Biofilms de *Pseudomonas putida*: conexión entre metabolismo y regulación mediada por diguanilato cíclico, y papel en la tolerancia a estrés derivado de compuestos tóxicos

Laura María Barrientos Moreno

Tesis Doctoral

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Memoria que presenta la Licenciada en Biología y Bioquímica

Laura María Barrientos Moreno

Para aspirar al Título de Doctor

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La doctoranda **LAURA MARÍA BARRIENTOS MORENO** y el director de la tesis **MANUEL ESPINOSA URGEL** garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección del director de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

En Granada, a 15 de junio de 2018

Director de Tesis

Fdo.: Manuel Espinosa Urgel

Doctoranda

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"Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar."

Antonio Machado

"Confía en el tiempo, que suele dar dulces salidas
a muchas amargas dificultades."

Miguel de Cervantes

"A veces sentimos que lo que hacemos es tan sólo una gota
en el mar, pero el mar sería menos si le faltara una gota."

Teresa de Calcuta

A mis padres y hermana

A mis abuelos

A Manu

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List of abbreviations

AHL	Acyl-homoserine lactone
ADI	Arginine deiminase
AST	Arginine succinyltransferase
Bp	Base pairs
CAA	Casamino acids
cAMP	3´-5´-cyclic adenosine monophosphate
c-di-AMP	Cyclic di-adenosine monophosphate or Bis-(3´-5´)-cyclic dimeric adenosine monophosphate
c-di-GMP	Cyclic di-guanosine monophosphate or Bis-(3'-5')-cyclic dimeric guanosine monophosphate
C.F.U.	Colony forming units
cGMP	3´-5´-cyclic guanosine monophosphate
DGC	Diguanylate cyclase
DNA	Deoxyribonucleic acid
ECF-α	Extra-cytoplasmic sigma factors
ECM	Extracellular matrix
eDNA	Extracellular deoxyribonucleic acid
EPS(s)	Exopolysaccharide(s)
fEMSA	Fluorescence-based electrophoretic mobility shift assay
GGDEF	Motif Gly-Gly-Asp-Glu-Phe
GFP	Green fluorescent protein
Gm	Gentamicin
KDa	Kilodalton
Km	Kanamycin
LB	Luria-Bertani medium
ml	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid

M. U.	Miller Units
OD	Optical density
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
(p)ppGpp	Guanosine pentaphosphate and tetraphosphate
PVD(s)	Pyoverdine(s)
QS	Quorum sensing
RBS	Ribosome binding site
r.p.m	Revolutions per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Tc	Tetracycline
TCS	Two component system
WT	Wild type

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RESUMEN

En el medio ambiente, las bacterias pueden encontrarse viviendo de forma libre o constituyendo poblaciones sésiles, siendo esta última el modo de vida preferente de estos organismos. De hecho, algunas estimaciones sugieren que más del 90% de las bacterias se encuentran viviendo en forma de biopelículas o biofilms (Geesey *et al*., 1977; Costerton *et al*., 1995). Las biopelículas son comunidades multicelulares y multidimensionales, constituidas normalmente por más de una especie bacteriana, en la que los organismos viven embebidos en una matriz producida casi en tu totalidad por ellos mismos, conocida como matriz extracelular. Estas biopelículas pueden contener una elevada densidad celular, que se ha estimado entre 10^8 y 10^{11} células por gramo de peso seco o por mililitro (Griebler *et al*., 2001; Morgan-Sagastume *et al*., 2008; Balzer *et al*., 2010). Normalmente, las biopelículas se encuentran asociadas a superficies, tanto de tipo biótico como abiótico, donde la primera capa de células está en contacto directo con el sustrato. Sin embargo, también pueden encontrarse en forma de flóculos, que son biopelículas móviles formadas por la agregación celular sin estar unidas a ningún tipo de sustrato (Flemming *et al*., 2016).

La matriz extracelular es esencial para la formación de las biopelículas puesto que actúa como una plataforma de anclaje de la estructura tridimensional de las mismas y puede llegar a constituir hasta el 90% de la biomasa que las constituye (Flemming and Wingender, 2010). Esta matriz extracelular está compuesta por proteínas, exopolisacáridos, lípidos y ADN extracelular, en diferentes proporciones según la composición bacteriana de las biopelículas (Martínez-Gil *et al*., 2010; Mann and Wozniak, 2012; Castillo-Pedraza *et al*., 2017). Sin embargo, es el agua el principal componente, el cual puede llegar a constituir más del 97% de la matriz extracelular (Flemming *et al*., 2016).

Las biopelículas son claves para la colonización, adaptación y supervivencia de las bacterias a muy diferentes y cambiantes hábitats debido a distintos factores. En primer lugar, dentro de la biopelícula se establecen interacciones sociales tales como cooperatividad metabólica, donde se produce un intercambio, eliminación y redistribución de los nutrientes, facilitado por canales acuosos formados dentro de las biopelículas (Davey and O´Toole, 2000; Flemming *et al*., 2016). En segundo lugar, es un ambiente muy favorable para adquirir nuevas características genéticas como resultado del intercambio genético entre las bacterias, lo cual puede llegar a conferir ventajas adaptativas (Ghigo, 2001; Stalder and Top, 2016). En último lugar, la matriz extracelular proporciona protección frente a una

gran cantidad de condiciones adversas tales como la toxicidad provocada por metales (Teitzel and Parsek, 2003; Grumbein *et al*., 2014; Dranguet *et al*., 2017), la exposición a ácidos (McNeill and Hamilton, 2003), la deshidratación y salinidad (Le Magrex-Debar *et al*., 2000; Nielsen *et al*., 2011), así como protección ante sustancias antimicrobianas (Høiby *et al*., 2010; Hall and Mah, 2017; Gupta *et al*., 2018) y a las defensas del hospedador (Kai-Larsen *et al*., 2010; Tian *et al*., 2018; Biesecker *et al*., 2018).

Para facilitar el estudio de la formación de biopelículas en el laboratorio normalmente se ha recurrido al empleo de modelos monoespecíficos, lo cual ha facilitado profundizar en el conocimiento de los determinantes moleculares, así como en la caracterización de rutas y componentes reguladores implicados en la formación de las mismas. De hecho, el desarrollo de estas biopelículas está alta y finamente regulado. Un primer nivel de regulación es debido a señales ambientales, tales como propiedades de la superficie donde se va a establecer la biopelícula y factores externos como el pH, la temperatura o la disponibilidad de nutrientes, como el calcio o el hierro (Chavant *et al*., 2002; Palmer *et al*., 2007). No se conoce completamente cómo son detectadas estas señales, pero en muchas bacterias se encuentran implicados sistemas de dos componentes, los cuales detectan el estímulo ambiental y transducen la señal al interior celular. Un segundo nivel de regulación es debido a la comunicación intracelular mediante lo que se conoce como *quorum sensing*, el cual está basado en moléculas pequeñas difusibles que una vez alcanzan una determinada concentración, activan mecanismos que coordinan la expresión de genes específicos de forma dependiente de la densidad celular (Whiteley *et al*., 1999; Rampioni *et al*. 2016), lo que permite sincronizar determinados comportamientos e influye en el desarrollo de la biopelícula. Por último, un tercer nivel de regulación es debido a moléculas intracelulares conocidas como segundos mensajeros, que permiten amplificar intracelularmente una señal externa detectada por la bacteria, lo que lleva a que se desencadene una transducción de la señal que conduce a una respuesta biológica, donde multitud de genes pueden ser regulados a distintos niveles. Uno de los segundos mensajeros clave para la formación y regulación de una biopelícula es el diguanilato cíclico o c-di-GMP (Römling *et al*., 2013).

Todos estos niveles de comunicación llevan a rutas reguladoras que a menudo están interconectadas, existiendo reguladores transcripcionales y post-transcripcionales que actúan a modo de controles adicionales.

Esta Tesis Doctoral se ha centrado en varios aspectos de la formación de biopelículas por *Pseudomonas putida*, una especie ubicua en el medio ambiente y que presenta gran versatilidad metabólica, siendo microorganismo modelo en estudios de degradación de contaminantes. En nuestro laboratorio, la cepa más ampliamente estudiada es KT2440, la cual es una eficiente colonizadora de la espermosfera y del sistema radicular de plantas con intereses tanto básicos como agronómicos, estableciendo una interacción beneficiosa con las plantas (Espinosa-Urgel *et al*., 2000; Molina *et al*., 2000; Matilla *et al*., 2010).

Trabajos previos del grupo habían permitido identificar genes que se encuentran preferencialmente expresados en poblaciones de *P. putida* KT2440 en la rizosfera de maíz (Matilla *et al*., 2007b). Uno de estos genes codifica un regulador de respuesta con actividad diguanilato ciclasa, actualmente denominado CfcR. Esta proteína parece ser la que principalmente contribuye a aumentar los niveles de c-di-GMP en *P. putida* KT2440 (Huertas-Rosales *et al*., 2017a). La sobreexpresión de CfcR causa un aumento del segundo mensajero que está asociado a cambios fenotípicos en la bacteria, incluyendo un incremento en la formación de biopelículas y una morfología de colonia alterada, que denominamos "de encaje". Mediante una mutagénesis al azar en condiciones de sobreexpresión de CfcR, se han obtenido mutantes afectados en la vía de señalización por c-di-GMP o/y en genes implicados en fenotipos asociados con la sobreexpresión de CfcR. Dos de los mutantes obtenidos, cfcK-66 y cfcK-74, se encuentran afectados en los genes *argG* y *argH* respectivamente, los cuales codifican para enzimas que catalizan los dos últimos pasos de la ruta biosintética de arginina (Ramos-González *et al*., 2016), siendo éste el punto de partida de esta Tesis Doctoral.

En el capítulo 1, "**Connecting amino acid metabolism with c-di-GMP levels and associated phenotypes in *Pseudomonas putida* KT2440"** se han construido los mutantes nulos en *argG* y *argH*, profundizándose en su caracterización en cuanto a niveles de c-di-GMP, formación de biopelículas y morfología de colonias. A diferencia de la cepa silvestre, estos mutantes presentaron bajos niveles de c-di-GMP y habían perdido el fenotipo de encaje, al igual que ocurría con los respectivos mutantes por transposición, aunque CfcR estuviera presente en multicopia. Sin embargo dichos fenotipos eran recuperados en presencia del aminoácido arginina. Así pues, en este capítulo se ha analizado en detalle la implicación de este aminoácido sobre dichos fenotipos, encontrándose que es capaz de

causar un incremento en los niveles de c-di-GMP tanto en la cepa silvestre como en los mutantes, del mismo modo que se ha perfilado estar implicado en el fenotipo de encaje, y además provoca un aumento en la formación de biopelículas, tanto adicionando el aminoácido de forma exógena como sobreexpresando los genes *argG* y *argH*. Por otro lado, el aminoácido ácido aspártico, uno de los precursores para la síntesis de arginina, desencadena los efectos opuestos, es decir, provoca una disminución en los niveles de c-di-GMP, reduce la capacidad de formación de biopelículas y reduce la capacidad de formar encaje en la cepa silvestre. Para determinar si el efecto del aminoácido arginina sobre los niveles de c-di-GMP era específico, se ensayaron el resto de aminoácidos proteinogénicos, encontrándose que la respuesta era bastante específica, puesto que aunque otros aminoácidos provocaron también un aumento en los niveles del segundo mensajero, no fueron tan importantes como en el caso de la arginina.

Dado que los mutantes por transposición fueron originalmente seleccionados en presencia de múltiples copias de CfcR, se determinó si la alteración fenotípica y en cuanto a los niveles de c-di-GMP en los mutantes *argG* y *argH* era debida a una alteración en la expresión de *cfcR*. Las diferencias obtenidas con respecto a la cepa silvestre fueron escasas, además un mutante en *cfcR* y en el "multisensor" híbrido con actividad histidina quinasa, encargada de fosforilar a esta diguanilato ciclasa para que sea activa, mantuvieron la respuesta a arginina en cuanto al incremento de los niveles de c-di-GMP, lo cual indicó que otros elementos son necesarios para explicar la respuesta al aminoácido. Este hecho nos ha permitido seguir profundizando sobre ello en el siguiente capítulo.

En el capítulo 2, "**Identification of the regulatory network associating arginine metabolism and c-di-GMP signalling in *Pseudomonas putida* KT2440"** se ha estudiado en más detalle la conexión entre el metabolismo de la arginina y la señalización mediada por c-di-GMP, a nivel de regulación. Para ello se ha estudiado el papel de diferentes reguladores descritos previamente en el grupo que modulan la formación de biopelículas de *P. putida* KT2440, bien porque tienen un papel sobre los elementos estructurales de la biopelícula como adhesinas y exopolisacáridos, o sobre la regulación de la diguanilato ciclasa CfcR. Así pues, se ha estudiado el efecto de los reguladores transcripcionales RpoS y FleQ y de las proteínas reguladoras post-transcripcionales pertenecientes a la familia Rsm (RsmA, RsmE y RsmI) sobre los genes *argG* y *argH*, así como el papel de estos genes o de su producto, la

arginina, sobre la expresión de alguno de ellos. También se ha explorado el papel que tiene el regulador transcripcional ArgR sobre la regulación de *argG* y *argH*, por ser el regulador central del metabolismo de la arginina. De esta forma, se ha determinado la existencia de una cascada regulatoria que involucra a las proteínas Rsm, RpoS, FleQ y ArgR que conlleva a la regulación de *argG* y *argH* y se han establecido mecanismos de retroalimentación sobre alguno de los elementos reguladores. Además, se ha demostrado la influencia de altos niveles de c-di-GMP en la expresión de *argR* a través de FleQ. Por otra parte, se ha caracterizado en *P. putida* KT2440 el operón *argT-hisQMP-argR*, donde se encuentra localizada la principal proteína de unión a arginina implicada en su trasporte (ArgT), así como ArgR, estudiándose la influencia de ArgR sobre su propia expresión y sobre *argT*. Los resultados de este capítulo nos han permitido proponer un modelo de regulación mediante el cual se pone de manifiesto la existencia de la conexión del metabolismo de la arginina y el segundo mensajero c-di-GMP.

En el capítulo 3, "**Exploring the connection between arginine metabolism and production of the siderophore pyoverdine in *Pseudomonas putida* KT2440"** se hace una primera aproximación a la conexión entre el metabolismo de la arginina y la producción del sideróforo pioverdina, implicado en la captura de hierro. Este estudio se inició debido a que en el transcurso de la caracterización de los mutantes *argG* y *argH* en el Capítulo 1, se observó menor producción de pioverdina en estos mutantes. Así, se ha demostrado que mutantes auxótrofos en la ruta de biosíntesis de arginina están afectados en la producción del sideróforo y que la arginina restaura esa capacidad. Sin embargo, este aminoácido no forma parte estructural de las pioverdinas de *P. putida* KT2440 (Wei y Aristilde, 2015). Además, se ha demostrado que los mutantes *argG* y *argH* realmente producen pioverdina, pero queda retenida en el interior celular, probablemente debido a que la expresión del transportador *pvdE,* encargado de transportar el precursor inmaduro de la pioverdina desde el citoplasma al espacio periplásmico, está reducida en estos mutantes. La deficiencia en la liberación de pioverdina al medio extracelular provoca que los mutantes *argG* y *argH* sean más sensibles a la escasez de hierro y a estrés oxidativo.

En el capítulo 4, "**Influence of stress caused by toxic hydrocarbons on development and resilience of *Pseudomonas putida* biofilms: a preliminary analysis"** se pretende iniciar una línea de trabajo sobre el papel de los biofilms en la resistencia a estrés, explorando

cómo se afecta la formación de biopelículas en distintas cepas de *P. putida* en presencia de un hidrocarburo aromático como es el tolueno, ya que está poco estudiado cómo el metabolismo de estos compuestos puede influenciar el estilo de vida de esta bacteria. Para ello, se han utilizado tres cepas de *P. putida*, mt-2, KT2440 y DOT-T1E, con distintas capacidades de tolerancia y metabolismo de compuestos aromáticos. Se ha comparado la formación de biopelículas en presencia de tolueno, tanto en cultivos pre-expuestos al tóxico, como no pre-expuestos, además de estudiar cómo la presencia del tóxico afecta a biopelículas ya preformadas. Los resultados obtenidos en este capítulo determinaron que en cultivos inicialmente no pre-expuestos al tóxico, la presencia de bajas concentraciones del mismo favoreció la formación de biopelículas en todas las cepas estudiadas, aunque altas concentraciones provocaron el efecto opuesto. Además, en cultivos pre-expuestos al tóxico, las biopelículas persistieron más tiempo que en ausencia del estresor, indicando que la presencia del tóxico puede favorecer la vida sésil de las bacterias, como un mecanismo de defensa frente a estrés. Sin embargo, la adición de tolueno a biopelículas preformadas en ausencia de tolueno, provocó un rápido desprendimiento de las biopelículas, probablemente a causa de que sus matrices extracelulares no están “preparadas”.

Esta Tesis Doctoral, aporta por una parte nueva información clave de cómo señales metabólicas pueden influir en el proceso de formación de las biopelículas en *P. putida* KT2440, permitiendo proponer un modelo más completo sobre la regulación de este proceso, en el cual se ha incluido la existencia de una conexión entre el metabolismo de la arginina y el c-di-GMP en esta bacteria. Por otro lado, revela una conexión entre este aminoácido, la captación de hierro y el estrés oxidativo, clave para la vida multicelular de *P. putida* y la colonización de la rizosfera. Por otro lado, ha permitido iniciar una nueva línea de investigación sobre cómo compuestos tóxicos metabolizables influyen en las biopelículas de *P. putida*, un campo de gran interés a nivel biotecnológico.

I. GENERAL INTRODUCTION

1. Biofilms: a strategy for adaptation, colonization and survival.

In the environment, bacteria can be found as planktonic, individual cells, or as sessile populations called biofilms, which are considered the prevalent mode of life of bacteria: some estimations suggest that more than 90% of bacteria exist within biofilms (Geesey *et al*., 1977; Costerton *et al*., 1995). Their development is a widespread trait in prokaryotes and probably was acquired early during their evolution (Hall-Stoodley *et al*., 2004). Biofilms are multicellular and multidimensional communities, usually formed by more than one species, in which microorganisms live embedded in a mostly self-produced extracellular matrix (ECM). They can contain a high cellular density, with an estimated population between 10^8 and 10^{11} cells per gram of wet weight or ml (Griebler *et al*., 2001; Morgan-Sagastume *et al*., 2008; Balzer *et al*., 2010). In nature, biofilms can be attached to biotic or abiotic surfaces, where only one layer of cells is in direct contact with the substratum, or can exist as flocs, mobile biofilms formed by cell-cell aggregation in absence of any substratum (Flemming *et al*., 2016). The ECM is essential for biofilm formation due to its role as a stabilizing scaffold for the three-dimensional biofilm structure and may constitute over 90% of biofilm biomass (Flemming and Wingender, 2010). The ECM is composed by proteins, exopolysaccharides (EPS), lipids and extracellular DNA (eDNA), in different proportions depending on the bacterial species (Martínez-Gil *et al*., 2010; Mann and Wozniak, 2012; Castillo-Pedraza *et al*., 2017), and water as the main component of the matrix (up to 97%) (Flemming *et al*., 2016); these elements, along with flagella, fimbriae, pili, and other adhesive fibers such as amyloids, can play a role as structural elements at different stages of biofilm development in some stages of its formation (Hobley *et al*., 2015; Das *et al*., 2017).

Biofilms are key for microorganisms to colonise, adapt and survive in very different and changing habitats due to several characteristics (Figure 1). Firstly, bacteria establish social interactions within the biofilm, such as competition or metabolic cooperativity, where they can exchange, remove and redistribute nutrients, as well as retain enzymes that provide digestive capabilities. These processes are facilitated by the existence of water channels inside the biofilm (Davey and O´Toole, 2000; Flemming *et al*., 2016). Secondly, biofilms constitute a favorable environment to acquire new genetic traits resulting from the exchange of genetic material between bacteria, which can confer an adaptive advantage by increasing genetic diversity and accelerating population evolution (Ghigo, 2001; Stalder and

Top, 2016). Finally, the ECM provides protection from a variety of environmental challenges: metal toxicity (Teitzel and Parsek, 2003; Grumbein *et al*., 2014; Dranguet *et al*., 2017), acid exposure (McNeill and Hamilton, 2003), dehydration and salinity (Le Magrex-Debar *et al*., 2000; Nielsen *et al*., 2011) and UV exposure (Espeland and Wetzel, 2001), as well as antibiotics and antimicrobial substances (Høiby *et al*., 2010; Hall and Mah, 2017; Gupta *et al*., 2018;) and host defences (Kai-Larsen *et al*., 2010; Tian *et al*., 2018; Biesecker *et al*., 2018).

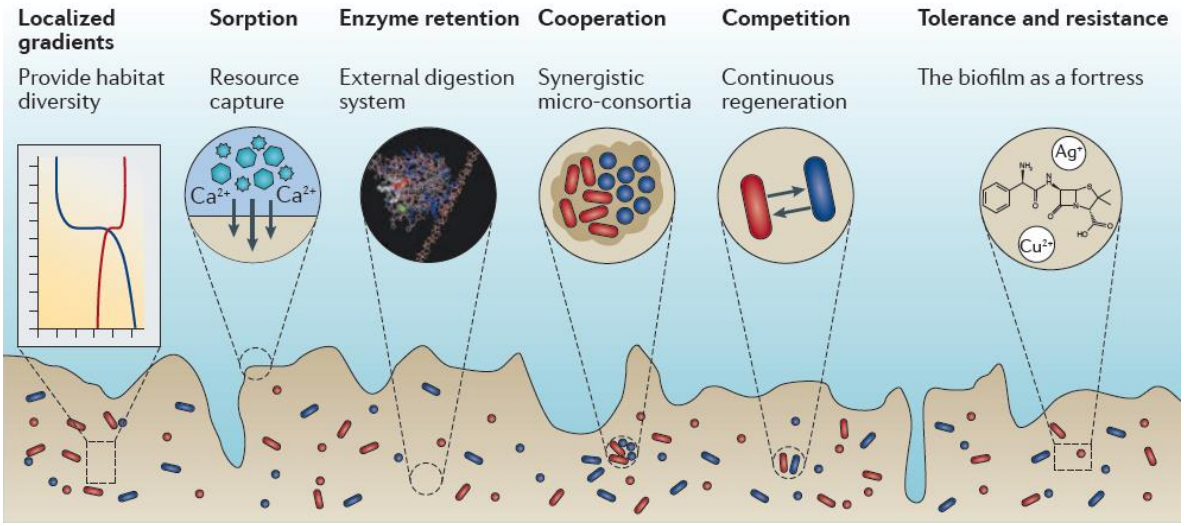


Figure 1. Advantageous properties of biofilm formation. See text for details (Adapted from Flemming *et al*., 2016).

1.1. Biofilm implications from an anthropological point of view.

The ability of biofilms to adapt and survive in very different habitats has significant implications, both negative and positive. Biofilms can cause obstruction in industrial pipelines or in air conditioning systems, reducing the life of equipment. Furthermore, biofilms can colonise health-related surfaces and thus can be found in intravenous catheters, heart valves, implants or medical instrumentation, and they are also responsible for nosocomial and chronic infections. Not surprisingly, biofilm research has focused mostly on relevant species in these aspects, such as *Pseudomonas aeruginosa* (Valentini *et al*., 2018), *Bordetella* spp. (Cattelan *et al.,* 2016) or *Staphylococcus aureus* (van Wamel, 2017), among others. Furthermore, biofilms are a big problem in food industry where contaminations with

Salmonella spp. or *Lysteria monocytogenes* are common (Van Houdt and Michiels 2010; Coughlan *et al*., 2016).

However, biofilms can also have positive impacts. Many bacteria colonise roots and leaves of plants with economic interest, growing as biofilms. Some species can promote plant growth, activate plant defence mechanisms or act as biocontrol agents against phytopathogens. It is the case of certain strains of some *Pseudomonas* species like *P. putida* and *P. fluorescens*, for instance (Couillerot *et al*., 2009; Müller *et al*., 2016). Biofilms have also been used in bioremediation, and in fact, many systems for this purpose have been designed based on biofilms. Thus, biofilms are used in wastewater treatment systems (Venkata Mohan *et al*., 2009; de Beer *et al*., 2018; Peng *et al*., 2018) and for the removal of toxic compounds considered as recalcitrant, including xenobiotics such as toluene or benzopyrene, by means of single- or multispecies biofilms with biodegradation capabilities (Morales *et al.,* 2017; Rajamanickam and Baskaran, 2017).

I.2. The developmental model of biofilm formation: what are the elements implicated in each step?

After many years of biofilm research, particularly in model organisms such as *Pseudomonas aeruginosa*, it has been stablished that biofilm development occurs in five main stages (Figure 2). It is initiated by one or more free-swimming bacteria attaching to a surface **(1)**. This stage is in part stochastic, driven by Brownian movement, partly owing to flagellar and/or pilus motility, and influenced by gravitational and particularly hydrodynamic forces (Donlan, 2002; Beloin *et al*. 2008; Petrova and Sauer, 2012), as well as nutrient availability (Anderson *et al*., 2008; Marsden *et al*., 2017). This step may therefore be dynamic and reversible if the conditions are not adequate for bacterial establishment. After this initial contact, some bacteria attach strongly to the surface by their long axis in an irreversible manner, resulting in a monolayer of cells **(2a)** (Monds and O´Toole, 2009), allowing cell to cell interactions and multicellular aggregation **(2b)**. This process leads to microcolony formation **(3)**, which can happen by clonal growth of attached cells and/or by translocation of cells across the surface (Monds and O´Toole, 2009). In this stage, cells undergo phenotypic and metabolic changes. Microcolonies start producing the ECM,

containing EPS, proteins, lipids, eDNA and other cellular components, leading to macrocolony formation and therefore a true and mature biofilm **(4)**. This transition from microcolony to macrocolony is poorly understood, and it might only be the development of a microcolony over time (Ha and O´Toole, 2015). Within the mature biofilm, a heterogeneous physicochemical environment is created in which subpopulations of bacteria exist, with different genetic patterns and phenotypic differentiation according to their spatial distribution. Finally, due to changes in the local environment such as oxygen concentration, nutrient availability, nitric oxide, surfactants, and/or age and size of biofilm, some cells are released from the biofilm and return to a planktonic mode of life **(5)** (Sauer *et al*., 2004; Karatan and Watnick, 2009; Rowe *et al*., 2010). This release can occur in a passive way by external forces such as shear stress or in an active way by a phenotype switch (Petrova and Sauer, 2016; Kim and Lee, 2016). These free-swimming bacteria can initiate the colonization of new habitats, initiating a new cycle of biofilm formation.

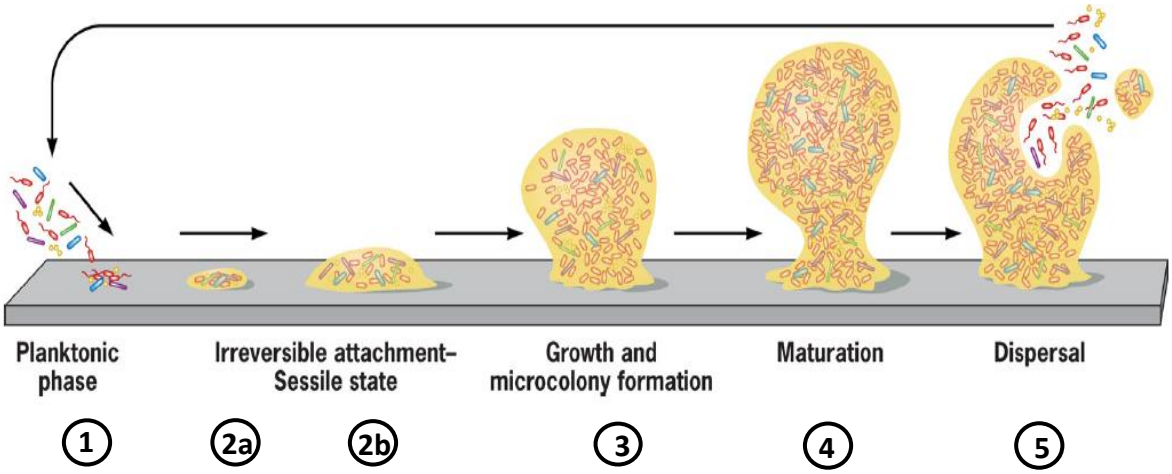


Figure 2. Model of biofilm formation cycle. See text for details (adapted from Magana *et al*., 2018).

This model of biofilm development is common among bacterial species, although the specific elements involved and their importance in each step can differ, even in strains of the same species, or as a consequence of specific environmental conditions. For example, flagella were observed by O'Toole and Kolter to be important to initiate cell-to-surface interactions in *P. aeruginosa* (O´Toole and Kolter, 1998a), whereas a different report indicated that pili and flagella are not critical for interactions with the surface (Klausen *et al*.,

2003). The explanation to this disparity is likely related to nutritional or conditional facts. In several strains of *Escherichia coli*, Type I fimbriae and curli play an important role in attachment, acting as adhesive molecules (Cookson *et al*., 2002; Beloin *et al*., 2004; Duncan *et al*., 2005). In other bacteria, large secreted proteins, usually called adhesins, are key at the initial stages of biofilm formation. In *P. putida* and *P. fluorescens*, the large adhesin LapA is involved in the transition from reversible to irreversible adhesion to the surface, and mutants impaired in its synthesis and transport are impaired in biofilm formation (Hinsa *et al*., 2003; Yousef-Coronado *et al*., 2008; Ivanov *et al*., 2012). Similar roles have been described for FrhA in *Vibrio cholerae* (Syed *et al*., 2009), SiiE in *Salmonella enterica* (Gerlach *et al*., 2007), RtxA in *Legionella pneumophila* (Cirillo *et al*., 2002), SagA and Acm in *Enterococcus faecium* or Ace in *Enterococcus faecalis* (Mohamed *et al*., 2006). However, in other species proteins are not the most relevant molecules implicated in attachment to surfaces. For example, in *P. aeruginosa* the specific EPS Psl is essential for initial attachment and adhesion (Ma *et al*., 2006; Jones and Wozniak, 2017) whereas a second EPS, Pel, is important in absence of type IV pili (Vasseur *et al*., 2005). In *Sinorhizobium meliloti*, this role is played by succinoglycan or EPSI (Fujishige *et al*., 2006).

Cell-to-cell interactions and subsequent cell aggregation to form microcolonies require proteins, EPS and other appendages. In *P. aeruginosa* Pel (Colvin *et al*., 2010), the adhesin CdrA (Borlee *et al*., 2010) and Cup fimbriae have been described to mediate cell-to-cell interactions and microcolony formation (Kulasekara *et al*., 2005; Ruer *et al*., 2007). In *E. coli* this role is due to curli (Prigent-Combaret *et al*., 2000) and specific adhesins such as TibA, that possess self-association characteristics and allow cell auto-aggregation (Sherlock *et al*., 2005), or the EPS PGA (homopolysaccharide composed of N-acetylglucosamine with β(1-6) glycosidic linkage), that promotes intercellular adhesion and promotes abiotic surface binding (Wang *et al*., 2004).

Finally, in a mature biofilm EPS gain special relevance. Thus, in *E. coli* and *Salmonella enterica* serovar Typhimurium biofilms, cellulose is an essential component of the extracellular matrix (Zogaj *et al*., 2001; Serra and Hengge, 2017). Recently, Thongsomboon and colleagues have described that these two species produce modified cellulose for ECM assembly and biofilm architecture (Thongsomboon *et al*., 2018). *Bacillus subtilis* (Roux *et al*., 2015), *Staphylococcus aureus* and *Staphylococcus epidermidis* (Izano *et al*., 2008),

Burkholderia sp. (Yakandawala *et al*., 2011) and *Acinetobacter baumannii* (*Choi et al., 2009*), among other bacteria, produce the EPS PIA (named also PNAG or PGA), which acts as an important scaffold for biofilm integrity. Also, in *S. meliloti* the EPS galactoglucan or EPSII is necessary to develop organized and structured biofilms (Rinaudi and González, 2009). In *P. aeruginosa*, the stability of mature biofilms is due to the interaction between the adhesin CdrA and the EPS Psl, functioning as structural elements within the ECM.

Apart from EPS, eDNA has an important role in mature biofilms of different bacteria. In *P. aeruginosa* it is important as scaffold, functioning as a cell-to-cell interconnecting and promoting bacterial self-organization within the ECM due to its role in maintaining cell alignment (Allesen-Holm *et al*., 2006; Gloag *et al*., 2013). Furthermore, eDNA can interact with other elements of ECM thus stabilizing the biofilm matrix (Jennings *et al*., 2015).

1.3. Biofilm regulation: a complex issue.

Planktonic and surface-attached cells show significant differences in terms of global gene expression, exemplified in a recent proteomic study where clear modifications were observed in *P. aeruginosa* between those two lifestyles, including proteins involved in two-component systems (TCSs), second messenger systems, appendages and outer membrane components, among others (Crouzet *et al*., 2017). Biofilm formation is a highly regulated process that is accompanied by gene expression changes at different stages of its development, or even in the same stage depending on the surrounding conditions and the spatial distribution of cells within the biofilm (Dötsch *et al*., 2012; Heacock-Kang *et al*., 2017). Although the regulation network involved in biofilm formation is very complex and far from undersood, with variations from one species to another, it is possible to stablish three broad levels of modulation of the process.

The first level is due to environmental cues. These include surface characteristics like charge or roughness, and external factors like pH, temperature or nutrient availability (Chavant *et al*., 2002; Palmer *et al*., 2007). Compounds such as glycerol or oils and microelements like calcium, magnesium, inorganic phosphate or iron have been reported to influence attachment (Newell *et al*., 2011; Portier *et al*., 2016; Tang *et al*., 2018; Tischler *et*

al., 2018; Crespo-Tapia *et al*., 2018; Rossi *et al*., 2018). It is not completely known how these signals are detected, but in many cases two-component systems (TCSs) are involved. TCSs are composed by a sensor histidine-kinase which senses the environmental stimuli and by a response regulator which transduces the signal inside the cell. Some examples are CarSR or PhoPQ, implicated in Ca^{2+} and inorganic phosphate response, respectively, involved in biofilm formation and virulence in *P. aeruginosa* (Gooderham *et al*., 2009; Guragain *et al*., 2016), or CpxRA involved in biofilm formation on hydrophobic surfaces in *E. coli* (Ma and Wood, 2009). Sometimes, the process is more complicated because different histidine-kinases interact, as it has recently been reported for the GacS/GacA TCS and the histidine-kinases LadS, RetS, and PA1611 in *P. aeruginosa* (Chambonnier *et al*., 2016). This pathway controls expression of different EPS.

The second level of regulation derives from intracellular communication via quorum sensing (QS). QS is based on small diffusible molecules called autoinducers produced by bacteria (Williams and Cámara, 2009) and released to the environment at a low basal rate. When a critical threshold concentration in the local environment is reached, QS mechanisms are activated, leading to coordinated expression of specific genes in a cell density-dependent manner (Whiteley *et al*., 1999; Rampioni *et al*. 2016). This process allows bacteria to synchronize particular behaviours, thus acting almost as a multicellular organism, as well as to control a wide range of functions, some of which influence biofilm development (Waters and Bassler, 2005; Rampioni *et al*., 2012; Inhülsen *et al*., 2012).

Finally, a third level of regulation comes from intracellular small molecules known as second messengers. These molecules amplify within the cell an external signal received, acting on intracellular signal transduction and leading to a biological response, often critical for survival. Usually, these second messengers are cyclic nucleotides, mononucleotides or dinucleotides (Pesavento and Hengge, 2009; Shanahan and Strobel, 2012), which are widely distributed in both prokaryotes and eukaryotes. These molecules are summarized in Figure 3.

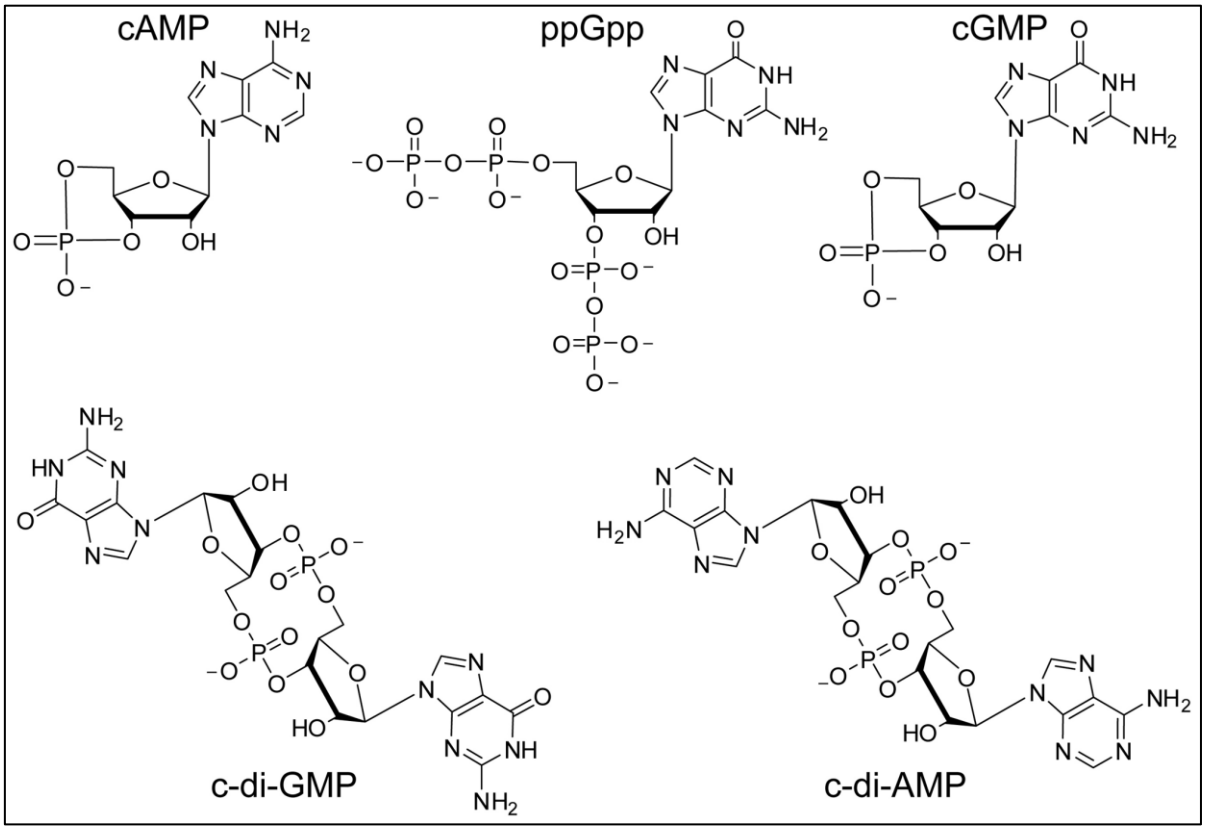


Figure 3. Chemical structures of second messengers based on nucleotides. Above, cyclic mononucleotides; Below, cyclic dinucleotides (Taken from Shanahan and Strobel, 2012).

It has been reported that bacteria use all of them (Gomelsky, 2011). For example, cAMP regulates catabolite repression, glucose sensing and the utilization of other carbon sources (McDonough and Rodríguez, 2012); cGMP regulates prokaryotic development (Gomelsky, 2011), and (p)ppGpp, which is produced in starvation and stress conditions, regulates many processes (Magnusson *et al*., 2005; Potrykus and Cashel, 2008). However, in terms of biofilm formation and the transition between lifestyles, cyclic di-guanosine monophosphate (c-di-GMP) has emerged as a key element widely used by bacteria and will be further described in Section 2.

Environmental, cell-cell communication and intracellular signals do not lead to independent regulatory pathways but are often interconnected, and there exist transcriptional and post-transcriptional regulators that allow additional control levels. Different regulatory proteins have been described to play a role in biofilm development. The key ones for the purpose of this work will be presented in the sections below.

2. Intracellular signalling and biofilms: the second messenger c-di-GMP.

About 30 years ago, the second messenger c-di-GMP was discovered as an indispensable activator of bacterial cellulose biosynthesis (Ross *et al*., 1987), although it started gaining importance around 2005 in terms of its cellular role/s and mechanisms of action as a novel bacterial second messenger, thus opening a new area of research (Römling and Galperin, 2017). C-di-GMP is involved in a wide range of processes, such as cell differentiation in *Caulobacter crescentus* (Paul *et al*., 2004), virulence in *V. cholerae* and *P. aeruginosa* (Tischler and Camilli, 2005; Kulesekara *et al*., 2006), multicellular development and antibiotic production in streptomycetes (Hull *et al*., 2012), or long-term nutritional stress survival and lipid metabolism and transport in mycobacteria (Bharati *et al*., 2012; Li *et al*., 2012). However, the key role of c-di-GMP is the transition between motile and sessile lifestyles in bacteria and the regulation of biofilm development (Simm *et al*., 2004). C-di-GMP is widely distributed among bacteria but not in archaea (Galperin, 2004; Römling, 2008), and c-di-GMP synthesis and degradation enzymes are found in most bacterial phyla (Römling *et al*., 2013). It is therefore considered as a universal second messenger in bacteria. These proteins also appear in plants and lower eukaryotes, but not in mammals (Römling *et al*., 2013).

2.1. C-di-GMP turnover.

C-di-GMP is synthesised by proteins with diguanylate cyclase activity (DGC), which is associated to the presence in the protein of a conserved GGDEF (or sometimes GGEEF) domain (Pei and Grishin, 2001). These proteins catalyse c-di-GMP formation in a two-step reaction from two molecules of GTP, releasing two pyrophosphate molecules, with 5´-pppGpG as intermediate of the reaction (Ross *et al*., 1987; Paul *et al*., 2004). For this reaction to take place, the formation of an active site (or A site) in which GGDEF domain proteins act as homodimers is necessary. Each GGDEF monomer contributes one GTP bound to the formation of an intermolecular phosphodiester bond, requiring two Mg^{2+} or Mn^{2+} (Chan *et al*., 2004; Ryjenkov *et al*., 2005; Wassmann *et al*., 2007).

The degradation of c-di-GMP is due to phosphodiesterase activity (PDE), which is associated to two types of protein domains: HD-GYP and EAL. HD-GYP domain proteins

degrade c-di-GMP into GMP (Ryan *et al*., 2010), while EAL domain proteins hydrolyse c-di-GMP in the presence of Mg^{2+} or Mn^{2+} into linear di-GMP, i.e., 5'-pGpG, which is subsequently degraded to monomeric pG by different enzymes (Ross et al., 1986; Christen *et al*., 2005). It has been reported that EAL domains may degrade c-di-GMP as monomers (Schmidt *et al*., 2005), but the dimeric state appears to be essential for the activation of PDE proteins (Rao *et al*., 2008; Sundriyal *et al*., 2014) so EAL domain proteins are supposed to act as dimers *in vivo*.

There are bifunctional enzymes containing GGDEF and EAL domains, or GGDEF and HD-GYP domains, often synthesised in the same polypeptide chain. However, most GGDEF-EAL proteins described so far contain one of the two domains active, the other one being enzymatically inactive. It is the case of the *P. putida* protein CfcR, in which only the DGC appears to be active (Matilla *et al*., 2011), or BifA, which only presents PDE activity (Jiménez-Fernández *et al*., 2015). Just a few GGDEF-EAL proteins have been demonstrated to have both activities. One example is ScrC from *Vibrio parahaemolyticus*, where DGC or PDE activities depend on expression of the ScrAB proteins, and is involved in biofilm formation or swarming depending on activation or inactivation of ScrAB (Ferreira *et al*., 2008). In *Mycobacterium smegmatis* protein MSDGC-1 is important for its survival under nutrient-depleted conditions, but not for biofilm formation (Bharati *et al*., 2012). Although they are broadly distributed in bacteria, GGDEF-HD-GYP proteins are less common than GGDEF-EAL proteins, and have not been experimentally characterised (Römling *et al*., 2013).

2.2. Roles of c-di-GMP in motility and biofilm formation

As mentioned above, c-di-GMP modulates the transition between motile and sessile lifestyles in bacteria. High intracellular levels of the second messenger have been associated with inhibition of motility and activation of biofilm formation, and *vice versa*. In fact, c-di-GMP is important in all steps of biofilm development (Figure 4), but its role may change at different stages.

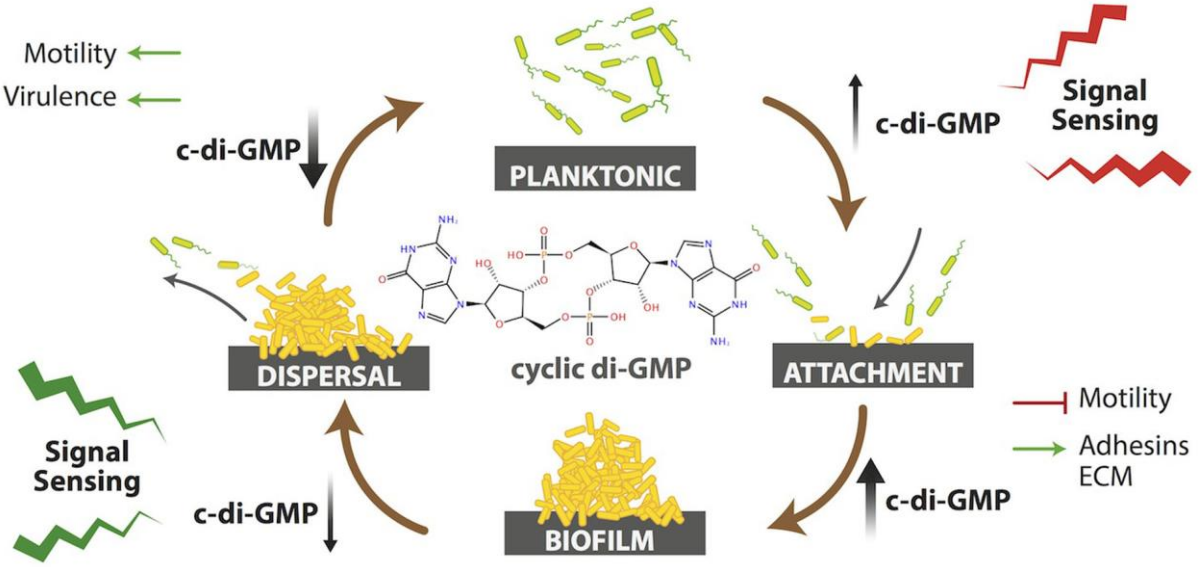


Figure 4. Schematic representation of c-di-GMP levels in biofilm formation. The thickness of grey arrows is indicative of c-di-GMP levels. More thickness, more c-di-GMP and *vice versa*. Green arrows shows processes activate by c-di-GMP and red lines indicate the opposite effect (Taken from Rinaldo *et al*., 2018).

First, motile cells need to reach the surface which is going to attach later. Flagella are implicated in initial and temporary attachment to the surface, but once cells are permanently surface-attached, synthesis and/or rotation of flagella has to be turned off. In *E. coli* and *S. enterica*, protein YcgR binds c-di-GMP via a PilZ-domain, and flagella function is inhibited by the interaction of this protein with the flagellar machinery (Ryjenkov *et al*., 2006; Boehm *et al*., 2010). Other filamentous appendages implicated in attachment to surfaces are pili or fimbriae and curli as shown in section 1.1. Production of these appendages is activated by c-di-GMP at the transcriptional level. In *K. pneumoniae*, the DGC YfiN stimulates expression of type 3 fimbriae (Wilksch *et al*., 2011), and in *E. coli*, the *csgBAC* operon, which encodes the structural subunits of curli, is subjected to transcriptional regulation by the DGC DosC and the PDE DosP (Sommerfeldt *et al*., 2009; Tagliabue *et al*., 2010). *P. aeruginosa* has five clusters of Cup fimbriae (CupA to CupE) which are required for cell-to-cell interactions. All of them, except CupE, are regulated by c-di-GMP at the transcriptional level (Giraud and de Bentzmann, 2012).

In *P. fluorescens* the mechanism that relates c-di-GMP with the turnover of the adhesin LapA is well characterised. At high intracellular concentrations of c-di-GMP, this second messenger is bound to the transmembrane protein LapD, which has a degenerate GGDEF domain with no enzymatic activity. This allows LapD to sequester the periplasmic protease LapG, which has specific proteolytic activity over LapA, preventing the adhesin from being cleaved and removed from the outer membrane of the cell. At low levels of c-di-GMP, a conformational change occurs in LapD, liberating LapG, which can then cleave LapA. This allows detachment of bacterial cells from the surface and dispersal of the biofilm (Newell *et al*., 2009; Newell *et al*., 2011).

Extracellular matrix (ECM) components known to contribute to biofilm formation at some step, including EPS, adhesins and eDNA can be regulated by c-di-GMP. As mentioned above, the synthesis of cellulose, an EPS produced in many bacterial biofilms, is regulated by c-di-GMP. In *G. xylinus*, the cellulose synthase BcsA has a PilZ domain where c-di-GMP is bound, acting as an allosteric activator of the enzyme (Ryjenkov *et al*., 2006). Similarly, in *E. coli* or *Yersinia pestis*, biosynthesis of PAG is activated by the second messenger (Kirillina et al., 2004; Boehm *et al*., 2009). The production of Pel and Psl polysaccharides in *P. aeruginosa* biofilms is positively regulated by c-di-GMP through the transcriptional regulator FleQ. In the absence of c-diGMP, FleQ forms a complex with another protein, FleN, and binds to the *pel* promoter, inhibiting transcription (Hickman and Harwood, 2008; Baraquet *et al*., 2012). When c-di-GMP levels are increased, the second messenger associated with FleQ, causing a conformational change that releases repression and results in *pel* and *psl* transcription (Matsuyama *et al*., 2016). On the other hand, eDNA production from cell-lysis, which is important for three-dimensional biofilm structure and maintenance, is dependent on low c-di-GMP (Ueda and Wood, 2010). Re-activation of cell motility during biofilm dispersal is also induced by low levels of c-di-GMP and requires specific PDEs such as DipA of *P. aeruginosa* (An *et al*., 2010; Roy *et al*., 2012).

2.3. Regulation of c-di-GMP signalling.

Although there is still limited knowledge regarding the connections between environmental stimuli and intracellular c-di-GMP levels, in many cases GGDEF, EAL and HD-GYP domains are combined with sensory domains such as PAS, GAF, PHY, H-NOX, CACHE and/or HAMP (in the same protein or by interaction with others) (Tuckerman *et al*., 2011; Plate and Marletta *et al*., 2012). These sensory domains respond to intracellular or extracellular ligands, allowing signal transduction and modulation of DGC or PDE activities (Henry and Crosson, 2011; Schirmer, 2016). In other cases, GGDEF, EAL and HD-GYP domains are present in proteins with REC domains that act as response regulators of TCS, modulating c-di-GMP levels in response to signals received by their cognate sensor histidine kinases, as the cases of WspR, PleD or PvrD proteins (Galperin, 2010). In most cases, the ligands remain unknown, but there are several cases in which signals have been identified. Thus, some of these protein can sense redox state (Qi *et al*., 2009), light (Tarutina *et al*., 2006; Barends *et al*., 2009), nitric oxide (Cashel and Gallant, 1969; Williams *et al*., 2017), oxygen (Chang *et al*., 2001; Tuckerman *et al*., 2009), or L-arginine (Mills *et al*., 2015).

Quorum sensing signalling has also been shown to participate in regulation of c-di-GMP-metabolizing proteins. One example is the hybrid protein RpfR, which has PAS and GGDEF-EAL domains. This protein senses the fatty acid BDSF, a QS signalling molecule required for swarming motility and biofilm formation in *Burkholderia cenocepaia* (Deng *et al*., 2012).

Other mechanism that regulates c-di-GMP contents is due to the interaction of proteins with mRNA transcripts that are involved in c-diGMP signalling but do not have GGDEF, EAL or HD-GYP domains *per se*. One example is the CsrA/RsmA family of proteins, RNA-binding proteins that play key roles in gene regulation. In *P. aeruginosa*, RsmA negatively controls the DGC SadC, which is an important player in biofilm formation in this bacterium (Moscoso *et al*., 2014). Furthermore, in many bacteria, the regulation of c-di-GMP-metabolizing proteins occurs through transcriptional regulators. For instance, the alternative sigma factor RpoS (σ^S), which regulates genes upon entry into the stationary phase, has been reported to control genes encoding GGDEF/EAL domain proteins such as

yaiC, *ydaM* or *yddV* in *E. coli* or *cfcR* in *P. putida* (Sommerfeldt *et al*., 2009; Matilla *et al*., 2011). Hence, factors that affect RpoS influence c-di-GMP content (Wang *et al*., 2011).

An additional mechanism of regulation that affects activity of DGC, and consequently c-di-GMP levels, is allosteric control through feedback inhibition by product. One example is diguanylate cyclase PleD, in which the binding of c-di-GMP to DGC inhibitory site (I site) blocks enzymatic activity (Chan *et al*., 2004; Christen *et al*., 2006; Schirmer, 2016).

These regulatory systems involved in c-di-GMP signalling are often interconnected, leading to a complex network that in many cases remains poorly characterized.

3. Environmental signals and biofilms: Iron.

Among the environmental stimuli that influence the lifestyles of bacteria, iron has been reported to play an important role. Iron is an essential nutrient for almost all forms of life, including most of bacteria. It is a constituent of enzymes implicated in electron transfer, oxygen metabolism and removal of reactive oxygen species, amino acids biosynthesis, and RNA synthesis, among others (Earhart, 1996; Paul and Duvey, 2015). Although iron is a very abundant element on Earth, its availability is limited in most of environments. This is because iron is oxidized to iron(III) at physiological pH and aerobic conditions, precipitating as a polymeric oxyhydroxide and being highly insoluble (Paul and Duvey, 2015). Because of this, the concentration of iron obtained by passive diffusion through membranes is not sufficient to support bacterial demand (Braun and Killmann, 1999; Andrews *et al*., 2003). To overcome iron limitation, bacteria have developed strategies for the acquisition, solubilisation, and transport of iron. One of the most important strategies is the secretion of siderophores, which are small secondary metabolites with a low molecular weight (less than 15 KDa) that allow chelating iron(III) from the environment with high affinity, making iron bio-available from bacteria and allowing its uptake through specific receptors and transport systems present on the cell surface. These molecules have been reported in bacteria, but also in fungi, and in some plants and mammals (Devireddy *et al*., 2010; Tyrrel and Callaghan, 2016; Khan *et al*., 2018). Once in the cytoplasm, iron(III) is reduced to iron(II) and siderophore is released (Figure 5) (Paul and Duvey, 2015; Khan *et al*., 2018; Leinweber *et al*.,

2018). Iron uptake needs to be tightly regulated, since free iron promotes the formation of toxic reactive oxygen species, causing oxidative stress in the cell, and therefore damage in proteins, DNA and lipids (Vinckx *et al*., 2008). Thus, production of siderophores decreases when iron concentration increases in the surroundings (Singh *et al*., 2008).

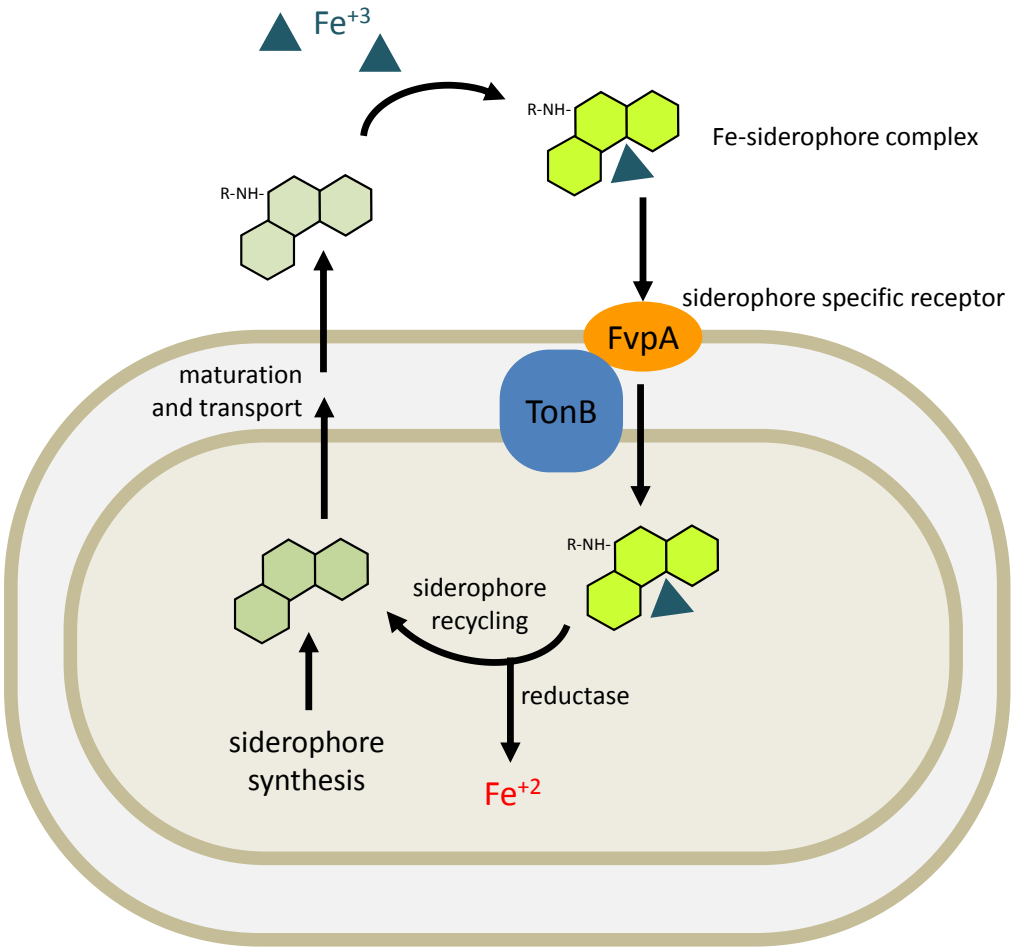


Figure 5. Schematic representation of siderophore mediated iron uptake (modified from Khan *et al*., 2018).

3.1. Pyoverdine synthesis, transport and regulation.

There are many different types of siderophores, pyoverdine being among the best characterized. Pyoverdines (PVDs), also called pseudobactins, are yellow-green, fluorescent, iron(III) high-affinity siderophores produced by all fluorescent *Pseudomonads* (Mossialos *et al*., 2002; Ravel and Cornelis, 2003). PVDs represent the primary iron uptake system in these bacteria, although many species can also synthesise additional siderophores in lower

amounts and with lower affinity for iron(III), such as (thio)quinolobactin, pyochelin, pyridine-2,6-bis thiocarboxylic acid (PDTC), or can acquire iron bound to a variety of exogenous chelators, including many heterologous siderophores (Cornelis and Matthijs, 2002; Poole and McKay, 2003; Matthijs *et al*., 2007; Mossialos and Amoutzias, 2007). One exception is represented by *P. putida* KT2440 which does not seem to produce any other siderophore at least in the conditions tested (Matthijs *et al.,* 2009). More than 60 PVDs have been identified (Meyer *et al*., 2008), but all of them are comprised by three different structural parts (Yeterian *et al*., 2010; Cézard *et al*., 2015; Wei and Aristilde, 2015):

- A chromophore: derived from 2,3-diamino-6,7-dihydroxyquinoline, responsible for the fluorescence of PVDs and iron(III) binding. This part is conserved in *Pseudomonas*.
- A peptide chain: 6 to 14 amino acids, bound by its N-terminus to the carboxylic group of the chromophore and interacting with iron(III). The chain can be linear or partially or completely cyclic and have L- and D- amino acids, some of which are unusual such as hydroxyornithine or hydroxyaspartate, for instance.
- An acyl side-chain consisting of a dicarboxylic acid (succinic, glutamic, malic, or α-ketoglutaric acid, depending on the strain and/or growth conditions) or its monoamide derivative. This chain is linked to the chromophore at position C-3.

The peptide chain is unique to each strain or specie, being its composition and length specific to it, and co-ocurring PVDs in the same strain or specie only differ in the side chain identity (Abdallah and Pattus, 2000; Meyer *et al*., 2008; Cézard *et al*., 2015). Pyoverdine synthesis and transport has been mainly studied and is best understood for *P. aeruginosa* PAO1, although homologs of most of the elements involved seem to be present in other fluorescent *Pseudomonas* (Ravel and Cornelis, 2003).

PVD synthesis begins in the bacterial cytoplasm by the combination of activities of non-ribosomal peptide synthases (NRPS) and enzymes that provide non-conventional moieties (Mossialos *et al*., 2002; Ackerley *et al*., 2003; Schalk and Guillon, 2013; Visca *et al*., 1994; McMorran, *et al*., 2001; Vandenende *et al*., 2004; Olucha *et al*., 2011), allowing chromophore and peptide chain biosynthesis in many complex enzymatic reactions. Eventually, the peptide moiety is terminated by the PvdD enzyme, rendering ferribactin (the inactive precursor of mature PVDs). This molecule is then exported through the inner

membrane to the periplasm by the PvdE ABC-transporter (McMorran *et al*., 1996; Yeterian *et al*., 2010). Maturation of the ferribactin chromophore takes place in the periplasm, before the functional PVD is released to the extracellular millieu (Lamont and Martin, 2003; Yeterian *et al*., 2010; Hannauer *et al*., 2012; Nadal-Jimenez *et al*., 2014). A recent report indicates that in *Pseudomonas taiwanensis* type VI secretion system (T6SS) participates in secretion of mature PVD from the periplasmic space into the extracellular medium (Chen *et al*., 2016).

It has been proposed that each of the classes/groups of PVDs, is recognised by a specific transporter, namely FpvA, once iron(III) is chelated. FpvA belongs to the TonB-dependent family of proteins and is located on the external membrane (Cobessi *et al*., 2005; Greenwald *et al*., 2009). Iron(III) is released and reduced in the periplasm without chemical modification or degradation of the PVD (Schalk *et al*., 2002; Greenwald *et al*., 2007), which is newly redirected to the extracellular medium.

In Gram-negative bacteria the Fur protein is the major iron-response regulator. It represses the transcription of genes involved in siderophore synthesis and iron uptake under iron-rich conditions (Vasil *et al*., 1999; Hantke, 2001; Troxell and Hassan, 2013). Promoters under direct control of Fur have a conserved sequence feature (Fur box), where the regulator binds above a certain threshold of intracellular iron(II), blocking transcription. Under iron limiting-conditions, Fur has no bound iron(II) and cannot bind DNA, allowing transcription. However, many genes are indirectly regulated by Fur via extra-cytoplasmic sigma factors (ECF) or other regulators (Cornelis *et al*., 2009; Cornelis, 2010), resulting in a complex cascade of regulatory systems related to iron uptake. In this cascade, a relevant role has been ascribed to PvdS, an ECF that controls pyoverdine synthesis genes and is in turn regulated by Fur.

Recently, it has also been reported in *Pseudomonas aeruginosa* that pyoverdine production is dependent on the activation state of the Gac/Rsm signal transduction pathway through PvdS and biosynthesis genes (as *pvdA*). This regulation seems to involve c-di-GMP (Frangipani *et al*., 2014). Transcriptional regulators belonging to the LysR-family (CysB, OxyR and PA2206), also control *pvdS* expression under iron deficiency or oxidative stress (Imperi *et al*., 2010; Wei *et al*., 2012; Reen *et al*., 2013; Llamas *et al*., 2014).

3.2. Pyoverdine and iron implications in sessility vs. motility.

Several studies have demonstrated the relationship between pyoverdine and biofilm formation. Once again, this connection has been mostly analyzed in *P. aeruginosa* due to its pathogenicity, although there are studies in other species.

In this bacterium, iron deficiency stimulates twitching motility, a form of surface motility that is incompatible with the formation of microcolonies and mature biofilms (Singh *et al*., 2002), while pyoverdine biosynthesis is necessary for full biofilm formation under iron-starved conditions (Banin *et al*., 2005). Thus, mutants defective in PVD form thin layers of bacterial aggregates that do not develop further. Also, PVD-dependent iron transport functions at a late stage of biofilm formation (Banin *et al*., 2005). Recently, it has been demonstrated that cell aggregation promotes pyoverdine-dependent iron uptake and this fact is essential to develop a mature biofilm (Visaggio *et al*., 2015). Another recent work showed that mutants in genes implicated in early attachment and biofilm formation, as flagellin or Type IV pili synthesis, show reduced production of PVD (Kang and Kirienko, 2017). However, the inverse regulation also occurs, that is, PVD is regulated by biofilm formation when bacteria are not iron-starved. Regulation of iron uptake via PVD contributes to virulence of *P. aeruginosa* (Kang and Kirienko, 2017; Kang *et al*., 2017).

Extracellular DNA (eDNA) has an important role in stabilization of biofilms in *P. aeruginosa*. Low levels of iron promote eDNA release and hence biofilm formation is favoured, while the opposite occurs in iron-rich conditions. This process is regulated by QS signalling (Yang *et al*., 2007). Also, recent reports indicate that the EPSs Pel and Psl play an important role in PVD regulation. Mutants that do not produce EPS show lower levels of PVD (Chen *et al*., 2015). It has also been recently shown that the Gac/Rsm signal transduction pathway regulates PVD production through modulation of c-di-GMP, indicating a connection between iron uptake and biofilm formation through c-di-GMP (Chen *et al*., 2015; Visaggio *et al*., 2015).

In other species of *Pseudomonas* iron and PVD have an important effect in the lifestyle of the bacterium. In *P. putida* KT2440, for example, surface motility is inhibited if iron concentration decreases below a certain level or there is an excess of iron (Matilla *et al*., 2007a). In this strain surface motility requires pyoverdine-mediated iron acquisition. This

kind of motility seems to be dependent of an active transport of ferripyoverdine rather than pyoverdine *per se* and pili are involved in it (Matilla *et al*., 2007a). The presence of iron influences biofilm formation in *P. fluorescens* (O´Toole and Kolter, 1998b), and iron acquisition is important for seed and rhizosphere colonization in *P. putida*, being essential for surface attachment and biofilm formation (Molina *et al*., 2005; Ponraj *et al*., 2012).

4. Metabolic signals and biofilms: the importance of L-arginine.

One aspect that remains poorly investigated is how cellular metabolism influences biofilm development and *vice versa*. Recent research points to amino acids as pontentially relevant metabolic signals in bacterial lifestyles. Amino acids have an essential role as constituents of proteins, although not all are proteinogenic. Besides, amino acids can serve as sources of carbon, energy, and nitrogen (Palmer *et al*., 2005; Corral-Lugo *et al*., 2016), and can have additional functions, such as serving as osmoprotectant molecules, like L-proline (von Blohn *et al*., 1997; Hoffmann *et al*., 2012), or act as a signal cues as is the case of L-serine, L-arginine, L-asparagine, or L-proline (Nishiyama *et al*., 2012; Mills *et al*., 2015). In recent years, among all the amino acids, L-arginine has been acquiring relevant importance due to its implications in multicellular behaviors of bacteria, as described later in this section. Arginine metabolism has a high degree of complexity and different catabolism pathways exist. The best knowledge of these pathways is because of its economic interest, since L-arginine has applications in flavour and pharmaceutical industries (Ikeda, 2003).

4.1. Arginine metabolism.

4.1.1. Biosynthesis of L-arginine.

L-arginine biosynthesis proceeds from L-glutamate in eight enzymatic steps (Figure 6; see also Figure 4 in Chapter 1 for further details), with ornithine as a key intermediate. The conversion of ornithine to arginine requires three steps, the first of which involves carbamoylphosphate utilization (Lu *et al*., 2006). The pathway is similar in different bacteria, although another pathway involving a novel family of transcarbamylases has been described (Shi *et al*., 2005). In *P. aeuginosa* PA01, as well as *P. putida* KT2440, ArgA and ArgJ catalyse

N-acetylglutamate synthesis from glutamate. ArgA essentially initiates arginine biosynthesis, *argA* mutants being auxotrophs, while ArgJ helps to minimize the amounts of acetyl-coA consumed by ArgA, by recycling the acetyl group and transferring it to glutamate. The same occurs in the step catalysed by ArgE (Itoh and Nakada, 2004; Lu *et al*., 2006). However, *E. coli* has only ArgA and *B. subtilis* has only ArgJ that probably assumes the anaplerotic function of ArgA (Glansdorff, 1996; Belitsky, 2002).

Arginine synthesis genes are generally not clustered in the chromosome in a single functional unit (Bachmann, 1990; Blattner *et al*., 1997; McClelland *et al*., 2001). In *E. coli*, the only genes to be clustered are *argECBH* and *carAB*. However the cluster *argECBH* constitute a divergent operon with two arms, *argE* and *argCBH* (Panchal *et al*., 1974; Pouwels *et al*., 1974; Weerasinghe *et al*., 2006). However, in *P. aeruginosa* and *P. putida* almost all of genes are spread in the genome, except for *carA* and *carB* (see at *Pseudomonas* Genome Database; Haas *et al*., 1977; Kwon *et al*., 1994).

4.1.2. Catabolism of L-arginine.

Five catabolic pathways for L-arginine utilization have been reported in bacteria:

a) The arginine deiminase (ADI) pathway.

This pathway is widespread among eubacteria and archaea, being the most important one for arginine utilization under anaerobic conditions (Maghnouj *et al*., 1998; Arena *et al*., 2002; Hong, 2006). L-arginine is converted into ornithine, ammonia and carbon dioxide by three enzymes encoded by *arcA*, *arcB* and *arcC* (Figure 6) that form an operon along with *arcD*, which encodes a membrane-bound arginine-ornithine antiporter that transports ornithine produced in the cytosol out of cells in exchange for L-arginine present in the medium (Verhoogt *et al*., 1992; Bourdineaud *et al*., 1993).

b) The arginine succinyltransferase (AST) pathway.

The AST pathway is the major catabolic route of arginine and ornithine utilisation under aerobic conditions as carbon and nitrogen sources or only nitrogen source depending on the strain/species. It was first reported in *P. aeruginosa* (Vander Wauven and Stalon, 1985; Stalon *et al*., 1987), although the AST enzymes have been identified in other species of *Pseudomonas* and different bacteria (Lu *et al*., 2006). In this pathway, arginine suffers

different transformations in several steps and the carbon skeleton is essentially degraded in the tricarboxylic acid cycle (Figure 6). The enzymes implicated in this route are encoded by the *aruCFGDBE* operon (Itoh, 1997), located immediately downstream of the *aotJQMOP-argR* operon (Nishijyo *et al*. 1998), which encodes components of an ABC transporter for arginine and ornithine as well as the arginine regulatory protein ArgR. This organization is highly conserved among *Pseudomonads* and *Burkholderia* (Itoh and Nakada, 2004), allowing exogenous L-arginine utilization very efficiently.

c) The arginine transaminase/oxidase/dehydrogenase (ADH) pathway.

The ADH pathway was first reported in *P. putida*. AST and ADH pathways function simultaneously and contribute to arginine utilisation in the same grade in this bacterium (Tricot *et al*., 1991). Four enzymes are implicated in this route, which convert L-arginine to 4-aminobutyrate with the concomitant formation of urea and carbon dioxide (Figure 6) (Chou and Rodwell, 1972; Fan and Rodwell, 1975; Vanderbilt *et al*., 1975; Nakada and Itoh, 2003).

d) The arginase pathway.

The arginase pathway hydrolyses arginine to urea and ornithine. Ornithine is then hydrolysed to production of glutamate and used as a carbon and/or nitrogen sources whereas urea is catalysed to ammonia and used as nitrogen source if the bacterium possesses a urease system (Cunin *et al*., 1986; Xiong *et al*., 2016). This pathway is well studied in bacilli and *Agrobacterium*, although it is also present in *Proteus* spp., *Streptomyces* spp. and *Thermus aquaticus* (Cunin *et al*., 1986).

e) The arginine decarboxylase (ADC) pathway.

Through this pathway, L-arginine is decarboxylated to agmatine by an arginine-inducible ADC. Then agmatine is converted into putrescine, ammonia and carbon dioxide in two steps (Nakada and Itoh, 2003), being the starting point of polyamines biosynthesis. It has been reported that this pathway does not contribute to arginine utilisation as a carbon source in many bacteria (Schneider *et al*. 1998; Nakada and Itoh 2003).

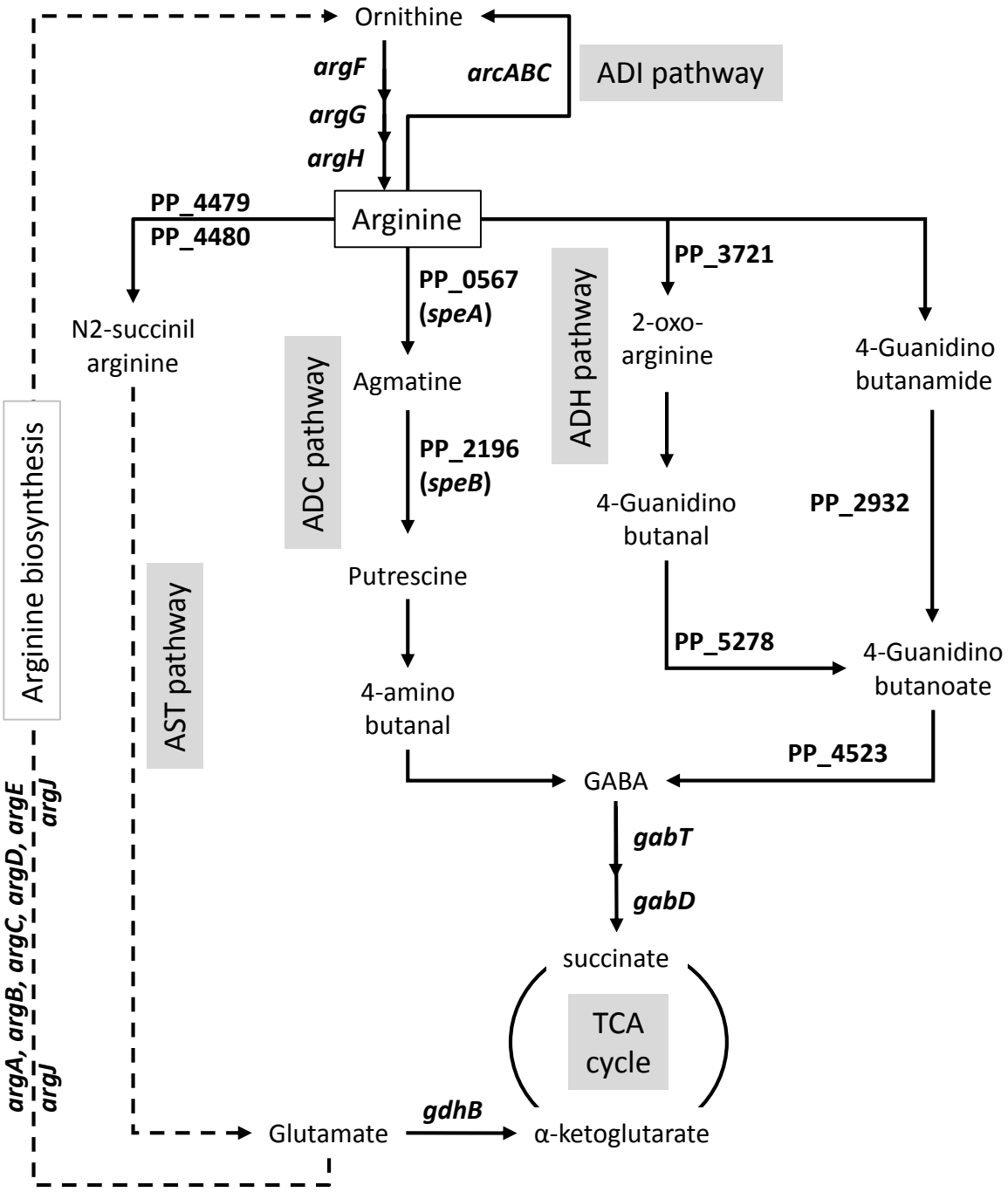


Figure 6. Schematic representation of arginine metabolism pathways in *Pseudomonas putida*, indicating some intermediaries, based on data obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) and modified from Lu *et al*., 2004. Broken lines indicate multiple steps are involved.

4.2. Arginine uptake.

Different transport systems for arginine uptake have been identified in bacteria, including ABC transporters and antiporters. ABC transporters typically are composed of two transmembrane permease domains, each of them spanning the membrane four to eight times, and two nucleotide-binding domains (Wilkens, 2015). In enteric bacteria, it has been described two different ABC transported involved in arginine uptake encodes in separate operons. One of them is *argT-hisJQMP*, in which *argT* encodes a periplasmic binding protein with affinity for arginine, as well as, for lysine and ornithine (Higgins and Ames, 1981; Das *et al*., 2010). This system transport is controlled positively under conditions of nitrogen starvation (Zimmer *et al.*, 2000) and is not subjected to arginine regulation (Glansdorff, 1996). However, the second ABC transport encoded by *artPIQMJ* operon (Wissenbach *et al*. 1995) -being ArtJ the arginine-binding protein- is specific to arginine and ArtP and ArtJ are repressed by the amino acid (Caldara *et al*., 2006). This transport system functions under aerobic conditions and in presence of sufficient nitrogen.

As mentioned previously, the *aotJQMP* operon encodes components of an ABC transporter for arginine and ornithine uptake in *P. aeruginosa* (Nishijyo *et al*., 1998), which is induced by arginine. Besides, it has been reported that another ABC transporter system (Lu *et al*., 2004) and at least two porins -named OprD and OpdP- located in the outer membrane are involved in arginine uptake (Tamber and Hancock, 2006).

Antiporters also play an important role on arginine uptake, being found in different bacteria. As shown in section of ADI pathway, in *P. aeruginosa* has been reported the existence of *arcD* gene that encodes a membrane-bound arginine-ornithine antiporter which mediates energy-independent exchange between ornithine produced in the cytosol and arginine present in the medium (Lüthi *et al*., 1990; Verhoogt *et al*., 1992; Bourdineaud *et al*., 1993). Recently, it has been reported the existence of this antiporter in *P. putida* (Udaondo *et al*., 2016) and *S. gordonii* (Sakanaka *et al*., 2015) as well. In *E. coli* and *S. enterica* serovar Typhimurium, it was found that AdiC protein of the adiCYA operon functions as an arginine/agmatine antiporter under low pH and anaerobic conditions (Gong *et al*., 2003).

4.3. ArgR: the major regulator in arginine metabolism.

L-arginine metabolism is mainly regulated by the transcriptional regulator ArgR. This protein and its cognate target sites are highly conserved among Gram-negative and Gram-positive bacteria (Lu, 2006). ArgR is classified as belonging to the ArgR/AhrC family in *E. coli*, *S. enteria* serovar Thypimurium or *B. subtilis* (Maas, 1994; Miller *et al*., 1997; Dennis *et al*., 2002; Griswold *et al*., 2004; Choi *et al*., 2012), and to the AraC/XylS family (Egan, 2002) in *Pseudomonas* spp. (Park *et al*., 1997a; Lu et al., 1999). In general, regulation is exerted by binding of ArgR to its operator sites, named ARG box, which precede the target genes, leading to positively control of arginine catabolic genes and negatively control of biosynthetic genes in the presence of L-arginine. ArgR synthesis itself is induced by exogenous arginine (Maas, 1994; Park *et al*., 1997a).

In *Pseudomonas*, ArgR acts as a dimer, possessing two helix-turn-helix (HTH) DNA-binding domains. Its binding site has been described as an imperfect tandem repeat, where sequences of the first-half sites are less conserved than those of the second-half sites (Nishijyo *et al*., 1998; Lu *et al*., 2004). AST and ADI pathways are positively controlled by ArgR and *argF*, *argG* and *carAB* biosynthetic genes are negatively regulated by this regulator (Lu *et al*., 2004). Arginine controls cellular amounts of ArgR by modulating the transcription of the *aotJQMOP-argR* operon. This operon has two promoters (P1 and P2). In the absence of arginine, transcription from the upstream promoter P1 occurs at low levels. When arginine is present in the medium, transcription from P1 ceases and expression of the operon from P2 takes place actively at high levels. This process is due to the ArgR binding to the ArgR box which is located adjacent to the -35 region of P2 and overlaps with the -10 region of P1 (Nishijyo *et al*., 1998). Arginine and ArgR are essential but not sufficient for P2 activation, which requires the two component system CbrA/CbrB. It seems that CbrB modulates P2 promoter indirectly by means of transcription activator of σ^{70}-RNA polymerase (Nishijyo *et al*., 2001). ArgR can bind to the ArgR box independently of arginine, so if ArgR has binding affinity to arginine is yet unknown (Park *et al*., 1997b; Nishijyo *et al*., 2001).

ArgR also activates the expression of the *aru* (AST pathway) and *arc* (ADI pathway) operons, and represses expression of biosynthetic genes due to its binding to the ArgR boxes within the -10 and -35 regions of *argF* and *carA*, and presumably of *argG* (Park *et al*., 1997b;

Lu *et al*., 2004). So, ArgR regulates negatively the biosynthetic pathway in response to arginine in the medium. A difference between *P. aeruginosa* and most bacteria is the fact that in the former not all biosynthetic genes are repressed by arginine (Lu, 2006).

4.4. L-arginine: implications in motility and biofilm formation.

In recent years, different reports have shown that L-arginine plays an important role in biofilm formation in many bacteria, although there are other amino acids that might also take part in it. For instance, in *S. enterica* serovar Thypimurium, all genes of the tryptophan biosynthetic pathway are up-regulated in mature biofilms. In fact, a mutant in *trpE* gene, which encodes the first enzyme of the biosynthetic pathway, shows reduced biofilm formation. Biofilm formation is promoted in the presence of this amino acid, increasing the number of adherent cell to the surface, as well as enhancing biofilm formation at later stages (Hamilton *et al*., 2009). However, in *E. coli* the effect of tryptophan favouring biofilm formation only occurs at early stages when cells are attaching to the surface, being tryptophan biosynthesis genes up-regulated in this moment (Ren *et al*., 2004; Domka *et al*., 2007).

In *P. aeruginosa*, it has been reported that several amino acids promote robust biofilm formation. These amino acids are arginine, ornithine, isoleucine, leucine, valine, phenylalanine, and tyrosine (Bernier *et al*., 2011). Furthermore, swarming motility are reduced in all these amino acids, but in the case of L-arginine is completely abolished. This suggests that arginine may be an essential environmental signal that favours a sessile lifestyle. This is support by the fact that in this bacterium, the QS precursor 2-Heptyl-4-quinolone, in presence of L-arginine, represses swarming motility (Ha *et al*., 2011). Furthermore, L-arginine increases c-di-GMP levels, being the effect dependent of two DGC required for biofilm formation, SadC and RoeA (Merritt *et al*., 2007; Merritt *et al*., 2010; Bernier *et al*., 2011). This effect has also been recently shown in *S. enterica* serovar Thypimurium, where L-arginine provokes an increase in c-di-GMP levels associated to the DGC encoded by STM1987, leading to an increase in cellulose production, which is the most important EPS in *Salmonella* biofilm matrix (Mills *et al*., 2015).

Several reports have shown that some mutants altered in biofilm formation are affected in genes related to L-arginine metabolism. These include *P. aeruginosa* mutants in biosynthetic genes like *carAB*, *argG* and *argH*, genes implicated in catabolism (*gabT* and *aruG*), and *cbrA* (Müsken *et al*., 2010). In *E. coli* arginine metabolism genes (*artJ*, *argC* and *argF*) are highly expressed under biofilm conditions (Schembri *et al*., 2003), and *carAB*, *argDABC* and *argGH* are highly expressed in *S. aureus* biofilms (Beenken *et al*., 2004). It seems that a functional arginine metabolism is necessary to biofilm formation in several bacterial species.

5. *Pseudomonas putida* KT2440: a model organism for metabolic studies and biofilm formation.

Due to their genetic plasticity and their broad metabolic versatility (Martínez-Bueno *et al*., 2002), strains of *Pseudomonas putida* can colonize many different environments and have been widely studied from biochemical, genetic and physiological points of view (Domínguez-Cuevas *et al*., 2006; del Castillo *et al*., 2007; Nogales *et al*., 2008; Belda *et al*., 2016). In our laboratory, *P. putida* KT2440 is the most studied strain. It is an efficient coloniser of the spermosphere and the root system of different plants of basic and agronomic interest, being beneficial in plant interactions (Espinosa-Urgel *et al*., 2000; Molina *et al*., 2000; Matilla *et al*., 2010). KT2440 is a plasmid-free derivative of *P. putida* mt-2 (Bayley *et al*., 1977), which was isolated from a vegetable orchard soil in Japan based on its ability to metabolise 3-methylbenzoate. It harbours plasmid pWW0, containing genes for aerobic catabolism of the aromatic compounds xylene and toluene (Nakazawa, 2002). Both strains can survive in presence of aromatic compounds (Fernández *et al*., 2009), although they are not as tolerant as strain DOT-T1E, a model organism in studies of biodegradation and tolerance to toxic organic compounds such as toluene and ρ-hydroxybenzoate (García *et al*., 2010; Udaondo *et al*., 2012). How the metabolism of these compounds may influence the lifestyle of *P. putida* remains poorly studied.

Biofilm formation on abiotic and plant surfaces has been an ongoing interest in our group. Previous work has allowed identifing iron metabolism as relevant in swarming motility and plant surface colonization, as mentioned previously. Also, research in our group

and other laboratories has led to the characterization of the structural elements involved in biofilm formation. In *P. putida* KT2440, the first key elements are the large adhesins LapA and LapF. The former is necessary for cell-surface interactions (Martínez-Gil *et al*., 2014) and the latter mediates cell-to-cell interactions (Martínez-Gil *et al*., 2010), although both are also important in the matrix of mature biofilms, along with EPSs. *P. putida* KT2240 produces four EPSs: alginate (alg), cellulose (bcs), Pea and Peb, being the last two specific of this strain. It has been reported the potential existence of interactions between EPS and adhesins in a mature biofilm and there are evidences of a cross-talk mechanism by which bacteria can modulate the lack of some components by modulating the expression of others in order to maintain biofilm integrity (Martínez-Gil *et al*., 2013).

Efforts have also been made to understand in detail how these elements are regulated (Figure 7). Thus, the GacS/GacA two component regulatory system modulates LapA and LapF expression indirectly and it is implicated in biofilm formation in *P. putida* KT2440 (Martínez-Gil *et al*., 2014). GacS is a sensor histidine kinase which senses a still-unknown environmental signal and activates by phosphotransfer the transcriptional regulator GacA, which subsequently activates target genes (Heeb and Haas, 2001). The main regulatory targets of GacA are the small RNAs rsmY and rsmZ, identified in *P. putida* KT2440 as well as in other bacteria, which sequester the post-transcriptional regulators of the Rsm family of proteins (Huertas-Rosales, PhD. Thesis, 2017b). Rsm proteins are small RNA-binding proteins that play key roles in the regulation of gene expression by altering translation, RNA stability and/or transcript elongation (Romeo *et al*., 2013). There are three Rsm proteins in *P. putida* KT2440 (RsmA, RsmE and RsmI). Recent work has shown that Rsm proteins play a negative role upon the expression of the four EPSs and LapF adhesin, having a slight effect on LapA (Huertas-Rosales *et al*., 2016). At least part of this effect can be attibuted to negative regulation of the sigma factor RpoS and the diguanylate cyclase CfcR (Huertas-Rosales *et al*., 2017a, our unpublished data), leading to reduced biofilm formation. RpoS (σ^S) is an alternative RNA polymerase sigma factor which controls the transcription of a high number of genes at the onset of the stationary phase of growth and in response to environmental stresses in many bacteria (Potvin *et al*., 2008; Chiang and Schellhorn, 2010). It positively controls the expression of LapF and CfcR upon entry in stationary phase in *P. putida* KT2440 (Martínez-Gil *et al*., 2010; Matilla *et al*., 2011) (Figure 7).

CfcR, previously named Rup4959, is the unique response regulator containing GGDEF and EAL domains in *P. putida* KT2440. It is responsible for up to 75% of c-di-GMP content in stationary phase (Huertas-Rosales *et al*., 2017a), and is RpoS-dependent (Matilla *et al*., 2011).

CfcR was found preferentially expressed in this bacterium when it colonises the rhizosphere of corn plants (Matilla *et al*., 2007b). Under laboratory conditions, overexpression of *cfcR* increases c-di-GMP levels, giving rise to a pleotropic phenotype that includes flocculation of cultures in liquid medium, increased biofilm formation and pellicle formation at the air-liquid interface in static cultures, and crinkle colony morphology (Matilla *et al*., 2011). A genetic screen designed to identify elements involved in the development of this phenotype, revealed that mutants deficient in arginine biosynthesis lost the crinkly colony morphology and showed reduced c-di-GMP levels. This opened the way to analyse for the first time the potential connection between metabolic signals and c-di-GMP regulation in *Pseudomonas putida*, one of the main topics in this Thesis.

In *P. putida* KT2440, c-di-GMP likely influences the presence of LapA on the cell surface in an indirect way by a mechanism involving the c-di-GMP-binding protein LapD and the protease LapG as in *P. fluorescens* (described in section 2.3). On the other hand, some biofilm structural elements are regulated by c-di-GMP through FleQ (Figure 7), in a way similar to other bacteria. Thus, low levels of c-di-GMP favour transcription of flagellar genes, while high levels lead to regulate positively biofilm formation (Jiménez-Fernández *et al*., 2016; Wang *et al*., 2017). FleQ through c-di-GMP binding positively modulates the expression of *lapA* and *pea* whereas it negatively affects expression of the *bcs* operon (Martínez-Gil *et al*., 2014; Xiao *et al*., 2016; Molina-Henares *et al*., 2017). Interestingly, the activity of FleQ upon *lapA* and *bcs* has been described to be positively modulated by FleN, which would act synergistically (Nie *et al*., 2017), in contrast with *P. aeruginosa*, where FleN and FleQ have an antagonistic role in regulating EPS *pel* and flagellar genes (Baraquet *et al*., 2012). Besides, it has recently shown that FleQ can bind directly to *peb* and *alg* promoters independently of c-di-GMP levels, showing an opposite effect on their expression. So, FleQ positively regulates *peb* expression whereas negatively regulates *alg* expression (Molina-Henares *et al*., 2017). On the other hand, FleQ does not have much effect upon LapF,

although some negative effect has been reported at transcriptional level but, in any case, c-di-GMP negatively affects the expression of LapF (Martínez-Gil *et al.*, 2014).

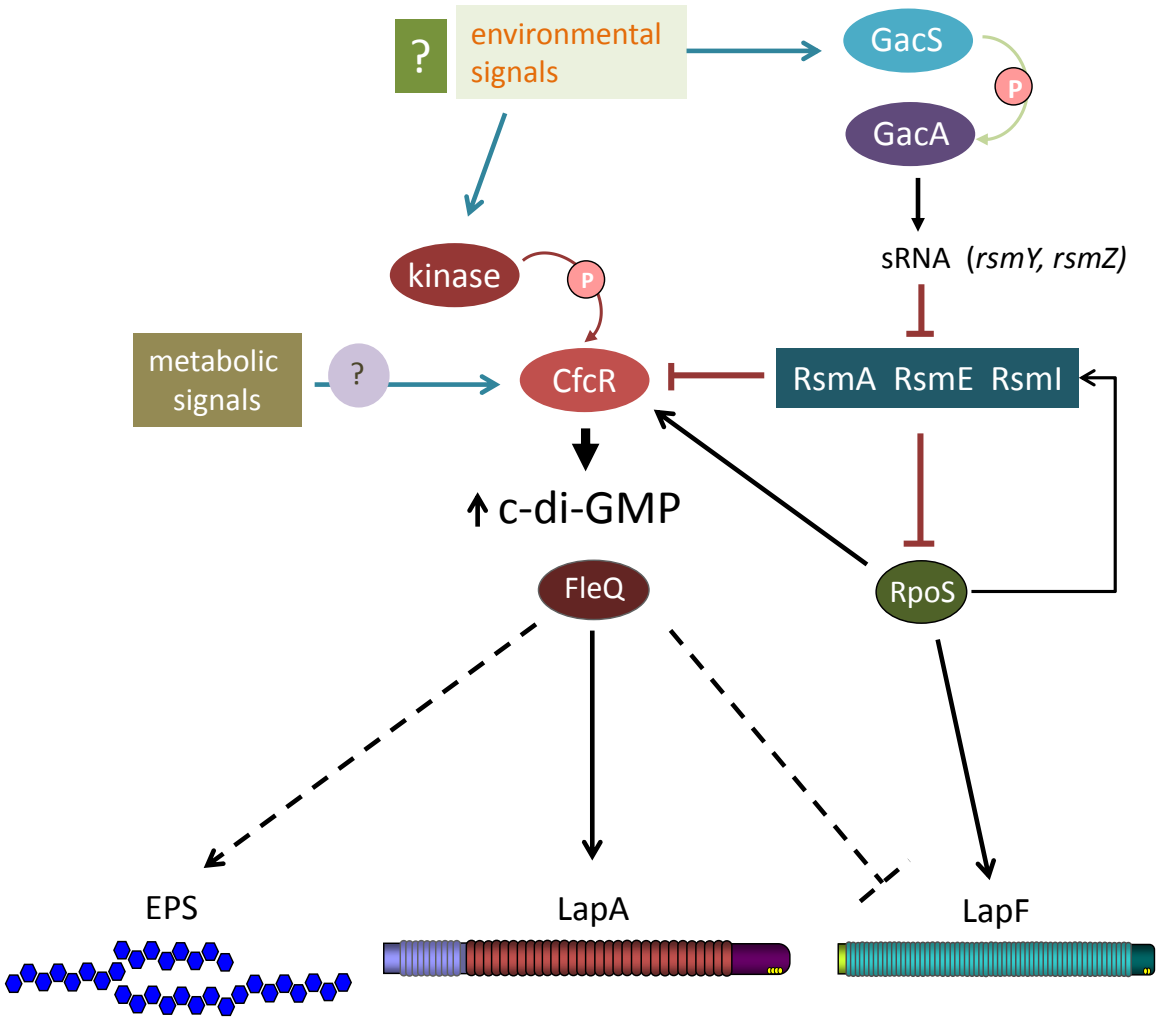


Figure 7. Schematic representation of the regulatory cascade involved in the regulation of structural elements implicated in biofilm formation in *P. putida* KT2440, as postulated at the beginning of this Thesis. Black arrow-ended and red T-shape-ended lines are indicative of positive and negative regulation, respectively. Broken lines indicate that the regulation is not fully confirmed or is not known to be direct.

II. AIMS OF THE THESIS

The metabolic versatility of *P. putida*, and an array of stress tolerance mechanisms, allow strains of this species to thrive in different environments and utilize a wide variety of substrates for growth, even toxic organic compounds. Our group is particularly interested in the adaptation mechanisms involved in colonization of plant surfaces by *Pseudomonas putida* KT2440, and how this process correlates with biofilm formation, which is acknowledged as one of the main colonization and persistence mechanisms of bacteria in the environment. In the last years, efforts have focused more specifically on decyphering the regulatory factors that control expression of the structural elements involved in attachment and biofilm maturation in this bacterium.

One particular aspect that so far has not been studied in detail is the influence of metabolic signals on the transition between planktonic and sessile lifestyles. In addition, there is limited information on how toxic but potentially metabolizable hydrocarbons influence the process of biofilm development. The overall aim of this Thesis was to begin exploring these questions. The starting point has been a high throughput screen carried out to find genes involved in the signalling pathway associated to high levels of the second messenger c-di-GMP (Ramos-González *et al.*, 2016). In this analysis, two mutants affected in the arginine biosynthesis pathway were identified, and a potential connection was observed with siderophore-dependent iron acquisition, which is relevant for multicellular behaviours of *P. putida* KT2440.

Thus, the aims of this work have been oriented towards the following questions:

1. To determine the effect of amino acids in c-di-GMP levels and biofilm formation in *P. putida* KT2440, focusing mostly on the characterization of mutants affected in the arginine biosynthesis pathway.
2. To identify the regulatory network associating arginine metabolism with c-di-GMP signalling.
3. To explore the connection between arginine metabolism and production of the siderophore pyoverdine.
4. To investigate the influence of toxic organic hydrocarbons on biofilm formation by strains of *P. putida* with different metabolic and tolerance capabilities.

III. MATERIALS AND METHODS

GENERAL METHODS

Bacterial strains, culture media and growth conditions.

Strains and plasmids used in this work are listed in the Tables indicated in the specific Materials and Methods sections for each Chapter. *Pseudomonas putida* KT2440 is a plasmid-free derivative of *P. putida* mt-2, originally isolated from a vegetable orchard in Japan and whose genome is completely sequenced (Nakazawa, 2002; Nelson *et al*., 2002). *Pseudomonas* strains were routinely grown at 30°C in Luria-Bertani (LB) medium (Lennox, 1955) or in different minimal media depending on the objective [M9 minimal medium (Yousef-Coronado *et al*., 2008) or modified FAB medium with glucose as a carbon source (Heydorn *et al*., 2000)]. Modified FAB medium was used for biofilm studies due to the presence of calcium in its composition, which is important for adhesins (Theunissen *et al*., 2010; Martínez-Gil *et al*., 2012). M9 minimal medium with $MgSO_4$, Fe citrate and trace metals as described previously (Yousef-Coronado *et al*., 2008) with glucose (20 mM) or sodium citrate (15 mM) as carbon sources was used for routine work. *Escherichia coli* strains were grown at 37°C in LB. When appropriate, antibiotics were used at the following concentrations (μg/ml): chloramphenicol (Cm) 30; kanamycin (Km) 25; tetracycline (Tc) 10; gentamicin (Gm) 10 (for *E. coli*) or 100 (for *P. putida*); piperacillin (Pip) 30; ampicillin (Ap) 100; streptomycin (Sm) 50 (for *E. coli*) or 100 (for *P. putida*); nitrofurantoin (Nit) 100.

Growth curves.

Growth of the different strains in liquid media was analysed either in flasks or using a Bioscreen apparatus. In the first case, cultures were grown overnight in the specified media, turbidity was measured and inocula were prepared by adjusting to an OD_{660} of 0.05 at the start of the experiment. Turbidity of cultures was measured at 660 nm every hour during the first 11 hours and one point at 24h. Experiments were done in triplicate with two technical measures every time. For Bioscreen experiments, inocula were adjusted to an initial OD_{660} of 0.02. Turbidity of cultures was monitored at 420-550 nm every 30 min during 24 hours. Experiments were done in triplicate.

Molecular biology techniques.

DNA preparation, digestion with restriction enzymes, plasmid dephosphorylation, ligation and cell transformations were done using standard protocols (Ausubel *et al*., 1987; Sambrook and Russell, 2001). Electrotransformation of *Pseudomonas* cells was performed as previously described (Enderle and Farwell, 1998). Plasmid purification and gel extraction from agarose gels were carried out following manufacturers´ instructions (NZYTech and QIAgen kits, respectively). The DIG-DNA labelling and detection kit (Roche) was used for Southern hybridization as recommended by the manufacturer.

Triparental conjugation.

Transfer of plasmids from *E. coli* to *P. putida* strains was performed by triparental mating using *E. coli* (pRK600) as a helper. For each strain, 0.5 ml of overnight LB cultures were collected by centrifugation, resuspended in 50 µl of fresh LB medium after several washing steps and spotted on mating filter (0.22 µm of pore diameter) on LB-agar plates, which were incubated overnight at 30°C. Then, cells were scraped off the mating filter and resuspended in 1.5 ml of diluted M9 salts medium. Serial dilutions were plated on selective LB medium or M9 minimal medium with citrate as a carbon source supplied with the appropriate antibiotics to select exconjugants and counterselect donor, helper, and recipient strains.

Generation of null mutants.

Null mutants were generated by gene replacement via homologous recombination. Upstream and downstream fragments of the gene to replace were amplified by PCR using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). To amplify each fragment, primers were designed as follows (see Tables indicated in the specific Materials and Methods sections for each Chapter): to amplify upstream fragment of the gene to be removed, one primer with NotI restriction site and other with a complementary tail to another primer used to amplify the downstream fragment were used. The same design was used for primers to amplify downstream fragment. So, PCR was done in two steps. Firstly, flanking regions of the gene to be eliminated were amplified separately and then these regions were used as template for the second PCR thanks to the overlapping tail present in both amplicons. Finally, one amplified fragment was obtained flanked with NotI site. This

PCR product was cloned into pCR2.1-TOPO vector after its adenilation and sequenced to ensure the absence of mutations. The null allele was subcloned into the NotI site of pKNG101 plasmid, which is a suicide vector unable to replicate in *Pseudomonas* that allows the generation of double recombinant events (Kaniga *et al*., 1991). Plasmid pKNG101 containing the mutation was mobilized from *E. coli* CC118 (λpyr) to *P. putida* KT224 by conjugation using HB101 (pRK600) as a helper as previously described. Merodiploid exconjugants were selected in minimal medium with citrate and streptomycin. One of these clones was grown in LB medium with 14% of sucrose to obtain clones in which double recombination event had taken place. Mutants were sucrose-resistant and streptomycin-sensitive. Null mutants were checked by PCR, subsequent sequencing and southern hybridization.

C-di-GMP quantification based on a bioreporter.

Bioreporter pCdrA::*gfp*^{C} was used to quantify c-di-GMP levels based on fluorescence emitted (Rybtke *et al*., 2012), using different plate readers (Synergy Neo2 Biotek, TECAN Infinite 200 or Varioskan Lux) and suitable 96-well plates (Greiner or Nunc 96 Flat Bottom Black Polystyrol well plates). To measure fluorescence of cultures in presence or absence of amino acid, we proceed as follows. Briefly, overnight cultures grown in LB diluted 1/3 or M9 minimal medium with glucose were diluted in freshly medium as previous used with 20 µg ml^{-1} Gm to a final OD_{600} of 0.02 in the wells. L-amino acids were added in different concentrations (0, 5, 15 and 25 mM). Growth (at 600 nm) and fluorescence (excitation at 485 nm and emission at 535 nm) were monitored every 30 min for 24 h using microplate reader. The assays were performed at least three times using three replicates per condition tested.

Biofilm assays.

Biofilm formation assays were performed using polystyrene 96-well microtiter plates as previously described (O'Toole and Kolter, 1998). Overnight FAB cultures were diluted to an OD_{660} of 0.02. Each microtiter well was inoculated with 150 µl of freshly FAB inoculated medium with glucose as a carbon source. To test the effect of different L-amino acids on biofilm formation, these were added in different concentrations (0, 5 and 15 mM), with four wells per condition. Microtiter plates were incubated at 30°C in static conditions during 24h, removing planktonic cultures from time to time and measuring the absorbance at 660 nm.

Unattached cells were washed away with destilled water and biomass attached to the surface was stained with crystal violet for 15 min and quantified after dye solubilization with glacial acetic acid (30% v/v). The crystal violet solution was measured at an absorbance of 595 nm using a Tecan Sunrise reader plate. Where indicated, biofilm assays were performed in borosilicate glass tubes in LB medium. Overnight cultures were diluted to an OD_{660} of 0.05 in 2 ml of the indicated medium and incubated under orbital shaking at 30°C. The procedure of staining and washing out was similar to the previous one.

Measurement of β-galactosidase activity.

β-galactosidase activity was assayed during growth in LB as described elsewhere (Miller, 1972). Overnight cultures were diluted (1:100) in fresh medium supplemented with 10 μg ml^{-1} Tc. After one hour of growth at 30°C and 200 r.p.m., cultures were diluted 1:10 to ensure proper dilution of β-galactosidase that might have accumulated after overnight growth. Then, samples were collected at the indicated times and β-galactosidase activity was measured indicating the results in Miller Units. Where appropriate, β-galactosidase activity was assayed during growth in minimal medium.

Crinkly morphology analysis and fluorescence imaging.

Bacterial streaks or drops of overnight cultures were grown on agar plates with the medium and supplements indicated in each case, at 30°C for 24-48h h. Images were taken using Fluorescent Stereo Microscope Leica M165 FC (Leica Microsystems). Excitation/Emission filter 480/510 nm was used for monitoring GFP fluorescence.

SPECIFIC MATERIALS AND METHODS – CHAPTER I

Strains, plasmids and primers.

Strains, plasmids and oligonucleotides used in this Chapter are detailed in Tables 1 and 2, respectively.

Construction of plasmids for ectopic expression of *argG* and *argH* under the control of their own promoter.

1.6 Kb sequence corresponding to promoter region and gene *argG* was amplified by PCR using primers with XhoI/BamHI restriction sites. Likewise, 1.8 Kb fragment was amplified to arg*H* and its promoter. These fragments were cloned into pCR2.1-TOPO and the resulting plasmids pLBM5 (for *argG* and its promoter) and pLBM6 (for *argH* and its promoter) were transformed into *E. coli* DH5α. Nucleotide sequencing was performed to confirm that no base changes had occurred during PCR amplifications. For ectopic expression of *argG* and *argH* under the control of their promoters, plasmids pLBM5 and pLBM6 were digested using XhoI/BamHI restriction sites incorporated by the PCR primers and resulting fragments were cloned in pBBR1-MCS5 vector cut with the same restriction enzymes to ensure the proper orientation of fragments. The resulting plasmids pLBM7 (for *argG* and its promoter) and pLBM8 (for *argH* and its promoter) were introduced into *E. coli* DH5α and subsequently transferred to *P. putida* KT2440 strains.

Table 1. Bacterial strains and plasmids used.

Strain or plasmid	Genotype/Relevant characteristics[a]	Reference or source
Strains		
E. coli		
CC118λpir	Rif^R, λpir	Herrero *et al*., 1990
DH5α	*supE44 lac*U169 (Ø80*lacZ*ΔM15) *hsd*R17 (r_k-m_k-) *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1	Woodcock *et al*., 1989
HB101 (pRK600)	Helper strain harbouring Cm^R *mob tra* plasmid	V. de Lorenzo
P. putida		
KT2440	Wild type; derivative of *P. putida* mt-2, cured of pWWO	Regenhardt *et al*., 2002
Δ*argG*	Null mutant derivative of KT2440 in PP_1088 (*argG*)	This study
Δ*argH*	Null mutant derivative of KT2440 in PP_0184 (*argH*)	This study
cfcK- 66	Km^R, *argG*::mini-Tn5[Km1] transposon mutant derivative of *P. putida* KT2440	Ramos-González *et al*., 2016
cfcK- 74	Km^R, *argH*::mini-Tn5[Km1] transposon mutant derivative of *P. putida* KT2440	Ramos-González *et al*., 2016
Δ*cfcA*	Null mutant derivative of KT2440 in PP_3761	Tagua *et al*., for publishing
Plasmids		
pCR2.1 TOPO	Km^R, cloning vector with β-galactosidase α-complementation	Invitrogen
pBBR1-MCS5	Gm^R, broad host range cloning vector, mobilizable	Kovach *et al*., 1995
pCdrA::*gfp*^{C}	Ap^R (Pip^R), Gm^R, FleQ dependent c-di-GMP biosensor	Rybtke *et al*., 2012
pKNG101	Sm^R, *oriR6K mobRK2 sacBR*	Kaniga *et al*., 1991
pLBM1	pCR2.1TOPO derivative with 1.5 Kb NotI fragment containing the *argG* null allele	This work
pLBM2	pCR2.1TOPO derivative with 1.8 Kb NotI fragment containing the *argH* null allele	This work
pLBM3	Sm^R, pKNG101 derivative for *argG* null allele replacement with the 1.5 Kb NotI fragment of pLBM1 cloned in pKNG101	This work
pLBM4	Sm^R, pKNG101 derivative for *argH* null allele replacement with the 1.8 Kb NotI fragment of pLBM1 cloned in pKNG101	This work
pLBM5	Km^R, pCR2.1TOPO derivative with 1.6 Kb BamHI/XhoI fragment containing the *argG* gene and its promoter region	This work
pLBM6	Km^R, pCR2.1TOPO derivative with 1.8 Kb BamHI/XhoI fragment containing the *argH* gene and its promoter region	This work
pLBM7	Gm^R, pBBR1-MCS5 derivative for the ectopic expression of *P. putida argG* with its own promoter	This work
pLBM8	Gm^R, pBBR1-MCS5 derivative for the ectopic expression of *P. putida argH* with its own promoter	This work
pMAMV1	Gm^R, pBBR1-MCS5 derivative containing *cfcR* expressed from its own promoter; it confers high c-di-GMP levels in KT2440	Matilla *et al*., 2011
pME0184	Ap^R (Pip^R), derivative plasmid of pMMB67HE for the ectopic expression of *P. putida argH*	Ramos-González *et al*., 2016
pME1088	Ap^R (Pip^R), derivative plasmid of pMMB67HE for the ectopic expression of *P. putida argG*	Ramos-González *et al*., 2016
pMIR178	Km^R, pBBR1-MCS2 derivative containing *cfcR* expressed from its own promoter; it confers high c-di-GMP levels in KT2440	Ramos-González *et al*., 2016
pMIR219	Tc^R, *cfcR´-`lacZ* translational fusion in pMP220-BamHI	Huertas-Rosales *et al*., 2017a
pMIR200	Tc^R, *cfcR´::`lacZ* transcriptional fusion in pMP220	Huertas-Rosales *et al*., 2017a
pMMB67HE	Ap^R (Pip^R), RSF1010 origin, *lacIq*	Fürste *et al*., 1986

[a]Rif, rifampin; Cm, chloramphenicol; Km, Kanamycin; Tc, tetracycline; Sm, streptomycin; Pip, piperacillin; Ap, ampicillin; Gm, gentamicin

Table 2. Primers used.

Primer name	Sequence (5´→3´)[a]	Use
argG-UpF	ATTGCGGCCGCGGTATCGAGCTGGACGCAGC	Null *argG* mutant construction
argG-UpR	**CCGGGGTTTTCGCACACGCCAGCAA**GCCATCACTCCACGGGGTTG	
argG-DwF	**CGTACAACCCCGTGGAGTGATGGC**TTGCTGGCGTGTGCGAAAACC	
argG-DwR	ATTGCGGCCGCTGGCGCGGCGATTTACCAG	
argH-UpF	ATTGCGGCCGCTCGGTCAGCAACTACTACA	Null *argH* mutant construction
argH-UpR	**CGCCACTTTTTCGTTCACGCCTGCA**TCCCCCAAGGCGAGGGCT	
argH-DwF	**AGGGCGAAGCCCTCGCCTTGGGGGA**TGCAGGCGTGAACGAAAAAG	
argH-DwR	ATTGCGGCCGCACATACTTGTGGTCGGCAA	
argG-XhoI-F	CTCGAGCTGAGATCTGGTCGGTGCAT	1,6-Kb XhoI/BamHI fragment for ectopic expression of *argG* with its own promoter
argG-BamHI-R	GGATCCCTATTAGCGAGACGCCAACAG	
argH-XhoI-F	CTCGAGCCTCATCGCCGTTGGTGG	1,8-Kb XhoI/BamHI fragment for ectopic expression of *argH* with its own promoter
argH-BamHI-R	GGATCCCTATTAGCGAGACGCCAACAG	

[a] Restriction sites inserted in the primer for the cloning strategy are underlined.
Base-complementary between upstream and downstream fragments of the gene to replace are shown in bold.

SPECIFIC MATERIALS AND METHODS – CHAPTER 2

Strains, plasmids and primers.

Strains, plasmids and oligonucleotides used in this Chapter are detailed in Tables 3 and 4, respectively.

Table 3. Bacterial strains and plasmids used.

Strain or plasmid	Genotype/Relevant characteristics[a]	Reference or source
Strains		
E. coli		
CC118λpir	Rif^R, λpir	Herrero *et al*., 1990
DH5α	*supE44 lac*U169 (Ø80*lacZ*ΔM15) *hsd*R17 (r_K-m_K-) *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1	Woodcock *et al*., 1989
HB101 (pRK600)	Helper strain harbouring Cm^R *mob tra* plasmid	V. de Lorenzo
P. putida		
KT2440	Wild type; derivative of *P. putida* mt-2, cured of pWWO	Regenhardt *et al*., 2002
Δ*argG*	Null mutant derivative of KT2440 in PP_1088	Chapter 1
Δ*argH*	Null mutant derivative of KT2440 in PP_0184	Chapter 1
Δ*argR*	Null mutant derivative of KT2440 in PP_4482	This work
Δ*PP_3593*	Null mutant derivative of KT2440 in PP_3593	This work
artJ	Km^R Rif^R, mutant derivative of *P. putida* KT2440R in PP_0282 obtained by random transposon mutagenesis with mini Tn5[Km1]	*Pseudomonas* Reference Culture Collection
argT	Km^R Rif^R, mutant derivative of *P. putida* KT2440R in PP_4486 obtained by random transposon mutagenesis with mini Tn5[Km1]	*Pseudomonas* Reference Culture Collection
C1R1	Lux^+; $RpoS^-$ derivative of *P. putida* KT2440	Ramos-González and Molin, 1998
R6C1	Sm^R, Suc^S, Lux^+; *P. putida* KT2440 cointegrate containing pMIR592	Ramos-González and Molin, 1998
Δ*rsmA*	Null mutant derivative of KT2440 in PP_4472	Huertas-Rosales *et al*., 2016
Δ*rsmE*	Null mutant derivative of KT2440 in PP_3832	Huertas-Rosales *et al*., 2016
Δ*rsmI*	Null mutant derivative of KT2440 in PP_1476	Huertas-Rosales *et al*., 2016
Δ*rsmEA*	Double null mutant derivative of KT2440 in PP_3832/PP_4472	Huertas-Rosales *et al*., 2016
Δ*rsmIA*	Double null mutant derivative of KT2440 in PP_1746/PP_4472	Huertas-Rosales *et al*., 2016
Δ*rsmIE*	Double null mutant derivative of KT2440 in PP_1746/PP_3832	Huertas-Rosales *et al*., 2016
ΔΔΔ*rsmIEA*	Triple null mutant derivative of KT2440 in PP_1746/PP_3832/PP_4472	Huertas-Rosales *et al*., 2016
cfcK-77	Km^R , fleQ mutant derivative of KT2440 obtained by random transposon mutagenesis with miniTn5[Km1]	Ramos-González *et al*., 2016
Δ*cfcR*	Null mutant derivative of KT2440 in PP_4959	Matilla *et al*., 2011
C1R1Δ*argR*	Double null mutant derivative of *P. putida* KT2440 in PP_1623/PP_4482	This work
Δ*cfcR*Δ*argR*	Double null mutant derivative of KT2440 in PP_4959/PP_4482	This work

Plasmids		
pCR2.1 TOPO	Km^R, PCR cloning vector with β-galactosidase α-complementation	Invitrogen
pBBR1-MCS5	Gm^R, broad host range cloning vector, mobilizable	Kovach *et al*., 1995
pBBR1-MCS2	Km^R, broad host range cloning vector, mobilizable	Kovach *et al*., 1995
pCdrA::*gfp*^{C}	Ap^R (Pip^R), Gm^R, FleQ dependent c-di-GMP biosensor	Rybtke *et al*., 2012
pKNG101	Sm^R, *oriR6K mobRK2 sacBR*	Kaniga *et al*., 1991
pMP220	Tc^R, *oriRK2'lacZ*	Spaink *et al*. 1987
pMP220-BamHI	Tc^R, pMP220 with a deletion of a 238-bp BamHI fragment containing ribosome binding site and ATG of *cat*	Matilla *et al*., 2011
pLBM9	pCR2.1TOPO derivative with 376 bp fragment BglII/PstI fragment containing *argG* promoter	This work
pLBM10	pCR2.1TOPO derivative with 395 bp fragment BglII/BamHI fragment containing *argG* promoter and first codons	This work
pLBM11	pCR2.1TOPO derivative with 367 bp fragment BglII/PstI fragment containing *argH* promoter	This work
pLBM12	pCR2.1TOPO derivative with 393 bp fragment BglII/BamHI fragment containing *argH* promoter and first codons	This work
pLBM13	Tc^R; transcriptional fusion *argG::´lacZ* in pMP220	This work
pLBM14	Tc^R; transcriptional fusion *argH::´lacZ* in pMP220	This work
pLBM15	Tc^R; translational fusion *argG´-`lacZ* in pMP220-BamHI	This work
pLBM16	Tc^R; translational fusion *argH´-`lacZ* in pMP220-BamHI	This work
pLBM17	Km^R, pCR2.1TOPO derivative with 362 bp BglII/EcoRI fragment containing the *argR* promoter	This work
pLBM18	Km^R, pCR2.1TOPO derivative with 229 bp BglII/PstI fragment containing the *argT* promoter	This work
pLBM20	Tc^R; transcriptional fusion *argR::´lacZ* in pMP220	This work
pLBM21	Tc^R; transcriptional fusion *argT::´lacZ* in pMP220	This work
pLBM22	Tc^R; translational fusion *argR´-`lacZ* in pMP220-BamHI	This work
pMAMV1	Gm^R, derivative plasmid of pBBR1-MCS5 containing *rup4959* of *P. putida* KT2440 and it is expressed from its own promoter; it confers high c-di-GMP levels in the wt strain	Matilla *et al*., 2011
pMIR178	Km^R, derivative plasmid of pBBR1-MCS2 containing *rup4959* of *P. putida* KT2440 and it is expressed from its own promoter; it confers high c-di-GMP levels in the WT strain	Ramos-González *et al*., 2016
pMIR125	Tc^R; transcriptional fusion *algD::´lacZ* containing RBS and first codons in pMP220	Molina-Henares *et al*., 2017
pMP220-bcs	Tc^R; transcriptional fusion PP_2629*::´lacZ* containing RBS and first codons in pMP220	Molina-Henares *et al*., 2017
pMP220-pea	Tc^R; transcriptional fusion PP_3132*::´lacZ* containing RBS and first codons in pMP220	Molina-Henares *et al*., 2017
pMP220-peb	Tc^R; transcriptional fusion PP_1795*::´lacZ* containing RBS and first codons in pMP220	Molina-Henares *et al*., 2017
pMMG1	Tc^R; transcriptional fusion *lapF::´lacZ* containing RBS and first codons in pMP220	Martínez-Gil *et al*., 2010
pMMGA	Tc^R; transcriptional fusion *lapA::´lacZ* containing RBS and first codons in pMP220	Martínez-Gil *et al*., 2014
pMIR219	Tc^R, *cfcR´-`lacZ* translational fusion in pMP220-BamHI	Huertas-Rosales *et al*., 2017a
pMIR200	Tc^R, *cfcR´::`lacZ* transcriptional fusion in pMP220	Huertas-Rosales *et al*., 2017a
pMAMV21	Tc^R, translational fusion *rpoS´-`lacZ* in pMP220-BamHI	Matilla *et al*., 2011

[a]Rif, rifampin; Cm, chloramphenicol; Km, Kanamycin; Tc, tetracycline; Sm, streptomycin; Pip, piperacillin; Ap, ampicillin; Gm, gentamicin.

Construction of transcriptional and translational fusions to lacZ.

To generate transcriptional fusions, promoter regions expanded 387 bp of *argG*, 357 pb of *argH*, 350 pb of *argR* and 217 of *argT* were generated by PCR amplifications. Primers used are listed in Table 4. Amplicons of *argG*, *argH*, *argR* and *argT* were cloned into pCR2.1 TOPO vector to generate pLBM9, pLBM10, pLBM17 and pLBM18 plasmids, respectively. Plasmids were transformed into *E. coli* DH5α and the absence of mutations was assessed by sequencing. Subsequently, pLBM9, pLBM10, and pLBM18 were double digest with BglII/PstI and pLBM17 with BglII/EcoRI. The resulting fragments were cloned in pMP220 (Spaink *et al*., 1987) to yield pLBM13, pLBM14, pLBM20 and pLBM21. New DNA sequencing step was carried out to confirm that fragments were correct in pMP220. Subsequently, plasmids were transferred to *P. putida* strains by triparental conjugation as previously described. RBS and ATG for *´lacZ* in pLBM13, pLBM14, pLBM20 and pLBM21 plasmids were those of *cat* gene in pMP220.

Translational fusions were generated by PCR amplification of a fragment covering the promoter regions plus initiation codon and the first codons of each gene (between 7 and 10 pb) designed to ensure in-frame cloning in pMP220-BamHI (Matilla *et al*., 2011). The primers used are listed in Table 4. PCR amplications of 401 bp for *argG* and 386 bp for *argH* were cloned into pCR2.1-TOPO vector and the resulting plasmids pLBM11 and pLBM12, respectively, were transformed into *E. coli* DH5α. Nucleotide sequencing was performed to confirm the absence of mutations. For the translational fusion to *´lacZ*, each fragment were digested using BglII and BamHI restriction sites incorporated by the PCR primers and cloned into the same sites of pMP220-BamHI to ensure the adequate orientation with respect to *´lacZ*. In the case of *argR*, product of 382 pb amplified by PCR was directly digested with BglII/EcoRI and cloned in pMP220-BamHI. The resulting plasmids pLBM15 (*argG´-`lacZ*), pLBM16 (*argH´-`lacZ*), and pLBM22 (*argR´-`lacZ*) were introduced into *E. coli* DH5α. New DNA sequencing step was carried out to confirm that fragments were correct in pMP220-BamHI. Subsequently, plasmids were transferred to *P. putida* strains by triparental conjugation as previously described.

Table 4. Primers used.

Primer name	Sequence (5´→3´)[a, b, c, d]	Use
argR-UpF	ATTGCGGCCGCGGTTCGGGCAAGTCGACCTT	Null *argR* mutant construction
argR-UpR	**ATACACCCCGAACCCACCCGAAATC**AGGGTACGGTCCGAAACCTG	
argR-DwF	**GACCTCAGGTTTCGGACCGTACCCT**GATTTCGGGTGGGTTCGGG	
argR-DwR	ATTGCGGCCGCACGCTGACGGTAAACAGGGT	
PP_3593-UpF	ATTGCGGCCGCCAAGACCTGCAGAACGATCTG	Null mutant construction in locus PP_3593
PP_3593-UpR	**CTGGGCGTTCGCACAACTTCCTGAAGA**CTCGCACCTCATTGCACTCT	
PP_3593-DwF	**TAGCAAGAGTGCAATGAGGTGCGAG**TCTTCAGGAAGTTGTGCGAACG	
PP_3593-DwR	ATTGCGGCCGCGAACATCGGTTCCTTGGTTACG	
RT-PCR argR-F	CCAGCGAATCGGTTTTCTCATC	Cotranscription experiment; amplified region between locus PP_4483 and *argR*
RT-PCR argR-R	CCTGCAGGAAGACCAACTCG	
RT-PCR argT-F	AGAAGCTCGCACTGCTTGG	Cotranscription experiment; amplified region between *argT* and locus PP_4485
RT-PCR argT-R	GGTTTTCATCTCTTCGCACAGC	
argG-BglII	CTGAGATCTGGTCGGTGCAT	*argG* transcriptional fusion
argG-PstI	CTGCAGCACTCCACGGGGTTGTACG	
argG-BglII	CTGAGATCTGGTCGGTGCAT	*argG* translational fusion
argG-BamHI	GGATCCTTTTTTACGTCCGCCATGCC	
argH-BglII	AGATCTCTCCAGTTCGCCGAGCAG	*argH* transcriptional fusion
argH-PstI	CTGCAGTGCAGGCGTGAACGAAAAAGTG	
argH-BglII	AGATCTCTCCAGTTCGCCGAGCAG	*argH* translational fusion
argH-BamHI	GGATCCGTCTTCTCGGTGCTCATGGA	
argR-BglII	AGATCTCTGTTCGACGAGCCGACCT	*argR* transcriptional fusion
argR-EcoRI	GAATTCAGGGTACGGTCCGAAACCTG	
argR-BglII	AGATCTCTGTTCGACGAGCCGACCT	*argR* translational fusion
argR-BamHI	GGATCCCAGATGAGAAAACCGATTCGC	
argT-BglII	AGATCTTGCACTGGCTGTCAACATC	*argT* transcriptional fusion
argT-PstI	CTGCAGCAGGTAACTCCATCGGTACG	
PT7-*argG*	*TTTTCTGCAGTAATACGACTCACTATAGG*GCCCCACTGTAAATTTATCC	fEMSA: *argG* promoter and first codons
argG-tail	AAAAAAAACCCCCCCCCGAAGTATCAAGGCCGCCGG	
PT7-*argGI*	*TTTTCTGCAGTAATACGACTCACTATAGG*CTCCATGGCGCGAGTGGGAC	fEMSA: *argG* inner zone
argGI-tail	AAAAAAAACCCCCCCCCGGACACCGCCCTCGTAGGAG	
PT7-*argR*	*TTTTCTGCAGTAATACGACTCACTATAGG*AAGCGCCCGTGCACGTGC	fEMSA: *argR* upstream region
argR-tail	AAAAAAAACCCCCCCCCGGCTCGTCGAACAGCAT	

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

[b] Base-complementary between upstream and downstream fragments of the gene to replace are shown in bold.

[c] Italics indicate T7 polymerase promoter

[d] Highlighted in grey indicate the sequence used to hybridize with labelled ATTO700

RNA purification.

Cells were grown in M9 minimal medium with different carbon sources (25mM of glucose and 25 mM of L-arginine) until OD_{660} of 0.3, prior to centrifugation, and were immediately freezing in liquid nitrogen. Pellets were stored at -80°C. Total RNA was extracted using TRI Reagent (Ambion, Austin, TX, USA) according to the manufacturer´s protocol, with the exception that the reactive was pre-heated at 65ºC before adding to the samples, which were incubated 10 min at 65°C immediately. Samples were subjected to treatment with RNase-free DNase I (Turbo RNA-free; Ambion) plus RNaseOUT (Invitrogen) followed by inactivation with Inactivation Reagent (Invitrogen). The RNA concentration was determined with a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples quality was evaluated through agarose gel electrophoresis and the absence of any residual DNA was checked by PCR.

Reverse transcription coupled to PCR (RT-PCR).

Cotranscription analyses were performed by using Titan One Tube RT-PCR System (Roche) following the manufacturer´s recommendations. For each reaction, 50 ng of total RNA was used. A positive and negative control was included. Thus, for positive control total DNA was used, and the negative control was a reaction with the same RNAs used but omitting the cDNA synthesis step. Primers were designed for the amplification of the 783 pb intergenic region between PP_4482 (*argR* gene) and PP_4483 (*hisP* gene) and the 761 pb intergenic region between PP_4485 (*hisQ* gene) and PP_4486 (*argT* gene). See primers used in Table 4.

RNA synthesis and fluorescence-based electrophoretic mobility shift assays (fEMSA).

RNA transcripts were generated by PCR amplification using primers that incorporates a T7 promoter at the 5´end and a 17 nucleotides tag at the 3´end (Table 4). PCR amplicons were purified using QIAquick PCR Purification Kit (QIAGEN) and then used for RNA probes synthesis using MAXIscript Kit (Applied Biosystems) according to the manufacturer´s instructions. After RNA purification using RNeasy MinElute Cleanup Kit (QIAGEN), RNA was hybridized with an ATTO700-labelled primer as described by Ying and co-workers (Ying *et al*.,

2007). This reaction was carried out using an excess of ATTO-labelled primer in proportion 1:20 with regard to the ARN. Then different concentrations of Rsm proteins purified as previously described by Huertas-Rosales and colleagues (Huertas-Rosales *et al*., 2017a) were incubated with 10 nM of RNA in 1x binding buffer (10 mM Tris-Cl pH 7.5, 10 mM of $MgCl_2$ and 100 mM KCl), 0.5 µg/µl of yeast tRNA (Life Technologies), 7.5% (vol/vol) glycerol, 0.2 units SUPERase In RNase Inhibitor (Life Technologies). Reactions were incubated for 30 min at 30°C and bromophenol blue was added at final concentration of 0.01 wt/vol. Samples were immediately subjected to electrophoresis on 7% polyacrylamide TBE gels (47 mM Tris, 45 mM boric acid, 1 mM EDTA, pH8.3) at 4°C. Images were obtained using a 9201 Odyssey Imaging System (LICOR Biosciences) with Image Studio V5.0 software.

SPECIFIC MATERIALS AND METHODS - CHAPTER 3

Strains, plasmids and primers.

Strains, plasmids and oligonucleotides used in this Chapter are detailed in Tables 5 and 6, respectively.

RNA purification and cDNA synthesis.

Cultures were grown in King´s B medium at 30° during 24 h, prior to centrifugation and were immediately freezing in liquid nitrogen. Pellets were stored at -80°C. Total RNA was extracted using TRI Reagent (Ambion, Austin, TX, USA) according to the manufacturer´s protocol, with the exception that the reactive was pre-heated at 65ºC before adding to the samples, which were incubated 10 min at 65°C immediately. Samples were subjected to treatment with RNase-free DNase I (Turbo RNA-free; Ambion) plus RNaseOUT (Invitrogen) followed by inactivation with Inactivation Reagent (Invitrogen). The RNA concentration was determined with a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples quality was evaluated through agarose gel electrophoresis and the absence of any residual DNA was checked by PCR. cDNA was generated by reverse transcription reactions of 500 ng of RNA using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamers as primers.

Quantitative real-time PCR (qRT-PCR).

Quantitative real-time PCR amplifications were performed to assess the gene expression using iCycler Iq (Bio-Rad, Hercules, CA, USA). Primers used are listed in Table 6. 16S rRNA was utilised as internal control for normalization, using primers previously described (Matilla *et al*., 2011). Each 12.5 µl reaction contained 1 µl of template cDNA and iQ^{TM} SYBR Green Supermix (Bio-Rad). Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, then 40-cycle amplification [95°C for 10 min, 61°C (for *algQ*, *pvdS* and *pvdA*), 58°C (for *pvdD*) or 56°C (for *pvdE*) for 30 s and 72°C for 20 s] and a final extension at 72°C for 1 min, with a single fluorescence per reading. Melting curve was obtained by gradually heating of PCR mixture from 55°C to 95°C at a rate of 0.5°C every 10 s

for 80 cycles, with continuous fluorescence scanning. Quantification was based on analysis of threshold cycle (Ct) value (Pfaffl, 2001). Analysis was made using three independent biological replicates with three technical replicates each.

Table 5. Bacterial strains and plasmids used.

Strain or plasmid	Genotype/Relevant characteristics[a]	Reference or source[b]
Strains		
E. coli		
CC118λpir	Rif^R, λpir	Herrero *et al*., 1990
DH5α	*supE44 lac*U169 (Ø80*lacZ*ΔM15) *hsd*R17 (r_K-m_K-) *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1	Woodcock *et al*., 1989
HB101 (pRK600)	Helper strain harbouring Cm^R *mob tra* plasmid	V. de Lorenzo
P. putida		
KT2440	Wild type; derivative of *P. putida* mt-2, cured of pWWO	Regenhardt *et al*., 2002
Δ*argG*	Null mutant derivative of KT2440 in PP_1088	Chapter 1
Δ*argH*	Null mutant derivative of KT2440 in PP_0184	Chapter 1
Δ*argF*	Null mutant derivative of KT2440 in PP_1079	This work
argA	Km^R, *argA*::mini-Tn5[Km1] transposon mutant derivative of *P. putida* KT2440R	PRCC[b]
argB	Km^R, *argB*::mini-Tn5[Km1] transposon mutant derivative of *P. putida* KT2440R	PRCC[b]
argD	Km^R, *argD*::mini-Tn5[Km1] transposon mutant derivative of *P. putida* KT2440R	PRCC[b]
argE	Mutant in *argE* using pChesi insertion	J. De la Torre, unpublished
argJ	Km^R, *argJ*::mini-Tn5[Km1] transposon mutant derivative of *P. putida* KT2440R	PRCC[b]
argR	Null mutant derivative of KT2440 in PP_4482	Chapter 2
pvdD	Km^R, Mutant derivative of KT2440R in PP_4219, previously annotated as *ppsD*	Matilla *et al*., 2007a
Plasmids		
pCR2.1 TOPO	Km^R, cloning vector with β-galactosidase α-complementation	Invitrogen
pKNG101	Sm^R, *oriR6K mobRK2 sacBR*	Kaniga *et al*., 1991
pLBM26	pCR2.1TOPO derivative with 1.4 Kb NotI fragment containing the *argF* null allele	This work
pLBM28	Sm^R, pKNG101 derivative for *argF* null allele replacement with the 1.4 Kb NotI fragment of pLBM26 cloned in pKNG101	This work
pME0184	Ap^R (Pip^R), derivative plasmid of pMMB67HE for the ectopic expression of *P. putida argH*	Ramos-González *et al*., 2016
pME1088	Ap^R (Pip^R), derivative plasmid of pMMB67HE for the ectopic expression of *P. putida argG*	Ramos-González *et al*., 2016

[a]Rif, rifampin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Pip, piperacillin; Ap, ampicillin

[b] PRCC, *Pseudomonas* Reference Culture Collection (http://artemisa.eez.csic.es/prcc/).

Table 6. Primers used.

Primer name	Sequence (5´→3´)[a]	Use
argF-UpF	ATTGCGGCCGCGTAATTGTGCACGCTCGGGT	Null *argF* mutant construction
argF-UpR	**TTGAGCAGTAGGGGTTGACTCAT**GAGTGACTACCTTATCTGCAAC	
argF-DwF	**CGTTGCAGATAAGGTAGTCACTC**ATGAGTCAACCCCTACTGCTC	
argF-DwR	ATTGCGGCCGCGATGAAACTCGCCACGAATG	
qRT*pvdS-F*	GCGGAACAACTATCCACAAG	qRT-PCR; *pvdS*
qRT*pvdS*-R	GAACGATGAGGTGATCTGCG	
qRT*pvdA-F*	GCACTGTTCATCGACAAGCAG	qRT-PCR; *pvdA*
qRT*pvdA-R*	GGTGCAGGTAGTTGACGAAG	
qRT*pvdE-F*	TGCTCAAACCCTTCTGGC	qRT-PCR; *pvdE*
qRT*pvdE-R*	AGCAGATCGAGCTGAACAG	
qRT*algQ-F*	GTTCACAAGCTGATCGACC	qRT-PCR; *algQ*
qRT*algQ-R*	GCTCGCAGACTTCGAAGT	
qRT*pvdD-F*	CTCGATTCCGTGGAGTCAC	qRT-PCR; *pvdD*
qRT*pvdD-R*	GTCCAGTTGCCAGAGAAAC	
qRT*16S-F*	AAAGCCTGATCCAGCCAT	qRT-PCR control 16S RNA
qRT*16S-R*	GAAATTCCACCACCCTCTACC	

[a] Restriction sites inserted in the primer for the cloning strategy are underlined.
Base-complementary between upstream and downstream fragments of the gene to replace are shown in bold.

Detection of siderophore production Chrome Azurol S (CAS) assay.

The CAS assay was used for siderophore detection, which is based on its high affinity for iron(III). CAS-agar plates were prepared according to Louden and colleagues (Louden *et al*., 2011). Plates has a blue colour due to chrome azurol S dye is complexed with iron(III). When bacteria were growing in this medium and produce siderophore, iron(III) is chelated by the siderophore and released from the dye, which is orange in colour. For that reason, if bacteria produce siderophore, an orange halo is produced in their surroundings. CAS assay was carried out as follows. Overnight LB cultures were diluted to an OD_{660} of 0.5 and 2µl-drops were put over CAS-agar plates. Plates were incubated at 30°C for 24 h, subsequently halo areas were measured and pictures were taken. Several compounds were added to the medium to test their influence upon siderophore production.

Pyoverdine detection in King´s B medium.

To visualise the fluorescence indicative of pyoverdine production, overnight cultures grown in liquid King's B medium (King *et al*., 1954) were diluted to and OD_{660} of 0.05 at starting the experiment. Arginine was added to the culture medium to test the influence by pyoverdine production. Cultures were incubated at 30°C for 24 h and the OD_{660} were adjusted to 1 to compare fluorescence under UV light.

2,2´-bipyridyl assay.

To determine the tolerance of *argG* and *argH* mutants to iron chelator 2,2´-bypiridyl, which has preference for iron(II), we proceed as follows. Overnight LB cultures were diluted to an OD_{660} of 0.05. 2 ml of these cultures were distributed into borosilicate glass tubes and different concentrations of the iron chelator were added to the culture medium. After 24 h of growth, OD_{660} was measured.

Intracellular and extracellular pyoverdine fractions.

Intracellular and extracellular pyoverdine determinations were made as described by Imperi and colleagues with modifications (Imperi *et al*., 2009). Cultures were grown in King´s B medium during 24 h. Three aliquots of each culture were taken to obtain extracellular pyoverdine, after centrifugation (9000 r.p.m., 5 min, 4°C) to remove bacterial contents. Other three aliquots were used to obtain intracellular pyoverdine as follows. Cultures were washed three times with 30 mM Tris-HCl pH 7, 150 mM NaCl, centrifuging between washing as previously indicated. Bacterial pellets were resuspended in 1,2 ml of 10 ml Tris-HCl pH8, 100 mM NaCl, and lysed by sonication. Cell debris were removed by centrifugation (9000 r.p.m., 5 min, 4°C).

Pyoverdine quantification.

Pyoverdine quantification was made using extracellular and intracellular supernatants obtained previously. When necessary, supernatants were diluted in 100 mM Tris-HCl pH8. Pyoverdine-based fluorescence was quantified using a Varioskan Lux microplate reader by recording emission at 455 nm upon excitation at 398 nm. Fluorescence readings were corrected for growth (OD_{600nm}).

Evaluation of hydrogen peroxide effects upon culture growth.

Overnight King´s B cultures were diluted in fresh King´s B medium to an initial OD_{660nm} of 0.05. Different concentrations of hydrogen peroxide ranging between 0-0.3% (w/v) were added to the cultures, which were incubated at 30° under 200 r.p.m. during 24 h, to evaluate their resistance to oxidative stress caused by the chemical. Then, optical density was measured at 660 nm.

Evaluation of polyamines effects upon pyoverdine production and oxidative stress.

Polyamines were used to evaluate their potential protective role upon oxidative stress caused by the presence of hydrogen peroxide in the culture medium, as well as their possible role on pyoverdine production. We proceed as indicated in the previous section. Agmatine, putrescine and spermidine were added to the cultures at final concentration of 5 mM. On the other hand, hydrogen peroxide was added along with polyamines.

Surface sterilization and germination of seeds.

Maize seeds were surfaced sterilised using ethanol 70% and sodium hypochlorite 20%. After several distilled-water washes steps, seeds were germinated on Musharige and Skoog MS-phytagel (0.2%) medium supplemented with glucose (0.5%) to detect microbial contamination (Matilla *et al*., 2007b). Maize seeds were incubated for 48 h at 30°C and subsequently used in plant root colonization assays.

Plant root colonization assays.

For competitive colonization assays, overnight cultures grown in LB medium were diluted to an OD_{660} of 1 and both cultures which are to be used in competitive colonization were mixed in proportion 1:1 and drop-platting was done to check this proportion by colony forming units (C.F.U) enumeration. 48 h seedlings were incubated with bacterial mix during 30 min at 30°C under static conditions and subsequently planted in 50 ml Sterilin tubes filled with 40 g of sterilised silica sand and 10 ml of rich PNS medium. Some incubated-seedlings were used to check C.F.U. enumeration at start of the experiment. The inoculated seedlings were maintained in a controlled chamber at 24°C during the day and 18°C during the night

and between 55 and 65% of relative humidity with a daily light period of 16 h. After 7 days, bacterial cells were recovered from the rhizosphere. Root tips were placed into 2 ml Eppendorf tubes filled with glass beads and 1X M9 salts. Rest of the roots were placed in 50 ml Sterilin tubes containing glass beads. In both cases, tubes were vortexed to resuspend bacterial cells attached to roots and C.F.U. were determined by drop-plating on LB-agar plates supplied with the appropriate antibiotics.

SPECIFIC MATERIALS AND METHODS - CHAPTER 4

Bacterial strains, culture media and growth conditions.

Different strains of *Pseudomonas putida* were used in this chapter: mt-2, which harbours the pWW0 plasmid containing genes for aerobic catabolism of xylenes and toluene; KT2440, a plasmid-free derivative of *P. putida* mt-2 (Nakazawa, 2002; Nelson *et al*., 2002), and DOT-T1E, a model organism in studies of biodegradation and tolerance to toxic organic compounds, due to its elevated resistance to these chemicals. *Pseudomonas* strains were routinely grown at 30°C in Luria-Bertani (LB) medium (Lennox, 1955).

Biofilm assays.

Biofilm formation assays were performed in flasks or borosilicate glass tubes. Overnight LB cultures were adjusted at optical density of 0.05. 2 ml or 10 ml of adjusted cultures were distributed into the tubes or flasks, respectively. Tubes were incubated in a tube rotor under orbital shaking at 40 r.p.m. at 30°C and flasks were incubated in a shaker at 150 r.p.m at 30°C. At indicated times, liquid cultures were removed and non-adherent cells were washed away using distilled water. Biomass attached to the surface was stained with crystal violet for 15 min and quantified after dye solubilization with glacial acetic acid (30% v/v) (O'Toole and Kolter, 1998). The crystal violet solution was measured at an absorbance of 590 nm. Pictures were taken before and after staining.

To test the impact of toluene upon biofilms, different approaches were carried out:

1. Different concentration of chemical ranging between 0-0.2% (w/v) was added to not preexposed overnight cultures and biofilm formation was analysed at different times.
2. Overnight cultures were preexposed to toluene. Biofilms were performed during 4 h, and toluene ranging between 0-0.2% (w/v) was added into the culture medium at this point. After 1 and 2 hours, biofilms were analysed.
3. To test the effect of toluene in gas phase, toluene-stuffed glass rods were introduced in the culture medium and biofilm were analysed at indicated times.

IV. RESULTS AND DISCUSSION

CHAPTER I

Connecting amino acid metabolism with c-di-GMP levels and associated phenotypes in *Pseudomonas putida* KT2440

Parts of this chapter have been adapted from:

Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Genetic dissection of the regulatory network associated with high c-di-GMP levels in *Pseudomonas putida* KT2440. Front. Microbiol. 7:1093.

BACKGROUND

In the last decade, cyclic diguanylate (c-di-GMP) has emerged as a broadly conserved bacterial intracellular second messenger, both in Gram-negative and Gram-positive bacteria (Römling *et al*., 2013). It is involved in a wide range of bacterial processes such as cell differentiation, virulence or antibiotic production, among others (Paul *et al*., 2004; Hull *et al*., 2012; D'Alvise *et al*., 2014). However, its most important role is related to the transition between motile and sessile lifestyles in bacteria and the regulation of biofilm formation (Simm *et al*., 2004). In general terms, elevated intracellular levels of c-di-GMP have been associated with inhibition of motility and activation of biofilm formation and *vice versa* (Boyd and O´Toole, 2012).

C-di-GMP turnover is due to the activity of two antagonistic enzyme families. It is synthesised by diguanylate cyclase activity (DGC), which is associated to the presence of a GGDEF domain in the protein, and hydrolysed by phosphodiesterase activity (PDE), linked to two alternative domains, EAL and HD-GYP (Jenal *et al*., 2017). DGCs and PDEs are found in members of all major bacterial phyla, representing important families of signalling proteins in bacteria (Römling *et al*., 2013). Bifunctional enzymes with DGC and PDE activities have also been described in many bacteria. Usually, these proteins contain one of the two domains active, being the other one enzymatically inactive.

In *Pseudomonas putida* KT2440, the gene *cfcR* (previously named *rup4959*), encodes a multidomain response regulator with a REC-PAS-GGDEF-EAL architecture, similar to FimX of *P. aeruginosa* (Huang *et al.*, 2003). Unlike FimX, which exhibits PDE activity (Kazmierczak *et al.*, 2006), CfcR has been shown to present only DGC activity under all the conditions tested (Matilla *et al.*, 2011). This gene was identified by transcriptomic analysis as being preferentially expressed in *P. putida* populations colonizing the corn rhizosphere (Matilla *et al*., 2007b). In laboratory conditions, *cfcR* shows its maximum activity in the stationary phase of growth, its transcription being dependent on the alternative sigma factor RpoS (σ^S). Overexpression of this gene from its own promoter in plasmids pMAMV1 or pMIR178 —only differing in the antibiotic resistance— results in high levels of c-di-GMP and causes a pleiotropic phenotype that includes pellicle formation in the air-liquid interface, crinkly colony morphology, flocculation of liquid cultures and increased biofilm formation (Matilla

et al., 2011; Ramos-Gónzalez *et al*., 2016).

Taking advantage of the crinkly colony phenotype, which is clearly evident after 48 h of growth in LB, a broad screen was carried out to identify elements modulating CfcR expression and/or activity, as well as those involved in the transduction of the c-di-GMP signal to downstream processes. For that purpose, mini-Tn5[Km1] or mini-Tn5[Tc] were used for random mutagenesis of KT2440. The resulting clones were pooled and masse mating experiments were used to transfer pMAMV1 to the mutants. About 15,000 exconjugant clones were then streaked on LB-agar plates and analysed for colony morphology (Figure 1).

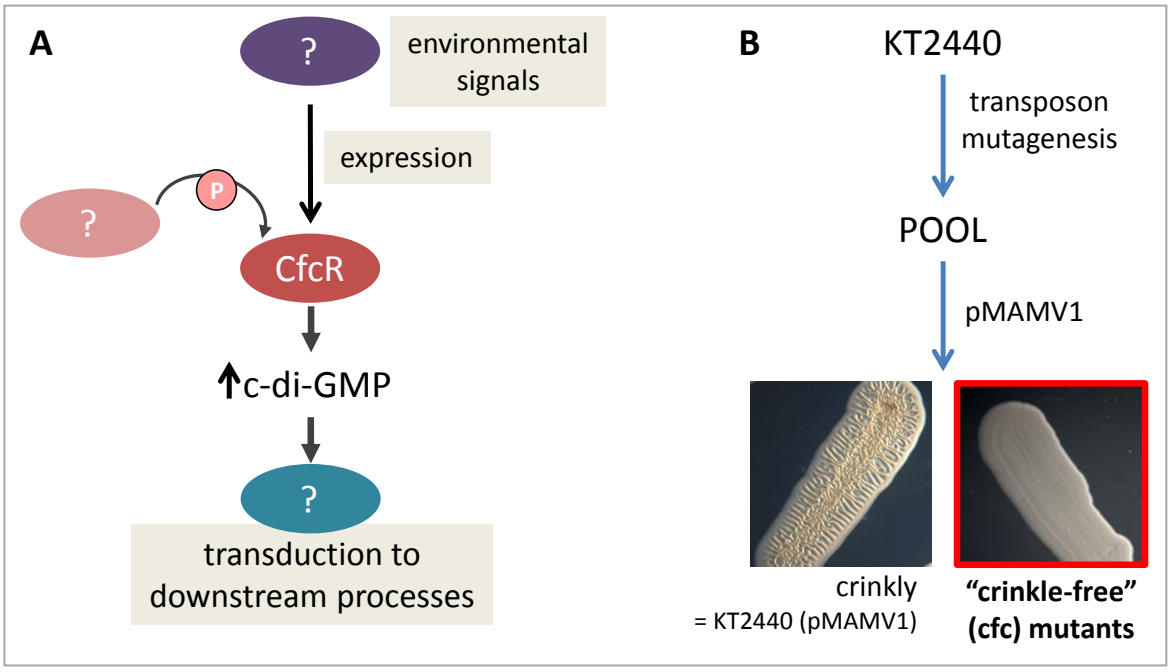


Figure 1. Conceptual (A) and experimental (B) design of the genetic screen to identify functions related to c-di-GMP regulation via *cfcR* in *P. putida* KT2440. The schemes have been adapted from the description published in: Ramos-González MI, Travieso ML, Soriano MI, Matilla M, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093.

Approximately eighty clones were initially isolated as having lost the crinkly colony morphology after 48h in LB, despite carrying *cfcR* in multicopy. These mutants were termed cfcK or cfcT (for crinkle-free colony / Km^R or Tc^R). Selected mutants were cured of plasmid pMAMV1 and the same plasmid was newly transferred into them by conjugation in order to confirm their cfc phenotype, as well as flocculation and biofilm formation (Figure 2). In all cases the presence of the intact pMAMV1 plasmid was confirmed, ensuring that the loss of

the phenotype was not due to plasmid deletions or rearrangements.

Figure 2. Streak morphology, biofilm formation and flocculation phenotypes of cfc derivatives *of P. putida* KT2440 harbouring *cfcR* in multicopy in plasmid pMAMV1. Mutants studied in detail in this chapter are highlighted with red squares. Mutant in the histidine kinase CfcA is indicated with a blue square (adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093).

This screen allowed identifying a mutant (cfcK-54), disrupted in PP_3761, which encodes a hybrid sensor histidine kinase. This protein, renamed as CfcA, is required for phosphorilation of CfcR at the Asp65 residue in its REC domain, essential for the DGC activity of CfcR (Ramos-González *et al*., 2016). Accordingly, mutant cfcK-54 does not display any of the phenotypes associated to overexpression of *cfcR*.

The results described in this Chapter have as starting point two other mutants, cfcK-66 and cfcK-74. These mutants were affected in the *argG* and *argH* genes, respectively, encoding the enzymes that carry out the two last steps in the arginine biosynthesis pathway (see below). Both showed loss of crinkly phenotype despite *cfcR* being overexpressed. However, while cfcK-74 retained increased biofilm formation and flocculation, two subpopulations were obtained for cfcK-66 when it was newly transformed with pMAMV1, one maintaining flocculation and increased biofilm (cfcK-66F subpopulation) and the other losing all the phenotypes (cfcK-66 subpopulation) (Figure 2). Analysis of the c-di-GMP contents in these mutants by means of a fluorescence-based bioreporter showed that cfcK-74 and the two variants of cfcK-66 had reduced levels of the second messenger even in the presence of *cfcR* in multicopy (Figure 3 and Supplementary Figure 1).

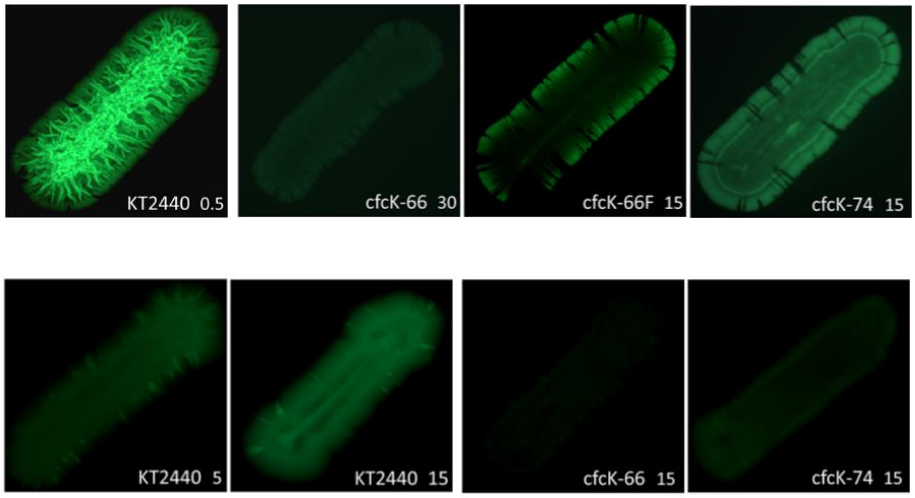


Figure 3. Fluorescence-based analysis of c-di-GMP levels in *P. putida* KT2440 and *cfc* derivatives harbouring (top) or not (bottom) pMAMV1. All strains carry the bioreporter pCdrA::*gfp*^{C} (Rybtke *et al*., 2012), which contains a fusion of the c-di-GMP-responsive *cdrA* promoter to *gfp*, so that fluorescence intensity correlates with levels of the second messenger. Images correspond to streaks of each strain after 48 h of growth on LB-agar plates, and were taken using a Leica stereomicroscope, as detailed in Materials and Methods. Numbers next to the names indicate the exposure time in seconds (adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093).

Some amino acids have been recently shown to influence biofilm formation in *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* (Hamilton *et al*.,

2009; Bernier *et al*., 2011). Although these and other factors, such as light or nitric oxide, have been related to c-di-GMP signalling (Tarutina *et al*., 2006; Williams *et al*., 2017), there is still little information about the environmental signal(s) that initiate the regulatory pathway(s) modulating c-di-GMP levels in different bacteria. The results presented above indicated that besides outside stimuli, there exists a connection between certain metabolic routes and c-di-GMP turnover. Based on the characteristics shown by cfcK-66 and cfcK-74 mutants, in this Chapter we have explored the roles of arginine and other amino acids upon c-di-GMP levels, associated phenotypes and biofilm formation in *P. putida* KT2440.

RESULTS

Characterization of mutants hampered in the arginine biosynthetic pathway.

As mentioned above, mutants cfcK-66 and cfcK-74 showed the transposon insertion in genes *argG* and *argH*, respectively, required for the last two steps of the arginine biosynthesis pathway (Figure 4).

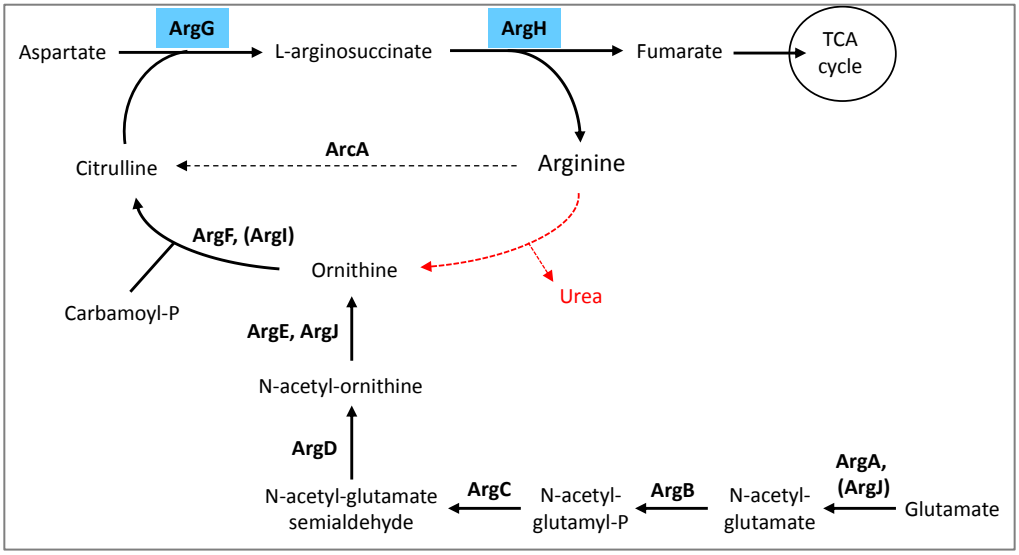


Figure 4. Arginine biosynthesis in *P. putida* KT2440, based on the annotation in the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/). The genes identified in the *cfc* screen are highlighted. Conversion of arginine to citrulline (broken black line) has been described in *P. aeruginosa* under anaerobic conditions (Gamper *et al*., 1991). The step shown by the broken red line would be absent in KT2440 (adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093).

To confirm the results obtained with the transposon mutants, we generated null mutants in the *argG* and *argH* genes by complete deletion of the ORF without introducing any antibiotic resistance cassettes, as indicated in Materials and Methods. Growth of these mutants was assessed in different culture media, both rich and defined minimal media. The two mutants were auxotrophs for arginine, being unable to grow in minimal medium with glucose as the sole carbon source (Figure 5A), as was the case of cfcK mutants (Figure 5B). Auxotrophy was overcome by supplementing M9-glucose with 1 mM of L-arginine, both in cfcK and null mutants (Figure 5).

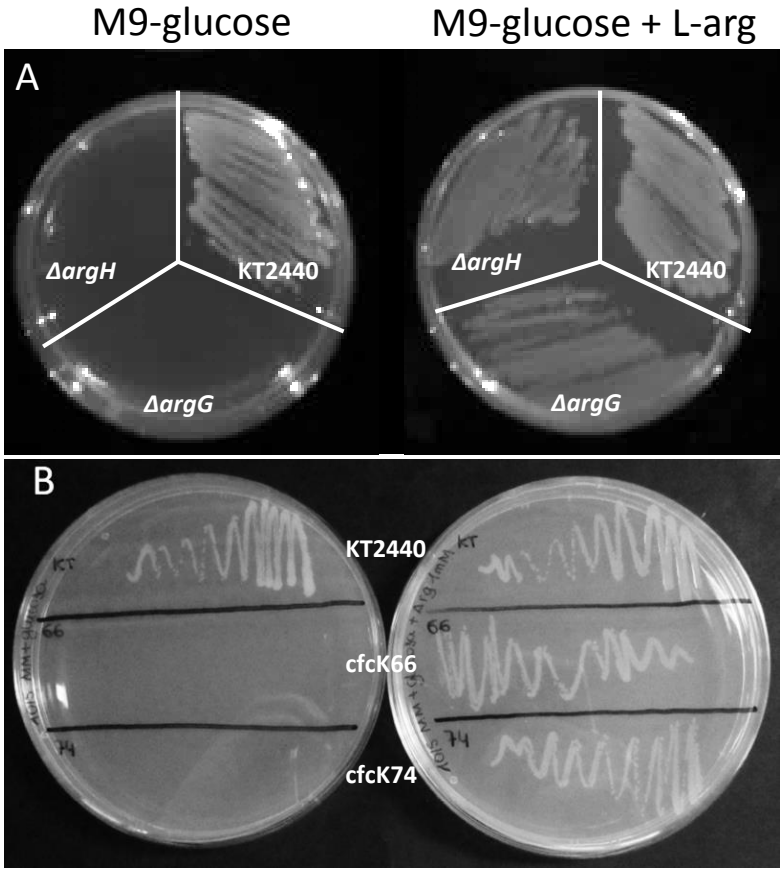


Figure 5. Growth of KT2440 and the *argG* and *argH* mutants on minimal medium with glucose, supplemented or not with 1 mM arginine. Plates were incubated at 30°C during 24 h. (**A:** null mutants; **B:** transposon mutants; panel adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M., 2016. Front. Microbiol. 7:1093).

Growth curves showed no differences between the wild type and the mutants in liquid LB, whereas in minimal medium with glucose and supplemented with 0.5% casamino acids (CAA), the mutants grew more slowly than the WT, suggesting there is a limited supply of arginine in CAA. Addition of L-arginine (5 mM) allowed normal growth of the mutants in this medium (Figure 6).

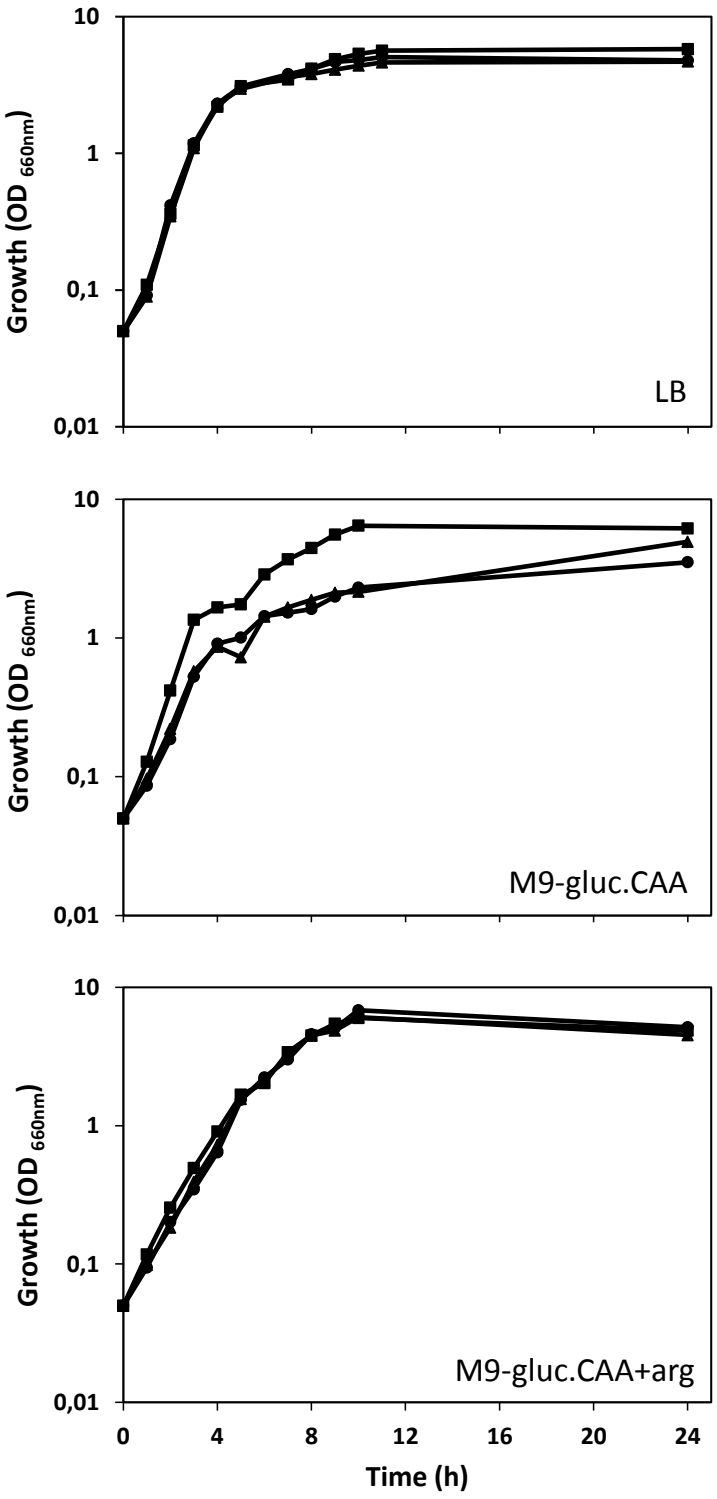


Figure 6. Growth of *P. putida* KT2440 (squares) and the Δ*argG* (triangles) and Δ*argH* (circles) strains in LB (top), M9 medium with glucose supplemented with 0.5% CAA (middle) or 0.5% of CAA and 5 mM L-arginine (bottom). Cultures were grown overnight and inoculated in each medium at an initial OD_{660} of 0.05. Turbidity was measured every hour during growth at 30°C and 200 r.p.m. Experiments were made in triplicate with two technical measures each time. One representative experiment is shown.

When plasmid pMIR178 was introduced in the *argG* and *argH* null mutants, both showed the same lack of crinkly colony phenotype as the cfcK mutants, and retained increased biofilm and flocculation (Figure 7). However, the two subpopulations identified in cfcK-66 (flocculating and non-flocculating), were not observed in the Δ*argG* strain, suggesting additional alterations may be present in cfcK-66.

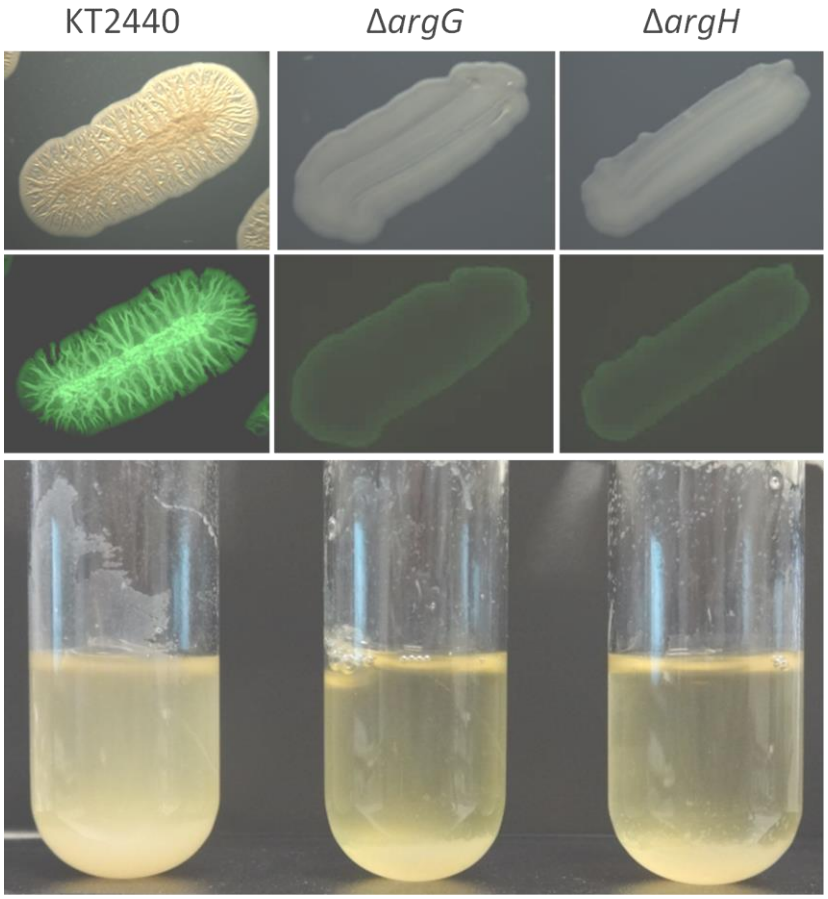


Figure 7. Streak morphology, fluorescence-based c-di-GMP detection (exposure time of 1.3 s) and flocculation phenotypes of *P. putida* KT2440 and the null mutants in *argG* and *argH* harbouring *cfcR* in multicopy in plasmid pMIR178.

As mentioned in the Introduction, analysis of the cfck-66 and cfck-74 mutants with the pCdrA::*gfp*^{C} bioreporter revealed reduced intracellular levels of c-di-GMP in both mutants. Consistently, the null mutants also showed this reduction in c-di-GMP, observed qualitatively in solid medium even in the presence of *cfcR* in multicopy (Figure 7). Quantitative analysis during growth of liquid cultures in microtiter plates revealed that the physiological levels of

second messenger (i.e., without overexpressing *cfcR*) were also clearly reduced in the null mutants compared to the wild type (Figure 8), the differences being more evident in late stationary phase, which is when *cfcR* shows its maximal expresion (Matilla *et al*., 2011). Interestingly, the Δ*argG* mutant was more severely affected than the Δ*argH* strain.

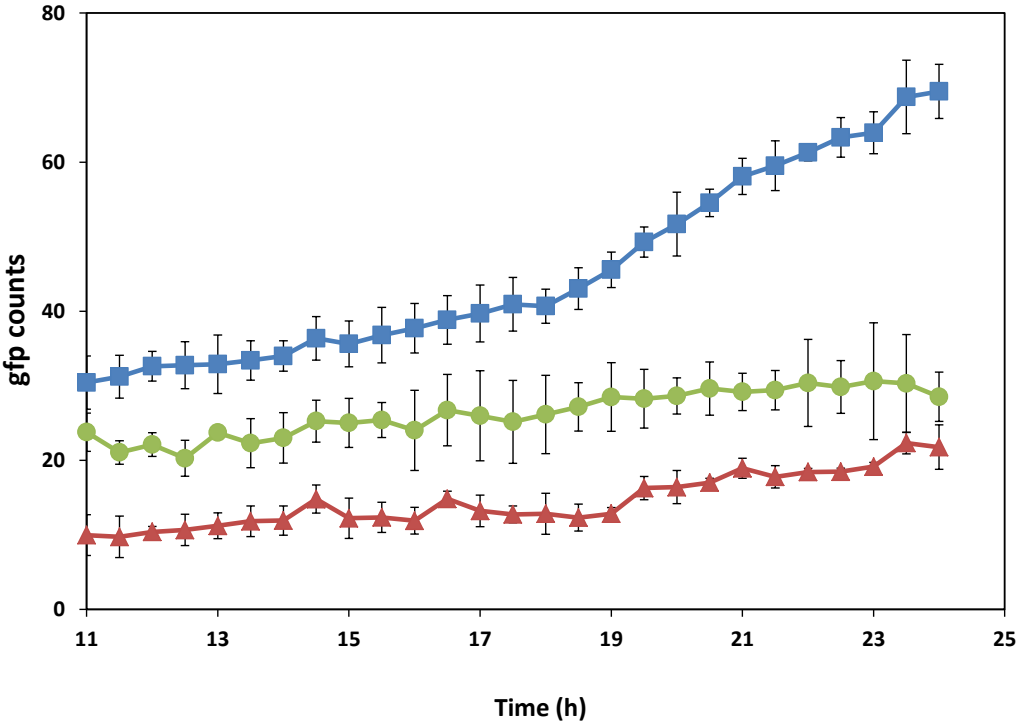


Figure 8. Fluorescence-based analysis of c-di-GMP contents in *P. putida* KT2440 (blue), Δ*argG* (red) and Δ*argH* (green) strains harbouring pCdrA::*gfp*^{C} during growth in LB 1/3, using a Synergy Neo2 Biotek fluorimeter. GFP counts indicate the fluorescence values corrected by culture growth (OD_{600}). Averages and standard deviation of two biological replicates, with three experimental replicates, are plotted.

Genetic complementation was also analysed. To this end, plasmids pME1088 and pME0184, which harbour the *argG* and *argH* genes, respectively, were constructed and introduced into the corresponding mutants. The presence of these plasmids restored growth of the null mutants in the absence of arginine (data not shown). Remarkably, an intact *argG* allele supplied via pME1088 only complemented the arginine auxotrophy of cfcK-66 when this strain harboured pMAMV1, even though to our knowledge there is no previous evidence that arginine biosynthesis is regulated by c-di-GMP. The presence of pMAMV1 was not a requirement in order to complement the auxotrophy of cfcK-74 with an intact *argH* allele provided via pME0184. In both cases, recovery of the crinkly colony morphology was

observed in the presence of the complementing plasmid, although again two subpopulations arose in the case of cfcK-66, one completely recovering the phenotype and the other remaining mostly smooth after 48h of growth (Figure 9).

Figure 9. Morphology of cfcK mutants harbouring pMAMV1, complemented with the corresponding wild type alleles. Pictures were taken after 48h of incubation. About 20% cfcK-66 (pMAMV1) colonies carrying pME1088 (P_{lac}::*argG*) exhibited the phenotype marked as **1** and the rest were similar to that marked as **2** (adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093).

These results further suggested that additional alterations might be present in the cfcK-66 mutant. However, the constructs in plasmids pME1088 and pME0184 harbour the *argG* and *argH* open reading frames under the control of a heterologous promoter (P_{lac}), so we could not exclude that non-native expression patterns of the genes might also influence the observed phenotypes. Therefore, null mutants were also complemented with the wild type alleles under control of their own promoters using plasmids pLBM7 and pLBM8, as described in Materials and Methods. These plasmids complemented the auxotrophy and also the crinkly colony morphology of the null mutants in the presence of pMIR178, although this phenotype was not as marked in the complemented mutants as in the wild type strain (Figure 10).

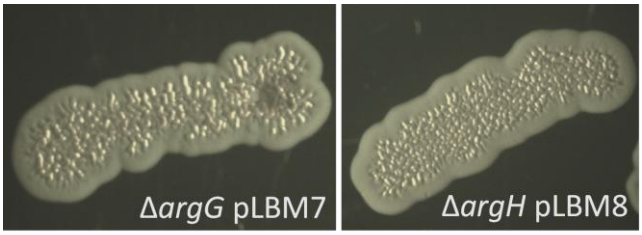


Figure 10. Complementation of the colony morphology of *argG* and *argH* mutants harbouring pMIR178 (*cfcR* in multicopy) by their respective wild type alleles with their native promoters.

L-arginine specifically restores the crinkly phenotype in the mutants and is required for its appearance in *P. putida* KT2440.

As indicated in Figure 4, the arginine biosynthesis pathway renders fumarate as a second end-product. Also, although the cycle that converts arginine into ornithine, releasing urea, would not be present in *P. putida*, arginine can potentially be metabolized back to citrulline via ArcA [which in *P. aeruginosa* has been shown to function under microaerobiosis, being completely active under anaerobiosis (Mercenier *et al*., 1980; Lüthi *et al*., 1990)]. We therefore decided to test if arginine and/or any other related compound in the pathway could restore the crinkly colony morphology of the mutants. As shown in Figure 11, addition of arginine reverted the loss of the crinkly colony morphology of the mutants, although concentrations ≥10 mM were required for this complementation.

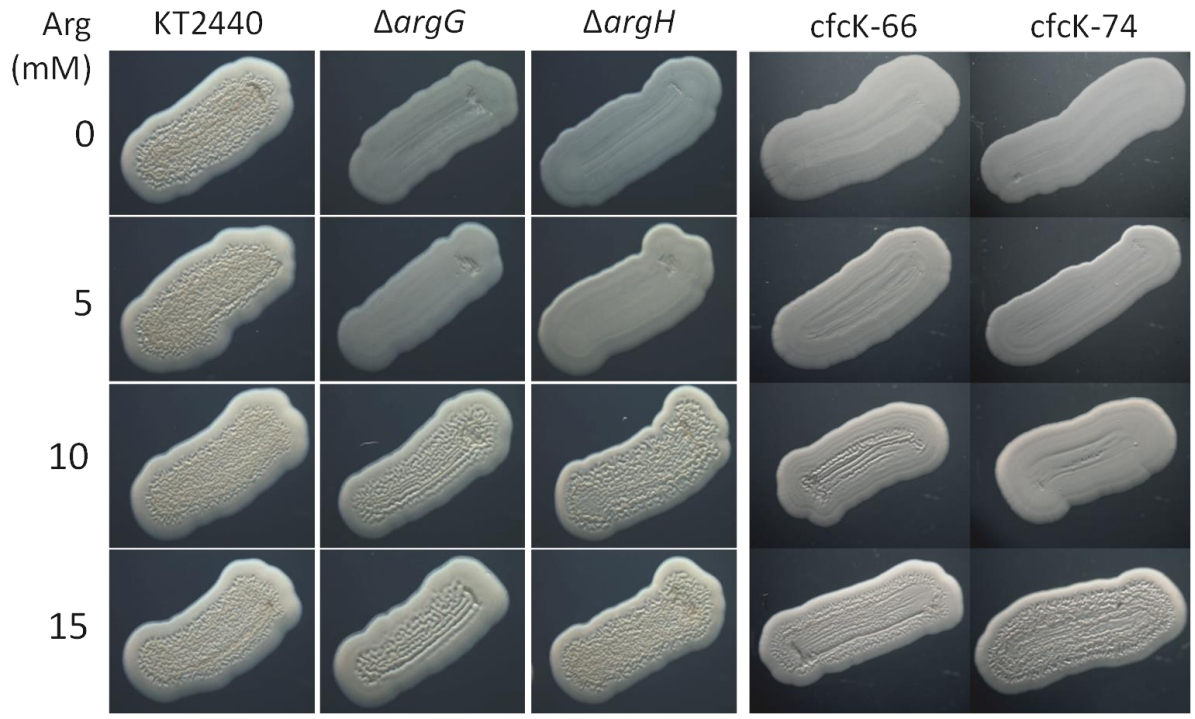


Figure 11. Recovery of the crinkly phenotype resulting from *cfcR* overexpression in pMAMV1 in mutants hampered in the arginine biosynthetic pathway by increasing concentrations of arginine exogenously added to LB (partially adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093).

Similar assays were done with fumarate and with different intermediaries of the pathway (ornithine, citrulline, L-aspartate, and L-arginosuccinate), as well as with products derived from further metabolism of L-arginine (agmatine, putrescine and GABA; guanidine hydrochloride was also tested as an analog of other intermediaries). These molecules were added to LB medium and crinkly phenotype was observed in bacterial streaks or drops of KT2440 and the null mutants. With the exception of arginine, none of the molecules tested restored the crinkly phenotype in LB medium (Figure 12). This was surprising in the case of the Δ*argG* strain in the presence of arginosuccinate, which would be predicted to restore the phenotype. However, it should be noted that neither this compound nor citrulline seem to be efficiently taken up by the bacterium, since they do not support its growth (data not shown), and therefore would not be available for ArgH to convert it to arginine.

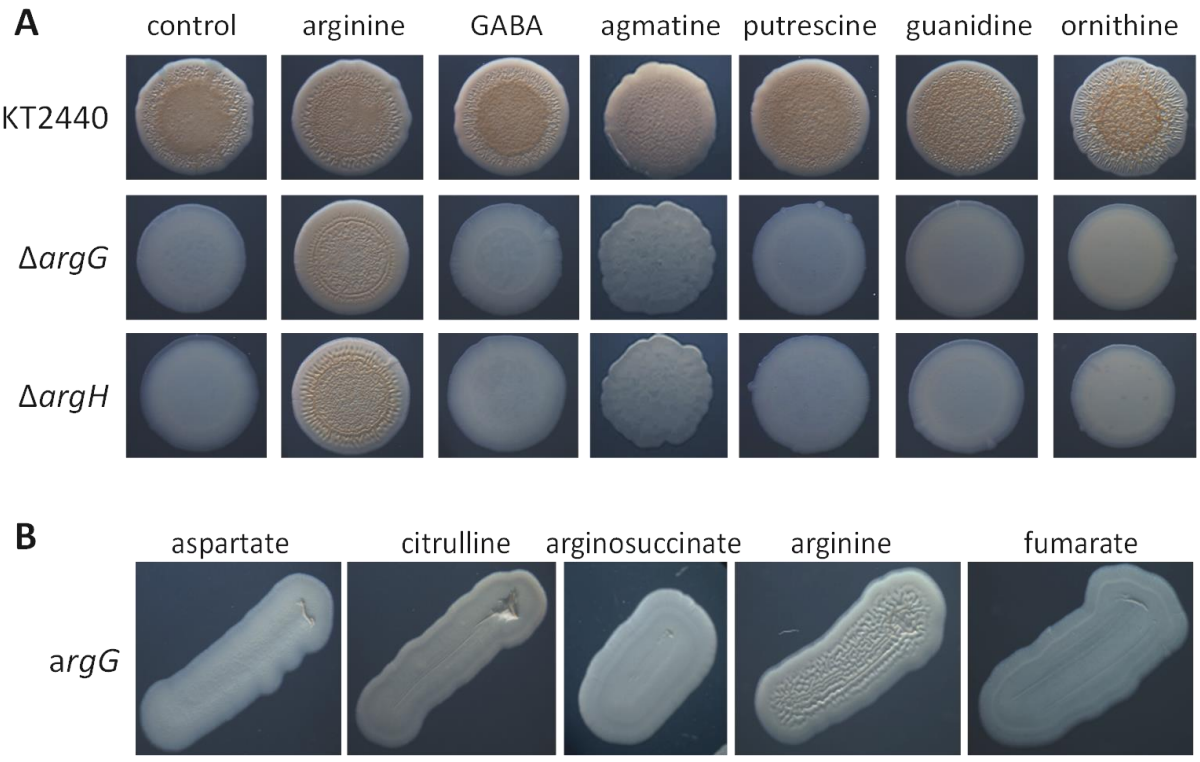


Figure 12. A. Analysis of the crinkly phenotype in KT2440 and *argG* and *argH* mutants harbouring pMIR178 in the presence of different compounds related to arginine metabolism. **B.** Analysis of the *argG* strain harbouring pMIR178 in the presence of intermediates and products of the arginine biosynthesis pathway. Images were taken after 48 h of growth on LB plates with 10 mM of each compound.

These results indicated that L-arginine is specifically associated to the appearance of the crinkly phenotype. To further analyse its role, we tested the effect of L-arginine in KT2440 harbouring *cfcR* in multicopy during growth in minimal medium with glucose, to avoid possible interferences with other molecules present in rich medium. Other amino acids were also tested: L-aspartate (negatively charged), L-lysine (positively charged) and L-alanine (without charge). Results presented in Figure 13 show that in minimal medium high concentration of L-arginine is essential to develop crinkly morphology after 48h of growth, the phenotype starting to be evident at with 20 mM of the amino acid, and completely developed at 25 mM. None of the other amino acids promoted the phenotype after 48 h of growth at any concentration tested (data not shown).

In the course of all these assays, we noticed that increasing concentrations of L-aspartate provoked a reduction in the crinkly phenotype of the wild type in LB medium, indicating that this amino acid exerts the opposite effect with respect to arginine (Figure 13).

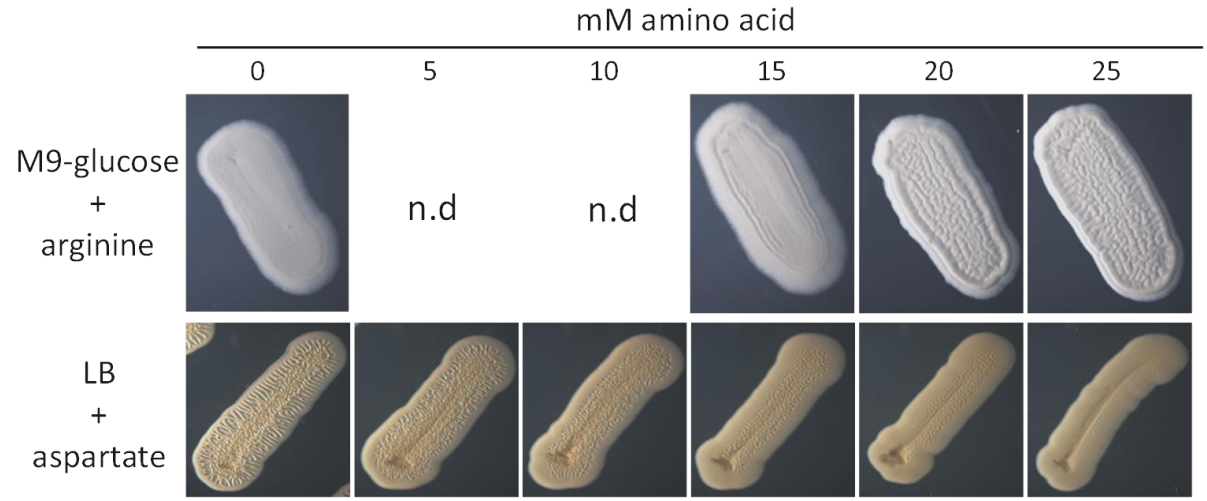


Figure 13. Arginine is required for the crinkly phenotype of KT2440 (pMIR178), whereas aspartate inhibits its appearance. Images were taken after 48 h of growth in minimal or rich medium, respectively, in the presence of increasing concentrations of the amino acids (n.d.: not determined).

Inverse modulation of free c-di-GMP bacterial content by arginine and aspartic acid.

Given all the results presented above, it seemed logical to test if exogenous arginine and/or aspartic acid influenced c-di-GMP levels in *P. putida* KT2440. As shown in Figure 14, increasing concentrations of arginine in the growth medium resulted in a rise in the intracellular c-di-GMP contents, whereas addition of aspartic acid had the opposite effect.

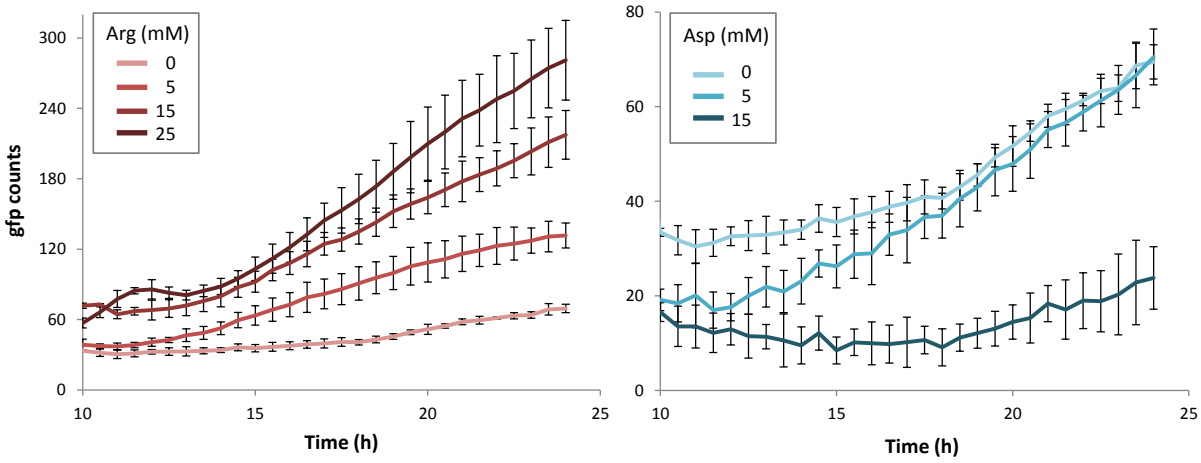


Figure 14. Inverse modulation of c-di-GMP cell content by arginine and aspartic acid. GFP counts indicate the fluorescence values corrected by culture growth (OD_{600}) of KT2440 harbouring pCdrA::*gfp*^{C} in LB 1/3. Left: Effect of arginine; right: Effect of aspartic acid. In both panels, average of two biological replicates, with three experimental replicates, and standard deviation are plotted. To measurements Synergy Neo2 Biotek fluorimeter was used (adapted from Ramos-González MI, Travieso ML, Soriano MI, Matilla MA, Huertas-Rosales Ó, Barrientos-Moreno L, Tagua VG, Espinosa-Urgel M. 2016. Front. Microbiol. 7:1093).

Since we had established that arginine restored the c-di-GMP associated phenotype in the arginine biosynthesis mutants, we also analysed the influence of both amino acids in the intracellular contents of the second messenger in the Δ*argG* and Δ*argH* strains harbouring the pCdrA::*gfp*^{C}. Results are presented in Figure 15 and confirm that in both cases increasing concentrations of arginine also increase c-di-GMP, an effect that is more evident in stationary phase of growth. On the contrary, aspartic acid causes a reduction in the levels of second messenger, this being more obvious in the Δ*argH* strain (which also has overall higher fluorescence levels in the control situation than the Δ*argG* mutant).

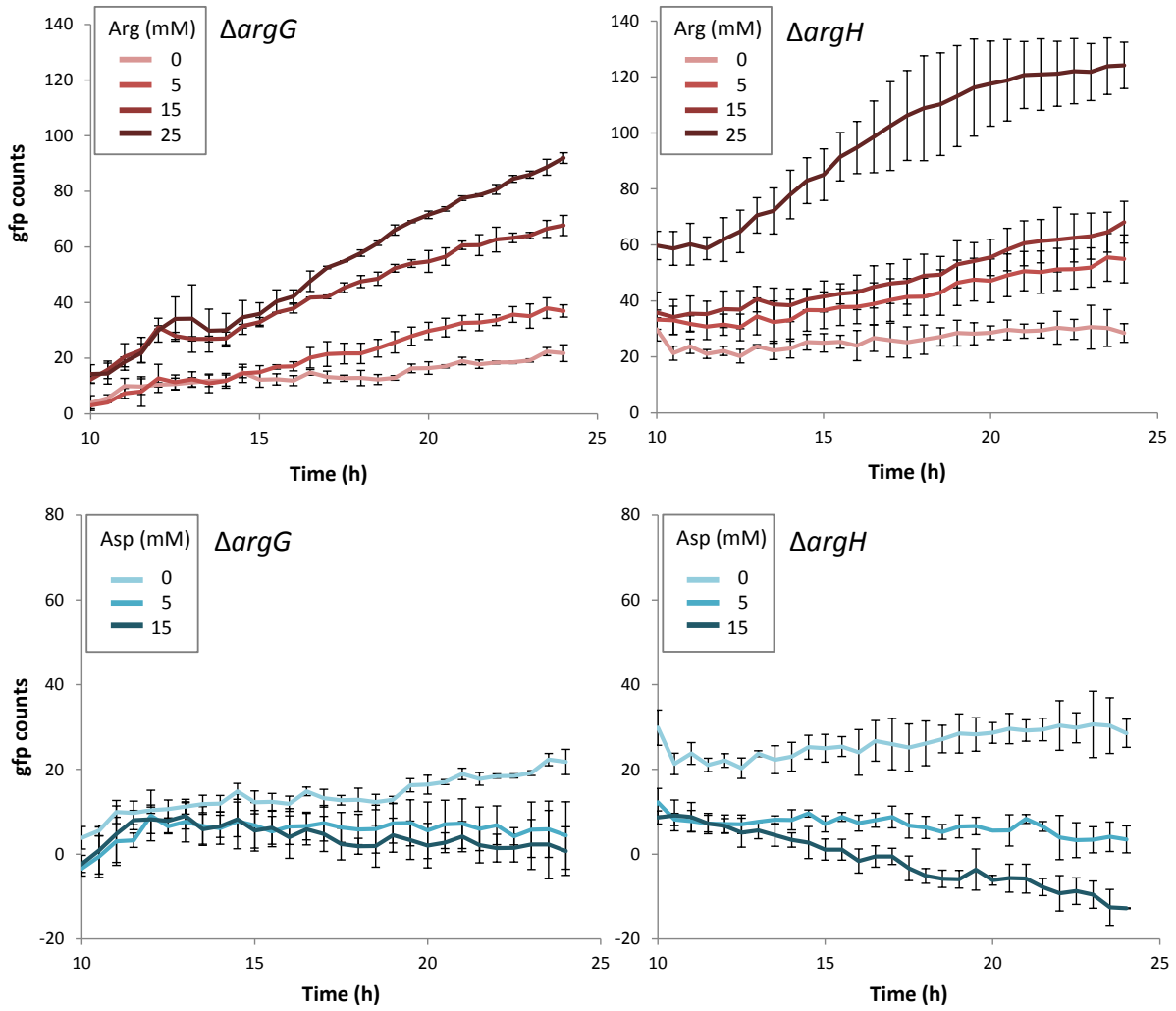


Figure 15. Arginine (top panels) and aspartic acid (bottom panels) modulate c-di-GMP levels in the *argG* (left) and *argH* (right) mutants harbouring pCdrA::*gfp*^{C}. GFP counts indicate the fluorescence values corrected by culture growth (OD_{600}) in LB 1/3. To measurements Synergy Neo2 Biotek fluorimeter was used. Average of two biological replicates, with three experimental replicates, and standard deviation are plotted.

Next, we wanted to evaluate if the increase in c-di-GMP was an specific effect of arginine, or if other amino acids could have a similar role. For this purpose, *P. putida* KT2440 (pCdrA::*gfp*^{C}) was grown in microtiter plates, either in diluted rich medium (LB) or in minimal medium with glucose as a carbon source, supplemented with each of the 20 proteinogenic L-amino acids at two different concentrations, 5 and 15 mM. As shown in Figure 16 arginine caused a very notorious rise in c-di-GMP levels compared to the rest of amino acids tested in rich medium. A significant increase was also observed in the presence of tryptophan, but

only at 15 mM. Besides aspartic acid, other amino acids provoked a decrease of c-di-GMP levels, particularly proline, but also histidine, isoleucine and phenylalanine.

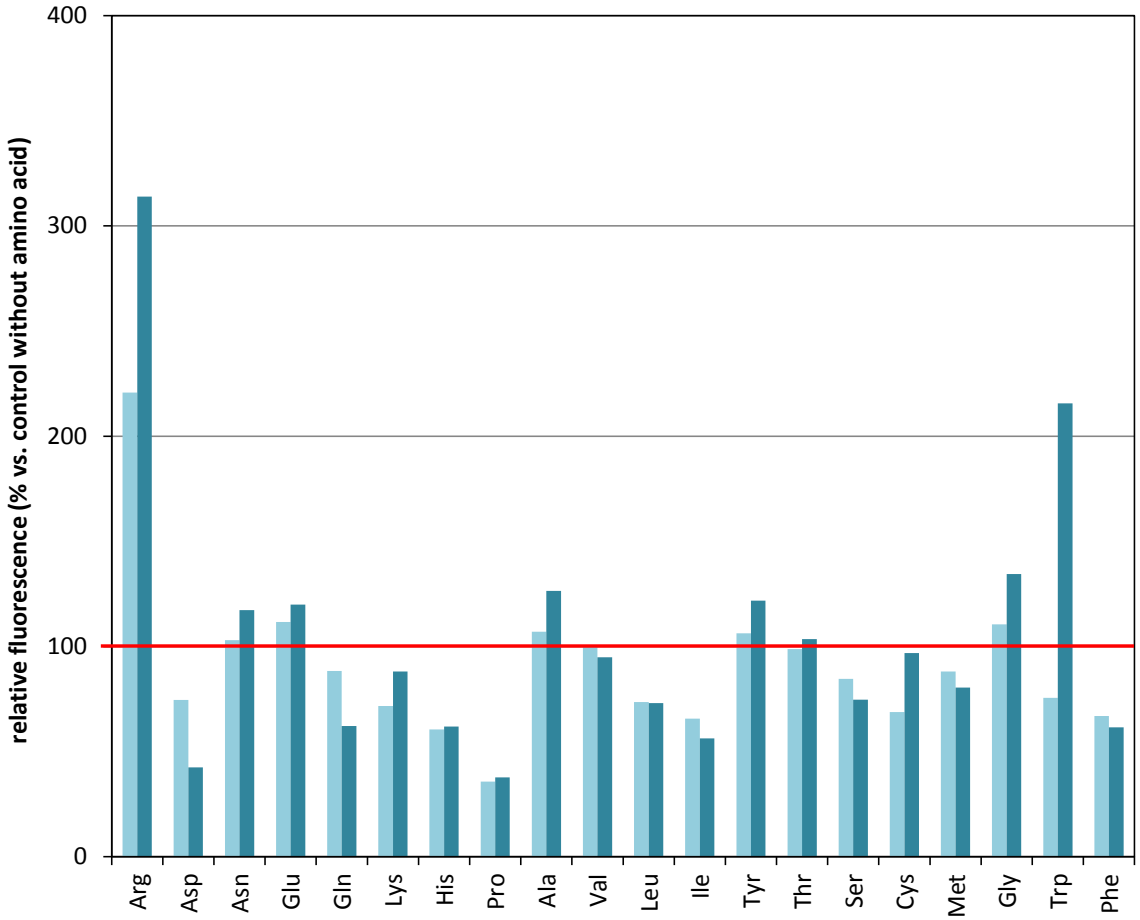


Figure 16. Fluorescence of *P. putida* KT2440 harbouring pCdrA::*gfp*^{C} after 24h of growth in LB 1/3 and each amino acid at 5 mM (light bars) or 15 mM (dark bars). Fluorescence was quantified using a TECAN Infinite 200 fluorimeter. Data are given as percentage compared to the control without added amino acid (=100), and are the averages from three biological replicates with three technical replicates each.

The results obtained in minimal medium with glucose confirmed the positive and dose-dependent role of arginine (Figure 17). In this medium, some of the negative effects were not observed, and in fact aspartic acid slightly increased fluorescence, as did histidine and asparagine, whereas valine, tyrosine and cysteine had a negative effect. However, the data from this experiment were somewhat difficult to interpret, since addition of certain amino acids caused changes in the growth rate and/or pattern of KT2440. Thus, for example, aspartic acid accelerated growth whereas cysteine caused a longer lag phase (not shown).

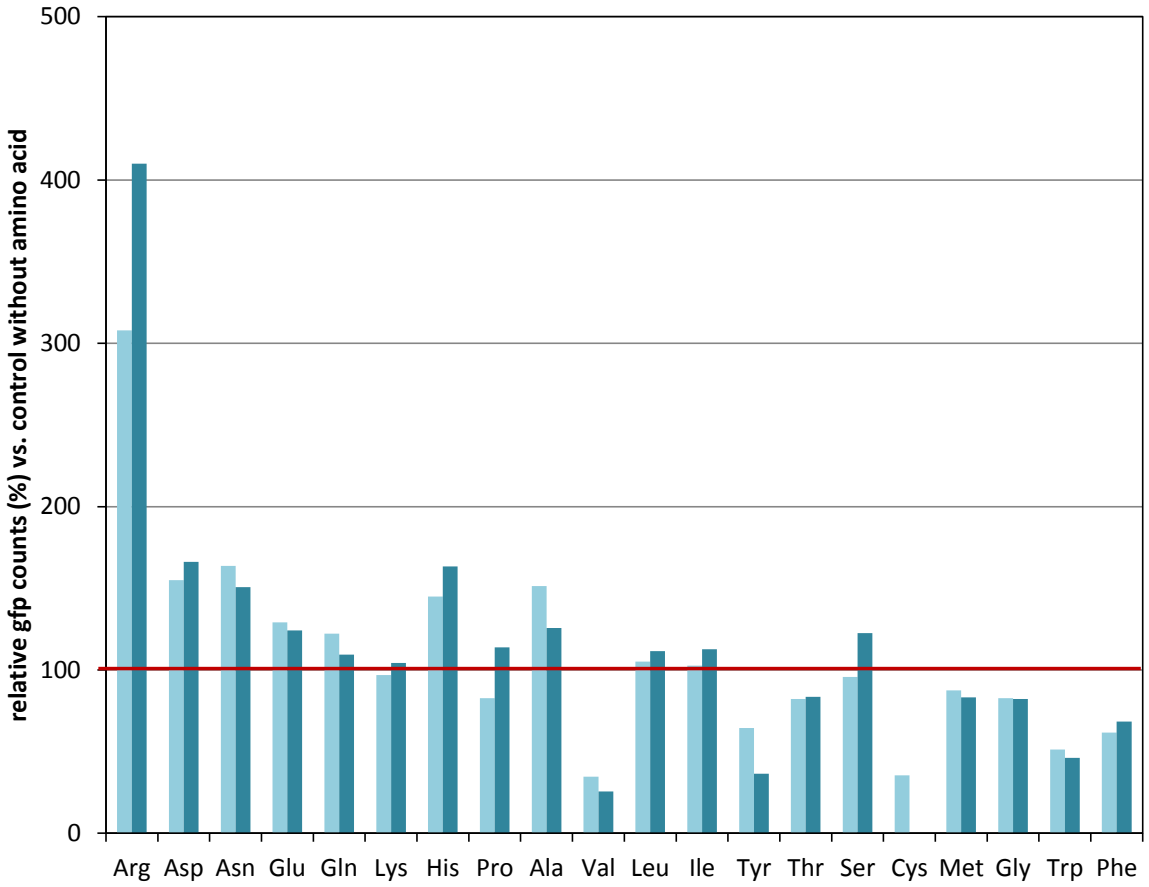


Figure 17. Fluorescence of *P. putida* KT2440 harbouring pCdrA::*gfp*^{C} after 24h of growth in M9+glucose and each amino acid at 5 mM (light bars) or 15 mM (dark bars). Fluorescence was quantified using a TECAN Infinite 200 fluorimeter. Data are given as percentage compared to the control without added amino acid (=100), and are the averages from three biological replicates with three technical replicates each.

L-arginine and L-aspartate have opposite effects on biofilm formation by *Pseudomonas putida* KT2440.

As described earlier, c-di-GMP levels directly correlate with biofilm development in many bacteria, including *P. putida* KT2440 (Figure 2). We therefore presumed that L-arginine could promote biofilm formation, while L-aspartate might have the opposite effect. Thus, we evaluated biofilm development in *P. putida* KT2440 in polystyrene multiwell plates under static conditions in minimal medium with glucose as a carbon source and supplemented with different concentrations of either amino acid. As shown in Figure 18, the presence of arginine increased the amount of attached biomass, this effect being larger at higher

concentrations (15 mM) and between 8 and 11 hours of growth. In contrast, addition of L-aspartate caused a reduction of approximately 50% in biofilm formation between 7 and 11 hours of growth, regardless of the concentration used.

These results indicate that both amino acids have an impact in biofilm formation although in opposite ways. Thus, L-arginine has a positive effect upon biofilm formation capacity in *P. putida* KT2440 whereas L-aspartate has a negative impact. Furthermore, these results correlate positively with data obtained with respect to c-di-GMP levels in presence of both amino acids.

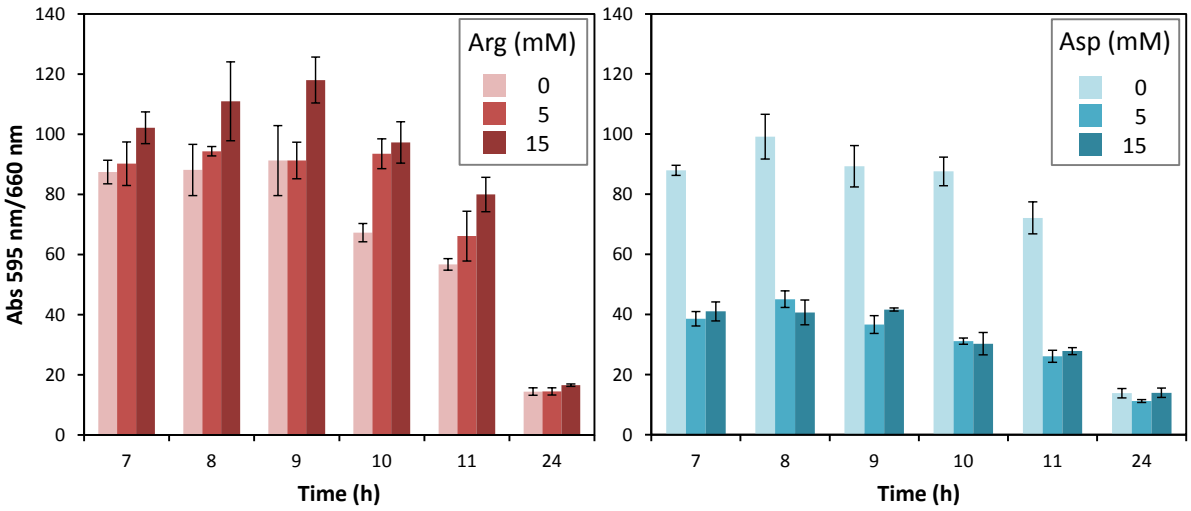


Figure 18. Effect of arginine (left) and aspartate (right) on biofilm formation by *P. putida* KT2440 in FAB medium. Growth (turbidity) was measured at 660 nm, and attached biomass was quantified at 595 nm after staining with crystal violet and solubilization of the dye as described in Materials and Methods. Results are averages and standard deviations of two experiments with three technical replicates.

Overexpression of *argG* and *argH* genes increases biofilm formation.

In view of the above results, we hypothesised that *argG* and *argH* mutants should be affected in their biofilm formation ability. Since these mutants are auxotrophs, it was impossible to evaluate their biofilm formation capacity in minimal medium. Experiments carried out in rich medium did not show any clear differences between the wild type and the mutants (data not shown). As an alternative approach, we decided to test the effect of overexpressing *argG* and *argH* in the wild type strain using pME1088 and pME0184 plasmids (Ramos-González *et al.*, 2016).

We evaluated biofilm formation in *P. putida* KT2440 carrying each of these plasmids or the empty vector as a control. Assays were done as described for Figure 18, in polystyrene multiwell plates using FAB minimal medium with glucose as a carbon source. Quantification of the attached biomass revealed an increase in biofilm formation when *argG* and *argH* were overexpressed after 10 hours of growth, the effect being more noticeable for *argH* (Figure 19).

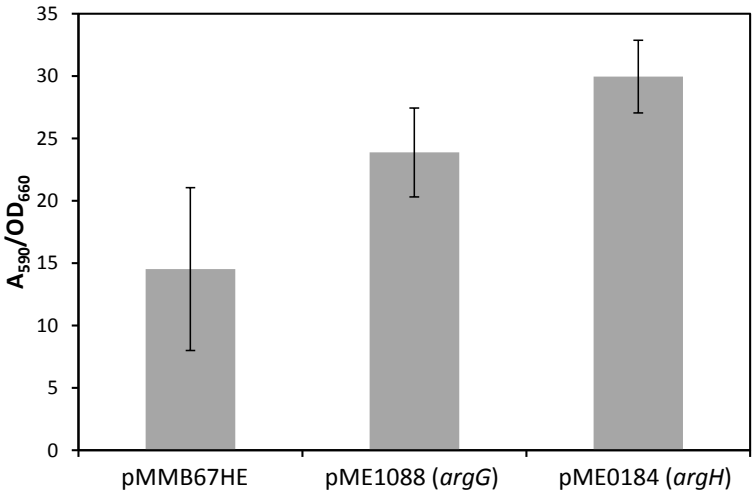


Figure 19. Biofilm formation by *P. putida* KT2440 overexpressing *argG* or *argH* after 10 h of growth. Results are averages and standard deviations of two experiments with three technical replicates. Differences between each of the two overexpressing strains and the control are statistically significant (Student's t test; p≤0.05).

The altered c-di-GMP levels in *argG* and *argH* mutants cannot be fully explained by changes in *cfcR* expression.

Since the cfcK-66 and cfcK-74 mutants were originally selected in the presence of multiple copies of the diguanylate cyclase CfcR, one possibility to explain the observed alterations in phenotype and c-di-GMP content would be that *cfcR* expression was altered in the *argG* and *argH* mutants. To test this hypothesis, transcriptional and translational fusions of *cfcR* to promoterless ´*lacZ*, cloned in plasmids pMIR200 and pMIR219, respectively (Ramos-González *et al*., 2016; Huertas-Rosales *et al*., 2017a) were used, and β-galactosidase activity was measured in the wild type strain and both null mutants, as well as in cfcK-66 and cfcK-74 mutants in LB medium as described in Materials and Methods.

No obvious differences in expression patterns of *cfcR* were observed in any of the mutants with respect to the wild type, with the exception of a small but statistically

significant reduction in the mutants early in stationary phase (Figure 20). Thus, the altered c-di-GMP levels in these mutants may not simply be ascribed to a modification in *cfcR* expression.

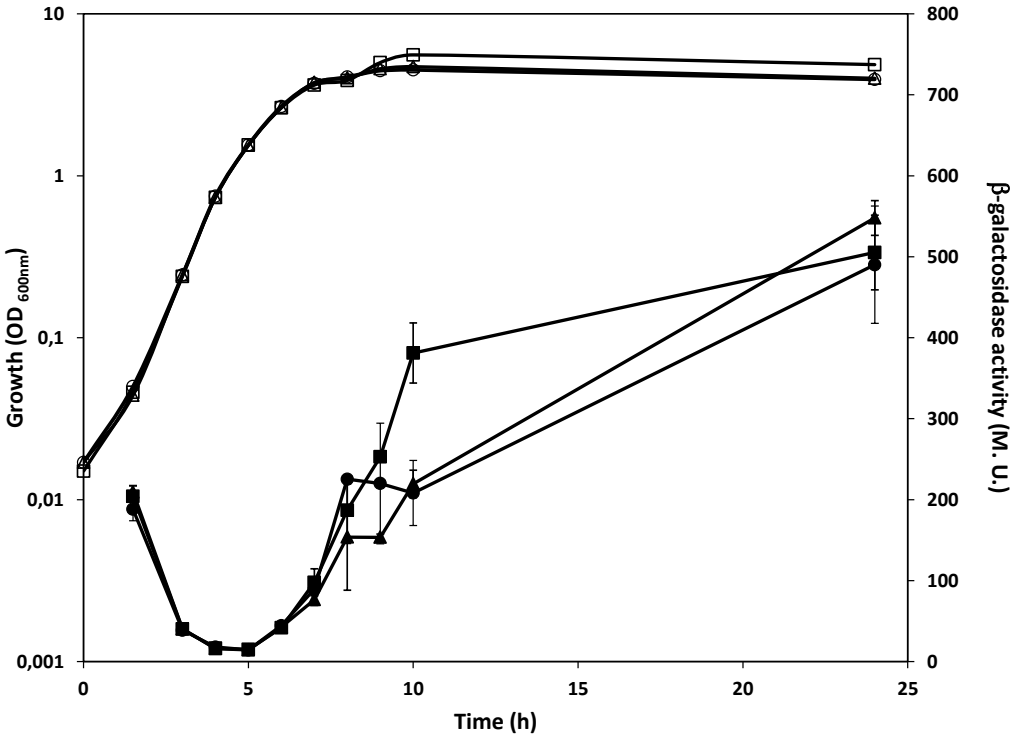


Figure 20. Growth (open symbols) and β-galactosidase activity (closed symbols) of *P. putida* KT2440 (squares), Δ*argG* (triangles) and Δ*argH* (circles) mutants harbouring pMIR200 (*cfcR*::*lacZ* transcriptional fusion). Values correspond to a representative experiment and are averages of two biological replicates with two technical replicates. Differences between the wild type and the two mutants are statistically significant at 10 h (Student's t test; p≤0.05).

The response to L-arginine involves additional c-di-GMP-related elements besides the CfcR/CfcA pathway.

All the results presented so far indicated that arginine has a stimulatory effect on c-di-GMP synthesis, presumably through the DGC activity of CfcR, which, as previously mentioned, requires phosphorylation of CfcR by the histidine kinase CfcA. The importance of the CfcA/CfcR system for the intracellular contents of second messenger in *P. putida* KT2440 in stationary phase is shown in Figure 21A. Using the pCdrA::*gfp*^{C} bioreporter, we monitored c-di-GMP levels in the wild type strain, Δ*cfcR* and Δ*cfcA* mutants and a double mutant Δ*cfcA*Δ*cfcR*. Quantitative data showed that Δ*cfcR* and Δ*cfcA*Δ*cfcR* mutants had low levels of

free c-di-GMP pool, as expected, whereas c-di-GMP in the Δ*cfcA* mutant was higher than in the wild type strain at the beginning of stationary phase, but did not raise later on, being maintained over time. In contrast, a boost in c-di-GMP levels was observed in the wild type strain later into stationary phase, reaching maximum after 24 hours of incubation (Figure 21A).

Similar results were observed in LB-agar medium after longer incubation times (Figure 21B), where both the Δ*cfcR* and Δ*cfcA* mutants showed much lower fluorescence intensity than the wild type. Interestingly, when arginine was added to the medium we could observe an increase in fluorescence in all the strains, particularly at the higher concentration used (15 mM). To explore this result in more detail, liquid cultures of KT2440 and the three mutants, harbouring pCdrA::*gfp*^{C} were grown in microtiter plates with increasing concentrations of arginine, and fluorescence was monitored over time. Results are presented in Figure 22. Interestingly, c-di-GMP contents increased in all the genetic backgrounds in the presence of L-arginine, with a dose-dependent response to the amino acid observed in all the mutants, as well as in the wild type. Nonetheless, the Δ*cfcR* and Δ*cfcA*Δ*cfcR* mutants only reached at most the c-di-GMP basal content of the wild type, at the maximum arginine concentration tested. Surprisingly, *ΔcfcA* showed an important boost of c-di-GMP contents in all L-arginine concentrations at early stationary phase of growth, whereas these increments were observed later in the wild type (Figure 11B and 11C). However, at late stationary phase, levels of c-di-GMP at 15 and 25 mM of L-arginine in *ΔcfcA* were in all cases lower than in the wild type.

These results show that changes in c-di-GMP levels influenced by L-arginine cannot simply be explained by the contribution of the CfcA/CfcR pathway, since all the mutants maintain at least to a certain degree the response to L-arginine.

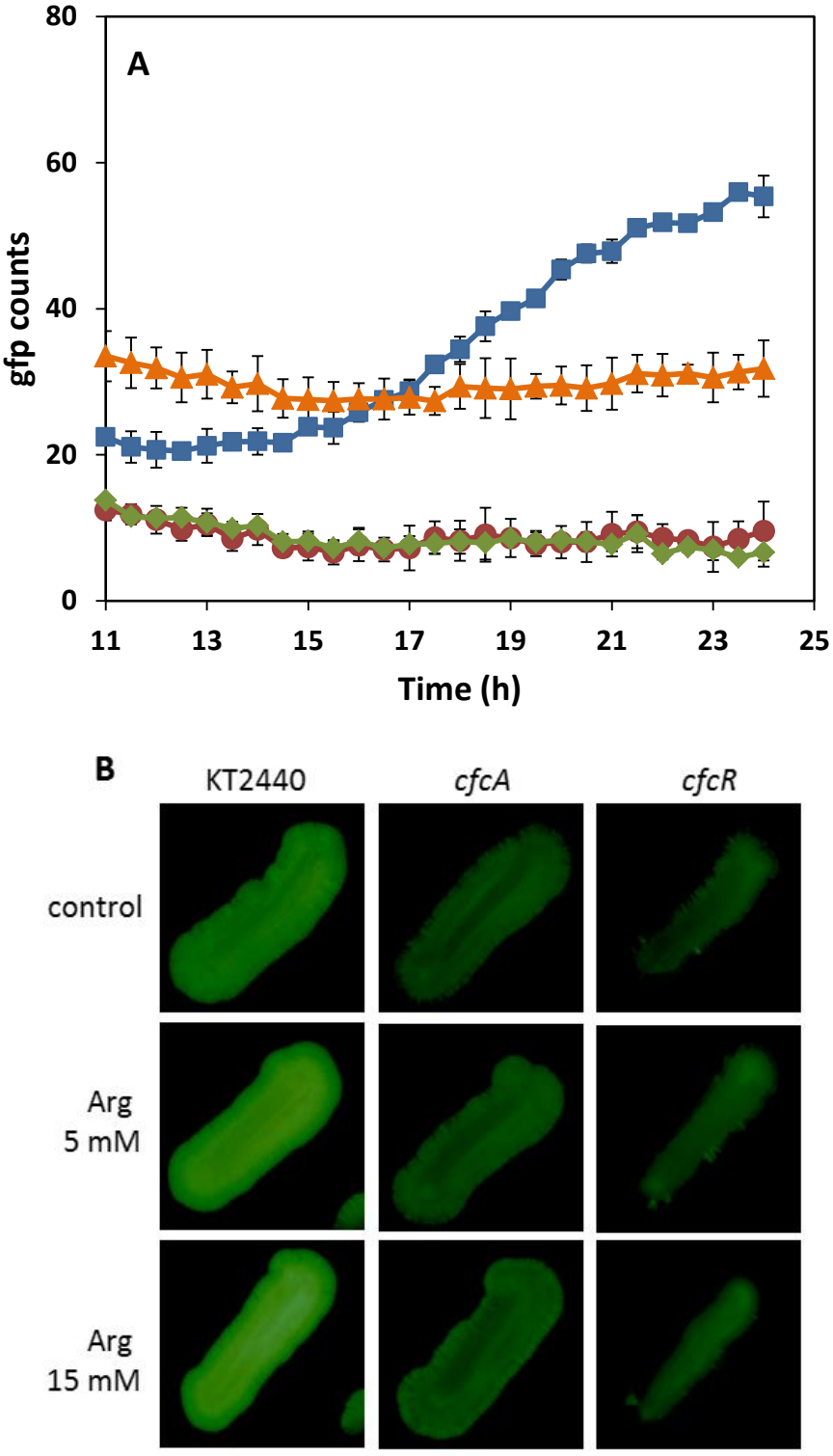


Figure 21. A. c-di-GMP levels in *P. putida* KT2440 (blue), and the *cfcA* (orange), *cfcR* (red) and double *cfcA/cfcR* (green) strains harbouring pCdrA::*gfp*^{C}. GFP fluorescence was quantified from liquid cultures grown in microtiter plates using a Synergy Neo2 Biotek fluorimeter. GFP counts indicate the fluorescence values corrected by culture growth (OD_{600}) in LB 1/3. Average of two biological replicates, with three experimental replicates, and standard deviation are plotted. **B.** Epifluorescence images of KT2440, *cfcA* and *cfcR* streaks after 48 h of growth in LB supplemented with different arginine concentrations. Exposure time of 5,4 s.

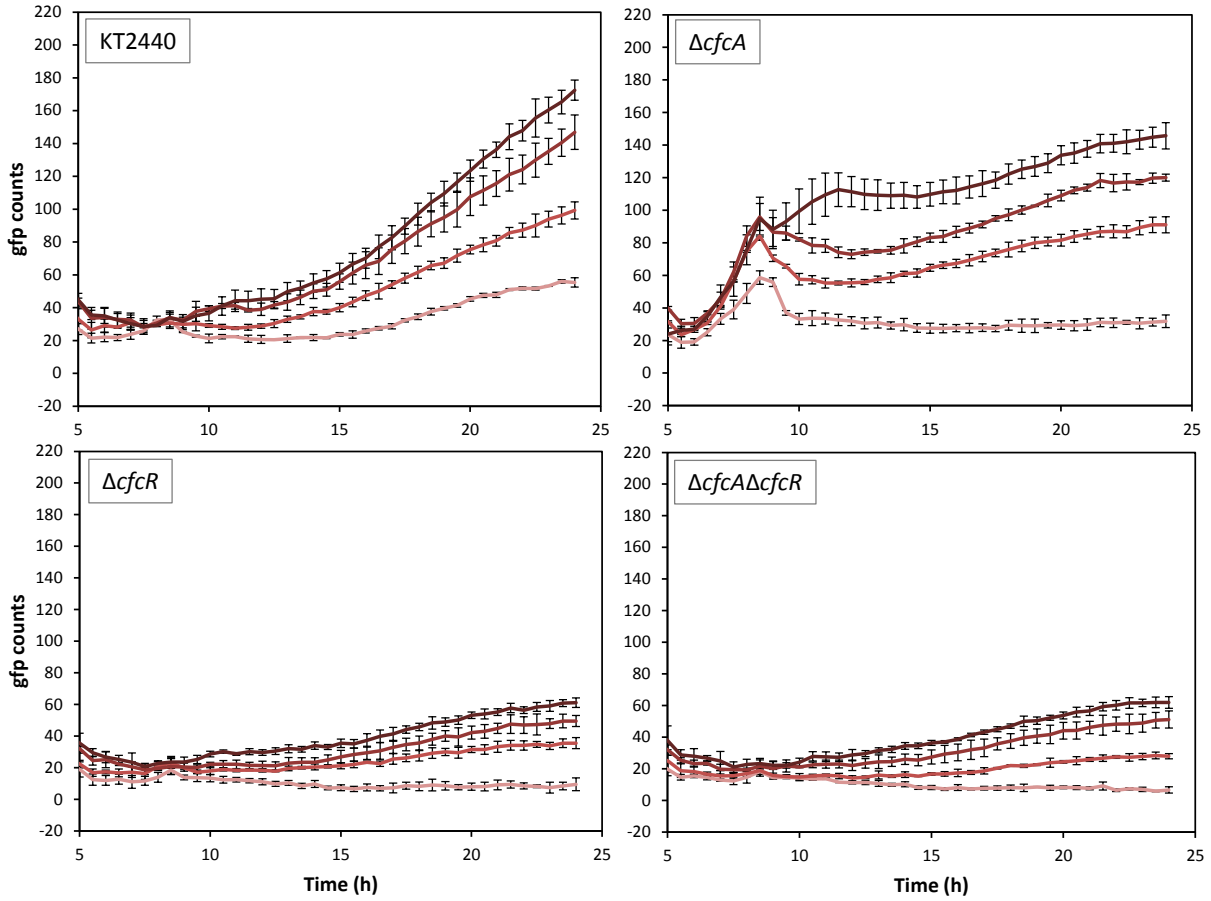


Figure 22. Accumulation of c-di-GMP in response to increasing arginine concentrations in *P. putida* KT2440 and mutants in the *CfcA/CfcR* route harbouring pCdrA::*gfp*^{C}. GFP counts indicate the fluorescence values corrected by culture growth (OD_{600}) in LB 1/3. Average of two biological replicates, with three experimental replicates, and standard deviation are plotted. The colour code is identical to the one used in Figure 15.

DISCUSSION

The work presented here shows the implications of amino acids, more specifically L-arginine and L-aspartate, upon c-di-GMP levels and related phenotypes in *P. putida* KT2440. Mutants in *argG* and *argH* genes, obtained in a transposon mutagenesis, and the corresponding *ΔargG* and *ΔargH* knock-out mutants, lack the crinkly morphology associated to high levels of c-di-GMP as a consequence of CfcR overexpression. This phenotype is recovered by addition of exogenous L-arginine and by genetic complementation of the

mutants, although this entails the intact genes to be under the control of their native promoters. This suggests that the regulation and timing of expression of the arginine biosynthesis genes is important for phenotype development. Furthermore, we have shown that the wild type strain requires arginine to develop the crinkly phenotype, which otherwise does not appear in minimal medium with glucose. Interestingly, L-aspartate, one of the immediate precursors of arginine synthesis provokes disorganization of the phenotype.

After analysing the role of the 20 L-amino acids on c-di-GMP levels, other amino acids also show a minor positive or negative influence that will deserve attention in future works, but arginine is the most influencial one in terms of free c-di-GMP pool in *P. putida* KT2440.

Previous studies have also indicated a connection between social behaviours and amino acids. Kolodkin-Gal and co-workers demonstrated that D-amino acids favour biofilm dispersal in *Bacillus subtilis* (Kolodkin-Gal *et al*., 2010). On the other hand, some reports have shown the role of L-amino acids on biofilm formation. Several L-amino acids reduce swarming motility of *P. aeruginosa* and promote biofilm formation when added exogenously to the growth medium; of these, arginine at a concentration of 4.8 mM completely inhibited swarming (Bernier *et al*., 2011). Although it was not the most prominent in terms of biofilm promotion, L-arginine supplementation resulted in a 1.7-fold increase in c-di-GMP content of *P. aeruginosa* cells compared to the content of cells grown solely on glucose. In *Escherichia coli*, tryptophan promotes attachment increasing the number of surface-adherent cells, as well as enhancing biofilm formation at late stages of its development (Ren *et al*., 2004; Domka *et al*., 2007; Hamilton *et al*., 2009). Our results show that L-arginine promotes biofilm formation whereas L-aspartate acts in the reverse way. Bernier and co-workers showed a positive effect of L-arginine on biofilm formation when *P. aeruginosa* PA14 was grown with the amino acid as the only carbon and nitrogen source, but no effect was observed when both L-arginine and glucose were present in the culture medium (Bernier *et al*., 2011). In contrast, in *P. putida* KT2240 L-arginine has an influence even when bacteria are grown in the presence of glucose. Nevertheless, in both cases high amounts of amino acid are required. In fact, several recent publications have demonstrated the effect of different concentrations of L-arginine in motility and biofilm formation. For example, in *Streptococcus gordonii* low arginine (between 0.5 and 500 µM) enhances biofilm development while high

levels (equal to or greater than 50 mM) lead to altered biofilm architecture (Jakubovics *et al*., 2015). Intermediate concentrations have no effect in biofilm formation in this organism.

Little is known about the mechanism of action of amino acids that lead to changes in second messenger levels. Recently, exogenous L-arginine has been found to induce the synthesis of c-di-GMP in *Salmonella enterica* serovar Typhimurium. Although the mechanism requires more research, the arginine transporter subunit ArtI and the diguanylate cyclase STM1987 are required for the amino acid response (Mills *et al*., 2015). In *P. putida* there is no equivalent of the above mentioned DGC STM1987 containing a periplasmic Arg-sensing domain, indicating that the mechanism by which arginine may control c-di-GMP levels in this bacterium is likely different. Bernier and co-workers reported that SadC and RoeA, two of the most important DGCs implicated in biofilm formation, are necessary for the response of L-arginine in *P. aeruginosa* PA14 (Bernier *et al*., 2011).

It should be noted that previous studies had focused on exogenous arginine. However, based on the c-di-GMP biosensor pCdrA (Rybtke *et al*., 2012), our data obtained with *argG* and *argH* mutants indicate that an intact arginine biosynthetic pathway positively influences free c-di-GMP content. This is consistent with the opposite effect of one of the precursors, aspartic acid, which might accumulate in the *argG* and perhaps the *argH* mutants.

Expression of *cfcR* was not significantly altered in the *arg* mutants, which was one of our first hypotheses. Since these mutants initially were selected in a screen when CfcR was overexpressed in and showed very low levels of c-di-GMP (Ramos-González *et al*., 2016), another possibility was that the histidine kinase CfcA, which has a CHASE3 sensor domain, might be implicated in L-arginine recognition, leading to its auto-phosphorylation and transferring the phosphate to CfcR. Once CfcR was phosphorylated and active, the DGC would synthesise c-di-GMP, increasing the second messenger content in the cell. However, mutants in the CfcA/CfcR pathway retained some capacity to respond to L-arginine, suggesting additional elements must participate in this response. Furthermore, isothermal tritation calorimetry (ITC) assays have shown no binding between L-arginine and the CHASE3 domain of CfcA (V.G. Tagua *et al*., unpublished data).

Thus, the connection between amino acid metabolism and c-di-GMP signalling appears to be a complex one, and the mechanism requires further analysis. The role of different regulatory elements and their influence in second messenger levels and downstream processes is analysed in the following Chapter.

CHAPTER 2

Identification of the regulatory network associating arginine metabolism and c-di-GMP signalling in *Pseudomonas putida* KT2440

Manuscript in preparation: Barrientos-Moreno, L., Molina-Henares, M.A., Ramos-González, M.I., Cámara M., Romero, M., Espinosa-Urgel, M. Global regulators and feedback loops connect arginine metabolism and c-di-GMP-mediated signal transduction in *Pseudomonas putida*.

BACKGROUND

As shown in Chapter 1, mutants in *argG* and *argH* showed reduced c-di-GMP levels even when the diguanylate cyclase CfcR was overexpressed, and they lost the associated crinkly phenotype. The phenotype was restored by the presence of the intact genes and by exogenous arginine, which also increased biofilm formation. In order to further explore this connection with c-di-GMP mediated signalling, experiments were designed to determine at which level arginine may be acting and what regulatory proteins are associated with this response.

RESULTS

Arginine biosynthesis influences the expression of structural elements of *P. putida* biofilms.

Previous work has established that the crinkly phenotype observed with high levels of c-di-GMP requires the species-specific exopolysaccharide Pea (Matilla *et al*., 2011). On the other hand, as mentioned in the General Introduction, the large adhesins LapA and LapF are essential for the development of mature biofilms in *P. putida*, a process in which EPS also would contribute to the biofilm matrix. These facts prompted us to investigate if expression of any of these structural elements were affected in *argG* and *argH* mutants. Strains were transformed with plasmids harbouring transcriptional fusions of *lapA*, *lapF* and the first gene in each EPS cluster –that include the promoter and the first codons- with the reporter gene *´lacZ* devoid of its own promoter, as previously described (Martínez-Gil *et al*., 2010; Martínez-Gil *et al*., 2014; Molina-Henares *et al*., 2017).

The results obtained are shown in Figure 1. A significant reduction in expression was observed for the *pea::lacZ* and the *lapF::lacZ* fusion in both mutants compared to the wild type, where expression activates upon entry on the stationary phase of growth. This reduction in *pea* expression is consistent with the loss of crinkly morphology in these mutants. Differences were also observed for the *peb* and *bcs* fusions, but they were significant only after 24 h of growth. In both cases, the mutants showed higher β-

galactosidase activity than the wild type at that time. Expression of *lapA* and *alg* was not modified in either mutant.

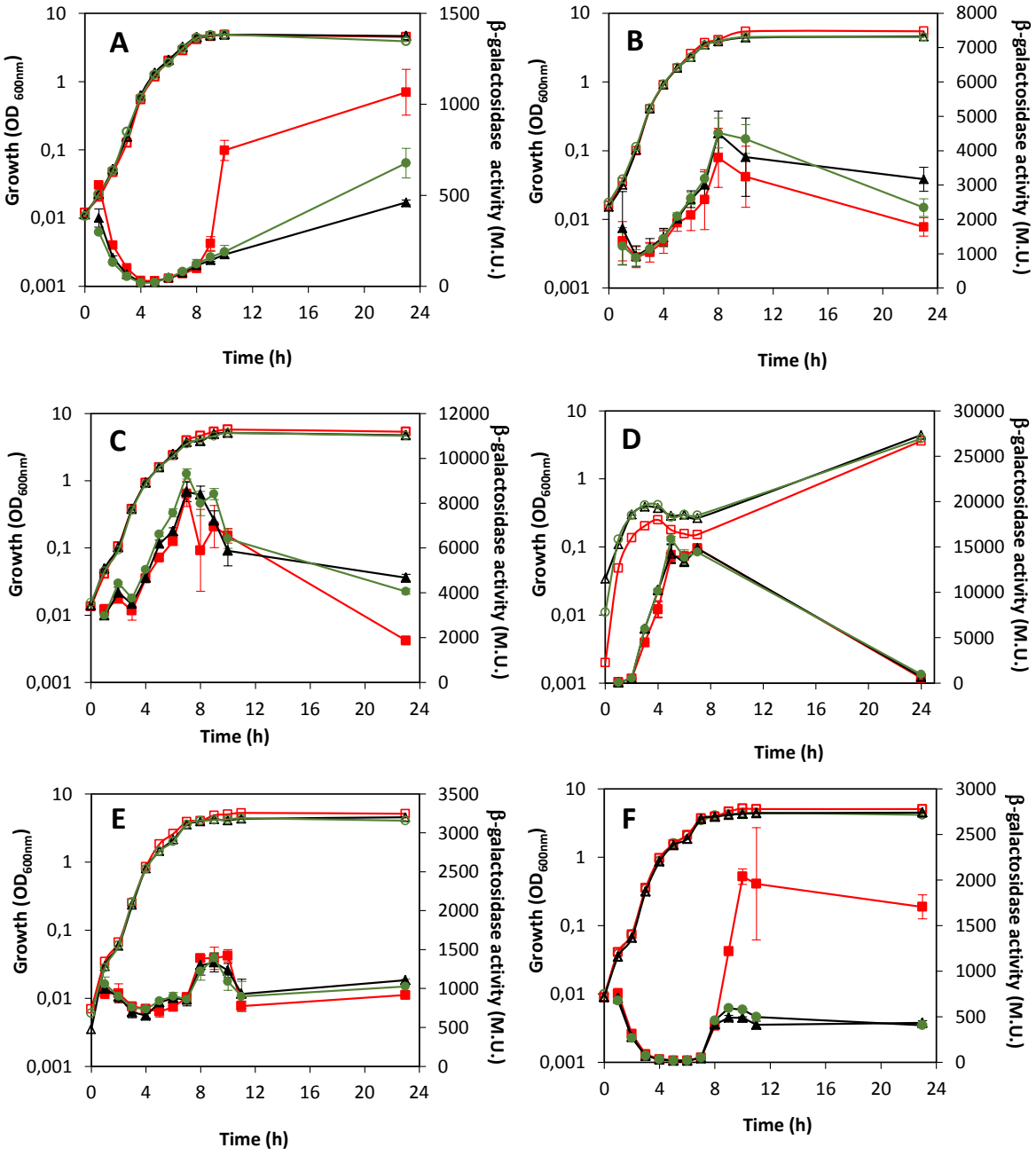


Figure 1. Influence of *argG* and *argH* mutations on expression of EPS- and adhesins-encoding genes during growth in liquid medium. Cultures growing in LB supplied with Tc as described in Materials and Methods were analysed for turbidity (hollow) and β–galactosidase activities (solid) at the indicated times. Data for the wild type (square), Δ*argG* (triangle) and Δ*argH* (circle) strains carrying reporter fusions corresponding to **(A)** Pea (PP_3132*::´lacZ*), **(B)** Peb (PP_1795*::´lacZ*), **(C)** Bcs (PP_2629*::´lacZ*), **(D)** Alg (algD*::´lacZ*), **(E)** LapA (pMMGA) and **(F)** LapF (pMMG1) are shown. D-cycloserine (75 µg/ml) was added in **D** after 1 hour of growth, since the *algD* promoter is inactive in the absence of cell wall stress in *P. putida* (Molina-Henares *et al*., 2017).

In the above experiments, it is noticeable that the expression patterns of *lapF* and *pea* are very similar. Expression of *lapF* has been shown to be controlled by the alternative sigma factor RpoS (Martínez-Gil *et al*., 2010), which regulates the transcription of genes upon entry in stationary phase of growth and in response to certain stresses (Potvin *et al*., 2008; Landini *et al*., 2014). This led us to test if *pea* was controlled by RpoS as well. The *pea::´lacZ* fusion was introduced in C1R1, a *rpoS* derivative of KT2440 and R6C1, a merodiploid derivative of KT2440 harbouring a mutated and wild type copy of *rpoS* in the chromosome (Ramos-González and Molin, 1998) and β–galactosidase activity was measured during growth in liquid medium. As shown in Figure 2, the expression of *pea* was almost completely abolished in the *rpoS* mutant and restored in the merodiploid, indicating that expression of the exopolysaccharide is dependent on this alternative sigma factor.

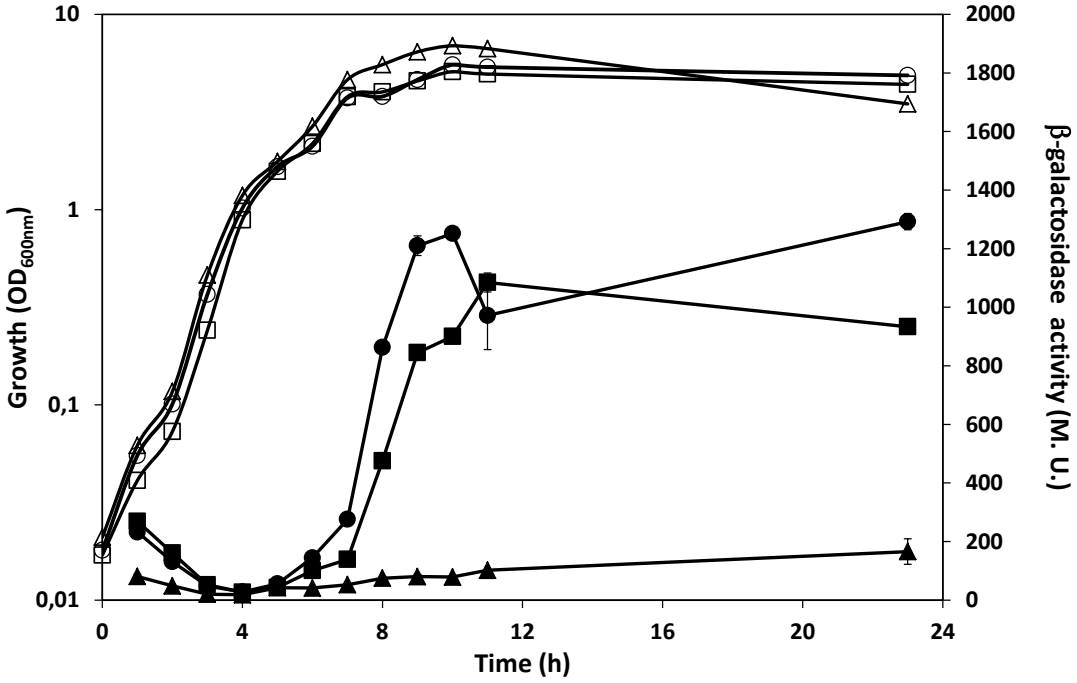


Figure 2. Expression of *pea* (PP_3132*::´lacZ*) is dependent on RpoS. Cultures growing in LB supplied with Tc as described in Material and Methods were analysed for turbidity (hollow) and β–galactosidase activities (solid) at the indicated times. Data for the wild type (square), C1R1 (*rpoS* mutant, triangle) and R6C1 (*rpoS* merodiploid, circle) strains are shown. Experiment was performed in duplicate with two technical repetitions each. A representative graph is presented.

Given the role shown in Chapter 1, in which L-arginine is necessary to develop crinkly morphology in the wild type strain after 48 h of growth in minimal medium agar plates and high concentration of the amino acid were required, we decided to check if the expression of *pea::´lacZ* was induced by L-arginine. For this purpose, β-galactosidase activity was measured in *P. putida* KT2440 cultures growing in minimal medium with different concentrations of the amino acid.

The results presented in Figure 3, show that L-arginine positively influences *pea::´lacZ* expression when cultures were in stationary phase of growth in liquid medium. The effect was more evident at high concentrations of the amino acid (15-25 mM), which correlated with the role of L-arginine on crinkly phenotype previously observed in agar plates (Figure 11, Chapter 1), although the expression of *pea* is advanced in liquid medium.

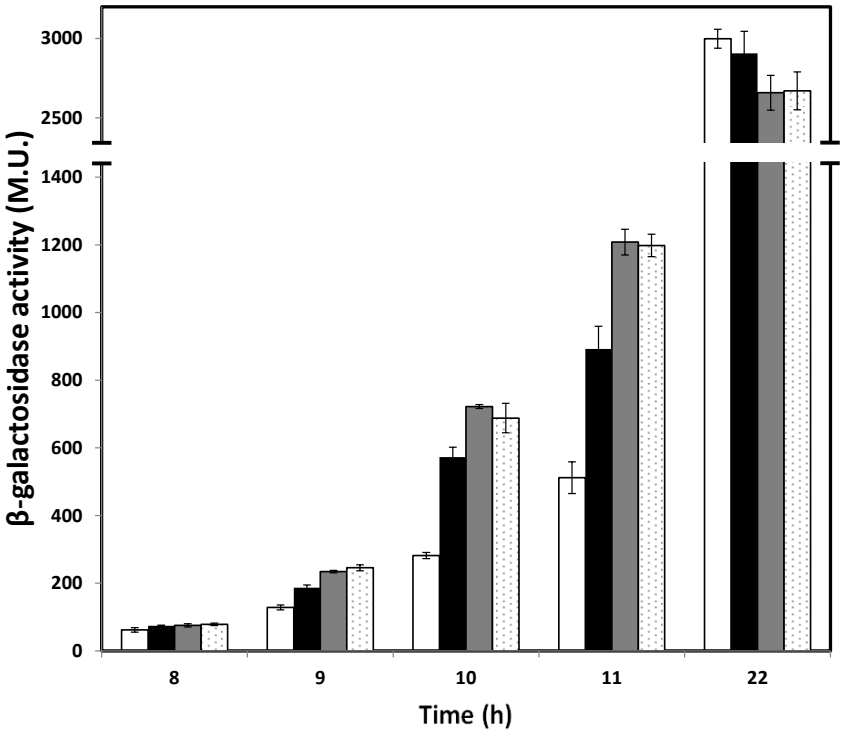


Figure 3. Arginine increases expression of *pea* (PP_3132*::´lacZ*) in *P. putida* KT2440. Cultures growing in M9 minimal medium with glucose as a carbon source and different concentrations of L-arginine were analysed for β–galactosidase activities at the indicated times. Data for the control (open bars), 5 mM (black bars), 15 mM (grey bars) and 25 mM (dotted bars) of L-arginine are shown. The experiment was done in duplicate with three technical repetitions each. Statistically significant differences are shown at 8, 9, 10 and 11 h between the absence and presence of arginine (Student´s *t* test; $\rho \leq 0.05$).

Arginine influences expression of RpoS.

The results presented above prompted us to investigate if the lack of a functional arginine biosynthetic pathway might influence *rpoS* expression. Thus, we checked *rpoS* expression in the *argG* and *argH* mutants using plasmid pMAMV21, which harbours a *rpoS´-´lacZ* translational fusion (Matilla *et al*., 2011). As shown in Figure 4, the lack of *argG* or *argH* had negative impact upon the expression of *rpoS*, being higher in the case of *argG* mutant. These results could suggest that a functional arginine biosynthesis pathway is required for expression of *rpoS* or maybe the final product of the pathway (arginine) influences its expression.

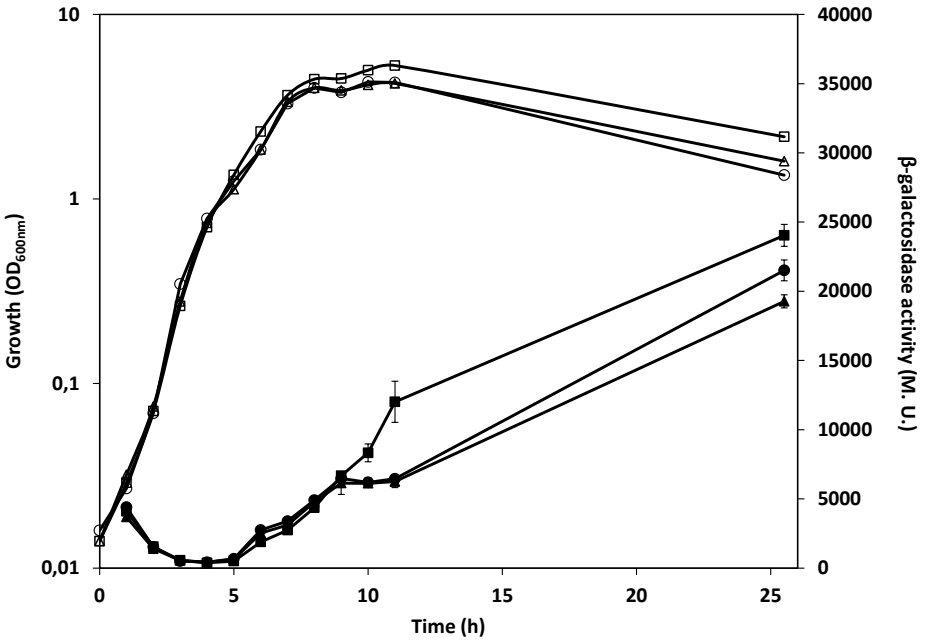


Figure 4. Activity of the translational fusion of *rpoS`-´lacZ* in *argG* and *argH* mutants. Cultures growing in LB supplied with Tc as described in Material and Methods were analysed for turbidity (hollow) and β–galactosidase activities (solid) at the indicated times. Data for the wild type (square), Δ*argG* (triangle) and Δ*argH* (circle) strains are shown. The experiment was made in triplicate with three technical repetitions each. A representative experiment is shown.

To investigate if arginine itself has a positive effect upon *rpoS* expression, we decided to measure β–glactosidase activity of the *rpoS´-`lacZ* in pMAMV21 by adding different concentrations of the amino acid to the culture medium. As shown in Figure 5, expression of *rpoS´-`lacZ* increased in KT2440 in the presence of 5 and 15 mM arginine, but was not

significantly different from the control at 25 mM. In the *argG* and *argH* backgrounds expression increased in all arginine concentrations tested, but the largest effect was observed at different concentrations of the amino acid depending on the mutant (15 mM for *argG* and at 5 mM for *argH*). It should be noted that these experiments were done in rich medium, since the mutants are unable to grow in minimal medium without arginine.

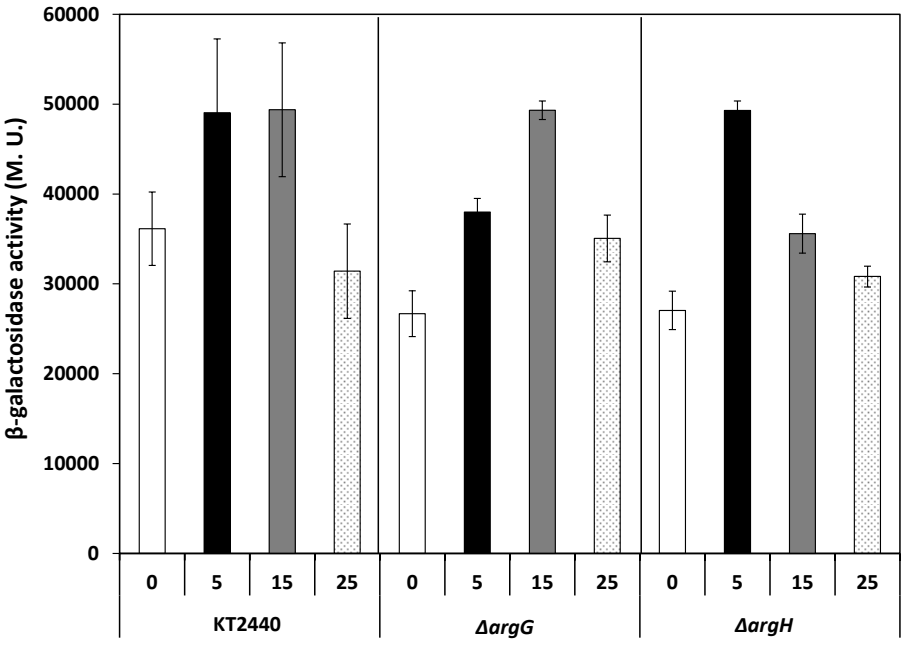
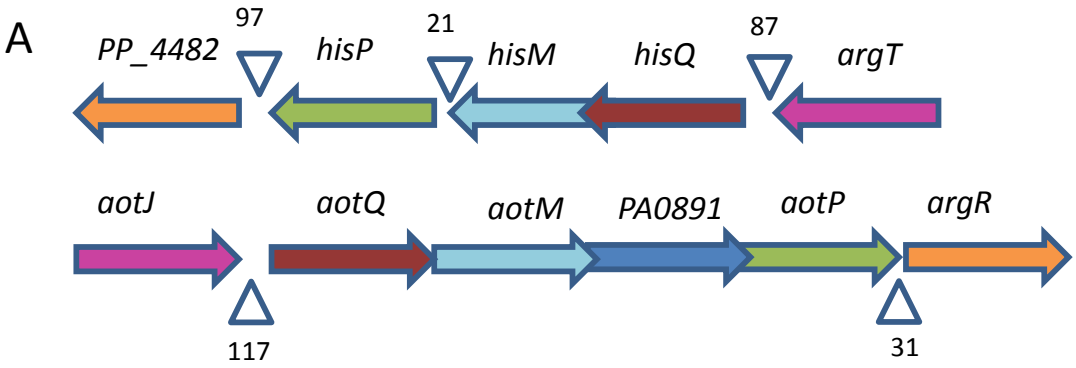


Figure 5. Effect of arginine upon *rpoS´-`lacZ* in the wild type and *argG* and *argH* mutants. Cultures growing in LB supplied with Tc as described in Material and Methods were analysed for β–galactosidase activity after 25h of growth. Different concentrations of arginine were added to the culture medium (0 mM, white bars; 5mM, black bars; 15 mM grey bars; and 25 mM, dotted bars). Data correspond to averages and standard deviations from two biological replicates with three technical repetitions each. Statistically significant differences are shown between 0 and 5 and 15 mM L-arginine in the wild type and between 0 mM and all L-arginine concentrations tested in both mutants (Student´s *t* test; $\rho \leq 0.05$).

Identification and role of ArgR, the arginine metabolism regulator, in *P. putida* KT2440.

As was mentioned in the Introduction, the transcriptional regulator ArgR is well characterised in *P. aeruginosa*, where it controls key steps in L-arginine metabolism and forms an operon with the *aotJQMOP* system for transport of arginine and ornithine, being

the last gene of the operon (Nishijyo *et al*. 1998). However, barely any previous work has been done in *P. putida*, and the gene encoding ArgR is not even annotated. Homology searches have allowed us to identify locus PP_4482 as the one corresponding to *argR.* As shown in Figure 6, the genetic organization of the chromosomal region containing locus PP_4482 in *P. putida* is similar to that of the *aotJQMOP-argR* operon in *P. aeruginosa*, with adjacent ORF that encode the components of a putative system for transport of arginine/lysine/histidine/ornithine. The protein encoded by PP_4482 shares 81.79% identical residues with ArgR protein of *P. aeruginosa* PAO1, and therefore this locus is named *argR* from now on.



B

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MTTQ--RIGFLIWPSTRPLTLALAEEVLLVAQRVHPDVVYELVFLQAE-PAQEGAWRLPGEPWTGR
MTAQPQRIGFLLWPATRALTLSLAEEALRAARRLHPEALYEPLFLLAEAPAEEEGWRLPGTAWNGR

LEGCHKLFLLADEPPAAMGAAFSAALKQLARSGCLIGGLSAGVYPLAMLGLLDGYRAAVHWRWQDD
LEQCSRLFLVADEAPAAVSPALGLALKQLARSGAAIGALSAGIYPLAQLGLLDGYRAAVHWRWHDD

FAERFPKVIATSHLFDWDRDRLTACGGMAVTDLLLAVLARDHGAELAGAVSEELVVERIREGGERQ
FTERFPKVIATNHLFEWDRDRMTACGGMAVLDLLLALLSRDHGAELAGAVSEELVVERIREGNERQ

RIPLQNRLGSSHPKLTQAVLLMEANIEEPLTTDEIAQHVCVSRRQLERIFKQYLNRVPSQYYLELR
RIPLKNRLGSSHPKLTQAVLLMEANIEEPLTTDEIAQHVCVSRRQLERIFKQYLNRVPSQYYLELR

LNKARQMLMQTSKSIIQIGLSCGFSSGPHFSSAYRNFFGATPREDRNQRRSSSPFELSSAPAEKG
LNRARQMLMQTSKSIIQIGLSCGFSSGPHFSSAYRNFFGVTPREDRNQRRGGSAFETTFTPVERG
```

Figure 6A. Comparison of genetic organisation of the *P. putida* KT2440 (above) chromosomal region containing PP_4482 (=*argR)* and putative arginine transport (*hispPMQ-argT*) with the *aotJQMOP-argR* operon in *P. aeruginosa* PAO1 (below). Arrows with the same colour indicate gene homology. Triangles and numbers indicate intergenic regions between adjacent genes and their length in bp. **B**. Sequence alignment between ArgR of *P. putida* (black letters) and *P. aeruginosa* (blue letters). Identical residues are highlighted in yellow and conservative residues in cyan.

To define the role of ArgR in the regulation of arginine biosynthesis and associated phenotypes in KT2440, a null *argR* mutant was generated by complete removal of the open reading frame, as described in Materials and Methods. Next, transcriptional fusions of *argG* and *argH* to ´lacZ were constructed using plasmid pMP220, named pLBM13 and pLBM14, respectively. The transcriptional constructs were introduced in KT2440 and the *argR* mutant, and β–galactosidase activity was assayed. As shown in Figure 7, activity of the *argG::´lacZ* fusion was higher at all times in the mutant, indicating that ArgR functions as a repressor of *argG*, as described in other organisms. In the case of *argH*, increased expression was only observed in the mutant at the beginning of stationary phase.

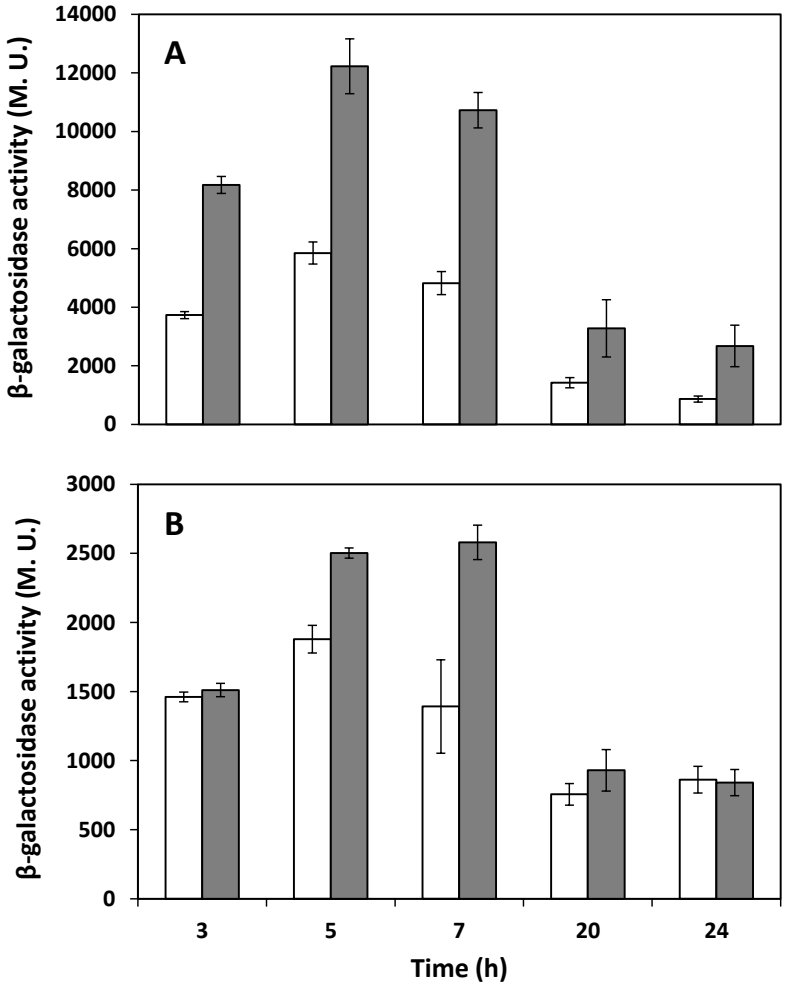


Figure 7. ArgR functions as a repressor of arginine biosynthesis genes. Activity of *argG::´lacZ* (A) and *argH::´lacZ* (B) transcriptional fusions was measured at different times during growth in LB of KT2440 (white bars) and a Δ*argR* mutant (grey bars). Data are averages and standard deviations of two independent experiments with three technical repetitions each. Statistically significant differences between the wild type and Δ*argR* were detected from 3h onward (A) and from 5h to 20h (B) (Student´s *t* test; $\rho \leq 0.05$).

ArgR influences expression of the diguanylate cyclase CfcR

In chapter 1 we showed that *argG* and *argH* mutants had very low levels of c-di-GMP. As seen above, ArgR is a negative regulator of these genes, particularly of *argG*. This, and the results shown in Figure 4 made us postulate that ArgR would influence the expression of *cfcR*. Plasmids pMIR200 and pMIR219 –harbouring transcriptional and protein fusions of *cfcR* to the reporter gene *´lacZ*, respectively– were introduced into the wild type and *argR* mutant and β-galactosidase activities were studied. As shown in Figure 8, the lack of *argR* influenced positively the expression of *cfcR*, although the effect observed was not as great as might be expected to explain the reduced levels of c-di-GMP in *argG* and *argH* mutants shown in Chapter 1.

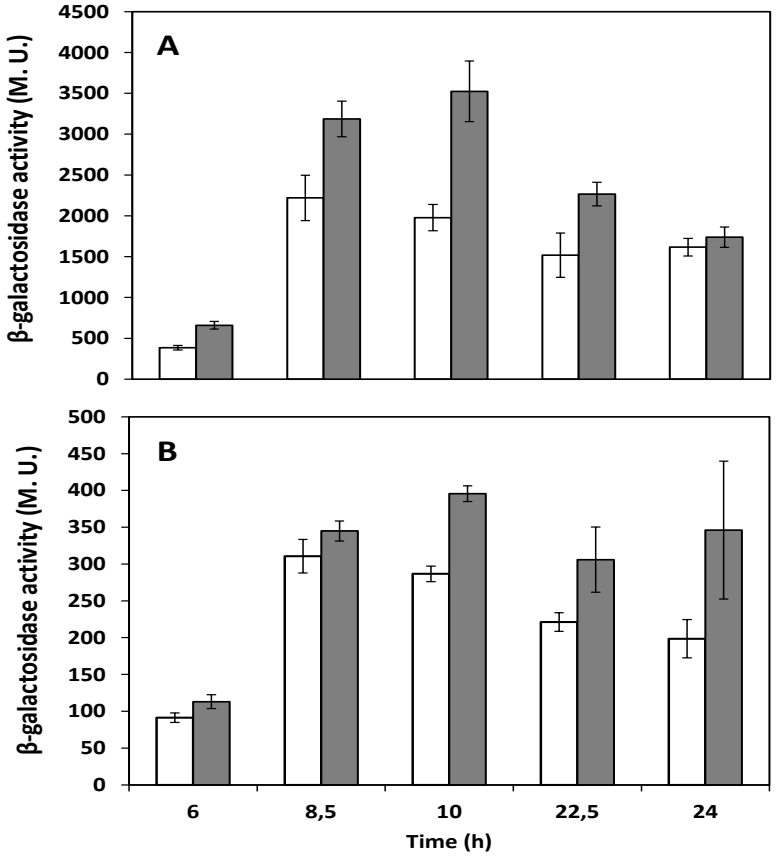


Figure 8. Expression of transcriptional (A) and translational (B) fusion of *cfcR* in the wild type strain (white bars) and *argR* mutant (grey bars). Cultures growing in LB supplied with Tc as described in Materials and Methods were analysed for β–galactosidase activity at indicated hours of growth. The experiment was made in duplicate with three technical repetitions each. Data correspond to averages and standard deviations from the two biological replicates. Statistically significant differences between the wild type and Δ*argR* were detected from 3h to 22,5h (A) and from 6h onward (B) (Student´s *t* test; $\rho \leq 0.05$).

ArgR is required for arginine-dependent modulation of c-di-GMP levels.

As shown in Chapter 1, the presence of L-arginine in the culture medium provokes an increase in the intracellular c-di-GMP pools. To test if ArgR plays a role in this response, plasmid pCdrA::*gfp*^{C} (Rybkte *et al*., 2012) was transferred to the *argR* mutant and fluorescence was measured using a TECAN Infinite 200 as previously described in Materials and Methods. As shown in Figure 9, the fluorescence profile of the mutant was similar to that of the wild type in the absence of exogenously added arginine, but the dose-dependent increase in c-di-GMP levels in the presence of arginine was almost completely abolished in the *argR* mutant. These results indicate that ArgR is involved in the response to L-arginine in relation to c-di-GMP levels.

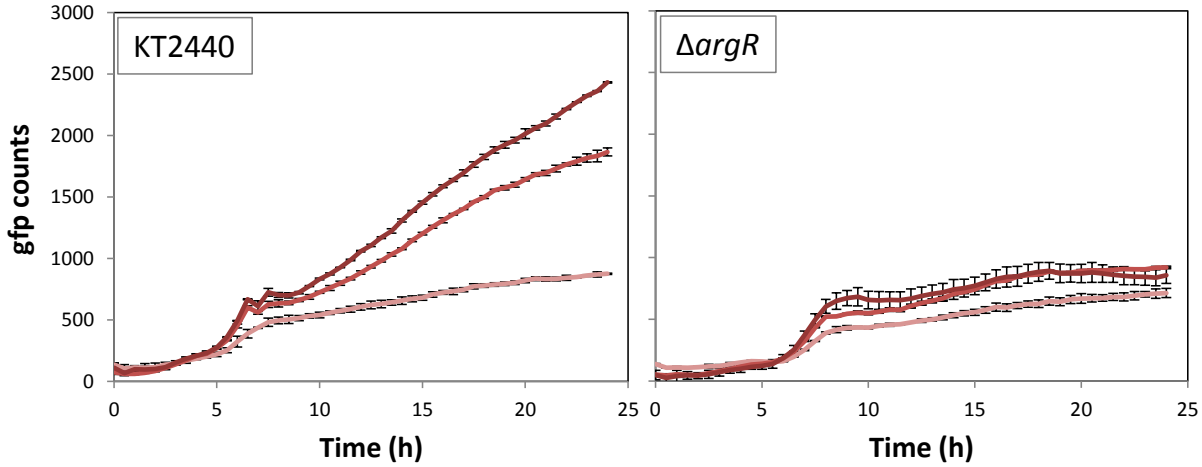


Figure 9. C-di-GMP free pool in KT2440 (left) and *argR* mutant (right) in response to arginine. GFP counts indicate fluorescence reading normalized with respect to growth in LB 1/3 (OD_{600nm}). Line colour intensity indicates increasing concentrations of the amino acid: 0 (pale pink), 5 (light red), and 15 mM (dark red). Experiments were made in triplicate and measurements for each strain and condition in triplicate using a TECAN Infinite 200 fluorimeter. Data are shown for one representative experiment.

Influence of L-arginine on c-di-GMP levels in different genetic backgrounds connected to the regulation of *cfcR*.

Expression of CfcR, and resulting c-di-GMP synthesis, is controlled by a complex regulatory cascade that includes the alternative sigma factor RpoS (Matilla *et al*., 2011), and the post-transcriptional regulatory proteins of the Rsm family (RsmA, RsmE and RsmI), which act directly on the mRNA of *cfcR* and indirectly through *rpoS* (Huertas-Rosales *et al*., 2017). Based on the above results and given the observed influence of arginine at certain concentrations on expression of *rpoS*, we next analysed if the response to arginine with respect to c-di-GMP levels was also altered in a *rpoS* mutant and in a triple *rsm* mutant, by introducing pCdrA*::gfp*^{C} in these strains and quantifying GFP fluorescence. Results are shown in Figure 10. As previously described (Huertas-Rosales *et al*., 2017a), the triple *rsm* mutant showed higher c-di-GMP levels than the wild type. These levels further increased in a dose-dependent manner by addition of arginine. In the *rpoS* mutant, on the other hand, c-di-GMP levels were lower than in the wild type, and the response to arginine was maintained but it was considerably less pronounced, in a way similar to what was observed for the *cfcR* mutant.

To further expand these data, double *rpoS/argR* and *cfcR/argR* mutants were generated, as described in Materials and Methods. Plasmid pCdrA*::gfp*^{C} was introduced in these strains and fluorescence was quantified as before. As shown in Figure 11, in both double mutants the c-di-GMP levels were almost completely undetectable using the bioreporter, and no response to arginine could be observed.

Several conclusions can be obtained from all these data: i) ArgR is the main element involved in the response to exogenous arginine in terms of c-di-GMP levels; ii) there is a cumulative effect of the *rpoS* and *argR* mutations, and the lack of both regulators severely reduces c-di-GMP levels in the conditions tested; iii) The lack of *cfcR* and *argR* also results in a severe reduction of c-di-GMP levels, suggesting that the regulatory role of RpoS is mainly through *cfcR* expression, while ArgR has some influence on this DGC and likely on other elements involved in c-di-GMP synthesis.

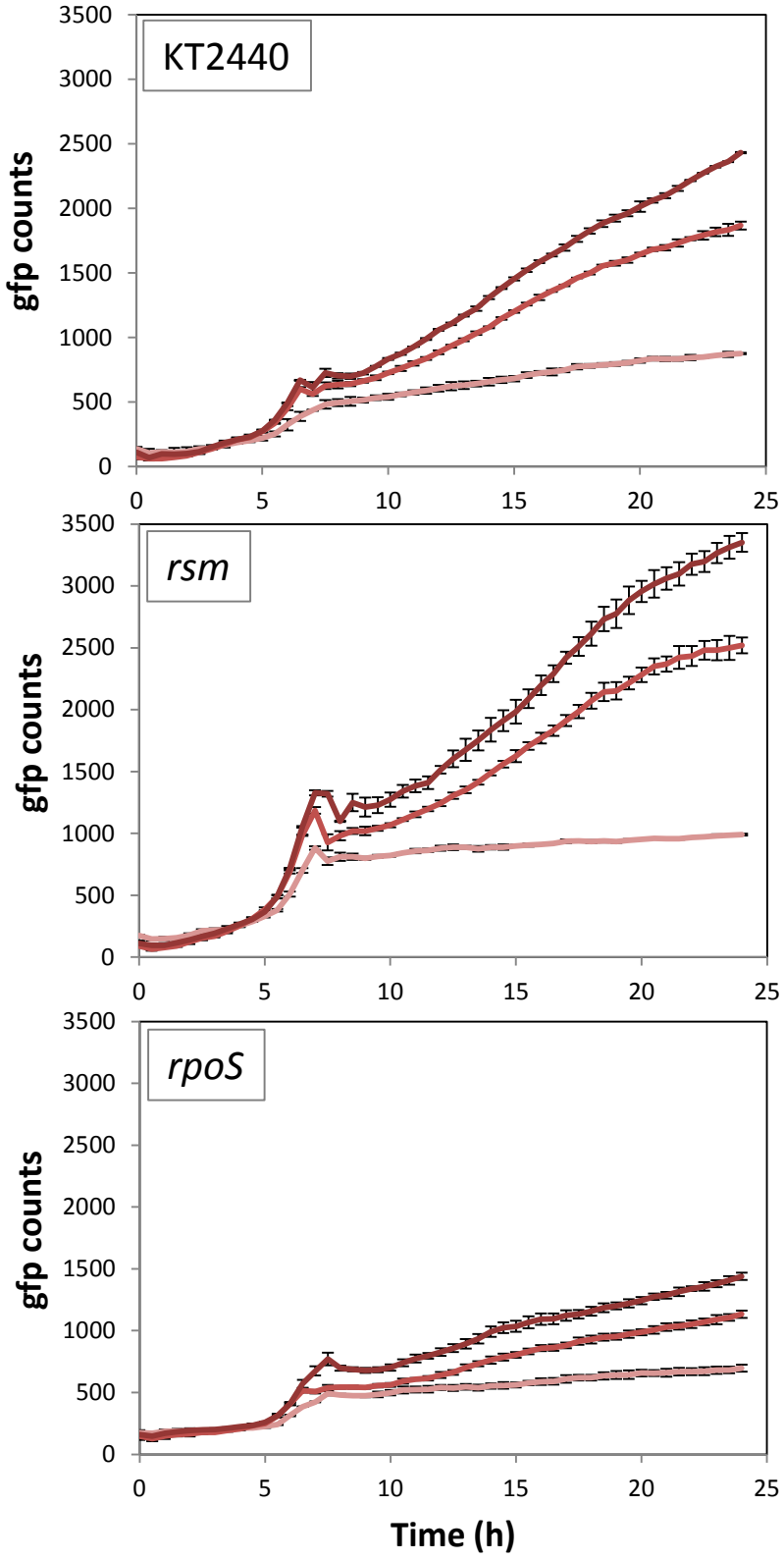


Figure 10. C-di-GMP free pool in KT2440, triple mutant *rsm* and *rpoS* mutant in response to arginine during 24h of growth in LB 1/3. GFP counts indicate fluorescence readings normalized with respect to growth (OD_{600nm}). Measurements were performed every 30 min. Line colour intensity indicates increasing concentrations of the amino acid: 0 (pale pink), 5 (light red), and 15 mM (dark red). Experiments were made in triplicate and measurements for each strain and condition in triplicate. Data are shown for one representative experiment. Measurements were made using a TECAN Infinite 200 fluorimeter.

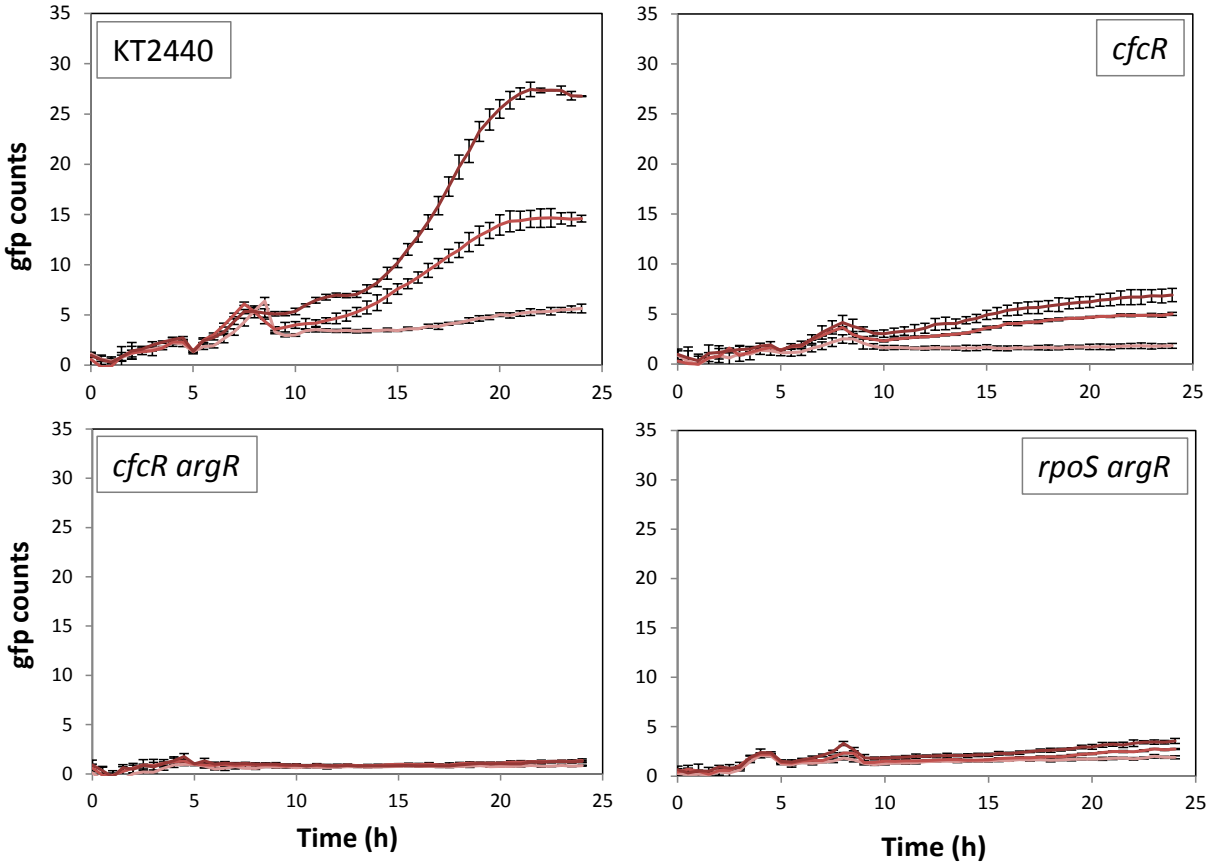


Figure 11. C-di-GMP free pool in KT2440 and double mutants Δ*cfcR*Δ*argR* and Δ*rpoS*Δ*argR* in response to arginine during 24h of growth in LB 1/3. The *cfcR* mutant is also included for comparison. GFP counts indicate fluorescence readings normalized with respect to growth (OD_{600nm}). Measurements were performed every 30 min. Line colour intensity indicates increasing concentrations of the amino acid: 0 (pale pink), 5 (light red), and 15 mM (dark red). Measurements were made using a Varioskan Lux plate reader.

ArgT is the main arginine binding protein implicated in L-arginine uptake in *P. putida* KT2440.

Searching for proteins showing similarities with the arginine binding protein ArtI of *S. enterica* serovar Typhimurium, which is involved in sensing extracellular arginine and is related with the DGC STM1987, causing an increase in c-di-GMP content in the cell (Mills *et al*., 2015), we identified three putative L-arginine binding protein as part of ABC transport systems. Genes which encode these putative binding proteins were *argT* (PP_4486), *artJ* (PP_0282) and locus PP_3593. As shown before, *argT* is the first gene in a cluster that includes *argR*.

To determine the potential role of these proteins in arginine transport, mutants were either generated by deletion of the ORF, or were obtained from the *Pseudomonas* Reference Culture Collection, when available. Growth of the mutants in minimal medium with L-arginine as the sole carbon source was evaluated. As shown in Figure 12, the *artJ* mutant showed somewhat longer lag phase than the wild type strain, but was able to grow in this medium, suggesting a minor role in the arginine uptake, while the mutant in PP_3593 behaved almost as the wild type. On the other hand, the *argT* mutant showed a lag phase twice as long as that of the wild type, although after 24 h of growth it reached similar turbidity. These results indicate that the substrate-binding protein ArgT is the most important for the L-arginine uptake in *P. putida* KT2440, although it is not the only one. Further analyses of growth of the mutants using different amino acids as carbon sources indicated that PP_3593 participates specifically in lysine transport, whereas ArtJ seems to be involved in non-specific transport of positively charged amino acids (data not shown).

ArgT is required for the arginine-dependent increase in c-di-GMP.

None of these proteins seems to be associated with any DGC, as it occurs in *Salmonella*, but we decided to check if the *artJ* and *argT* mutants were impaired in c-di-GMP free pool content in the cell. For this purpose, reporter plasmid pCdrA*::gfp*^{C} was introduced in the mutants. Cultures were grown by drop-plating on LB-agar plates for 24 h and fluorescence was visualised. As presented in Figure 13, *artJ* and *argT* showed reduced c-di-GMP content with regard to the wild type strain. When arginine was added into the culture medium, c-di-GMP content rised in the wild type and *artJ* mutant but the response was completely abolished in the *argT* mutant, further supporting its importance in arginine transport.

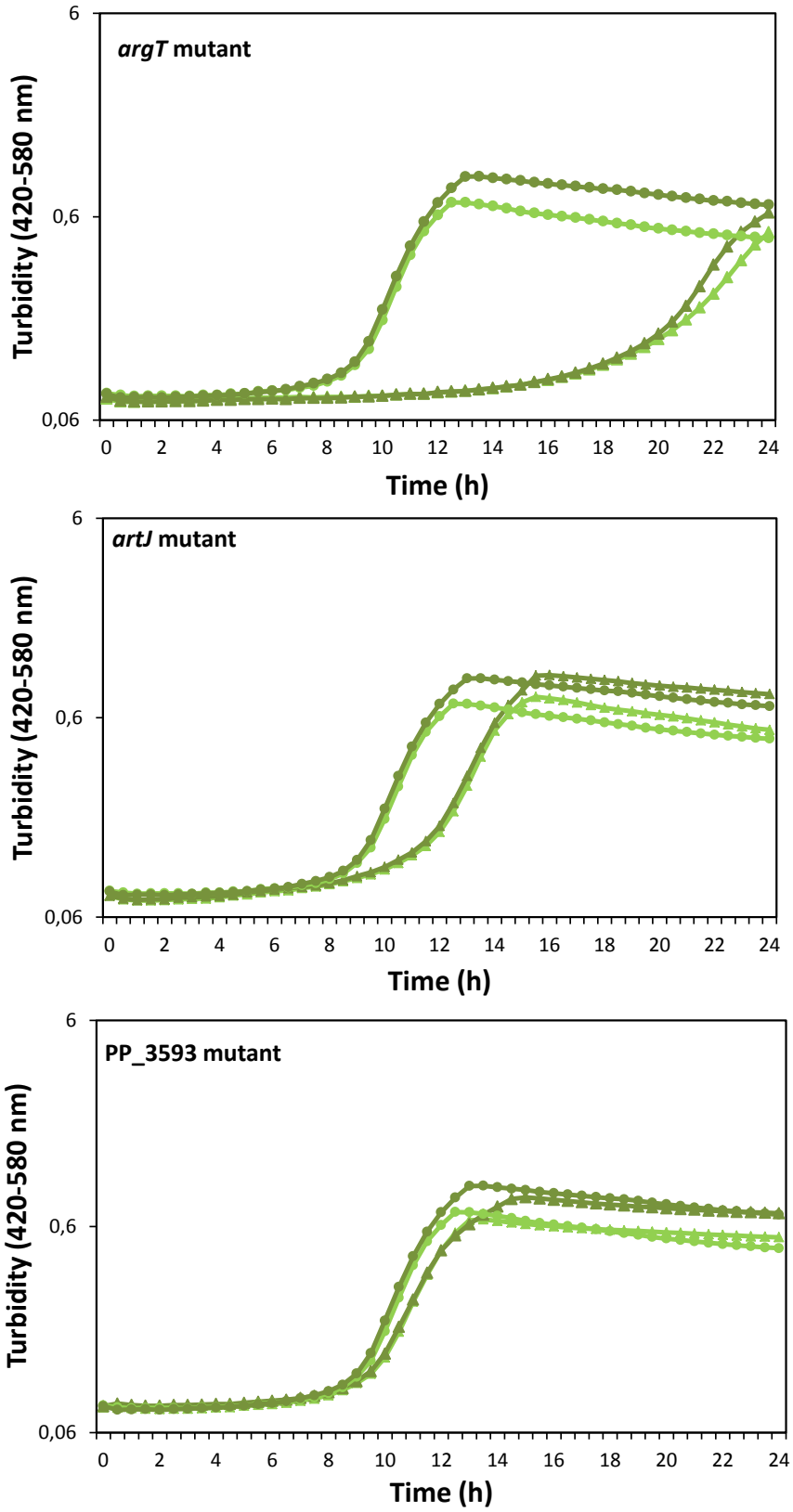


Figure 12. Growth curves of *argT*, *artJ* and locus PP_3593 mutants in minimal medium with L-arginine as a carbon source. Wild type strain (circles) and mutants (triangles) were grown at 10 mM (light green) and 15 mM (dark green) of L-arginine and turbidity was measured every 30 min at 30°C during 24h in a Bioscreen. Average growth curves of each strain are shown.

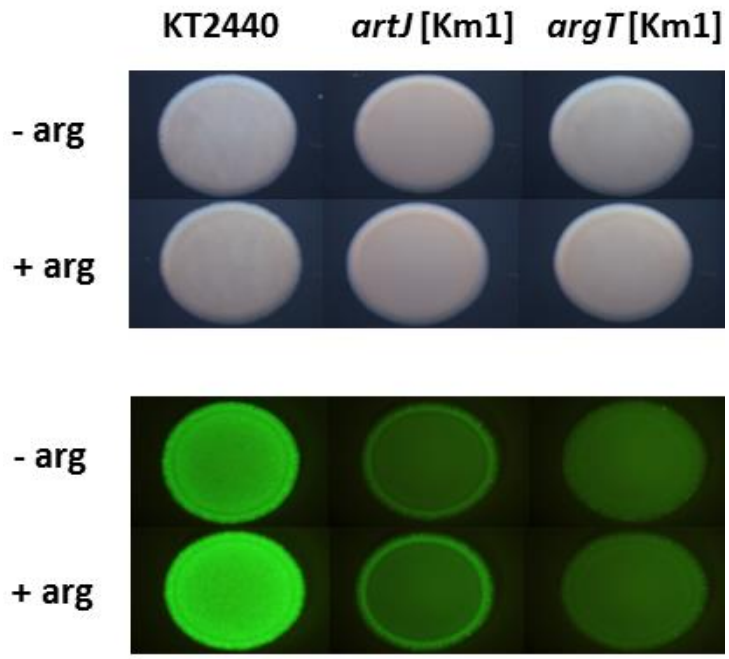


Figure 13. Fluorescence related to c-di-GMP content in different strains of *P. putida* KT2440 harbouring the biosensor plasmid pCdrA*::gfp*^{C}. LB-agar plates with 5 mM (indicated as + arg) or without (indicated as -arg) L-arginine were incubated at 30°C for 24h. Pictures of the visible field (above) were taken using a Fluorescent Stereo Microscope Leica M165 FC (Leica Microsystems). For dark field pictures (below), an excitation/emission filter 480/510 nm was used for monitoring GFP fluorescence with an exposure time of 519.7 ms.

Arginine and ArgR modulate expression of *argT* and *argR*.

Based on the genetic organization, we decided to determine if *argR* was forming an operon with the upstream genes, and to verify if *argT* and *hisQMP* genes (which we propose to re-annotate according to the similarity with *P. aeruginosa*) were forming an operon too. Reverse transcription coupled with PCR reaction (RT-PCR) was performed using RNA extracted from *P. putida* KT2440 cultures grown in minimal medium with glucose or L-arginine as carbon sources. Electrophoresis of the reaction products showed a band of the expected size corresponding to the cotranscription of *argT* and *hisQ* in all conditions tested, confirming the existence of a single transcript for *argT-hisQMP* genes (Figure 14). However, no cotranscription was observed for the region between *argR* and *hisP* in RNA obtained from cultures growing in glucose, whereas a band was observed with arginine (Figure 14), in the presence of the amino acid, indicating that arginine modulates transcription of *argR* as well as termination and/or stability of the mRNA corresponding to the whole gene cluster.

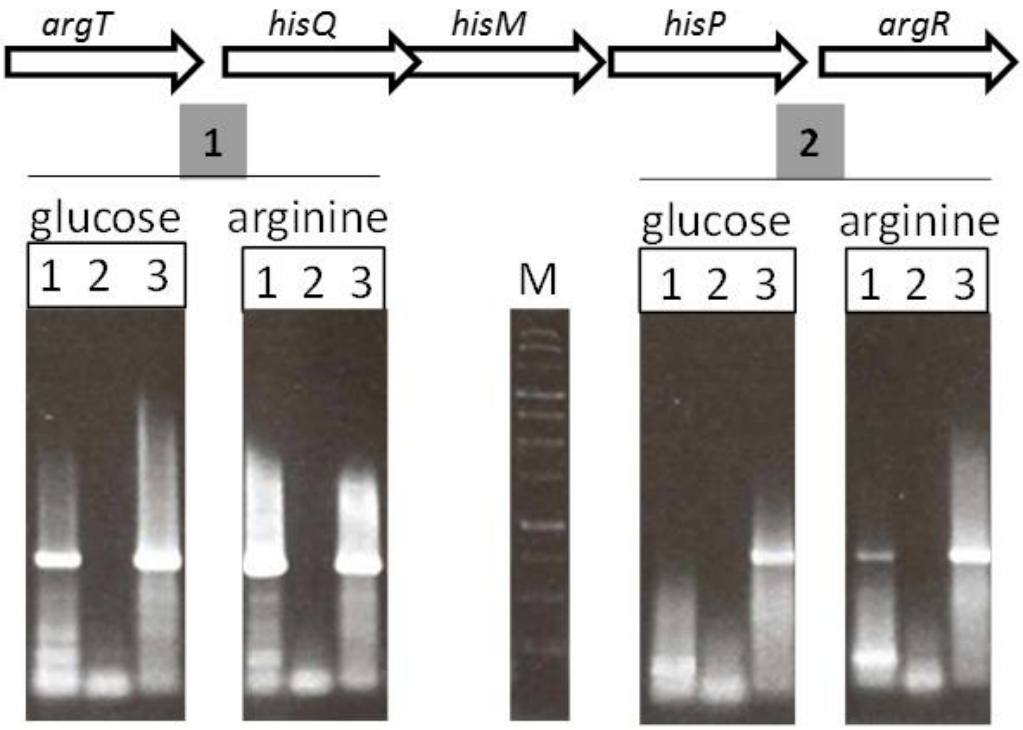


Figure 14. Electrophoresis of RT-PCR products amplified with primers designed to detect transcripts containing the intergenic regions between *argT* and *hisQ* (761 pb) and between *hisP* and *argR* (783 pb). 1: Template RNA obtained from KT2440 cultures; 2: Negative control without the reverse transcriptase; 3: Positive control using DNA as template of the reaction. Template RNA was obtained from KT2440 cultures grown to an OD_{660} of 0.3 in M9 with glucose or arginine as carbon source.

We next analysed if transcription of *argR* was solely dependent on the arginine-dependent cotranscription with preceding genes or if the intergenic region upstream of *argR* might contain an additional promoter. This intergenic region was cloned in pMP220 to generate a transcriptional fusion of that region to the reporter gene *´lacZ*, as described in Materials and Methods. Activity of this fusion was assessed in minimal medium with glucose and different concentrations of L-arginine. As shown in Figure 15, the fusion showed β-galactosidase activity in the absence of arginine, and this activity increased in the presence of arginine. This result shows that *argR* can be expressed from an arginine-inducible promoter in the intergenic region upstream the gene. This contrasts with results reported for *P. aeruginosa,* where *argR* gene is autoregulated from a single arginine-responsive promoter upstream *aotJ* (Nishijyo *et al*. 1998).

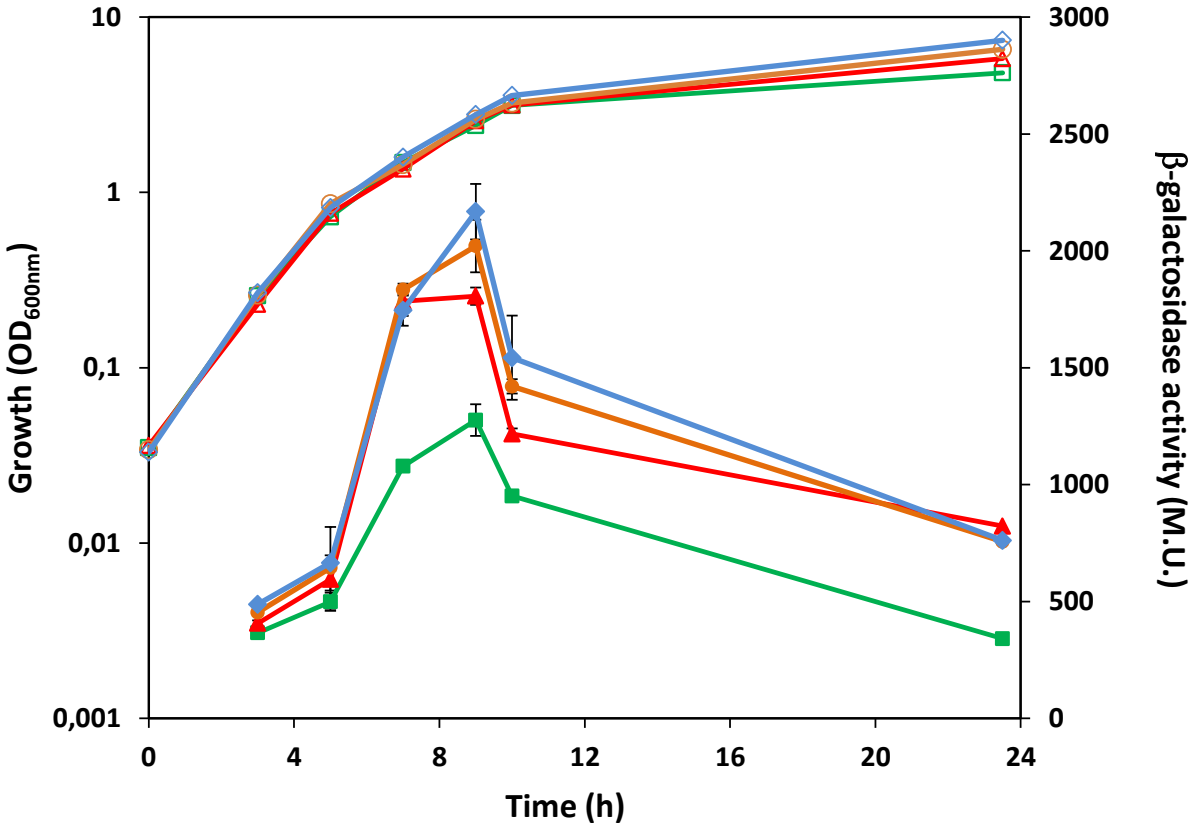


Figure 15. Expression of *argR::´lacZ* transcriptional fusion in KT2440 during growth in minimal medium with glucose and different concentrations of L-arginine. Cultures were analysed for turbidity (hollow symbols) and β–galactosidase activities (solid symbols) at the indicated times. Data for 0 mM (green lines), 5 mM (red lines), 15 mM (orange lines) and 25 mM (blue lines) of arginine are shown. Statistically significant differences between absence and the different concentrations of L-arginine were detected from 5h onward (Student´s *t* test $\rho \leq 0.05$).

To test if the arginine response of the *argR* promoter was mediated by ArgR, the activity of the transcriptional fusion was measured at different times in KT2440 and the *argR* mutant, in the presence or absence of arginine (Figure 16A). Results show a complex situation: early during growth there was no difference between strains. At 5 h, arginine increased expression in the wild type to a level similar to that observed in the mutant regardless of the medium; at 7 h, expression was higher in the mutant in both conditions; once into stationary phase, the mutant maintained higher expression levels than the wild type in the presence of arginine. These data suggest that ArgR represses its own promoter, but additional elements may also be involved in its regulation.

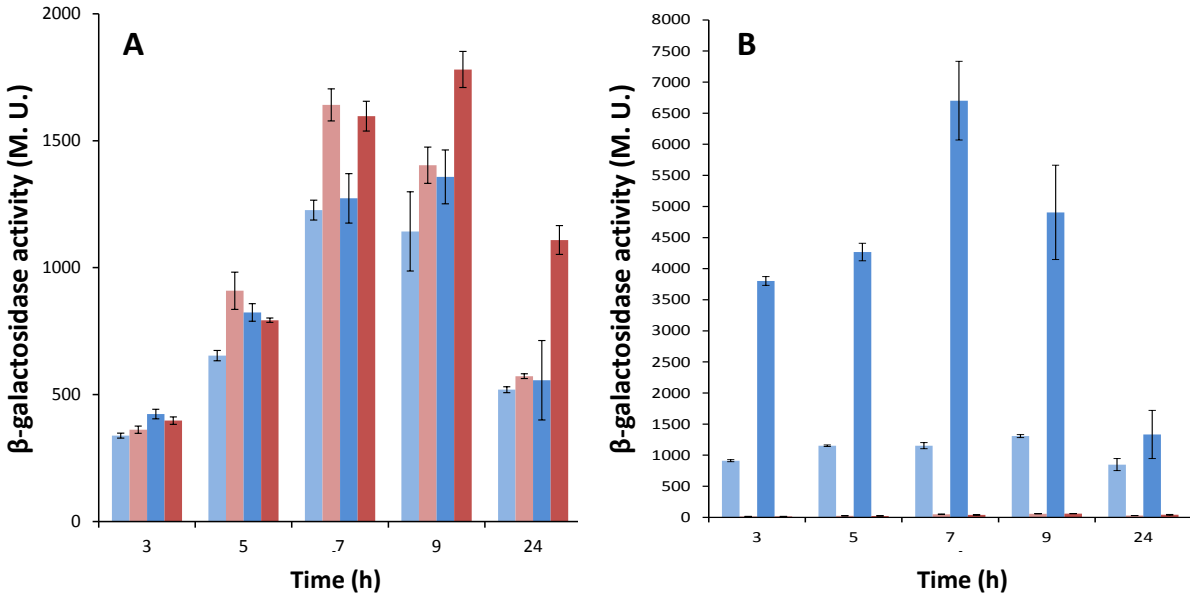


Figure 16. Expression of *argR::´lacZ* (**A**) and *argT::´lacZ* (**B**) in KT2440 (blue) and the *argR* mutant (red) during growth in minimal medium with glucose (light bars) or glucose and 10 mM arginine (dark bars). The experiment was made in duplicate with three technical repetitions each. Data correspond to one representative experiment.

To determine if the promoter region upstream *argT* showed also regulation by arginine and/or ArgR, a fusion of this region to *´lacZ* was also constructed in pMP220, as described in Materials and Methods, introduced in KT2440 and the *argR* mutant, and β–galactosidase activity was measured as above, in the absence or presence of L-arginine. Results are shown in Figure 16B. In this case, activity was completely abolished in the *argR* mutant, regardless of the growth medium. In the wild type strain, on the other hand, L-arginine caused a large increase in activity throughout the growth curve that could explain the RT-PCR data, whereas it remained more or less constant in the absence of the amino acid. These results indicate that L-arginine in the growth medium stimulates expression of its dedicated transporter, and that ArgR is strictly required for this response, which once more is different to what has been described in *P. aeruginosa*.

RpoS and ArgR establish a negative regulatory feedback loop.

Results presented so far indicate a role of ArgR in c-di-GMP regulation. Results in Figure 8 indicate that it modulates expression of *cfcR*, which is also under the control of RpoS, as mentioned previously. We therefore tested if there is a regulatory connection between both elements. For that purpose, plasmid pMAMV21, which harbours a *rpoS´-`lacZ* translational fusion, was introduced in KT2440 and the *argR* mutant and its expression was analysed in the presence of 5 mM L-arginine. Results presented in Figure 17 indicate that ArgR has a negative influence on expression of *rpoS* upon entry in stationary phase, an effect that is not observed at later times. This effect is only observed in the presence of arginine and can vary in intensity depending on the concentration (data not shown). Thus, ArgR emerges as an element that modulates the timing of RpoS synthesis in response to exogenous arginine.

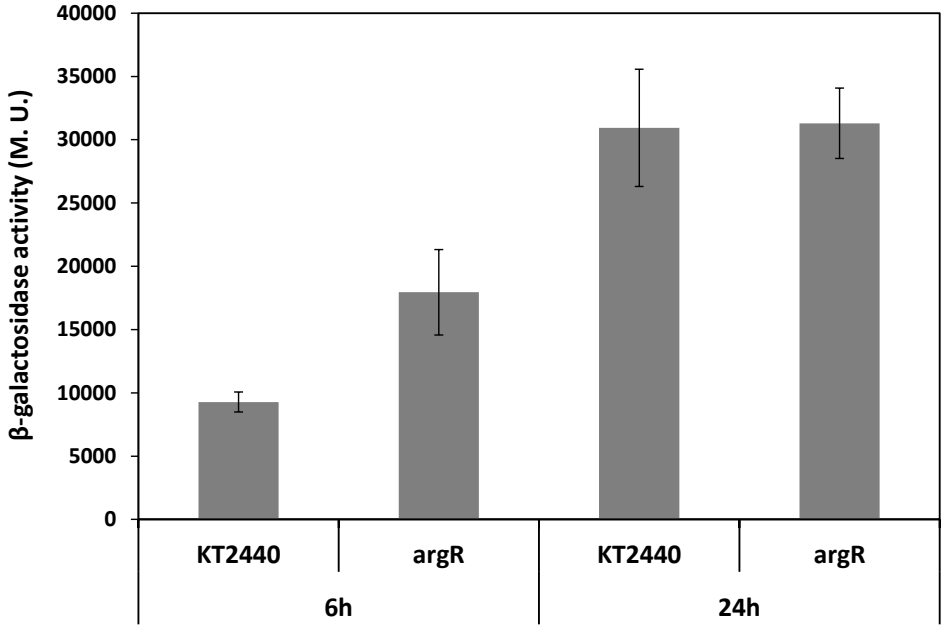


Figure 17. Expression of *rpoS´-`lacZ* in KT2440 and the *argR* mutant at different times of growth in minimal medium with glucose and 5 mM arginine. The experiment was made in duplicate with three technical repetitions each. Data correspond to averages and standard deviations from the two biological replicates. Statistically significant differences between the wild type and Δ*argR* were detected at 6h (Student´s *t* test $\rho \leq 0.05$).

On the other hand, the expression pattern of *argR* presented in Figures 15 and 16 was intriguing, since it showed an increase during growth and a sudden drop upon entry into

stationary phase and the potential existence of different regulators. One possible explanation could be that the *argR* promoter was itself under the control of the stationary phase sigma factor RpoS, and the increase in ArgR caused this drop in its own expression; alternatively, additional regulatory elements could contribute to silencing in stationary phase. We therefore analysed β-galactosidase activity of the *argR::´lacZ* fusion in KT2440 and the *rpoS* mutant. As shown in Figure 18, RpoS has a negative influence on *argR* expression in the presence of exogenous L-arginine, the mutant exhibiting higher expression levels than the wild type at all time points. This effect is likely indirect, being RpoS a sigma factor.

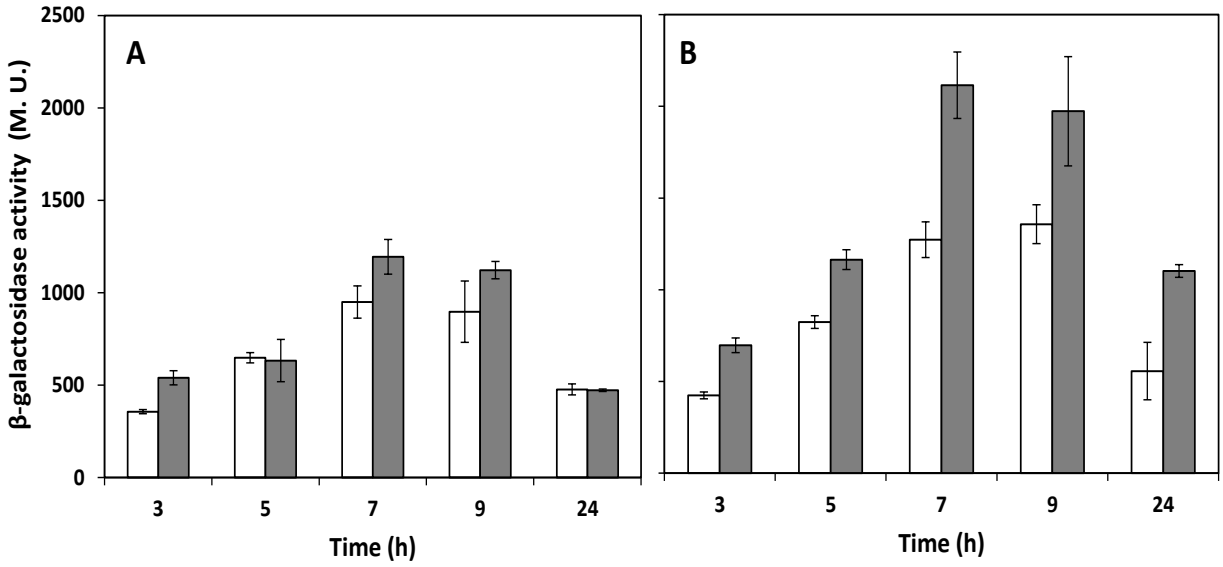


Figure 18. Expression of *argR::lacZ* in KT2440 (white bars) and the *rpoS* mutant (grey bars) at different times of growth in minimal medium with glucose and without (**A**) or with (**B**) 10 mM arginine. The experiment was made in quadruplicate with three technical replicates each. Data are shown for one representative experiment.

Rsm proteins influence expression of arginine-related genes.

Previous work has established that *rpoS* expression is modulated by the Gac/Rsm cascade (Huertas-Rosales *et al*., 2017a). Furthermore, a global analysis of the Rsm regulon in *P. putida* KT2440, suggested that *argG* might be a target for post-transcriptional modulation by Rsm proteins, specifically RsmA and RsmE (Huertas-Rosales, PhD Thesis, 2017b). This fact prompted us to investigate if these proteins exert some regulatory role on the expression of *argG* and *argH* genes. For that purpose, transcriptional and translational fusions of *argG* and

argH to ´*lacZ* were introduced in KT2440 and the triple *rsm* mutant and β-galactosidase activities were measured during growth in liquid medium.

The results obtained comparing the wild type and the triple mutant *rsm* are shown in Figure 19. The pattern of expression of *argH* did not show relevant differences between the wild type and the triple mutant *rsm*, neither at transcriptional nor translational level. However, the expression pattern of *argG* exhibited alterations at different times, being particularly clear in the translational fusion, which showed reduced activity in the triple mutant *rsm* with regard to the wild type, being the differences significant from 8h onward (Figure 19B). We tested the expression of translational fusion of *argG* in single and double *rsm* mutants and the major influence upon *argG* expression was observed when *rsmA* was mutated or in combination with the lack of *rsmE* (Figure Supplementary 1).

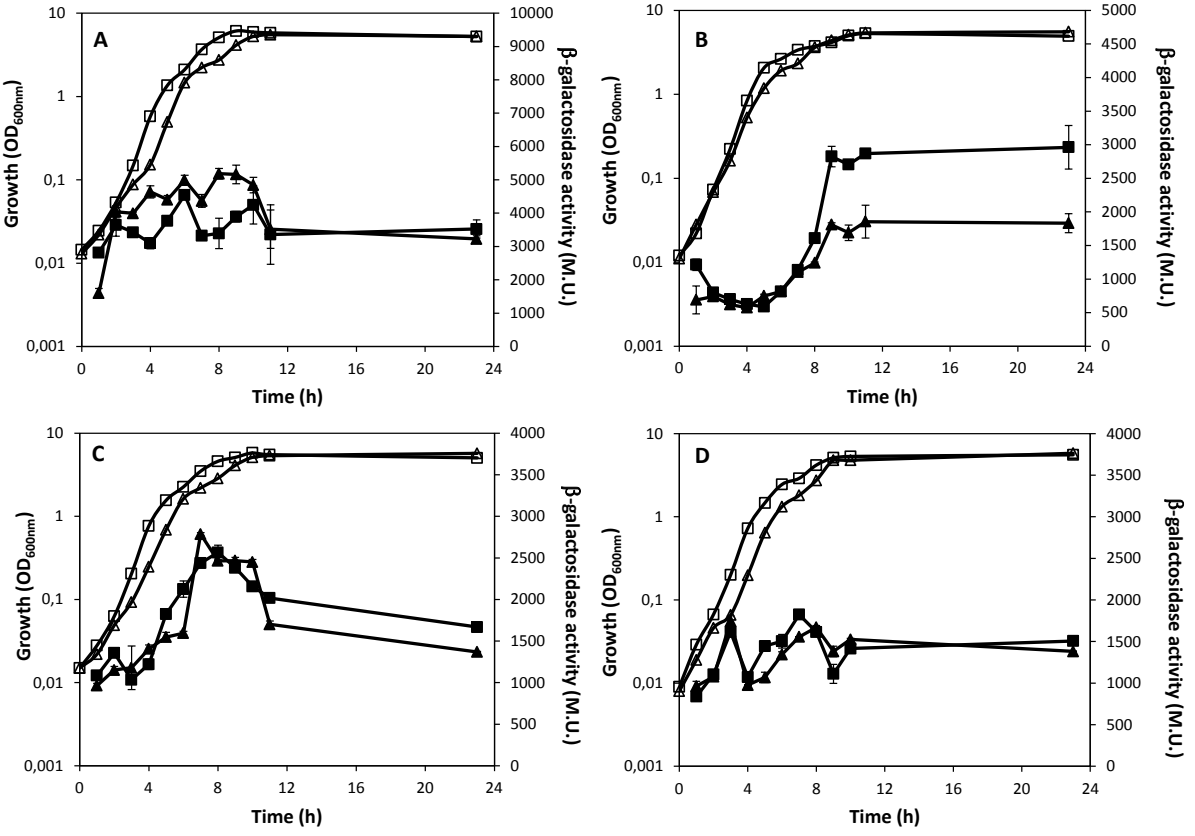


Figure 19. Activity of the transcriptional and translational fusion of *argG* and *argH* in triple mutant *rsm*. Cultures growing in LB supplied with Tc as described in Material and Methods were analysed for turbidity (hollow) and β–galactosidase activities (solid) at the indicated times. Data for the wild type (square) and the triple mutant *rsm* (triangle) are shown. A) transcriptional and B) translational fusion of *argG* to ´*lacZ*. C) transcriptional and D) translational fusion of *argH* to ´*lacZ*. Experiment was performed in duplicate with two technical repetitions each. A representative graph is presented.

This result was somewhat surprising, since Rsm proteins generally function as negative regulators. We therefore analysed if there was direct interaction between Rsm proteins and the *argG* transcript. Four possible binding sites of Rsm proteins have been found in the sequence of *argG*, one located in the promoter very close to the initiation codon (ATG), another after the ATG, and the other two inside the open reading frame (Figure Supplementary 2A). To enclose these putative binding sites, two different transcripts of RNA-*argG* were obtained using *in vitro* transcription as indicated in experimental procedures (Figure Supplementary 2B). The first transcript covered the putative binding sites in the promoter region and after the ATG and the second transcript the two putative binding sites located inside the gene. To test the *in vitro* interaction between Rsm proteins and *argG* transcripts, we performed fluorescence-based electrophoretic mobility shift assays (fEMSA). Fixed quantities of each transcript (10 nM) labelled with a fluorescent DNA-probe were incubated with increasing concentrations (0-1000 nM) of His-tagged Rsm (RsmA and RsmE) proteins, previously designed (Huertas-Rosales *et al*., 2016) and the electrophoretic mobility of the complexes was analysed in native TBE polyacrylamide gels. As shown in Figure 20, no binding of RsmA or RsmE with was observed, indicating that the regulation by Rsm proteins upon *argG* is likely indirect.

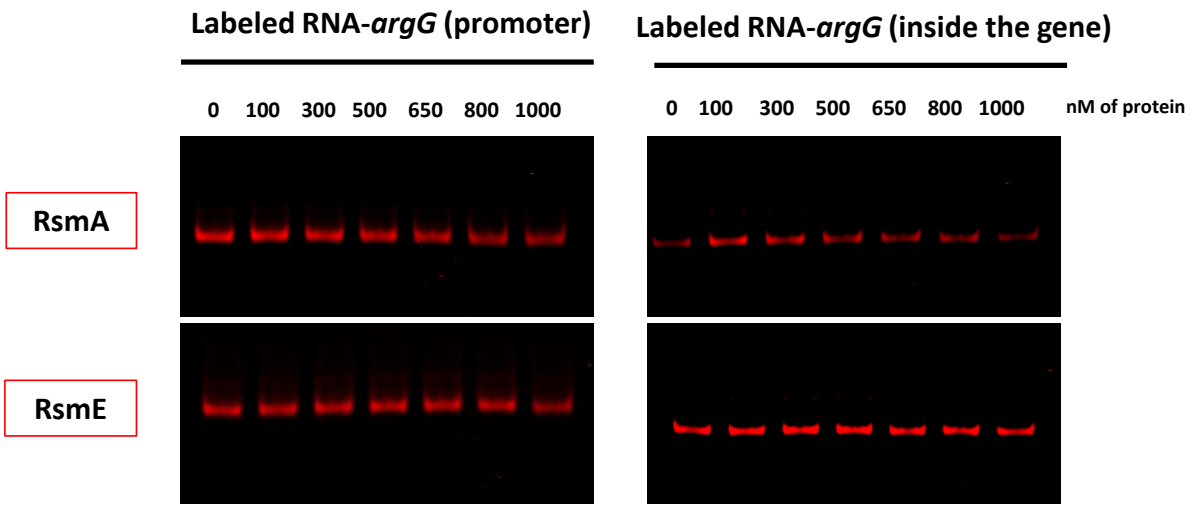


Figure 20. Fluorescence-based fEMSA of RsmA and RsmE upon RNA-*argG* transcripts. Two labeled RNA-*argG* were synthesised, one in the promoter close to the ATG start codon and other inside the gene. 10 nM of RNA was used in the assays.

We wondered if the effect of Rsm proteins could be through ArgR: since ArgR acts as negative regulator of *argG*, negative regulation of ArgR expression by RsmA would indirectly result in the observed positive effect. To test this possibility we analysed expression of a translational *argR'-'lacZ* fusion in the *ΔΔΔrsm* background. Surprisingly, the observed activity was lower in the mutant than in the wild type at all times tested (Figure 21) indicating a positive influence of Rsm proteins and suggesting additional, interposed elements in ArgR regulation.

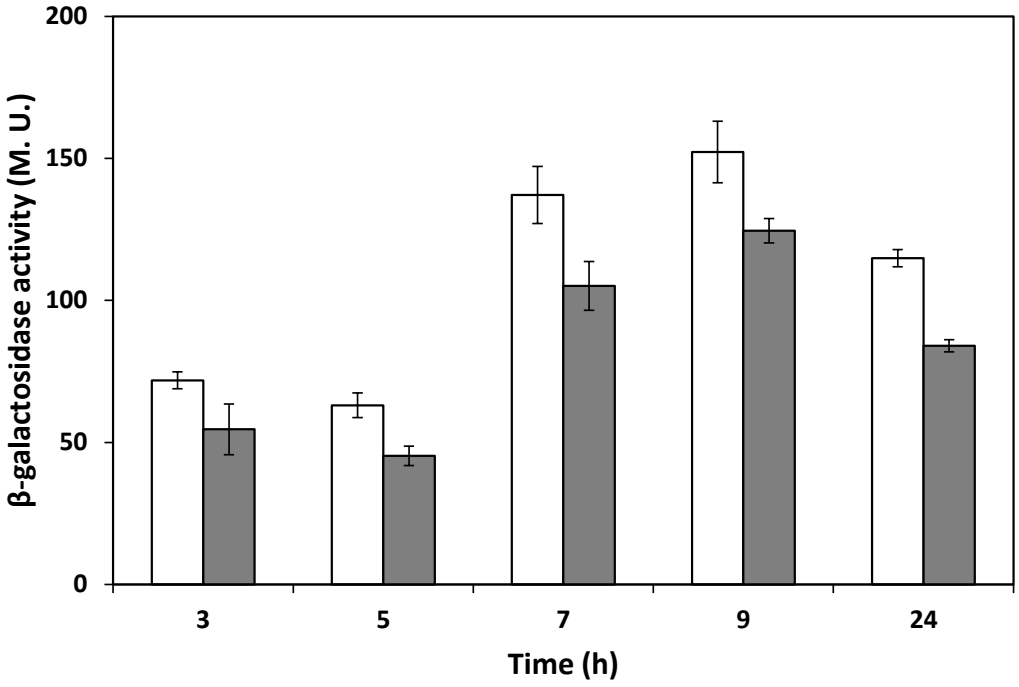


Figure 21. Expression of *argR'-'lacZ* in KT2440 (white bars) and the *rsm* mutant (grey bars) at different times of growth. Cultures were grown in minimal medium supplied with Tc as described in Materials and Methods. Data correspond to averages and standard deviations from two biological replicates with three technical repetitions each. Statistically significant differences between the wild type and triple *rsm* mutant were detected at all times assayed (Student´s *t* test $\rho \leq 0.05$).

c-di-GMP influences *argR* expression through the transcriptional regulator FleQ.

All the above data were conflicting with current knowledge, since Rsm proteins generally function as negative regulators and RpoS as a positive factor, and called for the identification of additional elements linking the mentioned ones. We considered the

possibility that c-di-GMP, the final element in this complex regulatory network, could play a role as a feedback modulator of its own synthesis. Hence, the potential modulation of *argR* expression by the second messenger was tested by transferring the *argR´-´lacZ* fusion to *P. putida* KT2440 harbouring pMIR178. This plasmid harbours the gene that encodes the diguanylate cyclase CfcR and confers high levels of c-di-GMP (Matilla *et al*., 2011; Ramos-González *et al*., 2016). As shown in Figure 22, expression of *argR* was around 2-fold higher in presence of high c-di-GMP levels in comparison to basal content of c-di-GMP, indicating that the second messenger modulates positively the expression of *argR*.

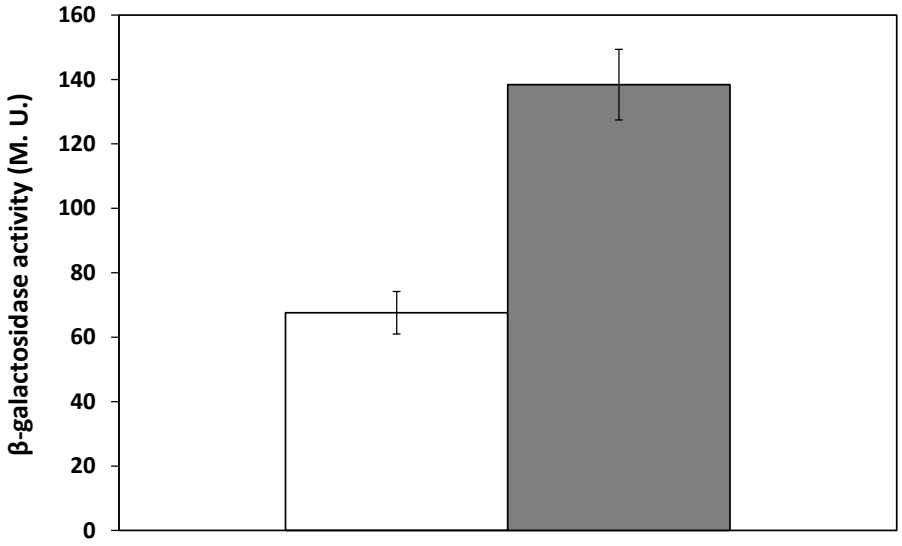


Figure 22. Expression of *argR'-'lacZ* in cultures of KT2440 harbouring the empty vector pBBR1-MCS2 (white bars) or pMIR178 (grey bars) after 25h of growth. Data correspond to averages and standard deviations from two biological replicates with three technical repetitions each. Statistically significant differences between the absence and presence of pMIR178 were detected (Student´s *t* test $\rho \leq 0.05$).

The transcriptional regulator FleQ has been shown to be a key element in the transduction of the c-di-GMP signal (Hickman *et al*., 2005; Martínez-Gil *et al*., 2014; Dahlstrom *et al*., 2018), through direct interaction with the second messenger (Jiménez-Fernández *et al*., 2016; Molina-Henares *et al*., 2017). To check if FleQ could play a role in *argR* regulation, transcriptional and translational fusions of *argR* were transferred to the wild type and cfcK-77 mutant, which is a *fleQ* mutant derivative of KT2440 obtained by mini-Tn5[Km1] insertion (Ramos-González *et al*., 2016). As illustrated in Figure 23, expression of

argR was significantly reduced in *fleQ* mutant in comparison to the wild type, both transcriptional and translational levels, being the effect higher in transcriptional fusion due to FleQ is a transcriptional regulator. So, we can conclude that FleQ influences positively *argR* expression. In fact, in the promoter region of *argR* it seems to be a putative target for FleQ (Figure Supplementary 3).

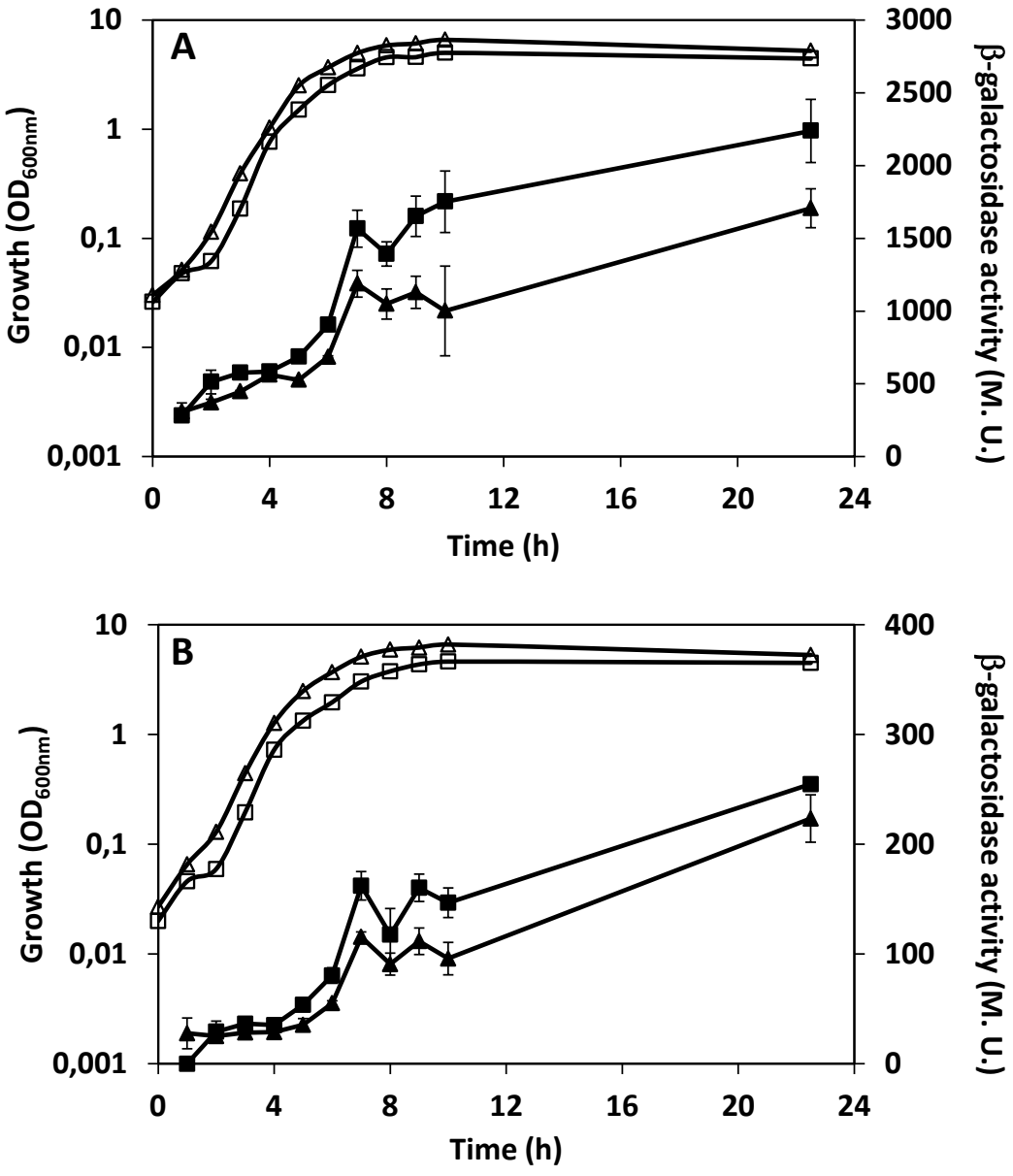


Figure 23. Activity of the transcriptional (A) and translational (B) fusions of *argR* in the wild type and *fleQ* mutant. Cultures growing in LB supplied with Tc as described in experimental procedures were analysed for turbidity (hollow symbols) and β–galactosidase activities (solid symbols) at the indicated times. Data for the wild type (square) and *fleQ* mutant (triangle) are shown. The experiment was made in duplicate with three technical repetitions each. A representative graph is presented.

To confirm that the role of FleQ on *argR* regulation is through c-di-GMP, we repeated the experiment shown in Figure 22 comparing the activity of the *argR`-´lacZ* fusion in KT2240 and the *fleQ* mutant. In this case, plasmid pMAMV1 (Matilla *et al*., 2011) was used because of the resistance incompatibility between plasmid pMIR178 and transposon insertion in *fleQ*, both harbouring kanamycin resistance cassettes. The results presented in Figure 24 show that the increase in expression of *argR`-´lacZ* in high concentrations of c-di-GMP did not take place in the *fleQ* mutant, indicating that FleQ is required for c-di-GMP-dependent activation of the *argR* expression.

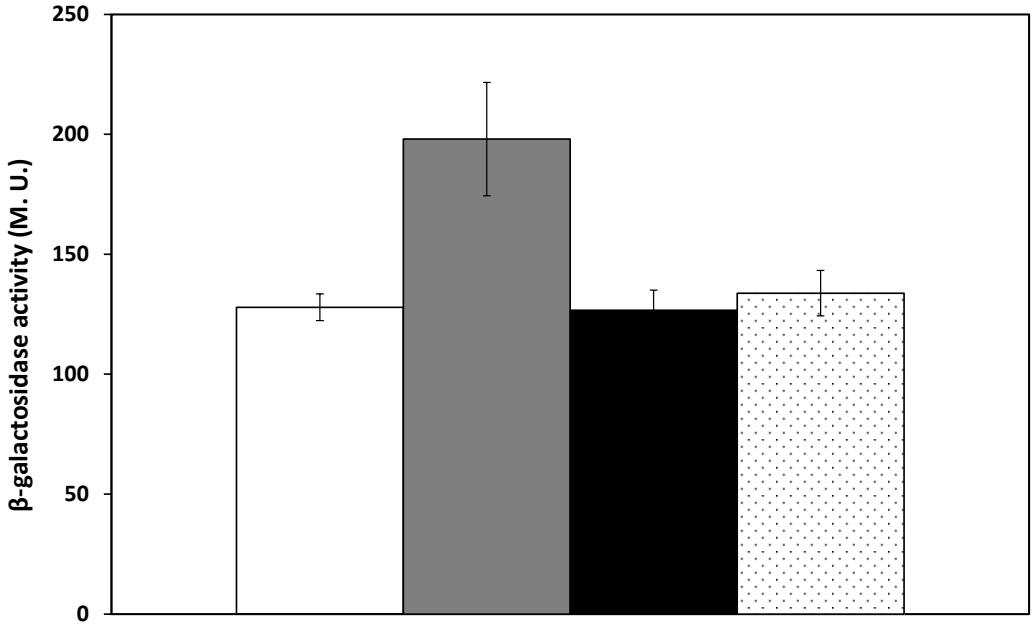


Figure 24. Expression of translational fusion of *argR* in the wild type strain in the absence (white bars) and presence (grey bars) of pMAMV1 and *fleQ* mutant in the absence (black bars) and presence (dotted bars) of pMAMV1. Cultures growing in LB supplied with Tc and Km as described in Materials and Methods were analysed for β–galactosidase activity at 6 and 24 hours of growth. The experiment was made in quadruplicate with three technical repetitions each. Data correspond to averages and standard deviations from two biological replicates with three technical repetitions each. Statistically significant differences between KT2440 harbouring pMAMV1 and the other strains were detected (Student´s *t* test $\rho \leq 0.05$).

DISCUSSION

In the last years, the amino acid arginine has gained increasing relevance due to its effect upon biofilm formation in different bacteria, acting as environmental cue (Bernier *et al*., 2011; Jakubovics *et al*., 2015). It has been reported that the effects of this amino acid can be both positive or negative depending on the microorganism and the concentrations used to test its impact on biofilms (Kolderman *et al*., 2015; Zheng *et al*., 2017; Huang *et al*., 2017). Nonetheless, little is known about the connection between arginine and the intracellular second messenger c-di-GMP. Recently, it has been reported that arginine increased c-di-GMP levels in *P. aeruginosa* PA14 and this increase is dependent on the SadC and RoeA diguanylate cyclases (Bernier *et al*., 2011). Furthermore, it has been proposed that the arginine-binding protein ArtI may bind arginine and through an unknown mechanism so far, it would activate the GGDEF domain of STM1987 diguanylate cyclase in *Salmonella enterica* serovar Typhimurium (Mills *et al*., 2015), leading to increased c-di-GMP content in the cell. In this work, we provide some evidences of the connection between arginine metabolism and signalling mediated by the intracellular second messenger c-di-GMP, and the influence of arginine biosynthesis on some structural components of the biofilm matrix in *P. putida* KT2440.

We have demonstrated that mutants in *argG* and *argH* genes show altered expression of exopolysaccharides, with a slight but significant increase in the expression of *peb* and *bcs* in the late stationary phase, whereas the expression of *pea* is highly reduced in both mutants. This correlates positively with the fact that *argG* and *argH* mutants do not show crinkly phenotype as shown in Chapter 1 and with the results previously obtained in our group demonstrating that EPS Pea is essential to acquire crinkly phenotype in *P. putida* KT2440 (Matilla *et al*., 2011). However, there is no indication that arginine might be present in Pea structure (Nielsen *et al*., 2011). This, along with the fact that we have demonstrated that arginine positively influences *pea* expression in stationary phase of growth (Figure 3), indicates that this amino acid might act as a signal molecule that influences other elements involved in the regulation of this exopolysaccharide. Recently, a study has demonstrated that *pea* promoter expression shows minor but positive regulation by the transcriptional regulator FleQ. FleQ binds to *pea* promoter *in vitro*, and binding is favoured by c-di-GMP (Molina-Henares *et al*., 2017). The expression pattern of *pea* pointed to the possibility that

the alternative sigma factor RpoS could be involved in its regulation. Here, we showed the first evidence that RpoS controls expression of *pea* in *P. putida* KT2440. This sigma factor regulates many genes in stationary phase, and positively influences EPS production in *Burkholderia pseudomallei* (Mongkolrob *et al*., 2015), *Yersinia pseudotuberculosis* (Guan *et al*., 2015) and *P. aeruginosa* (Irie *et al*., 2010), although it also has a negative impact on the expression of *wca* and *yjb* operons, related with the production of different EPS in *E. coli* (Ionescu and Belkin, 2009). A previous study showed that expression of the adhesin LapF is also under the control of RpoS in *P. putida* (Martínez-Gil *et al*., 2010), indicating the active role of RpoS on regulating structural elements of biofilm matrix in this bacterium. Here we have been able to link this regulatory role with arginine metabolism: *rpoS* expression is itself reduced in *argG* and *argH* mutants and positively modulated by addition of arginine. Given that transcription of the diguanylate cyclase-encoding gene *cfcR* is controlled by RpoS (Matilla *et al*., 2011), this work stablishes that arginine indirectly alters c-di-GMP levels through the modulation of *rpoS* expression in *P. putida* KT2440.

It has been reported that the transcriptional regulator ArgR controls key steps in L-arginine metabolism in different bacteria (Lu, 2006). However, in *P. putida* little was known about the role of this protein. We have identified the locus PP_4482 as the one corresponding to *argR* by homology with *argR* of *P. aeruginosa* and we also have studied ArgR implications in *argG* and *argH* regulation. In *P. aeruginosa*, ArgR exerts a negative influence upon *argG* expression (Lu *et al*., 2004), the next-to-last enzyme involved in arginine biosynthesis, while in *E. coli* the regulation falls on *argH* which is clustered in the *argCBH* operon, being the last gene of the pathway (Weerasinghe *et al*., 2006). In *P. putida*, we report that the main effect exerted by ArgR is upon *argG* as in the case of *P. aeruginosa*, however some influence has been observed upon *argH* at early stationary phase, indicating the possibility of an additional step of regulation in arginine biosynthesis in this bacterium.

On the other hand, we show the first evidence that ArgR influences the expression of a diguanylate cyclase, since ArgR exerts a negative impact upon *cfcR* expression. This adds an extra element in the already complex regulation of *cfcR* which includes transcriptional control by RpoS (Matilla *et al*., 2011) and posttranscriptional regulation by Rsm proteins (Huertas-Rosales *et al*., 2017a).

Furthermore, we report that ArgR is required to the response to L-arginine in relation to c-di-GMP levels in the cell, indicating a link between arginine and c-di-GMP metabolism. However, we also demonstrated that *ΔrpoSΔargR* and Δ*cfcRΔargR* have very low levels of c-di-GMP and the response to the amino acid was undetectable, suggesting that apart from ArgR other elements influence c-di-GMP contents in response to the presence of L-arginine, indicative of the complex regulation upon this second messenger, in which several elements are involved.

This study also showed that *argR* forms a cluster with the arginine ABC transport system encoded by *argT-hisPMQ*, where the binding protein *argT* is the main protein in arginine uptake in *P. putida* KT2440. This genetic organization is similar to the *aotJQMOP-argR* operon in *P. aeruginosa* (Nishijyo *et al*. 1998), and calls for a reannotation of the gene nomenclature in *P. putida*. Our results show that *argR* is cotranscribed with the ABC transporter genes in the presence of arginine, whereas the ABC transport system is also transcribed in the absence of the amino acid. Interestingly, we have been demonstrated the existence of a promoter upstream of *argT* and a second one upstream of *argR*, both arginine-inducible. In *P. aeruginosa*, *argR* expression occurs from the *aotJ* promoter (Nishijyo *et al*. 1998). Furthermore, we demonstrated that ArgR represses its own promoter and the transcription of *argT* is strictly dependent of ArgR. Taking all together, this indicates that different regulation patterns occur in both bacteria.

In a microarray analysis it was reported that RpoS influences *argR* expression during intracellular multiplication of *Legionella pneumophila* in its host, being its transcription increased during exponential phase and reduced during postexponential phase in the *rpoS* mutant (Hovel-Miner *et al*., 2009). Here, we have demonstrated that RpoS negatively influences *argR* expression in the presence of exogenous L-arginine. There is interconnectivity between ArgR and RpoS regulation in the presence of this amino acid, and ArgR also plays a negative role on *rpoS* expression upon entry in stationary phase.

Proteins belonging to CsrA/RsmA family are reported to be implicated in all major metabolic pathways like the carbon, amino acid and fatty acid metabolism as well as in transport and uptake of nutrients in many bacteria (Tatarko and Romeo, 2001; Häuslein *et al*., 2017; Sahr *et al*., 2017). Here, we demonstrated that Rsm proteins (RsmA, RsmE and

RsmI) have a positive effect on arginine biosynthesis in *P. putida* KT2440 through the regulation of the expression of *argG* gene. This effect is not direct, as shown by the fEMSA experiments. Recently, it has been reported that these proteins exert a negative impact upon the alternative sigma factor RpoS (Huertas-Rosales *et al*., 2017a). On the other hand, these proteins also regulate the expression of ArgR, but surprisingly in a positive way, while we expected a negative effect given their positive implication in *argG* expression and the fact that ArgR influences negatively this gene. fEMSA experiments showed no binding between Rsm proteins and the upstream transcript of *argR* (Figure Supplementary 4). Thus, additional mechanisms of regulation upon ArgR must exist.

In fact we have been able to identify another level of regulation upon ArgR: intracellular levels of the second messenger c-di-GMP also affect the expression of *argR.* Interestingly, this influence of the second messenger requires the transcriptional regulator FleQ. To complicate even more this interconnected network of regulators, we have preliminary evidence that FleQ might be regulated by Rsm proteins both directly and indirectly through RpoS.

All these results obtained in this Chapter suggest a finely tuned balance between arginine metabolism and c-di-GMP synthesis, highlighting the complexity of c-di-GMP signalling, where a large number of elements are involved. Some of these results lead us to propose a regulatory model in which transcriptional and post-transcriptional elements, arginine metabolism and c-di-GMP signalling are related (Figure 25).

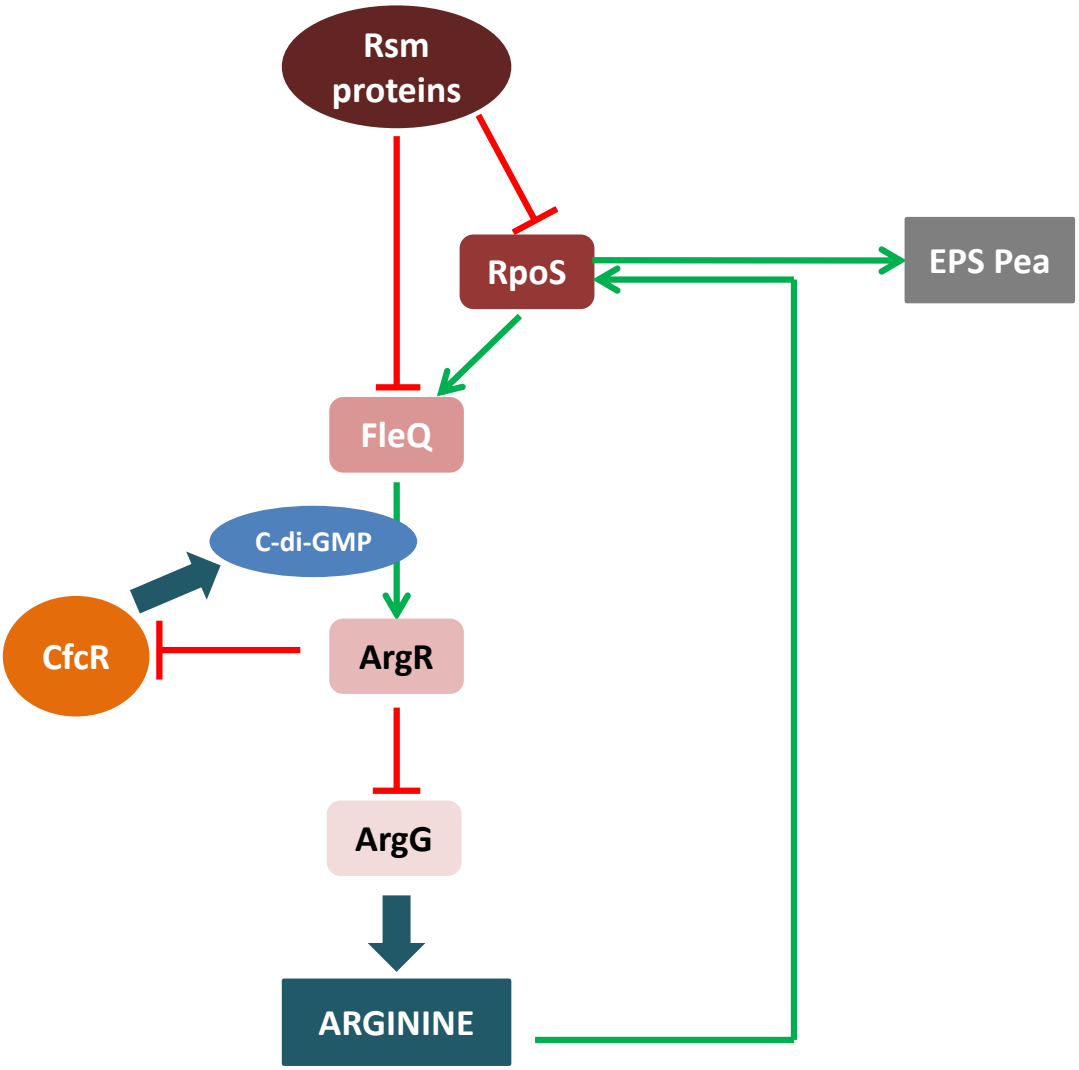
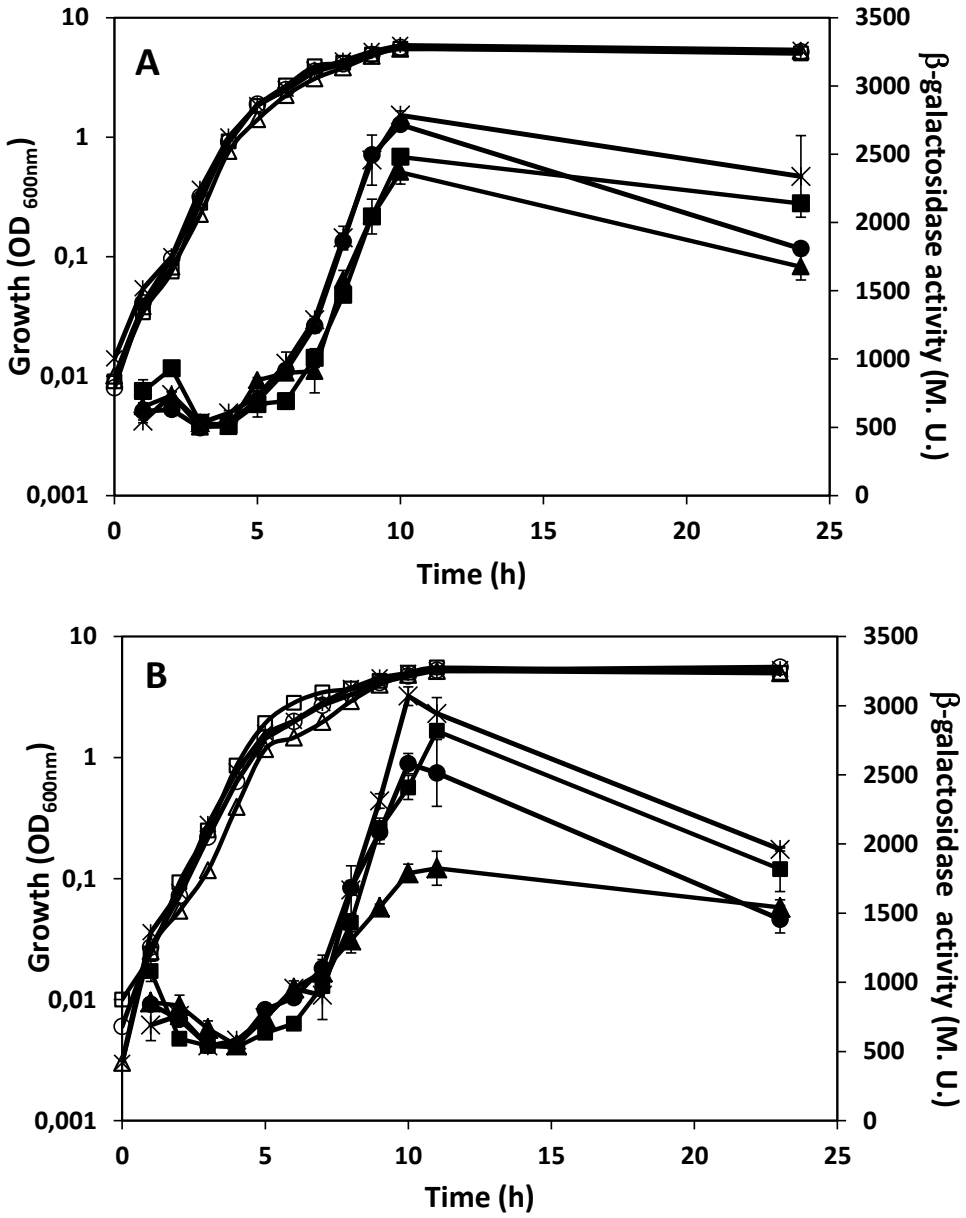


Figure 25. Schematic representation of elements and their regulation connecting arginine metabolism and c-di-GMP signalling in *P. putida* KT2440. Green arrow-ended lines and red T-ended lines represent positive and negative regulation, respectively. Blue arrows show the product of the preceded gene.

SUPPLEMENTARY MATERIAL



Supplementary Figure S1. Expression of the translational fusion of *argG* in simple (A) and double (B) *rsm* mutants. Cultures growing in LB supplied with Tc as described in Material and Methods were analysed for turbidity (hollow symbols) and β–galactosidase activities (solid symbols) at the indicated times. **(A)** Data for the wild type (square) Δ*rsmA* (triangle), Δ*rsmE* (circle) and Δ*rsmI* (asterisk) and **(B)** for the wild type (square) ΔΔ*rsmAE* (triangle), ΔΔ*rsmAI* (circle) and ΔΔ*rsmEI* (asterisk) are shown. A representative graph is shown in both experiments.

A

CGCUCGAUCUCACAGGCGCUGAACUCCUCAAGGCAUGCACGCUGCGGCUCGACGCCCCUCCAAGGUCCUCAUGCCACAUCCCUCAA
CCUACCGCCCGUCGCCCCUACGAAAGUUCCUGUCGCUUUGUCGUCAGAAGCUGUCGCCCCACUGUAAAUUUAUCCGCAACGCCGG
UAGAAUCGAUCCCUUUCCGUACAACCCCGUGGAGUGAUGGC**AUG**GCCCACGUAAAAAAGGUCGUACUGGCGUAUUCCGGCGGC
CUUGAUACUUCGGUGAUUCUCAAGUGGCUGCAGGAUACCUACAACUGCGAAGUGGUGACCUUCACCGCUGACCUGGGGCAGGGC
GAAGAGGUCGAACCGGCCCGUGCCAAGGCCCAGGCAAUGGGCGUUAAAGAGAUCUACAUCGACGACCUGCGCGAAGAAUUCGUGC
GUGAUUUCGUGUUCCCGAUGUUCCGCGCCAACACCGUCUACGAAGGCGAGUACCUGCUGGGUACUUCCAUCGCCCGUCCGCUGAU
CGCCAAGCGCCUGAUCGAAAUCGCCAACGAAACCGGCGCUGACGCCAUUUCCCAUGGCGCCACCGGCAAGGGUAACGACCAGGUGC
GCUUCGAGCUGGGUGCCUAUGCCCUGAAGCCAGGCGUCAAGGUCAUCGCUCCAUGGCGCGAGUGGGACCUGCUGUCCCGCGAAA
AGCUGAUGGACUACGCCGAGAAGCACGGCAUCCCGAUCGAGCGCCACGGCAAGAAGAAGUCGCCGUACUCGAUGGACGCCAACCU
GCUGCACAUCUCCUACGAGGGCGGUGUCCUGGAAGAUACCUGGACCGAGCACGAAGAAGACAUGUGGCGCUGGAGUGUCUCGCC
UGAGAAUGCCCCGGACCAGGCUACCUACAUCGAGCUGACCUACCGCAAUGGUGACAUCGUUGCCAUCGACGGCGUCGAGAAAUCC
CCGGCCACCGUCCUGGCAGACCUGAACCGUAUCGGUGGUGCCAACGGCAUCGGCCGUCUGGACAUCGUCGAAAACCGUUACGUCG
GCAUGAAGUCGCGCGGUUGCUACGAAACGCCUGGCGGUACCAUCAUGCUCAAGGCACACCGUGCCAUCGAGUCGAUCACCCUGGA
CCGCGAAGUCGCUCACCUGAAAGAUGAGCUGAUGCCAAAGUAUGCCAGCCUGAUCUACACCGGCUACUGGUGGAGCCCGGAGCGU
CUGAUGCUGCAACAGAUGAUCGAUGCUUCGCAGGUCAACGUGAAUGGUGUGGUGCGCCUGAAACUGUACAAGGGCAACGUGACC
GUGGUUGGCCGCAAGUCGGACGAUUCGCUGUUCGAUGCCAACAUCGCCACCUUUGAAGAAGAUGGUGGUGCCUACAACCAGGCA
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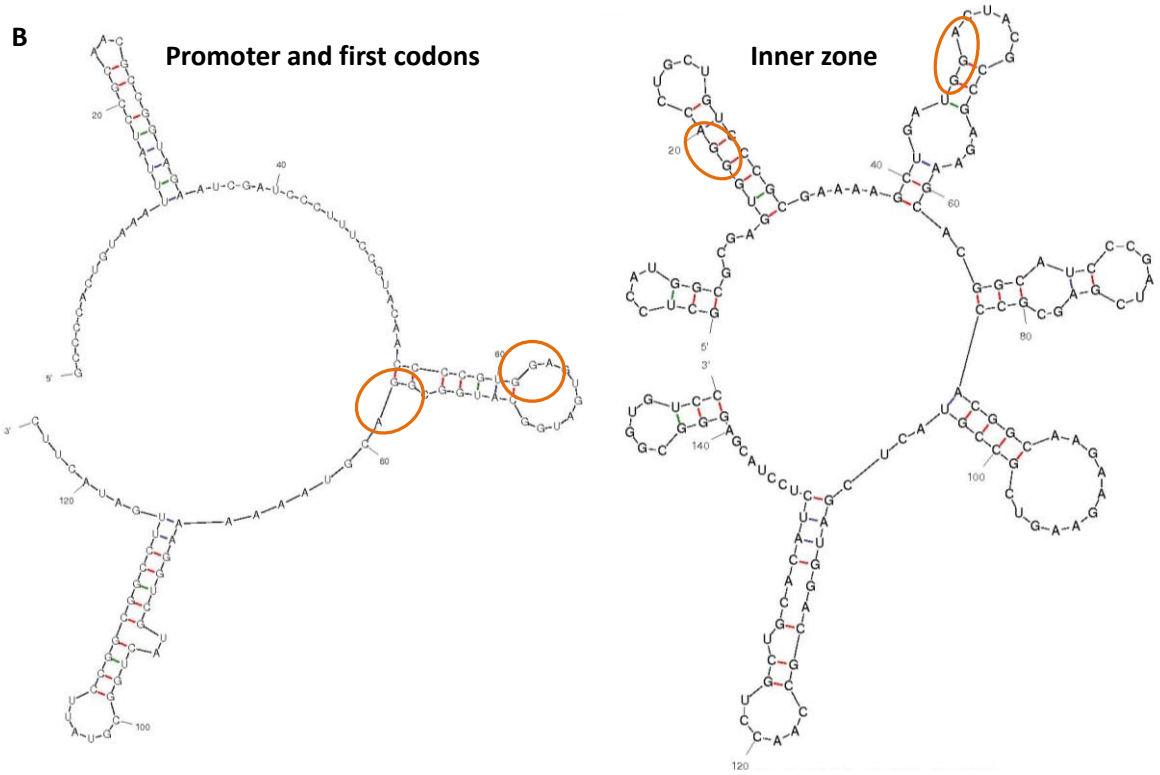


Supplementary Figure S2. RNA-*argG* transcripts used in fEMSA and their folding predictions. **A.** RNA-*argG* transcripts used in fEMSA are underlined and putative binding sites of Rsm proteins are highlighted in green. Promoter sequence are shown in black and *argG* sequence in orange. Start codon of *argG* is in bold orange. Both 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 ATTO700 fluorescent dye (see Materials and Methods). **B.** Secondary structure predictions of RNAs obtained at m-fold web server (Zuker, 2003) are shown. Circles show putative binding sites of Rsm proteins in RNA transcripts. Maximum distance between paired bases was established in 30.

A

B

GACGAGCCGACCTCGGCACTTGACCCTGAGCTGGTGGGTGATGTGCTGAAGGTGATGCAGG

CGCTGGCCCAGGAAGGTCGGACCATGGTAGTGGTGA**CCCACGAAATGGGC**TTTGCCCGCGA

GGTGTCCAACCAGTTGGTGTTCCTGCACAAAGGCCTGGTGGAAGAAACCGGCTGCCCACGTG

AAGTGCTGGCCAACCCGCAGTCGGAGCGCCTGCAGCAGTTCCTCTCCGGCAGCCTGAAGTAA

AAGCTGCATTTTTGCACCAGAATAGGGCATGCTGCGCGGCGAGCGCAGCCTGACCCGGCGCA

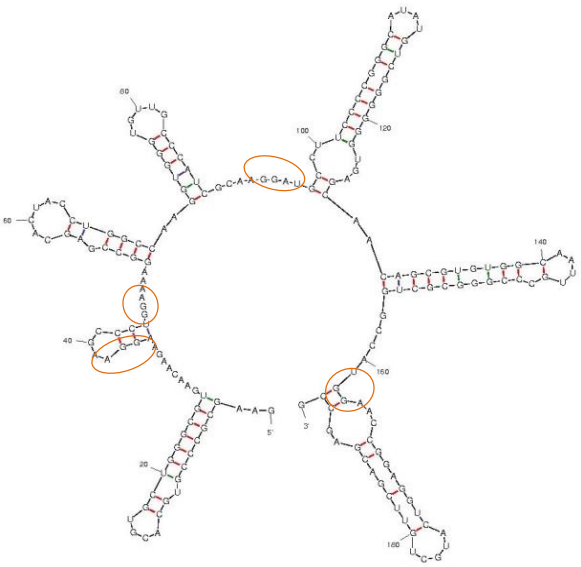
CAGACTCGAACGACCTCAGGTTTCGGACCGTACCCT**ATG**

Supplementary Figure S3. A. Predicted FleQ binding site in *P. putida* and *P. aeruginosa*. Sequence logo for the FleQ binding site in *P. putida* is shown at the bottom (Taken from Molina-Henares *et al*., 2017). **B.** Putative FleQ binding site in the promoter region of argR in *P. putida* KT2440 is underlined and equal bases to the consensus sequence are highlighted in grey. Start codon of argR is in bold.

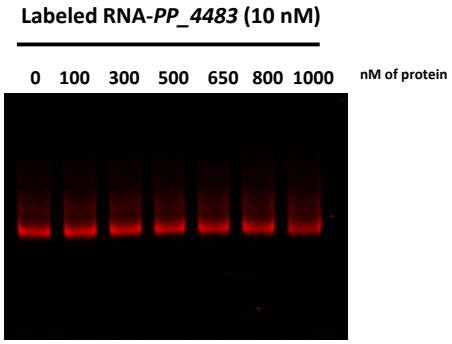
A

AUGACUGCGCUUGAGAACAUCAUCGAAGCGCCCGUGCACGUGCUGGGCGUGAACAAGAAGGAAGCCCUGGAAAAGGCCG
AGCACUACCUGGCCAAGGUGGGGUGUUGCCCAUCGCAAGGAUGCCUUCCCCGGGCAUAUGUCGGGGGGUGAGCAACAGC
GUGUGGCAAUUGCCCGGGCGCUGGCCAUCGAACCGGAGGUCAUGCUGUUCGACGAGCCGACCUCGGCACUUGACCCUG
AGCUGGUGGGGUGAUGUGCUGAAGGUGAUGCAGGCGCUGGCCCAGGAAGGUCGGACCAUGGUAGUGGUGACCCACGAA
AUGGGCUUUGCCCGCGAGGUGUCCAACCAGUUGGUGUUCCUGCACAAAGGCCUGGUGGAAGAAACCGGCUGCCCACGU
GAAGUGCUGGCCAACCCGCAGUCGGAGCGCCUGCAGCAGUUCCUCUCCGGCAGCCUGAAGUAAAAGCUGCAUUUUUGCA
CCAGAAUAGGGCAUGCUGCGCGGCGAGCGCAGCCUGACCCGGCGCACAGACUCGAACGACCUCAGGUUUCGGACCGUACC
CUAUGACCACCCAGCGAAUCGGUUUUCUCAUCUGGCCCAGCACCAGGCCUUUGACCCUGGCGUUGGCCGAGGAAGUGUU
GCUGGUGGCGCAGCGUGUGCAUC

B



C



Supplementary Figure S4. RNA-PP_4483 transcript used in fEMSA and its folding prediction. **A.** RNA-PP_4483 transcript used in fEMSA is underlined and putative binding sites of Rsm proteins are highlighted in green. **A** sequence region of locus PP_4483 is shown in red, intergenic region in black and the initial sequence of *argR* in blue, with the start codon highlighted in grey. Transcript 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 ATTO700 fluorescent dye (see Materials and Methods). **B.** Secondary structure prediction of RNA obtained at m-fold web server (Zuker, 2003) is shown. Circles show putative binding sites of RsmE protein in RNA transcripts. Maximum distance between paired bases was established in 30. **C.** Fluorescence-based EMSA of RsmE upon RNA-PP_4453 transcript. 10 nM of RNA and different protein concentrations (0-1000 nM) were used in the assay. No binding was observed at concentrations tested. The same result was obtained for RsmA.

CHAPTER 3

Exploring the connection between arginine metabolism and siderophore production in *Pseudomonas putida* KT2440

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BACKGROUND

Iron plays an essential role in many cellular processes, as a key element associated to proteins and enzymes participating in electron transport, energy metabolism, redox sensing or DNA repair (Oexle *et al*., 1999; Green and Paget, 2004; Rothery *et al*., 2008; Puig *et al*., 2017). Accordingly, expression of numerous genes is regulated by iron availability in different microorganisms, and many bacteria display a battery of iron sensing, uptake and scavenging mechanisms that often reflect their ecological niches and lifestyles (Braun and Hantke, 2011; Krewulak and Vogel, 2011; Kreamer *et al*., 2012; Frawley and Fang, 2014; Reinhart and Oglesby-Sherrouse, 2016). These mechanisms include the production and release of siderophores, a structurally diverse group of molecules, often produced through nonribosomal peptide synthetases, that allow bacteria to efficiently acquire iron from the environment (reviewed by Khan *et al*., 2018; Ronnebaum and Lamb, 2018). Siderophore production can influence bacterial fitness in environments as diverse as the rhizosphere of plants (Mirleau *et al*., 2001) or the human gut (Ellermann and Arthur, 2017), and has also been implicated in cross-talk between bacterial species (Weaver and Kolter, 2004; Galet *et al*., 2015; Sexton *et al*., 2017).

Despite its essential nature, excess free iron can have a detrimental effect on cell viability under aerobic conditions. It potentiates oxygen toxicity by causing an increase in hydroxyl (·OH) radicals through the Fenton reaction (Halliwell and Gutteridge, 1984), in which hydrogen peroxide can react with ferrous iron, rendering ·OH and OH^-. Further reactions of ferrous or ferric iron can also take place (Halliwell and Gutteridge, 1984), causing an overall increase in reactive oxygen species, which then leads to lipid, protein and DNA damage. Thus, it is not surprising that iron metabolism is not only tightly regulated, but also coupled with the regulation of defense mechanisms against oxidative stress (Touati, 2000; Imlay, 2015; Kreamer *et al*., 2015).

In the soil- and plant root-colonizing bacterium *Pseudomonas putida* KT2440, iron uptake has been shown to be relevant for attachment to corn seeds and bacterial fitness in the rhizosphere and in competition with other *Pseudomonas* species (Molina *et al*., 2005; Molina *et al*., 2006; Fernández-Piñar *et al*., 2011). Under iron-limiting conditions, this strain produces three forms of the siderophore pyoverdine, with slight structural differences (Wei

and Aristilde, 2015). The most abundant form (PVD2) shows the following composition: succinamide-chromophore(2,3-diamino-6,7-dihydroxyquinoline)-Asp-ornithine-[hydroxyAsp-diaminobutanoic]-Gly-Ser cyclohydroxyornithine, where the square brackets indicate a cyclic portion of the peptide chain. This portion is linear in the PVD1 variant, whereas the PVD3 variant contains succinic acid instead of succinamide (Wei and Aristilde, 2015). These are summarized in Table 1.

PVD	Identity of side chain and peptide sequence
1	succa-chr-Asp-Orn-OHAsp-Dab-Gly-Ser-cOHOrn
2	succa-chr-Asp-Orn-[OHAsp-Dab]-Gly-Ser-cOHOrn
3	succ-chr-Asp-Orn-[OHAsp-Dab]-Gly-Ser-cOHOrn

Table 1. Structural characterization of PVDs secreted by *P. putida* KT2440. Numbers indicate type of PVD. Brackets indicate a cyclic structure. Succ and succa represent succinic acid and succinamide, respectively. Unusual or modified amino acids: OHAsp (hydroxyaspartic acid), Dab (Diaminobutanoic acid), cOHOrn (cyclo-hydroxyornithine). The difference in PVDs structures are shown in bold (Adapted from Wei and Aristilde, 2015).

Pyoverdine-mediated iron uptake is essential for surface motility of *P. putida* KT2440 (Matilla *et al*., 2007a), and has been described as a key factor involved in growth inhibition of the plant pathogen *Xanthomonas fragariae* by KT2440 (Henry *et al*., 2016). Increased siderophore production has been reported to take place during growth on the aromatic compound benzyl alcohol as carbon and energy source (Joshi *et al*., 2014). Besides an increase in pyoverdine production, iron limitation also results in changes in the metabolic flux and increased gluconate secretion during growth of *P. putida* KT2440 on glucose (Sasnow *et al*., 2016).

In a previous work, the genes *argG* and *argH*, encoding the enzymes required for the last two steps of the arginine biosynthesis pathway (Figure 1A), were identified among a number of genetic elements involved in c-di-GMP signalling in *P. putida* KT2440 (Ramos-González *et al*., 2016). In the course of their characterization, we observed changes in the

colour and fluorescence of colonies formed by transposon mutants in these two genes, suggestive of altered siderophore production. The same observation was later done with deletion mutants (Figure 1B). These observations prompted us to explore the connection between arginine biosynthesis and pyoverdine production, which is presented in this work.

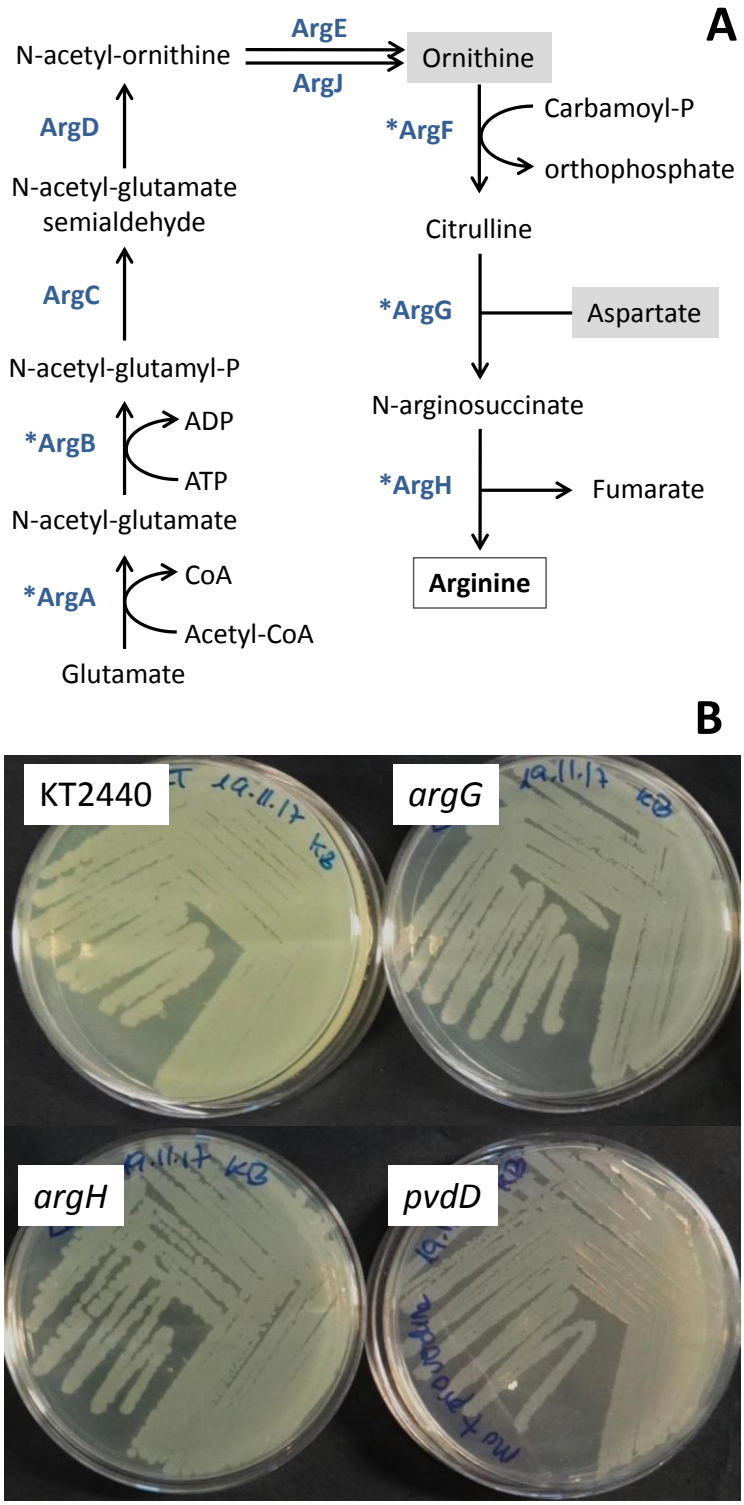


Figure 1. A. Arginine biosynthesis pathway in *P. putida* KT2440. Mutants defective in the enzymes indicated with an asterisk are auxotrophs for the amino acid. Structural components of pyoverdine are shaded in grey.

B. Visualization of yellow-green pigment in King´s B-agar plates indicative of siderophore production in *P. putida* KT2440, *ΔargG* and *ΔargH* mutants. A *pvdD* mutant is included as negative control.

RESULTS

Arginine auxotrophy causes reduced pyoverdine release and increased sensitivity to iron depletion.

As an initial step to establish the potential link between arginine and pyoverdine synthesis, the null mutants in *argG* and *argH* described in previous chapters were grown in King's B medium, where they showed colour differences with the wild type (Figure 1), as well as reduced fluorescence under UV light after overnight growth in liquid King's B medium (Figure 2A). Addition of 5 mM L-arginine to the growth medium restored fluorescence in the Δ*argG* and Δ*argH* mutants. A mutant in *pvdD* (PP_4219; previously annotated as *ppsD*), which does not produce pyoverdine (Matilla *et al*., 2007a), was used as a negative control.

Further analysis of siderophore production was done by a chrome azurol S (CAS) assay (Figure 2B). The Δ*argG* and Δ*argH* mutants did not produce the characteristic yellow/orange halo indicative of pyoverdine diffusion into CAS medium. This defect was restored by addition of 5 mM L-arginine to the growth medium, as well as by complementing the mutations with plasmids pME1088 and pME0184, bearing the respective wild type loci. Addition of fumarate (also produced in the last step of arginine biosynthesis), gamma-aminobutyrate (one of the main metabolites into which different arginine catabolism routes converge), or aspartate (required as precursor in the synthesis of arginine, and a structural constituent of pyoverdine) did not restore the appearance of the pyoverdine halo in either mutant (Figure 2B). Neither did ornithine –also a constituent of the pyoverdine molecule–, glutamic acid –precursor of ornithine and arginine synthesis–, or other positively charged amino acids like histidine or lysine (data not shown). These results indicate that arginine is specifically required for pyoverdine production.

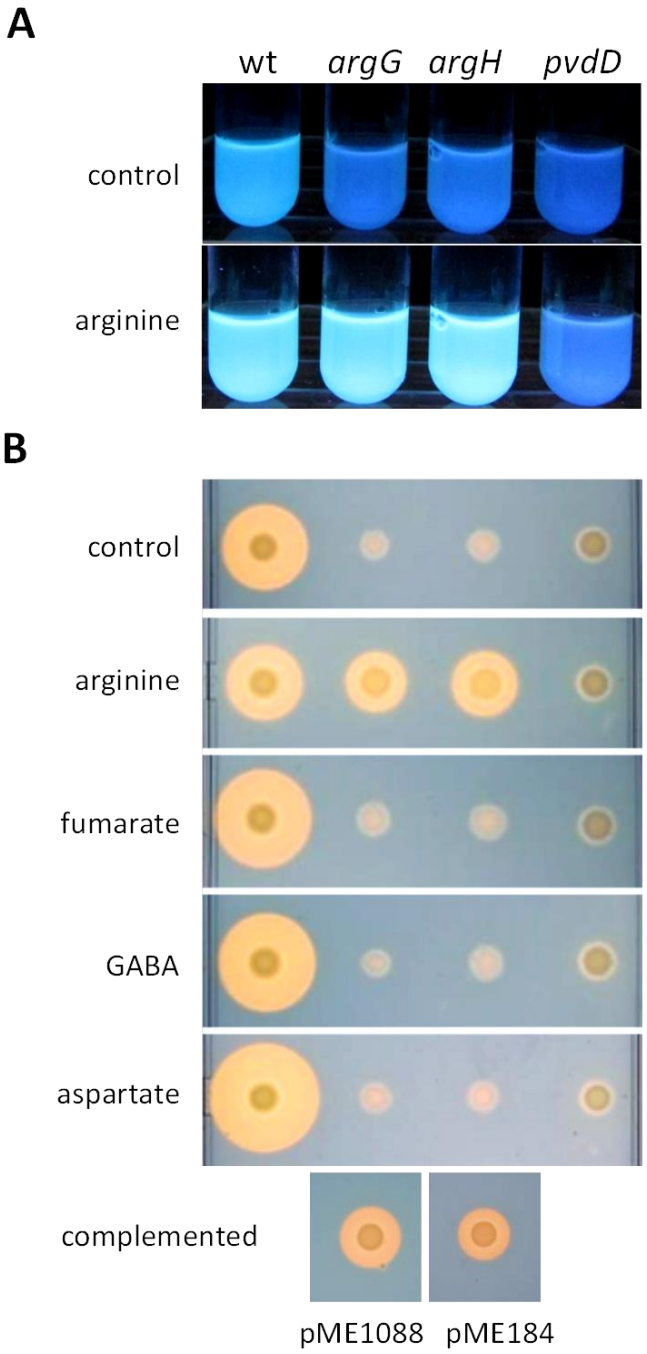


Figure 2. (A) Visualization of fluorescence under UV light in the wild type and mutants in *argG*, *argH* and *pvdD* of overnight cultures in liquid King's B medium in absence (upper panel) or presence (lower panel) of arginine. The OD_{660} of the cultures were adjusted to 1 to compare. **(B)** Production of yellow/orange halo indicative of pyoverdine diffusion into CAS medium supplied with different compounds involved in the arginine metabolism. Results obtained for Δ*argG* and Δ*argH* mutants were compared to the wild type and *pvdD* mutant, which was used as a negative control.

A similar assay was done measuring the area of the halo produced by several mutants affected in different steps of the arginine biosynthetic route. As indicated in Figure 3, mutations in *argA*, *argB* and *argF* caused the same reduction in pyoverdine release as the *argG* and *argH* mutations, and halo production was restored to different extents by addition of 5 mM L-arginine. Interestingly, mutations affecting *argD*, *argE* and *argJ* only resulted in a minor decrease in the halo area. It should be noted that these three mutants do not display arginine auxotrophy, contrary to the remaining mutants, which require exogenous arginine for growth in minimal medium (data not shown).

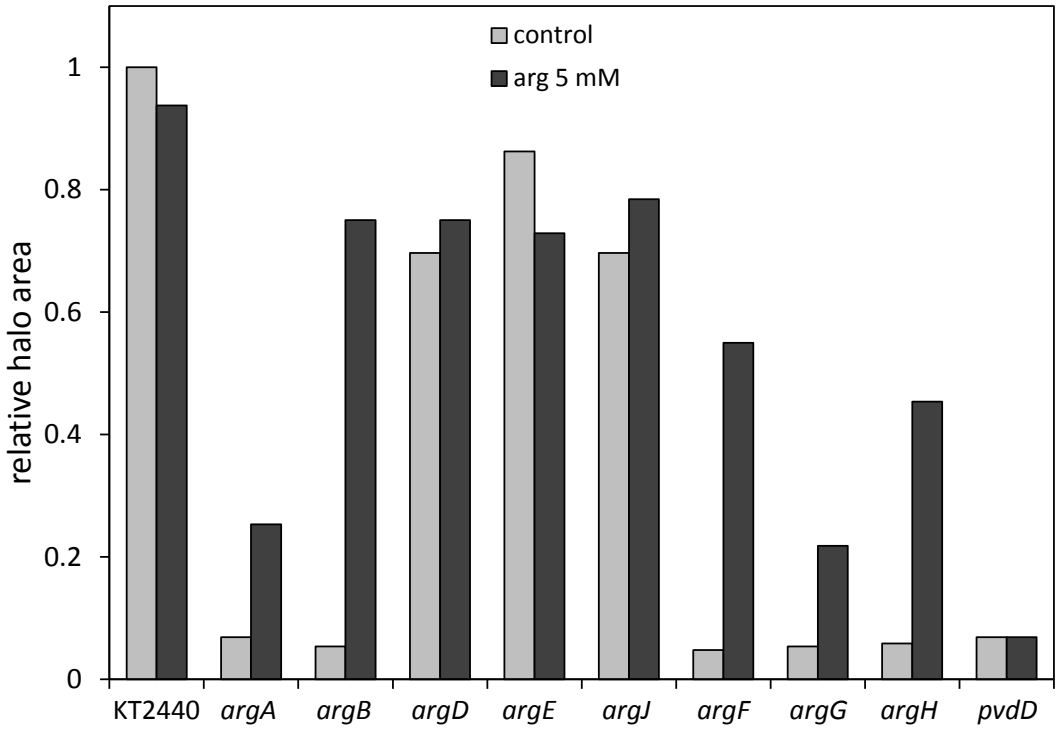


Figure 3. Quantification of halo area in mutants affected in different steps of arginine biosynthetic pathway growing in CAS medium in absence (light grey) or presence (dark grey) of 5mM L-arginine comparing to the wild type and *pvdD* mutant.

Bacterial strains that are defective or limited in iron uptake are generally sensitive to non-metabolizable iron chelators. We therefore decided to compare the growth of the Δ*argG* and Δ*argH* mutants with that of the wild type in the presence of increasing concentrations of 2,2'-bipyridyl. As shown in Figure 4, growth of both mutants was impaired at chelator concentrations above 1.3-1.5 mM, whereas the parental strain was able to grow at concentrations of 2,2'-bipyridyl above 2.5 mM.

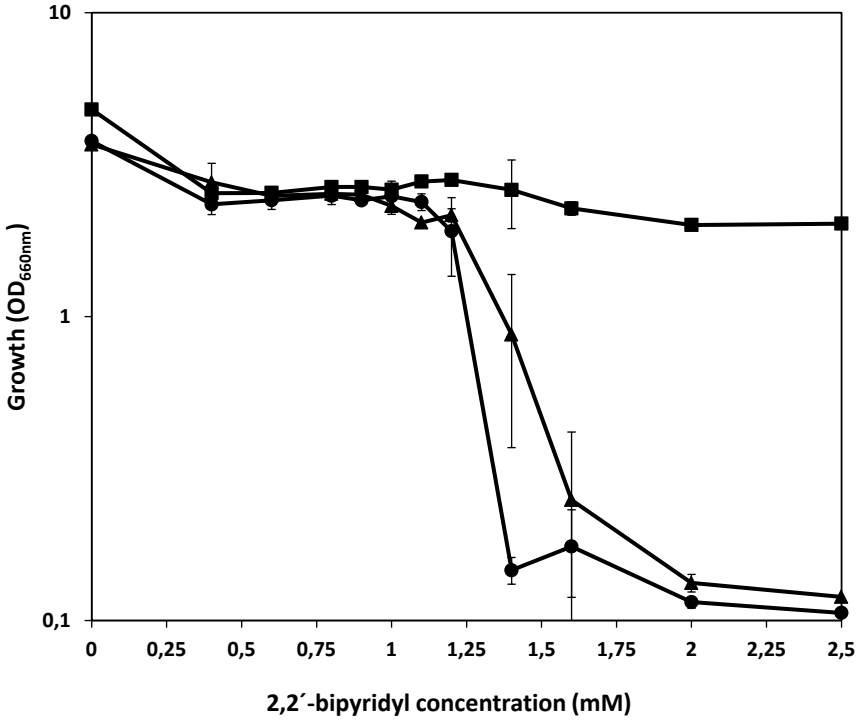


Figure 4. Growth of wild type (squares), Δ*argG* (triangles) and Δ*argH* (circles) in LB medium in presence of different concentrations of the iron chelator 2,2'-bipyridyl. A representative graph is presented.

Expression of some pyoverdine genes is altered in Δ*argG* and Δ*argH* mutants.

One possibility to explain the results described in the previous section could be that expression of pyoverdine-related genes was altered in the arginine auxotrophs. Genes involved in pyoverdine synthesis, transport and regulation (Supplementary Figure S1) were selected to analyse their mRNA levels in the Δ*argG* and Δ*argH* mutants compared to the parental strain, by qRT-PCR. The genes analysed were *pvdD* and *pvdA*, encoding a pyoverdine non-ribosomal peptide synthetase and a protein involved in pyoverdine side chain synthesis; *pvdE*, encoding an ATP-binding membrane protein presumably required for pyoverdine translocation to the periplasm (McMorran *et al*., 1996; Yeterian *et al*., 2010); and two regulatory genes: *pvdS*, encoding an alternative sigma factor that in *P. aeruginosa* constitutes the master transcriptional regulator for pyoverdine synthesis and transport (Leoni *et al*., 2000; Tiburzi *et al*., 2008), and *algQ* (named *pfrA* in *Pseudomonas fluorescens*, Venturi *et al*., 1993, 1995), which would sequester the housekeeping sigma factor σ^{70} (RpoD) to allow increased availability of RNA polymerase for PvdS binding (Ambrosi *et al*., 2005).

Results from the qRT-PCR analysis are shown in Figure 5. Intriguingly, both the Δ*argG* and Δ*argH* mutants showed increased expression of the pyoverdine structural genes *pvdA* and *pvdD* with respect to the wild type (between 3 and 5 fold), whereas expression of *pvdE* was reduced (around -2.5 fold). Expression of the regulatory genes *pvdS* and *algQ*, on the other hand, did not show significant change in the mutants with respect to KT2440.

In an attempt at finding a potential regulatory link between arginine and pyoverdine synthesis, the role of ArgR in these expression changes was also analysed. ArgR is a transcriptional regulator that positively controls arginine catabolic genes and other metabolic elements, and negatively regulates biosynthetic genes in the presence of L-arginine (Maas, 1994; Park *et al*., 1997a,b). As described in Chapter 2, we have identified ArgR in *P. putida* KT2440, confirmed its regulatory role and constructed a deletion mutant. This mutant was also included in the qRT-PCR analysis. As shown in Figure 5, no significant changes in expression of any of the analysed genes could be detected in the Δ*argR* strain, except for a slight increase in the case of *algQ* (1.7 fold).

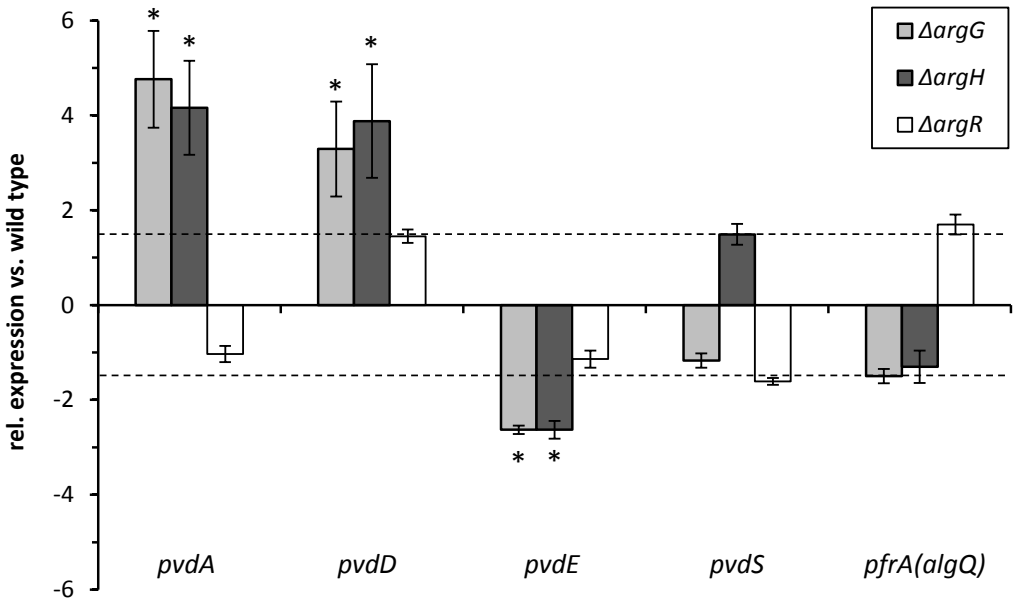


Figure 5. Relative expression of genes involved in pyoverdine synthesis (*pvdA*, *pvdD*), transport (*pvdE*) and regulation (*pvdS*, *pfrA* = *algQ*) in *argG* (light grey), *argH* (dark grey) and *argR* (white) mutants with respect to the wild type, measured by qRT-PCR. RNA was isolated from cultures grown overnight in King´s B medium. Asterisks indicate significant differences, considering a cutoff value of ±1.5-fold (dotted line). Data are the averages and standard deviations of three biological replicates with three technical repetitions.

Arginine biosynthesis mutants accumulate pyoverdine intracellularly.

The expression data suggested that the differences observed in the CAS assays and in liquid cultures were not really due to reduced pyoverdine synthesis, which could actually be increased in the Δ*argG* and Δ*argH* mutants, but rather to limited transport of the siderophore to the periplasm and further release from bacterial cells. To confirm this idea, extracellular and intracellular pyoverdine were separately quantified, as described in Materials and Methods, in overnight cultures of the *argG*, *argH* and *argR* mutants, with KT2440 and the *pvdD* mutant as positive and negative controls, respectively. Results are shown in Figure 6.

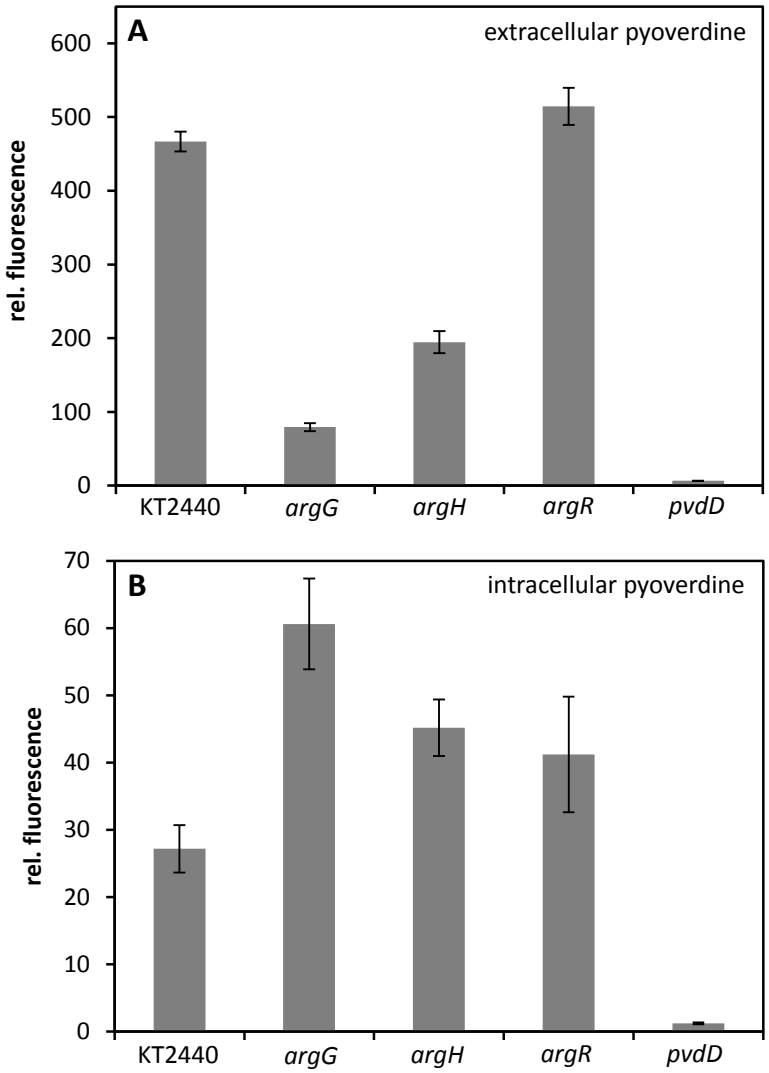


Figure 6. Quantification of fluorescence resulting from extracellular **(A)** and intracellular **(B)** pyoverdine in different strains of *P. putida* KT2440 after 24 h of growth in King´s B medium, using a Varioskan Lux fluorimeter. Fluorescence readings were normalized for growth (OD_{600nm}). Experiments were carried out in triplicate with three technical repetitons each. Representative graphs are presented.

As expected, both the Δ*argG* and the Δ*argH* mutants showed reduced fluorescence in the culture supernatant and increased fluorescence in disrupted cells, indicative of pyoverdine being retained inside the cell and not released to the medium. In the Δ*argR* mutant, an increase in intracellular pyoverdine was also observed, although in this case it was accompanied by a slight increase in extracellular siderophore.

Arginine biosynthesis mutants show increased susceptibility to oxidative stress.

As mentioned in the Introduction, despite its essential nature, free iron enhances oxidative damage in the cells. Several reports indicate that polyamines can have a protective function against oxidative stress (Rhee *et al*., 2007; Shah and Swiatlo, 2008), and arginine is the precursor of polyamine synthesis (Supplementary Figure S2). Hence, we hypothesized that arginine biosynthesis mutants would be more sensitive to oxidative agents, and that pyoverdine retention might be a protective response to try and reduce such stress by either limiting iron entry or ensuring iron sequestration inside the cell. To check this hypothesis, the *argG*, *argH* and *argR* mutants were grown in King´s B medium in the presence of increasing concentrations of hydrogen peroxide. As shown in Figure 7, growth of *argG* and *argH* mutants was impaired at lower concentrations of H_2O_2 than those required to inhibit growth of the wild type. A similar result was obtained with the *pvdD* mutant, whereas the *argR* mutant was able to tolerate higher concentrations of H_2O_2 although it still was more sensitive than the wild type strain.

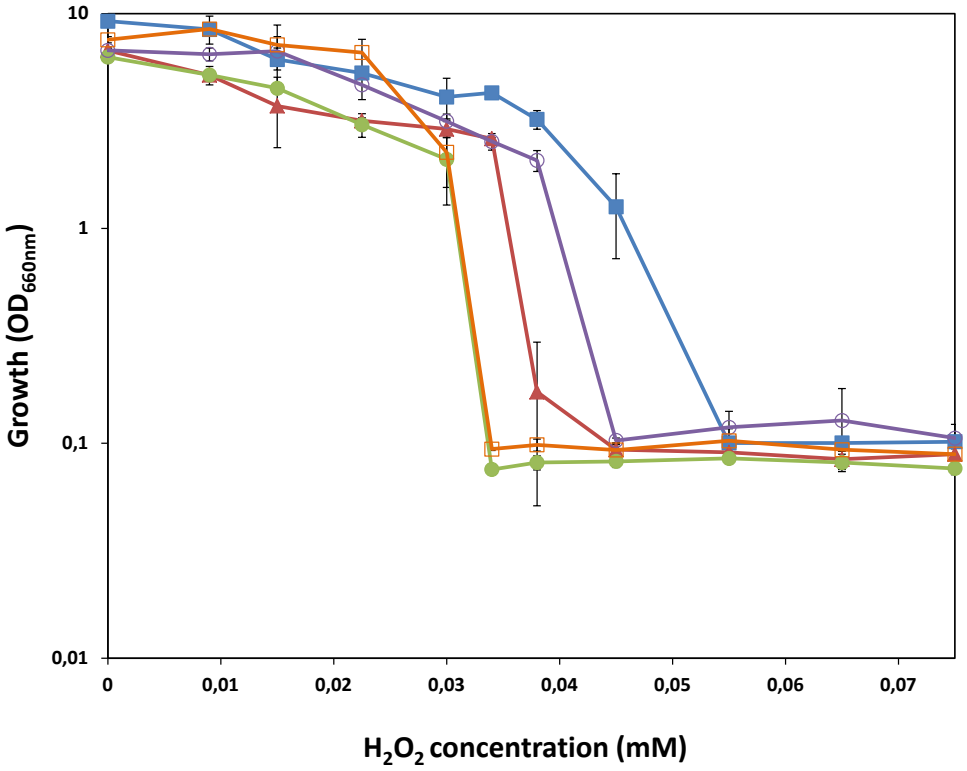


Figure 7. Growth of wild type (blue line), Δ*argG* (red line), Δ*argH* (green line) Δ*argR* (violet line) and *pvdD* (orange line) mutants in the presence of different concentrations of hydrogen peroxide (H_2O_2). Cultures were grown overnight in liquid King´s B medium and turbidity was measured. Data are from one representative experiment and correspond to averages and standard deviations from three replicas.

Some polyamines promote pyoverdine release and alleviate oxidative stress in Δ*argG* and Δ*argH* mutants.

Based on our previous hypothesis, we decided to check if polyamines had a similar effect as arginine in terms of pyoverdine production. As shown in Figure 8, addition of 5 mM agmatine or spermidine caused a significant increase in pyoverdine production in KT2440 and to a lesser extent in the *argG* and *argH* mutants. Intriguingly, putrescine had no clear effect.

We next tested if the increased sensitivity of the mutants to H_2O_2 might be alleviated by polyamines. Addition of 5 mM agmatine to the growth medium resulted in the mutants being able to tolerate higher concentrations of the stressor (Figure 9). Nonetheless, these data are preliminary and require further, more detailed analysis.

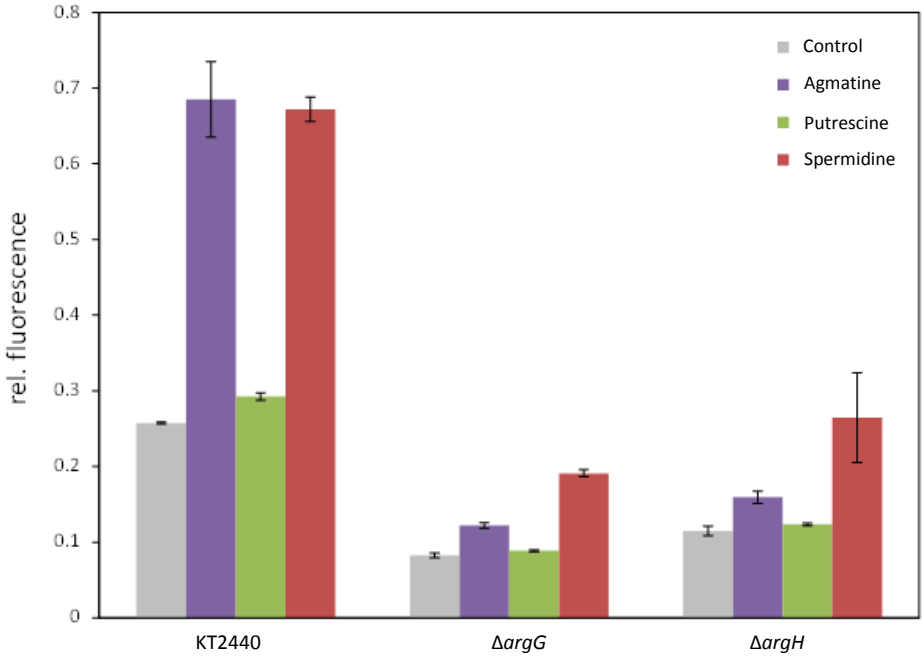


Figure 8. Quantification of fluorescence resulting from extracellular pyoverdine in KT2440 and the *arg* mutants after 24 h of growth in King´s B medium supplied with different polyamines, using a Varioskan Lux fluorimeter. Fluorescence readings were normalized for growth (OD_{600nm}). Experiments were carried out in triplicate with three technical replicates each.

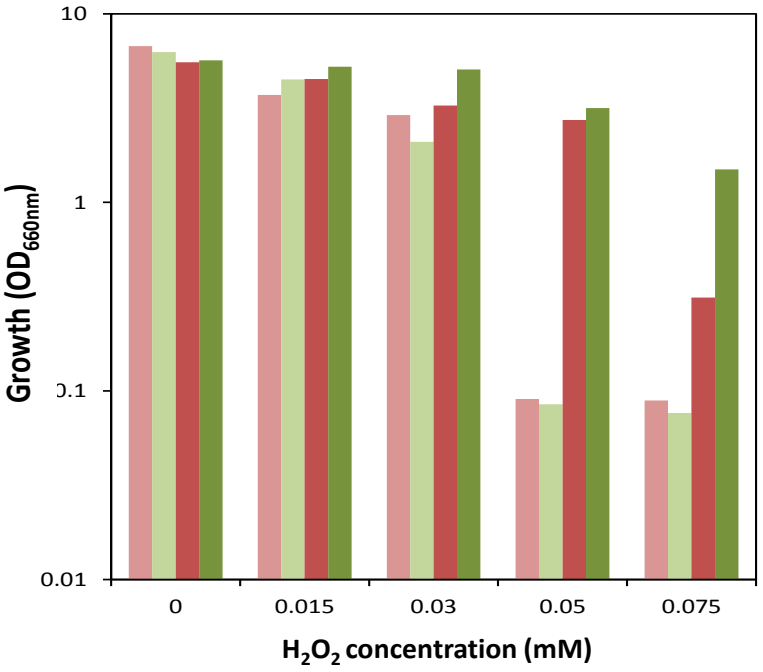


Figure 9. Growth of Δ*argG* (red) and Δ*argH* (green) mutants in increasing H_2O_2 concentrations, in the absence (light bars) or presence (dark bars) of 5 mM agmatine. Cultures were grown overnight in liquid King´s B medium and turbidity was measured. Data are from one experiment and correspond to averages from three replicas.

DISCUSSION

Iron is an essential metal for many biological processes. Previous work in our group revealed the importance of iron uptake in *Pseudomonas putida* KT2440, being a key for initial establishment on corn seeds and root colonization (Molina *et al*., 2005). Production of siderophores is essential for iron uptake in bacteria and plays many roles on bacterial behaviours (Saha *et al*., 2013).

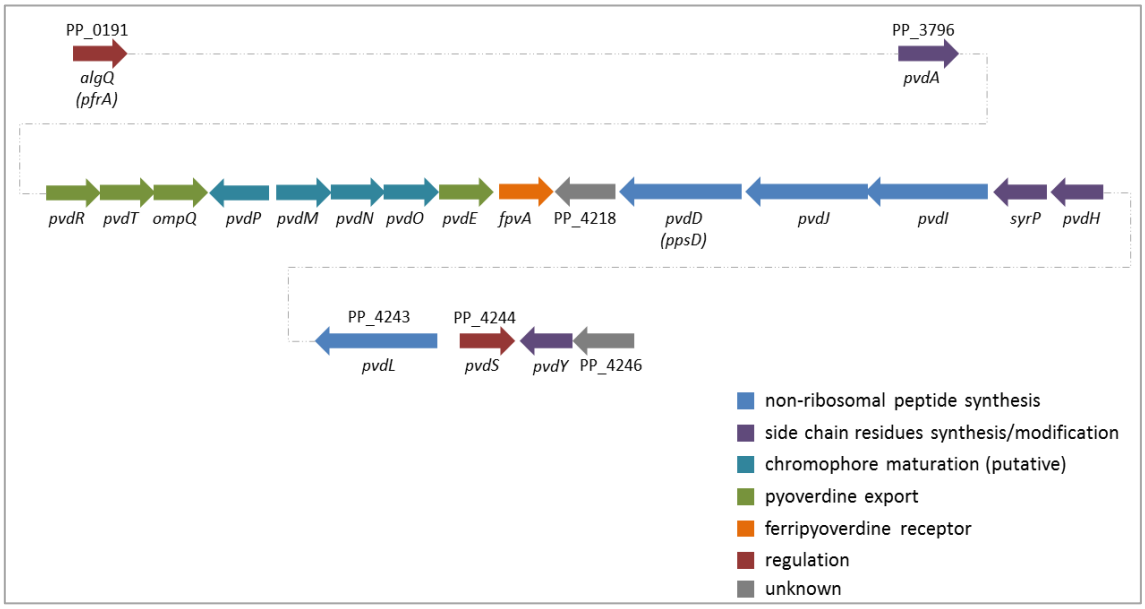
In this work we have begun to explore the possible connection between arginine metabolism and pyoverdine, a siderophore which has high affinity for iron (Ravel and Cornelis, 2003). Our results show that mutants deficient in arginine synthesis are also deficient in the production of extracellular siderophore, a defect that is restored by addition of the amino acid in the culture medium. It has been reported that arginine is a constituent of peptide chain of type 1 pyoverdine in *Pseudomonas aeruginosa* (Visca *et al*., 2007). However, as previously reported by Ravel and Cornelis (2003), an *in silico* analysis showed that pyoverdine produced by *P. putida* KT2440 do not possess this amino acid in its structure, a fact that has been recently demonstrated experimentally by Wei and Aristilde (2015). The same has been predicted for other strains of *P. putida* (Meyer *et al*., 2007). Thus, the lack of production of pyoverdine in these mutants is not due to limitation in one of its "building blocks", and is not restored by addition of compounds like aspartic acid or ornithine, which are actual constituents of the siderophore. In fact, our results show that *argG* and *argH* mutants actually produce pyoverdine, and in fact *pvdD* and *pvdA*, two genes related to siderophore biosynthesis, are overexpressed in these mutants. This, and the fact that *pvdE* had reduced expression, is consistent with the intracellular accumulation of pyoverdine observed in the mutants: PvdE has been reported to transport immature pyoverdine (PVDI) across the inner membrane into the periplasm, where full maturation of the molecule takes place (Yeterian *et al*., 2010). On the other hand, *argG* and *argH* mutants retain certain ability to release the siderophore to the extracellular medium as indicated in Figure 6B. One possible explanation to this is that *pvdE* is being translated at low level in these mutants, although mutation of *pvdE* almost completely prevents PVDI production in *P. aeruginosa* (Yeterian *et al*., 2010). Alternatively, this leads us to speculate with the possibility that another transport system currently uncharacterised might be involved.

The differences in expression of *pvdA*, *pvdD*, and *pvdE* in these mutants will need to be analysed in more detail. Expression of the main regulator involved in their transcription, PvdS (reviewed for Chevalier *et al*., 2018) is not altered in the mutants, and neither is the expression of *algQ*. One possibility that remains to be tested is if FvpI, another regulator implicated in *pvdD* and *pvdA* transcription (reviewed for Chevalier *et al*., 2018) shows altered expression in the mutants. In our analysis, no clear role could be ascribed to ArgR, the main regulator of arginine metabolism, except a minor increase in *algQ* expression, that might favour the availability of RNA polymerase for PvdS.

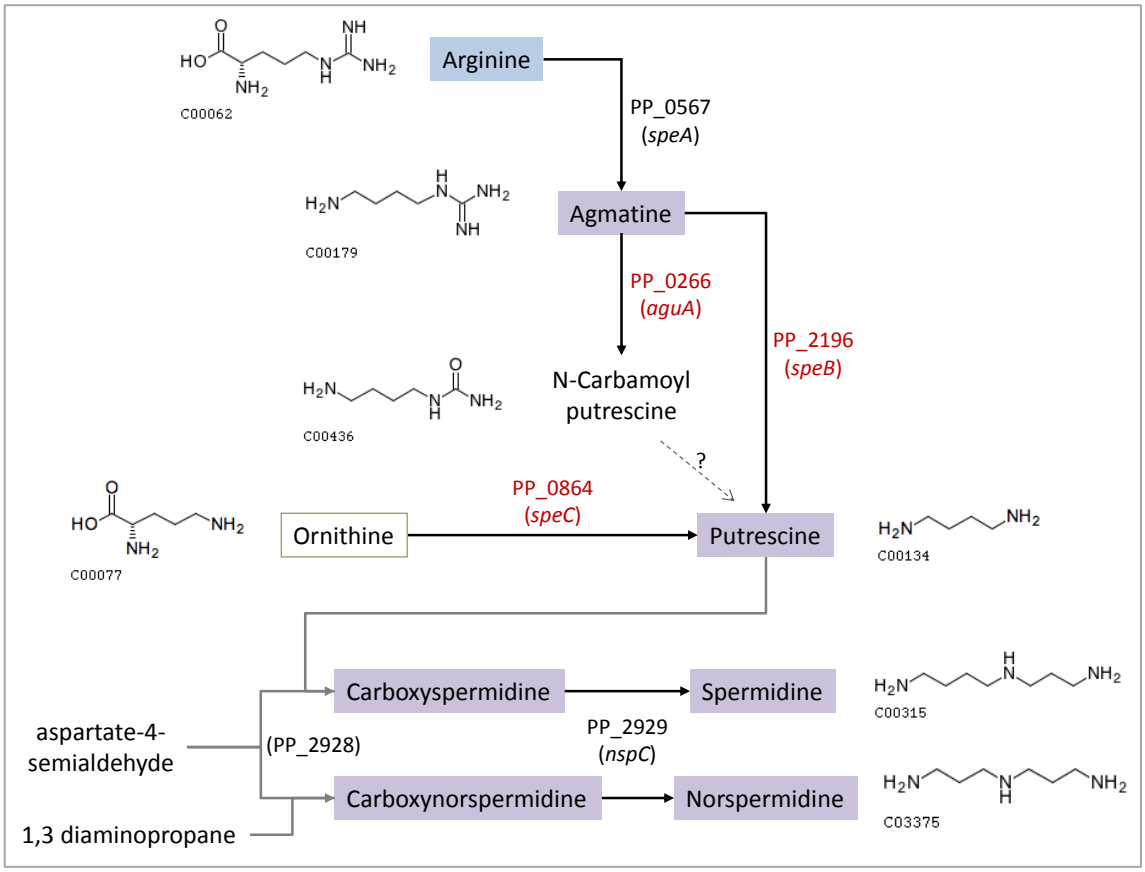
We have been able to establish a potential connection between arginine and iron metabolism and oxidative stress that will deserve more research. This might imply, for example, that siderophore production helps to reduce oxidative stress produced by plants during root colonization by *P. putida* KT2440 (Matilla *et al*., 2007b). Competitive rhizosphere colonization assays indicate that fitness of the *argG* and *argH* mutants in this environment is significantly reduced, and the mutants are almost completely displaced by the wild type (Supplementary Figure S3). We believe such dramatic effect is due to the combination of metabolic and stress factors accumulated in these mutants.

The results obtained in this Chapter, and those presented in the previous ones open the way for new avenues of research linking metabolism, multicellular lifestyles and stress in bacteria.

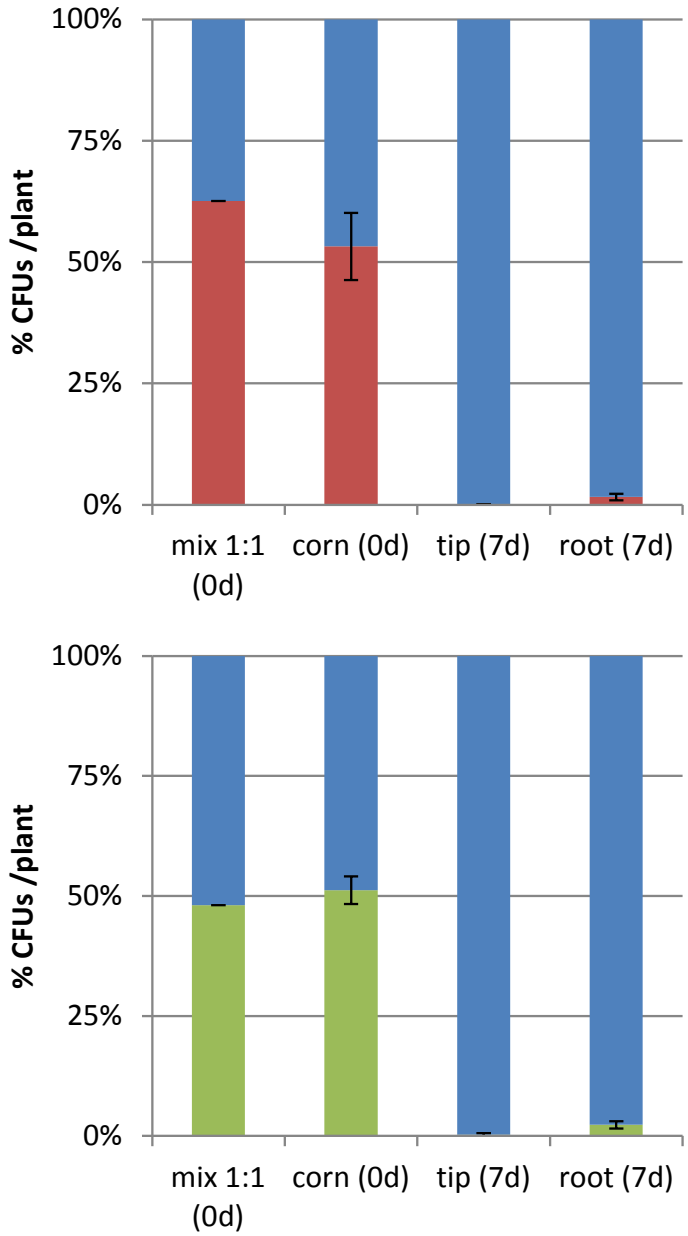
SUPPLEMENTARY MATERIAL



Supplementarry Figure S1. Genetic organization of genes involved in pyoverdine synthesis, transport and regulation in *P. putida* KT2440.



Supplementary Figure S2. Polyamine synthesis pathways in *P. putida* KT2440. Polyamines and intermediates are shaded in grey.



Supplementary Figure S3. Competitive corn root colonization assays by KT2440 (blue bars) and *argG* (red bars) and *argH* (green bars) mutants. Proportions of C.F.U. of each strain in the initial inoculum (mix 1:1), attached to corn seeds after incubation at 30°C for 30 min (corn) and recovered after 7 days in the corn rhizosphere (root) and the root tips (tip) are plotted. Results correspond to average and standard deviation from 10 plants.

CHAPTER 4

Influence of stress caused by toxic hydrocarbons on development and resilience of *Pseudomonas putida* biofilms: a preliminary analysis

Adapted from: Barrientos-Moreno, L., Espinosa-Urgel, M. Biofilm Stress Responses Associated to Aromatic Hydrocarbons. In: T. Krell (ed.), Cellular Ecophysiology of Microbe, Handbook of Hydrocarbon and Lipid Microbiology, Chapter 7, pp 105-115. Springer International Publishing AG 2017

BACKGROUND

In the environment, bacteria encounter a number of fluctuating factors, including temperature, nutrient and water availability, and the presence of toxic molecules produced in their abiotic and biotic surroundings or resulting from their own metabolism. Survival in such unpredictable conditions requires a wide range of adaptive responses. The acclimatization potential of bacteria relies on many different aspects that include metabolic versatility, the capacity to acquire new genetic information via horizontal transfer mediated by plasmids or other mobile elements, and a variety of stress resistance mechanisms (Goessweiner-Mohr *et al*., 2014 and references therein; Hughes and Andersson, 2017). Microorganisms able to combine all these features for physiological/biochemical adaptation are better suited to colonizing changing niches. At the molecular level, bacterial responses rely on a combination of constitutive, basal elements acting as a first line of defence, and the activation of the expression of genes encoding products that deal with a given physico-chemical stress in response to environmental or cellular signals.

One of the key strategies for colonization and persistence in many different environments is the ability to form biofilms. These multicellular communities associated to solid surfaces offer protection against predation, stress and the action of biocides, and thus are considered the predominant lifestyle for many bacterial species in the environment. Bacteria growing as biofilms are surrounded by an extracellular matrix with a high water content and usually composed of proteins, exopolysaccharides and eDNA (Costerton *et al*., 1995; Sutherland, 2001). The composition of the biofilm matrix varies depending on the bacterial species and the environmental conditions, and it is considered one of the main elements that determine biofilm protection (Balcázar *et al*., 2015, Gambino and Cappitelli, 2016). Different studies have shown that bacteria growing as biofilms show increased tolerance to a wide range of environmental challenges, such as antibiotics (Høiby *et al*., 2010; Hall and Mah, 2017; Gupta *et al*., 2018), metal toxicity (Grumbein *et al*., 2014; Dranguet *et al*., 2017), acid exposure (McNeill and Hamilton, 2003) or dehydration (Nielsen *et al*., 2011), among others. Whereas antibiotic resistance in biofilms has received significant attention, given the obvious clinical implications, the development of biofilms in the presence of toxic hydrocarbons and the potentially associated tolerance mechanisms are much less characterized. However, this knowledge would be of great interest in terms of

biotechnological applications of biofilms, such as remediation of sites contaminated with organic pollutants, the development of biosensors, or biotransformation reactions that could render added value products. The toxicity of compounds (substrates or products) can limit such reactions and thus reduce their biotechnological potential. This is particularly relevant in the case of biotransformations that involve toxic aromatic hydrocarbons (toluene, xylenes, etc.) to produce aromatic organic acids, or in the bioproduction of hydroxylated aromatic compounds from carbohydrates or alcohols (Gosset, 2009; Vargas-Tah and Gosset, 2015; Molina-Santiago *et al*., 2016). The toxicity of organic solvents is directly related with the logarithm of their partitioning coefficient (log P_{ow}) in a defined octanol–water mixture (Sikkema *et al*., 1995). Compounds with a log P_{ow} value below 4, such as toluene or styrene, are highly toxic because they accumulate in the cytoplasmic membrane, disorganizing its structure and altering its functionality. This result in a loss of ions and metabolites, disruption of the pH and electron gradient and eventually leads to cell death. Nonetheless, bacterial strains (mainly from the genus *Pseudomonas*) capable of enduring the presence of significant amounts of toxic hydrocarbons have been isolated, and several tolerance mechanisms have been reported (Segura *et al*., 1999; Segura *et al*., 2012; Ramos *et al*., 2015). In this chapter we will present new information on the role of biofilm formation in the persistence of *Pseudomonas* in the presence of toxic hydrocarbons such as toluene.

RESULTS

Biofilm formation varies significantly between closely related *P. putida* strains.

We analysed three closely related *P. putida* strains to test if the presence of toxic organic compounds such as toluene influences biofilm formation and persistence: DOT-T1E, which was isolated from a wastewater treatment plant and is an efficient degrader of benzene, ethylbenzene and toluene, and highly tolerant to these compounds, thanks to the presence of three efflux pumps (Duque *et al*., 2001); mt-2, which harbours plasmid pWW0, carrying catabolic and regulatory genes responsible for the complete transformation of toluene into Krebs cycle intermediates (Franklin *et al*., 1981); and KT2440, the plasmid-free derivative of mt-2 used as model organism in the rest of this Thesis. In this strain, two large

extracellular proteins, LapA (8682 amino acids) and LapF (6310 amino acids), are the main adhesins required for biofilm formation in different environmental conditions (Hinsa *et al*. 2003; Yousef-Coronado *et al*., 2008; Martínez-Gil *et al*., 2010; Martínez-Gil *et al*., 2014).

Initially, biofilm formation was compared in the absence of stressor. In these conditions, mt-2 formed more robust biofilms than KT2440, as previously observed (D'Alvise *et al*., 2010), whereas DOT-T1E showed limited adhesion to solid surfaces compared to the other two strains (Figure 1). An *in silico* analysis of extracellular matrix components comparing *P. putida* KT2440 with DOT-T1E revealed that this strain lacks most of the gene encoding LapA (Figure 2) and also has a shorter homolog of LapF (around 2000 amino acids less; data not shown). This loss of a key adhesin could explain the poor biofilm formation characteristics of this strain.

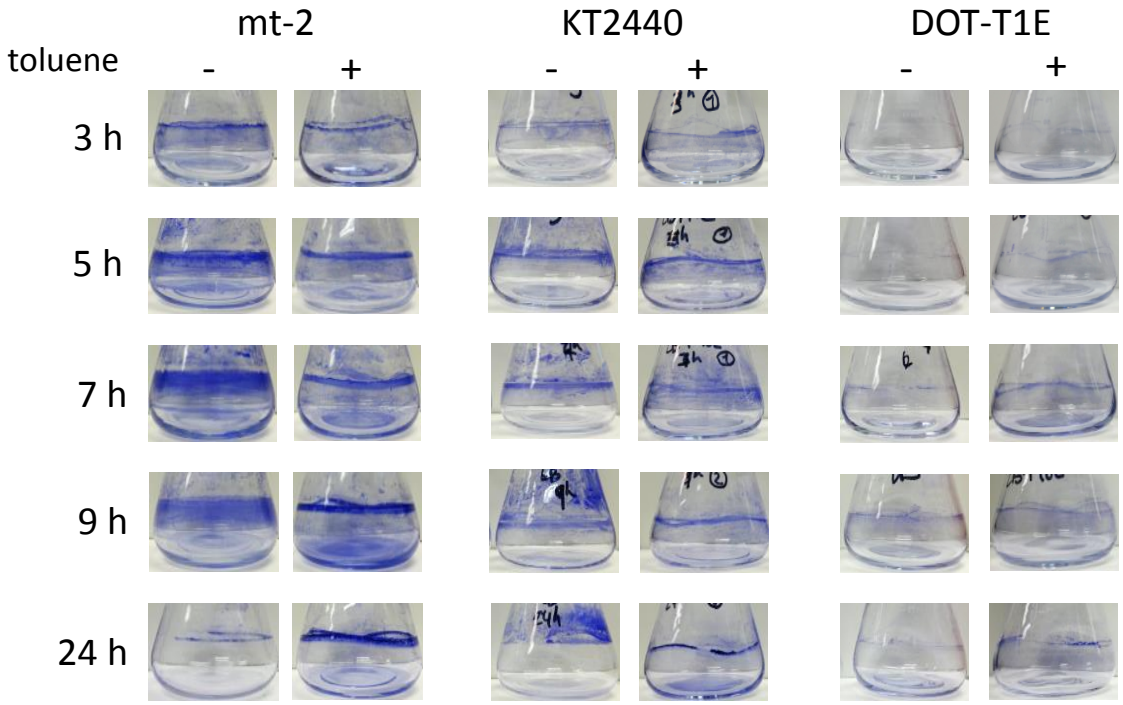


Figure 1. Biofilm formation by different strains of *P. putida* (mt-2, KT2440 and DOT-T1E) during growth in LB in the absence or presence of 0.05% toluene. At the indicated hours, medium was removed and biomass attached to the glass surface was stained with crystal violet, as described in Materials and Methods.

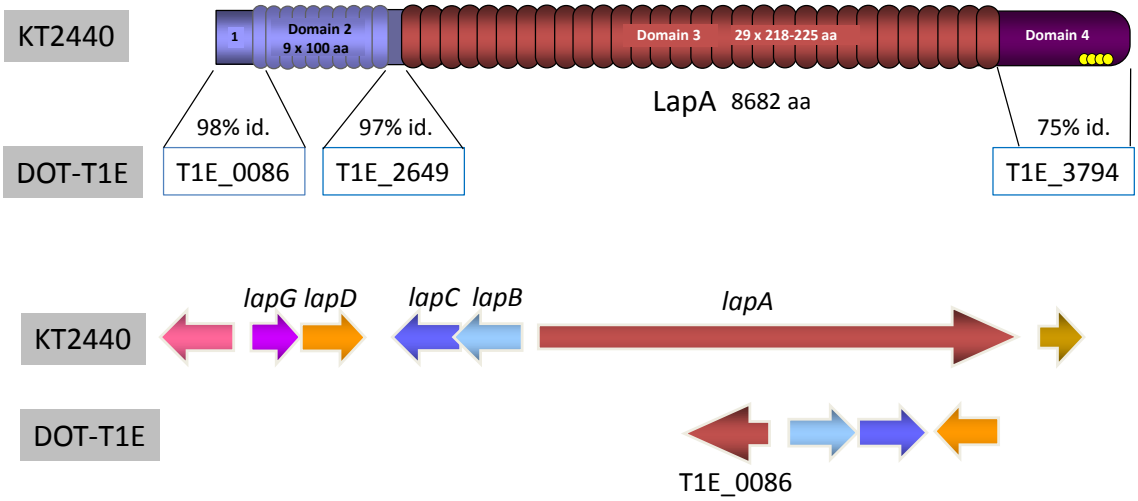


Figure 2. Top: comparison between LapA of KT2440 and proteins of DOT-T1E with sequence similarities. Bottom: KT2440 genomic region containing lapA and the equivalent region in DOT-T1E.

Influence of toluene on biofilm formation.

When cultures were not pre-exposed to low concentrations of toluene in the gas phase, addition of high concentrations of the compound to the medium limited growth and consequently hampered biofilm formation (data not shown). On the other hand, low concentrations of the hydrocarbon (0.05% v/v) resulted in increased biofilm development in all the strains (Figures 1 and 3), an effect that was observed in the tolerant strain DOT-T1E at concentration to up to 0.1% v/v toluene (Figure 4). In pre-exposed cultures, the most noticeable effect was that biofilms persisted after 24 hours, while in the absence of stressor they had already dispersed, as it is known to happen in the batch culture conditions used (Martínez-Gil *et al*., 2010). This could suggest that the hydrocarbon may favour the sessile lifestyle, as a protective state against stress, similarly to the effect of sub-inhibitory concentrations of antibiotics (Jones *et al*., 2013; Hathroubi *et al*., 2015; George and Halami, 2017).

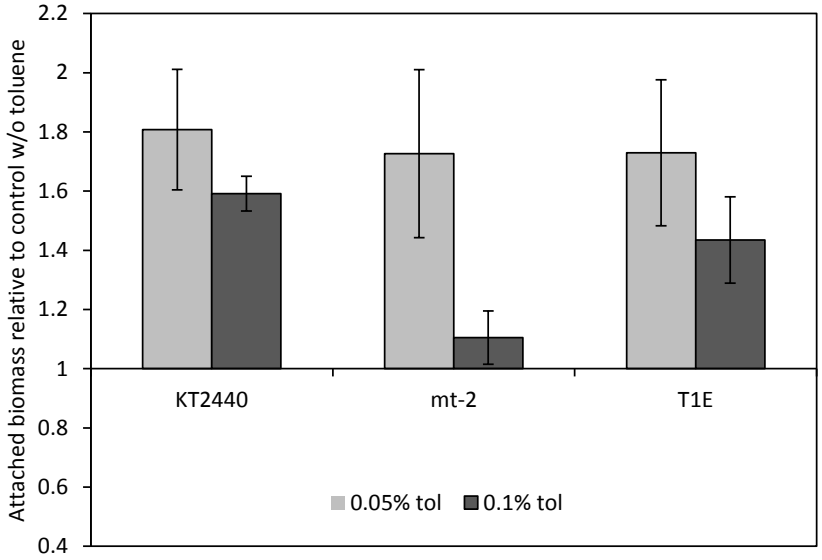


Figure 3. Effect of toluene on biofilm formation: comparison between strains at 6h of growth. The biomass attached to the surface, relative to the control without toluene (= 1), was measured spectrophotometrically after staining and solubilization of the dye with 30% acetic acid. Data correspond to averages and standard deviations from two independent experiments with three replicas each.

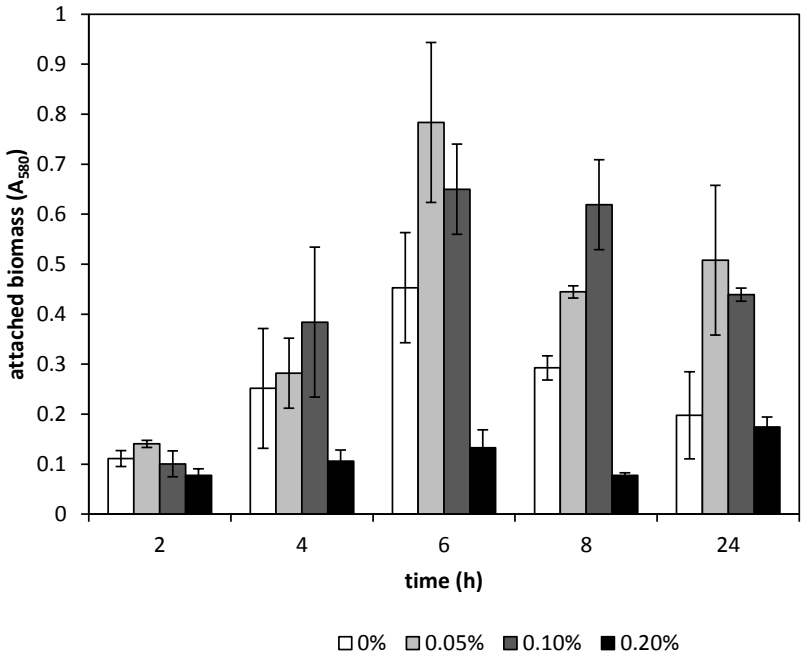


Figure 4. Details on the effect of increasing concentrations of toluene on biofilm formation by the tolerant strain DOT-T1E. The attached biomass was measured as in Figure 3. Data correspond to averages and standard deviations from two independent experiments with three replicas each.

Effect of toluene on preexisting biofilms.

Biofilms are generally considered a protective lifestyle for bacteria against environmental stress, biocides, etc. We decided to analyse the influence of toluene addition on sessile populations, using the strain that formed the more robust biofilms, mt-2. Paradoxically, addition of toluene to biofilms of mt-2 pre-formed in the absence of the compound caused accelerated detachment (Figure 5). The same was observed for KT2440 (not shown). A similar, but less pronounced effect was also observed for DOT-T1E although the limited attachment capacity of this strain in the absence of stressor did not allow a detailed analysis. We interpret these data as indicative that the presence of toluene from the early stages promotes attachment and gives rise to biofilms that are structurally different (in terms of matrix composition and probably hydrophobicity) from those produced under optimal growth conditions, so that when already established sessile populations are abruptly confronted with the solvent, their matrix is not "prepared" and the biofilm is disorganized. It is worth noting that in *P. putida* the four known exopolysaccharides seem to have different roles and importance depending on the surface colonised and the environmental conditions (Nielsen *et al*., 2011; Martínez-Gil *et al*., 2013).

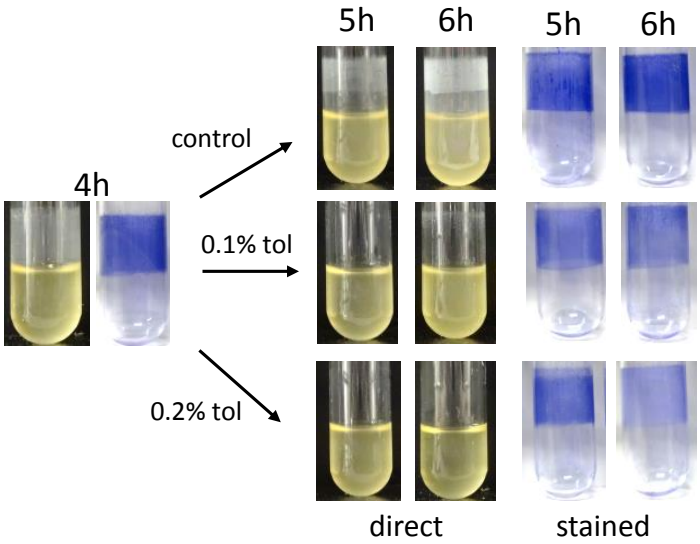


Figure 5. Effect of toluene on biofilms of *P. putida* mt-2 pre-formed in the absence of the hydrocarbon. Cultures were grown in LB in tubes at 30°C under orbital rotation (40 r.p.m.) for 4h and then toluene (0.1 or 0.2%) was added. The evolution of the attached biomass in the absence or presence of the hydrocarbon was followed for two more hours by direct visual inspection and crystal violet staining.

DISCUSSION

Several lines of research deserve further exploration that could potentially lead to optimized and expanded biotechnological uses of biofilms in relation with biodegradation or biotransformation of toxic hydrocarbons. In the past two decades there have been significant advances in understanding the mechanisms of bacterial biofilm formation and unveiling the resistance and tolerance strategies that microbes use to cope with toxic aromatic hydrocarbons. There is also solid information on the different parameters (kinetics, population dynamics, production rates) relevant to biofilm bioreactors applied to hydrocarbon biodegradation. However, these three (biofilm biology, tolerance mechanisms and bioreactor performance) are still rather isolated bodies of knowledge that need to be integrated for a full exploitation of microbial capacities. Analysing the expression and activity of tolerance mechanisms such as efflux pumps and other stress responses, or of catabolic genes in biofilms growing in close-to-real situations, would be important to model and predict the behaviour of the systems and ultimately to improve biotransformations. There is also little or no information on how the substrates and products of the desired reactions can affect biofilm growth and persistence and hence may limit productivity. From a more basic point of view, investigating the role and activity of tolerance mechanisms in natural environments could offer new information on the natural compounds that act as stressors and trigger these responses.

Finally, the preliminary data obtained with three closely related strains of *P. putida* open intriguing evolutionary questions. Catabolic genes for toluene and the most important element for tolerance to the compound, the TtgGHI efflux pump, are present in mobilizable plasmids, pWW0 and pGRT1, sharing some similar features (Segura *et al*., 2014), but not combined into one, which one would predict to offer increased advantages. On the other hand, it is noticeable that the most tolerant strain, DOT-T1E, lacks the essential element for robust biofilm formation present in mt-2 and its derivative KT2440. It is possible that the environmental conditions found by each microorganism during their evolution (waste water in the case of DOT-T1E and soil of a planted orchard in the case of mt-2) have favoured the selection of one or the other as the most efficient survival mechanism, taking into account the energetic burden of maintaining such big plasmids and the intact genes for large adhesins (nearly 27 kb for *lapA* and 19 kb for *lapF*, not counting the genes involved in their

transport and regulation). The existence of three predicted proteins encoded in different regions of the chromosome of mt-2 and sharing high identity with fragments of LapA suggest that this strain has actually lost the original *lapA* gene, rather than this element having been acquired by mt-2 later in its evolution. Nonetheless, this information can open the way to construct modified strains that carry all the relevant elements in the most compact possible way and test their performance in biofilm bioreactors. Well studied, non-hazardous strains such as *Pseudomonas putida* KT2440, generally recognized as safe, and for which many tools for genetic manipulation and gene expression are available, can provide the background for such modifications and their future use in the biotechnology industry.

V. GLOBAL DISCUSSION

In nature, most of bacteria tend to live forming biofilms in which many structural and regulatory elements are involved. However, little is known about the environmental signals that trigger the response that leads to transition between motile and sessile lifestyles, implying a great challenge for the understanding of biofilm development in most of the cases. In the last years, the amino acid arginine has emerged as a cue that regulates biofilm formation in different bacterial species. In this Thesis, we have explored the role of this amino acid in biofilm formation and its relationship with the intracellular second messenger c-di-GMP in *Pseudomonas putida* KT2440, as well as a preliminary study between this amino acid and pyoverdine siderophore production in this bacterium.

Random mutagenesis and transcriptomic analyses have been widely used as strategies to identify genes involved both in biofilm formation and regulation. Using both approaches, LapF and LapA have been identified and characterised (Espinosa-Urgel *et al*., 2000; Hinsa *et al*., 2003; Yousef-Coronado *et al*., 2008; Martínez-Gil *et al*., 2010), as well as CfcR (Matilla *et al*., 2007b). Recently in our group, using random mutagenesis, two genes involved in the last two steps of the arginine biosynthesis pathway, *argG* and *argH*, were identified as participating in c-di-GMP signalling (Ramos-González *et al*., 2016). Due to the emerging role of amino acids and, more specifically, arginine in biofilm formation in recent years, we decided to focus on the study of these two genes and the role of arginine upon different aspect of bacterial lifestyles.

We have demonstrated that L-arginine plays an important role on crinkly colony morphology, a phenotype that emerges as a consequence of high levels of c-di-GMP in *P. putida* KT2440. In fact L-arginine appears to be specifically related to this phenotype, which is corroborated by the fact that the expression of the EPS Pea, which was shown to be required for this phenotype (Matilla *et al*., 2011), is significantly reduced in *argG* and *argH* mutants. Furthermore, we also have shown that these mutants present reduced *lapF* expression and an increase in cellulose and Peb, which could imply an attempt to compensate the lack of Pea in a similar way as what happens between EPS and adhesins LapA and LapF in this bacterium (Martínez-Gil *et al*., 2013). This supports the idea of compensatory balance between extracellular matrix components showed by Martínez-Gil and coworkers (2013) due to the role of EPS as structural stabilizers and their implications in cell-to-cell interactions in biofilm formation (Nielsen *et al*., 2011; Nilsson *et al*., 2011), as the

case for LapF, which participates in microcolony development (Martínez-Gil *et al*., 2010). Similar results were obtained in the case of *Salmonella enterica* serovar Enteritidis where the overproduction of curli fimbriae compensates biofilm deficiency showed by a mutant in the cell-surface protein encoding gene *bapA* (Latasa *et al*., 2005) or in *P. aeruginosa*, where the absence of the EPS Psl leads to overproduction of a second EPS, Pel, and the lack of the latter induces alginate production (Ghafoor *et al*., 2011). This compensatory balance might explain why *argG* and *argH* auxotrophic mutants were not affected in biofilm formation in LB rich medium, added to the presence of L-arginine in this medium. However, overexpression of these genes, and therefore increase in intracellular levels of L-arginine, caused an increase in biofilm formation compared to the wild type strain. This, along with the fact that L-arginine added in minimal culture medium promotes biofilm formation in *P. putida* KT2440 is in agreement with data obtained for other bacteria as *P. aeruginosa* (Bernier *et al*., 2011), in spite of the different culture conditions. Furthermore, we have shown that L-arginine positively influences expression of the alternative sigma factor σ^S (RpoS), that the expression of EPS *pea* is *rpoS*-dependent and *pea* is positively influenced by L-arginine. In the same way, expression of LapF is under the regulation of RpoS (Martínez-Gil *et al*., 2010). This highlights the importance of L-arginine in biofilm formation and regulation in *P. putida* KT2440, acting in these cases indirectly upon biofilm-related elements.

It has been reported that the effect of exogenous L-arginine on biofilm formation varies among bacterial species, having both positive and negative implications. It is well known in bacteria that cause dental caries (Kolderman *et al*., 2015; Zheng *et al*., 2017) as *Streptococcus mutans* and *S. gordonii*. Thus, *S. mutans* rapidly colonises teeth and produces insoluble EPS (as glucans) which allow the establishment of other bacteria, forming a highly adherent and cohesive biofilm. *S. mutans* and other bacteria produce high amounts of insoluble EPS when sugars are fermented because of the increase in pH during early biofilm that stimulates EPS production. This favours proliferation of acidogenic bacteria which are involved in caries formation. It has been shown that L-arginine can modulate cariogenic biofilms. This is due to L-arginine repressing *S. mutans* genes associated with insoluble EPS production during early biofilm stages, leading to a reduction of *S. mutans* growth and promoting *S. gordonii* dominance in oral biofilms (He *et al*., 2016). L-arginine is metabolised by *S. gordonii* and other bacteria via the ADI pathway, producing ammonia that leads to pH

increase. Furthermore, L-arginine promotes biofilm formation in *S. gordonii* (Jakubovics *et al*., 2015). At the end, the amino acid causes a disruption in the EPS matrix assembly and tri-dimensional biofilm architecture. Thus, L-arginine can control the biofilm formed in oral cavity destabilising oral muti-species biofilms (Kolderman *et al*., 2015; He *et al*., 2016; Huang *et al*., 2017).

It is striking that high amount of the amino acid is required to observe the phenotypes in *P. putida*. However, amino acids are molecules that can be metabolised serving as carbon and nitrogen sources (Corral-Lugo *et al*., 2016) as well as being part of structural components of proteins. Thus, from the concentration of arginine added to the culture media, we do not really know which amount is transported, which part is metabolised by bacteria and which part acts as a signal molecule. In fact, the effect of L-arginine in bacterial lifestyles as biofilm formation and motility not only depends on the bacterial strains, as shown previously, but also on its concentration in the medium. Thus, high levels of L-arginine have been demonstrated to inhibits swimming motility (ranging between 250 and 750 mM), whereas low levels favour this type of motility (100 mM) in *P. aeruginosa* PAO1 (Everett *et al*., 2017). Everett and co-workers demonstrated that in burn wound tissues the levels of L-arginine are depleted due to the arginase activity of the eukaryotic cells, and on the other hand, L-arginine acts as a chemoattractant for *P. aeruginosa*. Taking all together, bacteria are attracted to the burn wound tissues, where they arrive by swimming motility from uninjured tissues. However, high levels of the amino acids reduce bacterial spread. So, L-arginine can modulate *P. aeruginosa* pathogenicity and transition from motile-to-sessile lifestyles (Everett *et al*., 2017). Another example is the case of *S. gordonii* in which L-arginine enhanced biofilm development, but only in a specific range (Jakubovics *et al*., 2015). Thus, concentrations between 0.5 and 500 µM (low arginine) resulted in increased biofilm formation and biofilms were structured. However, at high levels of amino acid (50-500 mM), biofilms shown altered architecture, thickness and biomass, and the presence of microcolonies were reduced.

Based on our findings with respect to crinkly morphology and biofilm formation, we have highlighted the opposite effect exerted by L-aspartic acid, being to our knowledge the first demonstration that other L-amino acid acts in the opposite way to L-arginine. Previously, D-amino acids have only been shown to prevent biofilm formation in *Bacillus*

subtilis, *Staphylococcus aureus* and *P. aeruginosa* (Kolodkin-Gal *et al*., 2010). This observed effect is extended to c-di-GMP levels, a key element in the transition from planktonic to sessile mode of growth. Besides, other L-amino acids tested in this Thesis showed positive or negative impact upon the second messenger content, which lead us to speculate with the possibility that other proteinogenic amino acids might modulate c-di-GMP pool, even though their effect may be altered by other molecules, given the differences found between their effects in minimal and rich media. Further analyses are required to determine the role and importance of some of them in *P. putida* KT2440 lifestyles, mainly in the case of tryptophan, which significantly increases c-di-GMP content in LB medium. This amino acid has previously been reported to have a positive impact on biofilm development in *E. coli* and *S. enterica* serovar Typhimurium (Ren *et al*., 2004; Domka *et al*., 2007; Hamilton *et al*., 2009), but not in the related species *P. aeruginosa* PA14 (Bernier *et al*., 2011). Thus, advancing knowledge of the role of these molecules, it might be possible to modulate the mode of growth of bacteria.

At the time we initiated this work, almost nothing was known about the relationship between amino acids, and more specifically of L-arginine, and the second messenger c-di-GMP. Bernier and coworkers have shown that arginine-dependent biofilm and swarming phenotypes require the activity of diguanylate cyclases SadC and RoeA in *P. aeruginosa* PA14, indicating that an increase in c-di-GMP content in the wild type strain in the presence of 4.8 mM of L-arginine is due to the activity of both DGC (Bernier *et al*., 2011). To date, the DGC activity of CfcR is the most important to contribute to increase c-di-GMP level in *P. putida* KT2440 (Huertas-Rosales *et al*., 2017). A *cfcR* mutant still retains certain ability to increase the second messenger pool in the presence of increasing concentrations of L-arginine, as well as a mutant in its multi-sensor hybrid histidine kinase *cfcA*. This, and the fact that *cfcA* does not show L-arginine binding, and that *argG* and *argH* mutants are little affected in *cfcR* expression, prompted us to suggest that other elements may be involved in the response to the amino acid. In order to further understand the role of L-arginine in c-di-GMP content, we have demonstrated that its effect on the intracellular second messenger is due to a cumulative effect in which several elements are involved, including *rpoS* and *argR*, being the latter required for the response to the amino acid. Likely this is the consequence of RpoS and ArgR acting upon *cfcR* expression (Huertas-Rosales *et al*., 2017; this Thesis) and

maybe over other elements. These results lead us to speculate that the effect of L-arginine on c-di-GMP levels in *P. putida* are more complex than in *P. aeruginosa*.

In an attempt to expand our knowledge regarding the regulation of *argG* and *argH*, we have demonstrated that the regulation of these genes is the result of a cascade in which many elements related with biofilm control are involved, as well as the existence of regulatory loops among some of them. Thus, using transcriptional fusions to the reporter gene *lacZ*, we have demonstrated that the Rsm proteins exert a positive effect on *argG*, and our results indicate this is most likely an indirect regulation. However, no effect was observed upon *argH*. Therefore, the control of Rsm proteins is over the second-to-last gene of L-arginine biosynthesis pathway, responsible of the L-arginosuccinate synthesis from citrulline and aspartate. Generally, proteins belonging to Csr/Rsm family act as negative regulators, although it has been reported that they can also act as a positive regulators, directly or indirectly. In that way, CsrA activates the operon for flagellum biosynthesis necessary for motility in *E. coli* (Wei *et al*., 2001), RsmA controls positively lipase and rhamnolipid production in *P. aeruginosa* (Heurlier *et al*., 2004) and T3SS in *Xanthomonas* (Andrade *et al*., 2014). Another level of regulation is due to ArgR, a key regulator of arginine metabolism, which controls some genes implicated both in catabolism and anabolism in many bacteria (Maas, 1994; Park *et al*., 1997a,b; Fulde *et al*., 2011; Cheng *et al*., 2017). We have shown that this regulator influences the expression of *argG* and *argH*, and found an ArgR binding box in the promoter of *argG*; there seems to be another one in *argH*, although it is less conserved (data not shown). This might explain why a stronger regulation is exerted on *argG*. In *P. aeruginosa*, ArgR only influences *argG* expression (Lu *et al*., 2004).

We have studied in more detail the role of ArgR in *P. putida*, since this protein was not characterised, or even annotated in this bacterium. We have determined that *argR* forms part of an operon together with the genes encoding the arginine-binding protein ArgT and the rest of the putative components of the ABC transporter, as occurs in *P. aeruginosa* (Nishijyo *et al*., 1998). However, contrary to this bacterium, we have shown that ArgR modulates its own expression from an arginine-inducible promoter in the intergenic region upstream the *argR* gene, besides the promoter located at the beginning of the operon as in the case of *P. aeruginosa*. Furthermore, we have been able to demonstrate for the first time the existence of a negative feedback loop between ArgR and RpoS, which is dependent of

the presence of exogenous L-arginine and, as is the case for *argR*, arginine itself positively influences *rpoS* expression. Furthermore, Rsm proteins and the transcriptional regulator FleQ are also involved in ArgR regulation. C-di-GMP participates in the modulation exerted by FleQ: high levels of this second messenger increase expression of *argR*, but this effect is not observed in a *fleQ* mutant. This is similar to what happens in some elements involved in biofilm formation in different bacteria, as the case of *lapA* in *P. putida* (Martínez-Gil *et al*., 2014) and *pel* in *P. aeruginosa* (Baraquet *et al*., 2012; Matsuyama *et al*., 2016).

All our results reflect the degree of interconnectivity and complexity in arginine-related regulation of *P. putida* lifestyles, including feedback loops, which probably reflects the need for a fine control of the different elements as a consequence of the natural changing environment in which this bacterium live.

Despite the roles associated with L-arginine, the mechanism of action of this amino acid remains unknown and almost no research has been done in this aspect. Only in *S. enterica* serovar Typhimurim two possible models (Figure 1) have been proposed, based on data obtained by Mills and co-workers (Mills *et al*., 2015). The first model (model A) implicates a periplasmic sensing pathway in which the periplasmic putative L-arginine-binding protein ArgI interacts with the periplasmic sensing domain CACHE1 of STM1987 DGC, directly or being part of a protein complex. ArtI binds L-arginine and together promotes the GGDEF activity domain of STM1987. As a result of this activity, levels of c-di-GMP are increased in the bacterium cytoplasm. The second model (model B) proposes that a putative ABC transporter present in the inner membrane of the cell transport L-arginine into the cytoplasm and requires the ArgI protein to sense L-arginine. Once in the cytoplasm, L-arginine activates GGDEF activity domain of STM1987 by an unknown mechanism, leading to increase c-di-GMP levels as in the first model. When c-di-GMP levels are high, synthesis of EPS cellulose required to root colonization is activated (Cowles *et al*., 2016).

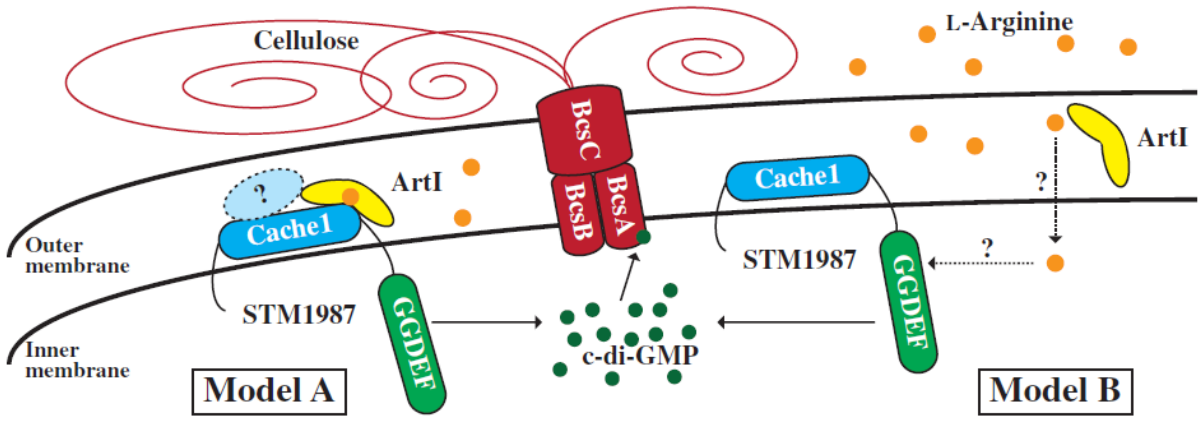


Figure 1. Possible models for sensing L-arginine and its mechanism of action in *S. enterica* serovar Typhimurium. See details in the text (Taken from Mills *et al*., 2015).

To date, no homologs to this system have been found in *P. putida* KT2440, since the most important arginine-binding protein ArgT does not seem to be related to any diguanylate cyclase. Furthermore, ITC experiments using FleQ and CHASE3 domain of CfcA in an attempt to find out if these proteins are responsible of L-arginine binding showed negative results (our unpublished data), as well as the search for other elements connecting c-di-GMP and arginine using random transposon mutagenesis. Thus, the mechanisms in *P. putida* seem to be fundamentally different from the models proposed for *S. enterica*. No other mechanisms of action of L-arginine have been proposed in other bacteria. Given the implication of Rsm proteins observed in relation to the regulatory network that associates arginine metabolism and c-di-GMP signalling shown in Chapter 2, and the role of the TCS GacS/GacA on activating the expression of small non-coding RNAs that sequester Rsm proteins (Kulkarni *et al*., 2006; Lapouge *et al*., 2008; Brencic *et al*., 2009), it is tempting to speculate that L-arginine could activate GacS. The signal to which GacS responds remains yet unknown.

Iron availability is an environmental cue that has a high impact on bacterial physiology. When the level of iron is low, *P. aeruginosa* modifies its gene expression profile activating different pathways involved in iron uptake (Ochsner *et al*., 2002). In *P. putida*, iron metabolism is essential for plant root colonization and to adapt to multiple niches (Martínez-

Bueno *et al*., 2002). Due to its significance, bacteria have the ability to produce siderophores which are able to bind iron present in the environment and are equipped with a series of iron-capturing systems that sense the specific siderophores. Our results indicate a link between the amino acid arginine biosynthesis and the production of the siderophore pyoverdine in *P. putida* KT2440. As we have shown, *pvdS* and *pvdA* are overexpressed in *argG* and *argH* mutants while *pvdE* is downregulated, which implies an intracellular accumulation of the siderophore. We speculate that the Gac/Rsm pathway might be involved in this process because of several reasons. Firstly, we have reported an indirect positive effect of Rsm proteins upon *argG* expression. Secondly, the Gac/Rsm pathway has been recently characterised in *P. putida* KT2440 (Huertas-Rosales, PhD Thesis, 2017b) and different reports have demonstrated the implication of this route in pyoverdine production in several bacteria. However, opposing results on the relationship between this pathway and iron uptake have been reported. Thus, a *gacA* mutant showed reduced pyoverdine levels in *Pseudomonas marginalis* (Liao *et al*., 1997); in *P. aeruginosa* PAO1, a *rsmA* mutant presented an increase in pyoverdine content and some control of siderophore production is exerted by the Gac/Rsm pathway, where RsmA negatively affects *pvdS* and *pvdA* expression (Frangipani *et al*., 2014); and the overexpression of *rsmA* of *P. aeruginosa* in *Pseudomonas syringae* led to a pyoverdine decrease in this strain (Kong *et al*., 2010). On the other hand, a transcriptomic analysis of *Pseudomonas fluorescens* Pf-5 indicated that *pvdS* was highly increased in a *gacA* mutant (Hassan *et al*., 2010) and mutants in Rsm proteins in *P. putida* KT2440 show reduced levels of pyoverdine (Huertas-Rosales *et al*., 2016).

Based on the results presented in Chapter 3 and those obtained by Huertas-Rosales (Huertas-Rosales *et al*., 2016, Huertas-Rosales, PhD Thesis, 2017b), it is probable that Rsm proteins positively influence pyoverdine production and release in *P. putida* KT2440, although the mechanism remains unknown. Perhaps different regulatory mechanisms could exist, since *argH* is not regulated by Rsm proteins and an *argH* mutant shows the same phenotype as an *argG* mutant. In any case, the regulation seems to be different from *P. aeruginosa* and other related *Pseudomonas* and perhaps the Gac/Rsm pathway would partly explain the relationship between arginine metabolism and pyoverdine production in *P. putida* KT2440.

The presence of free iron in excess can contribute to increase reactive oxygen species. Siderophores have been extensively reported to reduce oxidative stress in microorganisms producing them and there is growing evidence suggesting that siderophores may have other physiological roles apart from iron acquisition (Peralta *et al*., 2016; Khan *et al*., 2018). Our results from oxidative stress assays showed the susceptibility of *argG* and *argH* mutants to hydrogen peroxide. The production of reactive oxygen species is a plant response after microorganism recognition (Torres, 2010). Furthermore, oxidative stress is a predominant condition in the rhizosphere and surface-adhered bacteria are exposed to high levels of this stress (Honma *et al*., 2009; Nanda *et al*., 2010). Since *P. putida* KT2440 is a plant-root colonizer, one can speculate that pyoverdine production might serve as a mechanism to reduce oxidative stress, contributing to bacterial fitness in this environment. In prokaryotes, polyamines indirectly exert protective functions by induction of stress responsive regulons through activation of RpoS, among others (Rhee *et al*., 2007). Considering that polyamines are synthesised from arginine and that we have demonstrated that this amino acid plays a positive role on *rpoS* expression and that polyamines are presumed to alleviate oxidative stress, it is tempting to speculate that these molecules (polyamines and/or arginine) can act upon *rpoS* expression triggering the induction of oxidative stress tolerance systems. Taking all together, this is in accordance with the susceptibility of *argG* and *argH* mutant to iron capture and oxidative stress, which is alleviated by some polyamines. In Figure 2, a hypothetical model that summarizes these aspects is shown.

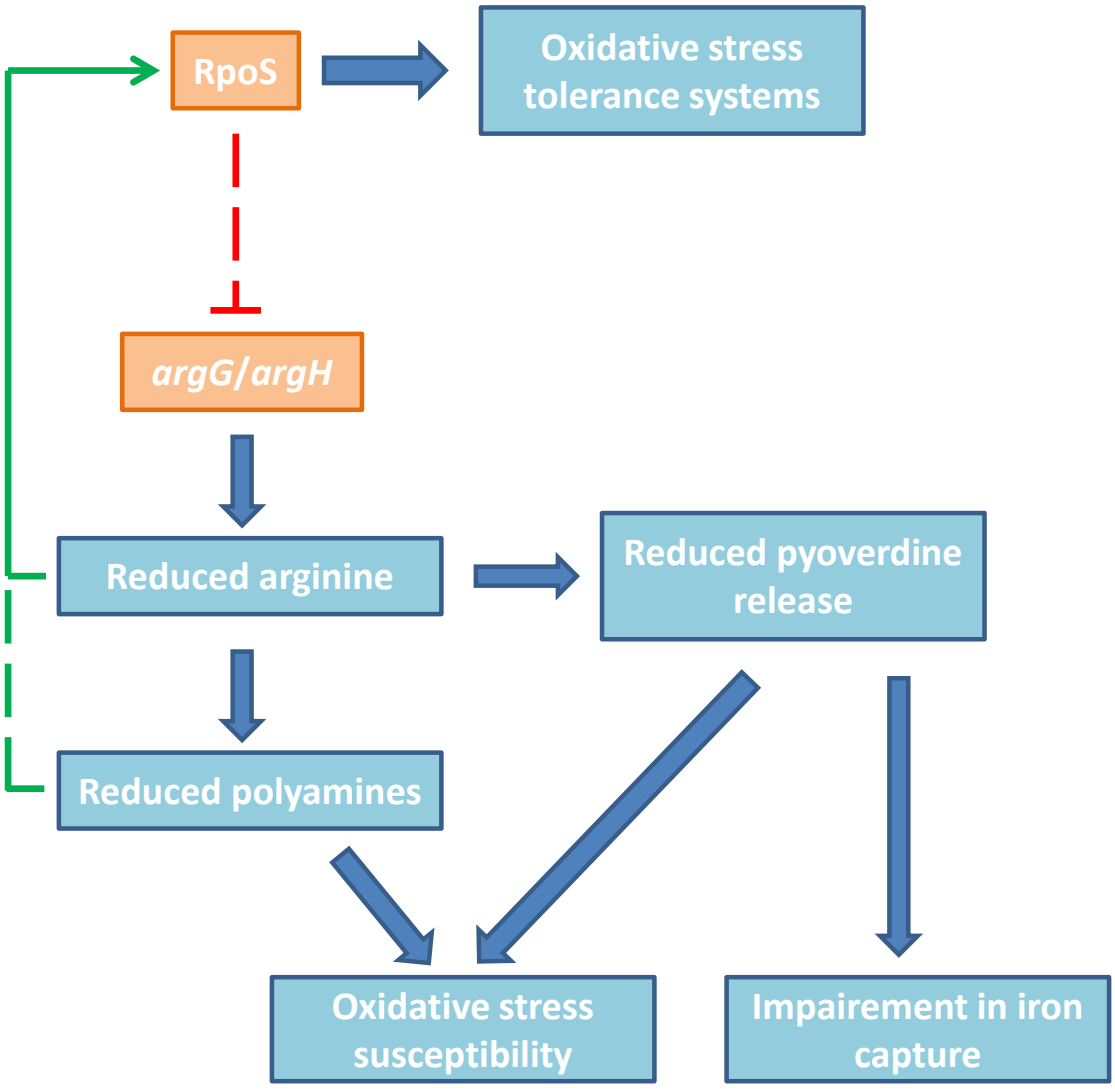


Figure 2. General hypothetical model linking arginine/polyamines metabolism with oxidative stress and pyoverdine release. Green arrow-ended and Red T-shape-ended lines show positive and negative regulation, respectively. Blue arrows are indicative of effects. Dashed lines indicate indirect effect.

Inhabiting as biofilms allow bacterial populations to be protected and tolerate stress situations more easily. This ability is in part due to the extracellular matrix which makes difficult the distribution of molecules responsible of stress as antibiotics, reactive oxygen species and biocides, among others, inside the biofilm (Flemming *et al*., 2016), allowing as a last resort to colonize changing niches. The *P. putida* biofilm matrix is mainly composed of four different EPS (pea, peb, alginate and cellulose) and two large adhesins (LapA and LapF) which have been shown to play different roles on biofilm development (Hinsa *et al*., 2003; Martínez-Gil *et al*., 2010; Martínez-Gil *et al*., 2014; Nilsson *et al*., 2011; Nielsen *et al*., 2011).

Biofilm formation is well studied in presence of different compounds like antibiotics due to their impact on health. However, little is known about the effect of toxic hydrocarbons. As a first approximation, we have started to evaluate the effect of toluene on biofilm formation and persistence in three related *P. putida* strains. Our preliminary results have shown that this compound promotes initial attachment, unlike its effect over preformed biofilms on which it favours biofilm disruption. The tolerance to the hydrocarbon is probable due to the low cell surface hydrophobicity and alterations in membrane components, as well as the role exerted by the extracellular matrix, which could be important in preventing the accumulation of organic solvent molecules in the membrane (Aono and Kobayashi, 1997). It is noticeable the reduced biofilm formation by the strain DOT-T1E, probably due to the lack of most of the adhesin LapA and the shorter LapF. In spite of this fact, *P. putida* DOT-T1E retains some ability to develop biofilm. It is possible that EPS, are present in this strain as well as in KT2440, and the shorter LapF compensate in part the lack of LapA. More detailed analyses are required to understand the implication of such compounds in biofilms.

Taken together, the results of this Thesis have contributed to greater knowledge about the link among arginine metabolism, c-di-GMP signalling, associated phenotypes and stress, as well as in understanding the effect of hydrocarbons over biofilms in *P. putida*. Besides, this work opens up several avenues of research that will allow more detailed and deeper knowledge about *P. putida* lifestyles.

VI. CONCLUSIONS

The results obtained in this Thesis lead us to the following conclusions:

1. The amino acid arginine is an environmental cue for *P. putida* KT2440 that promotes an increase in the c-di-GMP pool and positively modulates related phenotypes, like biofilm formation and crinkly colony morphology. Aspartic acid, one of the precursors of arginine synthesis, also acts as an environmental cue, but has the opposite effect with respect to c-di-GMP contents and related phenotypes.
2. The response to L-arginine involves the diguanylate cyclase CfcR and additional elements related to c-di-GMP yet to be identified, since a *cfcR* mutant still retains some response to exogenous arginine in terms of increasing second messenger levels.
3. The arginine biosynthesis pathway functions as a metabolic signal for c-di-GMP turnover. Mutants impaired in the two last steps of arginine biosynthesis show low levels of c-di-GMP even when CfcR is in multicopy, although this reduction cannot be fully explained by changes in *cfcR* expression. Exogenous addition of intermediaries or products related to arginine metabolism have no effect, with the exception of aspartic acid.
4. Arginine has a positive effect on expression of the stationary phase sigma factor RpoS, which regulates expression of the diguanylate cyclase CfcR, the adhesin LapF and the exopolysaccharide Pea. This explains the influence of arginine on the crinkly colony phenotype, which requires Pea, and on biofilm formation.
5. The connection between arginine metabolism and c-di-GMP signalling involves several elements that conform a complex regulatory network. The transcriptional regulator ArgR is a key element in this network, controlling arginine biosynthesis and transport and modulating c-di-GMP contents. Additional regulatory elements include the sigma factor RpoS, the transcriptional regulator FleQ, and the post-transcriptional regulatory proteins of the Rsm family (RsmA, RsmE, RsmI).
6. Arginine biosynthesis is necessary for the correct processing and release to the medium of the siderophore pyoverdine. Mutants impaired in arginine synthesis retain the siderophore inside the cell, and addition of exogenous arginine or complementation of these mutations restore normal pyoverdine release.

7. We propose a connection between arginine and iron metabolism and oxidative stress, based on the susceptibility of arginine biosynthesis mutants to hydrogen peroxide and the protective role that polyamines (derived from arginine) seem to exert.
8. The presence of low concentrations of a toxic hydrocarbon (toluene) from the early stages promotes biofilm formation, but its sudden addition to already established biofilms leads to dispersal. This suggests biofilms formed under stress and under optimal growth conditions can be structurally different. Comparison between *P. putida* strains indicates that toluene tolerance capacity and biofilm formation capacity are not linked features.

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