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Caracterización de elementos estructurales y reguladores implicados en la formación de biofilms de *Pseudomonas putida*

Tesis Doctoral

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Memoria que presenta la Licenciada en Biología

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para aspirar al Título de Doctor

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"Si al franquear una montaña en la dirección de una estrella, el viajero se deja absorber demasiado por los problemas de la escalada, se arriesga a olvidar cual es la estrella que lo guía"

A. Saint-Exupery

A mi Familia

A Diego

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A todos de corazón GRACIAS!!

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List of Abbreviations

AHLs	Acylated Homoserine Lactones
ATP	Adenosine Tri-Phosphate
Bap	Biofilm associated protein
bp	Base pair
C-terminal	Carboxilic terminal
DGC	Diguanilate cyclase
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
CFU	Colony-forming units
c-di-GMP	3',5'-Cyclic diguanylic acid
EAL	Motif Glu-Ala-Leu
EPS	Exopolysaccharides
FPLC	Fast Protein Liquid Chromatography
g	grams
GGDEF	Motif Gly-Gly-Asp-Glu-Phe
GFP	Green Fluorescent Protein
ITC	Isothermal Titration Calorimetry
kb	Kilobase
Lap	Large Adhesion Protein
LB	Luria-Bertani media
mg	Milligram
ml	Milliliter
mm	Millimeter
µl	Microliter
µm	Micrometer
mus	Mutant unattached to seeds

N-terminal	Amino terminal
O.D.	Optical Density
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PDE	Phosphodiestarase
PGA	β-1,6-*N*-acetyl-D-glucosamine
PGPR(s)	Plant Growth Promoting Rhizobacteria
QS	Quorum-sensing
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
rpm	Revolutions per minute
Rup	Rhizosphere up regulated
RT-PCR	Reverse transcription polymerase chain reaction
TCS	Two-component system
TEM	Transmission Electron Microscopy
WT	Wild type

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RESUMEN

Resumen

En el ambiente las bacterias se encuentran predominantemente formando comunidades microbianas adheridas a una gran variedad de superficies, formando lo que se conoce como biopelículas o biofilms (Costerton, 1999). Aunque estas comunidades microbianas suelen estar formadas por multitud de especies, para su estudio en el laboratorio se ha simplificado el modelo utilizando mayoritariamente biofilms monoespecíficos. Esto ha permitido profundizar en el conocimiento de los determinantes moleculares implicados en el desarrollo de un biofilm que han resultado ser sorprendentemente específicos. La estrategia de vida en forma de biofilm aporta numerosas ventajas adaptativas a las bacterias, en cuanto a la colonización y persistencia en distintos ambientes. A grandes rasgos, la formación de un biofilm confiere mayor resistencia a una gran variedad de factores ambientales como la desecación, radiación ultravioleta, moléculas con actividad antimicrobiana y protección frente al sistema inmune. Estas ventajas suponen numerosos problemas a la hora de la erradicación de biofilms bacterianos no deseados en superficies como el instrumental hospitalario (sondas, catéteres, campanas de oxígeno, etc.), lugares de procesado en la industria alimentaria y plantas purificadoras de agua. Uno de los casos más estudiados es la colonización de tejido pulmonar en pacientes enfermos de fibrosis quística por numerosas especies bacterianas entre ellas *Pseudomonas aeruginosa*, que es capaz de establecerse en forma de biofilms y provocar infecciones crónicas (Costerton *et al.*, 1995); los tratamientos con antibióticos son bastante ineficaces debido a la elevada resistencia que presenta *P. aeruginosa* en forma de biofilms. Sin embargo, el estudio de los biofilms tiene un lado positivo en el que el establecimiento de biofilms bacterianos puede aportar numerosos beneficios. Este es el caso de la colonización de superficies vegetales por parte de bacterias protectoras frente a fitopatógenos, donde se ha visto que la formación de biofilms es clave para que ejerzan su papel

protector (Chen *et al.*, 2012) y del uso de biofilms para el tratamiento de aguas residuales (Nicolella *et al.*, 2000). Una característica muy importante de esta forma de vida sésil, que la diferencia de la vida planctónica, es la producción de una matriz extracelular producida por las células bacterianas y en la que ellas mismas se encuentran embebidas. Se han sugerido numerosas funciones para la matriz extracelular, entre ellas que ejerce un papel protector frente a multitud de amenazas ambientales y por otro lado permite el desarrollo de comunidades estructuralmente complejas. Su composición es muy variable según la especie bacteriana aunque generalmente está constituida por proteínas, exopolisacáridos y ADN.

Esta Tesis Doctoral se ha centrado en el estudio de la formación y regulación de biofilms bacterianos usando como modelo *Pseudomonas putida* KT2440. *P. putida* KT2440 es una bacteria ubicua que coloniza eficientemente la rizosfera de diversas plantas con un elevado interés agroalimentario. Esta bacteria es capaz de activar la resistencia sistémica inducida frente a patógenos y promover el crecimiento de la planta. Trabajos previos en el grupo han permitido la identificación de numerosos determinantes moleculares implicados en la adhesión a semillas y estudios transcriptómicos han revelado la existencia de genes que se expresan preferentemente en la rizosfera (Matilla *et al.*, 2007, Yousef-Coronado *et al.*, 2008, Espinosa-Urgel *et al.*, 2000). Uno de esos trabajos permitió el aislamiento de numerosos mutantes afectados en la adhesión a semillas a los que se denominó mus, mutants unattached to seeds (Espinosa-Urgel *et al.*, 2000). Uno de los mutantes, mus-20, afectado en el gen PP_0806 que codifica una supuesta proteína de superficie de gran tamaño a la que se le ha llamado LapF, ha sido uno de los principales objetos de estudio en esta Tesis Doctoral.

En el capítulo **"LapF, the second largest *Pseudomonas putida* protein, contributes to plant root colonization and determines biofilm architecture"** se ha estudiado el papel de LapF en la colonización de la rizosfera de plantas de maíz y alfalfa así como la localización de la proteína y su

contribución en la formación de biofilms de *P. putida* KT2440. El gen PP_0806 codifica una proteína de 6.310 aminoácidos que puede ser dividida en 3 dominios principales. El domino N-terminal comprende los primeros 152 aminoácidos, seguido del segundo dominio que corresponde al 85% de la proteína y está formado por 64 repeticiones imperfectas cada una de 85-91 aminoácidos cada una. El dominio C-terminal está compuesto por 691 aminoácidos; es un dominio putativo de unión a calcio y contiene dos dominios GGXGXD característicos de proteínas secretadas por sistemas de secreción Tipo I. Se han llevado a cabo numerosas técnicas de formación de biofilm con las que se ha demostrado la implicación de LapF en etapas tardías del desarrollo del mismo: formación de microcolonias y un biofilm maduro. Un mutante afectado en *lapF* es además menos competitivo en colonización de la rizosfera de maíz y alfalfa en comparación con la cepa salvaje. Mediante la utilización de diversas técnicas se ha demostrado la localización subcelular de LapF en la superficie bacteriana y entre células formando parte de las microcolonias dentro de un biofilm. El patrón de expresión del promotor de *lapF* tanto en cultivos líquidos como en biofilms es característicamente de fase estacionaria y se ha demostrado que su expresión es dependiente del factor de transcripción alternativo RpoS. Los resultados obtenidos en este capítulo han permitido proponer el rol de LapF en la formación de microcolonias de *P. putida* KT2440 probablemente estableciendo interacciones célula a célula y permitiendo el desarrollo de un biofilm maduro.

En el siguiente capítulo, "**Calcium causes multimerization of the large adhesin LapF and modulates biofilm formation by *Pseudomonas putida*"** se ha estudiado el papel del calcio en la formación de biofilms de *P. putida* y en la funcionalidad de LapF a través de su dominio C-terminal. Como se ha mencionado anteriormente, LapF está constituida por 3 dominios diferentes, encontrándose dos supuestos motivos de unión a calcio en el dominio C-terminal de la proteína. Tras la purificación del dominio C-terminal de LapF (CLapF), se ha confirmado mediante ITC (Isothermal Titration Calorimetry) la unión específica

de calcio por parte de CLapF. Así mismo, se ha demostrado por medio de distintas técnicas (DLS "Dynamic Light Scattering", FPLC analítico y microscopía electrónica de transmisión) la tendencia de la proteína a formar grandes agregados en presencia de calcio. Mediante técnicas de inmunolocalización con microscopía electrónica de transmisión se ha estudiado con mayor detalle la localización de LapF en el biofilm, donde se ha detectado la presencia de agregados de LapF entre células. Los resultados obtenidos en este capítulo nos han llevado a proponer un modelo de acción en el que LapF mediaría la unión célula a célula mediante su dominio C-terminal y a través del calcio.

En el siguiente capítulo, "**Expression of the large *Pseudomonas putida* adhesins LapA and LapF is part of a complex global regulatory network governing biofilm formation"** se ha profundizado en la regulación de las dos adhesinas claves en la formación de biofilms de *P. putida*, LapA y LapF. El primer capítulo de esta tesis da pie a la propuesta de un modelo secuencial de formación de biofilm de *P. putida*. Este modelo sugiere que LapA (la primera adhesina identificada en *P. putida*) es esencial para la primera etapa de la formación de un biofilm, la unión irreversible al sustrato (Hinsa *et al.*, 2003) mientras que LapF es clave para el proceso de desarrollo de microcolonias y la maduración del biofilm. En este capítulo se compara el patrón de expresión de *lapA* y *lapF* tanto en cultivos planctónicos como en biofilms. Se ha demostrado que ambas adhesinas están bajo la regulación del sistema de dos componentes GacS/GacA pero tan solo *lapF* es dependiente del factor de transcripción RpoS. Igualmente se ha demostrado la influencia de altos niveles intracelulares del segundo mensajero di-GMPc en la expresión de ambas adhesinas tanto a través del "master regulator" del flagelo, FleQ, como en una ruta independiente. Los resultados de este capítulo apoyan el modelo secuencial propuesto anteriormente para la formación de biofilms de *P. putida* y así mismo proponen un modelo de regulación transcripcional independiente para *lapA* y *lapF*.

Recientemente y en paralelo al transcurso de esta Tesis Doctoral se han identificado 4 operones implicados en la síntesis de cuatro exopolisacáridos diferentes y se ha sugerido su participación en la estabilidad del biofilm de *P. putida* KT2440 (Nilsson *et al.*, 2011). En el último capítulo de resultados de esta Tesis Doctoral **"Compensatory balance between extracellular matrix components of *Pseudomonas putida* biofilms"** nos hemos centrado en estudiar cómo la ausencia de determinados componentes de la matriz extracelular de *P. putida* provoca una alteración a nivel transcripcional de la expresión de los componentes restantes. En este capítulo hemos demostrado que la ausencia de alguna de las dos adhesinas principales, LapA o LapF provoca una alteración en la producción de exopolisacáridos. Se ha estudiado el efecto de altas concentraciones del segundo mensajero di-GMPc en la producción de los distintos componentes de la matriz extracelular y se ha observado el fenotipo de mutantes afectados en LapA o LapF en estas condiciones, lo que sugiere una relación directa entre adhesinas y exopolisacáridos. Finalmente se propone que todos los elementos implicados en la matriz extracelular, tanto exopolisacáridos como adhesinas, están íntimamente regulados de una manera "alostérica" de modo que se asegura la habilidad de la bacteria para establecer interacciones célula-célula.

Esta Tesis Doctoral aporta información clave del proceso de formación de biofilms por parte de *P. putida* KT2440 que ha permitido la propuesta de un modelo secuencial de la formación de biofilms. Así mismo introduce nuevos componentes en cuanto a la regulación transcripcional de los factores claves de la matriz extracelular y en el que se propone un modelo de regulación que sugiere una relación estrecha entre todos los componentes de la matriz extracelular del biofilm de *P. putida* KT2440.

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I. GENERAL INTRODUCTION

1. Looking into the past: the concept of bacterial biofilm

Bacteria have acquired diverse mechanisms to survive in a wide variety of habitats, including inhospitable environments with extreme and changing conditions. One of the most important mechanisms is aggregation or attachment to develop surface-associated communities called biofilms (Davey & O'Toole, 2000). Although the idea that bacteria live preferentially as aggregates on surfaces has been present from early times in Microbiology, for many decades bacteria have been predominantly studied as unicellular organisms that grow as independent and planktonic individuals.

One of the first observations of bacteria in the XVIII century, as "aggregates of animalcules" was made by A. van Leeuwenhoek, the "Father of Microbiology", when he described his isolated teeth deposits (Percival *et al.*, 2011). Between 1928 and 1933, many microbiologists detected bacteria growing attached to glass slides and observed that water bacteria were for the most part adhered to surfaces rather than floating. They reported that bacteria attached to surfaces grew and formed microbial films (Winogradsky, 1928; Cholodny, 1930; Conn, 1932; Henrici, 1933). Afterwards, from 1933 to 1943, the marine microbiologist C. E. ZoBell made relevant contributions to the study of marine bacteria attached to surfaces (ZoBell e*t al.*, 1933; ZoBell *et al.*, 1935; ZoBell *et al.*, 1943). ZoBell observed a wide number of events taking place during biofilm formation. Some of them are currently being discussed. Many of these contributions could be summarized in the following list:

- Nutrients are concentrated on surfaces
- Bacterial attachment to surfaces is very rapid and active rather than passive
- Microcolonies can develop on surfaces
- Sessile bacteria are covered by a "sticky" material absent in planktonic cells

- The attachment tendency is influenced by nutrient availability
- It is difficult to ascertain the attachment mechanism

Later, in 1971, P.S. Meadows studied the attachment of gram-negative bacteria (*Pseudomonas fluorescens*, *Escherichia coli*, *Aeromonas liquefaciens*, among others) to solid surfaces and the effect of basic and acidic proteins on attachment (Meadows, 1971). No previous studies had questioned whether dead bacterial cells were able to attach to solid surfaces, which would mean that the physico-chemical forces are solely responsible for the attachment. To answer this relevant question, different bacteria were killed by ultraviolet irradiation, heat or formalin. The two later treatments would provoke changes in the cell wall of the bacterial cell, while ultraviolet exposure kills by damaging the DNA. The results revealed that bacteria killed by heat and formalin presented very little attachment in comparison to UV killed bacteria. Therefore, bacterial attachment depends on the integrity of the cell wall. He concluded that bacteria killed by most forms of physical and chemical stress in the environment are unlikely to attach, and so solid surfaces are colonized by living bacteria (Meadows, 1971). He also suggested that the physicochemical requirements of mammalian cells for adhesion to glass differ from those of bacteria.

Around the early 80´s the term biofilm started to arise from journals specialized in clinical and industrial microbiology. In 1990, the first review in methods for the study of bacterial biofilms is published (Ladd & Costerton, 1990), which triggered an increase in the number of articles focused on the study of biofilms, mainly in *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Giwercman *et al.*, 1991, Anwar *et al.*, 1992). Almost 10 years after the publication of the first articles specialized in the study and control of biofilms, the first review on the biology of microbial biofilms is published by Costerton and colleagues (Costerton *et al.*, 1995). From that moment, a general agreement is reached on the definition of biofilms as matrix-enclosed bacterial communities attached to each other and/or

to surfaces. Subsequently, the first screenings to isolate mutants affected in biofilm formation in different bacteria were developed (O'Toole & Kolter, 1998, Heilmann *et al.*, 1996) and served as the basis for the relevant contributions to the genetics of biofilm development made by numerous researchers in the past decade.

2. Advantages of biofilm formation that make it a widespread microbial lifestyle

As mentioned above, ZoBell was one of the first microbiologists to study the process of bacterial attachment to solid surfaces and he introduced the idea of an ecological advantage to the bacterial cell (ZoBell, 1943). The ecological success of bacteria possibly involves the ability to grow as surface-attached communities, embedded in a protective extracellular matrix (Davey and O´Toole, 2000). In addition, bacteria frequently colonize the environment forming multispecies consortia that are ideal for syntrophic relationships, where metabolically different bacterial species depend on each other to utilize certain substrates (Davey & O'Toole, 2000).

One of the hypotheses as to why biofilm formation is a common strategy in almost all microbes is that it affords protection from a variety of environmental challenges. It was first discovered that cells within a biofilm were at least 500 times more resistant to antibacterial agents (Costerton *et al.*, 1995). It has been suggested that protection against biocidal agents could be achieved through three different mechanisms (Hall-Stoodley *et al.*, 2004): (i) the extracellular matrix acts as a physical barrier which impairs the direct contact of different agents with the bacterial cells; (ii) within the biofilm there exist stationary-phase dormant zones which contribute to antibiotic resistance, given that many antimicrobial compounds need some cellular activity to be effective and (iii) the existence of

different phenotypes and the presence of a small subpopulation of resistant cells called "persisters" contribute to biofilm protection.

As discussed later in this chapter (The architecture of biofilms: the extracellular matrix), cells within a biofilm are surrounded by a complex extrapolymeric network, composed of a mixture of polysaccharides, proteins and nucleic acids. It has been demonstrated that the extracellular matrix is able to neutralize and dilute different antimicrobial compounds to sublethal concentrations (Hall-Stoodley *et al.*, 2004). This effect is enhanced with hydrophilic and positively charged antibiotics, such as aminoglycosides (Nickel *et al.*, 1985, Nichols *et al.*, 1988). A recent report has demonstrated that the exopolysaccharide Pel in *P. aeruginosa* confers resistance to aminoglycoside antibiotics (Colvin *et al.*, 2011). The extracellular matrix also confers protection from UV exposure, dehydration and salinity (Flemming, 1993), metal toxicity (Teitzel & Parsek, 2003), acid exposure (Welin-Neilands & Svensäter, 2007) and phagocytosis (Leid *et al.*, 2002).

Throughout the biofilm and surrounding the microcolonies, there often are permeable water channels that have been compared with a primitive circulatory system. These channels provide areas for exchanging nutrients and metabolites and for the removal of toxic molecules (Davey & O'Toole, 2000, Costerton *et al.*, 1995, O'Toole *et al.*, 2000). Biofilms have been considered an optimal environment for adaptation and subsequent evolution of new bacterial traits because they are ideal to the exchange of genetic material (Ghigo, 2001). Numerous evidences have shown the interconnection between biofilm formation and horizontal gene transfer (Roberts *et al.*, 1999, Clark & Warren, 1979, Madsen *et al.*, 2012). This was also demonstrated by a quantitative in situ analysis that revealed 1,000-fold higher conjugation rates in biofilms than in classical mating techniques (Hausner & Wuertz, 1999).

3. The Dr Jekyll and Mr Hyde of biofilms

Bacteria can form biofilms on a wide variety of surfaces. Frequently, these biofilms cause the obstruction of industrial pipelines, air conditioning systems and water distribution systems, reducing the life of this equipment. Implant surfaces and other medical devices are ideal substrates for bacterial colonization, therefore biofilms are frequently found in intravenous catheters, cardiac pacemakers and prosthetic heart valves, among others. Biofilms on medical instruments are responsible for over 80% of chronic inflammatory and infectious diseases of soft tissues (Costerton *et al.*, 1999), play an important role in the development of peri-implant infections (Badihi Hauslich *et al.*, 2011) and cause destruction of local tissues, patient weakness and morbidity (Antoci Jr *et al.*, 2008).

The colonization and biofilm formation of the opportunistic pathogen *P. aeruginosa* on human tissues has been widely studied. Cystic fibrosis patients are frequently infected by this bacterium, which colonizes the lung and forms persistent biofilms (Rudkjøbing *et al.*, 2012). This is not the only microorganism that develops biofilm-related infections; *Staphylococcus epidermidis, S. aureus*, *E. coli* and *Klebsiella pneumoniae*, among others, are also found in biofilms associated with nosocomial infections (Antoci Jr *et al.*, 2008, Singh *et al.*, 2011, Stahlhut *et al.*, 2012). Therefore, biofilm formation is considered a virulence trait given that it contributes to the bacterial ability to cause an infection and to resist to a variety of antimicrobial compounds. Biofilms in the agro-alimentary industry are of special interest given that biofilms of *E. coli*, *Listeria monocytogenes* and *Salmonella* sp., among other species, have shown to be an important source of persistent contamination that could lead to food putrefaction and transmission of infectious diseases (Berger *et al.*, 2010, Hall-Stoodley *et al.*, 2004, Van Houdt & Michiels, 2010).

However, not all biofilms have a negative impact. Beneficial biofilms are found in many environments and are responsible for numerous biological processes as nitrogen fixation and fermentation (Davey & O'Toole, 2000). This is

the case of marine bacterial communities which have been shown to be involved in biogeochemical transformation of organic carbon (Paerl & Pinckney, 1996). The study of biofilms has become a topic of increasing interest over the last decade in agriculture due to the current environmental problems we have to face. Biofilms have been used for decades in bioremediation of wastewater and biofilm-based biofertilizers are a possible method to improve soil quality and to reduce chemical fertilizers (Vlamakis, 2011). Fluorescent pseudomonads with the ability to form biofilms on plant tissues could be used as biocontrol agents against phytopathogens and for rhizoremediation of contaminated soils (Couillerot *et al.*, 2009, Fernández *et al.*, 2012). Microorganisms can also be used as new sources of energy in the form of microbial fuel cells. A recent study describes new methods for the characterization of electro-active biofilms (Connolly *et al.*, 2011).

4. Biofilm development: from planktonic cells to a coordinated community

Typically, biofilms in natural habitats are composed by a large number of different microbial species, although single-species biofilms are commonly studied in the laboratory. It is assumed that organic molecules concentrate at certain areas of surfaces, which are preferentially colonized by bacteria (Costerton *et al.*, 1995). Biofilm formation requires coordination, interaction and communication between bacterial cells (Davey & O'Toole, 2000), leading to a developmental cycle that comprises both biofilm formation and biofilm dispersal processes. Five stages have been proposed in biofilm formation, which are common in different bacteria, although the specific molecular mechanisms involved in each stage may differ (Figure 1).

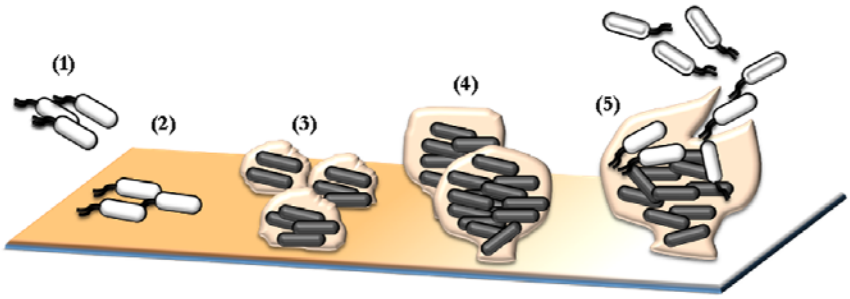

Figure 1. A model for biofilm development. **(1)** Planktonic cells use motility to reach a surface. **(2)** They first interact with the substratum by a pole, followed by lateral, "irreversible" attachment. **(3)** Cells become more firmly attached and form microcolonies by cell division and aggregation, producing the extracellular matrix. **(4)** The biofilm becomes thicker and mature. **(5)** In response to environmental conditions some cells may be released from the biofilm matrix and become planktonic cells, which will swim and colonize new niches.

The formation of bacterial biofilms necessarily begins with the adhesion of a small number of bacterial cells to a surface (Costerton, 1999). Costerton (1999) speculated that bacterial cells are able to "sense" their proximity to surfaces and/or interfaces by calculating diffusion of signalling molecules or protons released by them. (1) The first contact to the surface is mediated by pili and/or flagella (Pratt & Kolter, 1999, Petrova & Sauer, 2012), a process that many authors describe as a reversible union to the surface by planktonic cells; some of those cells then attach irreversibly to the surface (2) allowing the formation of microcolonies and the production of an extracellular matrix (3); these microcolonies could develop into macrocolonies making up a mature biofilm (4); some cells of the macrocolonies could start a planktonic life triggering the dispersion of the biofilm (5). Dispersion happens under specific environmental conditions and it is a key step for the colonization of new habitats (Stoodley *et al.*, 2002, Lasa, 2006, Sauer *et al.*, 2002).

Biofilms are not simply planktonic cells that have adhered to a surface, since cells embedded in the extracellular matrix differ significantly from planktonic cells at various levels, from the morphological to the physiological and metabolic state (Costerton *et al.*, 1995, Landini, 2009). In addition, the development from the first stages of attachment to a mature biofilm also comprises observable phenotypic changes and alterations in gene expression (Petrova & Sauer, 2012). Advances in genomics have contributed to the study of gene expression using DNA microarray techniques which allow the study of the global transcriptomic profile of different bacterial populations. Unexpectedly, a microarray study in *P. aeruginosa* showed that gene expression in biofilm cells is similar to those in planktonic cells, where only about 1% of genes presented differential expression. Among these genes they found the cytochrome *c* oxidase and *rpoS* genes repressed, which have been demonstrated to be involved in antibiotic sensitivity (Whiteley *et al.*, 2001, Bryan *et al.*, 1980). Two subsequent publications identified another group of genes which were up-regulated in biofilms and were preferentially involved in adhesion, autoaggregation, transport proteins and putative oxidoreductases among others (Waite *et al.*, 2005, Schembri *et al.*, 2003). In addition, other results showed a different protein pattern expression in *P. putida* biofilms where 30 proteins were down-regulated and 15 were up-regulated (Sauer & Camper, 2001).

The term "microbial development" has been defined as stable changes in form and function that play a prominent role in the life cycle of the organism where environmental signals are essential for the development process (O'Toole *et al.*, 2000, Monds & O'Toole, 2009). Biofilm formation has been suggested to result from a developmental programme of gene expression, supported by the identification of a wide variety of genetic determinants which are highly regulated by different mechanisms and triggered by environmental conditions (O'Toole *et al.*, 2000, Danese *et al.*, 2001). The regulated transition from a planktonic and solitary lifestyle to a coordinated community and evidences in functional changes in biofilm-grown cells support the idea of biofilms as a part of a developmental

programme. However, for many authors there are still some aspects of this model that have to be demonstrated with experimental data (Monds & O'Toole, 2009, Ghigo, 2003, Klausen *et al.*, 2006). In addition, some authors have suggested that surface attachment may evolve as a response to the environmental conditions rather than being part of a special developmental program (Klausen *et al.*, 2006).

5. Biofilms: a highly regulated process

Biofilm formation is a highly regulated process which responds to different environmental signals. A large and complex regulatory network connects the environmental stimuli with changes in gene expression (both at the transcriptional and posttranscriptional levels) in order to respond to those environmental conditions. The main role of this highly regulated network is to allow the coordinated synthesis of the different components that are needed in each step.

Many of these regulators are two-component systems (TCSs) which consist of a sensor kinase and a response regulator. These systems are the predominant signalling mechanism in most bacteria that allow them to sense a wide variety of conditions as nutrients, temperature, pH, osmolarity and toxic substances. Two-component systems have been widely studied in *P. aeruginosa* biofilms and it has been shown that different TCSs control different stages of the biofilm and regulate the transcriptional profile of the community (Figure 2). This is the case of the RetS/LadS/GacS pathway in *P. aeruginosa* which controls the expression of different exopolysaccharides and determines the switch from acute to chronic infection (Mikkelsen *et al.*, 2011). The Cup fimbriae of *P. aeruginosa*, extracellular appendages that promote attachment to abiotic surfaces, are regulated by different TCSs as RocS1A1R and PprBA (Mikkelsen *et al.*, 2011). In *Enterobacteriaceae* the RcsCBD pathway coordinates biofilm development and in *E. coli* K-12 the activation of this pathway resulted in increased levels of extracellular polysaccharide production (Clarke, 2010, Gottesman & Stout, 1991).

Recently, it has been shown that RcsB phosphorylation is essential for the switch from planktonic to biofilm life-style in *Salmonella enterica* serovar Typhimurium (Latasa *et al.*, 2012).

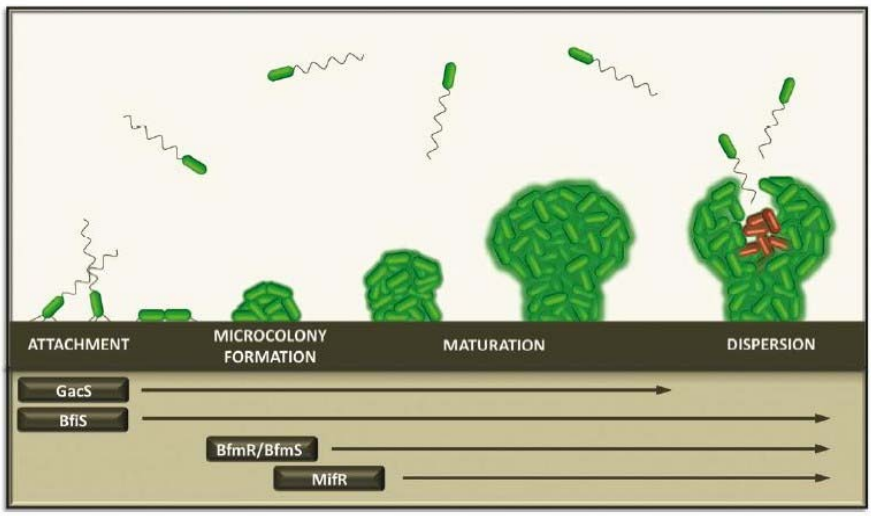

Figure 2. Biofilm life cycle and regulation. Stage-specific phosphorylation of partners of two component regulatory systems during biofilm formation of *P. aeruginosa* (Petrova & Sauer, 2009, Mikkelsen *et al.*, 2011). BfiSR (biofilm initiation), BfmSR (biofilm maturation) and MifSR (microcolony formation).

Biofilm regulation also includes global regulators and sigma factors that regulate the transcription of genes involved in matrix production. In *P. putida*, the global regulator Fis (factor for inversion stimulation) when it is overexpressed reduces swimming motility and enhances biofilm formation in barley roots (Jakovleva *et al.*, 2012). In *P. aeruginosa* the alternative sigma factor AlgT is essential for the biosynthesis of the exopolysaccharide alginate and probably activates a negative effector of flagellum synthesis upon surface attachment (Garrett *et al.*, 1999). The stationary-phase sigma factor RpoS is a positive regulator of the

expression of *psl* exopolysaccharide that when overexpressed results in higher transcription of *psl* and a phenotype consistent with Psl overproduction (Irie *et al.*, 2010). The process of biofilm formation involves other modulatory elements such as c-di-GMP and quorum sensing that will be discussed later on (sections 5.2. and 5.3.).

5.1. Environmental cues involved in biofilm regulation

Surfaces may provide advantages over the liquid column to the establishment of bacterial communities. Some relevant surface factors that influence bacterial attachment are mass transport, surface charge, hydrophobicity, surface roughness and growth medium (Palmer *et al.*, 2007). Bacterial contact to the surface induces biofilm formation in *P. aeruginosa* and *E. coli* (Toutain *et al.*, 2007, Otto & Silhavy, 2002) and it has been recently demonstrated that initial contact with the surface induces adhesin production in *Caulobacter crescentus*, *Asticcacaulis biprosthecum* and *Agrobacterium tumefaciens* (Li *et al.*, 2012).

Different environmental factors and chemical cues have an influence on biofilm formation. The role of nutrient concentration in bacterial attachment has been studied and different authors have concluded that either excess or depletion of nutrients lead to biofilm disassembling (Rochex & Lebeault, 2007, Sauer *et al.*, 2004). Monds and colleagues demonstrated that extracellular inorganic phosphate below a threshold impaired biofilm formation by *Pseudomonas aureofaciens* (Monds *et al.*, 2001). In addition, it has been shown that low availability of inorganic phosphate provokes a regulatory cascade which causes the loss of the critical outer-membrane adhesin LapA in *P. fluorescens* (Monds *et al.*, 2007). Interestingly, in *Sinorhizobium meliloti*, inorganic phosphate induces biofilm formation in two different ways; high levels of inorganic phosphate trigger the overproduction of succinoglycan exopolysaccharide while phosphate starvation enhanced biofilm

formation by galactoglucan exopolysaccharide (Mendrygal & González, 2000, Zhan *et al.*, 1991).

In *E. coli* K-12, acetate metabolism functions as a metabolic sensor from environmental conditions to biofilm regulation (Prüß *et al.*, 2010). In *Vibrio cholerae* the presence of monosaccharides induces *vps* (*Vibrio* polysaccharide) transcription and *vps*-dependent biofilm formation (Kierek & Watnick, 2003a, Mueller *et al.*, 2009), whereas high levels of Ca^{2+} promote *vps*-independent biofilm (Kierek & Watnick, 2003b). In *E. coli* O157:H7, indole reduces attachment to abiotic surfaces and epithelial cells while it induces *vps*-dependent biofilm in *V. cholerae* (Bansal *et al.*, 2007). A recent work has identified that Mg^{2+} limitation acts as an environmental signal that promotes *P. aeruginosa* biofilm formation by transcriptional repression of *retS* (Mulcahy & Lewenza, 2011). Numerous evidences have demonstrated that iron is another key element in bacterial attachment and biofilm formation: it is important for attachment of *P. putida* to corn seeds (Molina *et al.*, 2005) and *P. aeruginosa* mutants affected in biofilm formation are able to restore its biofilm capacity by the addition of extracellular iron (O'Toole & Kolter, 1998).

The information presented above shows the highly diverse mechanisms that have evolved for biofilm regulation among different bacteria; different environmental signals can trigger the same biofilm formation pathway and similar environmental conditions could result in different responses depending on the species.

5.2. Quorum sensing: cellular communication in biofilms

The ability of many bacteria to communicate within and between species is critical for their survival in natural habitats (Miller & Bassler, 2001). An intrinsic trait of bacterial surface communities is the interaction between bacterial cells and the collective behaviour within the biofilm. Nealson & Hastings discovered in 1979

that light emission from *Vibrio fischeri* and *V. harveyi,* two luminous bacterial species, took place only at high cell-population density in response to the accumulation of secreted signalling molecules (Nealson & Hastings, 1979).

Fuqua *et al.,* introduced the term "quorum sensing" (QS) to describe the process through which this cell-cell communication occurs (Fuqua *et al.*, 1994). Bacteria produce a diffusible compound termed autoinducer that accumulates in the surrounding environment during growth. At low cell densities this substance is at low concentration, while at high cell densities it accumulates to the critical concentration required for the activation of certain genes (Figure 3). Thus, quorum sensing leads to the coordinated regulation of gene expression in response to fluctuations in cell-population density. The evolution of quorum sensing as a communication system, could have been one of the early steps in the development of multicellularity (Miller & Bassler, 2001).

Bacteria use quorum sensing communication to regulate many physiological activities such as virulence, conjugation, antibiotic production, motility, sporulation, plant-microbe interactions and biofilm formation (Dubern *et al.*, 2006). In *P. aeruginosa* it positively regulates the major operon involved in exopolysaccharide production, *pel* (Sakuragi & Kolter, 2007). In contrast, in *V. cholerae* QS regulates in a negative way the production of exopolysaccharides, therefore avoiding biofilm formation (Zhu & Mekalanos, 2003).

While the most common Gram-negative autoinducers are acylated homoserine lactones (AHL) (Williams *et al.*, 2007), Gram-positive bacteria use processed oligo-peptides as signal molecules (Solomon *et al.*, 1996). AHLs are synthesized as the result of the activity of LuxI family proteins and are recognized by LuxR-type transcriptional regulators. In the human pathogen *P. aeruginosa*, three primary quorum sensing signals have been described: two AHLs (butyryl homoserine lactone and 3-oxo-dodecanoyl homoserine lactone) and PQS (quinolone-like signal). The *las* QS system controls expression of the *rhl* QS system and the two AHLs are able to regulate the PQS system (Latifi *et al.*, 1996).

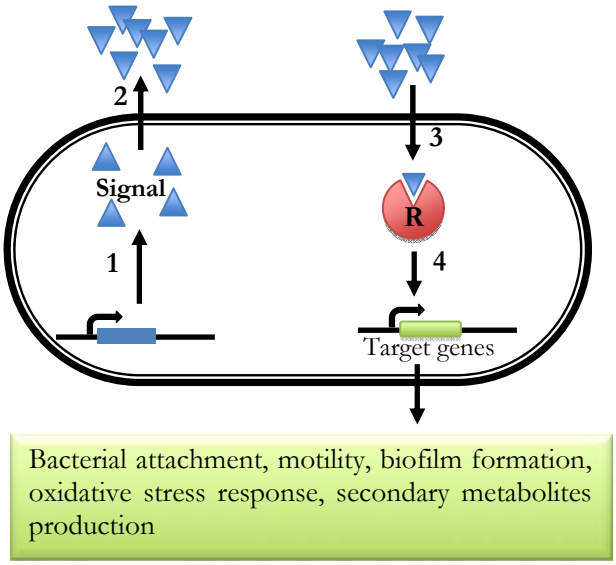

Figure 3. Scheme for a quorum sensing circuit. **(1)** A specific gene is responsible for the synthesis of a molecule called autoinducer (blue). **(2)** As the cell density increases the concentration of the autoinducer increases intra- and extracellularly. **(3)** At a critical autoinducer concentration a cytoplasmic receptor (R) of the familiy of LuxR binds the autoinducer. **(4)** The receptor-autoinducer complex binds the promoter of target genes and activates their transcription.

P. putida WCS358 posseses an orthologous system of the *las* QS system from *P. aeruginosa*, *ppu*, which regulates relevant processes for its activity as a plant growth promoter, like biofilm formation and siderophore production (Rampioni *et al.*, 2012). The *ppuI* is indirectly responsible for the production of the signal molecule (3-oxo dodecanoyl homoserine lactone) while *ppuR* is the receptor of this signal. The transcriptional regulator RsaL, which represses the production of the signal molecule (Bertani & Venturi, 2004), is located between these two genes. In some bacteria, there is an "orphan" LuxR regulator, but no LuxI homolog, suggesting that they can sense AHL signals from other species. As an example, in *P. putida* KT2440, there is a *ppuR* homolog, but no gene for the production of

AHL. However, a gene involved in adhesion to seeds and colonization of root surfaces, *ddcA*, is expressed in a cell density-dependent manner in this bacterium. It is controlled by the two-component system RoxS/RoxR (Fernández-Piñar *et al.*, 2008) and responds to signals related to fatty acids.

A recent work has reported new data on the connection between QS and biofilm formation through the secondary messenger c-di-GMP; the activation of the QS pathways causes the reduction in intercellular c-di-GMP and subsequent inhibition of matrix components production in *V. cholerae* (Waters *et al.*, 2008).

5.3. Role of the secondary messenger c-di-GMP in biofilm formation

The secondary messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) has been shown to regulate transition from planktonic-motile cells to the formation of a biofilm in different Gram-negative bacteria (Römling & Amikam, 2006, Simm *et al.*, 2004, Tischler & Camilli, 2004). Low levels of intracellular c-di-GMP inhibited biofilm formation and stimulated swimming and swarming motility. In contrast, high c-di-GMP concentrations stimulated biofilm formation and synthesis of components involved in adhesion such as surface proteins and exopolysaccharides (Gjermansen *et al.*, 2006, Matilla *et al.*, 2011, Morten *et al.*, 2006, Nakhamchik *et al.*, 2008) (Figure 4). C-di-GMP is synthesized by diguanylate cyclases (DGC), which typically show conserved GGDEF motifs, and is degraded by phosphodiesterases (PDE), which contain EAL or HD-GYP motifs. Proteins containing GGDEF/EAL domains represent a complex signalling mechanism which is able to transduce different signals into the production or degradation of the secondary messenger c-di-GMP (Galperin, 2004). This secondary messenger is able to bind a wide variety of effectors which cause changes at different levels including enzymatic activity, protein/protein interaction, gene transcription and translation (Baraquet *et al.*, 2012).

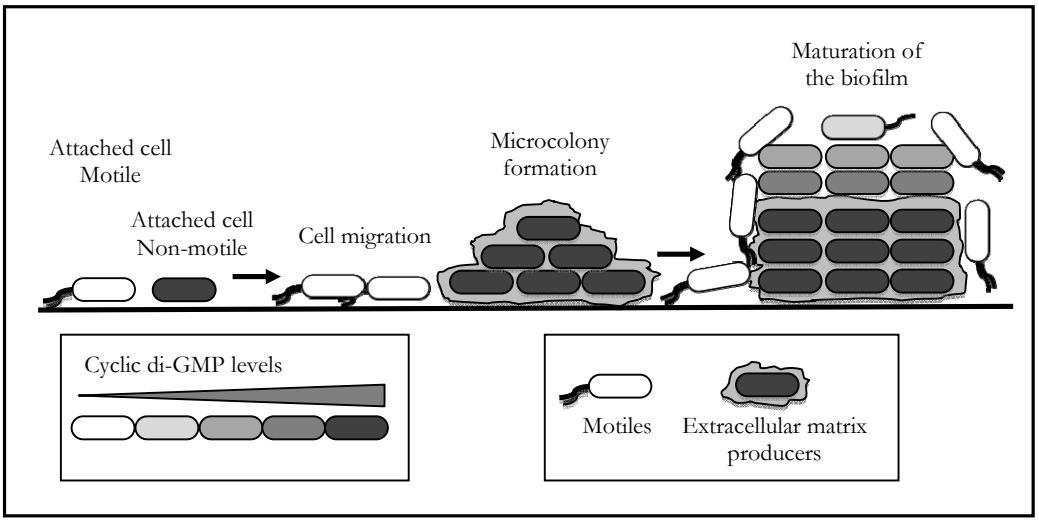

Figure 4. Correlation of intracellular levels of c-di-GMP with biofilm formation. Modified from (Römling & Amikam, 2006).

One of the consequences of c-di-GMP binding to its effectors is the regulation of the transcription of genes directly involved in biofilm formation and matrix production through different mechanisms. In *P. aeruginosa* the transcriptional regulator FleQ binds to c-di-GMP and induces the transcription of genes involved in the production of the exopolysaccharides Psl and Pel and the surface protein CdrA (Hickman & Harwood, 2008), Borlee *et al.*, 2010). A recent publication provides evidences of the involvement of c-di-GMP in a cell signalling system in the Gram-positive bacterium *Bacillus subtilis* that is directly or indirectly involved in biofilm formation (Chen *et al.*, 2012).

In *Pseudomonas putida* KT2440 up to forty-three genes are putatively involved in the synthesis and degradation of c-di-GMP (Matilla *et al.*, 2011). The response regulator *rup4959*, containing a GGDEF domain, has been shown to exhibit diguanylate cyclase activity and in multicopy it causes a pleiotropic phenotype

characteristic of high levels of c-di-GMP: particularly, enhanced biofilm formation, exopolysaccharide biosynthesis, reduced motility, and wrinkly colonies among others (Matilla *et al.*, 2011).

The localization of the surface protein LapA, essential for surface attachment of *P. putida* and *P. fluorescens*, is controlled by intracellular levels of this secondary messenger (Monds *et al.*, 2007, Newell *et al.*, 2011, Gjermansen *et al.*, 2010). Different works have elucidated the complex regulatory network which controls the retention of the surface protein LapA (Boyd *et al.*, 2012, Monds *et al.*, 2007, Navarro *et al.*, 2011, Newell *et al.*, 2011, Newell *et al.*, 2009). Under environmental conditions of low Pi, the phosphodiesterase RapA causes a reduction in the intracellular levels of c-di-GMP provoking the loss of LapA from the cell surface (Figure 5).

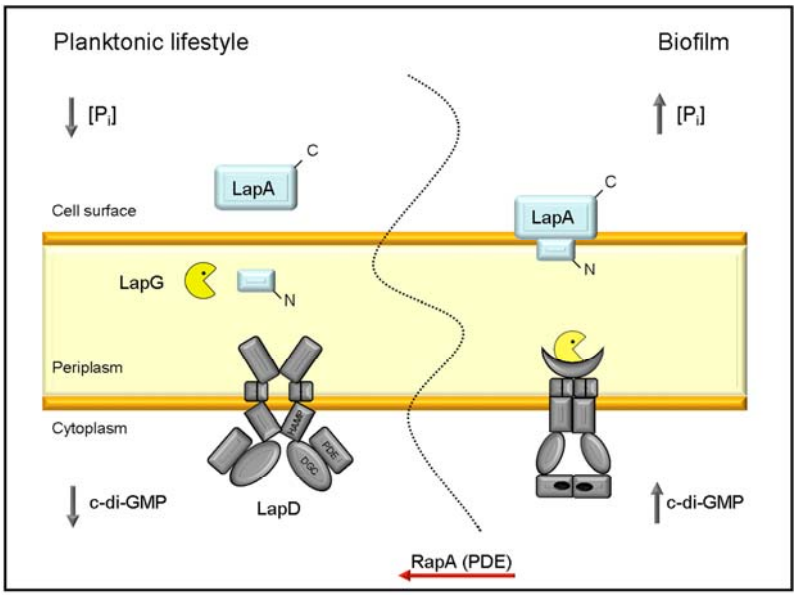

Figure 5. Model for inorganic phosphate (Pi) and LapA-mediated control of biofilm formation in *Pseudomonas fluorescens*. This model is based on structural and functional analysis (Gjermansen *et al.*, 2010, Hinsa *et al.*, 2003, Monds *et al.*, 2007, Navarro *et al.*, 2011, Newell *et al.*, 2009)

When intracellular amounts of this second messenger are high, an inner membrane protein called LapD interacts with the periplasmic protease LapG and inhibits its activity, which targets LapA (Figure 5).

6. The architecture of biofilms: the extracellular matrix

After planktonic bacterial cells have sensed a surface, cells start to activate the process of adhesion and biofilm formation. Initial bacterial adhesion is governed by specific interactions while a mature biofilm may involve a combination of specific and non-specific interactions. A "specific interaction" includes adhesins, spatially well-organised groups of specific molecules that require close approach of the interacting groups provoking immobilised adhesion. The "non-specific" type of interactions involves electrostatic and Van der Waals forces, which originate from the entire body of the cell and specific molecules are not required (Busscher *et al.*, 1992).

Molecular determinants involved in biofilm formation may vary among different bacterial species; however a common characteristic is the production of an extracellular matrix by the cells embedded within the biofilm structure. Endodontic biofilms that cause oral inflammatory disease mainly consist of extracellular material (van der Waal & van der Sluis, 2012). The composition of the extracellular matrix is complex and different among the bacterial diversity. These matrices provide multiple functions for the community, serving as a scaffold that holds cells together and providing protection from antimicrobial compounds and environmental stresses. Dramatic differences in biofilm morphology result when small changes in environmental conditions occur and are possibly due to a change in the matrix composition (Branda *et al.*, 2005).

The most representative components of the extracellular matrix of bacterial biofilms are surface proteins, nucleic acids and exopolysaccharides which may play different roles during the life cycle of bacterial biofilms or even under different

environmental conditions (Davey & O'Toole, 2000). Among these various components, exopolysaccharides and surface proteins have been extensively studied in a variety of bacteria. However, other components such as rhamnolipids, lipopeptides, lipopolyssacharides and other surfactants also have an influence (positive or negative) on bacterial adhesion to surfaces and cell-cell adhesion.

6.1. Exopolysaccharides

Exopolysaccharides (EPSs) are essential for biofilm formation and stabilization and provide the structural scaffold in many bacterial communities (Lasa, 2006, Branda *et al.*, 2005). Indeed, mutants affected in EPS production have been shown to be severely impaired in biofilm formation in either Gram-positive or Gram-negative bacteria (Friedman & Kolter, 2004, Laue *et al.*, 2006, Nakhamchik *et al.*, 2008, Ghafoor *et al.*, 2011, Kolodkin-Gal *et al.*, 2012). It has been proposed that EPSs contribute to prevent desiccation in soil-dwelling *Pseudomonas* sp (Roberson & Firestone, 1992, Chang *et al.*, 2007), or are able to absorb dissolved organic compounds, as is the case with herbicides and other xenobiotic compounds, allowing the concentration of essential nutrients and growth components within the biofilm (Wolfaardt *et al.*, 1998). Additional protective functionality of EPSs have been also demonstrated, given that they may serve as a physical barrier to a wide variety of antimicrobial compounds (Nichols *et al.*, 1988), chlorine (Ryu & Beuchat, 2005, Shemesh *et al.*, 2010) and protect bacterial pathogens against the human immune system (Leid *et al.*, 2005).

Among the variety of polysaccharides involved in biofilm formation, cellulose and homoglycans of β-1,6-linked *N*-acetylglucosamine, are the most common components of the exopolysaccharide of many different bacteria (Lasa, 2006). *E. coli* produces two different exopolysaccharides, colanic acid and β-1,6-*N*-acetyl-D-glucosamine (PGA); PGA has been shown to be required for irreversible attachment and cell-cell interaction (Agladze *et al.*, 2005) while colanic acid was

found to be essential for the development of three-dimensional structures (Danese *et al.*, 2000). In *P. aeruginosa* PAO1 three types of extracellular polysaccharides are implicated in biofilm development: alginate (β-*D*-mannuronate and α-*L*-guluronate residues), Psl (d-mannose, d-glucose and l-rhamnose) and Pel (glucose-rich polymer). Recently, Pel has been reported to be involved in initial attachment, maintenance of cell-cell interactions and resistance to aminoglycoside antibiotics (Colvin *et al.*, 2011, Friedman & Kolter, 2004, Byrd *et al.*, 2009). Psl polysaccharide has been localized in the cell surface following an helical distribution (Ma *et al.*, 2009) and is relevant for attachment to eukaryotic cells (Byrd *et al.*, 2009). *P. aeruginosa* strain PA14 does not produce Psl polysaccharide and Pel was shown to be responsible for biofilm maturation once the cells have made the initial contact with the surface (Colvin *et al.*, 2011). The overproduction of alginate is responsible for the mucoid appearance in many *P. aeruginosa* strains and is relevant for chronic infections in lungs (Mikkelsen *et al.*, 2011). Although alginate is not essential for *in vitro* biofilm development, it is partially responsible for the biofilm structure and resistance to tobramycin (Hentzer *et al.*, 2001).

Genome analysis of *P. putida* has revealed the presence of 4 clusters involved in the biosynthesis of putative EPSs: a cellulose synthesis operon (PP2634-PP2638); an alginate biosynthesis operon (PP1277-PP1288); and two additional polysaccharide biosynthesis operons, the *peb* gene cluster (PP1795-PP1788) and the *pea* gene cluster (PP3132-PP3142). The exopolysaccharide composition and the role of each polymer in biofilm formation have been recently investigated in separate studies (Nielsen *et al.*, 2011, Nilsson *et al.*, 2011). It is proposed that in *P. putida* KT2440 the products of the *pea* and *peb* clusters have a role in the stabilization of the biofilm structure and those of alginate and cellulose are not essential for biofilm formation and stabilization. In contrast, the study of Nielsen *et al.*, (2011) with a rifampin resistant derivative of *P. putida* mt-2 (the parental strain of KT2440 harboring plasmid pWWO) indicates that the *pea* cluster is

necessary for pellicle formation, cell-cell interaction and biofilm stability, and cellulose for rhizosphere colonization.

There are different reports on the connection between the secondary messenger c-di-GMP and exopolysaccharide production in many Gram-negative bacteria (Lasa, 2006, Matilla *et al.*, 2011, Nakhamchik *et al.*, 2008). Overexpression of GGDEF domain proteins in *P. putida* lead to massive production of a cellulose-like polymer (Gjermansen *et al.*, 2010; Matilla *et al.*, 2011). A recent work in *P. putida* KT2440 has shown that the overexpression of the diguanylate cyclase *rup4959*, a GGDEF/EAL response regulator, provoked a pleiotropic phenotype (wrinkly colony morphology, increased calcofluor staining and enhanced biofilm formation) due to the increase of intracellular levels of c-di-GMP. This phenotype was linked to more EPS production (Matilla *et al.*, 2011), and in particular the *pea* operon was essential for the appearance of the phenotype.

6.2. Adhesins: surface proteins involved in biofilm formation

In addition to the interaction of the exopolysaccharide layer in the firm adhesion to surfaces it has been demonstrated that surface proteins play an important role in biofilm formation in many bacterial species (Martínez-Gil *et al.*, 2010, Nilsson *et al.*, 2011, Lasa & Penades, 2006, Latasa *et al.*, 2005, Borlee *et al.*, 2010, Cucarella *et al.*, 2001, Romero *et al.*, 2010, Wagner *et al.*, 2011, Theunissen *et al.*, 2010). These surface proteins are regularly present in the biofilm extracellular matrix and their presence has been mainly related to the initial attachment of microbial cells to surfaces (Lasa & Penades, 2006, Mikkelsen *et al.*, 2011). Relevant examples are Bap in *S. aureus* (Cucarella *et al.*, 2001), LapA in *P. fluorescens* and *P. putida* (Hinsa *et al.*, 2003, Espinosa-Urgel *et al.*, 2000), CdrA in *P. aeruginosa* (Borlee *et al.*, 2010), BapA and SiiE in *S. enterica* (Wagner *et al.*, 2011, Latasa *et al.*, 2005), BpfA in *Shewanella oneidensis* (Theunissen *et al.*, 2010) and TasA in *B. subtilis* (Romero *et al.*, 2010).

Some of these adhesins are high molecular weight proteins present on the bacterial surface with 2-4 different domains. Characteristically they have a core domain of long tandem repeats. Lasa and Penadés (2006) proposed a group of Bap-related proteins based on many common features of some of these proteins (Table 1). The first member of this group, Bap (Biofim associated protein), was described as an essential surface protein involved in *S. aureus* biofilms (Cucarella *et al.*, 2001). Related proteins are involved in bacterial adhesion and biofilm formation and often play a relevant role in bacterial infectious processes.

As shown in Table 1 many Bap-related proteins present calcium-binding motifs and may be regulated by calcium. Variations in the number of repeats of some of these proteins and among different isolates have been described (Cucarella *et al.*, 2001, Toledo-Arana *et al.*, 2001). The surface protein Esp from *Enterococcus faecalis* is involved in biofilm formation but it probably has another mechanisms given that a strain of this species is able to form biofilms independently of Esp (Toledo-Arana *et al.*, 2001). In *S. enterica*, the deficiency of BapA could be compensated by overproduction of curli fimbriae, but not cellulose. This result supports the notion that BapA could play a role complementary to fimbriae in connecting cells. It has been suggested that BapA might interact with itself through homophilic interactions, thus acting both as a receptor and as a ligand between two bacterial clusters (Latasa *et al.*, 2005). Two novel adhesins involved in biofilm formation have been recently described: SiiE, a giant non fimbrial surface protein involved in cell adhesion of *S. enterica* to polarized epithelial cells (Wagner *et al.*, 2011) and BpfA from *Shewanella oneidensis* which promotes biofilm formation in a calcium dependent manner (Theunissen *et al.*, 2010).

Table 1. Bap related proteins

Name	Species	Size (aa)	Domains	Secretion	Ca^{2+} domains	Reference
Bap	*Staphylococcus aureus*	2276	4 *	43 aa signal peptide	Yes	Cucarella *et al.*, 2001
Esp	*Enterococcus faecalis*	1873	4 *	41 aa signal peptide	Yes	Toledo-Arana *et al.*, 2001
LapA	*Pseudomonas sp.*	8682	4 *	LapBCE	Yes	Hinsa *et al.*, 2003
BapA	*Salmonella enterica*	3824	3 *	BapBCD	No	Latasa *et al.*, 2005
BpfA	*Shewanella oneidensis*	2768	2 *	SO4318-20	Yes	Theunissen *et al.*, 2010
SiiE	*Salmonella enterica*	5559	3 *	SiiCDF	Unknown	Wagner *et al.*, 2011
LapF	*Pseudomonas putida*	6310	3 *	LapHIJ	Yes	This Thesis**

* One of them is a highly repeated domain

** Martínez-Gil *et al.*, (2010)

Large proteins (with an average length of around 3500 amino acids) predicted to be secreted and/or associated with the cell surface, including potential adhesins, filamentous hemaglutinins and others with unknown function are widespread among prokaryotes (Yousef-Coronado & Espinosa-Urgel, 2007). They are predicted or experimentally shown to be involved in bacterial attachment or cell-cell interactions. Maintaining and expressing genes of such length must be energetically costly for bacteria, and would imply that a significant selective pressure exists to retain them.

6.3. Extracellular DNA

Another important component of the matrix of many bacterial biofilms is extracellular DNA (eDNA). High concentrations of eDNA have been detected in *P. aeruginosa* PAO1, *P. putida* mt-2, *S. epidermidis*, *S. aureus* and *E. faecalis* biofilms (Steinberger & Holden, 2005, Izano *et al.*, 2008, Whitchurch *et al.*, 2002). The contribution of eDNA has been shown to be temporal given that mature biofilms (84 hours) treated with DNaseI were less affected than at earlier times, suggesting that eDNA is not a key component of the extracellular matrix in mature biofilms (Whitchurch *et al.*, 2002). However, it has been proposed that eDNA has a structural role for the formation of the characteristically mushroom-shaped microcolonies of *P. aeruginosa* biofilms (Allesen-Holm *et al.*, 2006).

It has been suggested that eDNA could come from membrane vesicles rather than cell lysis (Whitchurch *et al.*, 2002). However, recent results support that eDNA release occurs via cell lysis by different mechanisms, mediated by the autolysin AtlE in *S. epidermidis*, by the gelatinase GelE and serine protease SprE in *E. faecalis* or by a bacteriophage in *Streptococcus penumoniae* (Carrolo *et al.*, 2010, Thomas *et al.*, 2008, Qin *et al.*, 2007). Cell death in bacterial biofilms occurs inside the microcolonies, although this phenomenon contributes to dispersal and phenotypic diversification rather than to biofilm architecture (Mai-Prochnow *et al.*, 2006, Webb *et al.*, 2003).

7. *Pseudomonas putida* KT2440: a model bacterium for the study of biofilm formation

A number of bacteria have been demonstrated to promote the growth of different plants (Adesemoye *et al.*, 2009, Kloepper *et al.*, 1980, Kim *et al.*, 2011). Among plant growth-promoting rhizobacteria (PGPR) we find strains of the genus *Bacillus*, *Pseudomonas, Serratia* and *Azospirillum* (Vessey, 2003, Somers *et al.*, 2004, Koo & Cho, 2009, Combes-Meynet *et al.*, 2010). These bacteria develop their activity in many ways, like facilitating nutrients uptake (Desnoues *et al.*, 2003), producing fitohormones (Vessey, 2003) or antifungic compounds working as biological control agents (Montesinos *et al.*, 2002). The production of secondary metabolites, like siderophores, (Thomas *et al.*, 2004), cyclic lipopeptides and phenazines (D'Aes *et al.*, 2011) plays also important roles in the biocontrol of plant pathogens. The ability to provide plant protection depends directly on adhesion to plant seeds and on rhizosphere colonization (Kloepper *et al.*, 1988). This phenomenon depends at the same time on multiple bacterial functions as growth, motility and attachment.

P. putida has been widely used for biotechnology applications due to its ability to degrade aromatic compound as toluene (Contreras *et al.*, 1991, Pedersen *et al.*, 1997, Liang *et al.*, 2012). One of the first studies on biofilm formation of *P. putida* was focused on the effect of surface properties in biofilm accumulation (Shrove *et al.*, 1991) and studied toluene degradation by *P. putida* biofilms (Møller *et al.*, 1996). A global analysis of physiological changes in *P. putida* biofilms revealed differences in gene expression after 6h of attachment (Sauer & Camper, 2001).

The strain KT2440 is the model bacteria used in our laboratory for the study of plant-bacterial interactions and biofilm formation. It is a plasmid free derivative of *P. putida* mt-2, which was isolated from a planted orchard in Japan in 1960. It is a soil microorganism that colonizes the rhizosphere of a number of agronomically important plants at high population densities (Molina *et al.*, 2000). It has been shown that this strain promotes plant growth and induces systemic resistance

(Matilla *et al.*, 2007), therefore it is a good candidate for rhizorremediation and biocontrol. The sequencing of its complete genome (Nelson *et al.*, 2002) allowed us to study in detail several genes, described previously by Espinosa-Urgel *et al.*, (2000), involved in seed attachment and root surface colonization. Attachment of *P. putida* KT2440 to corn seeds appears to be strongly dependent on surface proteins, since the addition of proteinase *K* drastically reduces adhesion (Espinosa-Urgel *et al.*, 2000). In that work and a later one (Yousef-Coronado*, et al.*, 2008), *P. putida* KT2440 was mutagenized by random insertion of mini-*Tn5*-Km1 (Lorenzo *et al.*, 1990), and mutants showing a deficiency in attachment to seeds, called "mus" (mutants unattached to seeds), were isolated (Figure 6). The genes disrupted in each case were identified by arbitrary PCR and sequencing.

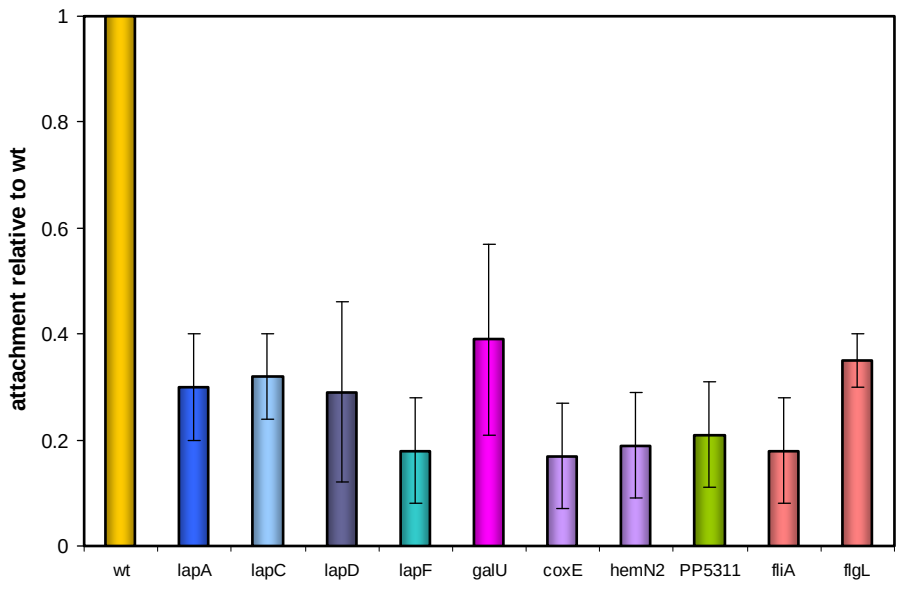

Figure 6. Quantification of the attachment to corn seeds of *Pseudomonas putida* KT2440 (wt) and different mutants. Results are presented as percentage of attached cells with respect to the number of cells inoculated.

Biofilm formation assays were performed with these mutants in different abiotic surfaces, to define if the functions disrupted were specific for attachment only to plant surfaces or if they represented a general adhesion pathway. After 3 hours, two mutants (mus-24 and mus-22) were deficient in surface attachment in all conditions tested (Figure 7). The mini-*Tn5*-Km insertions disrupted a gene encoding LapA (Large adhesion protein). Mutants affected in *lapA* were completely unable to form biofilms due to a deficiency in the early stage of irreversible attachment to the surface during biofilm formation (Hinsa *et al.*, 2003).

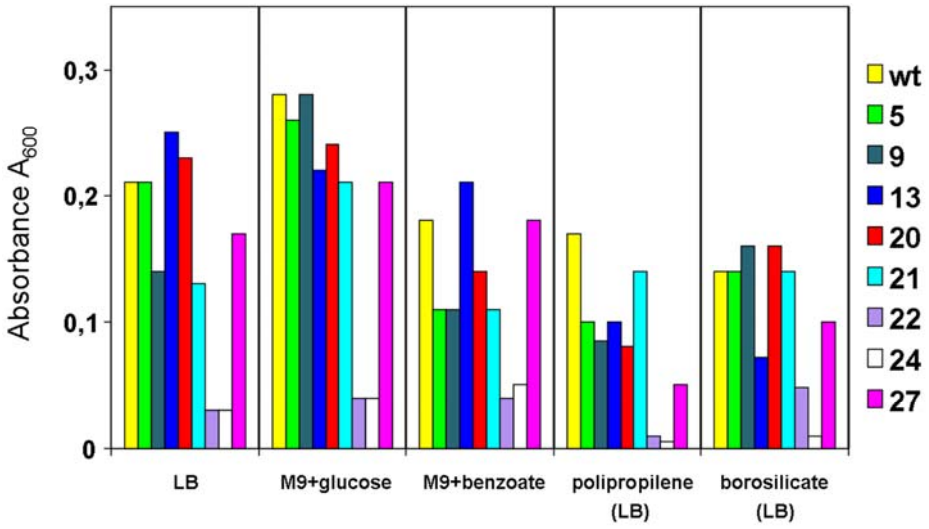

Figure 7. Attachment of *P. putida* mutants to different abiotic surfaces, quantified by staining attached cells after 3 h with crystal violet and measuring A_{600} after solubilisation of the stain with ethanol. Wt: wild type.

LapA is a large surface protein, that has homologues in other *Pseudomonas* species, *P. fluorescens*, *P. brassicacearum* and *P. entomophyla,* and has been characterized in separate works (Hinsa *et al.*, 2003, Yousef-Coronado *et al.*, 2008, Newell *et al.*, 2011). LapA is a Bap-related protein and it is also the first protein to be described

as essential in biofilm formation by fluorescent pseudomonads. LapA is secreted by a type I secretion system formed by LapB (inner membrane ATPase), LapC (periplasmic fusion protein) and LapE (outer membrane protein). In *P. fluorescens*, the three genes form an operon, while in *P. putida lapB* and *lapC* are clustered together but *lapE* is localized in a different position in the chromosome (Figure 8).

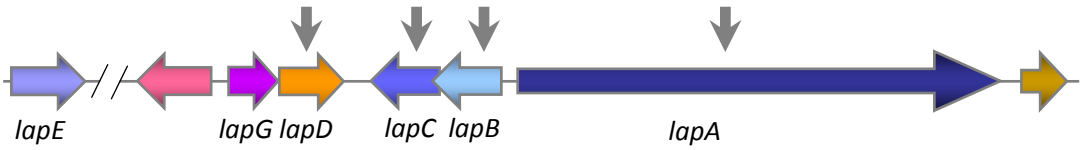

Figure 8. Genetic organization of the chromosomal region of *P. putida* containing *lapA*, its own ABC transporter system (*lapB* and *lapC*, with *lapE* in a different location), the c-di-GMP binding protein *lapD* and the periplasmic protease *lapG*. Mutants with reduced biofilm formation identified in *lap* genes are indicated (grey arrows).

Mutants in these different *lap* genes have similar phenotypes. Recent results, discussed above in section 5.3, have elucidated the molecular mechanism that controls the retention of the surface adhesin LapA by two additional proteins (LapD and LapG) and intracellular levels of the secondary messenger c-di-GMP. Details on these proteins are presented in Table 2.As shown in Figure 7, other mus mutants were less affected in biofilm formation at 4 hours. However, in one of them, mus-20, the gene interrupted (PP_0806) was found to encode a putative large surface protein, with no homologies in other *Pseudomonas*, but with similarities to LapA in terms of structure, size, and genetic environment (Table 2).

Table 2. Large adhesion proteins (Lap) and related proteins in *Pseudomonas putida* KT2440.

Name	Locus	Localization	Size (aa)	Function	Reference
LapA	PP_0168	Surface	8682	Adhesin	Hinsa *et al.*, (2003)
LapB	PP_0167	Inner membrane	718	LapA transport	Hinsa *et al.* (2003)
LapC	PP_0166	Periplasm	452	LapA transport	Hinsa *et al.* (2003)
LapE	PP_4519	Outer membrane	452	LapA transport	Hinsa *et al.* (2003)
LapD	PP_0165	Inner membrane	648	Binds to c-di-GMP/ Inside-out signalling	Newell *et al.*, (2009)
LapG	PP_0164	Periplasm	213	LapA protease	Navarro *et al.*, (2011)
LapF	PP_0806	Surface	6310	Cell-cell interaction, matrix component	This Thesis*
LapH	PP_0803	Outer membrane (p)	478	LapF transport (p)	This Thesis*
LapI	PP_0804	Inner membrane (p)	722	LapF transport (p)	This Thesis*
LapJ	PP_0805	Periplasm (p)	394	LapF transport (p)	This Thesis*

* Martínez-Gil *et al.*, (2010)

(p) = predicted

This mutant (mus-20) was defective in adhesion to seeds but in the first analysis of early attachment to abiotic surfaces it had little differences with the wild type. However, given the relevant role of surface proteins in biofilm formation, we decided to study in detail the role of this adhesin in root colonization and biofilm formation, its functionality and its regulation.

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II. AIM OF THE THESIS

Our research group has been studying the process of colonization of plant surfaces by the plant growth-promoting rhizobacterium *Pseudomonas putida* KT2440, and how this process correlates with biofilm formation. Molecular factors involved in adhesion to seeds and biofilm formation include a large secreted protein, LapA. A second putative large surface protein, LapF, was found to be important for seed colonization, but had not been studied in detail. Thus, we decided to address the following questions in the frame of this Thesis:

1. Characterization of the role of LapF in biofilm formation and rhizosphere colonization by *P. putida* KT2440.

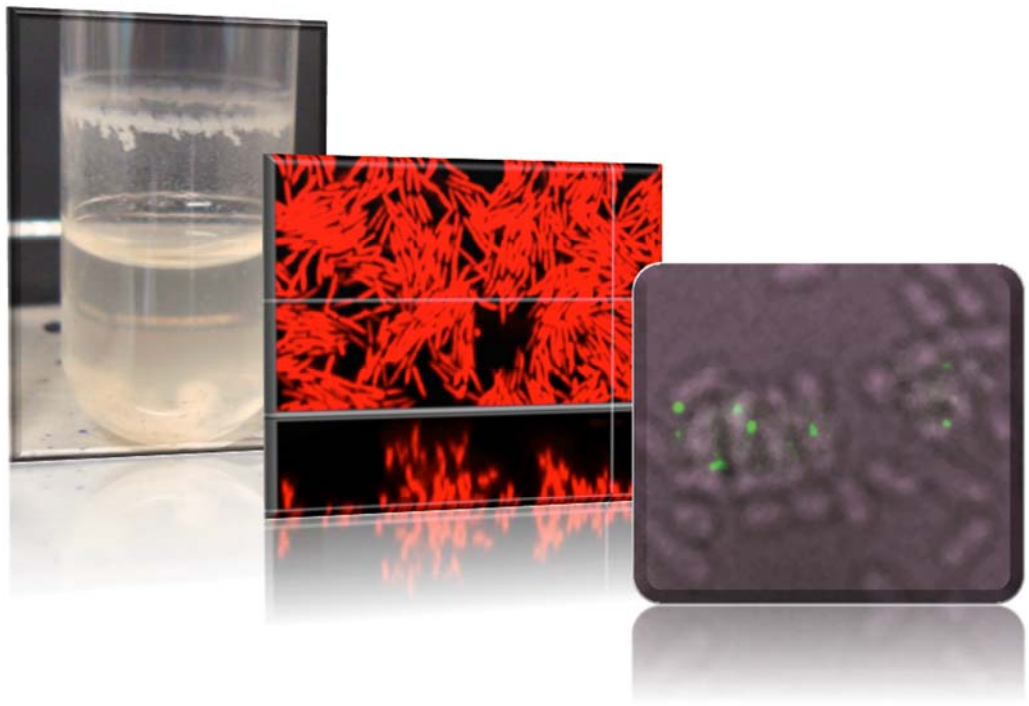
2. Analysis of putative calcium binding domains found in the LapF C-terminal region and study of the role of calcium in *P. putida* KT2440 biofilm formation.

3. Identification of regulatory pathways that control *lapA* and *lapF* expression.

4. Study of the relationship and possible interactions between large adhesion proteins and exopolyssacharides in *P. putida* biofilms.

III. RESULTS

Chapter 1



LapF, the second largest *Pseudomonas putida* protein, contributes to plant root colonization and determines biofilm architecture

Marta Martínez-Gil, Fátima Yousef-Coronado and Manuel Espinosa-Urgel. 2010. LapF, the second largest *Pseudomonas putida* protein, contributes to plant root colonization and determines biofilm architecture. *Molecular Microbiology*. 77 (3): 549-561.

Summary

We have investigated the role of LapF, one of the two largest proteins encoded in the genome of *P. putida* KT2440, in bacterial colonization of solid surfaces. LapF is 6310 amino acids long and is localized on the cell surface. The C-terminal region of the protein is essential for its secretion, which presumably requires the ABC transporter encoded by an operon (*lapHIJ*) adjacent to the *lapF* gene. Although the initial attachment stages are not different between the wild type and a *lapF* mutant, microcolony formation and subsequent development of a mature biofilm is impaired in the mutant. This is consistent with the expression pattern of *lapF*; activation of its promoter takes place at late stages of growth and is regulated by the alternative sigma factor RpoS. A *lapF* mutant is also affected in individual and competitive plant root colonization. In these assays, mixed microcolonies formed by cells of both the wild type and the mutant strains could be observed but microcolonies of the mutant alone were not found. These data and the localization of the protein at discrete spots in areas of contact between cells in biofilms suggest that LapF determines the establishment of cell-cell interactions during sessile growth.

Introduction

Colonization of solid surfaces seems to be a general persistence strategy for microorganisms. Single- or multispecies communities associated to a surface and surrounded by an exopolymeric matrix, referred to as biofilms, are known to be formed by a wide variety of bacteria under different environmental conditions and on diverse surfaces, both biotic and abiotic. The genetic basis of biofilm development has been the subject of much attention, and a number of genes have been found in various model microorganisms to play a role in this process. It is now clear that biofilm formation is a complex phenomenon that involves the integration of environmental and cellular signals into an intrincate regulatory network, leading to the adaptation of bacteria to multicellular life on a surface (for recent reviews, see Goller and Romeo, 2008; Hengge, 2009; Landini, 2009).

Bacteria of the genus *Pseudomonas* –particularly *Pseudomonas aeruginosa*– are among the better studied microorganisms with respect to phenotypic changes taking place throughout the process of biofilm formation and the genetic determinants involved. A general outline of the sequence of events leading from a planktonic culture to a mature biofilm has been assembled, going through the stages of initial adhesion of individual cells, followed by further colonization of the surface, growth and aggregation to form microcolonies, and production of an extracellular matrix.

Genes involved in *Pseudomonas* attachment to surfaces and biofilm structure determinants have been identified (O'Toole and Kolter 1998; Davey *et al.*, 2003; Hinsa *et al.*, 2003; Byrd *et al.*, 2009) and variations in the gene expression profile of *P. putida* and *P. aeruginosa* at the different stages have been reported (Sauer and Camper, 2001, Sauer *et al.*, 2002). This model of biofilm development with a specific genetic program following sequential steps has been the paradigm for the past years, but is being questioned (Ghigo, 2003), in part due to the incomplete

information existing with respect to the potential hierarchy of genetic elements and checkpoints during biofilm formation (Monds and O'Toole, 2009). Deciphering the precise mechanisms involved in the different stages of biofilm formation will not only help to understand microbial adaptation to this particular mode of life in a wide range of environments, but it may also provide relevant information with respect to the interaction of bacteria with eukaryotic hosts.

The Gram-negative bacterium *P. putida* KT2440 has been extensively studied in our laboratory as a model organism in plant-bacterial interactions. Such studies have provided evidences indicating that, while a number of genetic determinants are specifically relevant for the bacterium-plant interaction, biofilm formation and root colonization share certain common mechanisms (Yousef-Coronado *et al.*, 2008). As an example, previous work has established the importance of the surface-associated protein LapA in biofilm formation both by *Pseudomonas fluorescens* and *P. putida* (Hinsa *et al.*, 2003; Hinsa and O'Toole, 2006). This protein was identified as a key determinant of bacterial adhesion to corn seeds (Espinosa-Urgel *et al.*, 2000), and of root colonization by *P. putida* (Yousef-Coronado *et al.*, 2008). LapA is the largest protein encoded in the genome of *P. putida* KT2440, and closely related proteins are present in a wide variety of microorganisms (Lasa and Penadés 2006; Yousef and Espinosa-Urgel, 2007). Some of these large proteins containing long amino acid repeats have been shown to participate in biofilm formation in different organisms, as is the case with Bap in *Staphylococcus aureus* and *Salmonella enteritidis* (Cucarella *et al.*, 2001; Latasa *et al.*, 2005; Tormo *et al.*, 2005), or its *Acinetobacter baumanii* homolog (Loehfelm *et al.*, 2008). In the first two cases, the Bap protein is also relevant for colonization of eukayotic hosts by pathogenic strains (Cucarella *et al.*, 2004; Latasa *et al.*, 2005).

We now present evidences of the role played by a second large surface protein, which we have named LapF, in colonization of abiotic and biotic surfaces. While LapA determines the transition from reversible to irreversible attachment during biofilm formation (Hinsa *et al.*, 2003), LapF is important for further

development of a mature biofilm, as well as for the fitness of *P. putida* in the rhizosphere. Our results expose a key piece in the mechanism of biofilm formation by this plant-beneficial microorganism.

Material and Methods

Strains, plasmids and growth conditions

P. putida KT2440 is a plasmid-free derivative of *P. putida* mt-2, originally isolated from a vegetable orchard in Japan (Nakazawa, 2002). Strains mus-20 (*lapF* mutant) and mus-42 (*lapA* mutant) were obtained by random transposon mutagenesis with mini-Tn*5*[Km1] and identified as defective in attachment to corn seeds (Espinosa-Urgel *et al.*, 2000; Yousef-Coronado *et al.*, 2008). C1R1 is a *rpoS* null derivative of KT2440, while R6C1 is a merodiploid carrying an intact and a mutated copy of *rpoS* in the chromosome (Ramos-González and Molin, 1998). KT2440-Sm, streptomycin resistant derivative of KT2440 obtained by site-specific insertion of mini-Tn*7* ΩSm has been described elsewhere (Yousef-Coronado *et al.*, 2008). Other mini-Tn*7* derivatives (Koch *et al.*, 2001) were used to obtain fluorescently labeled strains KT2440*dsRed* [Gm] KT2440*gfp* [Km] and mus20*gfp* [Gm] by conjugation. *Escherichia coli* DH5α was routinely used in cloning experiments. Expression vectors pMP220 (Spaink *et al.*, 1987) and pRU1097 (Karunakaran *et al.*, 2005) were used to construct pMMG1 (*lapF::lacZ*) and pMMG2 (*lapF::gfp*), respectively (see below). *E. coli* was routinely grown at 37°C in LB. *P. putida* strains were grown at 30°C either in LB or in M9 minimal medium with $MgSO_4$, Fe-citrate and trace metals (Yousef-Coronado *et al.*, 2008), and glucose (20 mM) or citrate (15mM) as carbon and energy source. When appropriate, antibiotics were added at the following concentrations: kanamycin (Km), 25 μg/ml; tetracycline (Tc) 15 μg/ml; streptomycin 50 μg/ml; gentamycin (Gm) 10 or 100 μg/ml.

Molecular biology techniques

Plasmid DNA isolation, PCR amplification, digestion with restriction enzymes, gel electrophoresis and DNA ligation were done using standard protocols (Ausubel *et al.*, 1987). Plasmids pMMG1 and pMMG2 were constructed by PCR amplification of a 517 bp fragment that includes the intergenic region between PP_0807 and *lapF*, using primers LAPF1 (5'-GCGAATTCCAGCGACAGGTGATCGAAG-3') and LAPF2 (5'-GTCCACGGCGAAGAAGTTAC-3'). The PCR product was digested with *Eco*RI (underlined in LAPF1) and *Kpn*I, and the resulting 411 bp fragment was cloned in pMP220 and pRU1097. The absence of mutations in the cloned fragment was confirmed by sequencing.

To obtain antibodies against LapF, the Abie Pro 3.0: Peptide Antibody Design software (Chang Bioscience) was used to select an antigenic peptide (GRGEAGATVEVRNDQG), which corresponds to the best conserved region common to the 64 imperfect repeats of LapF. This peptide was synthesized and conjugated to Klh, and used to immunize SPF New Zealand rabbits. Immunization and antisera recovery were done at the Center for Animal Production of the Universidad de Sevilla.

For subcellular fractionation cells were grown overnight at 30ºC and B-PER Bacterial Protein Extraction Reagent (Pierce) was used for separating the soluble (cytosolic) fraction from 1.5 ml bacterial culture at an OD_{600} of 2. We followed a modified procedure developed by Cheng *et al.* (1973) to obtain outer-membrane proteins. 2ml overnight cultures were centrifuged and resuspended in a SL solution (20% sucrose; 1mg/ml lysozyme; 0,01M Tris 8.4) and incubated at 25ºC for 1h. After 10 min at 8000 rpm cells were resuspended with a Tris-Mg Solution (0,01M $MgCl_2$; 0,01M Tris pH 8.4), incubated at 25ºC for 20 min and centrifuged. The supernatant containing surface proteins was then collected. Cell fractions were quantified and detected after 5% SDS-PAGE. Western blot analysis of proteins was done following standard techniques (Ausubel *et al.*, 1987), after gel transfer onto PVDF membranes. Membranes were incubated with LapF-specific

polyclonal antiserum (1:4000) and detection was done with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad), using a chemiluminiscence-based detection system (Bio-Rad). A control experiment where incubation with the anti-LapF antibodies was omitted was also carried out to ensure the absence of non-specific binding of the secondary antibodies to the samples.

Rhizosphere colonization assays

Competitive colonization assays were done as previously described (Yousef-Coronado *et al.*, 2008), using 1:1 mixes of strains KT2440-Sm and mus-20, or each strain individually, for quantitative assays, and KT2440*dsRed* and mus-20*gfp* for visualization by fluorescence microscopy. For quantitative analysis of colonization, roots were cut, weighed and introduced in tubes with 20 ml of M9 basal medium and 4 grams of glass beads (Ø 3mm). Bacteria attached to the roots were recovered by vortexing for 2 min, and their numbers were calculated by plating serial dilutions of the resulting suspension on selective medium (M9 with citrate and Sm or Km). Alternatively, inspection of KT2440*dsRed* and mus-20*gfp* cells colonizing the root surface was performed on a Zeiss Axioscope fluorescence microscope with appropriate filter sets, coupled to a Nikon DS5-Mc CCD camera.

Biofilm formation analysis

Biofilm formation was examined during growth in polystyrene microtiter plates (Sterilin) or in borosilicate glass tubes, as described previously (Yousef-Coronado *et al.*, 2008). Biomass attached to the surface was visually inspected by staining with crystal violet, and quantified after solubilizing the dye with 70% ethanol and measuring absorbance at 590 nm (O'Toole and Kolter, 1998). Microscopy analysis of biofilm formation on glass was analyzed by obliquely placing 40 mm glass coverslips into the wells of a 6-well plate where cultures were allowed to grow in LB or minimal medium at 30°C. Visualization was done as described in the previous section. A modification of the method described by Gjermansen *et al.*

(2005) was used for biofilm formation under flow conditions, using LB diluted 1:10 as growth medium. Biofilms were grown at 30°C in three-channel flow chambers (BioCentrum-DTU, Technical University of Denmark), using a Watson-Marlow 205S peristaltic pump (Watson-Marlow Inc., Wilmington, MA). Fresh overnight cultures were diluted to an OD_{600}=0.5 and 300 µl were injected in the flow chamber. During the first hour, the flow was turned off in order to allow cells attach to the chamber; then, the flow was turned on and kept at a constant rate of 3 ml/h (laminar flow conditions). Biofilm structures were visualized with a Nikon C1 confocal laser scanning microscope. Images were analyzed with Imaris software (Bitplane), and biofilm parameters (biovolume, surface coverage, average thickness and maximum thickness) were calculated using COMSTAT (Heydorn *et al.*, 2000).

Collection of corn root exudates

Corn seeds were sterilized as above and hydrated for 48 hours at 30ºC in Phytagel (Sigma). After incubation, seeds were placed on a plastic floating structure in contact with 400 ml of sterile deionized water and Fe-EDTA, within a transparent container, where plants grew hydroponically in a sterile environment. Exudates released to the liquid were collected after 4 or 7 days, filtered into glass beakers and freeze-dried. Exudates were kept at -80ºC, and dissolved in 50% methanol immediately before use.

Reverse transcription coupled to PCR (RT-PCR)

RT-PCR analyses were performed by using the Titan One-Tube RT-PCR kit (Roche) in accordance with the manufacturer´s recommendations. For each reaction, 0.5 µg of total RNA was used. To rule out DNA contamination, a negative control was included. The mixture was heated for 5 min at 94ºC to inactivate reverse transcriptase before proceeding directly to the PCR amplification step. Primers were designed for the amplification of the 304bp intergenic region

lapF-lapH: RTLapFHFw 5´-GTACAGAACGGTATCCATGTG-3´ and the reverse primer RTLapFHRe 5´-GGTAAGGCTCAGCCGTTCGC-3´.

Measurement of β-galactosidase activity

β-galactosidase activity in *P. putida* cultures harboring pMP220 or pMMG1 was assayed as previously described (Espinosa-Urgel and Ramos, 2004). Experiments were repeated three times and results shown correspond to a representative experiment. Activity values are given in Miller units (Miller, 1972).

Immunofluorescence

LapF immunodetection was performed as described by Schmidt-Eisenlohr *et al.* (2001) with some modifications. Cells from static biofilms formed after 24 h of growth in 1:10 LB first washed with PBS and fixed with cold paraformaldehyde (4% v/v) for 30 min at 4ºC. After 3 subsequent washes in PBS, samples were incubated for blocking 30 min at room temperature with 1% BSA (w/v). Alternatively, cells were permeabilized with Triton X-100 prior to the blocking step. After 3 more washes in PBS, cells were incubated at 4ºC overnight with primary anti-LapF antiserum diluted 1:100. Samples were then washed with PBS and incubated with goat anti-rabbit Alexa Fluor 488 secondary antibodies (Sigma) for 3 h at room temperature, washed 3 times with PBS and treated with antifade reagent (Citifluor, Sigma) before visulization by fluorescence microscopy. A control omitting anti-LapF antibodies was also carried out to ensure the absence of non-specific binding of the secondary antibodies to the samples.

Results

lapF (PP_0806) encodes the second largest *P. putida* adhesin and forms an operon with its putative secretion system *lapHIJ* (PP_0805-PP_0804-PP_0803)

Strain mus-20 was originally identified in a screen for *P. putida* KT2440 random transposon mutants with reduced capacity to colonize corn seeds (Espinosa-Urgel *et al.*, 2000). At the time, the incomplete information available on the genome of KT2440 prevented further characterization of this mutant. Completion of the genome sequence allowed the identification of the disrupted locus, PP_0806, and the corresponding protein, LapF (large adhesion protein). LapF is 6310 amino acids long and is closely related to a group of large bacterial proteins that includes LapA (Yousef-Coronado *et al.*, 2008), a protein which has been shown to participate in bacterial adhesion to seeds and roots and biofilm formation by *P. putida* and *P. fluorescens* (Hinsa *et al.*, 2003, Yousef and Espinosa-Urgel, 2007).

Table 1. Comparison between the two Lap systems of *P. putida* KT2440. (1) LapE of P. putida KT2440 is encoded by locus PP_4519, which is not part of the *lapA*, *lapBC* cluster. In contrast, *lapE*, *lapB* and *lapC* form an operon in *P.fluorescens* (Hinsa *et al.*, 2003).

Function	Protein (aa)		% identities	% similarities
Adhesin	LapF (6310)	LapA (8683)	27	46
ATPase	LapI (722)	LapB (718)	34	53
Membrane fusion protein	LapJ (394)	LapC (458)	41	57
outer membrane protein (1)	LapH (478)	LapE (452)	20	41

As shown in Figure 1, the genetic organization of the chromosomal region containing *lapF* is similar to that of *lapA*, with adjacent open reading frames that encode the components of a putative type I secretion system. We have named these genes *lapH* (PP_0805, encoding a predicted outer membrane protein), *lapI* (PP_0804, a membrane-bound ATPase) and *lapJ* (PP_0803, a putative membrane fusion protein). The predicted start and stop codons of these three genes overlap, suggesting they are translationally coupled and form an operon. In the case of LapA, a similar transporter formed by LapB, LapC and LapE is required for translocation of this protein to the cell surface (Hinsa *et al.*, 2003).

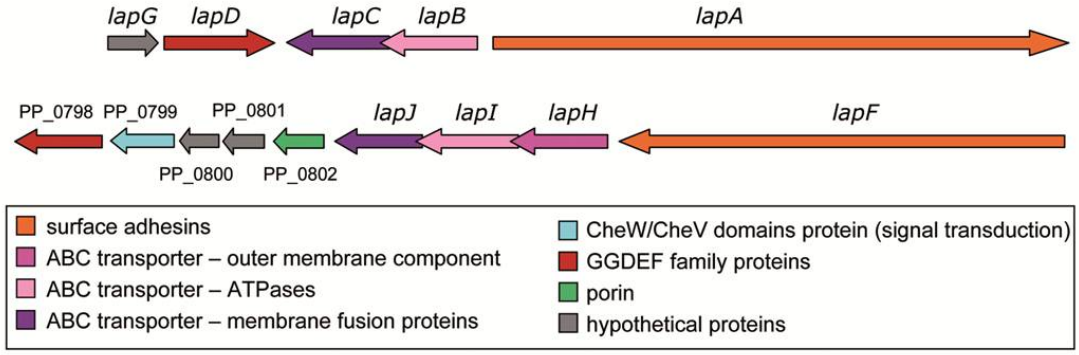


Figure 1. Genetic organization of the KT2440 chromosomal region containing *lapF* and comparison with the region comprising *lapA*.

Details on sequence similarities between the two Lap systems are described in Table 1. LapF presents three domains (Figure 2). Domain 1, comprising the first 152 amino acids, is followed by a repetitive region (Domain 2) covering over 85% of the protein. It consists on 64 imperfect repeats of 85-91 amino acids each (Figure 2). Domain 3, 691 amino acids long, corresponds to the C-terminal portion of the protein. It contains a predicted calcium binding region that includes two

copies of the GGXGXD motif (GGAGDD and GGSGTD), which has been proposed as a distinctive feature of proteins that are secreted via type I secretion systems (Delepelaire, 2004). The transposon insertion in mutant mus-20 is located in Domain 3, resulting in the protein being truncated 52 amino acids before the calcium binding region and secretion motifs.

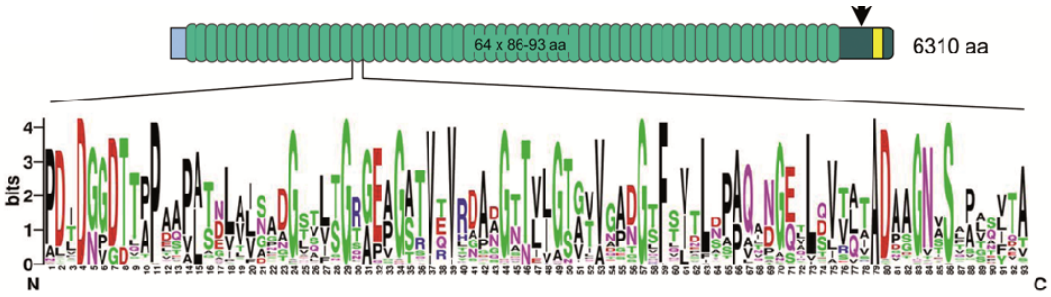


Figure 2. Schematic view of the LapF protein, showing the three structural domains and significant characteristics; secretion signal and calcium binding sites (yellow), and transposon insertion (arrow) are indicated. The sequence conservation (overall stack heigh) and relative frequency of each residue (heigh of each symbol) at every position of the 64 repeats are shown. The image was created using WebLogo software (University of California, Berkeley).

Reverse transcription coupled with PCR reaction (RT-PCR) was performed, using primers design to determine if *lapF* was forming an operon with the downstream genes which codify its putative ABC secretion system, *lapHIJ*. Total RNA was extraceted from KT2440 cultures grown in LB and use a template in RT-PCR reactions. After electrophoresis of the reaction products a band of the expected size was observed, confirming the existence of a single transcript corresponding to *lapFHI* (Figure 3).

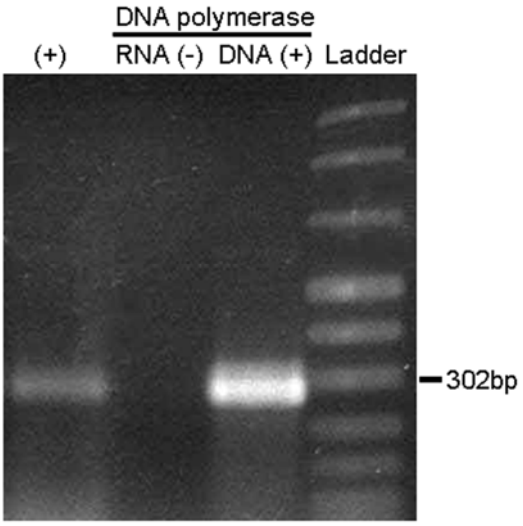


Figure 3. Electrophoresis of RT-PCR products obtained using RNA from KT2440 as template and primer pairs designed to verify the existence of a single mRNA molecule corresponding to *lapFHIJ*. From Left to right: template RNA obtained from KT2440 cultures, negative control to ensure amplification was not due to residual DNA and positive control of the DNA polymerase reaction.

LapF is localized on the cell surface and requires the C-terminal domain for translocation

In order to confirm the prediction that LapF is a surface-associated protein, polyclonal antibodies were obtained and used in a Western blot analysis of proteins obtained from the different subcellular fractions, as detailed in Experimental Procedures. Results are shown in Figure 4. A single band of high molecular weight reacting with the antibodies could be detected in the outer membrane fraction and in the cytoplasmic fraction of KT2440, indicating that after its synthesis in the cytoplasm, the protein is translocated to the cell surface.

In contrast, a band corresponding to LapF was only found in the cytoplasmic fraction in mutant mus-20 (Figure 4) and not in the outer membrane fraction, which indicates that the C-terminal domain disrupted by the transposon insertion is essential for translocation, as predicted, and that the role of LapF in adhesion to seeds requires its correct localization on the cell surface.

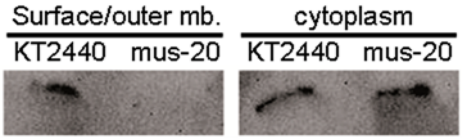


Figure 4. Localization of LapF in KT2440 and mus-20 by Western blot analysis.

Role of LapF in plant root colonization by *P. putida* KT2440

Whereas the large adhesin LapA and other determinants involved in attachment to seeds are also important for further colonization of plant roots, certain seed adhesion traits have no significant impact on bacterial fitness in the rhizosphere (Yousef-Coronado *et al.*, 2008). In order to establish the role of LapF, competitive colonization of corn roots by KT2440 and mus-20 was assayed. Seeds were inoculated with a 1:1 mix of KT2440-Sm and mus-20 before sowing in tubes containing sterile sand. Alternatively, the mix was added to the sand prior to sowing surface-sterilized seeds. Bacteria were recovered after 4, 7 and 12 days and the number of wild type and mus-20 cells was determined. In both types of experiment, the *lapF* mutant showed reduced competitive fitness (Figure 5). However, the number of both wild type and mus-20 cells increased over time and the differences between the two strains tended to decrease, from one order of magnitude at day four to approximately a 3:1 proportion of KT2440 versus mus-20 after 12 days.

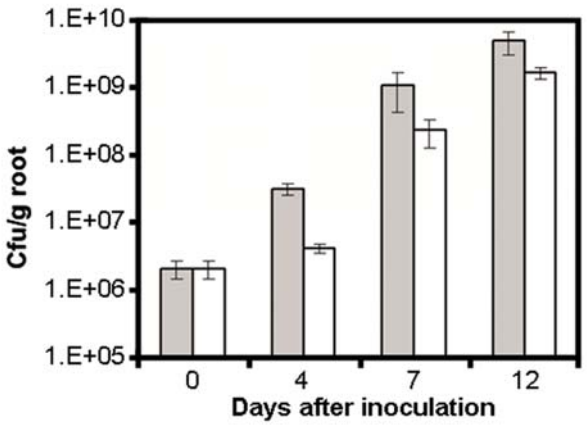


Figure 5. Competitive colonization of corn and alfalfa rhizosphere by KT2440 and mus-20. Quantitative analysis of corn root colonization. The number of cells of each strain recovered after 4, 7 and 12 days is shown. Grey bars: KT2440; white bars: mus-20.

Fluorescence microscopy was also used to examine colonization of 7 days old alfalfa roots by KT2440 and mus-20. For these experiments, strains were labeled with mini-Tn*7* derivatives harbouring the fluorescent protein-encoding genes *dsRed* and *gfp* (Koch *et al.*, 2001), respectively. Interestingly, microcolonies of KT2440 and mixed KT2440/mus-20 aggregates could be observed on different parts of the root (Figure 6), but microcolonies of the mutant alone were not found.

This result and the quantitative data suggest that LapF is involved in cell-cell interactions, and that the mutant can at least in part benefit from the protein produced and secreted by the wild type strain. If this interpretation was correct, it would be expected that mus-20 also showed reduced colonization capacity in the absence of competition. To test if this was the case, corn seeds were inoculated with both strain alone, and root colonization was quantitatively assessed as before. As with the competitive assays, the number of wild type bacteria per gram of root

recovered after 4 days was significantly higher than that of mus-20 ($10^9 \pm 4.5x10^8$ versus $2\ x10^8 \pm 8.2x10^7$ cfu/g root), a difference that was maintained after 7 and 12 days of incubation.

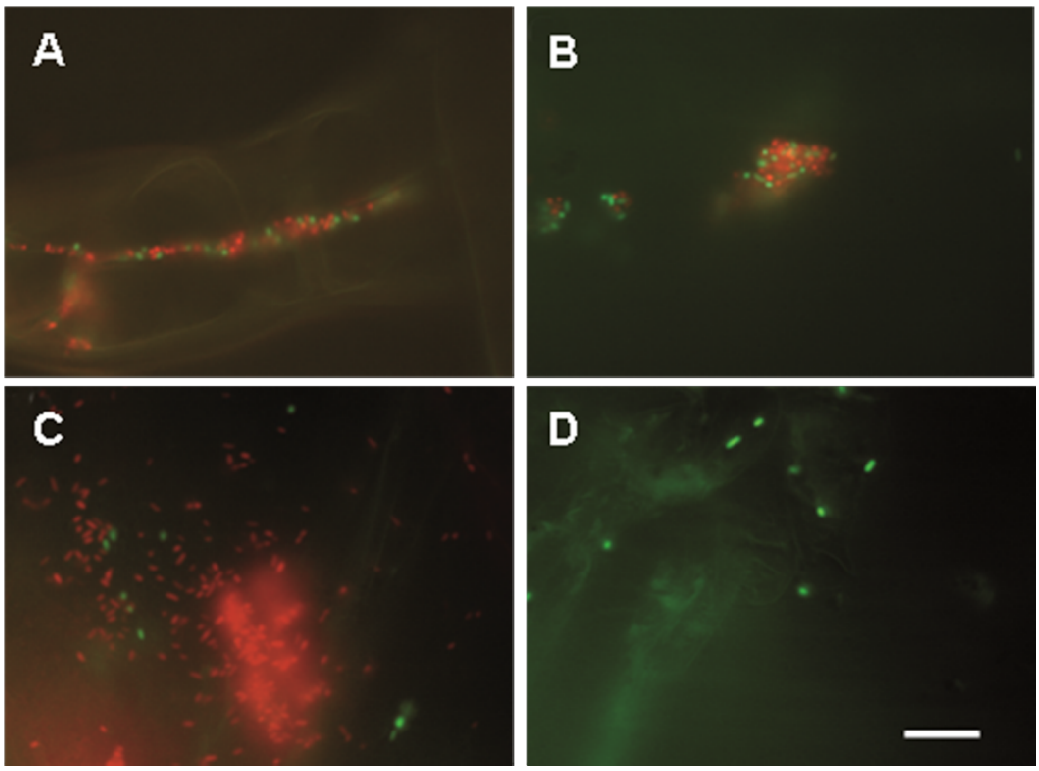


Figure 6. A-C. Visualization of alfalfa root colonization by fluorescence microscopy. Images are composites of each field observed with appropriate filters sets for dsRed (KT2440, red cells) and Gfp (mus-20, green cells). **D.** A field corresponding to mus-20gfp alone on alfalfa roots is shown (line: 10 μm)

LapF participates in biofilm development under environmental conditions favouring prolonged sessile growth

Previous results obtained with mutant mus-20 indicated that LapF was not a relevant factor for early bacterial attachment to abiotic surfaces (Espinosa-Urgel *et al.*, 2000), but an exhaustive follow-up of biofilm formation kinetics had not been performed. Biofilm formation by KT2440 and the *lapF* mutant in microtiter plates (polystyrene plastic) was monitored during growth in LB, LB diluted 1/10, and minimal medium with glucose or glucose plus casamino acids. Planktonic growth of the two strains was similar in all the media tested, with nearly identical duplication times (Table 2).

Table 2. Duplication times (minutes) of KT2440 and mus-20 in different growth media

Medium	KT2440	mus-20
LB	46±8	47±4
M9 glucose	76±3	72±6
M9 citrate	77±9	76±5
M9 glucose CAA	70±6	69±5

The biomass attached to the surface was evaluated by crystal violet staining, as described in Experimental procedures. Significant differences were only observed in M9 with glucose at late time points; as shown in Figure 7, the wild type strain continued developing a biofilm for 48 h, while the biofilm of the *lapF* mutant did not progress any further after 24 h.

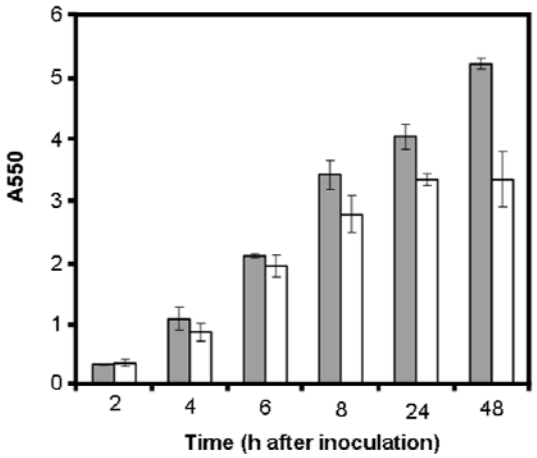


Figure 7. Quantification of biofilm formation by KT2440 (grey bars) and mus-20 (white bars) grown under static conditions in microtitre plates in M9 with glucose.

In the other media tested, mus-20 showed attachment kinetics similar to the wild type, although the maximum biomass associated to the surface was always slightly higher in the case of KT2440 (Figure 8).

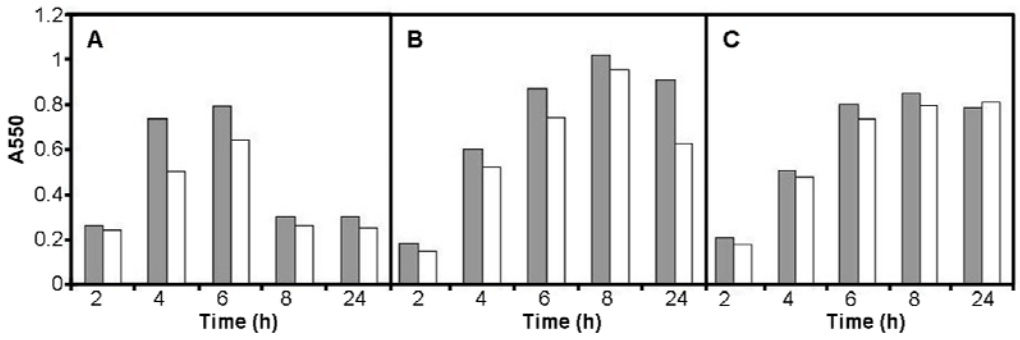


Figure 8. Biofilm formation by KT2440 (greys bars) and mus-20 (white bars) grown in LB **(A)**, 1/10 **(B)**, and M9+glucose+casamino acids **(C)** in microtiter plates.

As previously observed (Yousef-Coronado *et al.*, 2008), detachment of cells from the surface began to take place after 8 h of incubation in LB and LB diluted 1/10. Biofilm formation was also evaluated during growth in rotating glass tubes. As with the microtiter plates, the differences were not significant in LB medium, whereas in M9 with glucose and in 1/10 LB the mutant showed reduced attachment with respect to the wild type at late timepoints (Figure 9). These data suggested that LapF had no relevant role in the early attachment stages but could influence the development of a mature biofilm. However, as biofilm development progressed, KT2440 showed a more organized three-dimensional structure than mus-20, and the biofilm formed after 24h was significantly denser in the wild type than in the mutant.

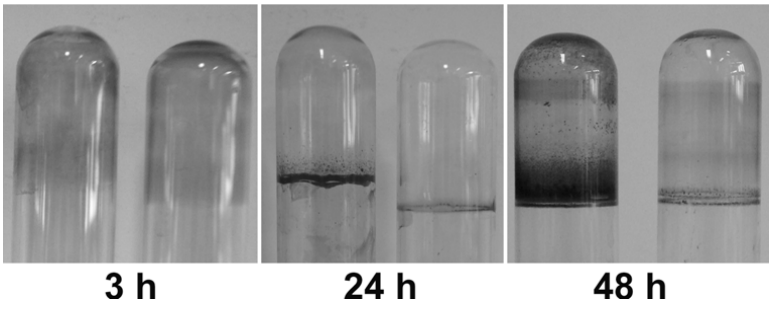


Figure 9. Three, twenty-four and forty-eight h biofilms of the wild type (left tube) and mus-20 (right tube) grown in M9 with glucose on borosilicate glass tubes.

To further explore this possibility, biofilm formation was followed on glass coverslips by fluorescence miscroscopy, using KT2440 and mus-20 labeled with *gfp* in single copy in the chromosome. Results are shown in Figure 10. As expected from the previous experiments, no differences were observed in the number of cells attached to the coverslip at early timepoints. This phenotype of the *lapF* mutant is clearly different from that of a *lapA* mutant, which under the same

conditions only showed scattered cells attached to the surface at all timepoints (Figure 10).

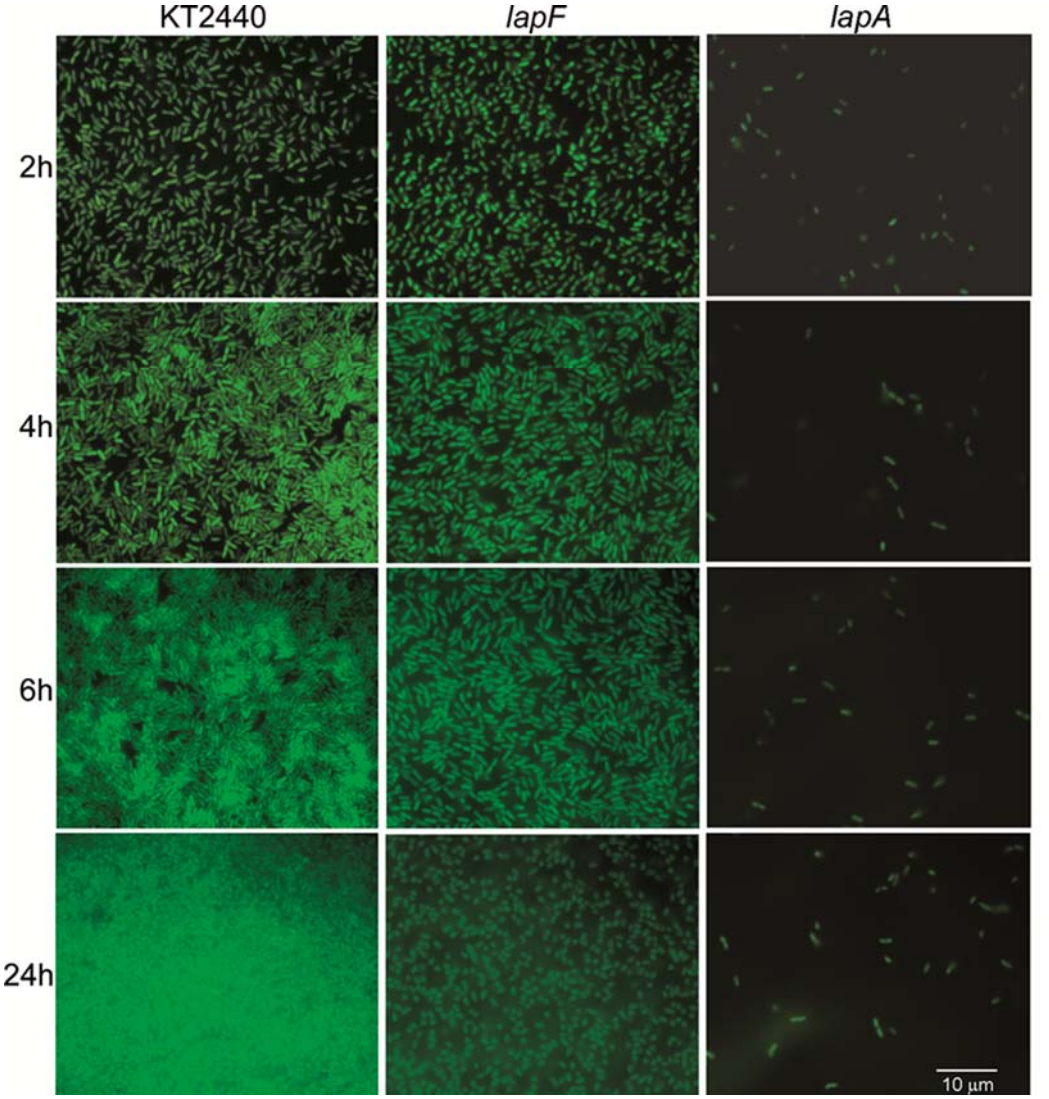


Figure 10. A Comparative analysis of the development of KT2440*gfp*, mus-20*gfp* (*lapF* mutant) and mus-42*gfp* (*lapA* mutant) biofilms on glass coverslips. Cultures were grown in six-well plates with a 40 x 20 mm glass coverslip obliquely palced in each well. Coverslips were removed every 2 hours and biofilm formation was followed by fluorescence microscopy (magnification: 1000x). Results shown correspond to cultures grown in M9 with glucose; similar results were obtained in 1:10 LB.

A detailed analysis of the biofilm structure formed by the wild type and *lapF* strains was then performed under flow conditions by confocal laser scanning microscopy (CLSM). As shown in Figure 11, the differences between the wild type and mus-20 were evident; KT2440 developed a mature biofilm, whereas in the case of the *lapF* mutant, cells attached to the surface appeared isolated and not forming microcolonies. Analysis of biofilm parameters using the COMSTAT software (Heydorn *et al.*, 2000) showed a 40-fold difference in biomass and 60-fold difference in average thickness between KT2440 and mus-20 biofilms (Table 3).

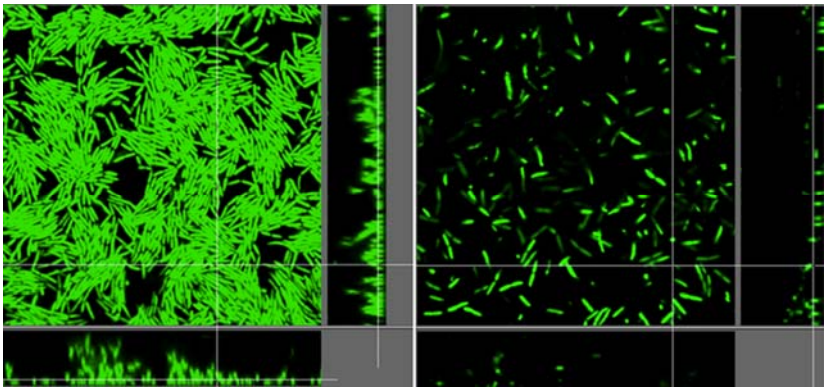


Figure 11. Confocal laser scanning microscopy images of KT2440*gfp* (left) and mus-20*gfp* (right) biofilms grown for 6 hours in 1:10 LB in flow cells.

Table 3. COMSTAT analysis of biofilm parameters in KT2440 and the *lapF* mutant mus-20.

Function	KT2440	*lapF*
Biomass ($\mu m^3/\mu m^2$)	2.29	0.06
Average thickness (µm)	8.27	0.14
Max. diffusion distance (µm)	3.22	1.24
Maximum thickness (µm)	29	10

Biofilm formation on glass coverslips was also examined in co-cultures of the wild type and the mutant tagged with fluorescent proteins. As in the pure culture experiments, although cells of both strains initiated attachment, mus-20 was unable to form microcolonies (Figure 12), and only individual cells of the mutant embedded in microcolonies of the wild type strain could be observed at late timepoints. This result supports a role for LapF in cell-cell interactions and suggests that cells unable to localize the protein on their surface cannot develop a mature biofilm by themselves. Differential effects due to the specific fluorescent protein used in each strain were discarded by switching the tags (Figure 12).

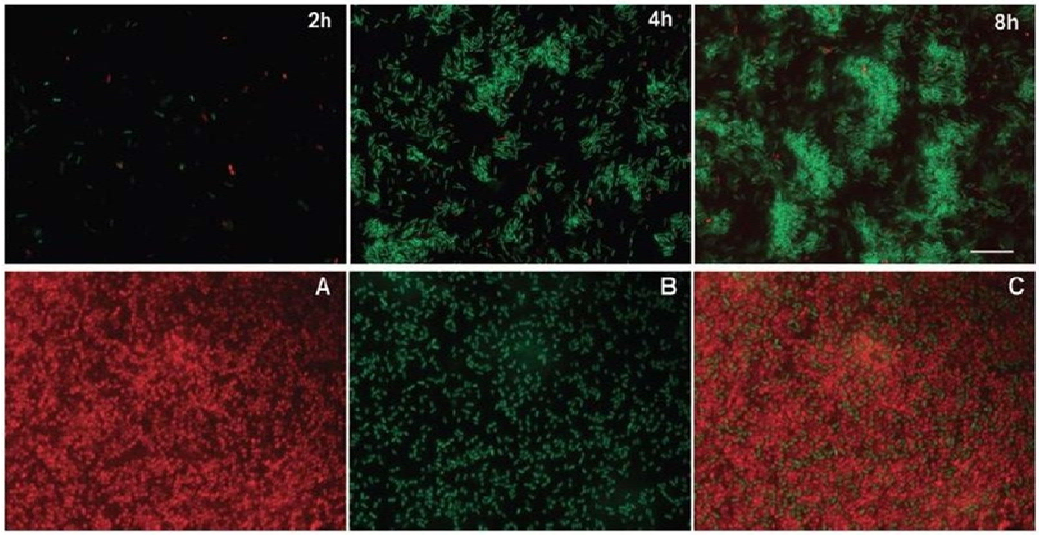


Figure 12. Top row: Mixed culture biofilms of KT2440*gfp* and mus-20*dsRed* on glass coverslips, followed at different times. Magnification: 400x. Bar: 10 μM. Bottom row: Mixed culture biofilms of KT2440*dsRed* **(A)** and mus-20*gfp* **(B)** after 24 hours of growth in 1:10 LB. **(C)** is a combined image of (A) and (B). Magnification: 1000x. Micrographs were taken with appropriate filter sets for each fluorescent protein.

Transcription of *lapF* is controlled by the alternative sigma factor σ^s

In order to analyze the expression pattern of *lapF*, a 411 bp fragment containing the upstream region and the first 32 codons of *lapF* was PCR-amplified and cloned in plasmid pMP220, as described in Methods, to generate a transcriptional fusion with the reporter gene *lacZ* devoid of its own promoter. The resulting plasmid, pMMG1, was electroporated into KT2440, and β-galactosidase activity was followed during growth in liquid LB medium. As shown in Figure 13, expression of the *lapF::lacZ* fusion was low in exponential phase and increased significantly upon entry in stationary phase.

This prompted us to test if expression from the *lapF* promoter was dependent on the alternative RNA polymerase sigma factor σ^S (RpoS), which controls the transcription of a number of genes upon entry in stationary phase and in response to environmental stresses (Hengge-Aronis, 2002; Potvin *et al.*, 2008). Plasmid pMMG1 was introduced in C1R1, a *rpoS* derivative of KT2440 (Ramos-González and Molin, 1998). Expression of *lapF::lacZ* was almost completely abolished in this mutant (Figure 13), indicating that the *lapF* promoter is directly or indirectly under the control of σ^S. Similar results were obtained with cultures grown in minimal medium with citrate or glucose as carbon sources, although the maximal level of β-galactosidase activity observed in the wild type was slightly higher than that obtained in LB (Table 4). Strain R6C1, a merodiploid derivative of KT2440 that carries a mutated and a wild type copy of *rpoS* in the chromosome (Ramos-González and Molin, 1998) was used to confirm the influence of σ^s upon *lapF::lacZ* expression. R6C1 harboring pMMG1 showed β-galactosidase activity kinetics similar to the wild type (Figure 13).

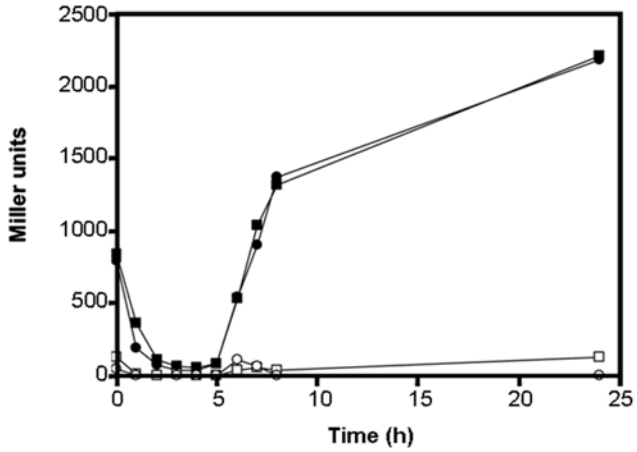


Figure 13. Expression of *lapF::lacZ* during growth in liquid medium. KT2440 (closed circles), its *rpoS* derivative C1R1 (open squares), and the merodiploid strain R6C1 (closed squares) harbouring pMMG1, were grown in LB and β-galactosidase activity was followed over time. KT2440 harbouring pMP220 (open circles) is shown as a negative control.

Given the role of LapF in the interaction with plants, the effect of root exudates on expression of the *lapF::lacZ* fusion was also tested. Corn root exudates were obtained from 4- and 7-days old plants and added at different concentrations to cultures of KT2440 (pMMG1). No significant influence on β-galactosidase activity could be detected with any of the concentrations tested (Table 4).

Table 4. Expression of the *lapF::lacZ* fusion in KT2440 grown in different culture media. Data correspond to β-galactosidase activity measured in overnight cultures (averages and SD of three experiments).

Medium	Miller Units
LB	3300±420
LB+ root exudate	3247±170
M9 glucose	3175±380
M9 citrate	3270±60

Expression of *lapF* in biofilms and the rhizosphere

As a tool to investigate *lapF* expression *in situ*, a transcriptional fusion was constructed by cloning the 411 bp fragment mentioned above in pRU1097, which harbors a promoterless *gfp* gene (Karunakaran *et al.*, 2005). The resulting plasmid, pMMG2, was introduced in KT2440 and expression of the fusion was confirmed in stationary phase cultures by fluorescence microscopy, whereas no expression was observed in exponentially growing cells (Figure 14).

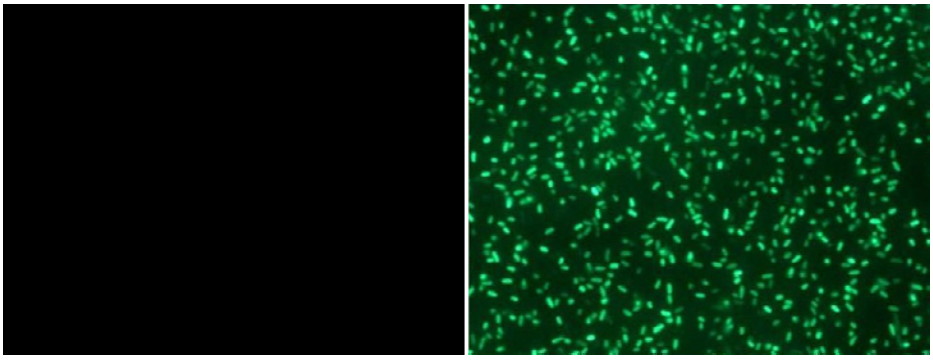


Figure 14. Expression of the *lapF::gfp* fusion in KT2440 harboring plasmid pMMG2 in liquid cultures. Left: exponential growth; right: stationary phase.

LapF expression was tested in the rhizosphere. Aggregates of cells expressing the *lapF::gfp* fusion could be detected in different colonization sites along the root and the root hairs (Figure 15). This is consistent with previous data indicating that transcription of the *rpoS* gene is induced in the rhizosphere with respect to exponentially growing cultures (Matilla *et al.*, 2007).

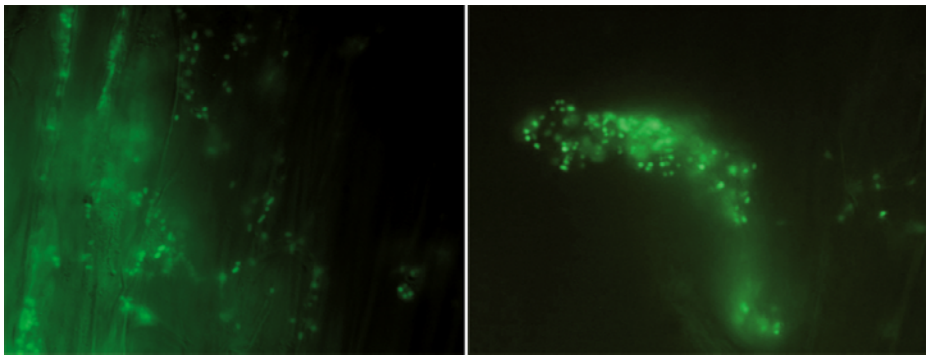


Figure 15. Expression of *lapF::gfp* in KT2440 colonizing alfalfa roots, analysed by fluorescence microscopy. Two root areas are shown: a zone of epidermal cells along the root (left) and a root air (right). Magnification: 1000x. Bar: 10 μM.

Expression of the *lapF::gfp* fusion was followed in biofilms under static conditions on glass coverslips by fluorescence microscopy. As with the liquid cultures, fluorescence of surface-associated cells was barely noticeable during the first 4 h, afterwards increasing as the biofilm developed (Figure 16). Interestingly, not all the cells in the population expressed the *lapF::gfp* fusion, fluorescence concentrating for the most part in densely colonized areas. Similar results were obtained in biofilms grown in flow-cells: expression of the *lapF::gfp* fusion was observed in the more densely colonized areas, particularly where microcolonies had already started to form, while no fluorescence was detected where only a few bacteria were attached to the surface (not shown).

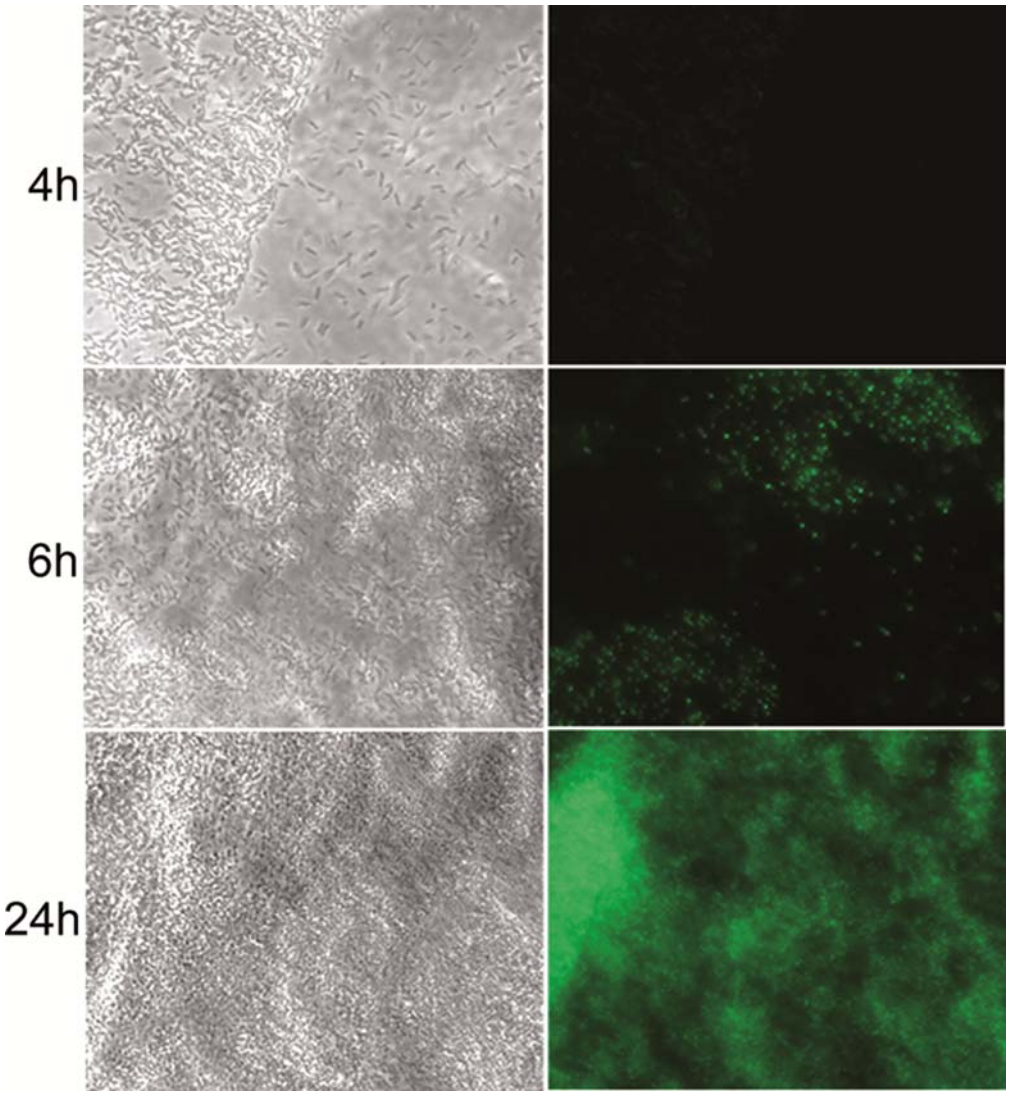


Figure 16. Expression of *lapF::gfp* in biofilms of KT2440 grown on glass coverslips under static condition, after 4, 6 and 24 hours. Phase-contrast (left panels) and fluorescence (right panels) microscopy were used to examine the same field. Magnification 400x.

In situ immunodetection reveals specific localization of LapF in *P. putida* biofilm cells

Taking advantage of the anti-LapF polyclonal antibodies, we decided to observe the localization of the protein in KT2440 biofilms, by immunofluorescence and microscopy. For this purpose, biofilms grown on glass coverslips for 24 h were fixed and incubated with the primary antibodies, followed by incubation with a secondary goat anti-rabbit antibody conjugated with Alexa Fluor 488 fluorophore. Cells were then observed by fluorescence microscopy. As shown in Figure 17, fluorescence was detected mainly in cells forming part of microcolonies, and not in isolated cells, a result that is consistent with the *lapF::gfp* expression data. Interestingly, fluorescence staining in KT2440 showed a highly punctuate appearance, with one or two spots per cell, and always localized between cells, so that the outer layer of cells in the microcolonies did not show staining. In agreement with the results obtained by Western blot, very faint fluorescence was detected in the mus-20 mutant after long exposure times (Figure 17). However, when bacteria were permeabilized before incubation with the anti-LapF antibodies, fluorescence could be observed in mus-20 cells (Figure 17), confirming that the mutant is unable to transport the protein outside the cell. All these data support a role of LapF in cell-cell interactions and suggest that the protein is concentrated at specific sites on the bacterial surface.

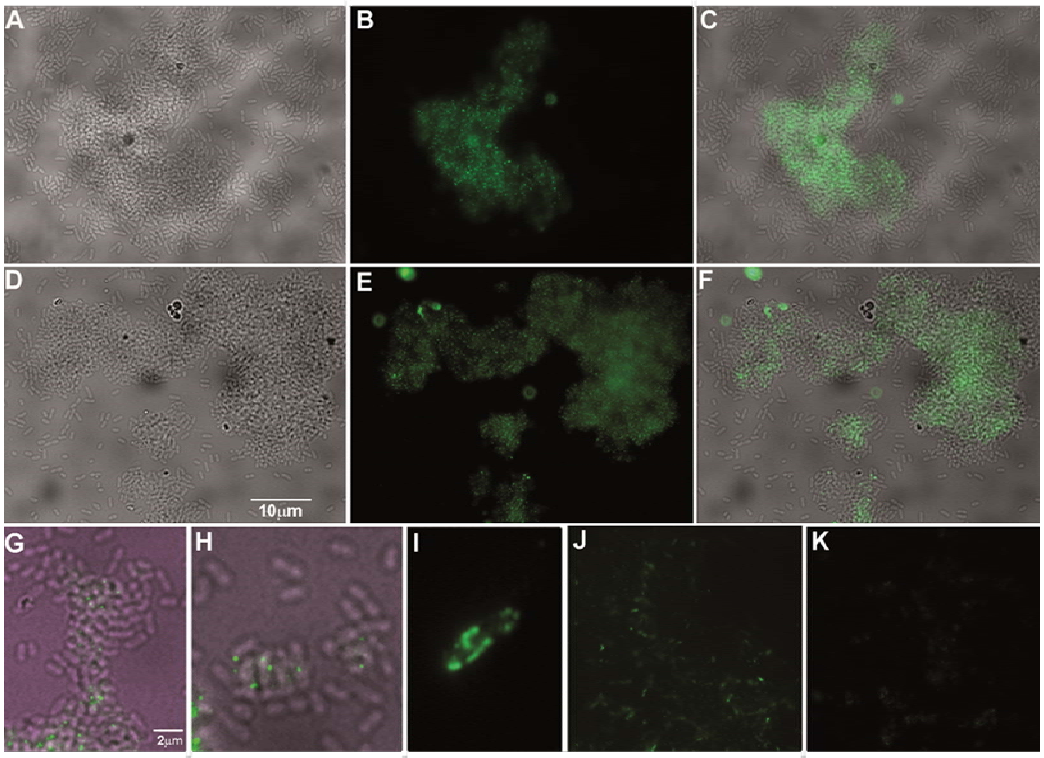


Figure 17. *In situ* localization of LapF in KT2440 biofilms by immunofluorescence.

A-F. Biofilms were grown for 24 hours on glass coverslips before being fixed and incubated with anti-LapF antibodies, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies, and visualization by phase-contrast (A and D) and fluorescence (B and E) microscopy (magnification: 1000x). Images (C) and (F) are composites of (A) and (B), and (D) and (E) respectively.

G-H. Enlarged images showing details of cell clusters and LapF localization in KT2440 (composite images).

I. Detail of a KT2440 cell permeabilized with Triton X-100 before incubation with antibodies.

J-K. Fluorescence images corresponding to mus-20 after identical incubations with anti-LapF and secondary antibody, with (J) or without (K) previous cell permeabilization. Exposure time for fluorescence in (k) was two times longer than the used in all other images.

Anti-LapF antibodies hamper biofilm development

To obtain further evidences of the role of LapF, biofilm formation assays were performed on glass coverslips in the presence of a 1:100 dilution of anti-LapF antibodies. As show in Figure 18, addition of the antibodies did not influence initial attachment of KT2440 to the glass surface. However, they obstructed the appearance of three-dimensional microcolonies, which were evident after 5 h of growth in the absence of antibodies; in their presence, small clusters of cells were only observed after 8 h of growth (Figure 18).

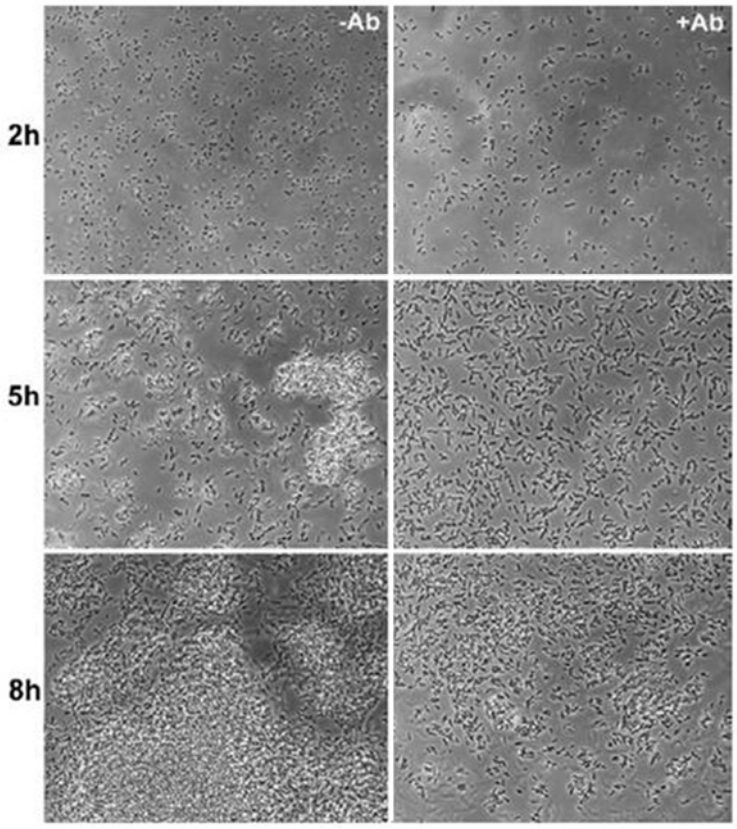


Figure 18. Anti-LapF antibodies hamper microcolony development by *P. putida* KT2440. Biofilm formation on glass coverslips was followed by phase contrast microscopy, in the absence (left, -Ab) or presence (right, +Ab) of a 1:100 dilution of anti-LapF antiserum. Magnification: 440x.

Thus, the behaviour, of the wild type strain in the presence of anti-LapF antibodies was somewhat similar to that of the *lapF* mutant. These data add weight to the involvement of LapF in cell-cell interactions during biofilm development.

Discussion

In this work we have characterized the role of LapF, a large repetitive protein that we have shown is localized on the surface of *P. putida* cells. As its "bigger sister" LapA, LapF participates in the process of colonization of both abiotic and plant surfaces. However, while LapA determines the progression of the biofilm beyond the initial attachment phase (Hinsa *et al.*, 2003), LapF seems to be required at later stages. Thus, a *lapF* mutant, while retaining its ability to go into a sessile mode of life, is unable to form a structured biofilm. This role had been previously missed (Espinosa-Urgel *et al.*, 2000) because attachment to abiotic surfaces had only been tested at early timepoints. For this reason, LapF was assumed to participate specifically in bacterial adhesion to plant seeds. We have now presented evidences of its importance for colonization of both abiotic and plant surfaces. This adds a new element to the previously identified functions required for the establishment of *P. putida* sessile populations regardless of the surface to be colonized, such as the above mentioned adhesin LapA, or HemN-2 a protein involved in heme biosynthesis (Hinsa *et al.*, 2003; Yousef-Coronado *et al.*, 2008). Thus, while bacterial populations associated to plant roots may not always be considered biofilms *sensu stricto*, colonization of abiotic and plant surfaces have essential mechanistic similarities.

The expression pattern of *lapF* is consistent with its observed participation in later stages of biofilm development, a role that suggests that the protein is involved in cell-cell rather than in cell-surface interactions. This is supported by the fact that, while the *lapF* mutant does not form three-dimensional

microcolonies on abiotic or biotic surfaces, mixed microcolonies of the wild type and the mutant strains are observed in competitive colonization assays in the rhizosphere. Microcolonies of the mutant alone are not found in these experiments. Similarly, in mixed culture biofilms the mutant does not form microcolonies, but isolated cells of mus-20 appear interspersed in wild type microcolonies. LapF could therefore be one of the protein components of the extracellular matrix of *P. putida* biofilms, helping bacteria to anchor to each other. Protease treatment is known to cause biofilm disruption even in the case of mutants showing defects in biofilm dispersal (Gjermansen *et al.*, 2010; Yousef-Coronado *et al.*, 2011), and it also prevents colonization of seeds by *P. putida* (Espinosa-Urgel *et al.*, 2000). The participation of the LapA protein in the composition of the matrix, with an exopolysaccharide-affixing role, has been recently postulated (Gjermansen *et al.*, 2010), although direct evidence of this function is still lacking. Based on our results and those published previously (Hinsa *et al.*, 2003), we propose that LapA is first required for surface colonization and then LapF, and probably LapA as well, participate in the buildup of a three-dimensional, structured biofilm. The effect of antibodies against LapF on biofilm development, and the results obtained in the immunolocalization assays support this function of LapF as mediator of intercellular adherence. We believe the specific localization of the protein at certain sites on the cell surface is an indicator of its architectural role. To our knowledge, this is the first time an element participating in biofilm buildup is thus charted.

The finding that *lapF* transcription is dependent on the stationary phase and general stress response sigma factor RpoS was somewhat surprising, since previous evidences indicated that starvation induces dispersal of *P. putida* biofilms (Gjermansen *et al.*, 2005), and the rhizosphere, where expression of *lapF* is also observed, is presumed to be a relatively nutrient-rich environment. However, global gene expression analysis of *P. putida* in the rhizosphere has revealed that plant roots not only provide nutrients but also impose stress on colonizing

bacteria, and *rpoS* transcription is increased in the rhizosphere compared to exponentially growing cultures or cells growing in microcosms in the absence of the plant (Matilla *et al.*, 2007).

The role of RpoS in biofilm formation has been studied mainly in *Escherichia coli* and remains controversial due to conflicting data with respect to the effect of *rpoS* mutations on biofilm structure and biomass. A recent study (Collet *et al.*, 2008) has shown that a *rpoS* mutant of *E. coli* forms biofilms with altered architecture, and that a set of proteins expressed in biofilms are under the control of σ^S. The contribution of a number of RpoS-regulated genes to the biofilm lifestyle of *Pseudomonas aeruginosa* has also been deduced from transcriptomic data (Waite *et al.*, 2006). These and other results (Landini, 2009) reveal that there is an overlap between environmental stress responses mediated by σ^S and traits important for biofilm formation.

In *E. coli*, this overlap involves the regulatory protein CsgD –which controls the synthesis of the fiber-like appendages known as curli under low temperature conditions– and several genes encoding GGDEF family proteins. These proteins are responsible for the synthesis of the secondary messenger cyclic-di-GMP, the levels of which are known to modulate motility, virulence and the transition between the planktonic state and biofilm formation (recently reviewed by Römling and Simm, 2009, and by Hengge, 2009) in different bacteria. Thus, a complex regulatory network modulates bacterial commitment to sessile life, and is likely to lie also underneath the physiological heterogeneity observed in biofilm cells (Spormann, 2008). In this respect, it is worth noting that differences in expression of a *lapF::gfp* fusion were observed among cells in different areas of *P. putida* biofilms, while more or less uniform expression could be detected in stationary phase liquid cultures (Figure 14). Since transcription from the *lapF* promoter seems to be strictly dependent on RpoS, one possibility is that RpoS is itself differentially regulated in various microenvironments within the biofilm, or at different developmental stages. Alternatively, an additional, biofilm-specific factor

could also participate in the regulation of *lapF* transcription, or in the localization of the protein. In *Staphylococcus aureus*, a phase variation mechanism appears to contribute, besides other regulatory elements, to control expression of the large repetitive adhesin Bap (Tormo *et al.*, 2007). It will be interesting to check if a similar phenomenon could be taking place in *P. putida* biofilms.

There is currently an ongoing debate regarding whether biofilm formation can be considered a true multicellular developmental process (Monds and O'Toole, 2009), a model that has been generally favored in the past decade and is now questioned by some authors. The sequential requirement of LapA and LapF at different stages of biofilm formation by *P. putida* would be in agreement with a developmental model, resembling the chronological checkpoints that exist in established examples of microbial development, such as fruiting body formation by *Myxococcus* or sporulation in *Bacillus* (Kroos, 2007). On the other hand, the fact that expression of *lapF* is under the control of the global transcriptional regulator σ^S, an alternative sigma factor involved in a variety of starvation- and stress-related processes, seems to speak against *lapF* being part of a dedicated biofilm regulatory program.

Yet, considering that LapF appears to mediate cell-cell interaction, its σ^s-dependent expression in stationary phase outside of a biofilm might be expected to result in the formation of bacterial aggregates in the liquid medium, a phenomenon that is not normally observed under standard culture conditions despite the presence of the protein on the bacterial surface. It is possible that post-translational modifications of LapF or its interaction with other extracellular components determine the ultimate function of the protein in different situations. Thus, it could be argued that for LapF to exert its intercellular linkage role, bacteria must first be associated to a surface and hence have gone through the previous stages of biofilm formation. Such history-dependent operation is compatible with a developmental process, although other possibilites cannot be excluded. An in-depth analysis of the temporal and/or environmental control of

LapF transport and functionality, and how these are integrated in the whole process of biofilm formation will contribute further to a mechanistic portrayal of the sessile lifestyle of *Pseudomonas putida*.

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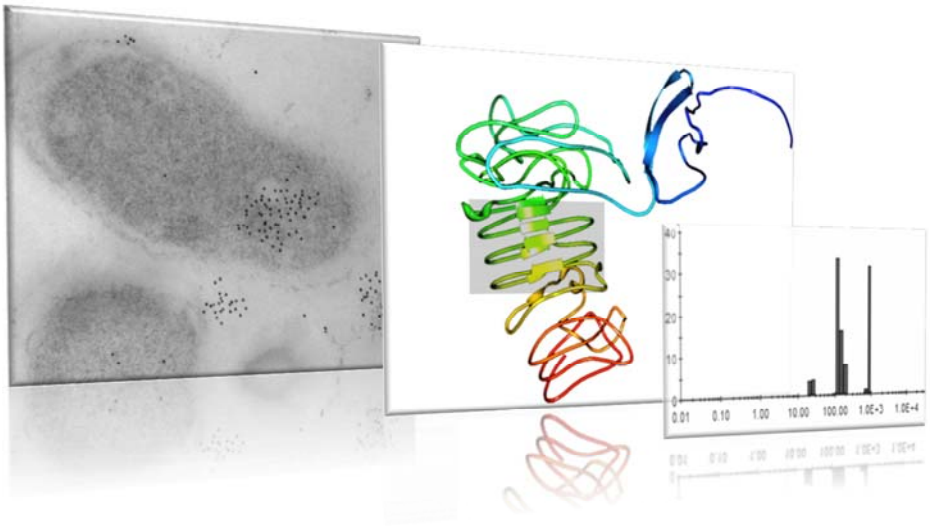
Chapter 2



Calcium causes multimerization of the large adhesin LapF and modulates biofilm formation by *Pseudomonas putida*

Marta Martínez-Gil, Diego Romero, Roberto Kolter, and Manuel Espinosa-Urgel. 2012. Calcium causes aggregation in the large adhesin LapF and modulates biofilm formation by *Pseudomonas putida*. . *Journal of Bacteriology* (In Press).

Summary

LapF is a large secreted protein involved in microcolony formation and biofilm maturation in *Pseudomonas putida*. Its C-terminal domain shows the characteristics of proteins secreted through a Type I secretion system and includes a predicted calcium binding motif. We provide experimental evidence of specific binding of Ca^{2+} to the purified C-terminal domain of LapF (CLapF). Calcium promotes the formation of large aggregates, which disappear in the presence of the calcium chelator EGTA. Immunolocalization of LapF also shows the tendency of this protein to accumulate in vivo in certain extracellular regions. These findings, along with results showing that calcium influences biofilm formation, lead us to propose a model in which *P. putida* cells interact with each other via LapF in a calcium-dependent manner during the development of biofilms.

Introduction

Bacteria are able to form surface-attached multicellular communities, called biofilms, which represent a protected mode of growth that allows cells to survive in hostile environments and colonize new niches (Hall-Stoodley *et al.*, 2004). Because biofilms can form on virtually any surface, they have a profound impact in medicine, industry and agriculture. Intensive research over the last decade has led to the identification of key factors for biofilm formation. Among these, the presence of an extracellular matrix produced by the bacterial cells contributes to the architecture, organization and maintenance of the three-dimensional structure of biofilms. The composition of the matrix depends on the bacterial species and the environmental conditions but it is generally made up by different exopolysaccharides (EPS) (Chang *et al.*, 2007, Nielsen *et al.*, 2011, Nilsson *et al.*, 2011), extracellular DNA (Whitchurch *et al.*, 2002) and secreted proteins (Espinosa-Urgel *et al.*, 2000, Latasa *et al.*, 2005, Nilsson *et al.*, 2011). The involvement of cell surface proteins in biofilm development has been described in detail for a variety of bacterial species. They include BapA in *Salmonella enterica* (Latasa *et al.*, 2005), Bap in *Staphylococcus aureus* (Lasa & Penades, 2006), TasA in *Bacillus subtilis* (Romero *et al.*, 2010) and LapA in *Pseudomonas putida* and *P. fluorescens* (Hinsa *et al.*, 2003). In a recent publication (Martínez-Gil *et al.*, 2010) we described that the adhesin LapF mediates cell-cell interactions which lead to microcolony formation and biofilm maturation in *Pseudomonas putida* KT2440. However, the molecular mechanisms by which LapF exerts its function remain unknown.

A significant number of large proteins involved in cell-surface and cell-cell interactions contain putative calcium binding domains (Arrizubieta *et al.*, 2004, Cruz *et al.*, 2012, Yousef-Coronado & Espinosa-Urgel, 2007, Josefsson *et al.*, 1998). Calcium has been related to a variety of biological processes in bacteria (Cruz *et al.*, 2012, Garrison-Schilling *et al.*, 2011, Johnson *et al.*, 2011), but its role in biofilm

development is somewhat controversial. Previous research has shown that increasing amounts of calcium disable Bap-mediated biofilm formation and intercellular adhesion of *Staphylococcus aureus* (Arrizubieta *et al.*, 2004). However, recent reports indicate that calcium promotes biofilm formation in other microorganisms such as *Xyllela fastidiosa* or *Vibrio vulnificus* (Cruz *et al.*, 2012, Garrison-Schilling *et al.*, 2011). Therefore, calcium may modulate biofilm formation in opposite ways in different bacteria, but the molecular basis for its role remains unclear. Its potentially direct connection with surface proteins has been explored in *Shewanella oneidensis*, where the presence of calcium within a certain concentration range promotes biofilm formation mediated by the Bap-related surface protein BfpA (Theunissen *et al.*, 2010). In contrast, recent data indicate a negative influence of calcium on the function of LapA, the main adhesin of *Pseudomonas fluorescens* (Boyd *et al.*, 2012). This effect would be indirect, through the activity of the protease LapG, which is modulated by calcium.

In this report we present evidence for the direct interaction of Ca^{+2} with the C-terminal domain of LapF. This interaction results in the aggregation of the protein. Interestingly, calcium depletion has a negative effect on biofilm formation by *P. putida*. This leads us to suggest that this cation promotes the interaction between the C-terminal domains of LapF molecules from adjacent cells and thus promotes the cell-cell interactions needed for microcolony formation and the development of the three-dimensional structure of the biofilm.

Material and Methods

Bacterial strains and growth conditions

Pseudomonas putida KT2440 is a plasmid-free derivative of the original soil isolate *Pseudomas putida* mt-2 (Regenhardt *et al.*, 2002). *Escherichia coli* DH5α and BL21 were used as hosts in cloning and expression experiments, respectively. Unless

otherwise specified, bacteria were grown in LB medium at 30°C (*P. putida*) or 37°C (*E. coli*). When appropriate, ampicillin (100 μg/ml) was added.

Construction of pMMGCa2 Expression Plasmid

A 2 kb DNA fragment corresponding to the C-terminal domain of LapF (amino acids 5622-6310) was PCR-amplified from the chromosome of *Pseudomonas putida* using KT2440 primers CaFw2 forward (5´-GGGCAGCCATATGGATGCCAACG CGCCGGTCA-3´) and CaRev reverse (5´-AAAA CTCGAGTCAAACCACATGGA TACC-3´) with Expand High Fidelity polymerase (New England). The resulting PCR product was digested with NdeI and XhoI (underlined sites) and cloned in frame into the plasmid pET-15b (Novagen) cut with the same enzymes. The resulting plasmid, pMMGCa2, was transformed into *E. coli* DH5α and the insert and flanking regions were verified by DNA sequencing.

Expression and Purification of the Recombinant C-terminal domain of LapF

The plasmid pMMGCa2 was used for the production of a fusion protein with 6 histidine residues at the N-terminus. The plasmid was transformed in *Escherichia coli* BL21 competent cells for protein expression. Cultures were grown in 250 ml LB with orbital shaking to an absorbance of 0.5 at 600 nm. The cells were induced with Isopropyl-1-thio-β-galactopyranoside (IPTG) to a final concentration of 1 mM and the cultures were incubated for additional 4 hours at 37ºC. Cells were harvested by centrifugation, resuspended in 20 ml of lysis buffer, 1 x CelLytic B cell lysis reagent (Sigma) diluted in 20mM Tris, 500mM NaCl, 20mM Imidazol and 1mM PMSF, and supplemented with 100 μg/ml of freshly prepared lysozyme solution, and incubated for 20-30 minutes. Further disruption and reduction of viscosity was done by sonication. 1.5 ml of nickel chelating resin (G-Bioscience) were washed and conditioned as indicated by the manufacturer prior to adding to

the samples and incubating with gentle agitation for 1 h at room temperature. The mixture was centrifuged and after decanting the supernatant, the lysate/resin mixture was washed with 5 volumes of binding buffer (20mM Tris, 500mM NaCl, 1mM Imidazol, 1mM PMSF), 3 volumes of washing buffer-I (20mM Tris, 500mM NaCl, 30mM Imidazol, 1mM PMSF). The proteins were eluted with elution buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazol and 1 mM PMSF). The purified protein was treated with 10mM EGTA and cleaned with Sephadex G-25 gel-filtration chromatography, followed by overnight dialysis against 10 mM Tris/HCl, 50 mM NaCl, pH 7.5. Protein concentration was determined as previously described (Whitaker & Granum, 1980). The integrity and homogeneity of the recombinant protein was confirmed by SDS/PAGE (4% stacking gel, 12% separating gel) and Western Blot analysis using anti-His tag antibodies (Santa Cruz Biotechnology, Inc.). The identity of the protein was confirmed by ESI-MS/MS analysis.

Molecular weight estimation by Analytical FPLC

The molecular weight of the protein before and after treatment with 10mM EGTA was estimated using a Superdex-200 HR 10/30 column with an AKTA explorer FPLC system (Amersham Biosciences). The column was equilibrated with 20 mM Tris, 50 mM NaCl buffer, pH 7.5. The molecular weight of the eluted protein was estimated by a calibration curve prepared using the elution times measured for standards (Bio-Rad, Hercules, CA) ranging from 1.35 to 670 kDa (thyroglobulin (670 kDa), α-globulin (158 kDa), albumin (67 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa)).

Dynamic Light Scattering

Experiments were performed at 25ºC using the DynaPro NanoStar (Wyatt). Protein solutions were dialysed overnight against a filtered buffer (10mM Tris/HCl, 50 mM NaCl, pH 7.5) and filtered before each experiment using a

sterile syringe filter w/0.45 μm cellulose acetate membrane (VWR). Protein solution was adjusted to a final concentration of 5 mg/ml of protein and injected in a 50μl cuvette and placed into the sample cell. The total sample injection volume was 60μl. To test the effect of calcium in the protein solution, a filtered $CaCl_2$ solution in 10mM Tris/HCl, 50 mM NaCl, pH 7.5, was injected to the sample to a final concentration of 10mM, using a Hamilton syringe (Hamilton Co). A filtered EGTA solution was subsequently added to the sample to a final concentration of 20mM. Data were collected from three independently prepared samples. Each sample measurement consisted in 4 repetitions, each one of 20 acquisitions of 15 seconds. Raw data were analysed by the DYNAMICS software package. The dispersity of the solution was assessed and the average of the hydrodynamic radius (R_H) was calculated.

Isothermal Titration Calorimetry

Reaction enthalpy measurements were done on a VP-ITC MicroCalorimeter (MicroCal) at 30ºC. The Ca^{2+} in the purified protein was removed by 10mM EGTA as describe above and subsequently dialyzed overnight against 10mM Tris/HCl, 50mM NaCl, pH 7.5 and adjusted to a final concentration of 8.5 μM and placed into the sample cell. A 100 μM $CaCl_2$ solution was prepared using the dialysis buffer and placed into the syringe injector. A typical experiment involved the automatic injection of 10 μl ligand into the protein solution over 25 injections with stirring at 300rpm. Heat produced due to dilution was measured by injecting ligand solution into the sample cell without protein. For each titration the heat of dilution was subtracted from the corresponding Ca^{2+} binding data of the protein. Data were fit to appropriate binding models and thermodynamic parameters determined from nonlinear least-squares fits, using the MicroCal version of the ORIGIN™ software.

Transmission Electron Microscopy and Immunogold labelling

Protein samples were treated with 10 mM, incubated 2 hours or 3 days at room temperature. After treatment with calcium, protein samples were diluted with distilled water and adsorbed onto a formvar/carbon coated grid (the grid surface was previously made hydrophilic with glow discharge in a vacuum evaporator) for approximately 10 min at room temperature. Excess of sample was discarded on a filter paper and the grid was incubated with 5 µl of negative staining solution (2% aqueous uranyl acetate) for 2 min at room temperature and the excess was discarded with filter paper. Samples were dried at room temperatures for 5-10 min and were visualized in a Tecnai G^2 Spirit BioTWIN microscope at an accelerating voltage of 80 KV. Images were taken with an AMT 2k CCD camera.

For immunolocalization studies of LapF, biofilms of *P. putida* KT2440 were grown on nickel grids for 8 h in LB medium in a 6-well microtiter plate. Grids were recovered from the culture and treated with blocking buffer consisting of 1% nonfat dry milk in PBS with 0.1% Tween 20 for 30 min, incubated for 2 h with anti-LapF primary antibody (Martínez-Gil *et al.*, 2010) diluted 1:150 in blocking buffer, rinsed in PBST, exposed to goat-anti-rabbit 20-nm gold secondary antibody diluted 1:50 (TedPella, Inc.) for 1 h and rinsed. All grids were stained with uranyl acetate and lead citrate and viewed as described above.

For subcellular localization studies of LapF, cells were grown for 8 h in LB medium in a 6-well microtiter plate. Cells were harvested and fixed with an equal volume of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Small pieces of cell pellet were infiltrated in 2.3 M sucrose in PBS with 0.15 M glycine. Samples were high pressure frozen in a Leica EM Pact2 high-pressure freezer and freeze-substituted in 0.2% glutaraldehyde and 0.1% uranyl acetate in Acetone at -90ºC for 72 h. Samples were warmed up at room temperature and embedded in LR White resin. Ultrathin sections were cut at -120°C with a cryo-diamond knife. Sections were transferred to a formvar/carbon coated grid for immunogold labelling. Grids were contrasted and visualized as described above.

Biofilm formation analysis

Biofilm formation was examined during growth in liquid LB in polystyrene microtiter plates (Sterilin) under static conditions as described previously (Yousef-Coronado *et al.*, 2008). Biomass attached to the surface was evaluated by staining with 0,5% crystal violet for 15 min (O'Toole & Kolter, 1998). Biofilms were quantified by solubilisation of crystal violet with 70% ethanol and measurement of absorbance at 580 nm on a spectrophotometer (Shimadzu UV-160A). To visually follow biofilm formation, 40 x 20 mm glass coverslips were placed obliquely into wells of a 6-well plate, with 5 ml of liquid LB diluted 1:10 and inoculated with the bacterial strain and the treatment to be tested ($CaCl_2$ or EGTA). Cultures were allowed to grow at 30°C for 24 h and biofilm formation evaluated visually or by fluorescence microscopy using a Zeiss Axioscope microscope after briefly washing the glass coverslips with deionized water.

Results

Calcium modulates biofilm formation by *Pseudomonas putida*

In order to analyze the role of calcium in biofilm formation by *P. putida* KT2440, this strain was first grown under static conditions in microtiter plates in LB with increasing concentrations of $CaCl_2$. As shown in Figure 1A, calcium caused a shift in the attachment/detachment kinetics of KT2440, with increasing concentrations promoting earlier attachment and early detachment of the cells. Addition of the calcium chelator EGTA, on the other hand, did not have a significant effect at early timepoints, but limited biofilm development later on (Figure 1B). EGTA also limited biofilm formation in minimal medium with glucose as carbon source (not shown) and in 1:10 strenght LB. This effect of the chelator was compensated by

addition of excess $CaCl_2$ (Figure 1B), even though cells began detaching earlier than in the control.

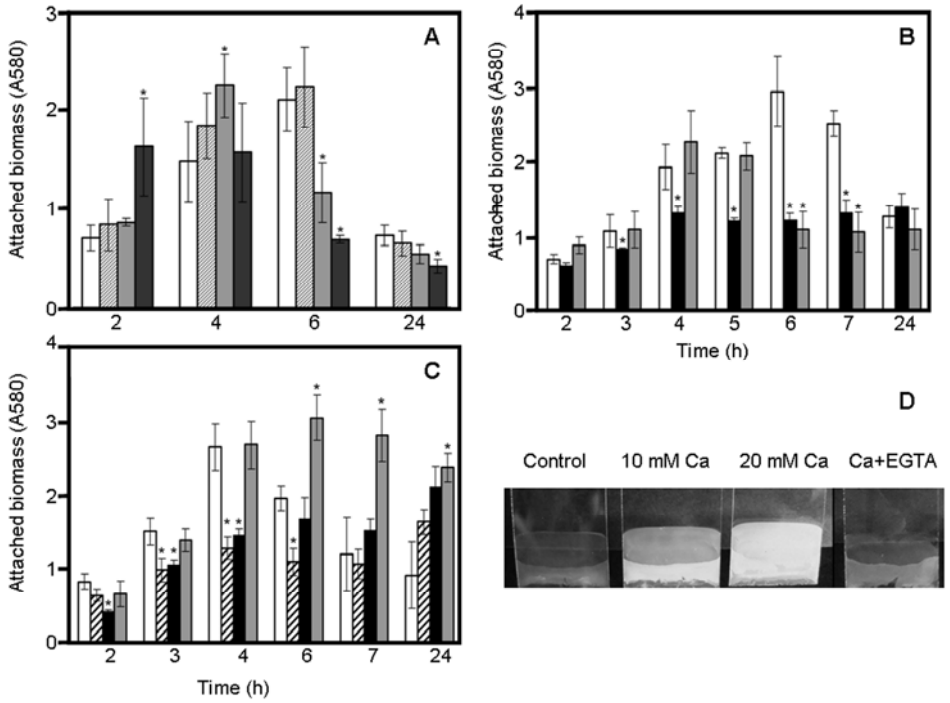


Figure 1. Influence of calcium, magnesium and the chelator EGTA on biofilm formation by *P. putida* KT2440. Attached biomass was determined by cristal violet staining, as described in Materials and Methods. Data are averages and standard deviations form four experiments. Asterisks indicate statistically significant differences with respect to the control. **(A)** Biofilm formation on microtiter plates during growth in LB with increasing concentrations of $CaCl_2$ (White bars: control without addition; hatched bars, 10 mM; light grey bars, 20 mM; dark grey bars, 50 mM). **(B)** Effect of EGTA on biofilm formation, determined as above (white bars, control; black bars LB with 0.1 mM EGTA; grey bars 0.1 mM EGTA and 10 mM $CaCl_2$). **(C)** Biofilm formation in LB (white bars) and in the presence of 0.1 mM EGTA (hatched bars), 10 mM $MgCl_2$ (black bars) or Mg+EGTA (grey bars). **(D)** Biofilm formation on glass coverslips during growth in 1:10 strength LB. Coverslips were directly photographed 24 h post-inoculation.

To determine if the effect of EGTA could also be explained by its potential chelation of other ions, similar experiments were performed in LB in the presence of 10 mM $MgCl_2$ with or without EGTA. Results are shown in Figure 1C. Addition of magnesium relieved the negative effect of EGTA on biofilm formation. However, when $MgCl_2$ was added alone, no increase in attachment was observed until 24 h after inoculation. This suggests that the effect of EGTA is indeed a consequence of calcium chelation, so that when Mg is added in excess it sequesters EGTA, and more free calcium is available. Surprisingly, the combination of $MgCl_2$ and EGTA caused cells to remain attached, an effect that was not observed with magnesium alone.

The role of calcium was also tested in 6-wells plates with glass coverslips partially immersed in the growth medium (1:10 LB with or without $CaCl_2$). Biomass attached to the coverslip during growth was evaluated directly. As shown in Figure 1D, addition of $CaCl_2$ caused an increase in biofilm biomass at 24 h. Conversely, addition of the calcium chelator EGTA at a concentration that did not affect planktonic growth caused a reduction in the amount of biomass attached to the coverslips. In these experiments, no significant influence of either treatment was observed at earlier timepoints. These data suggested that calcium has a time-dependent influence on attachment of *P. putida*. In this bacterium, two large adhesins showing putative calcium-binding motifs, LapA and LapF, participate in biofilm formation. LapA is key for initial attachment and LapF participates in later stages in a medium-dependent manner (Martínez-Gil *et al.*, 2010).

We decided to focus our attention on LapF, to define the potential role of calcium on its functionality. Initially, the biofilm phenotype previously observed for mus-20, a *lapF* mutant (Martínez-Gil *et al.*, 2010), was compared to the effect caused on KT2440 by addition of EGTA. Biofilms of *gfp*-tagged strains grown on glass coverslips were examined by fluorescence microscopy. As shown in Figure 2, KT2440 biofilms grown in the presence of EGTA showed an intermediate structure between the large, thick microcolonies formed by the wild type under

regular conditions and the flat, less structured biofilm of the *lapF* mutant. EGTA caused a clear reduction in the size of microcolonies, with many cells attached individually, as in the *lapF* mutant. EGTA also appears to weaken the adhesive force of KT2440, since cells were easily swept away upon washing. It is worth mentioning that addition of $CaCl_2$ did not increase attachment in the *lapF* mutant (data not shown).

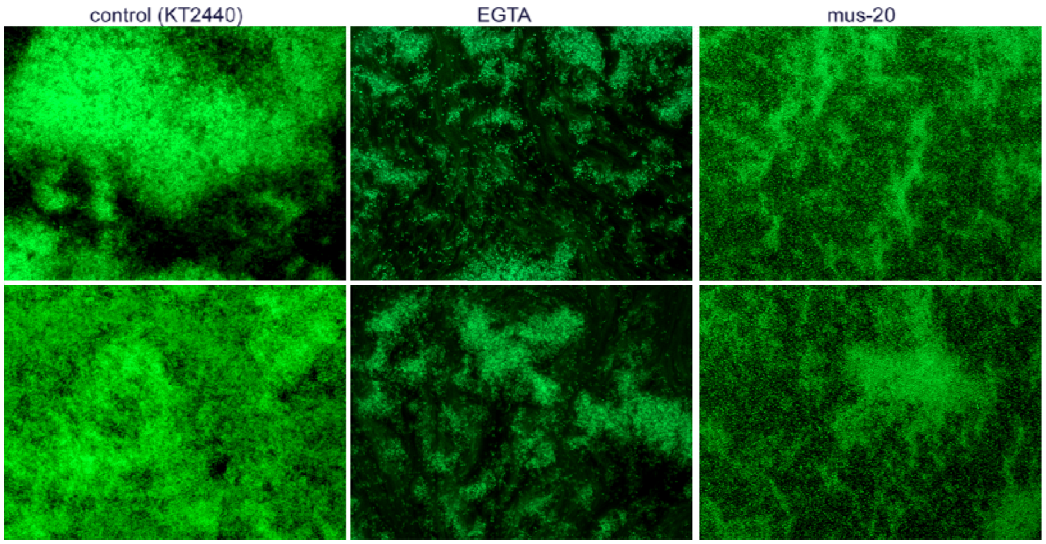


Figure 2. Biofilm formation on glass coverslips by KT2440 in the absence (control) or presence of EGTA, compared to the *lapF* mutant strain mus-20. Biofilms were grown in 1:10 strength LB, as described in Materials and Methods and observed after 24 h by fluorescence microscopy. Two different fields are shown in each case. Magnification: 400×.

The C-terminal domain of LapF (CLapF) selectively binds calcium

LapF consists of three domains: an N-terminal domain of 174 amino acids, followed by a long, repetitive region spanning most of the protein, and finally a C-terminal domain typical of proteins secreted through a type I secretion system

(Martínez-Gil *et al.*, 2010). Analysis of the sequence of LapF revealed the presence in the C-terminal region of the protein (CLapF) of putative calcium binding sites related to the hemolysin-type and those of the NodO protein (Economou *et al.*, 1990). The presence of these sites was further supported by *in silico* modelling of the three-dimensional structure of a region spanning the 300 C-terminal amino acids of LapF (Figure 3). The analysis with HHPred (Söding *et al.*, 2005) returned as best templates calcium-binding proteins and resulted in a predicted structure containing anti-parallel β-sheets that closely resemble those characteristically found in RTX toxins and known to bind calcium (Linhartová *et al.*, 2010).

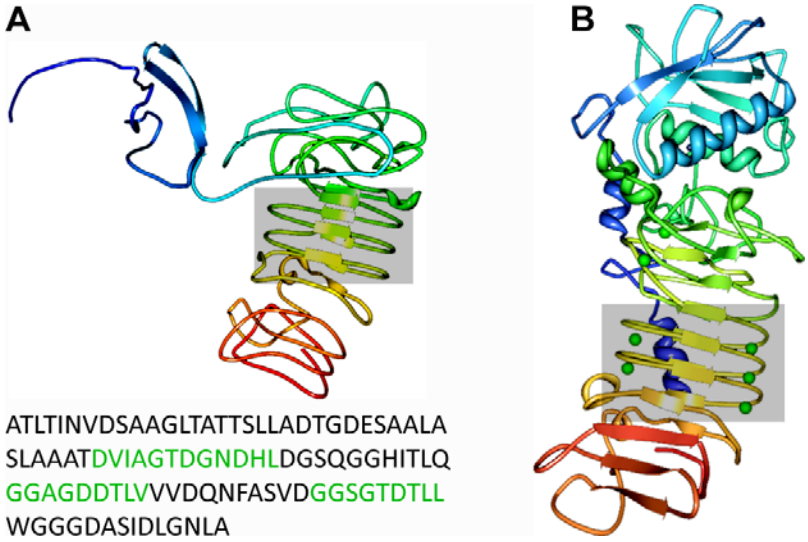


Figure 3. Bioinformatic analysis of the structure of the 300 C-terminal amino acids of LapF (left) compared to the *Serratia marcescens* metalloprotease (1SAT in the RCSB PDB database, which shows bound calcium as green balls (right). The calcium binding motifs are shown in green in the sequence and the corresponding region is boxed in the structural prediction. Model prediction was obtained using HHPred (Söding *et al.*, 2005) and the Protein Workshop toolkit (Moreland *et al.*, 2005) was used for visualization.

To begin defining the role of calcium in the functionality of the protein, a DNA fragment encoding CLapF was cloned in the expression vector pET15b. This construct was used to express and purify CLapF as a 690 amino acids fusion protein with a 6-His tag (Figure 4).

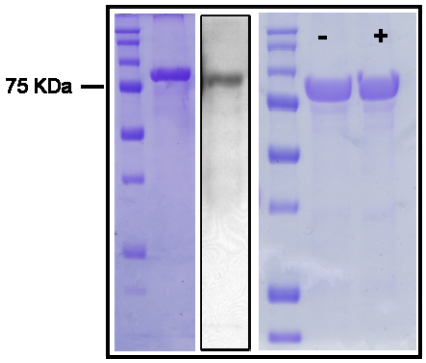


Figure 4. From left to right: Purification, Western-Blot and treatment with 10 mM EGTA of the C-terminal domain of LapF (CLapF). Fragment of 690 amino acids with an estimated molecular weight of 70kDa. Control without EGTA (-) and (+) treatment with 10 mM EGTA.

The purified polypeptide was treated with EGTA to remove any potential traces of calcium, and Isothermal Titration Calorimetry (ITC) assays were performed in order to test the interaction of CLapF with this cation. As shown in Figure 5 and Table 1, CLapF binds Ca^{2+} with moderate affinity (K_D=3.54±0.016 μM), calculated by fitting the experimental data to a single event model (N=1) for Ca^{2+}. No binding could be observed with other divalent cations such as Mg^{2+} or Mn^{2+} (data not shown), suggesting that the interaction of CLapF with Ca^{2+} is specific for this cation.

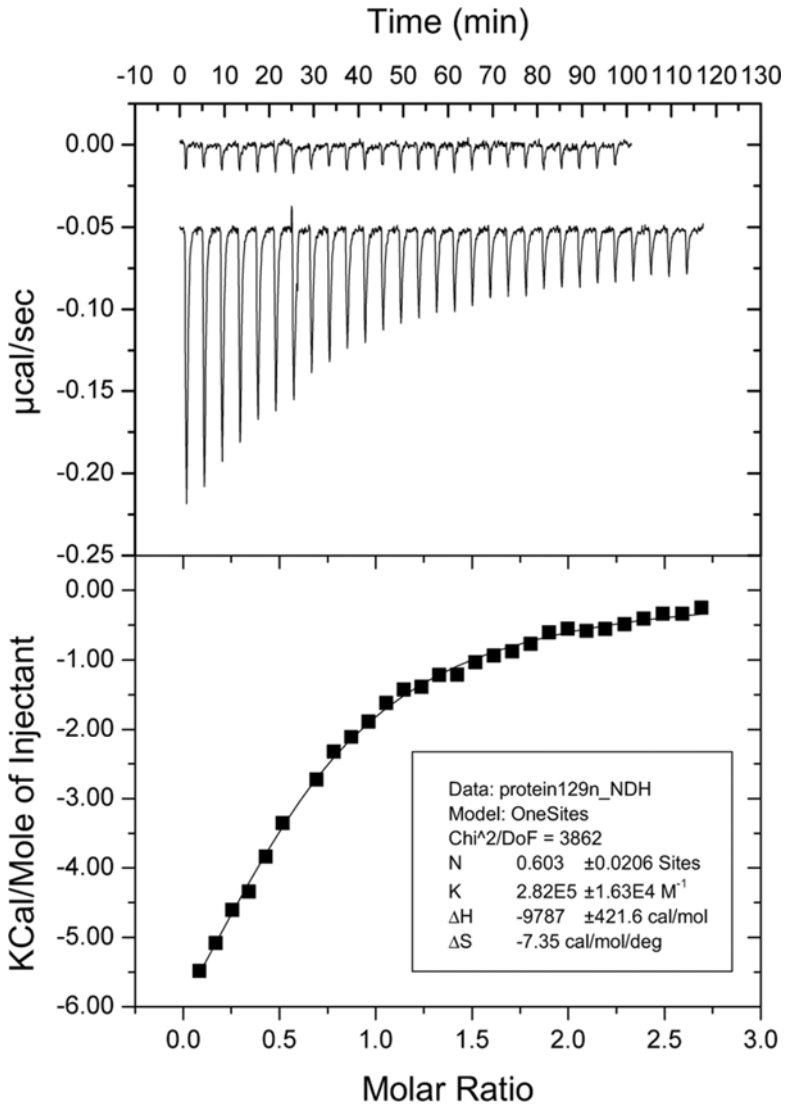


Figure 5. Isothermal titration of the C-terminal domain of LapF in the absence and presence of Ca^{2+}. Injection volume of 10 µl and a total number of 25 injections. **(A)** Injection of the ligand $CaCl_2$ into buffer. **(B)** Injection of the ligand into a 8,5 µM protein solution. Ligand and protein were in 0.1M Tris, 0.5M NaCl, pH 7.5. Experiments were carried out at 30ºC. Upper panel: Raw titration data. Lower panel: Integrated and dilution corrected peak areas of raw data. Data were fitted using the "One binding site model" of the MicroCal version of ORIGIN.

Table 1. Binding parameters derived from isothermal titration calorimetry experiments. All experiments were conducted at 30°C. Ligands were placed into the syringe and CLapF into the sample cell. Buffer: 10 mM Tris-HCl, 50 mM NaCl2 adjusted to a pH 7.5 by the addition of concentrated HCl.

K_A (M^{-1})	2.82 (±0.16)×10^5
K_D (μM)	3.54±0.016
ΔH (Kcal/mol)	-9.8±0.4
ΔS (cal/mol/deg)	-7.35
N (sites)	0.603±0.0206

Multimerization of CLapF in the presence of calcium

After purification and treatment of CLapF with 10mM EGTA, the protein was incubated for 3 days at room temperature with 10 mM $CaCl_2$ and a control without calcium. Samples were analysed by transmission electron microscopy (TEM). In samples treated with calcium, aggregates were clearly visible (Figure 6). Controls without calcium, or calcium in the absence of protein were devoid of any aggregates. During these in vitro experiments with purified CLapF, we noticed that prolonged incubation of CLapF in the presence of high calcium concentrations resulted in faintly visible precipitates in the samples, suggesting that calcium might cause aggregation of the protein. Addition of EGTA in the presence of calcium resulted in lack of aggregation (Figure 6), whereas increasing concentrations of calcium caused faster and more compact clustering of the protein, so that with 100 mM $CaCl_2$ large aggregates were visible after 3 h of incubation (data not shown).

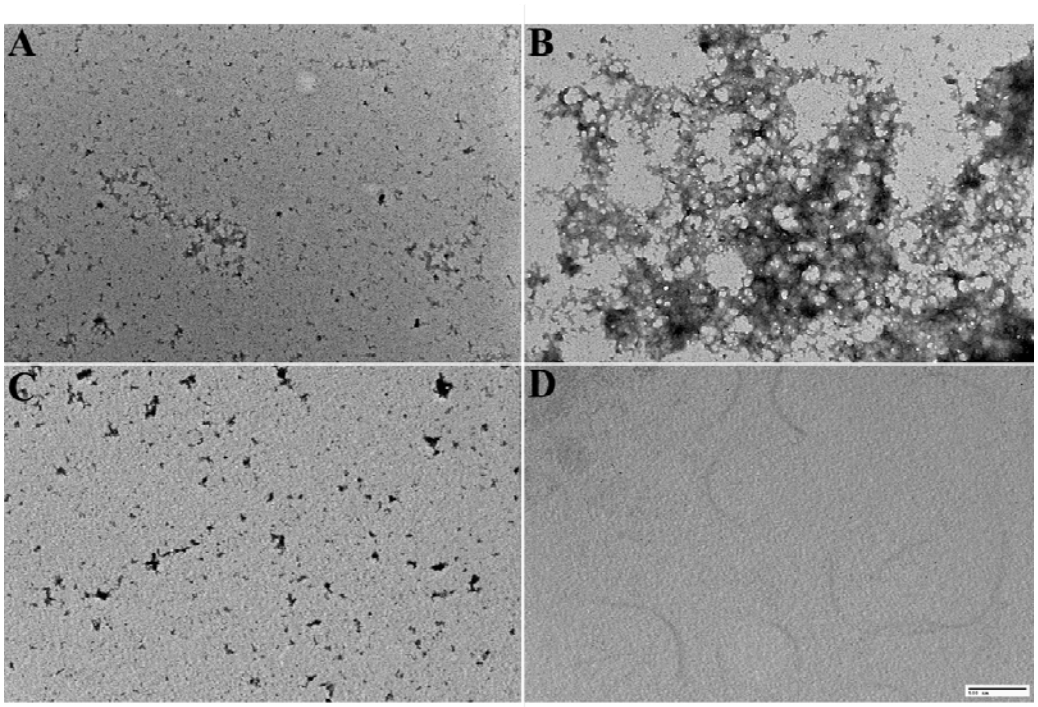


Figure 6. Transmission Electron Microscopy of CLapF incubated 3 days at room temperature without $CaCl_2$ **(A)**, with 10 mM $CaCl_2$ **(B)**, or with $CaCl_2$ and EGTA **(C)**. Panel D corresponds to a control with 10 mM $CaCl_2$ without protein, to ensure that the aggregates in panel B are not artifacts due to calcium precipitation. Scale bar: 500 nm.

Dynamic Light Scattering (DLS) was then used to analyse the size distribution of the hydrodynamic radius and the polydispersity of CLapF in the presence or absence of Ca^{2+} and EGTA (Figure 7). The polydispersity (Pd) value gives information about the homogeneity or heterogeneity of a given population (peak). The level of homogeneity is considered high when the population has a Pd less than 15%. Purified CLapF shows three different protein subpopulations in solution (Figure 7). The hydrodynamic radius (R_H) of each subpopulation is described in Table 2. After addition of 10 mM of $CaCl_2$ to the same sample, a new subpopulation of large aggregates is generated with a R_H of 5262 nm, nearly six times more than the biggest aggregate in the absence of calcium.

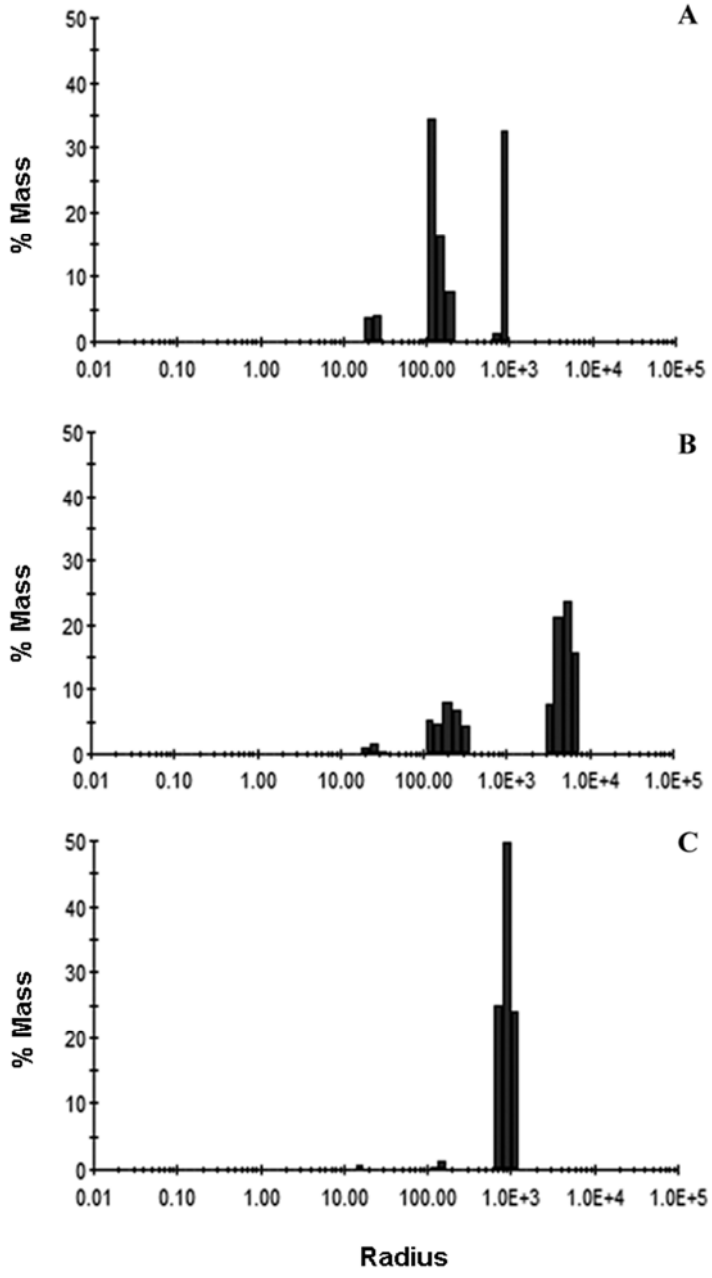


Figure 7. Size distribution histogram for **(A)** purified CLapF, treated with 10 mM EGTA and dialysed o/n against 10mM TrisHCl, 50mM NaCl, pH 7.5; **(B)** Protein from (A) with 10mM CaCl2; **(C)** Protein from (B) treated with EGTA to a final concentration of 20mM.

The new subpopulation of aggregates corresponds to more than 50% of the total mass of the protein sample (Table 2). This effect was completely reversed when EGTA was added to a final concentration of 20 mM, the subpopulation of higher R_H disappearing after 10 min of incubation with the chelator. Data from buffer and buffer with $CaCl_2$ and EGTA were acquired as control (data not shown).

Table 2. Dynamic light scattering results for CLapF. Three different parameters are given for each peak: hydrodynamic radius (nm), polydispersity (%Pd) and Mass (%).

	control			**$CaCl_2$**			**$CaCl_2$ +EGTA**		
Peak	Radius (nm)	%Pd	%Mass	Radius (nm)	%Pd	%Mass	Radius (nm)	%Pd	%Mass
1	24.313	11.2	7.8	25.233	17	3	16.183	8.8	0.6
2	148.346	18.7	58.4	136.969	12.8	9.9	153.293	5.7	1.3
3	917.719	4.7	33.8	251.616	20.5	19	913.999	18.5	98.1
4	-	-	-	5262	23.7	68	-	-	-

Based on the DLS results, analytical gel filtration was used to determine the molecular weight of CLapF and the different aggregates (Figure 8). Purified CLapF, after treatment with EGTA appears in only two different forms that eluted in the void volume of an analytical gel-filtration column with an exclusion size of 600 kilodaltons (Figure 8). The smallest peak detected (~77 kDa) corresponds to the monomer of the protein. A peak of approximately 630 kDa is also found in the purified protein solution. This peak may correspond to aggregates of eight CLapF monomers. After incubation with 10mM $CaCl_2$ the 77 kDA peak practically disappears and two new peaks corresponding to sizes of around 180 and larger than 900 kDa appear (Figure 8). These data further support multimerization of the protein in the presence of calcium.

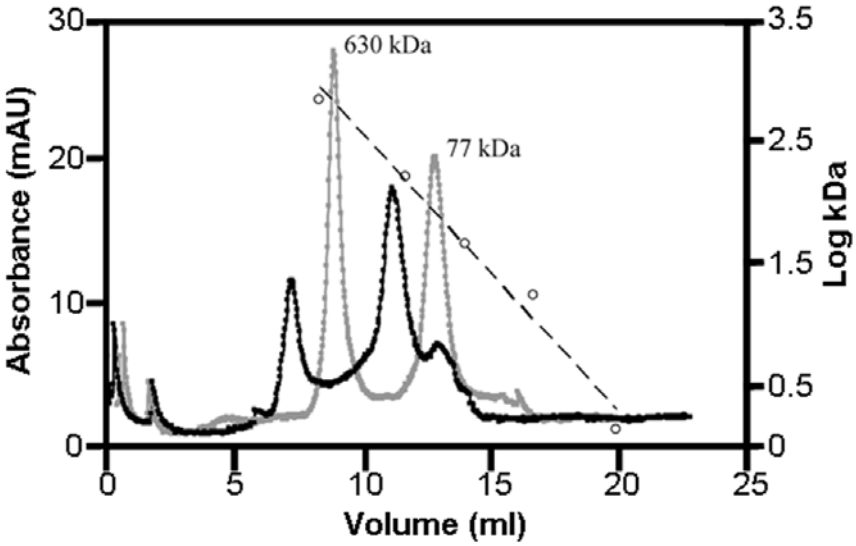


Figure 8. Study of the molecular weight of CLapF by Analytical FPLC. Purified CLapF after treatment with EGTA (grey line) and CLapF incubated with 10 mM $CaCl_2$ (black line) are shown, as well as the molecular weights (open circles) and linear trend (broken line) of the standards. The ~77 kDa and ~630 kDa peaks are indicated.

LapF clusters on the cell surface and between cells in biofilms

LapF had been previously localized in the cell surface of *P. putida* KT2440 and between cells in the biofilm using immunofluorescence and western blot analysis (Martínez-Gil *et al.*, 2010). To get a more precise localization of LapF in cells within a biofilm, *P. putida* KT2440 was grown in 6-well microtiter plates in LB, with a nickel grid in the middle of each well (Figure 9). Cells were incubated at 30°C and after 8 hours of incubation, grids were recovered and treated as described in Material and Methods, for electron microscopy observation with anti-LapF antibodies. As shown in Figure 9, clustering of LapF on the cell surface can be observed. In ultrathin sections of the cells recovered from the well, LapF is detected in the cytoplasm but is mainly localized in large aggregates between cells,

supporting its role in cell-cell interactions and as part of the extracellular matrix of the biofilm.

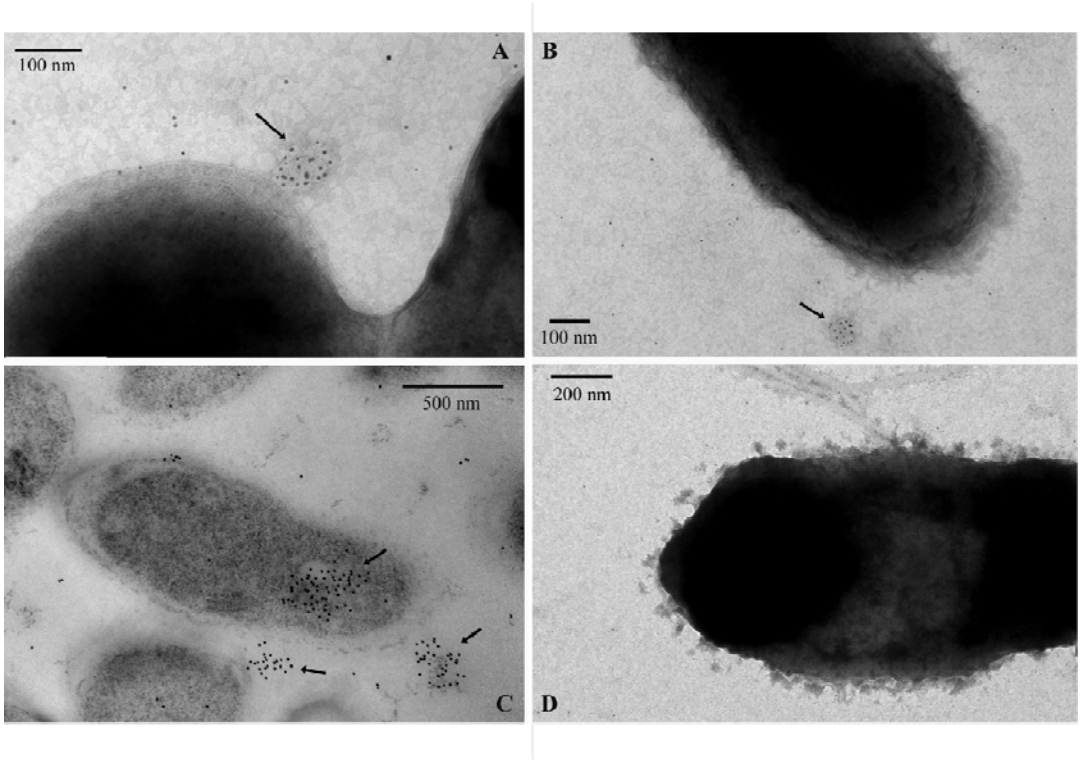


Figure 9. Electron transmission microscopy and immunodetection of LapF. **(A, B)** Wild-type cells after 8 h growth and immunogold labelling with anti-LapF antibody; **(C)** Thin sections of resin-embedded wild-type cells after 8 h growth in 6 well-microtiter plates. **(D)** Mutant strain mus-20 (*lapF*). Arrows point to the accumulation of gold particles in the cell surface and in the cytoplasm.

Discussion

Calcium is essential for many biological processes and has been previously shown to influence the multicellular behavior of different microorganisms. In this work we have provided evidence of calcium causing accelerated biofilm formation by *P. putida*, followed by early detachment under static conditions. This effect might be

in part due to calcium interaction with LapF, which causes the C-terminal portion of this adhesin to alter its conformation and form multimers. Among the variety of methods available to analyze the state of a macromolecular sample, DLS is a very sensitive technique frequently used to explore size and aggregation properties of macromolecules in solution (Murphy, 1997). In our results, the presence of calcium causes an increase in the polydispersity of the subpopulations of CLapF (Table 2). The effect of calcium in the sample heterogeneity is more evident in the new population of aggregates, whereas the homogeneity of the sample increases when EGTA is added. A lower proportion of multimers are present in the population of purified CLapF in the absence of calcium, and clustering can also be observed when the native protein is analyzed in vivo in biofilms through immunolocalization.

These results suggest a natural tendency of LapF to aggregate, an effect that is enhanced by increasing calcium concentrations. Thus, it seems possible that LapF contributes to the scaffolding of the extracellular matrix through its multimerization. This structural role based on polymerization would be somewhat similar to that reported for the formation of amyloid fibers by certain extracellular proteins. It is the case of TasA, which has been shown to polymerize to form amyloids and thus confer stability to *Bacillus subtilis* biofilms (Romero *et al.*, 2010). Such interpretation would be in contrast with the proposed influence of calcium in the functionality of other large surface proteins. In *Staphylococcus aureus*, for example, Ca^{+2} causes inhibition of biofilm formation in strains where the large adhesin Bap is present (Arrizubieta *et al.*, 2004). This protein contains four EF-hand-like calcium binding motifs; when these are mutated, the inhibitory effect of Ca^{+2} is lost. It is worth noting that the Ca^{+2}-binding sites in the C-terminal region of LapF do not correspond to EF-hand motifs but rather resemble those described in NodO (Economou *et al.*, 1990), a Ca^{+2}-binding secreted protein which has been proposed to mediate the interaction between *Rhizobium leguminosarum* cells and plant roots in a calcium-dependent way.

The influence of calcium in *P. putida* also contrasts with that observed in *P. fluorescens*, where this cation seems to have a negative effect on attachment mediated by the large adhesin LapA (Boyd *et al.*, 2012). It is worth noting that whereas LapA homologs are present in *P. putida* and *P. fluorescens*, LapF is exclusively found in the former. It could be that the role of calcium is different in the two species or that in *P. putida* calcium is somehow part of a "clock" for the attachment process. Our current model of sequential requirement of LapA and LapF in biofilm formation and the fact that calcium concentration alters the timing for attachment/detachment would favor this idea.

Calcium is a relatively abundant element in soils, with average concentrations ranging from 7 to 24 mg/g of soil, according to different surveys (Shacklette *et al.*, 1984). It is generally present as a component of soil minerals or in its cationic form adsorbed to soil particles, from which it is available to plant roots to be incorporated by mass flow. It is therefore likely that the plant root-colonizing bacterium *P. putida* KT2440 encounters a gradient of this element in its natural niche and has partly adjusted its multicellular behavior to the concentrations found on the root surface.

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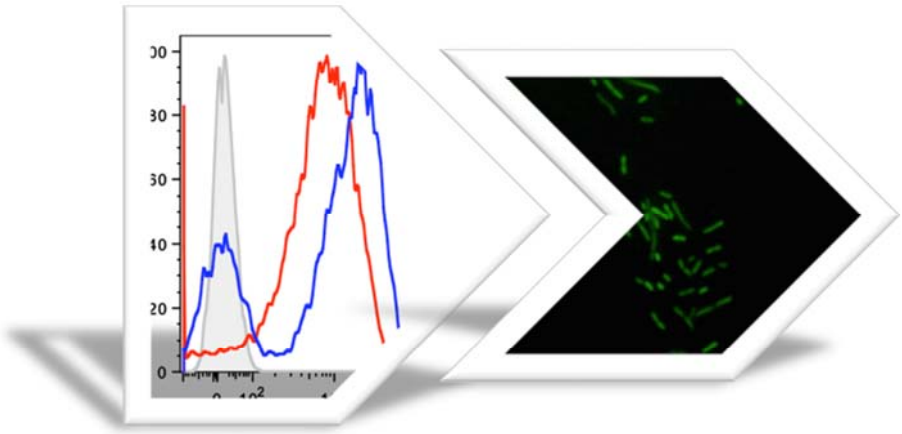
Chapter 3



Expression of the large *P. putida* adhesins LapA and LapF is part of a complex global regulatory network governing biofilm formation

Marta Martínez-Gil and Manuel Espinosa-Urgel. 2012. Expression of the large *P. putida* adhesins LapA and LapF is part of a complex global regulatory network governing biofilm formation. *Microbiology* (Submitted).

Summary

LapA and LapF are large extracellular proteins that play a relevant role in biofilm formation by *Pseudomonas putida*. Current evidence favours a sequential model in which LapA is first required for initial adhesion of individual bacteria to a surface, while LapF participates in later stages of biofilm development. In agreement with this model, *lapF* transcription was previously shown to respond to the stationary-phase sigma factor RpoS. We have now analyzed the transcription pattern of *lap*A, and what other regulatory elements influence expression of both genes. The *lapA* promoter shows a transient peak of activation early during growth, with a second increase in stationary phase that is independent of RpoS. The same pattern is observed in biofilms, but expression is not uniform in the population. Both *lapA* and *lapF* are under the control of the two-component regulatory system GacS/GacA, and their transcription also responds to the intracellular levels of the second messenger cyclic diguanylate (c-di-GMP), although in surprisingly reverse ways. Whereas expression from the *lapA* promoter increases with high levels of c-di-GMP, the opposite is true for *lapF*. The flagellar regulator FleQ is required for the modulation of *lapA* expression by c-di-GMP, but has a minor influence on *lapF*. This work represents a further step in our understanding of the regulatory interactions controlling biofilm formation.

Introduction

The Gram-negative bacterium *Pseudomonas putida*, is an efficient colonizer of plants rhizosphere with relevant agronomic interests: (i) it is considered a PGPR, (ii) it induces the systemic resistance in plants and (iii) it is widely used in rhizoremediation (Matilla *et al.*, 2011b, Molina *et al.*, 2000, Matilla *et al.*, 2011a, Kuiper *et al.*, 2002, Fernández *et al.*, 2012). *P. putida* is also able to develop multicellular communities on biotic and abiotic surfaces, which are commonly known as biofilms. Molecular determinants responsible for the development of *P. putida* biofilms are structural as surface proteins and exopolysaccharides, regulatory genes and genes involved in the synthesis, regulation and functionality of the flagella system (Hinsa *et al.*, 2003, Martínez-Gil *et al.*, 2010, Duque *et al.*, 2012, Yousef-Coronado *et al.*, 2008, Nilsson *et al.*, 2011, Nielsen *et al.*, 2011).

Research on diverse bacteria species have provided interesting insights into the molecular bases which control the establishment of biofilms, however more effort is necessary to elucidate the regulation networks that connect environmental signals and the subsequent differential gene expression that switch cells from planktonic lifestyle to biofilm development in *P. putida*. Two-component systems have been related to biofilm regulation in different ways and in diverse bacteria: ArsIRS in *Staphylococcus epidermidis* (Wu *et al.*, 2012); CpxRA in *Escherichia coli* (Ma & Wood, 2009, Otto & Silhavy, 2002); PprBA, PilRS, FleRS and RcsBC in *Pseudomonas aeruginosa* (Mikkelsen *et al.*, 2011) and GacSA in *P. chlororaphis* (Poritsanos *et al.*, 2006), *P. aeruginosa* (Davies *et al.*, 2007) and *P. putida* (Duque *et al.*, 2012). The GacS/GacA two-component system is known to regulate other many processes as the expression of virulence factors and the synthesis of secondary metabolites with antimicrobial activity (Heeb & Haas, 2001). In *P. fluorescens* F113 swimming motility is negatively control by GacS/GacA through the flagellar master regulator FleQ and high intracellular concentration of c-di-GMP represses

motility through WspR proteins (Navazo *et al.*, 2009, Martínez-Granero *et al.*, 2012). In addition, mutations in the Gac system causes a reduction in the biocontrol activity of *P. fluorescens* (Barahona *et al.*, 2011). In *Azotobacter vinelandii* this system regulates the synthesis of alginate, an important exopolysaccharide in the assembly of the extracellular matrix necessary for the formation of bacterial biofilms (Manzo *et al.*, 2011). A recent publication has reported the involvement of the two-component system in biofilm formation in *P. putida* as a mutant in *gacS* was defective in the formation of biofilms (Duque *et al.*, 2012).

In a variety of bacteria species, the control of the transition from planktonic life style to biofilm seems to be dependent on other important factor, the secondary messenger c-di-GMP (Gjermansen *et al.*, 2006, Hoffman *et al.*, 2005, Newell *et al.*, 2011). Indeed, changes in the intracellular concentration of c-di-GMP provoked by the overexpression of a diguanylate cyclase enzyme (DGC) led to variations in the global expression pattern in *E. coli* (Méndez-Ortiz *et al.*, 2006). In *P. putida* it has been also demonstrated that the overproduction of proteins with a GGDEF domain and DGC activity impaired the dispersion of the biofilm, resulting in a hyperbiofilm phenotype (Gjermansen *et al.*, 2006, Matilla *et al.*, 2011b). A recent study in *Pseudomonas fluorescens* has shown that intracellular levels of the secondary messenger c-di-GMP participate in the molecular mechanism that control the release of the surface protein LapA from the cell surfaces (Newell *et al.*, 2011).

In previous works we identified and characterized LapF as the second largest surface protein of *P. putida* involved in microcolony formation and biofilm maturation (Espinosa-Urgel *et al.*, 2000, Martínez-Gil *et al.*, 2010). From these results we proposed a sequential model for *P. putida* biofilm formation in which the surface protein LapA is mainly involved in the first stages of biofilm formation, facilitating the irreversible interaction cell-surface; later on, LapF mediates cell-cell interactions, providing support for microcolony formation and maturation of the biofilm (Hinsa *et al.*, 2003, Martínez-Gil *et al.*, 2010).

Transcriptional studies have shown that the *lapF* promoter is activated at the beginning of the stationary-phase of growth and completely dependent on the alternative sigma factor RpoS (Martínez-Gil *et al*., 2010). RpoS is a central regulator of stationary-phase gene expression (Stockwell & Loper, 2005) and it plays a relevant role in the control of the expression of genes involved in bacterial adhesion in *Escherichia coli* (Landini, 2009).

In this work, we analysed the expression pattern of these two adhesins at transcriptional level in the strain *P. putida* KT2440. The promoter of *lapA* was highly active at early stages and contrary to *lapF*, the expression was independent of RpoS. We also analysed the involvement of the two-component system GacS/GacA and the transcriptional analysis showed that the expression of both adhesins were down regulated in a *gacS* mutant. We also demonstrate that high intercellular levels of the secondary messenger c-di-GMP provoke a different effect in the expression of both adhesins: an increase of *lapA* and decrease of *lapF*. This work contributes to the current model of biofilm formation in *P. putida* KT2440 providing information of a possible regulatory network which governs biofilm formation in this strain.

Material and Methods

Bacterial strains, growth conditions

Pseudomonas putida KT2440 is a plasmid-free derivative of *P. putida* mt-2, originally isolated from a vegetable orchard in Japan (Nakazawa, 2002). Strains mus-20 (*lapF* mutant) and mus-42 (*lapA* mutant) were obtained by random transposon mutagenesis with mini-Tn*5*[Km1] and identified as defective in attachment to corn seeds (Espinosa-Urgel *et al*., 2000, Yousef-Coronado *et al*., 2008). C1R1 is a *rpoS* null derivative KT2440 and *gacS* mutant was obtained by random transposon mutagenesis with Tn*5* [Km1] and identified as deficient in biofilm formation to

abiotic surfaces. Expression vectors pMP220 (Spaink *et al.*, 1987) and pRU1097 (Karunakaran *et al.*, 2005) were used to construct pMMGA (*lapA::lacZ*) and pMMG6 (*lapA::mcherry*) and pMMG5 (*lapA::gfp*), respectively (see below). *P. putida* strains were routinely grown at 30ºC either in LB or in M9 minimal medium with $MgSO_4$, Fe-citrate and trace metals (Yousef-Coronado *et al.*, 2008), and glucose (20 mM) as carbon and energy source. When appropriate, antibiotics were used at the following concentrations: kanamycin (Km) 25 μg/ml; tetracycline (Tc) 15 μg/ml; gentamycin (Gm) 10 μg/ml.

Molecular biology techniques

To study the expression of *lapA*, transcriptional fusions of the promoter region of *lapA* to ´*lacZ* and ´*gfp* were made. To generate the plasmid pMMGA (*lapA::lacZ*), a PCR fragment of 404 bp from *lapA* promoter was obtained with primers LapAFwEcoRI (5´-AAAAAGAATTCTACGGCTGCTGAGGTGTATG-3´) and LapAReKpnI (5´- AAAAAGGTACCACAGGCGGTCACCTTCGATA-3´). The PCR product was digested with EcoRI and KpnI (underlined) and cloned into the plasmid pMP220 cut with the same enzymes. To obtain the plasmid pMMG5 (*lapA::gfp*), a PCR fragment of 403 bp from *lapA* promoter was obtained with primers LapAFwPstI (5´-TACGGCTGCAGAGGTGTATG-3´) and LapARevKpnI (5´-CAGGCGGGTACCTTCGATA-3´). The PCR product was digested with PstI and KpnI and cloned in pRU1097 cut with the same enzymes. Absence of mutations was confirmed by DNA sequencing.

Biofilm formation analysis

Biofilm formation was examined during growth in polystyrene microtiter plates (Sterilin) or in borosilicate glass tubes, as described previously (Yousef-Coronado *et al.*, 2008). Biomass attached to the surface was visually inspected by staining with crystal violet, and quantified after solubilizing the dye with 70% ethanol and measuring absorbance at 590 nm (O'Toole & Kolter, 1998).

β-galactosidase assay

β-galactosidase activity was measured as described before (Miller, 1972). Overnight cultures were inoculated (1:100 dilution) in fresh LB and grown for 1.5 h, diluting 1:1 every half hour before the collection of samples. These steps were done to ensure proper dilution of β-galactosidase that might have accumulated after overnight growth. Activity was measured in chloroform/SDS permeabilized cells. Experiments were repeated at least three times with two technical repeats per sample, and data were given in Miller units.

Flow Cytometry

Cells of *P. putida* KT2440 were grown in a six-well plate with a 40 mm glass coverslip placed obliquely into the well and grown in LB at 30ºC. For the analysis of planktonic cells, 2 ml of culture from the well were recovered and processed as described above. Cells attached to the coverslip were recovered and analysed as cells from biofilm, except at 2 h, when the number of cells from coverslips was insufficient. The coverslip was washed twice in sterile PBS buffer and then introduced in a 50 ml tube with 25 ml PBS buffer; cells were detached with 3 repetitive passes of 30 s of mild sonication. Cultures recovered from the wells and coverslips were centrifuge at 13,000 rpm for 15 min and the pellet was fixed in 4% paraformaldehyde for 7 min. After fixation, cells were washed with PBS, resuspended in GTE buffer (50 mM Glucose, 10 mM EDTA at pH 8, 20 mM Tris-HCl at pH 8), and stored at 4ºC. Prior to flow cytometry analysis, cells were subjected to mild sonication in conditions that disrupt cells from the extracellular matrix, but that not lyse cells at detectable levels (Branda *et al.*, 2006). This procedure gave a preparation of single cells. For flow cytometric analysis, cells were diluted in PBS and directly measured on a BD LSR II flow cytometer (BD Biosciences) operating a solid-state laser at 488 nm. For each sample, at least 30,000 events were analyzed. Data containing the fluorescent signals were

collected by a 505LP and a 530/30-bp filter, and the photomultiplier voltage was set between 300 and 500 V. Data were captured using FACS Diva software (BD Biosciences) and further analyzed using FlowJo 8.5.2 software (http://www.flowjo.com). Figures were prepared for publication using FlowJo 8.5.2 and Adobe Illustrator CS2 12.0.1.

Results

The *lapA* promoter is active at early and late stages of growth

In order to reinforce the sequential model of LapA and LapF activity proposed previously (Martínez-Gil *et al.*, 2010), the expression pattern of *lapA* was studied in *P. putida* KT2440 using plasmid pMMGA, which carries a *lapA*::*lacZ* transcriptional fusion. Expression from the *lapA* promoter was followed during growth in liquid LB medium. As shown in Figure 1, there was a peak of β-galactosidase activity at early timepoints, followed by a decrease until the culture entered the stationary phase, when activity increased again. The early transient peak of activity was consistently observed when sampling was done with sufficient frequency during the first hours, but easily missed when periods between samples were longer. We have previously reported that the *lapF* promoter is dependent on RpoS, being active in stationary phase and at late times during biofilm formation (Martínez-Gil *et al.*, 2010). The β-galactosidase activity observed after 24 h was around 3 times higher for the *lapF::lacZ* fusion than for the *lapA::lacZ* fusion (Figure 1), suggesting that the overall strength of the *lapF* promoter is higher than that of the *lapA* promoter.

We also considered whether RpoS could influence expression of the *lapA* promoter in stationary phase, as is the case for *lapF*. Plasmid pMMGA was introduced in strain C1R1, a *rpoS* mutant derivative of KT2440 (Ramos-González

& Molin, 1998). There were no differences in expression of the *lapA* promoter between the wild type and C1R1 was similar to that observed in the wild type strain (data not shown).

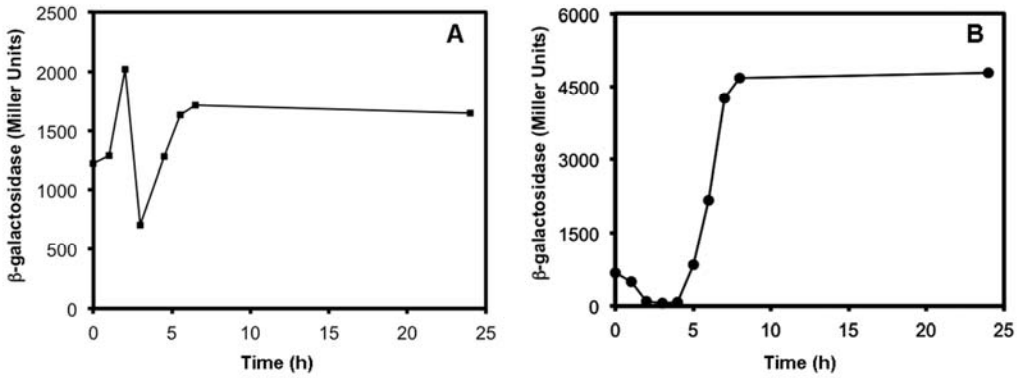


Figure 1. Expression of *lapA::lacZ* (pMMGA) and *lapF::lacZ* (pMMG1) during growth in liquid medium. **(A)** KT2440 harbouring pMMGA (closed squares) **(B)** KT2440 harbouring pMMG1 (close circles) Strains were grown in LB and β-galactosidase activity was followed during growth. Experiments were performed in triplicate with each sample measured twice. One representative experiment is shown.

Expression of *lapA* was also studied in biofilms grown under static conditions on glass coverslips. For that purpose, a *lapA::gfp* transcriptional fusion was constructed in plasmid pRU1097 (see Material and Methods), rendering pMMG5, which was introduced in *P. putida* KT2440. Expression during biofilm formation was followed by fluorescence microscopy (Figure 2). In agreement with the β-galactosidase activity results, expression of the *lapA::gfp* was evident at very early times (30 minutes after inoculation), with almost every cell attached to the coverslip showing green fluorescence. During the following stages of biofilm development, a decrease in fluorescence intensity was observed. However, after 24

h expression had increased again, although it was heterogeneous, so that there were groups of cells in the coverslip which were not showing fluorescence.

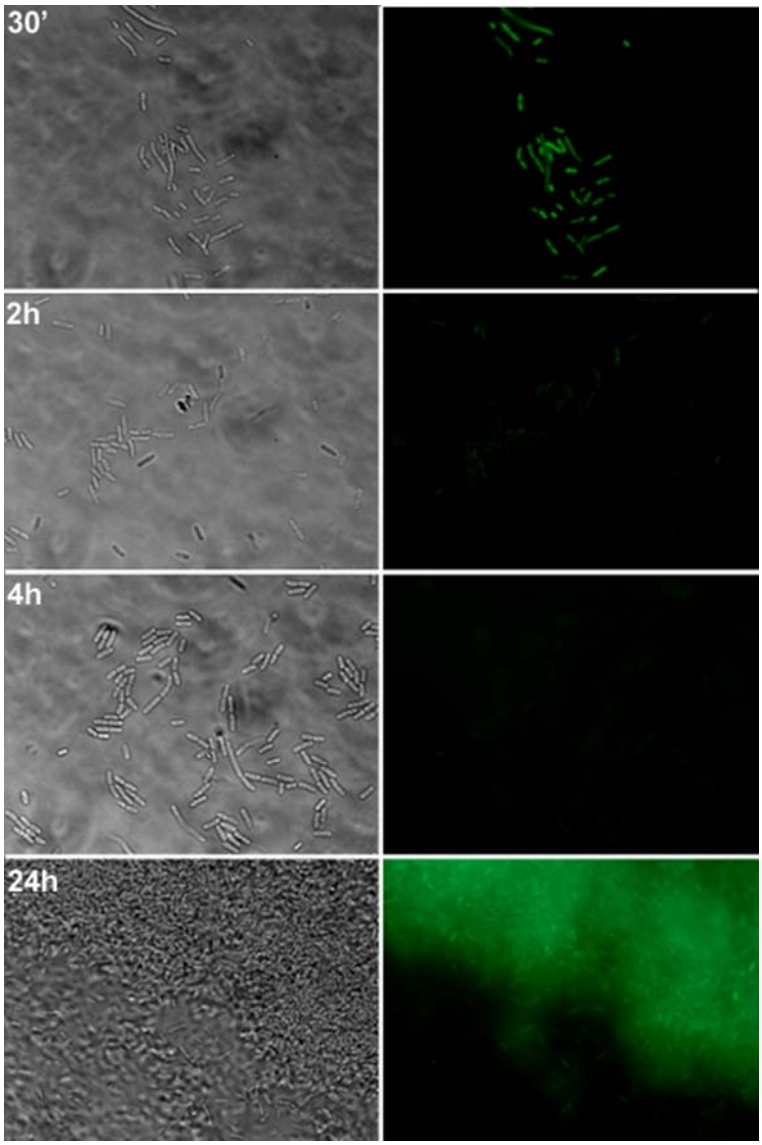


Figure 2. Expression of *lapA::gfp* in biofilms of KT2440 harbouring pMMG5. Cells were grown on glass coverslips under static conditions. Phase-contrast (left panels) and fluorescence (right panels) microscopy were used to examine the same field. Micrographs were taken after 30 min, 2 h, 4 h and 24 h. Magnification 30 min, 2 h and 4 h: 1000x. Magnification 24 h: 400x

Heterogeneous populations of cells expressing *lapA* or *lapF* within a biofilm

The results of fluorescence microscopy presented above revealed heterogeneity in the expression of *lapA* within a biofilm, especially at 24 h. Thus, we decided to follow the population dynamics of cells of *P. putida* KT2440 expressing either *lapA::gfp* (pMMG5) or *lapF::gfp* (pMMG2) using flow cytometry. Cells were grown under static conditions in 6-well plates with an immersed coverslip. At different time points cells were harvested from the well and coverslips (see Material and Methods).

Results show that after 2 h of incubation, a small subpopulation of cells from the well were expressing *lapA* and the expression of *lapF* was undetectable (Figure 3A). Small subpopulations of cells expressing *lapF* were detected at 4 and 6 h in both coverslips and wells (data not shown). These subpopulations increased at 8 h (Figure 3B). Interestingly, at this time we could not detect subpopulations of cells expressing *lapA* in the well, although the overall intensity of the signal was slightly higher compared to the negative control, suggesting that the entire population may be showing some basal expression of *lapA*. Nevertheless, a subpopulation expressing *lapA* in the coverslip was observed (Fig. 3B). This situation remained invariable over 24 h, with no changes in the expression of *lapA* (Figure 3C). At 24 h, the expression of *lapF* increased considerably, not only in the number of cells but also in the intensity of the fluorescence signal. Interestingly, we could differentiate two subpopulations in the coverslip, while the entire population in the well was expressing *lapF*. It is noteworthy that the intensity of *lapF* expression, once it started, continued increasing over the time, while *lapA* promoter presented its highest activity at early time points and decreased its intensity over time. These data support the results obtained by β-galactosidase assays, where the expression of *lapA* starts earlier than *lapF* and the later reached higher levels of expression.

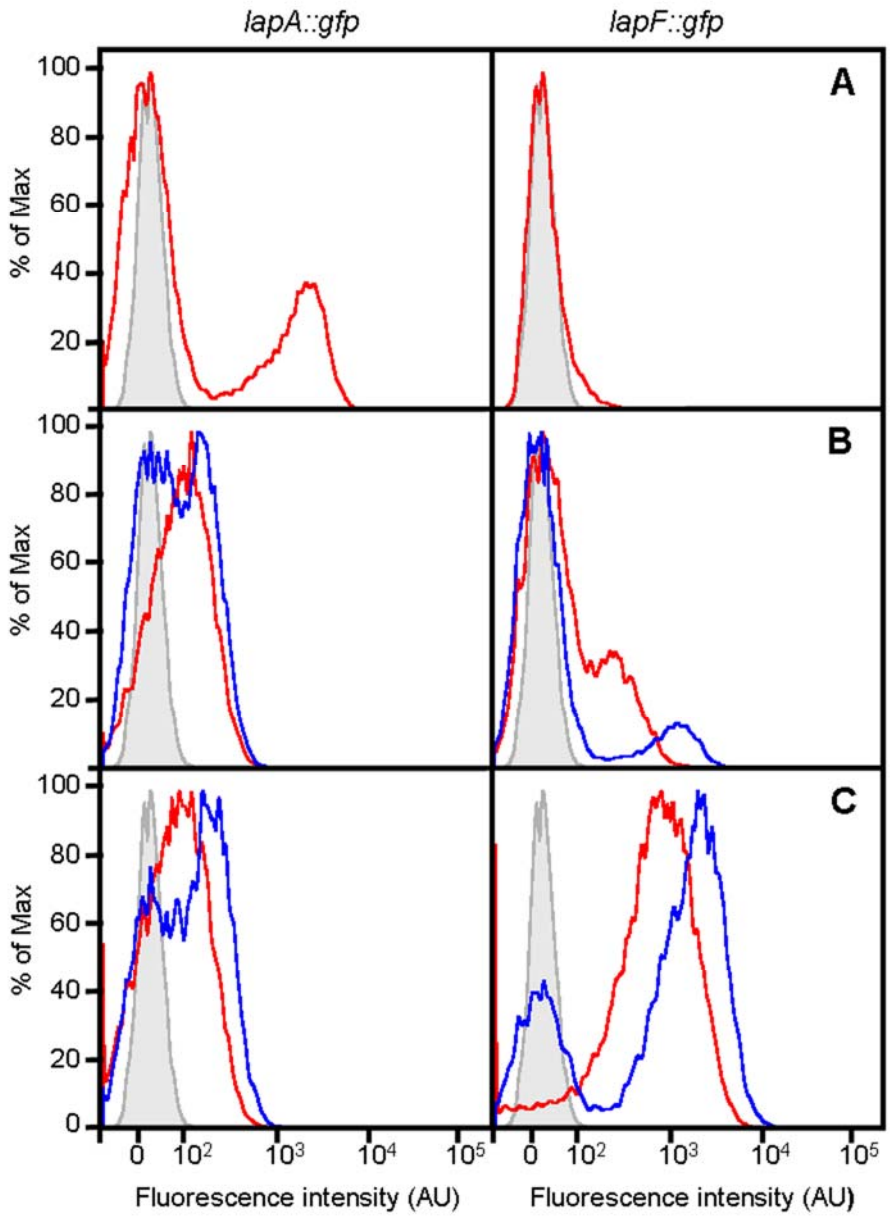


Figure 3. Flow cytometry analysis of cells expressing *lapA::gfp* (left panel) and *lapF::gfp* (right panel). Expression was followed over a period of 24 h. In grey is the peak for control cells with no *gfp* reporter. **(A)** 2 h; **(B)** 8 h and **(C)** 24 h. The Y-axis represents cell counts for each strain (30,000 cells were counted). The X-axis is arbitrary units (AU) of fluorescence in a logarithmic scale. Red: cells from the well; blue: cells recovered from the coverslip.

A mutant in the sensor kinase GacS is affected in biofilm formation due to reduced expression of *lapA* and *lapF*

The two-component system GacS/GacA regulates diverse processes in numerous Gram-negative bacteria (Pernestig *et al.*, 2001). GacS, the sensor kinase, responds to a still unknown environmental signal and activates by phosphotransfer the transcriptional regulator GacA, which subsequently activates target genes (Heeb & Haas, 2001). In *P. putida* KT2440 a recent work has identified *gacS* mutans which were affected in biofilm formation (Duque *et al.*, 2012). From a random mutagenesis of *P. putida* KT2440 using mini-*Tn5*-Km (Lorenzo *et al.*, 1990) we obtained a mutant in *gacS* which was affected in biofilm formation (Figure 4).

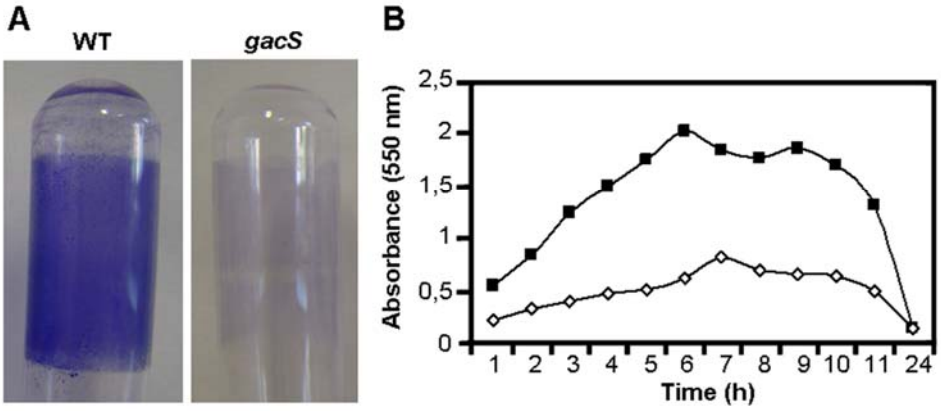


Figure 4. Biofilm formation of a *gacS* mutant in comparison to the wild type. **(A)** biofilm formation in borosilicate glass at 3h in LB medium; **(B)** Biofilm formation of wild type (closed squares) and *gacS* mutant (open circles) in microtiter plates in static conditions in LB medium. Both experiments were performed in triplicate. One representative experiment is shown. Experiments were also performed in minimal medium supplemented with citrate and similar results were obtained.

We therefore decided to analyze if this defect was due to altered expression of either adhesin in the *gacS* mutant. For that purpose, pMMGA (*lapA::lacZ*) and pMMG1 (*lapF::lacZ*) (Martínez-Gil *et al*., 2010) were introduced in this mutant and β-galactosidase assays were performed. As shown in Figure 5, the expression of both adhesins is affected although in different ways.

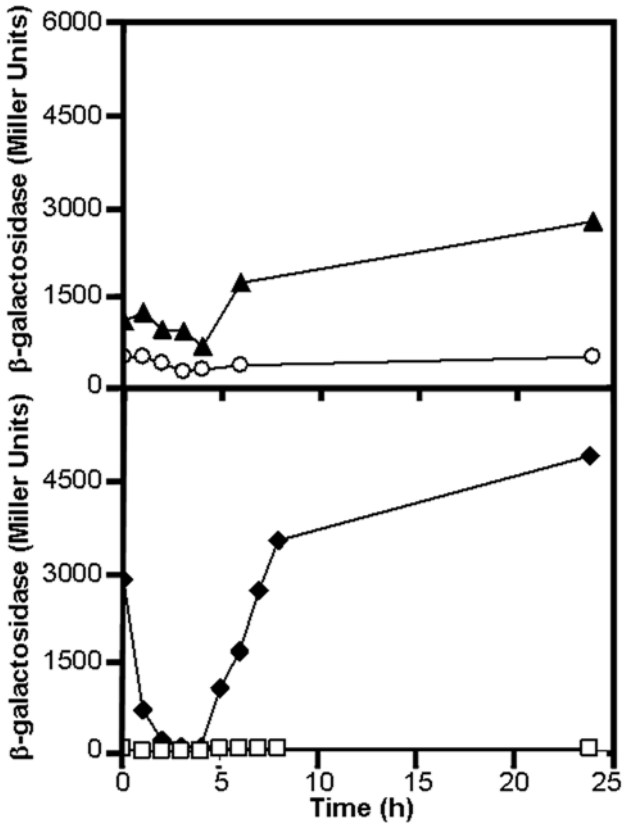


Figure 5. Upper panel: Expression of *lapA::lacZ* (pMMGA) in the wild type (closed triangles) and *gacS* mutant (open circles). Lower panel: Expression of *lapF::lacZ* (pMMG1) in the wild type (closed diamonds) and *gacS* mutant (open squares).

We observed a clear reduction in the level of activity of the *lapA::lacZ* fusion, although the expression pattern was not significantly altered (Figure 5; upper panel). However, expression of *lapF::lacZ* was completely abolished in the *gacS* mutant (Figure 5; lower panel).

The alternative sigma factor σ^s (RpoS) is positively regulated by the two-component system GacS/GacA in *P. fluorescens* Pf-5 (Whistler *et al.*, 1998). Given that the expression of *lapF* is under the regulation of this sigma factor (Martínez-Gil *et al.*, 2010), we checked *rpoS* expression in the *gacS* mutant using plasmid pMAMV21, which harbors a *rpoS*::*lacZ* fusion (Matilla *et al.*, 2011b) (Figure 6).

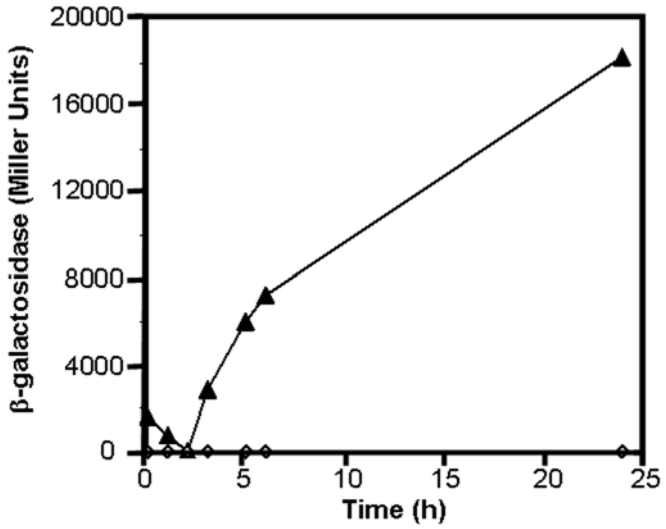


Figure 6. Transcription of *rpoS* is under the regulation of GacS. β-galactosidase assays were done during growth of the wild type (closed triangles) and the *gacS* mutant (open diamonds) harboring pMAMV21 (*rpoS::lacZ*).

As shown in Figure 6, expression of *rpoS* was abolished in the mutant, indicating that the two-component system GacS/GacA regulates *rpoS* at the transcriptional

level, and suggesting that the influence of the two component system on *lapF* is indirect, via RpoS.

High concentrations of c-di-GMP influence transcription of *lapA* and *lapF*.

It has been recently shown that the response regulator Rup4959 of *P. putida* KT2440 contains GGDEF/EAL domains and when the gene is present in multicopy it confers high levels of intracellular c-di-GMP due to diguanylate cyclase (DGC) activity (Matilla *et al.*, 2011b). As a consequence, a variety of pleiotropic effects are apparent, including enhanced biofilm formation, wrinkly colonies, pellicle formation in the air-liquid interphase, exopolysaccharide overproduction and cellular clumping.

In many bacteria the effect of the secondary messenger c-di-GMP also relies on gene expression (Hickman *et al.*, 2005), in particular genes involved in exopolysaccharide production and biofilm formation (Beyhan *et al.*, 2006, Lee *et al.*, 2007). Therefore, we next studied if high intracellular level of this secondary messenger could have an influence on the transcriptional regulation of *lapA* or *lapF*. For this purpose pMMG1 or pMMGA were introduced in KT2440 harbouring pMAMV1 (*rup4959* in multicopy) and β-galactosidase activity was studied. Although the profiles did not change significantly, we observed variations in the expression intensity of both promoters (Figure 7). Surprisingly, each was affected in different ways. In the presence of high intracellular levels of c-di-GMP, the expression of *lapA* was around 2-fold higher in comparison to normal intracellular concentrations of the secondary messenger (Figure 7A). In contrast, the expression of *lapF* was negatively affected by high levels of c-di-GMP (Figure 7B).

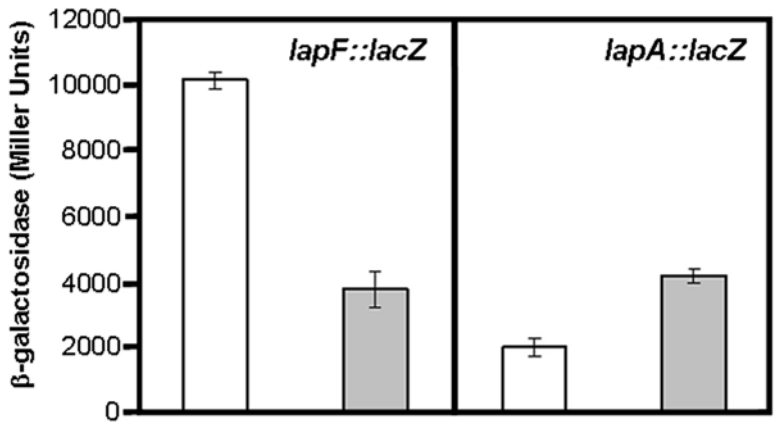


Figure 7. Expression of *lapF::lacZ* and *lapA::lacZ* fusions in KT2440 in the absence (open bars) or presence (grey bars) of plasmid pMAMV1. This plasmid confers high levels of c-di-GMP due to the diguanylate cyclase activity of Rup4959. Data correspond to averages and standard deviations from six replicas of cultures after overnight growth in LB.

In a recent report, the flagellar regulator FleQ has been shown to modulate expression of certain genes through its interaction with c-di-GMP (Baraquet *et al.*, 2012). A *fleQ* mutant of *P. putida* KT2440 has been reported to show reduced attachment to plant seeds and biofilm formation on abiotic surfaces (Yousef-Coronado *et al.*, 2008). Analysis of expression of the *lapA::lacZ* and *lapF::lacZ* fusions in this mutant (Figure 8) revealed that FleQ functions as a positive regulator of *lapA*, and has little effect on *lapF* (only a slight increase in β-galactosidase activity was observed in the mutant at 24 h). When plasmid pMAMV1 was introduced in this mutant, the increase in activity observed for the *lapA::lacZ* fusion in the wild type strain was lost, indicating that FleQ is required for c-di-GMP-dependent activation of the *lapA* promoter. In contrast, in the case of lapF, there was a similar reduction in β-galactosidase activity in the presence of

high c-di-GMP levels as that observed in the wild type, suggesting that the effect of the second messenger is not through FleQ.

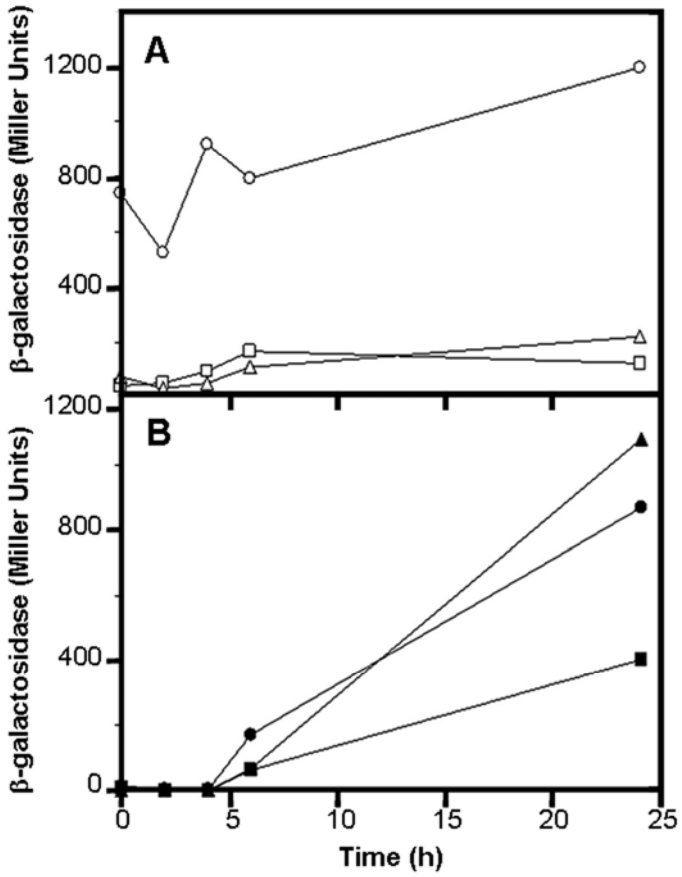


Figure 8. Expression of *lapA::lacZ* (A, open symbols) and *lapF::lacZ* (B, closed symbols) in a *fleQ* mutant in the presence or absence of high c-di-GMP levels. Circles, KT2440; triangles, *fleQ* mutant; squares, *fleQ* mutant harboring pMAMV1.

Lack of LapA induces changes in *lapF* expression

We have recently reported a pseudo-compensatory effect by which mutations in *lapA* or *lapF* result in increased expression and production of exopolyssacharide. To define if such effect could also exist between the two adhesins, expression of their promoters in either mutant was analyzed. The expression profiles of *lapA::lacZ* and *lapF::lacZ* in both mutants were similar to those observed in the wild type (data not shown). However, a significant reduction in the intensity of *lapF* expression was detected in the *lapA* mutant (Figure 9).

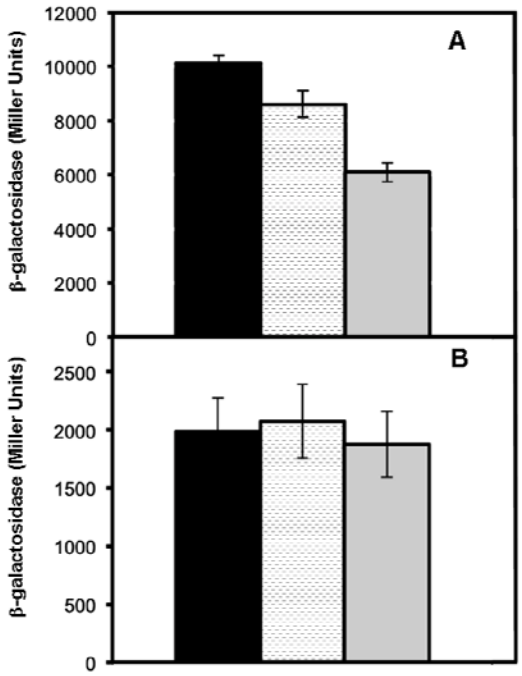


Figure 9. Expression of the *lapA* and *lapF* promoters at 24 h in adhesin-deficient backgrounds. **(A)** pMMG1 (*lapF::lacZ*) and **(B)** pMMGA (*lapA::lacz*) at 24 h in wild type (black bars), *lapF* mutant (dotted bars) and *lapA* mutant (grey bars).

These results were further explored in biofilm-growing conditions using flow cytometry. Plasmids pMMG5 (*lapA::gfp*) and pMMG2 (*lapF::gfp*) were introduced in mus-42 and mus-20 and cells were grown on 6-well plates under static conditions with a coverslip in each well. At different time points cells were harvested from the well and the coverslips and treated for the analysis as described in Material and Methods. It was not possible to recover cells from the coverslip in the *lapA* mutant (mus-42) given that this mutant is impaired in biofilm formation. For this mutant, we only compared the expression in cells recovered from the well. Surprisingly, *lapF* expression in the *lapA* mutant showed a dramatically altered pattern in comparison to the wild type (Figure 10). A new subpopulation of cells expressing *lapF,* absent in the wild type, appeared at 2 h (Figure 10). At 8 h, we observed a shift of the entire population towards increased fluorescence in comparison to the wild type where a small subpopulation of cells expressed the lapF promoter. Similar to the results obtained in β-galactosidase assays, expression of *lapF* was reduced in the *lapA* mutant with respect to the wild type after 24 h. Expression of *lapF* in mus-20 (*lapF* mutant) and expression of *lapA* in either mutant did not vary compared to the wild type (data not shown).

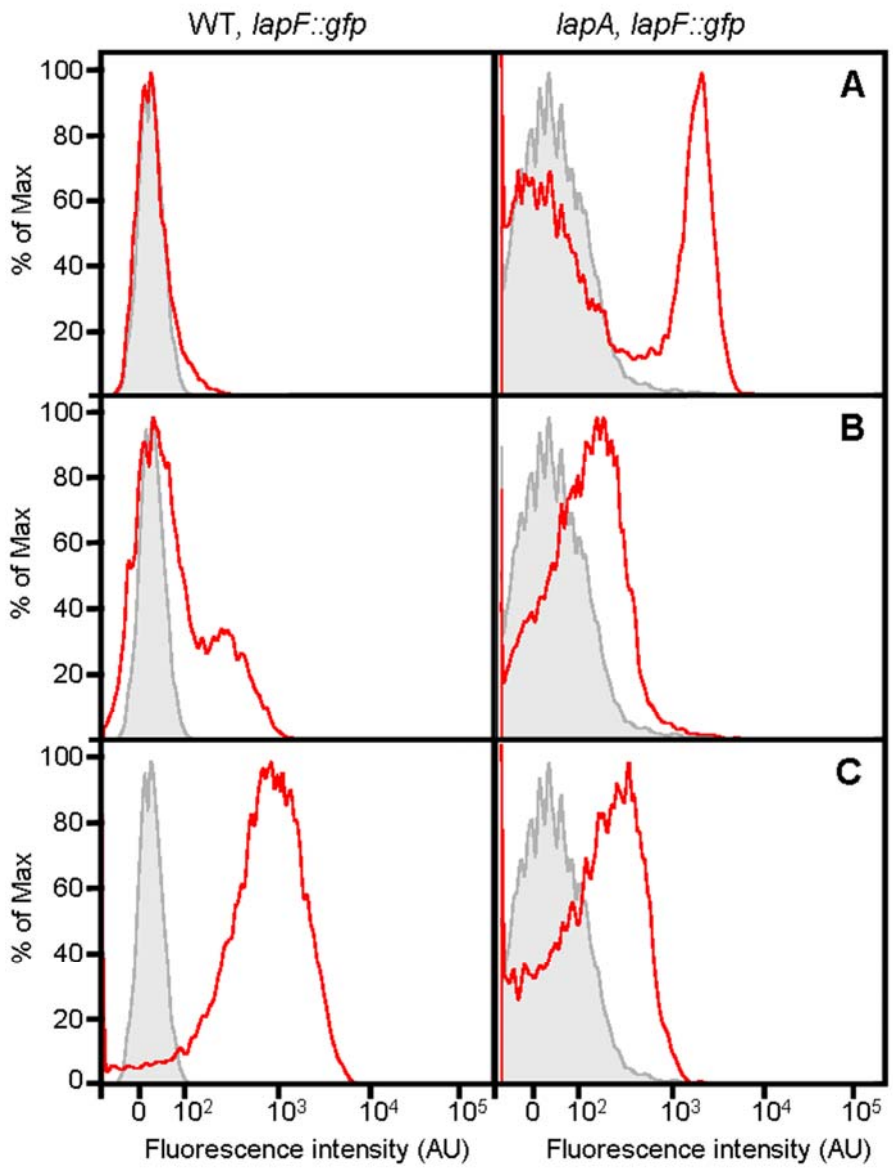


Figure 10. Flow cytometry analysis of cells expressing *lapF::gfp* in cultures of the wild type (left) and *lapA* mutant (right). Expression was followed over a period of 24 h. In grey is the peak for control cells with no *gfp* reporter. **(A)** 2 h; **(B)** 8 h and **(C)** 24 h. The Y-axis represents cell counts for each strain (30,000 cells were counted). The X-axis is arbitrary units (AU) of fluorescence in a logarithmic scale.

Discussion

Bacterial communities living on surfaces are commonly surrounded by an extracellular matrix composed mainly of polysaccharides and surface proteins, which confer adhesiveness, cohesiveness and stability. *P. putida* KT2440 biofilm formation is essentially governed by the surface adhesion proteins LapA and LapF which have been demonstrated to play different roles in biofilm formation (Hinsa *et al.*, 2003, Martínez-Gil *et al.*, 2010). In this work we provide evidences that these two adhesins are differentially expressed in planktonic cells and cells within the biofilm, where subpopulations of cells expressing *lapA* or *lapF* appear. The transcription pattern deduced for *lapA* correlates with its participation in the initial, irreversible attachment of bacteria to the surface (Hinsa *et al.*, 2003) and with a later role in the matrix of mature biofilms. In *P. fluorescens* and *P. putida* LapA is essential for the maintenance of the biofilm structure given that the activity of the periplasmic protease LapG on LapA releases the protein from the cell surface causing disassembly of the biofilm (Newell *et al.*, 2011, Navarro *et al.*, 2011, Morten *et al.*, 2009, Gjermansen *et al.*, 2010, Yousef-Coronado *et al.*, 2011). These data, in combination with the pattern observed for *lapF*, support a sequential model of events during biofilm formation. Results from flow cytometry experiments indicate that planktonic cells behave differently from cells recovered from the biofilm in relation with *lapA* and *lapF* expression, which is dynamic during biofilm development and based on the results from this work, seems to be finely regulated (Figure 11).

The two-component system GacS/GacA appears as a master regulator of the process, influencing expression of both adhesins. In the case of LapF, the influence is indirect, via RpoS. In the case of LapA, it remains to be determined what the exact pathway is.

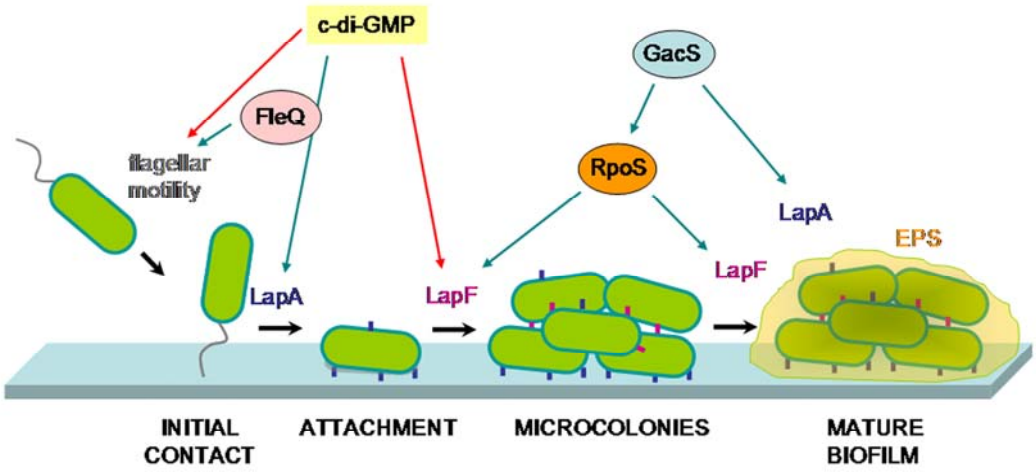


Figure 11. Overview of elements and their regulation involved in the development of *P. putida* biofilms. Red arrows represent downregulation and green arrows upregulation.

This is the first direct evidence in *P. putida* connecting this two-component regulatory system with genes involved in biofilm formation. A recent work has demonstrated that in *Azotobacter vinelandii* the GacS/GacA system regulates the synthesis of alginate, a common extracellular matrix component (Manzo *et al.*, 2011). It has been previously described that GacS/GacA control the synthesis of extracellular products which confer plant protection against pathogens (Heeb & Haas, 2001). Mutants in the two-component system in the opportunistic pathogen *P. aeruginosa* were less virulent (Jander *et al.*, 2000, Rahme *et al.*, 2000). In the plant root-colonizing strain *P. fluorescens* F113, GacS/GacA negatively regulate swimming motility and mutations in *gacS* or *gacA* cause an hypermotile phenotype (Navazo *et al.*, 2009, Martinez-Granero *et al.*, 2006) as well as the loss of biocontrol activity (Barahona *et al.*, 2011). Although the production of certain secondary metabolites is regulated by this system, biocontrol activity also requires efficient colonization of the rhizosphere and of root surfaces. Based on our results,

defectiveness in biofilm formation could also contribute to the reduction in biocontrol in those mutants.

The intracellular levels of the secondary messenger c-di-GMP also affect the expression of *lapA* and *lapF*, although in reverse ways. The role of c-di-GMP in the transition between motile and sessile lifestyles has been widely demonstrated, although many studies have focused on the aspects related to flagellar motility. Recent results have demonstrated that high intracellular concentrations of c-di-GMP contribute to maintain LapA in the cell surface (Newell *et al.*, 2011, Navarro *et al.*, 2011, Gjermansen *et al.*, 2010). Our results show an increased expression of the *lapA* promoter, suggesting that high levels of this secondary messenger regulate biofilm formation at the transcriptional and post-translational levels. Interestingly, the influence of c-di-GMP on *lapA* expression requires the master flagellar regulator FleQ, thus adding an extra level of complexity to the system. The fact that *lapF* expression is reduced when c-di-GMP is high could be indicative of a mechanism for controlling the timing of events during biofilm development. Furthermore, the lack of LapA alters the expression pattern of *lapF*, which suggests there is an internal mechanism sensing the balance of structural components of the biofilm. How this balance is sensed, and how the "biofilm clock" is controlled is the next challenge to be faced in the analysis of bacterial multicellularity.

Acknowledgements

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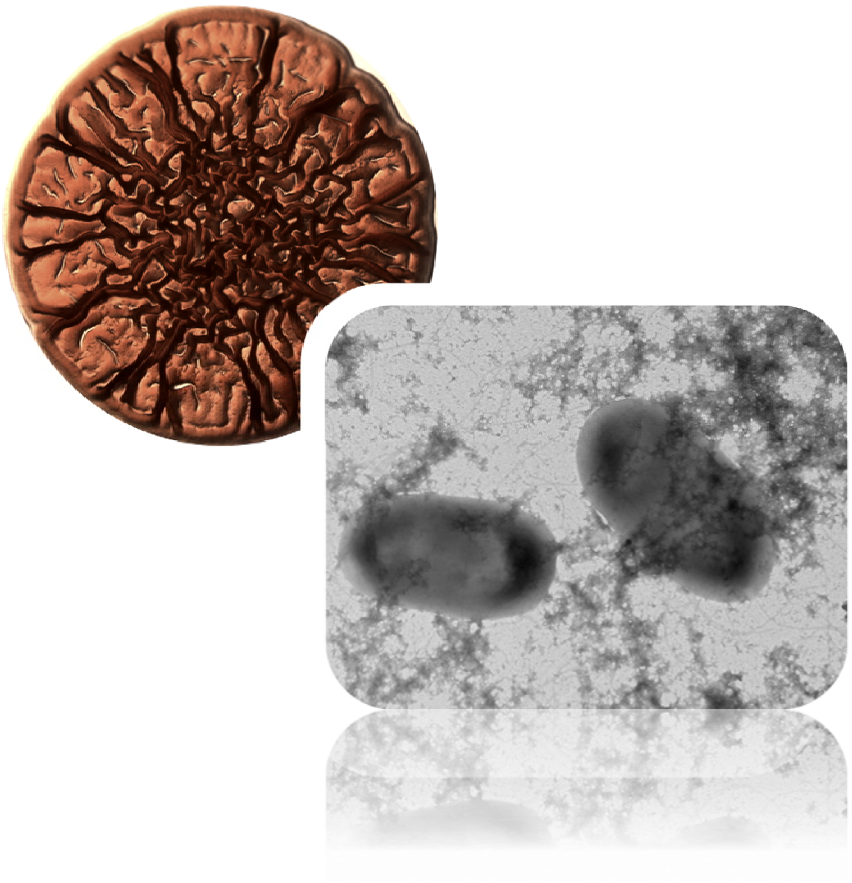
Chapter 4



Compensatory balance between extracellular matrix components of *Pseudomonas putida* biofilms

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Summary

The extracellular matrix of bacterial biofilms has at least two key functions, to serve as structural scaffold for the multicellular community and a protective role against external stress. In this work we report a compensatory effect whereby *Pseudomonas putida* reacts to the lack of either of the two main surface proteins involved in biofilm formation, LapA and LapF, by increasing the expression and production of a species-specific exopolysaccharide. Elevated levels of the second messenger molecule cyclic di-GMP alter the balance of extracellular matrix components, and the phenotypes of *lapA* and *lapF* mutants under these conditions are indicative of direct interactions taking place between large secreted proteins and exopolysaccharides. Our data suggest the existence of an "allostasis" mechanism by which bacteria would sense and counter alterations in the composition of the extracellular matrix, as a means to partly retain their cell-cell interaction ability.

Introduction

Biofilms, communities of bacteria attached to each other and/or to a surface, represent a common microbial life strategy. Within a biofilm, bacterial cells are embedded in a complex extracellular matrix that serves at least for two purposes: providing robustness and stability to the biofilm and protecting the cells against a variety of external aggressions (Chang *et al.*, 2007, Colvin *et al.*, 2011, Davey & O'Toole, 2000, Kovács *et al.*, 2012). The most representative components of the extracellular matrix of bacterial biofilms are proteins, nucleic acids and exopolysaccharides (EPS) which may play different roles during the life cycle of the biofilm or under different environmental conditions (Chang *et al.*, 2007, Hinsa *et al.*, 2003, Martínez-Gil *et al.*, 2010). EPS are key structural components of all known bacterial biofilms, and mutants affected in EPS production are often impaired in biofilm formation (Friedman & Kolter, 2004, Ghafoor *et al.*, 2011, Laue *et al.*, 2006). Additional protective functions of EPS have also been demonstrated, since they may serve as a physical barrier against antimicrobial compounds (Colvin *et al.*, 2011, Mah & O'Toole, 2001, Nichols *et al.*, 1988), disinfectants such as chlorine (Ryu & Beuchat, 2005, Shemesh *et al.*, 2010), and even the human immune system (Leid *et al.*, 2005).

In *Pseudomonas putida* KT2440 and its parental strain mt-2 (harboring the catabolic plasmid pWW0), the function of different matrix components in the development of biofilms has been studied to a certain extent. Two large secreted proteins, LapA and LapF, are key elements with distinct roles. LapA is essential for irreversible attachment to biotic and abiotic surfaces (Gjermansen *et al.*, 2010, Hinsa *et al.*, 2003, Yousef-Coronado *et al.*, 2008), while LapF is involved in cell-cell interactions, microcolony formation and development of a mature biofilm (Martínez-Gil *et al.*, 2010). Other authors have also reported the presence of high concentrations of extracellular DNA in *P. putida* mt-2 biofilms (Steinberger &

Holden, 2005), although our own observations in KT2440 suggest that DNA is not a key component of the matrix (Yousef-Coronado *et al.*, 2011).

The exopolysaccharide composition and the role of each polymer in biofilm formation have been recently investigated (Nielsen *et al.*, 2011, Nilsson *et al.*, 2011). *P. putida* KT2440 has four gene clusters dedicated to the production of EPS, *alg* (alginate), *bcs* (cellulose-like), *pea* and *peb* (Nielsen *et al.*, 2011; Nilsson *et al.*, 2011). These two appear to be specific for *P. putida*, although orthologues of at least some of the genes are present in a small number of closely related species. The products of the *pea* and *peb* clusters have a role in the stabilization of the biofilm structure, whereas those of *alg* and *bcs* are not essential for biofilm formation and stabilization under standard conditions, although alginate participates in biofilm architecture under water-limiting conditions (Chang *et al.*, 2007). In a rifampin-resistant derivative of *P. putida* mt-2, the *pea* cluster is important for pellicle formation, cell-cell interaction and biofilm stability, and *bcs* for rhizosphere colonization (Nielsen *et al.*, 2010). Pea has also been shown to contribute to the pleiotropic phenotype (increased biofilm formation, wrinkly colony morphology, reduced motility) associated to high levels of the second messenger cyclic diguanylate (c-di-GMP) in *P. putida* KT2440 (Matilla *et al.*, 2011).

It has been suggested that the surface proteins LapA and/or LapF could be interacting with different exopolysaccharides (Gjermansen *et al.*, 2010; Nielsen *et al.*, 2011) in order to maintain the biofilm structure. Evidences of this kind of association have been obtained in *Pseudomonas aeruginosa*, where a secreted protein regulated by c-di-GMP levels participates in stabilization of EPS components of the matrix (Borlee *et al.*, 2010). In this work we have explored the connection between exopolysaccharides and surface adhesive proteins in *P. putida* KT2440, and how this interplay is influenced by c-di-GMP.

Material and Methods

Bacterial strains, plasmids and growth conditions

Pseudomonas putida KT2440 is a plasmid-free derivative of the soil isolate *P. putida* mt-2 (Regenhardt *et al.*, 2002). Strains mus-20 (*lapF* mutant) and mus-42 (*lapA* mutant), are KT2440 derivatives obtained by transposon mutagenesis with mini-Tn*5* [Km1] (Espinosa-Urgel *et al.*, 2000, Yousef-Coronado *et al.*, 2008). KT2440-Sm, an otherwise isogenic KT2440 derivative carrying a streptomycin resistance gene in single copy in the chromosome (Yousef-Coronado *et al.*, 2008) was used for root colonization assays. Strains MN38-1 (*bcs* null mutant), MN39-1 (*pea* null mutant), MGJ214-1 (*alg* null mutant) and MGJ215-1 (*peb* null mutant) were gifts from T. Tolker-Nielsen (Nilsson *et al.*, 2011). Plasmid pMAMV1, harboring the diguanylate cyclase encoding gene *rup4959*, has been described elsewhere (Matilla *et al.*, 2011). Cultures were grown at 30 °C either in LB or M9 minimal medium with glucose (20 mM) as carbon and energy source. When appropriate, antibiotics were added at the following concentrations (in μg ml^{-1}): kanamycin (Km), 25; gentamicin (Gm), 20 or 100 (for chromosomally- or plasmid-encoded resistance, respectively); streptomycin (Sm), 100; tetracyclin (Tc), 15.

EPS extraction

Total extracellular polysaccharide of high molecular weigh was extracted as described by (Wingender *et al.*, 2001) with modifications. Cells recovered from LB agar plates were resuspended in liquid LB, and centrifuged at 13,000 rpm for 20 min at 4 °C. Supernatants were kept at 4 °C, while pellets were resuspended in 10 mM EDTA / 3% NaCl, vortexed and centrifuged again. Both resulting supernatants were treated with DNase (67 μg ml^{-1}) and RNase (330 μg ml^{-1}) for 1 h at 37 °C, followed by 30 min incubation at 55 °C with proteinase K. EPS were precipitated overnight with 3 volumes of isopropanol at -20 °C and recovered by

centrifugation for 20 min at 4 °C. Once dry, samples were resuspended in deionized water and SDS-PAGE followed by silver staining was performed after normalization according to protein content of the samples prior to treatments.

Transmission Electron Microscopy

Cells of *P. putida* KT2440, mus-42 and mus-20 were grown on nickel grids for 24 h in LB medium in a 12-well microtiter plate. Grids were recovered and excess of sample was discarded on a filter paper. Grids were incubated with 5 µl of negative staining solution (2% aqueous uranyl acetate) for 2 min at room temperature and the excess was discarded with filter paper. Samples were dried at room temperature for 5-10 min and were visualized in a Tecnai G^2 Spirit BioTWIN microscope at an accelerating voltage of 80 kV. Images were taken with an AMT 2k CCD camera.

RNA extraction and preparation of cDNA

Total RNA from cells grown on LB plates for 48 h was extracted with TRI Reagent (Ambion) as recommended by the manufacturer, except that the TriPure Isolation Reagent was preheated at 70°C. RNA was pretreated with RNase-free DNase I (Roche) plus RNaseOUT (Invitrogen), followed by purification with RNeasy columns (Qiagen) and a second DNase I treatment with the Turbo DNA-free kit (Ambion). Reverse transcription reactions to generate the corresponding cDNA were performed with 0.5 µg RNA using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamers as primers, according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR)

Primers used for real-time PCR analyses were as follows (5'-3' sequences): AlgFwd (GCTTCCTCGAAGAGCTGAA) and AlgRev (CTCCATCACCGCATAGTCA) for *algA*, encoding a guanylyltransferase involved in the synthesis of alginate;

PebFwd (GCAATGTCTCCACAGGCAC) and PebRev (TCATCTGATTGGCG ACCAG) for PP_1795, which is part of the *peb* cluster; CelFwd (GTCGAGAGCAGCCAGCTTC) and CelRev (GCCTCATACAGTGCCAGC TC) for PP_2629, involved in the production of bacterial cellulose; PeaFwd (TGCTCAGCACGCCGACACG) and PeaRev (GGTCTCGCTGTTCAGCA) for PP_3132, the first gene in the *pea* operon. The PCR products were 189, 252, 206 and 239 bp, respectively. 16S rRNA was used as internal control for normalization, using previously described primers (Matilla *et al*., 2011). Real-time PCR amplification was carried out on a MyiQ2 system (Bio-Rad) associated with an iQ5 Optical System Software (version 2.1.97.1001). Each 25 μl reaction contained 12.5 μl iQ SYBR Green Supermix [100 mM KCl, 40 mM Tris/HCl, pH 8.4, 0.4 mM of each dNTP, iTaq DNA polymerase (50 U ml^{-1}), 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers] (Bio-Rad) and 2 μl template cDNA (diluted 1- or 1000-fold). Thermal cycling conditions were as follows: one cycle at 95 °C for 10 min and then 40 cycles at 95 °C for 15 s, 68 °C (for *pea*, *peb* and *bcs*) or 65 °C (for *alg*) for 30 s and 72 °C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers´ recommendations. A final extension cycle (72 °C, 1 min) was performed. Melt curve analysis was performed by gradually heating the PCR mixture from 55 to 95 °C at a rate of 0.5 °C per 10 s for 80 cycles. The relative expression of each gene in the mutants was normalized to that of wild type RNA and the results were analysed by means of the comparative critical threshold ($\Delta\Delta C_t$) method (Pfaffl, 2001).

β-galactosidase activity assays

β-galactosidase activity was assayed in cultures of KT2440 and the *pea* and *peb* mutants harboring plasmids pMMG1 or pMMGA during growth in liquid LB or after 48 h of growth on plates. Experiments were repeated three times, each with two biological replicas. Values are given in Miller units (Miller, 1972).

Competitive root colonization

Corn root colonization experiments were done as described previously (Yousef-Coronado *et al.*, 2008). Briefly, strains grown overnight in LB were diluted in M9 to an OD_{660} = 1, and each mutant was mixed with KT2440-Sm in a 1:1 proportion (~$5x10^6$ cfu of each strain) in 10 ml M9. This mix was used to inoculate surface-sterilized germinated corn seeds. Plants were maintained in a controlled chamber at 24 °C with a daily light period of 16 h. After 1 week, plants were collected, shoots discarded and the roots placed in tubes containing 20 ml of M9 basal medium and 4 g of glass beads (diameter, 3 mm). Tubes were vortexed for 2 minutes, and dilutions were plated on selective media (LB with Sm or Gm, respectively).

Results

The absence of LapA or LapF causes increased EPS production

The extracellular components from KT2440, mus-20 and mus-42 grown on LB plates were extracted as described in Material and Methods and exopolysaccharides were analyzed by SDS-PAGE and silver staining. As shown in Figure 1, the amount of EPS recovered was higher for both mutants than for the wild type. These results were further supported by transmission electron microscopy analysis of KT2440, mus-42 and mus-20 cells, after growth in static conditions for 24 h on nickel grids. Considerable quantities of an electron-dense extracellular polymer could be observed between cells in the cases of the *lapA* and *lapF* mutants after negative staining, with much less stainable material being apparent in the wild type (Figure 1). These differences were not observed at earlier times (data not shown).

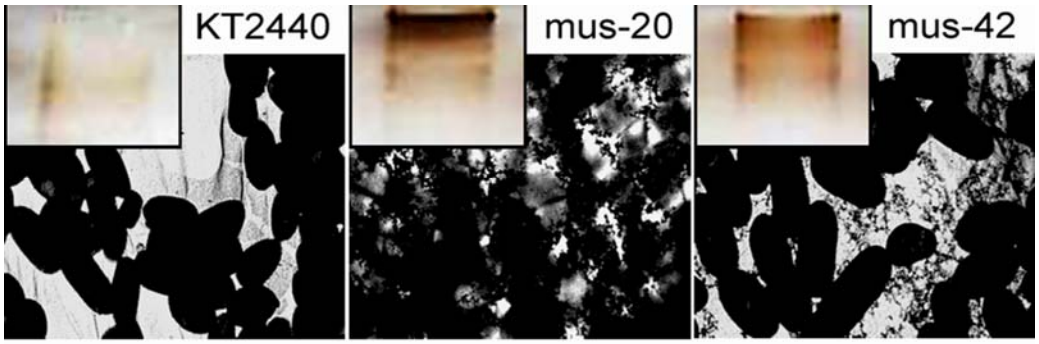

Figure 1. Increased production of exopolysaccharide in *lapF* (mus-20) and *lapA* (mus-42) mutants of *P. putida*. Top: transmission electron micrographs (negative staining with 2% uranyl acetate) of wild type (KT2440) and mutant cells grown on nickel grids in LB medium in a 12-well microtiter plate, showing the accumulation of electron-dense extracellular material. Samples were visualized in a Tecnai G2 Spirit Bio TWIN microscope at 80 kV. Insets correspond to SDS-PAGE of EPS extractions from cultures grown on LB plates, visualized after silver staining.

The above data suggested that production of one or more exopolysaccharides was enhanced in mus-42 and mus-20 as compared to the wild type. Therefore, we analyzed the expression of the *pea*, *peb*, *bcs* and *alg* operons in the different strains using quantitative real-time PCR (qRT-PCR). RNA was isolated from cells grown on LB plates and qRT-PCR was performed with primers designed for each of the four operons. Results show a 2.5-fold increase in *pea* mRNA in the *lapA* mutant with respect to the wild type (Figure 2). In contrast, we did not observe significant differences in *peb* or *bcs* transcription. Interestingly, expression of *alg* appears to be slightly reduced in mus-20 and increased in mus-42 with respect to the wild type.

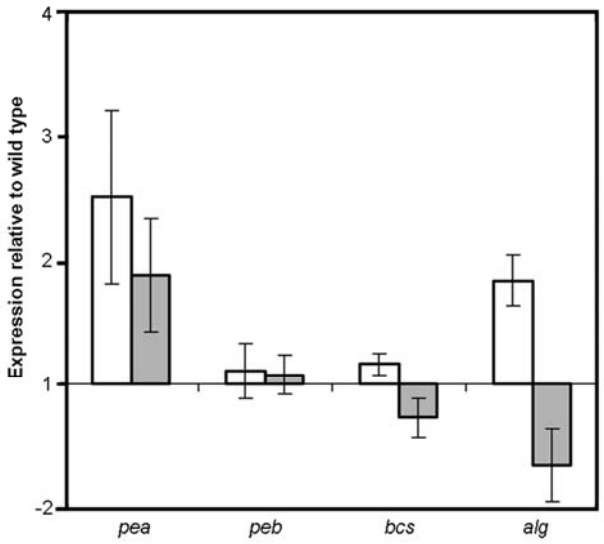

Figure 2. Relative expression of each of the four EPS-encoding operons in the *lapF* (open bars) and *lapA* (grey bars) mutants with respect to the wild type, measured by qRT-PCR. A value of 1 implies that no differences in expression were observed between wild type and mutant strains. The results are means and standard deviations of three independent experiments with three replicas each.

Lack of certain EPS influences transcription of *lapA* and *lapF*

We then decided to check if the absence of EPS previously described to participate in biofilm formation influenced expression of the two adhesins. For that purpose, plasmids pMMG1 or pMMGA were introduced in mutant derivatives of KT2440 deficient in each of the exopolysaccharides. These plasmids carry transcriptional fusions of the *lapF* and *lapA* promoters to *lacZ*, respectively (Martínez-Gil *et al.*, 2010; Martínez-Gil *et al.*, manuscript in preparation). Expression was analyzed in the same conditions as above, i.e. recovering cells from LB plates and measuring β-galactosidase activity.

Under these conditions, expression of the *lapF* promoter was reduced in all mutants compared to the wild type (Figure 3), the reduction varying from 3- to 19-fold. In the case of the *lapA* promoter, no significant differences were observed between the *alg* mutant and the wild type. In the remaining mutants there was also a reduction in β-galactosidase activity (Figure 3), although less pronounced than that observed for the *lapF::lacZ* fusion.

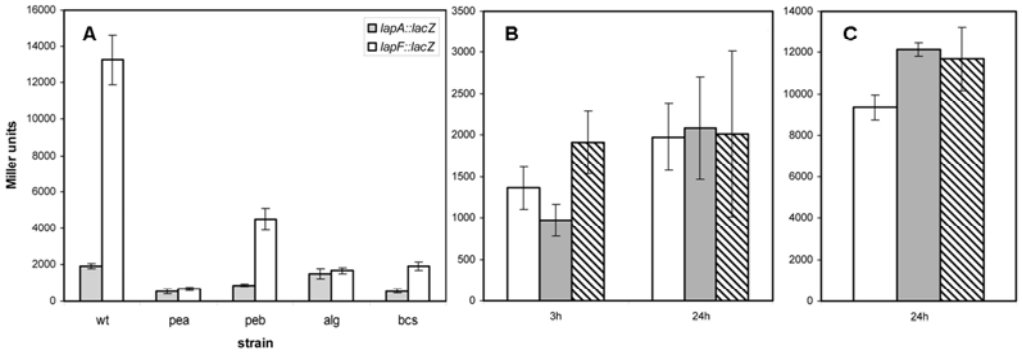

Figure 3. A. Expression of *lapA::lacZ* (grey bars) and *lapF::lacZ* (open bars) transcriptional fusions in KT2440 (wt) and the different EPS mutant strains (*pea*, *peb*, *alg*, *bcs*), grown on plates for 48 h. Cells were recovered from LB plates, resuspended in M9 salts and β-galactosidase activity was measured. **B.** Expression of *lapA::lacZ* in KT2440 (open bars), *pea* (grey bars) and *peb* (hatched bars) strains, grown in liquid LB to mid-exponential (3 h) and stationary phase (24 h). **C.** Expression of *lapF::lacZ* in KT2440 (open bars), *pea* (grey bars) and *peb* (hatched bars) strains, grown in liquid LB to stationary phase (24 h). All data are given in Miller units and correspond to averages and standard deviations from 3 biological replicas with 3 samples per replica.

Experiments were also done in liquid cultures of the *pea* and *peb* mutants, measuring β-galactosidase activity in mid-exponential and stationary phase. Expression of the *lapA::lacZ* fusion was slightly reduced in the *pea* mutant and slightly increased in the *peb* mutant in mid-exponential phase, while no differences were observed after overnight growth (Figure 3). In the case of the *lapF::lacZ*

fusion, only basal expression was observed in exponential phase (data not shown), as previously reported (Martínez-Gil *et al.*, 2010), whereas both the *pea* and the *peb* mutants showed increased expression at 24 h (Figure 3). It is worth noting that in stationary phase cultures there was a great variability in β-galactosidase activity between biological replicas in the *peb* mutant for both promoters.

LapA and LapF participate in the Pea-dependent multicellular phenotypes caused by increased c-di-GMP

The gene *rup4959* (locus PP4959) encodes a GGDEF/EAL response regulator with diguanylate cyclase activity, involved in the synthesis of the secondary messenger c-di-GMP (Matilla *et al.*, 2011). When *rup4959* is present in multicopy, a pleiotropic phenotype can be observed, directly related with the increase in intracellular c-di-GMP. This phenotype includes reduced swimming and swarming motility, wrinkly colony morphology, enhanced biofilm formation and pellicle development on the air-liquid interface (Matilla *et al.*, 2011). Some of these altered phenotypes, such as wrinkly colony development, require the production of Pea. Given the interplay between proteins and EPS evidenced in the previous results, we decided to evaluate the potential contribution of LapA and LapF to this multicellular phenotype. As described by Matilla *et al.* (2011), KT2440 harbouring plasmid pMAMV1 (*rup4959* in multicopy) started to develop wrinkly colony morphology after 24 h at 30°C, and the phenotype was fully evident after 48 h. The same was true for mus-20 harboring pMAMV1, whereas in mus-42 the alterations in morphology were clearly observed earlier on (data not shown). Although the two mutants showed this altered colony development, the morphology differed between them and from that of the wild type (Figure 4). These differences were more evident when Congo red was added to the medium (Figure 4).

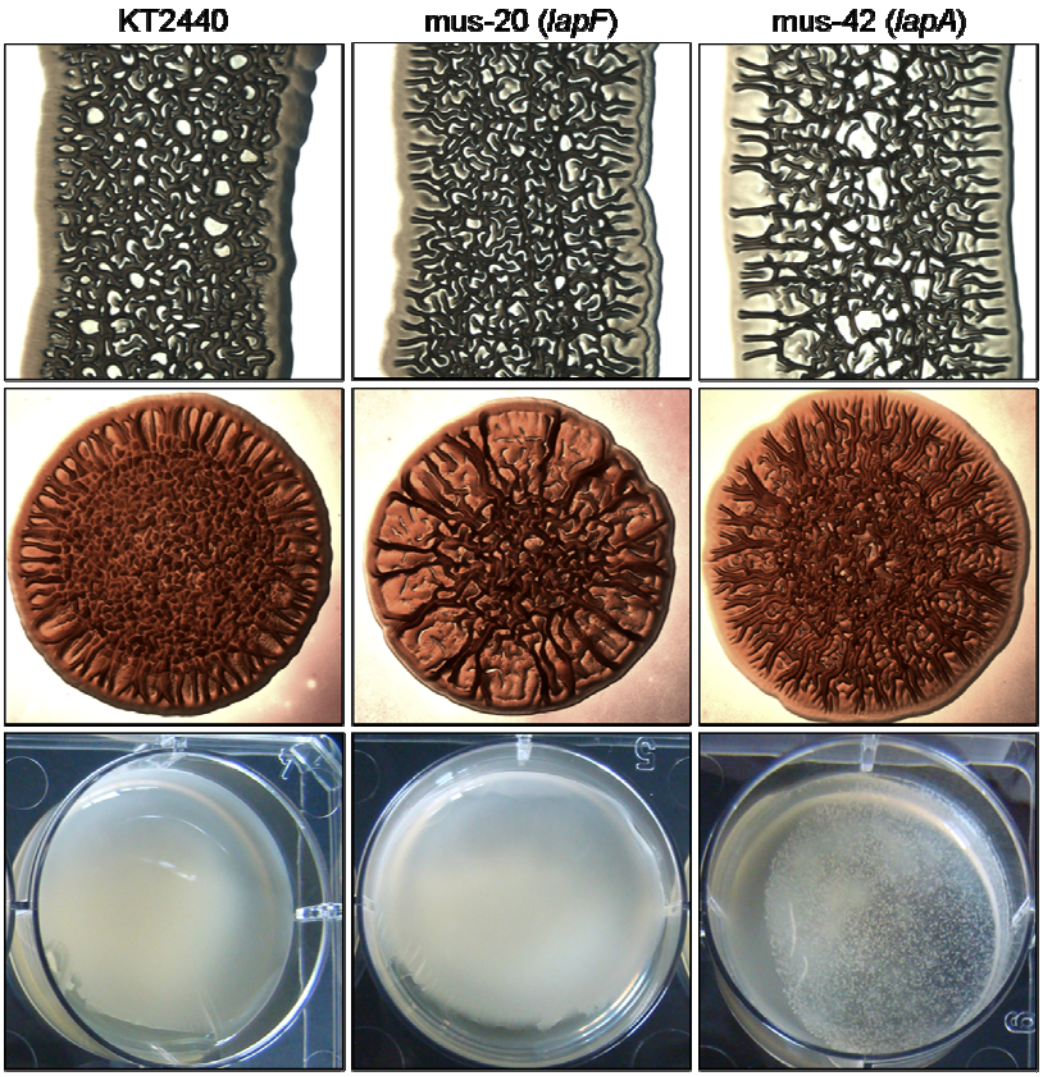

Figure 4. Colony morphologies, and pellicle formation by KT2440, mus-20 and mus-42 harboring plasmid pMAMV1. **A, B.** Cultures grown as streaks on LB agar plates **(A)** or as patches on LB containing 40 μg/ml Congo red **(B)**, and visualized with a stereomicroscope after 48 h of growth. Illumination and contrast were adjusted to highlight the different wrinkle patterns. **C.** Liquid cultures grown for 24 h in 6-wells plates under static conditions at 30 C in M9 with glucose as carbon source.

Pellicle formation at the air-liquid interface under static growth conditions was also analyzed in the two mutants harboring pMAMV1. As shown in Figure 4, the *lapF* mutant formed a similar, although slightly more frail, pellicle as the wild type. The *lapA* mutant, on the other hand, did not form a continuous pellicle, but presented a granular aspect (Figure 4).

Contribution of different EPS to competitive root colonization by *P. putida* KT2440

The two large surface proteins LapA and LapF were initially identified for their role in seed colonization (Espinosa-Urgel *et al.*, 2000) and their participation in the establishment of *P. putida* KT2440 populations on root surfaces (Yousef-Coronado *et al.*, 2008; Martínez-Gil *et al.*, 2010). It has also been previously reported that at least one EPS, Bcs, plays a role in corn root colonization by a rifampin-resistant derivative of *P. putida* mt-2, the plasmid-bearing parental strain of KT2440 (Nielsen *et al.*, 2011). However, there were significant fluctuations between experiments, perhaps related to the presence of the self-transmissible plasmid pWW0, which has been reported to influence biofilm formation (D'Alvise *et al.*, 2010). Thus, we decided to evaluate the fitness of KT2440 mutants in each of the EPS clusters in the rhizosphere in competition with their parental, plasmid-free strain. As shown in Figure 5, all the mutants are less competitive than the wild type, but the most significant differences were observed with the *alg* mutant, followed by the *pea*-deficient strain. Contrary to the results reported for *P. putida* mt-2, in this case the *bcs* mutant showed the lowest fitness loss of all four KT2440 derivatives.

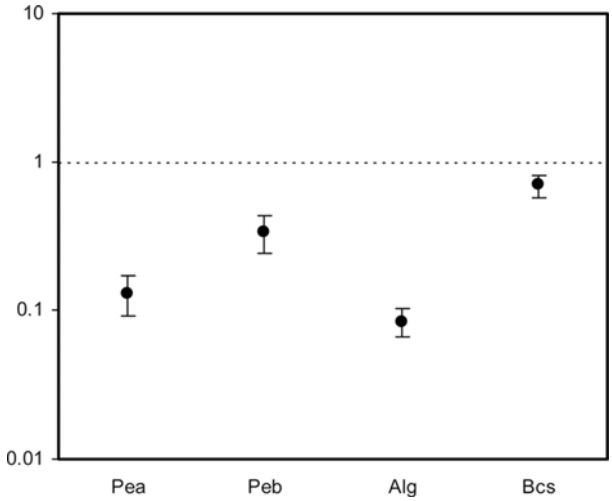

Figure 5. Competitive corn root colonization by KT2440 and EPS mutants. Results correspond to averages and standard deviations of duplicate data obtained from six plants, and are expressed as the index of colonization fitness (Ramos-González *et al*., in press), i. e. the proportion of mutant vs. wild type cells in the total root population at day 7 divided by the proportion of mutant vs. wild type cells in the initial population inoculated at day 0. An index of 1 implies that both strains are equally competitive in the rhizosphere; indexes below 1 indicate that those strains are less competitive than the wild type.

Discussion

In this work we have begun to explore the interplay between structural elements involved in cell-cell and cell-surface interactions in *P. putida* biofilms. Our results indicate that bacteria have a means to sense and modulate the balance of their extracellular components. In *P. aeruginosa*, the lack of the surface polysaccharide Psl induces overproduction of Pel, and in the same way, the production of alginate is enhanced in the absence of Pel (Ghafoor *et al*., 2011). In *P. putida*, a similar balancing effect takes place between EPS and surface proteins. However, the regulatory relationship seems to be more complex in this case, since the lack of LapA or LapF increases the production of the species-specific EPS Pea, but the

presence or absence of the different EPS influences the level of expression of the two adhesins in diverse ways depending on the growth conditions. It is likely that the overall composition of the matrix and the relative importance of each component varies depending on environmental cues. As an example, previous data indicate that alginate is relevant in *P. putida* under hydric stress (Chang *et al.*, 2007), and that in the absence of alginate other exopolysaccharides are overproduced in water-limiting conditions (Nielsen *et al.*, 2011). However, our results in the rhizosphere reveal that EPS are not redundant in this habitat, since individual null mutants in alginate, Pea or Peb are less competitive than the wild type. The role of alginate in rhizosphere fitness is in accordance with the promoter of the alginate biosynthesis operon having been identified for its preferential induction during competitive rhizosphere colonization (Ramos-González *et al.*, 2005).

The increased production of Pea in the *lapA* and *lapF* mutants does not compensate the lack of these two proteins in terms of biofilm formation, since both strains show reduced attachment and/or microcolony development (Yousef-Coronado *et al.*, 2008; Martínez-Gil *et al.*, 2010). It would therefore seem a futile energy expense to overexpress a component of the biofilm matrix when no biofilm can be formed. The most obvious interpretation is that both proteins are not only required for attachment but are also integral parts of the scaffold of mature biofilms. This is in agreement with the previously suggested association of LapA with an extracellular polysaccharide (Gjermansen *et al.*, 2010), and with the differences in wrinkly colony morphology between wild type *P. putida* and *lap* mutants observed here in the presence of increased c-di-GMP. The altered pellicle formation of a *lapA* mutant also indicates that under these conditions, either LapA is necessary for the organization of the pellicle in the air-liquid interface, or LapF produces agglutination of cells in the absence of LapA. It should be noted that in the *lapF* mutant the amount of extracellular substances observed by electron microscopy or gel electrophoresis is higher than in the *lapA* mutant, whereas the increase in expression of *pea* is less pronounced. This could imply that additional

surface structures or components yet to be defined are overproduced in the absence of LapF.

All these results highlight the requirement of interactions among diverse macromolecules during the assembly of a functional matrix. The composition of the matrix would be dynamic, so that changes affecting one element might be compensated by increasing the production of another, or by modifying the overall composition. Thus, to preserve the biofilm matrix even if with altered components, there exists what could be considered as an "allostasis" mechanism (i.e. a control circuit maintaining stability of a biological system through a dynamic balance of variable elements, in contrast to homeostasis, which would imply keeping a physiological parameter close to constant). Although the term is rather used in neurobiology (McEwen, 1998), the concept of allostasis can be adapted to other fields. Speculations about the actual mechanism by which these alterations are sensed by and transduced into the bacterial cells will require further research aimed at defining the regulatory pathways involved in biofilm formation in *P. putida*.

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IV. GLOBAL DISCUSSION

In most natural environments, bacteria are predominantly found forming structured communities called biofilms. In the last decade, numerous works have contributed to identify regulatory and structural factors involved in biofilm formation in a wide variety of species. We are starting to understand the complexity of the regulatory networks that govern the genetic program leading to biofilm development. The great diversity of factors involved in biofilm development demonstrates that bacteria have evolved different mechanisms in order to successfully colonize surfaces and survive to many environmental threats.

This Thesis has focused on the study of biofilm formation by the plant growth promoting rhizobacterium *Pseudomonas putida* KT2440. In the first two chapters, we have mainly focused on the structural factors that lead to maturation of biofilms, and in the second part of the work (chapters 3-4) we have studied how the expression of the different matrix components of *P. putida* biofilms is regulated. This work has contributed to our knowledge of the elements that determine the structure and regulation of *P. putida* biofilms.

Random mutagenesis has been a strategy widely used for the identification of key genes involved in biofilm formation in different species. However, as the selected phenotype is the absence of biofilm formation, many genes involved in different steps of biofilm development could be missed in those screenings. A random mutagenesis with the mini*Tn5*Km transposon was used to identify genes involved in *P. putida* KT440 attachment to corn seeds (Espinosa-Urgel *et al.*, 2000). One of the isolated mutants affected in attachment to seeds was called mus-20 (mutant-unattached to seeds) and it was found that the transposon was interrupting the gene encoding a putative surface protein with a high molecular weight that was called LapF. Prior to this Thesis, our group had identified and contributed to characterize another large protein, LapA, with an essential role in seed colonization and biofilm formation by *P. putida* and *Pseudomonas fluorescens* (Hinsa *et al.*, 2003). Given the role of large surface proteins in biofilm formation and that LapF does not have any homolog in other *Pseudomonas* species, we

decided to focused on the role of LapF in biofilm formation and rhizosphere colonization.

Based on our findings with respect to the biofilm phenotype of a *lapF* mutant, along with the cellular localization of the protein and gene expression data, we propose a role for LapF in cell-cell interaction, microcolony formation and biofilm maturation. We have confirmed the extracellular localization of LapF by subcellular fractioning of planktonic cultures and by immunolocalization in biofilms. LapF shows the expected features of a protein secreted by a TISS: (i) it is an acidic protein, with a theoretical pI of 3.67, (ii) the signal peptide is in the C-terminal domain, (iii) it has a low number of cysteine residues and (iv) it has two aspartate and glycine-rich domains. In addition, we proved that *lapF* is co-transcribed with its putative Type I secretion system (TISS), which we have named *lapHIJ*. The complete characterization of the LapHIJ system awaits further experimentation.

When differentially labeled cells of the wild type and a *lapF* mutant were co-cultured, we observed that a mixed biofilm of both strains was formed but cells of the mutant failed to form microcolonies. Instead, *lapF* cells appeared embedded in the wild type biofilm, which was characteristically dense and compact. Similarly, in root colonization assays, mixed microcolonies with a majority of wild type cells, were observed. These observations have prompted some questions: Is the *lapF* mutant taking advantage of LapF produced and secreted by the wild type in order to establish cell-cell interactions? And if so, how is the appearance of "cheaters" (i.e. cells lacking LapF and thus reducing their energetic costs) in a normal biofilm population prevented? Is the presence of LapF necessary on the surface of both cells to exert its function? In contrast to our results, in *S. aureus*, cells of *bapA* mutants were not recruited into the biofilm matrix (Latasa *et al.*, 2005). Thus, even though many Bap-related proteins have been reported in diverse bacteria species, and proposed to mediate cell-to-cell interactions, it still remains unknown if in this

interaction the protein needs to be present in both cells. It has been suggested that BapA might interact with itself through homophilic interactions, thus acting both as a receptor and as a ligand between two bacterial clusters (Latasa *et al.*, 2005).

The sequence of LapF can be divided into three clearly defined domains: N-terminal, a large central core and a C-terminal domain, which presumably should contribute to the localization and the functionality of the protein. As proposed for other large proteins associated to cell surfaces, we hypothesize that LapF is anchored to the cell-surface by its N-terminal domain. As an example, in *P. fluorescens*, LapA has been shown to be attached to the cell surface by its N-terminal domain. This linkage has been proved to be key for its functionality given that when it is released from the cell surface by the periplasmic protease LapG, the biofilm detaches from the surface (Newell *et al.*, 2011). In *Salmonella enterica*, the level of retention of the surface protein SiiE from by its N-terminal domain was directly correlated with the invasion of polarized cells (Wagner *et al.*, 2011). In contrast, the extracellular addition of the surface protein of *S. aureus* called Protein A is able to induce biofilm development (Merino *et al.*, 2009).

One characteristic of Bap-related proteins is the presence of a highly repeated domain in the central core of the protein. It has been suggested that these highly conserved repeats may be essential for the functionality of those proteins. However, the exact role of these domains has not been clarified (Toledo-Arana *et al.*, 2001). The ability of *S. aureus* to form a biofilm *in vitro* was not affected by the number of repeats within the Bap protein (Lasa & Penades, 2006, Cucarella *et al.*, 2001). In contrast, *P. fluorescens* expressing a modified allele of LapA containing the N- and C-termini domains of LapA and an internal myc-epitope tag was not able to restore the lack of LapA, suggesting the implication of the repetitive domain in the functionality of this protein (Newell *et al.*, 2011). In addition, it has been proposed that the repeated central region of the *Enterococcus faecalis* surface protein Esp, might function as a mechanism to retract the protein from the cell surface and hide it from the immune system of the host (Toledo-Arana *et al.*, 2001). An

interesting hypothesis suggests that the presence of Esp could increase cell surface hydrophobicity and promote hydrophobic interactions with surfaces (Shankar *et al.*, 1999, Toledo-Arana *et al.*, 2001).

It is noteworthy that a high number of surface proteins involved in bacterial attachment contain calcium-binding motifs. Proteins classified as LapA family proteins are characterized by the presence of hemolysin-type calcium binding motifs (Yousef-Coronado & Espinosa-Urgel, 2007). Proteins from the Bap family could contain cadherin domains. Cadherins are animal glycoproteins that interact with themselves and are involved in calcium dependent cell-cell adhesion. The C-terminal domain of LapF (CLapF) presents a calcium binding domain related to that of NodD, with two conserved motifs GGXGXD. We have confirmed by Isothermal Titration Calorimetry that CLapF specifically binds calcium. In addition, we have shown that EGTA, a calcium binding chelator, is able to decrease biofilm formation at a concentration that did not affect growth. We have studied the effect of calcium addition in biofilm formation and we observed a time-lapse effect at the first stages of biofilm formation. There are evidences indicating that calcium regulates adhesion processes in a wide range of bacteria; however the molecular mechanisms that involve calcium and surface adhesion proteins remain still unclear. The attachment of *Salmonella typhimurium* was inhibited by the chelating agent EDTA, but was enhanced in the presence of calcium and other divalent cations (Craven & Williams, 1998). The same effect was observed in the surface attachment of *Streptococcus mutans* and *Fusobacterium nucleatum* when calcium was removed from titanium surfaces (Badihi Hauslich *et al.*, 2011). In contrast, the addition of millimolar amounts of calcium to the growth medium inhibits intercellular adhesion and biofilm adhesion of *Staphylococcus aureus* in a Bap-dependent manner, suggesting that the binding of calcium and Bap protein impaired cell-cell interactions through Bap (Arrizubieta *et al.*, 2004, Lasa, 2006). In *Vibrio cholerae* has been suggested that calcium plays its role as an ionic

cross-bridging molecule for negatively charged bacterial polysaccharides, being particularly involved in the biofilm architecture (Kierek & Watnick, 2003). Our results of biofilm formation in the presence of calcium and EGTA and the fact that CLapF shows a tendency to form big aggregates in the presence of calcium led us to propose that LapF contributes to cell-cell interaction in the presence of calcium through its C-terminal domain. Calcium is an element that often accumulates on surfaces as sedimentary deposits (Patrauchan *et al.*, 2005), thus it is tentative to suggest the implication of this cation in a variety of bacterial physiological processes, as biofilm formation. It has been hypothesized that calcium is involved in biofilm formation at three different levels: (i) cell-environment interaction, conditioning the surfaces; (ii) cell-cell association, in the build up of the biofilm structure and (iii) within cells for biochemical reactions and physiological activities (van der Waal & van der Sluis, 2012). The central role for calcium in general biological processes is well established and its function depends on its ability to bind to other molecules and change their properties (Geesey *et al.*, 2000). Calcium also participates in the regulation of vital eukaryotic functions such as enzyme activity, motility, attachment, metabolic processes and signal transduction among others. In prokaryotes, calcium is exploited in cell cycle regulation, cell division and regulation of channels and transporters (Michiels *et al.*, 2002). The intracellular levels of calcium are maintained at physiological levels (<100nM) by dedicated influx and efflux systems (Linhartová *et al.*, 2010). We have demonstrated that calcium may have a structural role interacting with surface adhesive factors, but other roles can not be ruled out, for example as a sensory ion for gene expression of biofilm-associated components, although we could not detect changes of *lapA* or *lapF* expression in the presence of calcium. In contrast, calcium enhances the production of extracellular proteins and reduces the synthesis of flagella and flagellin in *Pseudoalteromonas* sp. (Patrauchan *et al.*, 2005) and in mucoid biofilms of *P. aeruginosa* calcium affects the expression and stability of bacterial extracellular products; an eightfold increase in expression of the *alg*

gene, responsible for the synthesis of the exopolysaccharide alginate, has been reported in the presence of calcium (Sarkisova *et al.*, 2005).

The switch from free-swimming cells to surface communities involves many physiological changes, and bacteria have developed complex regulatory networks to minutely regulate it. This includes the synthesis of an extracellular matrix composed by several different components. It is a process that would be expected to be intensively regulated in order to ensure their timely biosynthesis and correct assembly. The existence of multiple sensory systems that allow bacteria to respond to environmental alterations, poses considerable challenges in terms of unwanted crosstalk or conflicting pathways, and a number of solutions have evolved to avoid these effects. In our model, the extracellular matrix of *P. putida* KT2440 is constituted by two adhesins, LapA and LapF and one or more of four different exopolysaccharides. However, how these genes are regulated was not known at the time we initiated this work. In order to further understand the regulation of the matrix components in *P. putida* we have analysed gene expression of *lapA* and *lapF*, using transcriptional fusions to reporter genes that allow quantification by means of β-galactosidase activity or *in vivo* visualization using the green fluorescent protein, gfp. LapF expression showed an increase in activity at the beginning of the stationary phase when cells were grown either in biofilm or in planktonic cultures and we have shown that it is dependent on the alternative sigma factor RpoS (σ^{38} or σ^{S}). The alternative σ^{S} subunit of the RNA polymerase is encoded by the *rpoS* gene and is considered the master regulator of the general stress response in many bacteria, having been widely studied in *Escherichia coli* (Hengge-Aronis, 1999). In this bacterium, biofilm formation is favoured in non-optimal growth conditions or in response to nutritional stresses (Landini, 2009) and many genes involved in stress regulation appeared up-regulated in biofilms, connecting biofilm formation and cellular response to stress. In *E. coli* it has been shown that among 17 proteins with increased expression in biofilm cells, 14 were

encoded by *rpoS*-dependent genes (Collet *et al.*, 2008). The main surface component involved in biofilm formation in *E. coli*, known as curli fibers, has been shown to be completely dependent on RpoS and a deletion of *rpoS* negatively affects biofilm formation (Schembri *et al.*, 2003). In *E. coli* RpoS also controls the expression of some GGDEF proteins and the production of the exopolysaccharide cellulose in response to slow growth and other stress conditions (Landini, 2009). In *Pseudomonas aeruginosa*, a mutant affected in *rpoS* produced more biofilm biomass compared to the wild-type (Heydorn *et al.*, 2002), although in a p-nitrophenol degrading *P. putida*, a mutation in the stationary phase σ-factor gene, *rpoS*, causes suppression of biofilm development when co-cultured with the wild type (Maki *et al.*, 2009). In *Vibrio cholerae* RpoS positively controls HapR, a negative regulator of *vps*, therefore a mutant in *rpoS* is affected in biofilm detachment due to an overproduction of the exopolysaccharide VPS (Müller *et al.*, 2007). Contrary to *lapF* expression, the expression of *lapA* is independent on the alternative sigma factor RpoS. It resulted to be more active at the begging of the biofilm, possibly when cells are still not attached, which is consistent with its role in early attachment. An increase in *lapA* activity is also observed at 24 hours in biofilm conditions suggesting that it is possibly involved in later stages of biofilm formation too.

In *P. putida* it has been recently shown that a mutant in *gacS* is affected in biofilm formation (Duque *et al.*, 2012). GacS is the sensor component of the two component system, GacS/GacA, which is present in a wide variety of Gram-negative bacteria and regulates diverse bacterial processes such as expression of virulence factors and synthesis of secondary metabolites and antimicrobial compounds (Rich *et al.*, 1994, Pernestig *et al.*, 2001, Heeb & Haas, 2001). Once the response regulator GacA is phosphorylated by GacS, it activates or represses the expression of regulatory genes, at the transcriptional level. (Heeb & Haas, 2001). It was first described in *P. syringae* pv. *syringae* that a mutant in *gacS* presented reduced

virulence and ecological fitness (Hirano & Upper, 2000). GacA was discovered as essential for antifungal activity (antibiotic and cyanide production) in the plant beneficial bacteria *Pseudomonas fluorescens* CHA0 (Laville *et al.*, 1992). The GacS/GacA two component system is generally involved in either the biocontrol ability of plant-beneficial bacteria or in the virulence capacity of pathogenic bacteria. 20 GacS and GacA homologs have been identified in enteric bacteria, fluorescent pseudomonads, *Vibrio* and *Azotobacter* (Heeb & Haas, 2001). The expression of the cholera toxin depends on the GacS homolog, VarA, in *V. cholerae* (Wong *et al.*, 1998) and a *varA* mutant is impaired in skin and systemic infections by *Vibrio vulnificus* (Gauthier *et al.*, 2010). The opportunistic pathogen *P. aeruginosa* has shown less virulence in plant and animal tissues when *gacS* or *gacA* are mutated (Jander *et al.*, 2000, Rahme *et al.*, 2000). In the uropathogenic *E. coli* DS17 the two component system BarA/UvrY is responsible for the ability to switch between different carbon sources, thus a mutant in the two component system presented reduced fitness and less virulence (Tomenius *et al.*, 2006). In *P. fluorescens* F113 swimming motility is under negative control of this two-component system since mutants in *gacS* or *gacA* were almost 150% more motile than the wild-type (Martinez-Granero *et al.*, 2006). In addition, it has been demonstrated that this down-regulation takes place through the repression of FleQ, a flagellar master regulator (Navazo *et al.*, 2009). In a random mutagenesis screening for mutants affected in biofilm formation we isolated a mutant affected in *gacS* in which the ability to form a biofilm was significantly reduced. We have demonstrated that expression of *lapF* is fully abolished in the *gacS* mutant, as was the case by mutating *rpoS*. In fact, regulation by GacS seems to be via RpoS, since expression from the *rpoS* promoter is GacS-dependent. The connection between the two-component system and stress resistance has been previously reported in *P. fluorescens* given that *rpoS* was positively regulated by the two-component system (Whistler *et al.*, 1998). In *E. coli* the regulation of *rpoS* takes place at different levels although the transcriptional level seems less important, contrary to *Pseudomonas* spp. (Bertani *et*

al., 2003) where the transcriptional regulator PsrA binds to the DNA sequence and induces expression of *rpoS* (Kojic *et al.*, 2002). It is noteworthy that the phenotype of a mutant in *gacS* differs from a *lapF* mutant as the first one resulted to be more affected than the second. This mutant is able to attach to the surface but it is clearly impaired in the development of the biofilm. Interestingly, *lapA* expression is also reduced, although not completely abolished, in a *gacS* mutant. Given that *lapA* does not depend on RpoS, there must be another alternative route for this influence of GacS on *lapA*. The connection of the two-component system GacS/GacA with biofilm regulation has been recently reported in *Azotobacter vinelandii* where this system regulates the synthesis of alginate, an important exopolysaccharide in the assembly of the extracellular matrix necessary for the formation of bacterial biofilms (Manzo *et al.*, 2011). Two additional sensor kinases upstream the GacS/GacA system, RetS and LadS, have been shown to regulate biofilm formation in *P. aeruginosa* through the GacS/GacA pathway. The RetS via downregulates biofilm formation while LadS induces biofilm formation and a chronic state of the infection (Goodman *et al.*, 2004). It is still unknown if the expression of the different exopolysaccharides of *P. putida* is under the regulation of either RpoS or the two-component system. in *E. coli* K-12, for example, a mutant in *rpoS* overproduced exopolysaccharide (Ionescu & Belkin, 2009).

Based on our results it is tempting to speculate that the loss of biocontrol activity in *P. fluorescens* or the reduced virulence observed in *P. aeruginosa* when *gacS* is mutated could be due not only to the lack of production of secondary metabolites but also to reduced ability to colonize plant or human tissues. In accordance with this hypothesis, a recent work has demonstrated that the biocontrol competence of *Bacillus subtilis* depends on its ability to develop a biofilm (Chen *et al.*, 2012).

A relatively recent report has demonstrated an inverse relationship between flagellum synthesis and polysaccharide production in *P. aeruginosa* PAO1 given that the flagellar master regulator FleQ represses the transcription of the *pel* operon involved in the Pel exopolysaccharide biosynthesis (Hickman & Harwood, 2008). The relationship between flagella and polysaccharide production suggests that the flagellum functions as a mechanosensor for adhesion and biofilm formation, triggering the expression of genes involved in biofilm formation (Petrova *et al.*, 2012). Contributing to this idea, our results have demonstrated that FleQ modulates expression of *lapA*. The secondary messenger cyclic di-GMP seems to participate in this modulation. High levels of c-di-GMP increase expression of *lapA*, but this effect is not observed in a *fleQ* mutant, suggesting that FleQ is required for c-di-GMP control of *lapA*. It has been recently reported that in *P. aeruginosa* c-di-GMP determines whether FleQ functions as a positive or negative regulator of expression of Pel (Baraquet *et al.*, 2012)

The *P. aeruginosa* biofilm matrix is mainly composed of two types of exopolysaccharides which have been shown to play different roles in the development of the biofilm. A recent study has identified a novel surface protein involved in *P. aeruginosa* biofilms, called CdrA, whose functionality has been shown to be dependent on the Psl polysaccharide. It has been suggested that this protein is able to link Psl to the cell surface (Borlee *et al.*, 2010). In *Staphylococcus epidermidis*, it has been suggested that the adhesive protein Aap interacts with PIA (Polysaccharide intercellular adhesin) also called PNAG (polymer β-1-6-linked *N*-acetylglucosamine) (Hussain *et al.*, 1997). Two works published in parallel with the development of this Thesis contributed to the knowledge of the role played by four different exopolysaccharides (alginate, cellulose and two species-specific polysaccharides, Pea and Peb) in *P. putida* biofilms. High intracellular levels of the secondary messenger c-di-GMP produced by the overexpresssion of *rup4959*, an intracellular response regulator with diguanilate cyclase activity, causes a

pleiotropic phenotype that is Pea-dependent and it is relatively altered in *lapA* and *lapF* mutants. These results prompted us to suggest that LapA and/or LapF may be interacting with one or more exopolysaccharides. In addition, we have demonstrated the existence of an intriguing regulatory network connecting LapA and LapF with exopolysaccharide expression and production.

Compensatory balance in the production of matrix components has been recently reported in *P. aeruginosa*, where the lack of the surface polysaccharide Psl induces overproduction of Pel, and in the same way, in the absence of Pel the production of alginate is enhanced (Ghafoor *et al.*, 2011). In our case, the overexpression and overproduction of Pea in *lapA* and *lapF* mutants is not enough to restore the ability to form a biofilm in those strains. This result suggested that adhesins and exopolysaccharides are not redundant within the biofilm developmental process in *P. putida*. In addition, the pleiotropic phenotype caused by increased production of c-di-GMP (by *rup4959* overexpression) is LapA-dependent and the overexpression of exopolysaccharides in those conditions cannot restore biofilm formation in a *lapA* mutant (Matilla *et al.*, 2011). This may not be the same for exopolysaccharides, as a recent report has demonstrated that the exopolysaccharides Psl and Pel have redundant roles within the biofilm of *P. aeruginosa* (Colvin *et al.*, 2012). In *Salmonella enterica* the overproduction of curli fimbriae but not cellulose, is able to compensate biofilm deficiency of a mutant in *bapA* (Latasa *et al.*, 2005).

Results from competitive rhizosphere colonization have revealed that besides *lapA* and *lapF* mutants, all mutants affected in any of the four clusters involved in exopolysaccharide production were less competitive than the wild type, although this defect was less obvious in a cellulose-deficient mutant. However, only Pea and Peb have been shown to play a role in biofilm stabilization (Nilsson *et al.*, 2011). The mutant in the alginate cluster, although not affected in biofilm

formation, was the most affected in our competitive colonization assays, suggesting that alginate may be a specific exopolysaccharide for attachment to biotic surfaces. These results support the idea that environmental conditions influence directly the composition of the matrix and that the same exopolysaccharide could perform different roles according to those environmental conditions.

Our knowledge of the molecular mechanisms involved in biofilm formation in *Pseudomonas putida* has increased over the last years and accordingly with McEldowney & Fletcher (1986) adhesion can not be attributed to any specific type of interactions. Collectively, this Thesis provides a detailed view of the structure of *P. putida* biofilms and contributes to characterize novel elements involved in the complex process of biofilm formation and its regulation.

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V. CONCLUSIONS

The results obtained in this Thesis have prompted us to conclude the following:

1- LapF is a very large protein whose structural features (a long repetitive domain and a C-terminal secretion domain) indicate that it belongs to the heterogeneous family of bacterial large adhesins.

2- Once secreted, LapF stays associated to the cell surface, promoting cell-cell interactions that lead to microcolony formation necessary for the maturation of *P. putida* KT2440 biofilms and for the effective colonization of plant seeds and roots.

3- *lapF* is part of a four-gene operon that includes the genes encoding a predicted Type I secretion system (*lapHIJ*), thus ensuring efficient coordination between production and secretion.

4- Calcium modulates biofilm formation in *P. putida* KT2440 in a time-dependent manner. The C-terminal domain of LapF specifically binds calcium, and this binding causes the formation of large protein aggregates, which we propose could be the basis for the LapF-mediated interaction between cells.

5- The expression patterns of *lapA* and *lapF* are consistent with their respective roles in biofilm development. Transcription of *lapF* starts late during growth, as expected given its participation in microcolony formation, whereas *lapA* shows an early peak of expression and then a second increase in stationary phase. This suggests that LapA is not only involved in early attachment but also in biofilm maturation.

6- Expression of *lapA* and *lapF* is influenced by high intracellular levels of c-di-GMP in opposite ways. Positive regulation of *lapA* by high levels of c-di-GMP occurs through the flagellar master regulator FleQ while negative regulation of

lapF expression by c-di-GMP takes place in a FleQ-independent manner. Both genes are regulated by the GacS/GacA two-component system, indicating that environmental signals yet to be identified modulate the process of biofilm formation by *P. putida*.

7- There exist structural and regulatory interconnections between extracellular matrix components of *P. putida* biofilms. Lack of LapA or LapF provokes increased expression of the species-specific exopolysaccharide Pea, and the two adhesins contribute to the Pea-dependent pleiotropic phenotype observed with high intracellular levels of c-di-GMP.

8- The four different exopolysaccharides produced by *P. putida* KT2440 have non-redundant roles in competitive colonization of the rhizosphere.