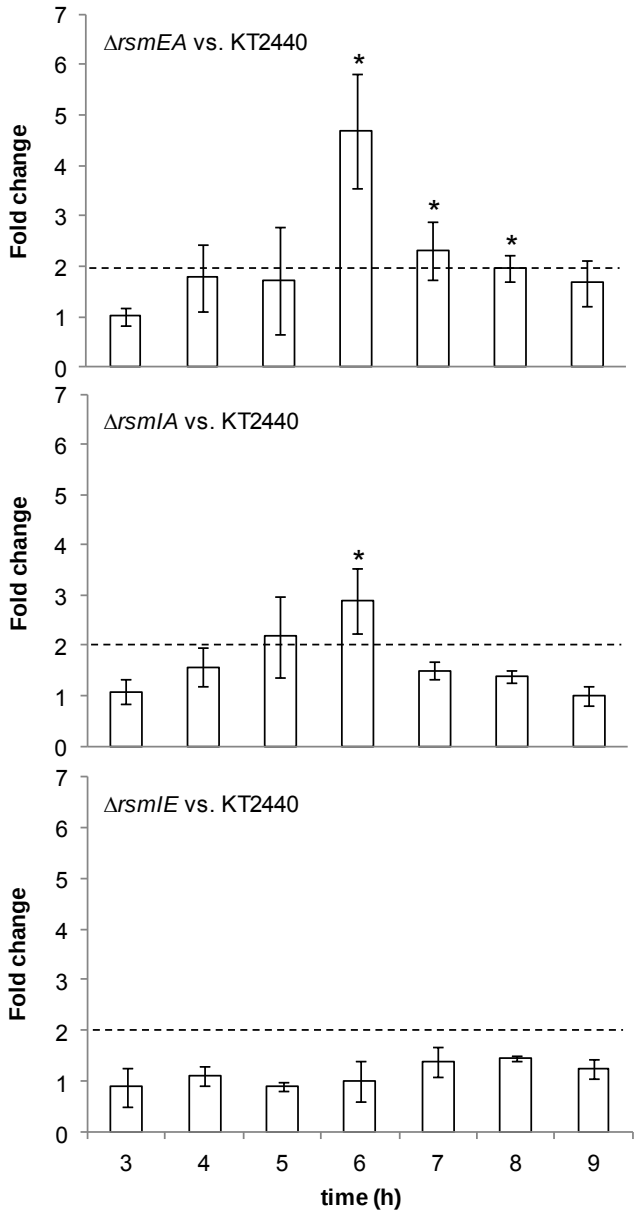

Fig. 5. Time course of the relative quantities of CfcR-RNA in the double *rsm* mutants versus the wild type *P. putida* KT2440 strain. Fold changes were calculated using qRT-PCR data. Averages and standard deviation of three biological replicates and three experimental replicates are plotted. Asterisks indicate when values for each mutant are significantly different from wild-type values (Student's t test, $p<0.05$). Fold change of 2 is indicated with a dotted line.

We hypothesized that the significant enhancement in *cfcR* transcripts observed in the *ΔrsmIEA* strain might perhaps be due to a modified expression pattern of the alternative transcription factor RpoS in this mutant, as a consequence of the release of repression by Rsm proteins upon RpoS. This could also explain that in a transcriptional *cfcR'::'lacZ* fusion there still remained a positive effect of deleting these post-transcriptional regulators (Fig. 2C). To investigate this possibility, the expression of RpoS was also analyzed in the seven mutants hampered in one, two and the three Rsm proteins, using a translational *rpoS'-'lacZ* fusion in the plasmid pMAMV21 (Matilla *et al.*, 2011). Expression of *rpoS* differed only in *ΔrsmIEA* background right at the beginning of the stationary phase, when a significant increase was observed in the mutant (Fig. 6). This may explain at least in part the transient accumulation of *cfcR* mRNA observed in the triple mutant *ΔrsmIEA* compared to the wild type. Moreover *rpoS* has been identified as a target of Rsm proteins in RIP-seq experiments (our unpublished results shown in chapter 3) as an indication that RpoS regulation by Rsm proteins is direct. In addition to this negative regulation of RpoS by Rsm proteins the possibility remained that these proteins directly repress the expression of *cfcR* and/or cause a reduction in its mRNA stability.

Binding of the Rsm proteins to the cfcR transcripts. To investigate the potential of a direct interaction between the Rsm proteins of *P. putida* KT2440 and the motifs matching the consensus for the binding of these proteins found in the leader sequence of the CfcR transcripts, we performed fluorescence-based electrophoretic mobility shift assays (fEMSA). Three different transcripts RNA-CfcR (a), RNA-CfcR(b), and RNA-CfcR(ab), obtained using *in vitro* transcription, were used in these experiments. The first transcript covered the putative Rsm-binding motif A whereas the latter two contained either motif B or motifs A and B, respectively. While motif B is located in a predicted stem-

heptaloop, an unorthodox Rsm-binding stem-loop was predicted around motif A (Fig. 7).

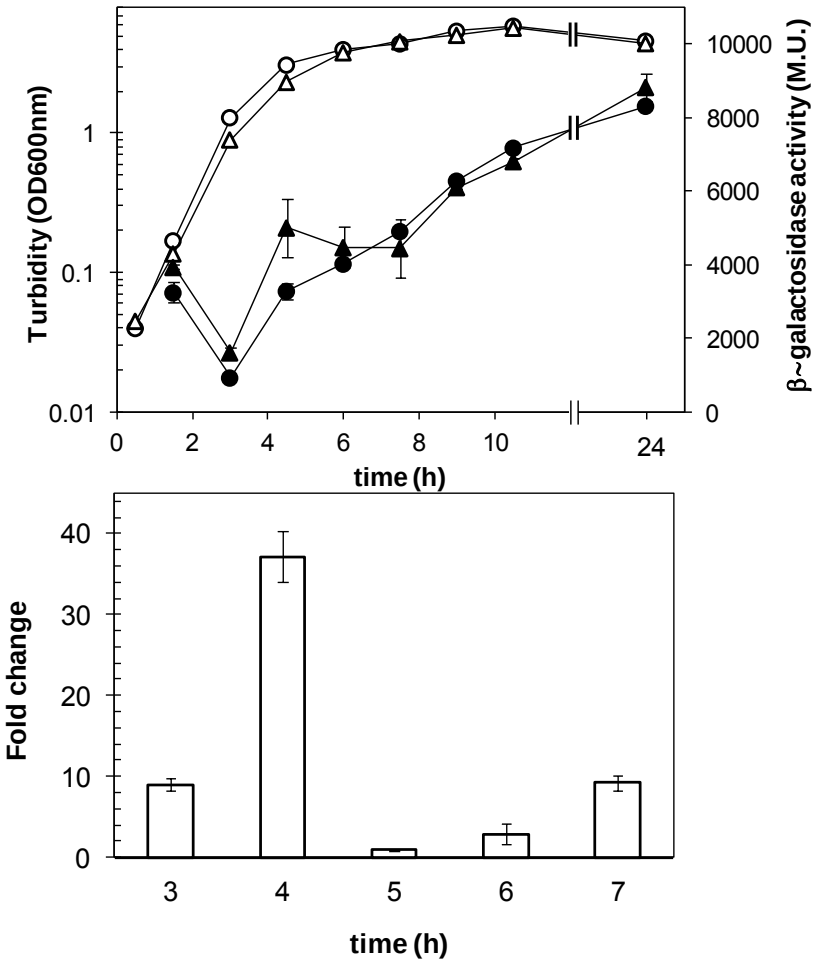

Fig. 6. RpoS expression in *P. putida* KT2440 (circle) and triple mutant *ΔrsmIEA* (triangle) strains. **Top**: Activity of the translational *rpoS'-'lacZ* fusion. LB cultures supplied with Tc were obtained as indicated in the experimental procedures section. Samples were analyzed for turbidity (hollow symbols) and β-galactosidase activity (solid symbols) at the indicated times. Experiments were performed in six biological replicates. Average and standard deviation of data from one representative experiment with two experimental replicates are plotted. Statistically significant differences in β-galactosidase values at 4.5 h were detected in every biological replicate (Student's *t* test; $P< 0.05$). **Bottom**: Time course of the relative quantities of RpoS-RNA in the triple mutant *ΔrsmIEA* versus the wild type *P. putida* KT2440 strain. Fold changes were calculated using qRT-PCR data. Averages and standard deviation of three biological replicates and three experimental replicates are plotted.

>RNA-CfcR(ab) (motif A and motif B)
GAUAGCGUUCUGAUUGCAUUGCGUGUUGCAGCCUCAGGUCUAGAGUACUAAUGGAUCCAACCGU
AUUUUGAAGUCGGUCACAUAACAGAGAAGCAGC***AUG***GAUGGCGCUUACCCUCAACAGCAGCCAC
UGGAUGGCAGCUCAGUUCUUCUGGUGGUAGACGAC

>RNA-CfcR(a) (motif A)
GAUAGCGUUCUGAUUGCAUUGCGUGUUGCAGCCUCAGGUCUAGAGUACUAAUGGAUCCAACCGU
AUUUUGAAGUC

>RNA-CfcR(b) (motif B)
UCCAACCGUAUUUUGAAGUCGGUCACAUAACAGAGAAGCAGC***AUG***GAUGGCGCUUACCCUCAAC
AGCAGCCACUGGAUGGCAGCUCAGUUCUUCUGGUGGUAGACGAC

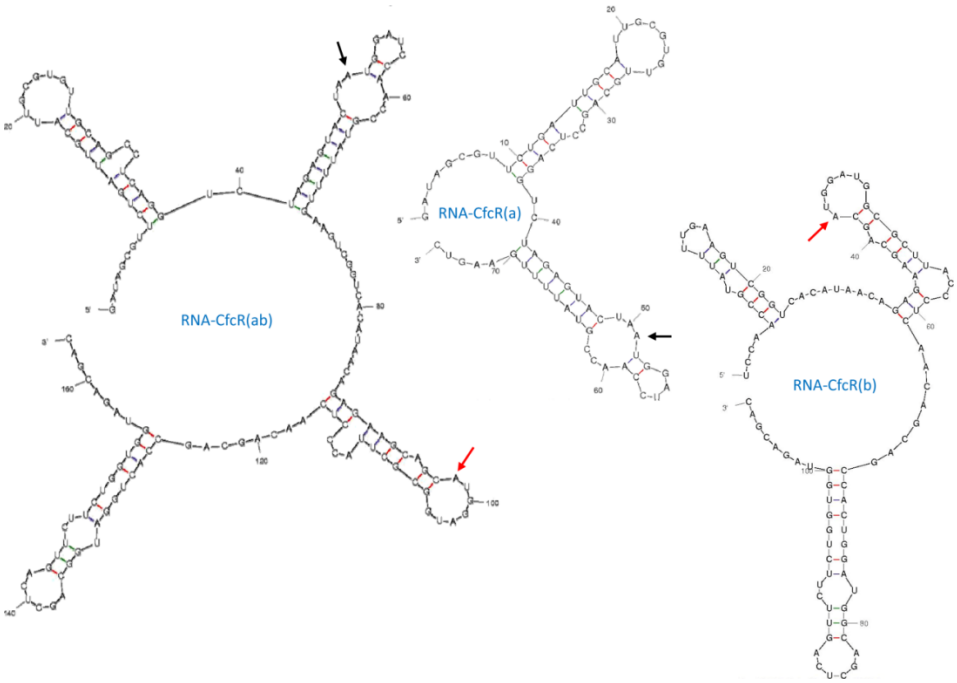

Fig. 7. RNA-CfcR transcripts used in fEMSA. All transcripts contain two tags that are not included in the sequences shown, one in their 5' ends with the sequence for the T7 polymerase promoter (5'UUUUCUGCAGUAAUACGACUCACUAUAGG3'), and another in their 3' ends with the sequence (5'UUUUUUUUGGGGGGGGG3') complementary to the DNA probe labeled with ATTO 700 fluorescent dye (See Materials and methods section). Nucleotides matching the consensus for Rsm binding in motifs A and B are boxed. The translation start codon of *cfcR* is in bold. Secondary structure predictions of RNAs obtained at m-fold web server (Zucker, 2003) are shown. Motif A and motif B are indicated by arrows black and red, respectively. Maximum distance between paired bases was established in 30.

Fixed quantities of each of the transcripts labeled with a fluorescent DNA-probe as described in the experimental procedures were incubated with increasing concentrations of His-tagged Rsm proteins (0-1000 nM) and the electrophoretic mobility of the complexes was analyzed in native TBE polyacrylamide gels. The effect of incubating RNA-CfcR(b) with purified RsmA (100-400 nM), RsmE (400-800 nM) and RsmI (400-800 nM) resulted in RNA-CfcR(b) shifts when compared to the same experimental condition without protein, indicating their binding to this transcript (Fig. 8).

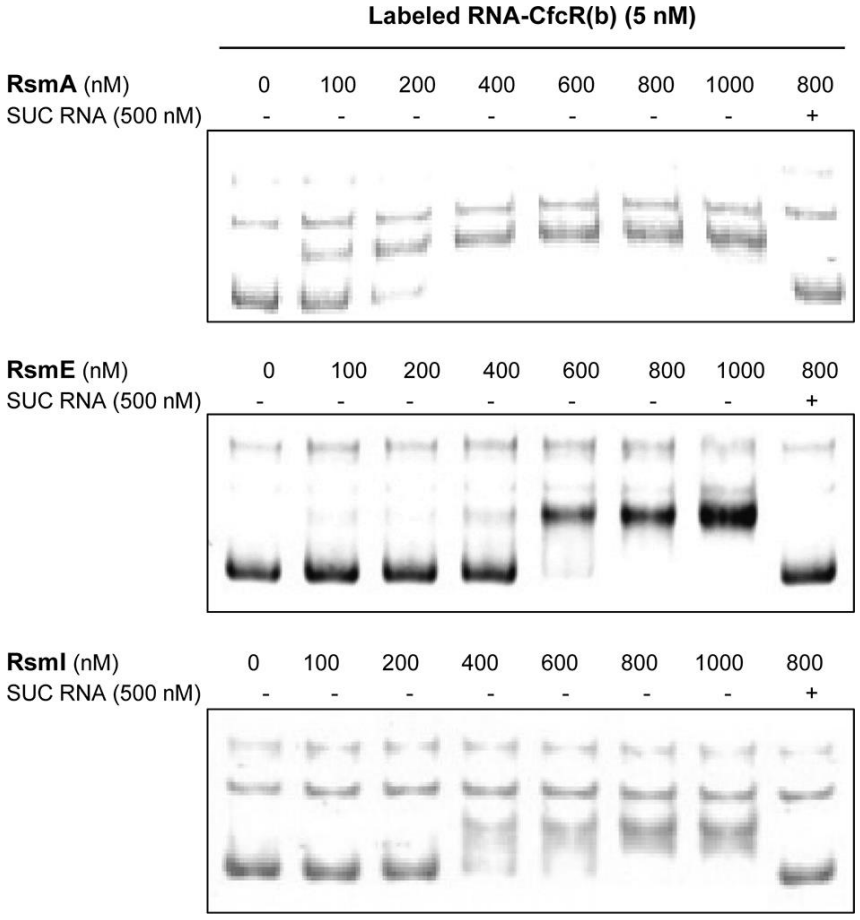

Fig. 8. Fluorescence-based EMSA of RsmE, RsmI and RsmA binding to RNA-CfcR(b). RNA-CfcR(b) includes the predicted Rsm binding motif B (see Figure 7). Note that specific unlabeled competitor (SUC) RNA-CfcR(b) prevented the formation of the labeled RNA-CfcR(b)-protein complexes.

These shifts disappeared when an excess (500 nM) of specific unlabeled competitor (SUC) RNA-CfcR(b) was added, indicating that RsmA, RsmE and RsmI proteins likely interact with the motif B and these interactions are specific (Fig. 8). No interactions were observed between RNA-CfcR (a) and any of the Rsm proteins (Fig. 9).

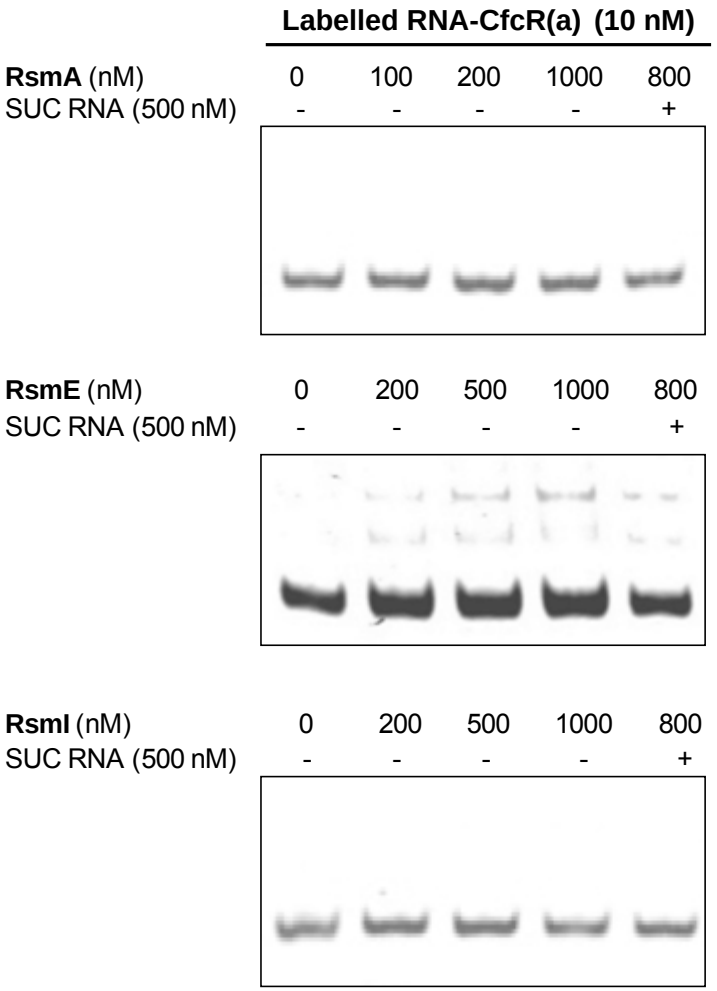

Fig. 9. Fluorescence-based EMSA of RsmE, RsmI and RsmA proteins binding to RNA-CfcR(a). RNA-CfcR(a) spans more nucleotides than the predicted Rsm binding motif A (see Figure 4). Note that specific unlabeled competitor SUC RNA-CfcR(a) did not prevent the formation of the labeled RNA-CfcR(a)-RsmE complexes, indicating non-specific binding.

With RsmA, no obvious shifts in RNA-CfcR(ab) were observed although by increasing RsmA concentration above 400 and up to 1000 nM the attenuation of a minor band was noticeable and shown to be specific (Fig. 10).

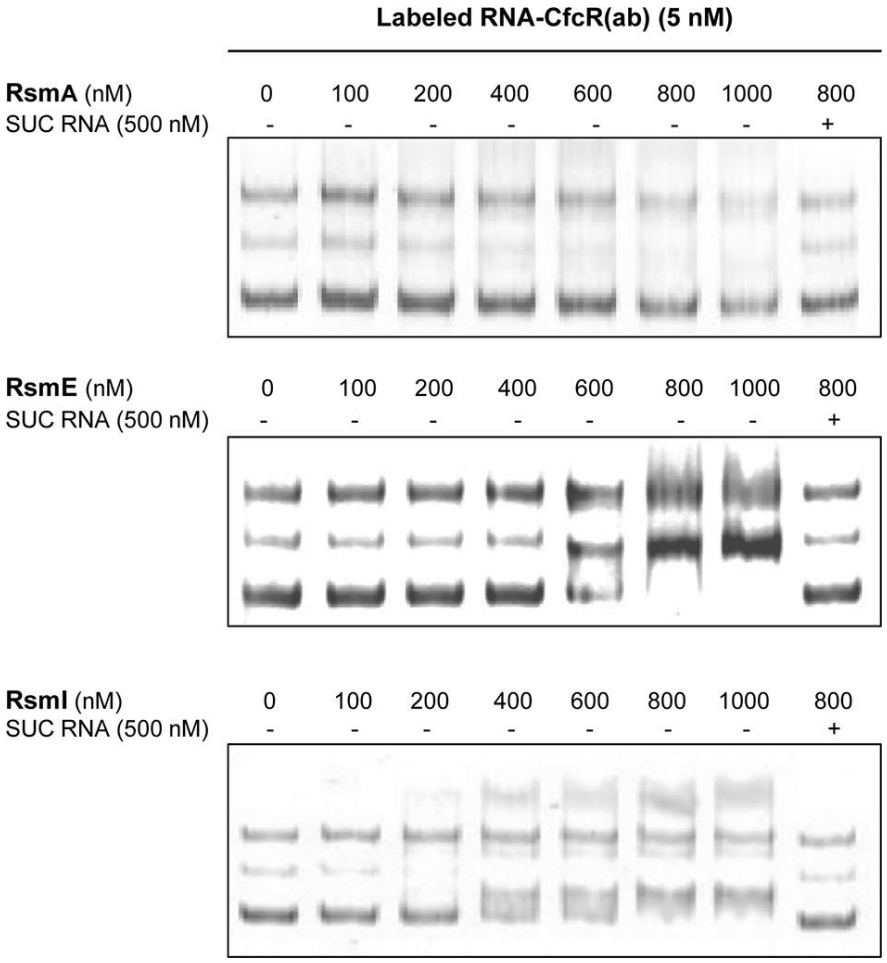

Fig. 10. Fluorescence-based EMSA of RsmE, RsmI and RsmA binding to RNA-CfcR(ab). RNA-CfcR(ab) includes the predicted Rsm binding motifs A and B (see Figure S5). Note that specific unlabeled competitor (SUC) RNA-CfcR(ab) prevented the formation of the labeled RNA-CfcR(ab)-protein complexes.

Specific RNA-Cfc(ab) shifts were observed after incubation with RsmE (600-800 nM) and RsmI (200-400 nM) indicating that these Rsm

proteins likely interact with motif B encompassed in this transcript. These *in vitro* results strongly suggest that there is a binding motif (motif B) for Rsm proteins at the translation start in the *cfcR* mRNA. Although the binding of RsmA to this motif in RNA-CfcR(b) transcript showed the highest affinity, the fact that the interaction of this protein with RNA-Cfc(ab) was not as clear as those of RsmE and RsmI suggests that perhaps the binding between RsmA and the Rsm-binding motif might be more easily impeded by changes in mRNA secondary structure.

Motif B overlapping the translation initiation codon in the RNA-CfcR transcript is essential for the repression of CfcR by Rsm. The involvement of motif B in the post-transcriptional repression of *cfcR* was tested *in vivo* using a translational fusion of *cfcR*, containing a modified motif B, to *lacZ* (*cfcR'*$_{(bmod)}$*-'lacZ* in pMIR220) (Fig. 1B). The replacement of CATGGATG for CATGTTAG in this fusion destroys the required core "GGA" in the consensus (Dubey *et al*., 2005). The activity of this fusion was compared to that of the fusion with an intact motif B (*cfcR'-'lacZ* in pMIR219) in the wild-type strain. The first construction with the modified motif B reached higher activity levels and its kinetics were very similar to that of *cfcR'-'lacZ* in the triple mutant *ΔrsmIEA* (Fig. 11).

This result confirmed that motif B is involved in CfcR repression and together with the *in vitro* evidence obtained with fEMSA further supports that Rsm proteins are direct repressors of *cfcR*. The activity of the *cfcR'*$_{(bmod)}$*-'lacZ* fusion was also tested in the triple *ΔrsmIEA* mutant and resulted in earlier and higher activity than in the wild type (Fig. 12A) indicating that Rsm-mediated repression still remained active independently of the modification made to motif B.

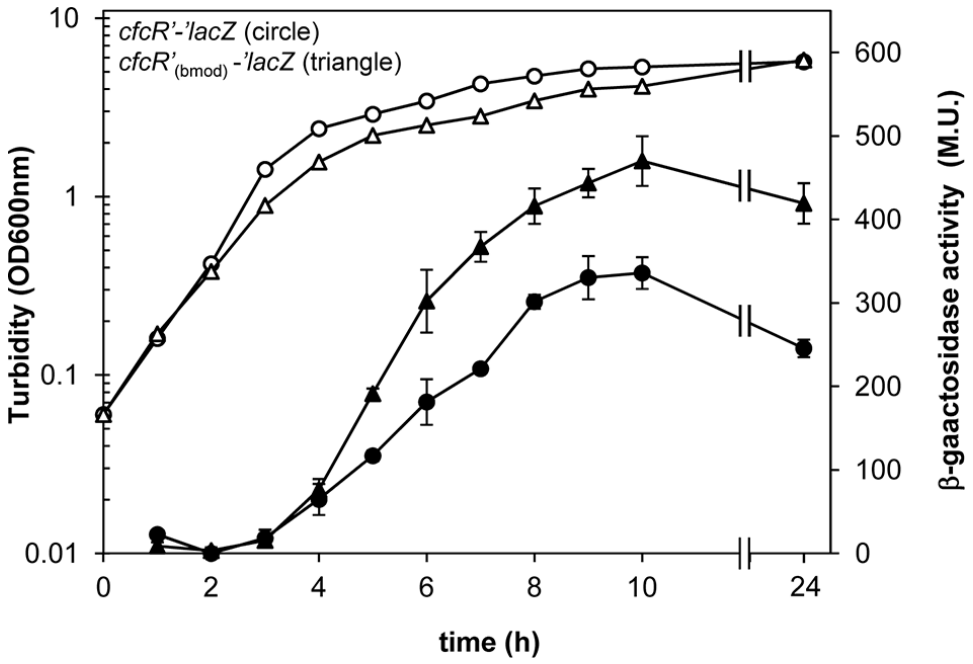

Fig. 11. Alteration of Rsm-binding motif B in RNA-CfcR affects the expression pattern of *cfcR* in *P. putida* KT2440. Growth (hollow) and β-galactosidase activity (solid) are plotted for strains with the *cfcR'-'lacZ* fusion in pMIR219 (circle) and the *cfcR'*$_{(bmod)}$-'*lacZ* fusion in pMIR220 (triangle). Sequence ATGGAT in pMIR219, being ATG the start codon of *cfcR*, was replaced with ATGTTA in pMIR220. Experiments were carried out in triplicate and activities were assayed in duplicate. Average data and standard deviation are plotted from one representative experiment. Statistically significant differences between activities of the fusions were detected from 5 h onwards (Student's *t* test; $P < 0.05$).

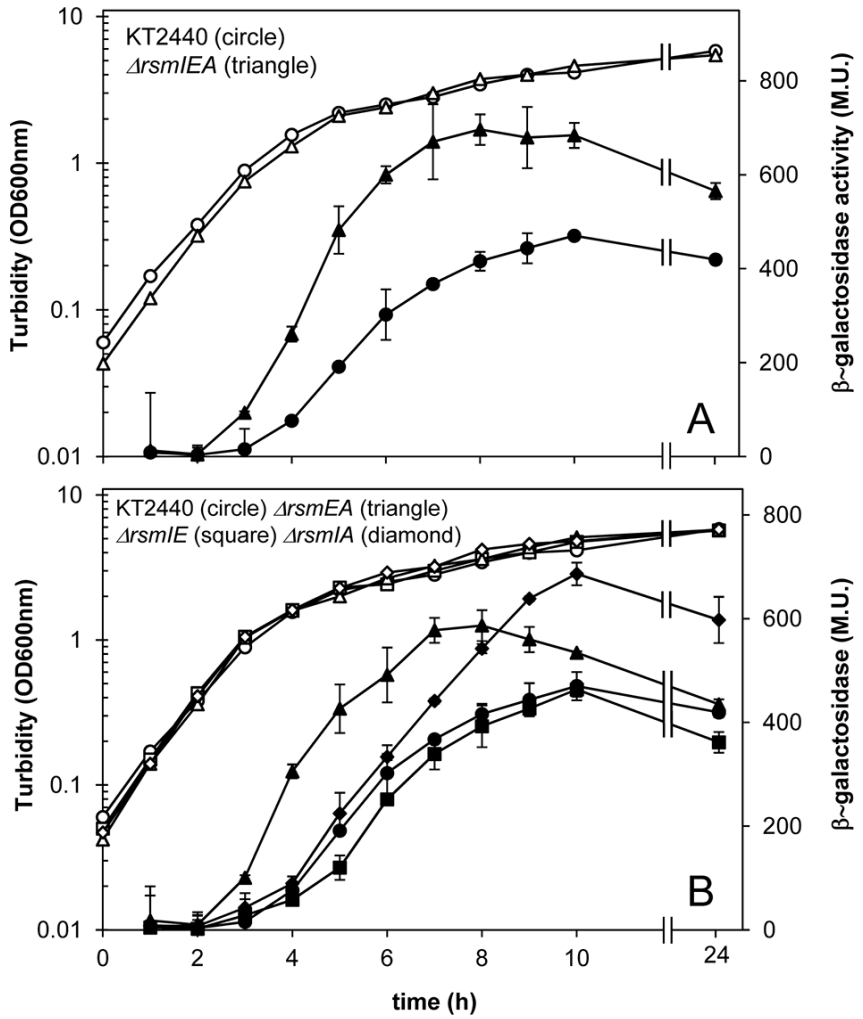

Fig. 12. Activity of the translational fusion *cfcR'*$_{(bmod)}$-*'lacZ* in *rsm* mutant genetic backgrounds. Cultures growing in LB supplied with Tc as described in the experimental procedures were analyzed for turbidity (hollow) and β-galactosidase activities (solid) at the indicated times. **A)** Data for wild type *P. putida* KT2440 (circle) and the *ΔrsmIEA* triple mutant (triangle) strains are shown. **B**) Data for *P. putida* KT2440 (circle), *ΔrsmEA* (triangle), *ΔrsmIE* (square) and *ΔrsmIA* (diamond) strains are shown. The experiments were performed in triplicate and activities were assayed in duplicate. Average data and standard deviations are plotted from one representative experiment. Statistically significant differences were observed between wild type and *ΔrsmIEA* (from 4 h onwards), *ΔrsmEA* (between 4 - 9 h) and *ΔrsmIA* mutant strains (from 7 h onwards). No differences were observed between wild type and *ΔrsmIE* (Student's *t* test; $P< 0.05$).

This fusion with a mutated motif B exhibited similar expression pattern in the three null *rsm* single mutants and the wild type (Fig. 13), and the same result was observed with the double mutant *ΔrsmIE*, where only RsmA is active (Fig. 12B).

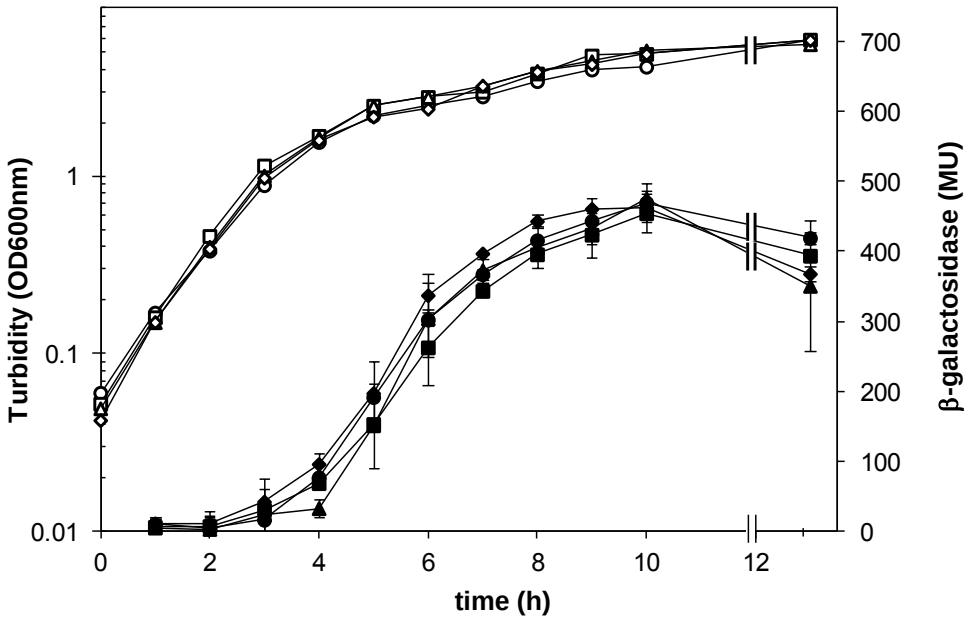

Fig. 13. Activity of the translational fusion *cfcR*$_{(bmod)}$*'-'lacZ* in single *rsm* mutant strains. LB cultures supplied with Tc were obtained as indicated in the Experimental procedures section. Samples were analyzed for turbidity (hollow symbols) and B-galactosidase activity (solid symbols) at the indicated times. Wild type KT2440 (circle), *ΔrsmE* (triangle), *ΔrsmI* (square) and *ΔrsmA* (diamond). Experiments were performed in triplicate. Average and standard deviation of data from one representative experiment with two experimental replicates are plotted.

However, certain derepression was observed in mutants *ΔrsmEA* and *ΔrsmIA* (Fig. 12B). These results indicate that the binding of RsmA was lost after the modification of motif B and suggest together with those obtained in the fEMSA experiments that the repression of *cfcR* exerted at

its translation start takes place mainly through RsmA. Certain repressive role of RsmE and RsmI was maintained independently of motif B likely by an indirect way.

The free c-di-GMP pool of Pseudomonas putida is negatively regulated by Rsm proteins largely through CfcR. As a consequence of the enhancement of *cfcR* expression in the *ΔrmsIEA* background we hypothesized that the level of c-di-GMP should be increased in this mutant. To investigate this possibility we monitored c-di-GMP levels in the wild type strain and compared them to those from the *ΔcfrR, ΔrsmIEA* and *ΔrsmIEAcfcR* mutant genetic backgrounds using the biosensor plasmid pCdrA::gfp^C (Rybtke *et al.*, 2012). GFP fluorescence was quantified from cultures grown in microtiter plates using an Infinite 200 Tecan fluorimeter and also directly observed from bacterial streaks on agar plates as indicated in experimental procedures. In the wild type strain a boost in c-di-GMP was observed in the stationary phase of growth which reached a maximum after 20 hours of incubation (Fig. 14A).

Interestingly in the triple *ΔrsmIEA* mutant the c-di-GMP boost was observed earlier (6 hours in advance) than in the wild type and although the values at some stages were up to 6-fold higher than in the wild type, they were not sustained over time (Fig. 14A). In the *cfcR* mutant the levels of c-di-GMP remained significantly lower than in the wild type strain during the stationary phase (Fig. 14A), which was in agreement with *cfcR* being RpoS dependent (Matilla *et al.*, 2011). The fact that CfcR was responsible for up to 75% of c-di-GMP cell content in this growth stage suggested that the diguanylate cyclase activity of this protein is a major determinant of the free global pool of this second messenger in *P. putida* during the stationary phase.

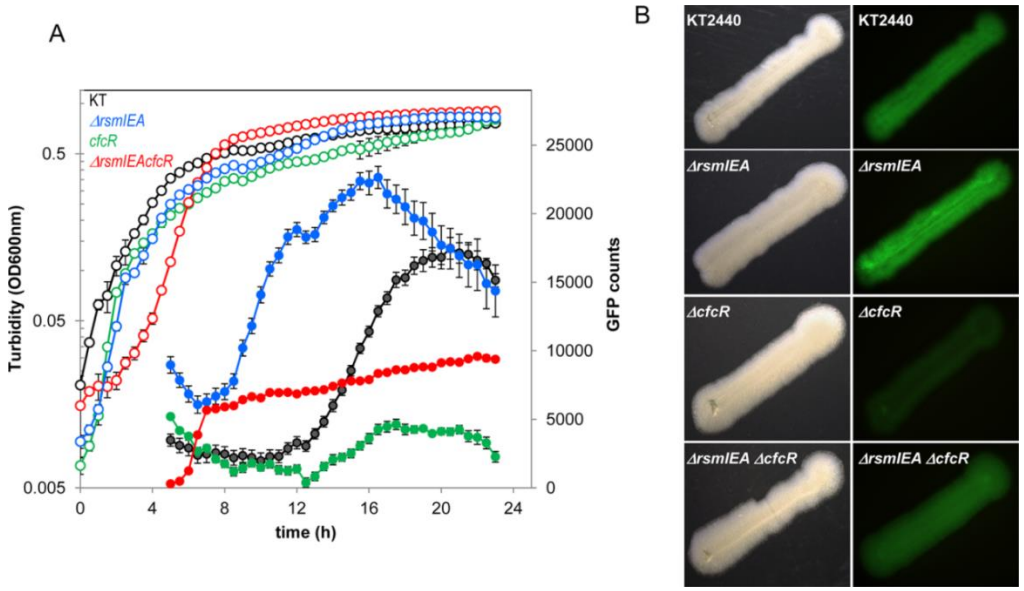

Fig. 14. Time course of c-di-GMP free pool in *P. putida* strains. All strains harboring the biosensor plasmid pCdrA::*gfp*^{C}. **A)** Growth (hollow symbols) and GFP counts (solid symbols) indicating fluorescence readings normalized for growth in LB 1/10 (OD_{600nm}). Experiments were carried out in duplicate and measurements were made in triplicate. Average data and standard error is plotted for *Pseudomonas putida* KT2440 (black), *ΔrsmIEA* (blue), *ΔcfcR* (read) and *ΔrsmIEAcfcR* (green) strains. **B)** LB-agar plates were incubated at 30°C for 24 h; pictures of the visible field (left panels) were taken using a Leica stereomicroscope M165FC and for dark field pictures (right panels), an excitation/emission filter 480/510 nm was used with an exposure time of 1.1 seconds.

This could be confirmed with the quadruple *ΔrsmIEAcfcR* mutant where the c-di-GMP boost in the triple *ΔrsmIEA* genetic background was replaced by a more moderate and earlier increase, at the onset of the stationary phase (Fig. 14A). Visualization by stereomicroscopy of the fluorescence produced by these strains gave results that positively correlated with the quantitative data (Fig. 14B). The kinetics of c-di-GMP were also analyzed in the single *rsm* mutants and no important differences were observed between these strains and the wild type (Fig. 15).

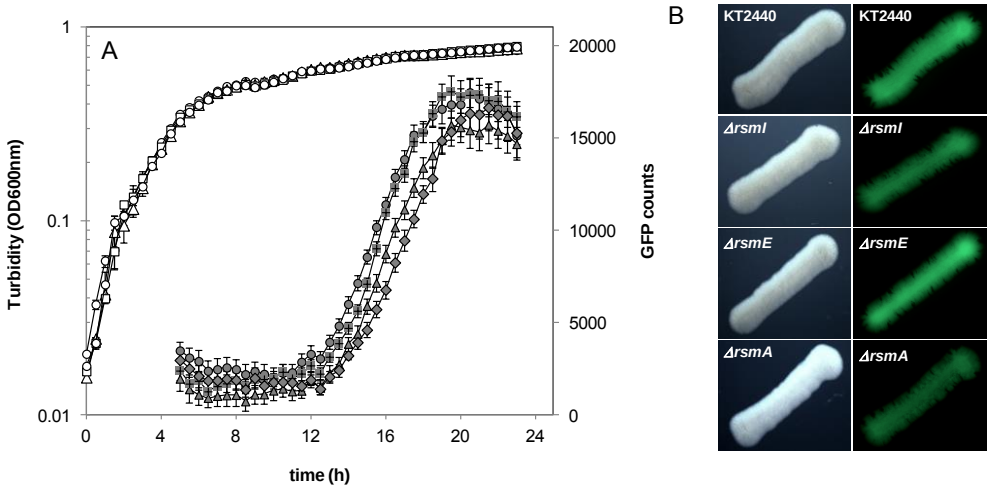

Fig. 15. Modulation of c-di-GMP cell content by Rsm proteins in single *rsm* mutants. All strains harbor biosensor plasmid pCdrA::*gfp*^{C}. A, Growth (hollow symbols) and GFP counts (solid symbols) that indicate fluorescence readings corrected for growth in LB 1/10 (OD_{600nm}). Experiments were carried out in duplicate with three experimental replicates. Average data and standard error are plotted for *Pseudomonas putida* KT2440 (circles), *ΔrsmI* (triangle), *ΔrsmE* (square) and *ΔrsmA* (diamond). B, LB-agar plates were incubated at 30°C for 24 h; pictures of the visible field (left panels) were taken using Leica stereomicroscope M165FC and for dark field pictures (right panels), an excitation/emission filter 480/510 nm was used with an exposure time of 1.3 seconds.

Similar kinetics were observed in the double Δ*rsmIA* mutant, with only RsmE active, although with a slight decrease in c-di-GMP levels at the end of the experiment (Fig. 16). Different c-di-GMP profiles were observed for *ΔrsmEA* and *ΔrsmIE*; in the first strain, with only RsmI active, the boost in the level of the second messenger was delayed in comparison with the wild type, while in the latter, where only RsmA remained active, the maximum values were almost 50% below those attained by the wild type (Fig. 16). This seems to be an indication that the repression exerted by RsmA upon c-di-GMP levels (through *cfcR*) is

stricter in the absence of RsmE and RsmI and that the last acts later in the stationary phase of growth.

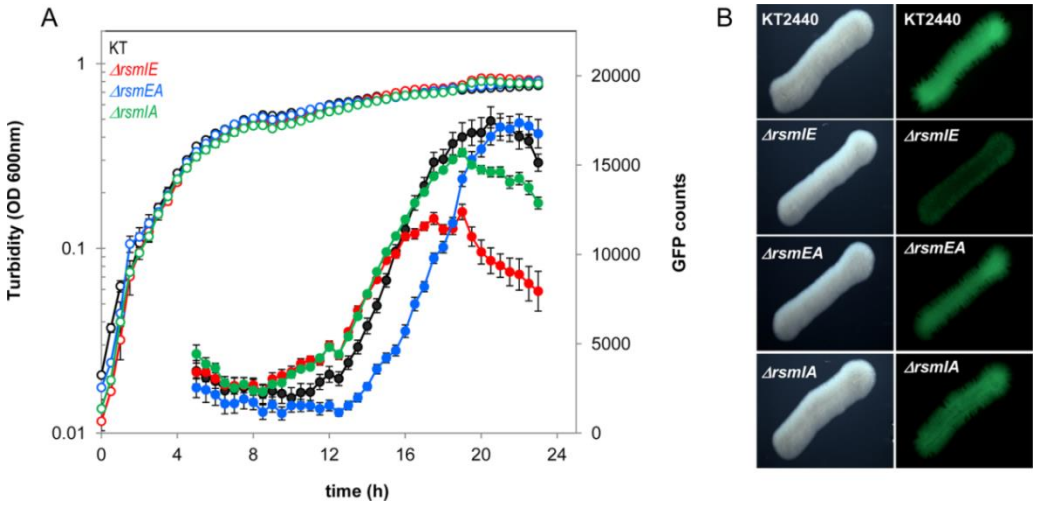

Fig. 16. Time course of c-di-GMP free pool in double *rsm* mutants. All strains harbor biosensor plasmid pCdrA::gfp^{c}. **A**, Growth (hollow symbols) and GFP counts (solid symbols) that indicate fluorescence readings corrected for growth in LB 1/10 (OD600nm). Experiments were carried out in duplicate with three experimental replicates. Average data and standard error are plotted for *Pseudomonas putida* KT2440 (circles), *ΔrsmIE* (triangle), *ΔrsmEA* (square) and *ΔrsmIA* (diamond). **B**, LB-agar plates were incubated at 30°C for 24 h; pictures of the visible field (left panels) were taken using Leica stereomicroscope M165FC and for dark field pictures (right panels), an excitation/emission filter 480/510 nm was used with an exposure time of 1.3 seconds.

CfcR is responsible for the enhanced biofilm formation capacity of the triple mutant ΔrsmIEA. It was previously shown that the triple *ΔrsmIEA* mutant formed more biofilm than the wild type when this capacity was analyzed with slight rotation or under static conditions (Huertas-Rosales *et al.*, 2016). In addition, as seen above, the increase in c-di-GMP observed in this strain was compromised when a *cfcR* deletion was added to *ΔrmsIEA* mutant. In order to evaluate the contribution of CfcR to the increased biofilm formation capacity observed

in the triple *ΔrsmIEA* mutant we evaluated the biofilm formed by the quadruple mutant *ΔrsmIEAcfcR* in polystyrene multiwell plates under static conditions and found that at the onset of stationary phase biofilm formation was reduced by half in comparison to the triple mutant (Fig. 17).

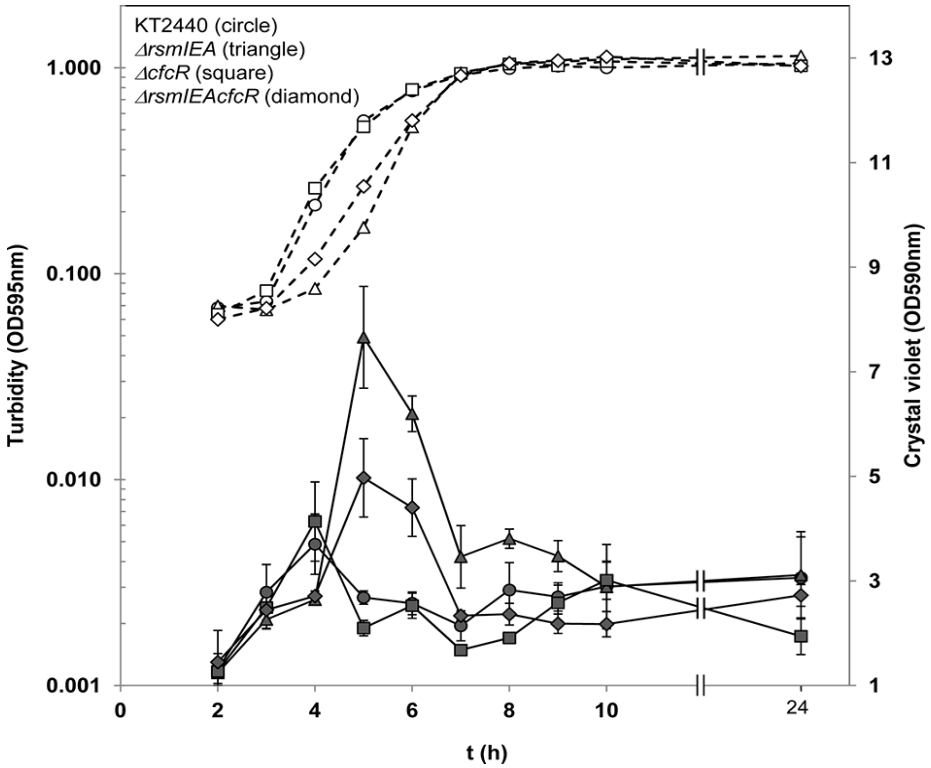

Fig. 17. Biofilm formation capacity of *P. putida* strains measured using multiwell plates. Growth (hollow symbols) and biofilm measurements (solid symbols) are shown. Data for wild type *P. putida* KT2440 (circle), triple mutant *ΔrsmIEA* (tringle), *ΔcfcR* mutant (square) and quadruple *ΔrsmIEAcfcR* mutant (diamond) strains are shown. The experiment was carried out in quadruplicate. Average data and standard deviations are plotted from a representative experiment using data measured in triplicate. Statistically significant differences were detected at 5 and 6 h between triple and quadruple mutants, between wild type and triple mutants, and between wild type and quadruple mutants (Student's *t* test; $P < 0.05$).

No difference in biofilm formation capacity between the triple and the quadruple mutant was observed in polycarbonate tubes with optimal aeration (Fig. 18).

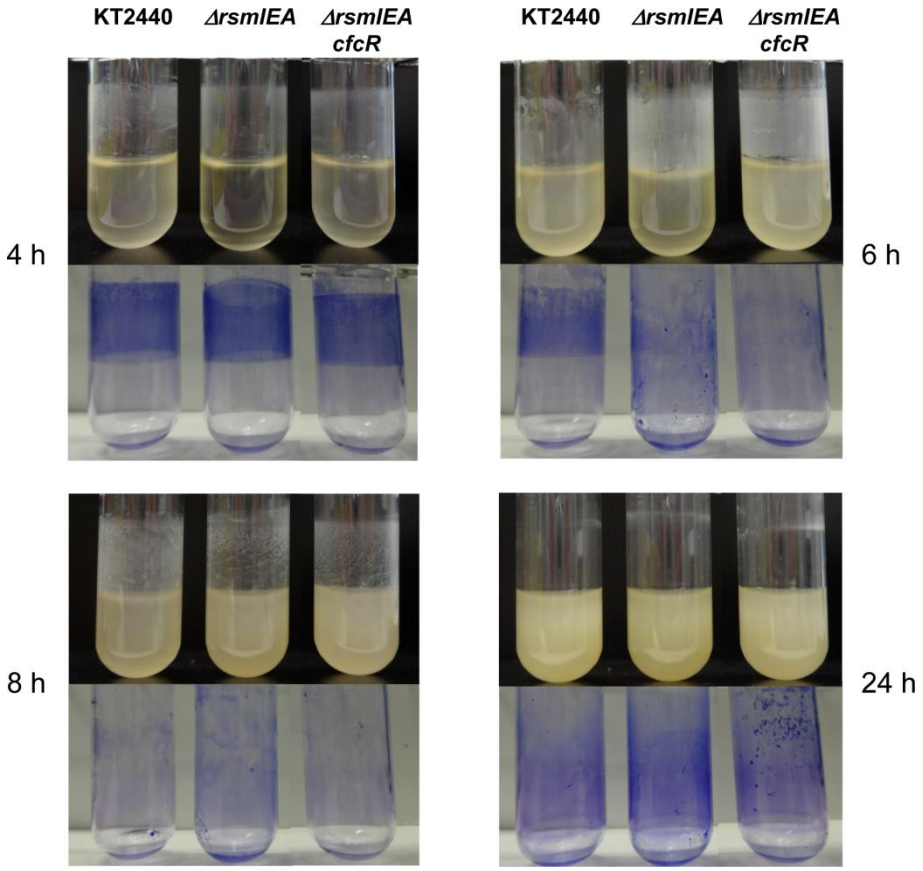

Fig. 18. Time course of biofilm formation capacity in wt, triple null *rsm* and quadruple *ΔrsmIEAcfcR* mutant strains in borosilicate glass tubes. Experiments were performed as indicated in the experimental procedures and photos were taken at the indicated times.

Discussion

In this work we aimed to unravel the role of the RNA-binding post-transcriptional regulators of the CsrA/RsmA family (RsmA, RsmE and RsmI) from *P. putida* (Huertas-Rosales *et al*., 2016) in the regulation of the diguanylate cyclase CfcR and the impact this control exerts on c-di-GMP levels and biofilm formation. We therefore tested if single and combined deletions of the *rsm* genes affected the expression of the translational fusion *cfcR'-'lacZ*. Single mutants allowed us to evaluate the effect upon *cfcR* expression of the loss of individual Rsm proteins, while double mutants allowed to investigate the role of each Rsm protein when present in the cells alone (i.e. *ΔrsmIE* for active RsmA; *ΔrsmIA* for active RsmE; and *ΔrsmEA* for active RsmI). Expression of *cfcR* was only notably altered (induced) in the triple *ΔrmsIEA* mutant, indicating that Rsm proteins repress *cfcR* but single mutations may be compensated by the other two Rsm homologues and therefore their roles can be interchangeable. In support of this, we confirmed that the three Rsm proteins could specifically bind the transcript RNA-CfcR(b) containing motif B, which matches a consensus for Csr/Rsm proteins binding at the start codon of *cfcR*. These *in vitro* results together with the results obtained *in vivo* with the *cfcR'-'lacZ* fusion allowed us to confirm the negative regulation of *cfcR* via direct RNA-binding regulators of the CsrA/RsmA family. Furthermore, the specific role of motif B 5'C**ATG**GATG3' (start codon in bold) was established after verifying the positive effect of replacing the nucleotides GGA, which are highly conserved (Dubey *et al*., 2005), for GTT in the activity of the fusion *cfcR'*$_{(bmod)}$*-'lacZ* in the wild type strain. This confirmed that the repression of *cfcR* by Rsm proteins takes place at the initiation of translation. Given that the activity of this mutated fusion was very slightly reduced in the double mutant *ΔrsmIE*, with an RsmA active, whereas it was enhanced in

the other two double mutants with active RsmE or RsmI, we came to conclude that RsmA had a major role in the inhibition of translation initiation, and in fact it bound with the highest affinity to RNA-CfcR(b). However this binding was easily impeded as a consequence of increasing the length of the RNA target likely because of changes provoked in its secondary structure. A heptaloop-stem is predicted at the binding site in the case of *cfcR* mRNA, instead of the penta- or hexaloop more commonly found (Schubert *et al.*, 2007; Lapouge *et al.*, 2013). Yet to our knowledge although RsmA structure of *P. putida* has been resolved (Rife *et al.*, 2005), a structural model of this protein, or any CsrA/RsmA, contacting target RNA is not available.

We have discarded that Rsm proteins interact with another putative motif (motif A) upstream in the leader of mRNA *cfcR*. In fact, the sequence of this motif A 5'TAATGGATCC3' differs more than motif B from the consensus 5'$^{A}/_{U}$CANGGANG$^{U}/_{A}$3', based on the optimal contacts of the RsmE homodimer with its two RNA-binding sites (Schubert *et al.*, 2007). Thus in a genetic background with all *rsm* genes deleted no differences in post-transcriptional regulation of *cfcR* would be expected from a fusion with an altered motif B. However, increased activity was detected from *cfcR'*$_{(bmod)}$*-'lacZ* compared to *cfcR'-'lacZ* in the triple mutant *ΔrsmIEA*. This might be explained if the transcription of *cfcR* was activated in this strain. We have confirmed that the expression of RpoS, which was previously shown to positively regulate transcription of *cfcR* (Matilla *et al.,* 2011), was anticipated in the *ΔrsmIEA* strain compared to the wild type. This early activation of RpoS could be responsible not only for the enhanced activity of the fusion *cfcR'*$_{(bmod)}$*-'lacZ* in the mentioned strains. It could also contribute to the *cfcR* mRNA level enhancement observed in the double mutants *ΔrsmEA* and *ΔrsmIA* (not in *ΔrsmIE*) and to the shortening in the incubation times necessary to achieve a great boost in the triple mutant. We have identified *rpoS* as a target of Rsm

proteins in RIP-seq experiments (our unpublished results) as an indication that RpoS regulation by Rsm proteins is direct. In *P. protegens* CHA0, RpoS was also found to be negatively regulated by RsmA (Heeb *et al.*, 2005). Thus we can conclude that *cfcR* is not only negatively regulated by Rsm proteins directly at the initiation of its translation, but also indirectly at the transcriptional level through RpoS. Nevertheless it cannot be ruled out that Rsm proteins binding to their target mRNA might alter (shorten) their stability.

We have reported previously that the transcriptional regulators ANR (Matilla *et al.*, 2011) and FleQ (Ramos-González *et al.*, 2016) positively modulate the transcription of *cfcR*. Since in the same RlP-seq experiments we have identified that ANR is a target of Rsm proteins (our unpublished results), it is tempting to speculate that increased levels of ANR are attained as a consequence of the loss of Rsm proteins. Thus, enhanced ANR levels might also contribute to the changes in the *cfcR* expression observed in the triple *ΔrsmIEA* mutant and to a lesser extent in the double *rsmEA* and *rsmIA* mutants.

Given the low amount of c-di-GMP present in *P. putida* KT2440, quantification of the levels of this second messenger in this strain has not been feasible to date using analytical methods. However, identification of mutants with lower levels of c-di-GMP has recently been made possible using a c-di-GMP biosensor (Ramos-González *et al.*, 2016). In this work we confirm that c-di-GMP values are severely reduced in a *cfcR* mutant during stationary phase, indicating that this DGC is of major relevance to the free pool of c-di-GMP in *P. putida* during this growth stage. In addition, the earlier and enhanced expression of *cfcR* observed in the triple mutant *ΔrsmIEA* correlated with an earlier boost of free c-di-GMP in this strain, which was compromised when *cfcR* was deleted. While *ΔrsmIEA* had been shown to form more biofilm at earlier stages than wild type strain when grown with slight rotation or under static conditions

(Huertas-Rosales *et al.*, 2016), in the quadruple mutant *ΔrsmIEAΔcfcR* this increased biofilm formation was also observed although to a lesser extent in static conditions. Therefore the contribution of CfcR to biofilm formation is more important under conditions with limited aeration—a finding that is in agreement with *cfcR* expression being enhanced under O_2 depletion (Matilla *et al.*, 2011). These observations indicate that out of the 36 genes that have been annotated as encoding GGDEF domain containing proteins in *P. putida* (Ulrich and Zhulin, 2010), *cfcR* is a key player and required for the *ΔrsmIEA* mutant to display both a c-di-GMP boost and increased biofilm formation capacity in the conditions tested. C-di-GMP values were significantly higher in *ΔrsmIEAcfcR* than in the *cfcR* mutant at earlier stages. This indicates that diguanilate cyclase(s) other than CfcR might also be de-repressed as a consequence of the loss of Rsm proteins. Nevertheless c-di-GMP levels were maintained during the stationary phase of growth in *ΔrsmIEAΔcfcR* below those observed in wild type (Fig. 14) indicating that, even if de-repressed, these putative DGCs likely play only a minor role in the regulation of free c-di-GMP during this stage and under the conditions used in our studies. It should be mentioned that other proteins that contribute to regulate c-di-GMP levels, such as PDE, might also become differentially regulated as a consequence of *rsm* genes deletion. In *E. coli*, two GGDEF containing proteins (YcdT and YdeH) are post-transcriptionally regulated by CsrA (Jonas *et al.*, 2008). In *P. aeruginosa*, SadC is the only DGC involved in biofilm formation that is controlled by the Gac/Rsm pathway (Moscoso *et al.*, 2014). Interestingly, CfcR has no orthologs in *P. aeruginosa* strains and there is no orthologue of SadC in *P. putida* KT2440 (Winsor *et al.*, 2016). Thus our study confirms the link between the Gac/Rsm cascade and c-di-GMP signaling, although the proteins involved in each bacterium are different. It should be noticed that the expression of RpoS in *P. putida* requires an active Gac system (Martínez-Gil *et al.*, 2014), therefore the inactivation of Gac impedes the expression of *cfcR*.

C-di-GMP kinetics were similar in the single Rsm mutants and the wild type consistently with the lack of difference found in *cfcR* expression between these strains. When RsmA remained as the only Rsm protein in the double mutant *ΔrsmIE,* it promoted a great decay in the c-di-GMP free pool at the stationary phase of growth to the point that values of this second messenger were lower in this strain than in the WT. Since under the conditions tested, most of the c-di-GMP free pool of *P. putida* in the stationary phase was due to the presence of CfcR, the low value of this second messenger in the mutant *ΔrsmIE* is in agreement with RsmA being the key inhibitor at the initiation of *cfcR* translation, as it has been mentioned above. We hypothesizes that RsmA protein levels in the cell are tightly regulated. With three proteins sharing the same RNA target, if RsmA, with the highest affinity, was under-represented compared to the other two, removing its less efficient competitors RsmE and RsmI, might result in increased repression, which was in fact our observation in the double mutant *ΔrsmIE*. Consistently RsmE expression levels were higher than those of RsmA (above 5-fold) and although the expression of RsmI was inferior to that of RsmA (Huertas-Rosales et al., 2016) an induction on RsmA expression was observed when RsmE and RsmI were deleted. Thus the co-existence of three members of the CsrA/RsmA family in *P. putida* seems to allow a finer modulation of *cfcR* regulation especially since self-regulation and cross-regulation among the Rsm proteins have been reported (Huertas-Rosales *et al.*, 2016). The phenotype related with a reduced *cfcR* expression in the double mutant having still an RsmA active was noticeable under static conditions (c-di-GMP values). However under shaking with optimal O_2 availability this effect was not observable, either for second messenger (Fig. 19) or gene expression values obtained with *cfcR'-'lacZ*, which is in agreement with the reduced biofilm formation capacity observed for this mutant specifically in microtitter plates in static (Huertas-Rosales *et al.*, 2016).

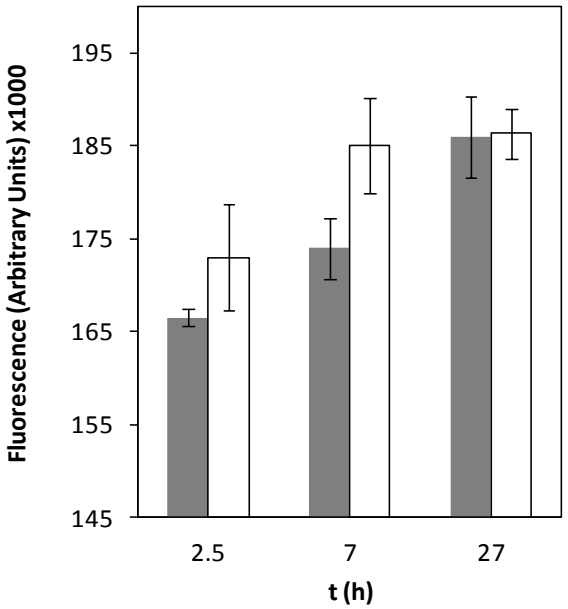

Fig. 19. Time course of c-di-GMP free pool of *P. putida* KT2440 (pCdrA) and *ΔrsmIE* (pCdrA) strains under shaking. LB cultures of KT2440 (pCdrA) (grey) and *ΔrsmIE* (pCdrA) (white) supplied with Gm50 and Pip30 were incubated in flasks under shaking (200 r.p.m) and diluted at the indicated times at an OD_{660nm}= 0.15 before measuring fluorescence in a LPS-220B fluorometer (Photon Technology International) with λ_{ex} 485 nm and λ_{em} 510 nm. Average and standard deviation of data from two experiments with two experimental replicates are plotted. Cultures without pCdrA exhibited a background of 145 ±3 (x1000). Statistically significant differences in fluorescence were detected between wild type and *ΔrsmIE* at 7 h (Student's *t* test; $P< 0.05$).

The results presented in this work provide evidence that CfcR is a key determinant for the generation of the free pool of c-di-GMP in stationary phase in *P. putida*, especially when O_2 is depleted, and also for the increased biofilm formation observed when Rsm proteins are absent. This central role for CfcR, and the impact that c-di-GMP levels have on different phenotypes, may explain why *cfcR* expression is tightly regulated at multiple levels, transcriptionally via RpoS, ANR and FleQ, post-transcriptionally via direct interaction with Rsm proteins, and post-translationally via phosphorylation, very likely by the multi-sensor hybrid histidine kinase CfcA (Fig. 20).

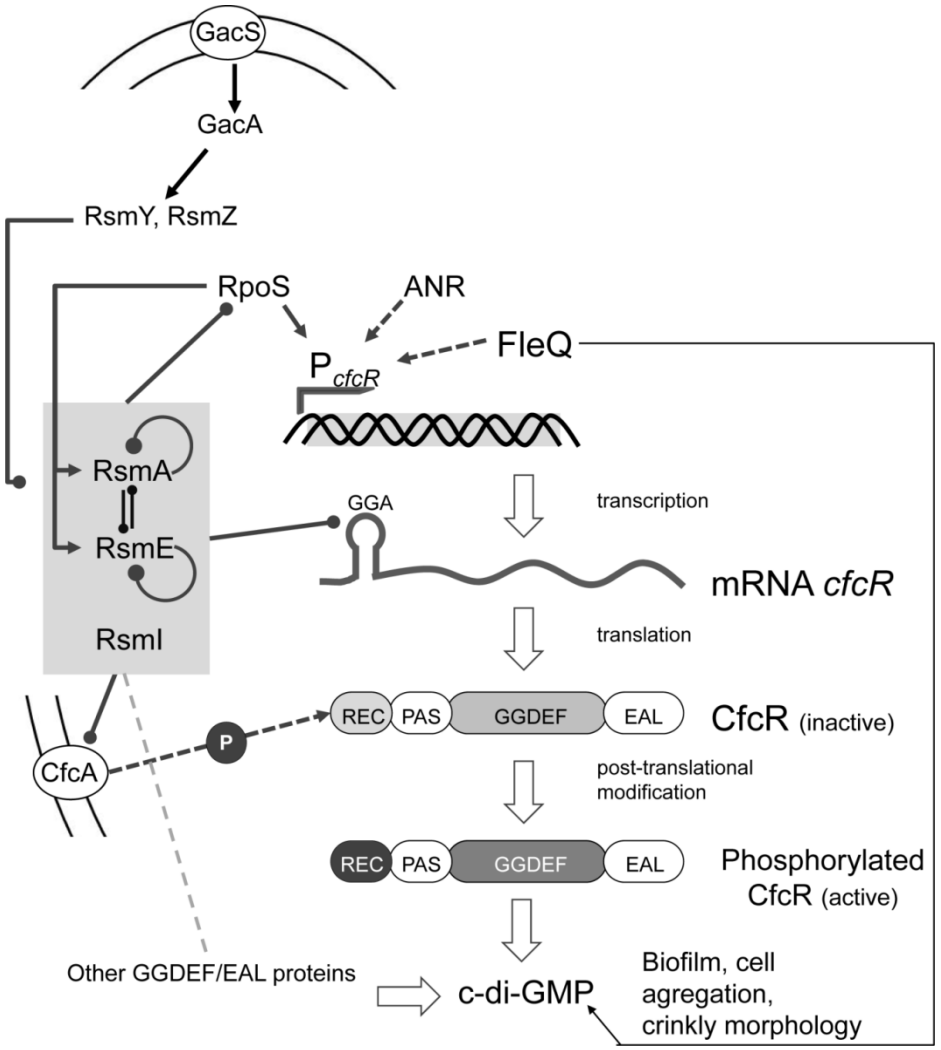

Fig. 20. Schematic summary of the regulatory circuits that influence expression and diguanylate cyclase activity of CfcR, ultimately leading to changes in c-di-GMP levels. Arrow-ended and round-ended lines indicate positive and negative regulation, respectively. Broken lines indicate regulatory processes for which evidence exists but need to be studied in further detail. Binding of c-di-GMP to FleQ and regulation of Rsm proteins as recently reported by Huertas-Rosales and coworkers (Huertas-Rosales *et al.,* 2016) is included. Rsm proteins regulate the expression of other GGDEF/EAL proteins (Huertas-Rosales *et al.*, in preparation). See text for more details.

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Chapter 3

Global analysis of the Rsm regulon in *Pseudomonas putida* KT2440

Summary

We have carried out a global transcriptome analysis to identify RNA sequences that are bound by Rsm proteins in *Pseudomonas putida* KT2440, in order to identify potential regulatory targets for each of these proteins. A variant of RIP-seq analysis with strict selection parameters was used to identify 410 different targets, 38 of them common to the three proteins. The targets were assigned to genes involved in several functional classes including transport and secretion, iron homeostasis, carbon, nitrogen, phosphorous and sulfur sources metabolism, signal transduction, transcriptional regulation, and genes encoding proteins involved in biofilm formation and in the turnover of c-diGMP.

Introduction

Bacteria use genetic regulatory mechanisms to adapt, compete and survive in response to changing environmental and physiological conditions. Besides, global regulatory network permit bacteria to co-ordinate expression of large sets of genes in multiple operons (Gottesman, 1984; Beisel and Storz, 2010).

The destiny of RNA molecules in the cell is largely determined at the post-transcriptional level by RNA-proteins interactions. RNA-binding proteins (RBPs) are responsible for essential traits such as RNA stability, structure, translatability, export and localization. Recent screens in human cells have suggested that the number of proteins with RNA-binding properties may be vastly underestimated (Baltz *et al.*, 2012; Castello *et al.*, 2012; Kramer *et al.*, 2014), prompting new systematic searches for RBPs in many eukaryotic systems (Ascano *et al.*, 2012). By comparison, our knowledge of the scope and binding preferences of prokaryotic RBPs is clearly behind eukaryotic systems, and new approaches are needed to fully elucidate the roles of RBPs in post-

transcriptional control in bacterial pathogens (Barquist and Vogel, 2015). That is, although the structural details of the interactions of many positively and negatively acting proteins with DNA have been established, the shortage of understanding with respect to RBPs has retarded the field of bacterial gene regulation.

In the plant-beneficial bacterium *Pseudomonas putida* KT2440 three genes have been identified that encode posttranscriptional regulators belonging to the CsrA/RsmA (acronyms for carbon storage regulator and regulator of sedondary metabolism) family and playing important roles in the motile and sessile lifestyles (Huertas-Rosales, *et al.,* 2016). Proteins belonging to the CsrA/RsmA family are small sequence specific RNA-binding regulators that active or repress gene expression by altering translation, RNA stability and/or transcript elongation (Romeo *et al.*, 2013). Csr/Rsm proteins primarily affect translation of mRNAs by binding to 5´untranslated regions (UTRs). A plethora of genetic, biochemical and structural data shown that these proteins generally recognize GGA motifs in apical loops of RNA secondary structures (Dubey *et al.,* 2005; Duss, *et al.*, 2014). Other reported mechanisms of CsrA activity in the cell include promotion of Rho-dependent transcription termination, or mRNA stabilization by masking of RNase E cleavage sites (Yakhnin *et al.*, 2013; Figueroa-Bossi *et al.*, 2014). CsrA may also govern a large post-transcriptional regulon, as inferred from transcriptomic and RNA co-purification data in *Salmonella* and *E. coli*, respectively (Lawhon *et al.*, 2003; Edwards *et al.*, 2011).

The Csr/Rsm proteins are themselves regulated by sRNAs such as RsmY and RsmZ, which contains multiple GGA sites that titrate the protein away from mRNA targets (Brencic *et al.,* 2009; Weilbacher *et al.,* 2003; Valverde *et al.*, 2004). Structural studies of RsmE from *Pseudomonas fluorescens*, a CsrA-like protein, revealed a sequential and

cooperative assembly of the proteins and antagonistic sRNAs (Duss *et al.*, 2014a). Antagonist of CsrA activity also includes the Hfq-dependent sRNA McaS in *E. coli* (Holmqvist and Vogel, 2013; Jørgensen *et al.*, 2013) and a sponge-like mRNA in *Salmonella* (Sterzenbach *et al.,* 2013). Despite the strong interest in these proteins, the global binding preferences o Csr/Rsm *in vivo* remains unknown. In chapter two we reported the regative regulation of *cfcR*, which encodes the only response regulator with GGDEF-EAL domain in *P. putida* KT2440 by the three Rsm proteins present in this strain to a greater of lesser extent.

In this study, we use a high-throughput sequencing approach to screen for direct targets of RsmA, RsmE and RsmI regulation. Our data suggest a greatly expanded global regulatory role for Rsm proteins.

Results and Discussion

Preliminary transcriptome analysis of targets for RsmA, RsmE and RsmI post-transcriptional regulation. A variant of RNA immunoprecipitation and sequencing (RIP-Seq) analysis was done to identify genes that could potentially be regulated post-transcriptionally by RsmA, RsmE or RsmI (Figure 1). For this purpose, each recombinant His-tagged Rsm protein (RsmA-His_6, RsmE-His_6 and RsmI-His_6) was expressed in *P. putida* KT2440 as described in Materials and Methods. Instead of immunoprecipitation, proteins were purified by affinity column chromatography and RNA non-covalently bound to them was subsequently isolated. In all cases, protein yield and purity was checked. In parallel, total RNA was isolated from the same cultures. Total and Rsm-bound RNA were analyzed for purity, quantified and converted to cDNA, and analyzed by Ilumina sequencing. Filtered sequences were aligned with the reference genome of *P. putida* KT2440 obtained from GeneBank and analyzed to detect the regions corresponding to those

sequences that were significantly enriched in the Rsm-bound RNA population with respect to the total RNA population.

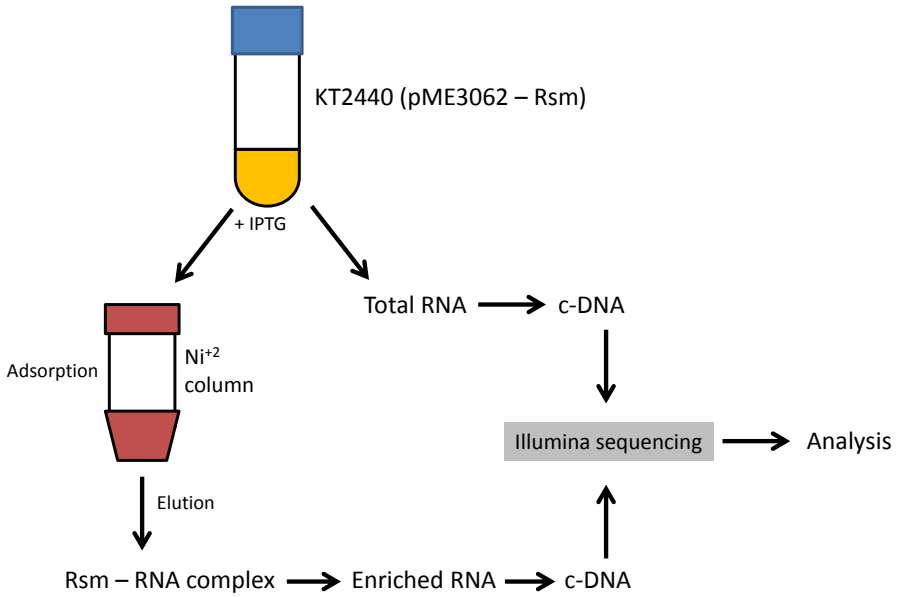

Figure 1. Schematic representation of the "RIP-Seq" method used for Rsm-bound RNA. See text for details.

Those peaks that did not appear in the three technical replicates of the experiment were discarded for later analysis. The number of peaks corresponding to RsmE-bound RNA sequences significantly overrepresented with respect to total RNA was much higher than those associated to RsmA or RsmI. Data were grouped in intervals and histograms were built to analyze the distribution of values in each case (Figure 2). The distribution of fold enrichment values was similar for RsmA and RsmI, with slightly lower values in the first case. For RsmE, the distribution was different and the average values clearly higher. In all cases, the *P*-value data distribution was similar between the biological replicates of each protein RNA purification. The analysis of distributions

was the basis to establish cut-off values for further analysis of Rsm targets. In this preliminary analysis we have opted for rather strict parameters in order to take into account sequences strongly overrepresented in the Rsm-bound RNA population, and also to minimize the number of potential false positives, at the expense of missing real targets that do not pass the established cut-off values. Thus, enriched regions with a fold enrichment ≥3 or4 score -10·Log_{10}(*P*-value) ≥ 200 or 300, for RsmA/RsmI or RsmE respectively were selected for further study. Again, peaks that did not pass the cut off in one of the three replicas were discarded.

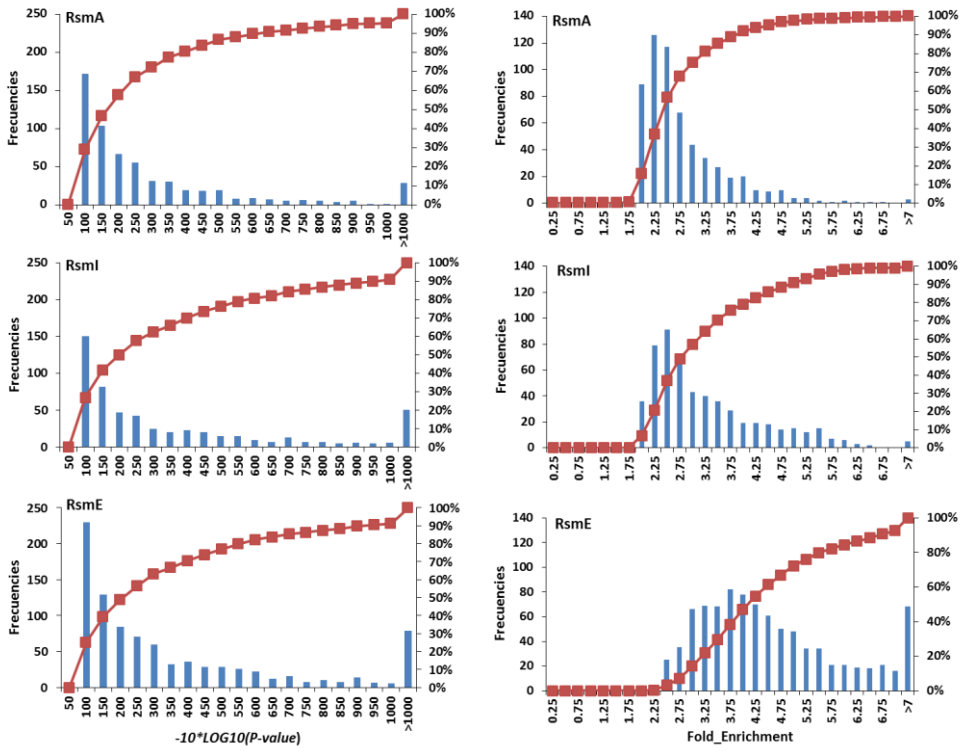

Figure 2. Distribution of -10xLog_{10}(*P*-value) (left) and fold enrichment (right) data of overrepresented RNA sequences associated to each Rsm protein with respect to total RNA. Data were grouped in the value intervals indicated in the X axis. The histograms (blue bars, primary Y axis) show the frequency of targets in each interval. The red lines (secondary Y axis) correspond to the cumulative percent values.

Using these parameters, a total of 102, 311 and 168 including genes and intergenic regions were identified as potential targets for RsmA, RsmE and RsmI, respectively (Figure 3). Of these, 38 appear as common targets for the three Rsm proteins and 95 are common to two of them. In total, 410 different targets were identified that passed the cut-off.

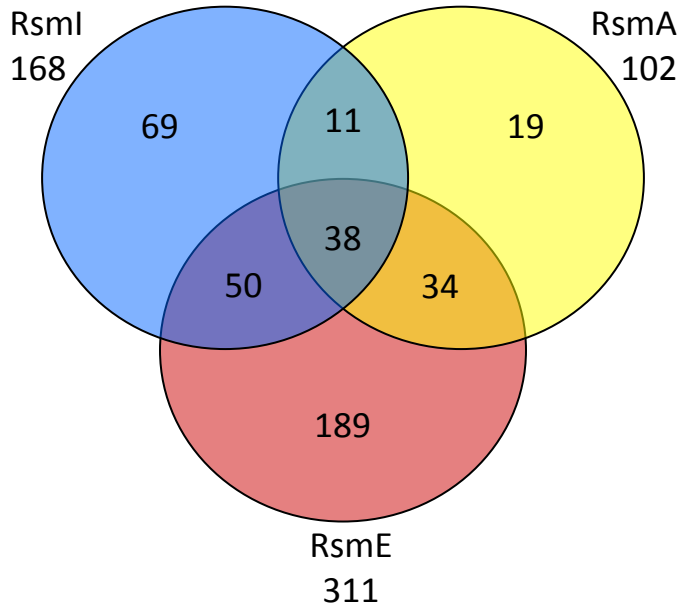

Figure 3. Venn diagram summarizing the number of targets identified for Rsm proteins showing fold enrichment and *P*-values above the cut-off.

Interestingly, 60% and 40% of the RsmE and RsmI targets are unique for each protein, respectively, while only 10% of the RsmA targets are not shared with other proteins. It should be noted that due to the way the cDNA libraries were constructed the analysis does not allow distinguishing the DNA strand to which the transcript corresponds. This means that in some cases where divergently transcribed genes are

adjacent in the genome, it is not possible to discern which of them the actual target is. Despite this limitation, the results indicate that over 10% of the transcripts in *P. putida* KT2440 are bound *in vivo* by Rsm proteins. The data also provide a broad overview of the functions that this type of post-transcriptional control shows.

These 38 targets common to the three Rsm proteins are compiled in table 1, broadly classified according to their functions. These include the small, non-coding RNA rsmY, known to bind and titrate Rsm proteins, and which therefore serves as positive control that the RIP-Seq methodology used was successful.

Besides RsmY, we have found the second small RNA involved in titration of Rsm proteins previously identified in *P. putida*, rsmZ, among those common targets to RsmA and RsmE. Three other annotated small non-coding RNAs (PP_mr44, PP_mr53 and PP_mr57) and five previously unannotated intergenic regions potentially corresponding to non-coding RNAs have also been identified as bound to one, two or the three Rsm proteins (Table 2). These will deserve future detailed analysis. It is also worth noting that both RsmA and RsmE are targets for RsmE, which confirms the in vivo data presented in chapter 1 on self-regulation of these proteins.

Table 1. List of targets common to all three Rsm proteins

Locus	Description
Metabolism	
PP_0063	Putative Lipid A biosynthesis lauroyl acyltransferase
PP_0370	Acyl-CoA dehydrogenase family protein
PP_0712	Polyphosphate kinase
PP_1032	GMP synthase [glutamine-hydrolyzing]
PP_1434	GTPase
PP_2437	Putative Acyl-CoA dehydrogenase
PP_2640	Acetyltransferase, GNAT family
PP_2681	PqqD - Coenzyme PQQ synthesis protein
PP_3547	Putative NAD(P)-binding oxidoreductase
PP_3972	Oxidoreductase, short chain dehydrogenase/reductase family
PP_4558	Acetyltransferase, GNAT family
PP_4559	Peptide deformylase
Transport and secretion	
PP_1200	Probable potassium transport system protein
PP_0906	Putative multidrug efflux RND transporter
PP_0907	RND efflux membrane fusion protein-related protein
PP_3548	Multidrug efflux transport system-permease subunit
PP_3010	Secretion
PP_3108	Secretion
PP_4542	Putative ABC transporter, ATP-binding protein/permease protein
DNA binding	
PP_1813	DNA binding protein
PP_1865	Transposase
PP_2114	Transposase
PP_4796	HolA - DNA polymerase III, delta subunit
Other functions	
PP_0563	GGDEF domain containing protein
PP_2397	EF hand domain protein (calcium binding)
PP_2641	Iron-sulfur cluster-binding protein
PP_4794	LeuS - Leucine-tRNA ligase
PP_4795	LptE - LPS-assembly lipoprotein
Uncharacterized	
PP_1814	Uncharacterized protein
PP_1887	Uncharacterized protein
PP_2396	Uncharacterized protein
PP_3007	Uncharacterized protein
PP_3901	Uncharacterized protein
PP_3908	Uncharacterized protein
PP_3909	Uncharacterized protein
Intergenic / non-coding RNA	
PP_1865-1866	Intergenic sequence
PP_3547-3548	Intergenic sequence
mr05	rsmY – non-coding RNA

(Annotated according to Winsor et al., 2016)

Table 2. Non-coding RNAs and intergenic regions identified as Rsm targets

locus	RsmI	RsmE	RsmA	
PP_mr05	x	x	x	**rsmY** - ncRNA
PP_mr22		x	x	**rsmZ** - ncRNA
PP_mr44	x	x		ncRNA
PP_mr53	x		x	ncRNA
PP_mr57			x	ncRNA
PP_1865-PP_1866	x	x	x	intergenic secuence
PP_1989-PP_1990		x		intergenic sequence
PP_3547-PP_3548	x	x	x	intergenic secuence
PP_3962-PP_3963		x	x	intergenic sequence
PP_4318-PP_4319	x	x		intergenic sequence

(Annotated according to Winsor *et al*., 2016)

CfcR-related targets. Intriguingly, *cfcR* RNA is not among the 38 common targets listed in Table 1. Although we have shown in the previous chapter that all three Rsm proteins bind *in vitro* a specific sequence overlapping the ATG of this gene, *cfcR* is only found as target for RsmE and RsmI. In reality, it is also among the RNA sequences overrepresented in the case of RsmA, but below the established cut-off values (Table 3). Two compatible possibilities can explain this. Perhaps the threshold established for RsmA is too strict, or that *in vivo* binding of RsmA to the mRNA of *cfcR* is hampered by competition with the other two proteins, a possibility that we have already suggested in chapter 2, based on *in vivo* expression data compared to the high affinity observed for the RsmA/*cfcR* mRNA interaction *in vitro*. We have also analyzed the data for elements involved in CfcR expression and digunylate cyclase activity, namely the sigma factor RpoS, the transcriptional regulator ANR, and the sensor histidine kinase CfcA (Table 3). Expression of RpoS has been shown to be modulated by Rsm proteins (chapter 2), and as

shown in Table 3, the -10*$\log_{10}$(*P*-value) is above the threshold in most samples while the fold enrichment values are somewhat below the cut-off. In the case of ANR, data indicate the RNA sequence is overrepresented for RsmE and RsmA, but the values are below the established cut-off. For *cfcA*, all three Rsm proteins are identified as binding its mRNA, although in one of the three replicas for RsmE the -10*$\log_{10}$(*P*-value) is slightly below the threshold. These results again suggest that the selection criteria here used are overly strict, and the number of potential false positives is likely insignificant.

Table 3. Values obtained for *cfcR, rpoS*, *anr* and *cfcA* RNA associated to each Rsm protein.

		-10*$\log_{10}$(*P*-value)	Fold_enrichment
cfcR	RsmI	437, 342	3.58, 3.1
	RsmE	722, 885, 1000	6.45, 6.31, 8.29
	RsmA	141, 93	1.82, 1.88
rpoS	RsmI	247, 139	2.01, 1.84
	RsmE	832, 1080, 1645	2.87, 3.3, 4.26
	RsmA	716, 736	2.16, 2.24
anr (fnrA)	RsmI	77, nd	2.06, nd
	RsmE	267, 392, 328	3.33, 4.37, 3.38
	RsmA	145, 188	2.12, 2.25
cfcA	RsmI	475, 414	4.64, 4.17
	RsmE	308, 250, 326	6.78, 6.22, 5.81
	RsmA	353, 232	3, 3.08

nd: not detected in the enrichment experiment
Red values – Values under the stablished cut-off

Other c-di-GMP and biofilm-related targets. Besides *cfcR*, four uncharacterized genes encoding proteins potentially related with c-di-GMP turnover were identified in this analysis, one of them as target of the three proteins, and the rest as targets of RsmE. Other relevant biofilm-related genes are targets for RsmE: *lapA*, and the operon

encoding the species-specific EPS Pea. Neither *lapF* nor the other three EPS operons were identified in this analysis. These results are somewhat surprising, since altered expression of *lapF* and EPS genes (alginate, Peb and to a lesser extent cellulose), was observed in the triple *rsm* mutant (chapter 1). It is likely that the observed expression changes are due to an indirect effect of Rsm proteins through other regulators. In fact, *lapF* expression is dependent on RpoS, and the influence of Rsm proteins on *rpoS* expression has been described in this Thesis.

Influence of Rsm proteins on metabolism and nutrient utilization. A significant number of targets identified correspond to functions involved in transport and potential utilization of carbon and nitrogen sources, particularly amin oacids/amino acid derivatives, glucose, and glycerol. In order to correlate the existence of these targets with a regulatory role of Rsm proteins on metabolic routes, 192 compounds were assayed as carbon sources, 96 as nitrogen sources, 48 as phosphate sources and 48 as sulphur sources, using BIOLOG plates and comparing the wild type KT2440 strain and the triple Δ*rsmIEA* mutant derivative. Growth differences were observed in several cases between the two strains, the most evident shown in Figure 4.

These results are consistent with the identification of three RsmE targets annotated as related to aspartic acid metabolism (PP_1001, PP_3721, PP_4473), and eight targets related to glutamic acid metabolism (PP_1000, PP_1001, PP_4823 and PP_4999 for RsmE; PP_5184 for RsmI; PP_2080 for RsmI and RsmE; and PP_1815, PP_4822 for RsmE and RsmA). In the case of xanthine, known functions related to its metabolism do not appear as targets, suggesting the observed differences may be due to an effect on expression of transport systems. L-cysteic acid is an intermediary in cysteine metabolism, but the enzymes involved in its conversion into 3-sulfopyruvate or 3-sulfolactate

do not seem to be characterized in *P. putida*. However, the enzyme encoded by PP_0654, a target for RsmE, is annotated as responsible for the conversion of 3-sulfopyruvate to render 3-sulfolactate.

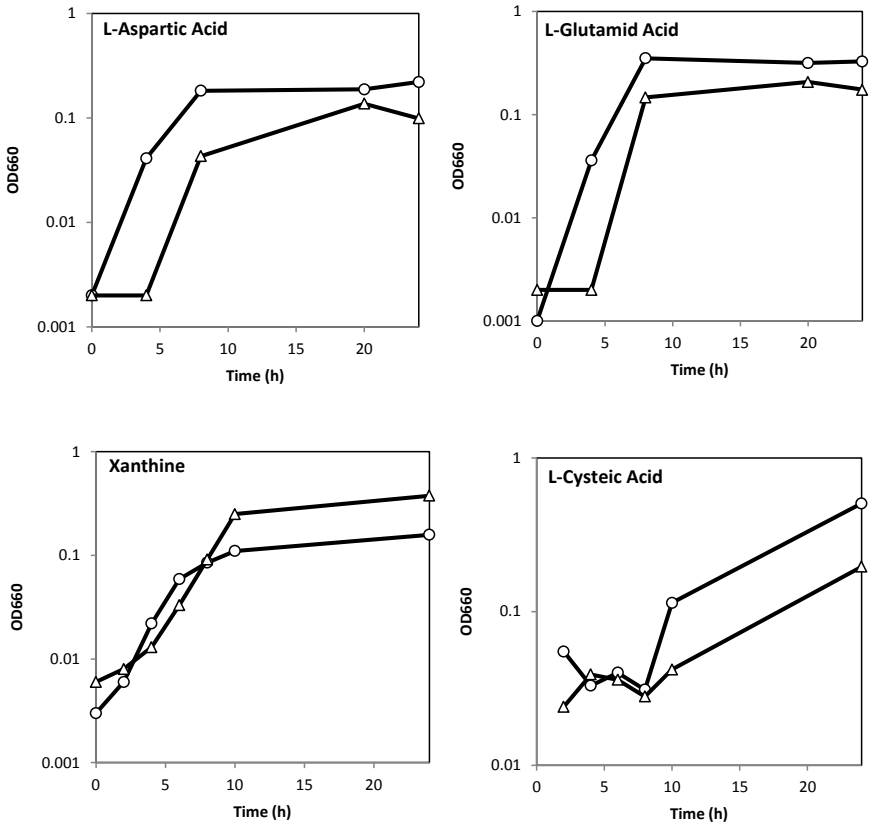

Figure 4. Growth of KT2440 (circles) and ΔIEA (triangles) mutant in BIOLOG plates with aspartic or glutamic acid as carbon sources, xanthine as nitrogen source, and cysteic acid as sulphur source. Experiment don´t show error bars because were made only ones.

The results presented in this chapter are preliminary and will have to be further pursued to obtain a more detailed view of the influence of Rsm regulation on the metabolic and environmental adaptation of *P. putida*.

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V. GLOBAL DISCUSSION

The regulatory cascade involving the RNA-binding post-transcriptional regulators of the CsrA/RsmA family has gained increasing relevance in recent years, due to the fact that these proteins are widespread in many bacterial species and have been recognized as global regulators of bacterial gene expression. In this Thesis we have explored the roles of the Rsm family of proteins in biofilm formation and c-di-GMP turnover in *P. putida* KT2440, where three homologs (RsmA, RsmE and RsmI) exist. We have also done a preliminary analysis at the global level of the target genes regulated by these proteins.

We have shown that Rsm proteins act as negative regulators in the process of biofilm development. This influence is exerted at different levels; results in chapter 1 indicate that Rsm proteins negatively affect *lapF* expression at the transcriptional level (and therefore likely in an indirect way), while RIP-Seq data revealed that *lapA* and the operon corresponding to the specific EPS Pea are both direct targets of RsmE. These three elements and especially the two large adhesins LapA and LapF are key structural components for biofilm formation by *P. putida*, with sequential roles in the process. LapA is needed for bacteria-surface attachment (Hinsa *et al*., 2003), while LapF participates in microcolony development (Martínez-Gil *et al.,* 2010). The expression of both adhesins was already known to be regulated by the GacS/GacA two component regulatory system (Martínez-Gil *et al.* 2014). In the case of *lapF*, its expression depends on the alternative sigma factor RpoS (σ^S), which in turn requires an intact GacS/GacA system to be expressed. The results of this Thesis add a missing link in this regulatory network (Figure 1).

GacA has been described in other *Pseudomonas* to activate expression of the small RNAs (RsmX, RsmY, RsmZ) that sequester Rsm proteins, (Kulkarni *et al.,* 2006). In *P. putida* KT2440, we have identified rsmY and rsmZ and shown in vitro binding of Rsm proteins to rsmY. Besides, in the upstream region of both genes, a sequence coincident with

the consensus described for GacA regulation can be found (Figure 2), indicating that these two small RNAs are under the control of the GacS/GacA system. Furthermore, a negative feedback loop is likely to exist, since rsmY and rsmZ are targets for Rsm proteins, as shown in this work. This would be similar to what has been proposed in other *Pseudomonas*, as *P. aeruginosa*, where SadC is the only DGC involved in biofilm formation that is controlled by the Gac/Rsm pathway (Moscoso *et al.,* 2014), and completes the cascade linking the sensory protein GacS with the structural elements that participate in biofilm development and form the biofilm matrix of *P. putida*.

The generation of the seven (three single, three double and one triple) mutant strains in *rsmA*, *rsmE* and *rsmI* as well as the overexpression constructs for each protein, and the combination of all these elements has allowed to make the first integrative analysis of these proteins in an organism having more than one homolog. It has also revealed the complexity of the system, since there are regulatory interconnections between them, as well as autoregulation. We have also been able to establish that expression of the three Rsm proteins is sequentially turned on and then off during growth, and that their expression levels seem to be quite different, with *rsmE* showing higher expression than the other two genes. It remains to be established how these expression patterns correlate with the expression of small regulatory RNAs, and perhaps even more importantly, what are the environmental and/or metabolic signals that trigger the cascade.

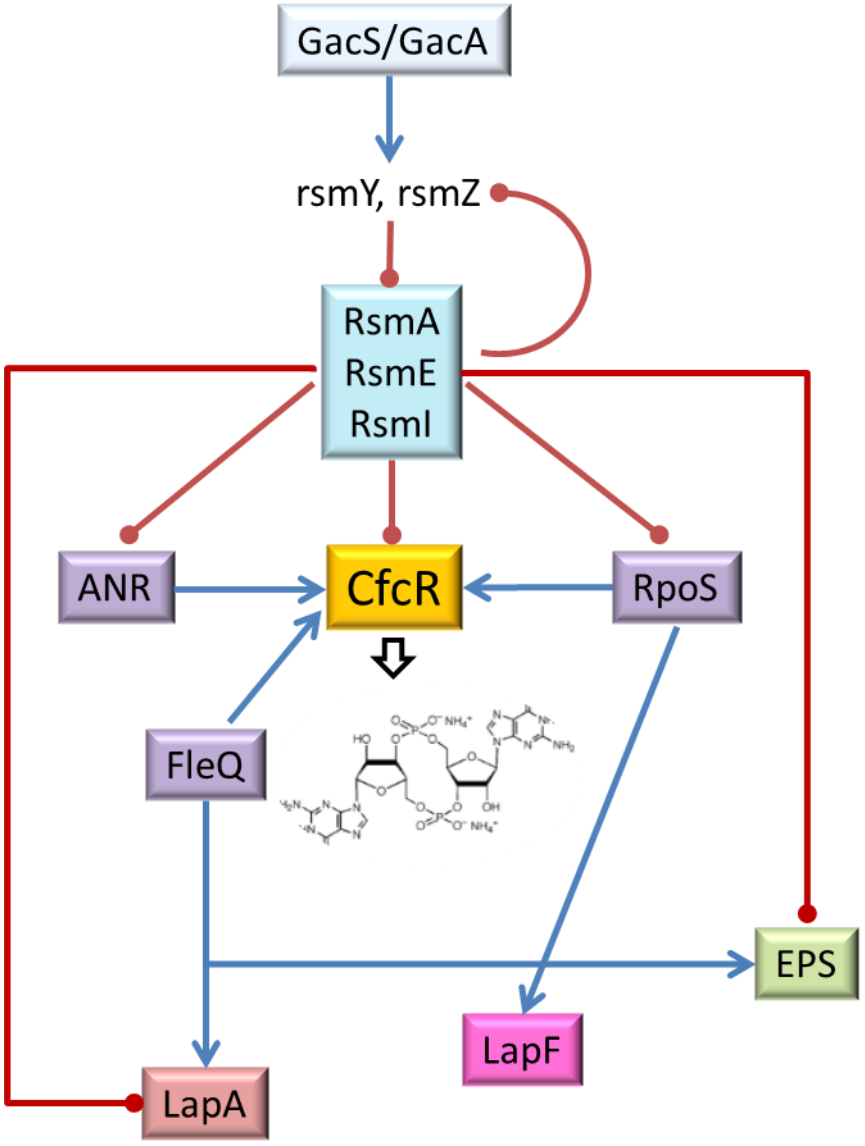

Figure 1. Schematic summary of the regulatory cascade involving Rsm proteins that modulates c-di-GMP levels through CfcR and the different stages of biofilm formation in *Pseudomonas putida* KT2440. Arrow-ended and round-ended lines indicate positive and negative regulation, respectively.

Consensus	TGTAAGNNNNNNCTTACA
P. putida rsmY	**TGTAAG**CTTTTT**C**C**TACA**
P. putida rsmZ	**TGTAAG**CCTTTG**CTTAC**T
P. brassicacearum rsmZ	TGTAAGCCTTTGCCTAGT

Figure 2. Putative GacA recogintion sites in the upstream regions of *rsmY* and *rsmZ* of *P. putida* KT2440. Bases identical to the proposed consensus for *P. fluorescens* (Humair *et al.* 2010) are shown in bold. The equivalent sequence described in *P. brassicacearum rsmZ* (Lalaouna *et al.*, 2012) is shown for comparison.

A key element in this transition from planktonic, motile life to the biofilm mode of growth is the intracellular second messenger c-di-GMP. We have therefore explored in detail the role of RsmA, RsmE and RsmI in the regulation of the response regulator with diguanylate cyclase activity CfcR, and the impact this control exerts on c-di-GMP levels. Overexpression of *cfcR* when cloned in multicopy leads to increased c-di-GMP levels and causes a pleiotropic phenotype that includes increased biofilm formation, crinkly colony morphology on solid medium, and flocculation in liquid cultures (Matilla *et al.,* 2011).

By using a combination of in vivo and in vitro techniques, we were able to show that the three Rsm proteins specifically bind the transcript RNA-CfcR at a motif overlapping the start codon of *cfcR* (5'C**ATG**GATG3', where the start codon is shown in bold). This confirmed that the repression of *cfcR* by Rsm proteins takes place at the initiation of translation, and the in vivo data showed that the GGA motif is essential for recognition, as described in other systems (Lapouge, 2008) while the secondary structure differs slightly from the canonical penta- or hexaloop. The absence of the three Rsm proteins results in increased and earlier expression of *cfcR*, leading to a rise in c-di-GMP content. Our results show a certain parallelism between a plant-beneficial bacterium like *P. putida* KT2440 and what has been described in the best studied *Pseudomonas* species, the oportunistic pathogen *P. aeruginosa*. However, the proteins involved in both bacteria are different, since SadC,

the only diguanylate cyclase involved in biofilm formation controlled by the Gac/Rsm pathway in *P. aeruginosa*, is absent in *P. putida* (Moscoso *et al.*, 2014; Winsor *et al.*, 2016). On the other hand, CfcR has no orthologs in *P. aeruginosa*. Furthermore, the intricacy of the system is higher in *P. putida*, with three Rsm homologs whereas there is only two in *P. aeruginosa* (Marden *et al.* 2013). The regulatory interplay between Rsm proteins is also evident here, and our data suggest they likely compete for the same targets with different efficiencies: removing RsmE and RsmI and leaving RsmA as the only one present leads to enhanced repression of *cfcR* and results in lower c-di-GMP levels.

Besides this direct interaction with *cfcR* mRNA, Rsm proteins also function indirectly, via RpoS, which is required for transcription of *cfcR*. Additional control elements include the transcriptional regulators ANR and FleQ (Fig. 1). The first has been identified in our RIP-Seq analysis as a target of RsmE and RsmA; it also explains that CfcR contribution to biofilm formation is more important under conditions with limited aeration and that *cfcR* expression is enhanced under O_2 depletion (Matilla *et al.*, 2011). In *P. aeruginosa*, Anr activity contributes to biofilm formation on abiotic surfaces and epithelial cells (Jackson, 2013), and FleQ functions as a positive or negative regulator of exopolysaccharide synthesis depending on its binding to c-di-GMP (Baraquet *et al*, 2012). Our group has previously shown that in *P. putida*, FleQ binds c-di-GMP and positively modulates expression of *lapA* and the operons for the species-specific EPS Peb and, to a minor extent, Pea (Martínez-Gil *et al.*, 2014; Molina-Henares *et al.*, 2016).

A third level of modulation is exerted in terms of the activity of CfcR, which requieres phosphorylation, likely through the multi-sensor hybrid histidine kinase CfcA (Ramos-González *et al* 2016), which our analysis shows is also a target for RsmI and RsmA.

An interesting question is the biological significance of such tight and complex control of CfcR expression and activity. It is worth noting that *cfcR* was identified as a gene with preferential expression in *P. putida* populations in the rhizosphere (Matilla *et al* 2007), and we have shown that it is the main contributor to c-di-GMP levels in stationary phase cultures of this bacterium. We believe this complexity reflects the need for a fine control of when and how the bacterial population switches from planktonic to sessile life style. This may also be related to the need for diverse environmental adaptations, which can be particularly relevant in the rhizosphere, where different microniches exist and it is likely that one or the other mode of life is favoured in each of them. Consistent with the up-regulation of *cfcR*, in the transcriptomic work of Matilla et al. (2007), PP_3832 (*rsmE*) appeared as down-regulated (-3.3 fold) in rhizosphere populations in comparison with stationary phase cultures in rich medium. The other two genes do not appear in that study as differentially regulated, but this might be due to the fact that they appear to be expressed at much lower levels than *rsmE*, and may not have passed the signal intensity cut-off established in that work.

Finally, the RIP-Seq analysis of targets done in this Thesis provides a general view of the global role of Rsm proteins. Preliminary results indicate that several metabolic routes are modulated by them, and we have confirmed that growth on certain carbon or nitrogen sources is altered in the triple *rsm* mutant. In particular, reduced growth of the mutant is observed with some amino acids and amino acid derivativesIt will also be of great interest to explore the role of Rsm proteins in stress tolerance mechanisms, since several efflux pumps, heat shock and universal stress proteins, and gluthatione peroxidase appear as targets, and also in the functionality of the type VI secretion system, which has barely been studied in *P. putida*. These aspects are open avenues for future research in our group.

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VI. CONCLUSIONS

1. *Pseudomonas putida* KT2440 has three functional proteins of the CsrA/RsmA family of RNA-binding post-transcriptional regulators: RsmA, RsmE and RsmI. These proteins influence motility and the dynamics of biofilm formation, regulating expression of adhesins and exopolisacharides.
2. The *P. putida* KT2440 Rsm proteins negatively affect c-di-GMP pools and biofilm formation through the GGDEF/EAL response regulator CfcR. CfcR seems to play an equivalent role to SadC, the only diguanylate cyclase involved in biofilm formation controlled by the Gac/Rsm pathway in *P. aeruginosa*.
3. The Rsm proteins regulate expression of the response regulator CfcR at different levels: post-transcriptionally, by direct binding to *cfcR* mRNA, and transcriptionally in an indirect way through RpoS. A third level of control is exerted through the histidine kinase CfcA, which is required for CfcR diguanylate cyclase activity.
4. The motif 5'-CAUGGAUG-3' overlapping the translation initiation codon in the mRNA CfcR transcripts is essential for the repression of CfcR by Rsm proteins.
5. The three Rsm proteins likely have a global regulatory role that can often be interchangeable: they bind more than 400 genes or intergenic regions and small RNAs (sRNAs), a significant percent of targets being shared by more than one Rsm protein. Five potentially new sRNAs have been identified as targets of these proteins.
6. Besides the demonstrated regulation of CfcR, five other GGDEF domain containing proteins are targets of Rsm proteins, suggesting additional effects of these proteins on the intracellular c-di-GMP pool of *P. putida*.
7. The RIP-seq data, and altered growth of the triple *rsm* mutant on certain carbon, nitrogen and sulphur sources, indicate that several metabolic routes are modulated by Rsm proteins.