~ Tesis Doctoral ~

Bases genéticas de la formación de biofilms bacterianos en superficies abióticas y vegetales

Fátima Yousef Coronado

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ESTACIÓN EXPERIMENTAL DEL ZAIDÍN

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BASES GENÉTICAS DE LA FORMACIÓN DE BIOFILMS BACTERIANOS EN SUPERFICIES ABIÓTICAS Y VEGETALES

Memoria que presenta la licenciada Fátima Yousef Coronado para optar al título de Doctora

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INTRODUCCIÓN

1. Biopelículas

Los estudios fisiológicos, proteómicos, genómicos y comportametales bacterianos se han llevado a cabo tradicionalmente usando cultivos planctónicos, que aunque han proporcionado gran cantidad de información sobre las actividades celulares, han dado de lado una de las formas de persistencia más extendidas y reconocidas en ecología bacteriana, el biofilm o biopelícula. Estas biopelículas se defininen como "comunidades asociadas a superficies rodeadas de una matriz extracelular producida por ellas mismas" (Costerton *et al.,* 1995) y son un fenómeno habitual en la naturaleza, ya reseñado por Arthur Henrici en 1933, al observar que en su mayor parte, los microorganismos acuáticos no se encontraban en forma de células individuales nadando libremente, sino agrupadas sobre superficies sólidas sumergidas (Henrici, 1933). Su composición puede ser mono-especie o multi- especie, formándose sobre un amplio número de superficies bióticas o abióticas. En la naturaleza, generalmente las biopelículas se componen de varias especies, siendo el caso más conocido el de los biofilms formados en la boca, donde la especie dominante es *Streptococcus mutants* pero se han detectado más de 200 especies diferentes.

La formación de biopelículas es una de las características esenciales del ciclo de desarrollo bacteriano (Davey & O'Toole, 2000). Es una actividad colectiva, en la que se presentan fenómenos de comunicación intercelular, y una de las primeras en aparecer entre los procariotas. Stoodley et al. (2002) infieren esto de observaciones que revelan que la capacidad de formar biopelículas por archeas y bacterias pertenecientes a las líneas filogenéticamente más antiguas. Los registros fósiles revelan la existencia de biopelículas con una antigüedad estimada de 3.25 billones de años. Stoodley et al. (2002) hipotetizan que en contra de la asunción general de que las células con estilo de vida planctonico se desarrollaron primero, ambos sistemas, biopelícula y vida planctónica, pudieron evolucionar de forma simultánea, en las condiciones fluctuantes y extremas de la Tierra primitiva que acogió los primeros pasos de la evolución bacteriana y donde las biopelículas habrían proporcionado una protección contra temperaturas extremas, exposición a UV y variaciones en el pH entre otros.

A pesar de que las observaciones de estas comunidades se remontan al principio del siglo XX, ha sido en la última década cuando se ha empezado a reconocer de forma generalizada la importancia de la formación de biopelículas como estrategia de supervivencia microbiana. El interés por su estudio ha hecho que en los últimos años se hayan desarrollado un gran número de técnicas y métodos diseñados específicamente para el análisis de la vida bacteriana en comunidades multicelulares.

1.1 Biopelículas en la esfera antropológica

La formación de biopelículas como parte del programa de desarrollo celular confiere serias ventajas adaptativas relacionadas con la colonización y persistencia. Desde un punto de vista antropológico se convierte tanto en un problema, como en una ventaja o herramienta. A continuación se exponen algunos ejemplos:

<u>Los biofilms como problema</u>

La capacidad de los microorganismos para colonizar superficies sólidas con eficacia tiene gran impacto en numerosas actividades humanas y constituye un serio inconveniente en sanidad humana y animal (Furukawa et al., 2006), debido a su papel como reservorios de patógenos y al hecho de que estas poblaciones sésiles son más resistentes que las bacterias planctónicas a la acción de antibióticos y biocidas (Mah et al., 2003).

- Se ha puesto de manifiesto la importancia de biofilms en relación con infecciones derivadas de implantes médicos (Ramage et al., 2006), oclusión de catéteres (Zhang et al., 2011), queratitis asociadas al uso de lentes de contacto (Zegans et al., 2002), o colonización de tejido pulmonar por el patógeno oportunista *Pseudomonas aeruginosa* en enfermos de fibrosis quística (Costerton et al., 2003), entre otras.

- Los biofilms pueden causar problemas de biodeterioro de materiales (Gu et al., 1998) o biocorrosión (Beech et al., 2005), como consecuencia de la combinación de reacciones biológicas y abióticas de transferencia de

electrones (por ejemplo reacciones redox de metales, favorecidas por la actividad microbiana).

- Afectan a actividades industriales y agrícolas: el establecimiento de poblaciones sésiles de bacterias fitopatógenas sobre superficies vegetales (raíces, hojas o semillas), favorece la persistencia y constituye la primera etapa para la posterior invasión de tejidos (Wilson et al., 1999; Monier & Lindow, 2003). Además, es cada vez más evidente el posible papel de los productos frescos como reservorios y transmisores de patógenos humanos (como en el reciente caso de dispersión de *E. coli* enterohemorrágica en Europa). A nivel industrial, además de los posibles problemas de biodeterioro, en ocasiones se hace imposible deshacerse de biopelículas que cubren las maquinas y exponen a infecciones repetitivas a los operarios (S. Kirkelumd, comunicación personal)

Utilidad de biopelículas

La capacidad de las bacterias de formar comunidades multicelulares asociadas a superficies tiene también aplicaciones biotecnológicas (biorreactores), agrícolas y en salud humana. Las bacterias establecen sistemas simbiontes con otros organismos y se sabe que determinados equilibrios entre comunidades son necesarios para evitar procesos patogénicos.

- Biopelículas de microorganismos con capacidad degradadora de distintos compuestos se emplean rutinariamente en procesos como la depuración de aguas residuales, o en sistemas de biorremediación en reactores, con membranas o distintas matrices porosas, elásticas, etc. como sustratos para el desarrollo del biofilm (Nicolella et al., 2004).

- Un ejemplo de relación mutualista en la que la colonización de superficies es importante, es la que se establece entre las PGPRs (plant growth promoting rhizobacteria) y las plantas. Estas bacterias colonizan la superficie de las raíces, de donde obtienen nutrientes provenientes de los exudados radiculares, y a su vez tienen un efecto beneficioso sobre la planta, a menudo convirtiéndose en un elemento clave en procesos de control biológico de patógenos (Chin-A-

Woeng et al., 2000; Espinosa-Urgel, 2004). Matilla y colaboradores (Matilla et al., 2010) han demostrado, por ejemplo, que la colonización de raíces por *Pseudomonas putida* KT2440 protege a *Arabidopsis thaliana* activando la resistencia sistémica de la planta, tal como se detalla más adelante. Otros ejemplos incluyen la producción de metabolitos antifúngicos como el 2,4-diacetilfloroglucinol, o la solubilización de fosfato favoreciendo su biodisponibilidad para la planta.

- En infecciones por candidiasis se recurre habitualmente a repoblación masiva con *Lactobacillus* para restablecer una flora equilibrada en la superficie de la mucosa y eliminar los efectos adversos de la proliferación de *Candida*.

1.2. Técnicas de estudio de las biopelículas

Uno de los métodos más extendidos y que permite un screenning masivo es la cuantificación de la biopelícula de forma indirecta mediante la tinción con cristal violeta en placas multipocillo (O'Toole & Kolter, 1998). También se ha usado la morfología de colonia en placas de agar que contienen el tinte rojo congo, como una referencia de la producción de EPS (Friedman & Kolter, 2004).

Para visualizar la formación de la biopelicula en condiciones dinámicas, con el fin de evaluar las interacciones celulares, las distintas etapas de desarrollo del biofilm, o el efecto de alterar las condiciones ambientales, el sistema usado de forma preferente es el denominado "flow cell" (celda de flujo), en combinación con microscopía láser confocal (CLSM, confocal laser scanning microscopy). El sistema se ilustra en la figura 1 (Stenberg & Tolker-Nielsen, 2006). Mediante proteínas reporteras fluorescentes se puede examinar el desarrollo de la biopelícula así como la organización espacial, la expresión génica o la resistencia a antibióticos en tiempo real y bajo diferentes condiciones. Además, el uso de tinciones vitales fluorescentes permite analizar el estado fisilógico de las células. La base del CLSM es la captura de imágenes de alta precision a intervalos de profundidad determinados, que posteriormente se ensamblan mediante software específico para generar imágenes tridimensionales del

biofilm. En combinación con programas matemáticos como el COMSTAT (Heydorn), los datos obtenidos permiten además calcular parámetros como la biomasa, superficie cubierta por el biofilm, volumen, distancia de difusión, etc.

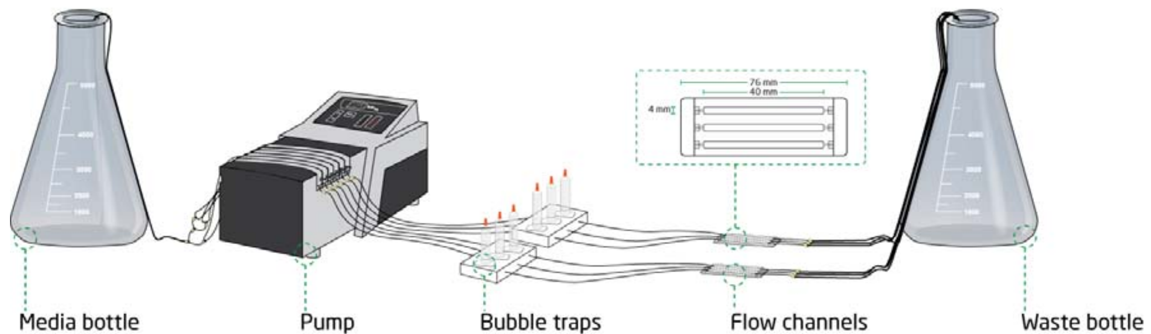

Figura 1. Esquema del sistema de celdas de flujo para el estudio de biofilms.

Otros métodos de extrema resolución son la microscopia de fuerza atómica (AFM) y la microscopia electrónica de barrido (SEM), pero la necesidad de tratar las muestras para cada medida no permite un seguimiento de la dinámica del biofilm. Se han empleado también métodos de estudio de la expresión génica a nivel global, mediante proteómica (Sauer & Kamper 2001) o microarrays (Whiteley et al., 2001; Lazazzera, 2005). Estos métodos permiten obtener información general de genes inducidos o reprimidos, pero tienen la limitación de no reflejar la heterogeneidad poblacional que se da en biofilms.

1.3. Etapas de formación de biopelículas en superficies abióticas.

En todos los tipos de biopelículas los pasos fundamentales son básicamente tres: la asociación a una superficie de manera estable, la agregación de las células en microcolonias y la maduración de la biopelícula. Uno de los ejemplos más estudiados y a la vez relevante para esta Tesis Doctoral es el modelo de formación de biopelículas en *Pseudomonas aeruginosa*, en condiciones de flujo de nutrientes (Figura 2). Está dividido en varios pasos:

1. Aproximación individual de las bacterias a la superficie gracias a la movilidad mediada por flagelo, **“swimming”.**
2. En condiciones de abundancia de nutrientes, una subpoblación de bacterias inician la **adhesión reversible** por un polo de la célula. En este primer contacto, la bacteria puede abandonar la superficie, o permanecer asociadas a ella.
3. Las células se unen a la superficie por el eje longitudinal, iniciando la formación de una monocapa de bacterias sobre la misma (**adhesion irreversible**).
4. Formación de **microcolonias**, mediante agragación y división de las bacterias sobre la superficie.
5. Las microcolonias se desarrollan y quedan envueltas en una matriz de polímeros extracelulares, formando el **biofilm maduro,** con una arquitectura que se caracteriza por macrocolonias en forma de seta, separadas por canales, por los que circula fluido y que suplirían de nutrientes y oxígeno al biofilm.
6. Cuando las condiciones nutricionales empeoran, o el biofilm crece en exceso, se produce la dispersión del mismo, bien por desprendimiento de fragmentos del biofilm maduro, o porque células individuales recuperan su carácter móvil y pasan al estado planctónico.

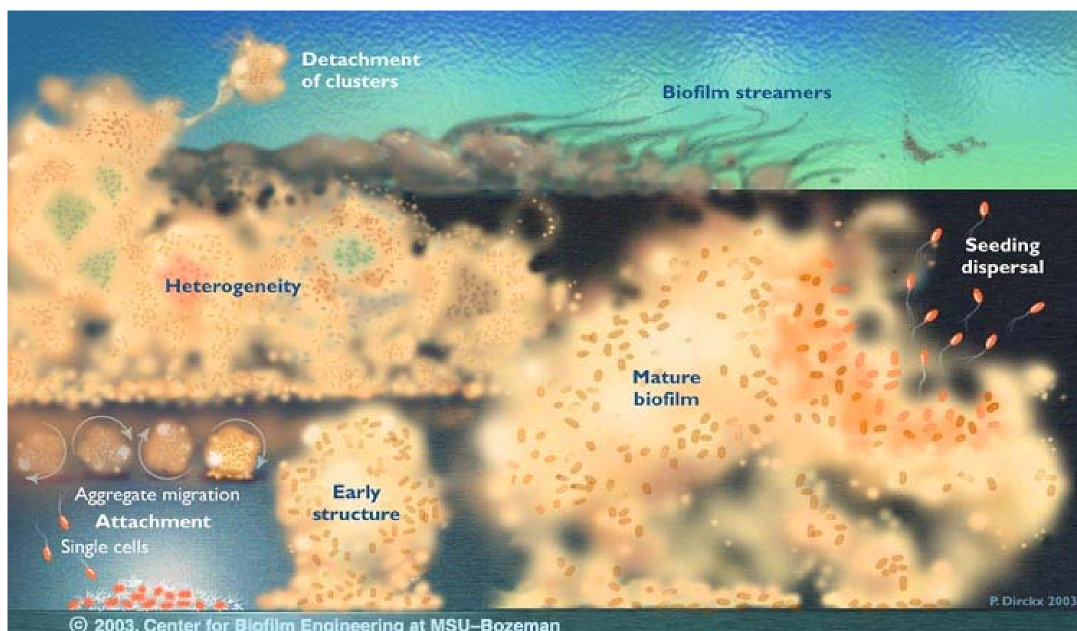

Figura 2. Etapas de la formación de biopelículas en *Pseudomonas aeruginosa.*

En *Pseudomonas putida* KT2440, la bacteria objeto de estudio en esta Tesis Doctoral, las etapas de formación de biopelículas son muy similares a las descritas para *P. aeruginosa*. Sin embargo, a diferencia de esta última, los biofilms maduros de *P. putida* no suelen presentar una estructura de macrocolonias tan definida como en *P. aeruginosa*, siendo las macrocolonias más irregulares y la cobertura de la superficie más uniforme (Figura 3).

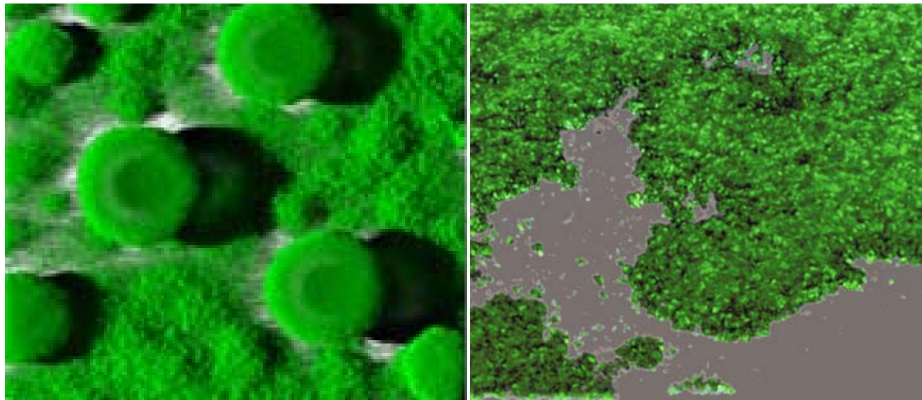

Figura 3. Reconstrucción tridimensional de biofims maduros de *P. aeruginosa* PAO1 (izquierda) y *P. putida* KT2440 (derecha).

El interés del estudio de biofilms en *Pseudomonas* viene dado por la gran ubicuidad de estas bacterias (Figura 4). Los miembros de esta familia se han muestreado en una gran variedad de ambientes terrestres y acuaticos, en plantas, en insectos y como patógenos humanos o saprófitos en diversos sitemas. Su amplia capacidad para la adaptación a diferentes medios y condiciones fluctuantes deriva de su versatilidad metabólica. Esta capacidad adaptativa se ha atribuido además a la potencial flexibilidad de su genoma en el que hay presentes elementos geneticos moviles como fagos, plásmidos, islas génicas y transposones. En el caso de *Pseudomonas putida* KT2440, su importancia radica en la capacidad de promover el crecimiento vegetal y el hecho de ser un microorganismo ampliamente utilizado en aplicaciones biotecnológicas. Con un genoma de tamaño 6.18 Mb, KT2440 carece de los genes correspondientes a determinantes de virulencia (sistema de secreción tipo III, exotoxina A, proteasa alcalina, elastasa, síntesis de rhamnolípidos, exolipasas o

fosfolipasa C). Sin embargo posee una gran versatilidad a nivel de metabolismo. En su genoma hay 350 que codifican transportadores citoplásmicos y de membrana externa, un 25% más que PAO1, utiles para la incorporación o excreción de un amplio rango de de sustratos.

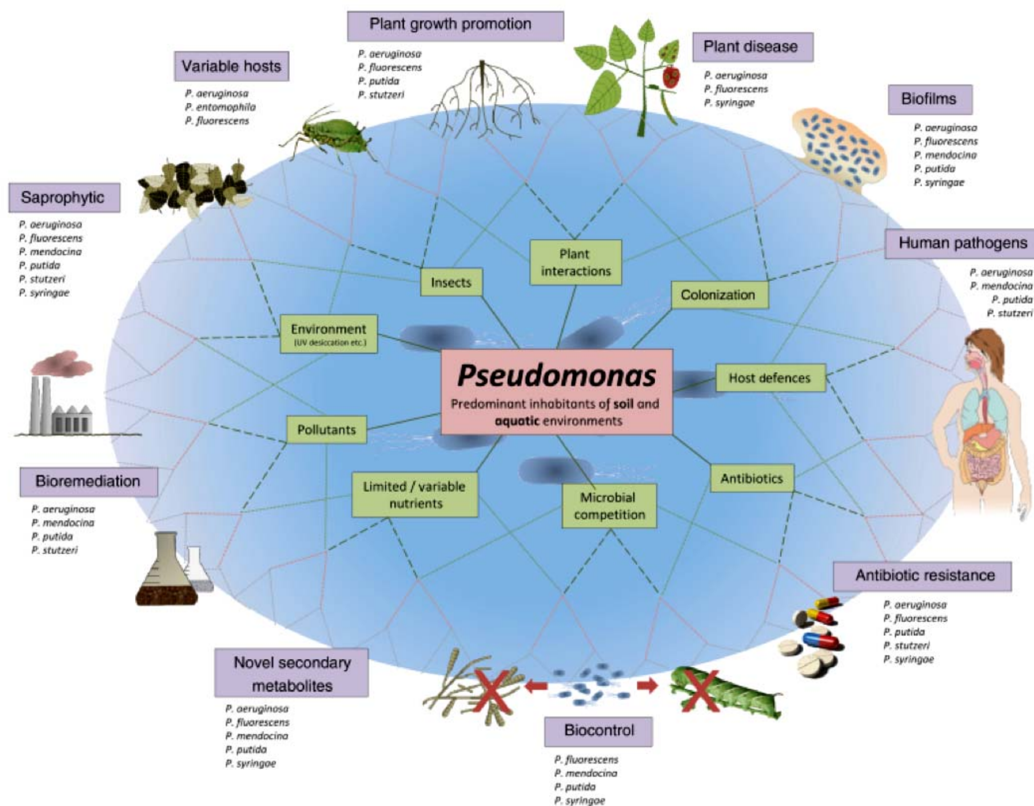

Figura 4. Ubicuidad de *Pseudomonas*

1.4. La matriz extracelular

A la matriz que rodea las macrocolonias en biofilms maduros se la denomina habitualmente EPS (sustancia polimérica extracelular). Aunque a menudo el término EPS se usa como sinónimo de exopolisacáridos, ya que estos son componentes importantes de la matriz, la composición de ésta varía en cada especie. Así, en biofilms de *Pseudomonas aeuruginosa* estudiados en laboratorio uno de los principales componentes que estabilizan la matriz es el DNA (Yang et al., 2011). Sin embargo, en

Pseudomonas putida, el rol principal parecen tenerlo determinadas proteínas. Otros ácidos nucleicos y lípidos también se han descrito como componentes de la matriz en distintos organismos.

1.4.1 Matriz de la biopelícula en *Pseudomonas putida*

Proteínas

Trabajos previos de nuestro grupo de investigación han permitido identificar dos proteínas (Figura 5) que se exportan al exterior celular con un papel importante en la formación de biofilms por *P. putida*. Ambas fueron identificadas en una primera búsqueda de funciones relevantes para la adhesión de *P. putida* KT2440 a semillas de maíz (Espinosa-Urgel et al., 2000):

- LapA (large adhesion protein A) es la mayor proteína codificada en el genoma de *P. putida* con un tamaño estimado de más de 800 KDa. Estructuralmente consta de cuatro dominios, dos de ellos compuestos por repeticiones imperfectas de 100 (Domino 2) y 218-225 aminoácidos (Dominio 3), respectivamente. Participa en las primeras etapas de formación de biopelículas, siendo esencial para la transición entre las etapas de adhesión reversible e irreversible. En *Pseudomonas fluorescens* existe una proteína homóloga, aunque de menor tamaño. En este microorganismo se ha descrito el mecanismo que controla su recambio en la superficie de la bacteria, el cual se detalla más adelante. En colaboración con el laboratorio del Dr. George O'Toole, donde el gen *lapA* había sido identificado en *P. fluorescens*, se ha determinado el mecanismo de secreción de LapA. Es transportada a la superficie celular a través de un transportador de tipo I específico, cuyos componentes están codificados por los genes *lapE*, *lapB* y *lapC*. Mutantes en cualquiera de estos genes presentan defectos en adhesión a semillas, raíces y partículas de suelo, así como en la transición de la etapa de adhesión reversible a irreversible durante la formación de biofilms sobre superficies abióticas (Hinsa et al., 2003).

- LapF, la segunda proteína en tamaño en *P. putida* KT2440, contiene 64 repeticiones imperfectas de 86-93 aminoácidos. Se localiza en las regiones internas de microcolonias, de manera que es posible detectar focos específicos de localización entre bacterias adyacentes, lo que sugiere un papel de LapF en el establecimiento de interacciones célula-célula (Martínez-Gil et al., 2010). Dicho papel se ve apoyado por su patrón de expresión, dependiente del factor sigma de fase estacionaria RpoS. Esto indica que la proteína LapF ejerce su función en etapas tardías de la formación de biofilms. La caracterización detallada de esta proteína está siendo objeto de una Tesis Doctoral actualmente en realización en el grupo.

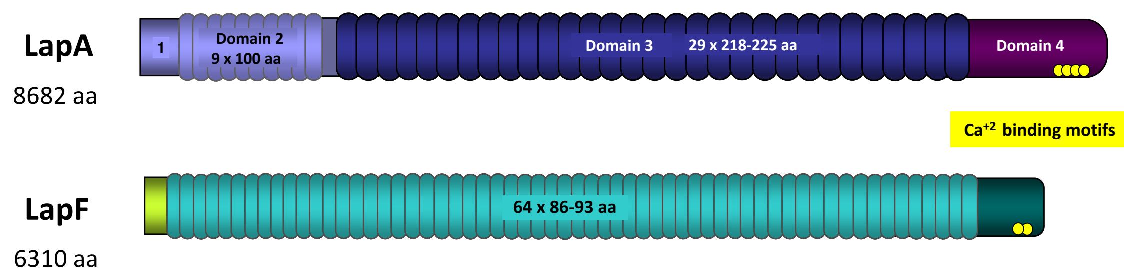

Figura 5. Estructura de LapA y LapF

Ácidos nucleicos

Los acidos nucléicos, aunque presentes en la matriz de *P. putida*, no parecen tener un papel prioritario, tal como se detalla en el Capítulo 3 de esta Tesis Doctoral. El origen de los ácidos nucléicos en la matriz extracelular no está bien definido, pero podría deberse a la lisis de algunas bacterias en el interior de las microcolonias.

Exopolisacáridos

Tienen un papel importante en la formación y estabilidad de la biopelícula en *P. putida* KT2440 aunque no son esenciales para la formación de ésta, y su contribución puede

variar en función de las condiciones ambientales. Los grupos de genes hasta ahora descritos relacionados con la síntesis de exopolisacáridos en esta bacteria son:

- ***pea* (putida exopolysaccharide A) :** operón que codifica las proteínas necesarias para la síntesis de un polímero rico en glucosa, manosa y galactosa que se ha sugerido podría estar envuelto en la interacción célula-célula y participa en la formación de biofilms (Nielsen et al., 2011). Su expresión se induce por estrés hídrico.
- ***peb* (putida exopolysaccharide B):** junto con el polisacárido pea desempeña un papel mayoritario en la estabilización de la matriz (Nilsson et al., 2011).
- ***alg* (síntesis de alginato):** a diferencia de otras bacterias, el alginato solo es importante en KT2440 en determinadas condiciones ambientales, especialmente en respuesta a estrés hídrico - en caso de deshidratación de la envuelta celular aumenta la expresión del operón de forma transitoria. En condiciones normales de laboratorio tiene una contibución minoritaria a la matriz del biofilm, y apenas se aprecia producción de alginato.
- ***bcs* (síntesis de celulosa) :** al igual que el alginato, la celulosa tiene un rol minoritario en la formación de biofilms sobre superficies abióticas, aunque mutantes en *bcs* presentan ligeramente inferior formación de biofilms que la cepa silvestre. Sin embargo este operón es importante para la colonización de raíces de maíz (Nielsen et al., 2011).

1.5. Ventajas evolutivas de la formación de biopelículas

Las células que forman parte de una biopelícula están embebidas en una matriz que varía según la especies que componen el biofilm, pero que en general, se encarga de proteger a las bacterias frente a la desecación, oxidación, acción de algunos antibióticos, cationes metálicos, en cierto grado de la radiación ultravioleta y de la acción de elementos del sistema inmune, en el caso de biofilms relacionados con infecciones. Como consecuencia también de la estructura tridimensional, hay diferencias en el patrón metabólico y de expresión génica en base a la posición de las células en la matriz. Aparecen, por ejemplo, células en estado latente, que son más

resistentes a la acción de antibióticos como los beta-lactámicos, que actúan durante la división celular. Los diversos micro-nichos, favorecen la aparición de mutantes y diferentes fenotipos, confiriendo una ventaja adaptativa a nivel poblacional en un ambiente fluctuante.

La organización tridimensional de la comunidad permite la proximidad entre las células y la presencia de hebras de ADN atrapadas en la matriz, facilitando mediante la interacción célula-célula la incorporación e intercambio de material genético. Se ha observado que el proceso de transmisión de plásmidos por conjugación está favorecido en biofilms, y a su vez la presencia de plásmidos conjugativos en la población parece tener un efecto positivo sobre la formación y estabilidad de biofilms (Dudley et al., 2006). Este efecto probablemente sea debido a las conexiones célula-célula mediadas por pili, aunque también se ha sugerido que se debe al incremento en DNA extracelular observado en estas poblaciones (D'Alvise et al., 2010).

1.6. Eligiendo estilo de vida: mecanismos de regulación

Tal como se ha mencionado más arriba, el cambio de estilo de vida bacteriano está relacionado con el aporte de nutrientes y por tanto el nivel energético de la célula o la comunidad. A nivel molecular, se ha demostrado en varios estudios en *Bacillus subtilis*, *Pseudomonas aeruginosa*, o *Vibrio cholerae*, una relación inversa entre la síntesis del flagelo y la producción de EPS. Ademas se ha comprobado que este cambio esta mediado por los niveles intracelulares del segundo mensajero diguanilato cíclico (di-GMPc): altas concentraciones de esta molécula promueven la transición hacia la vida sésil, mientras que bajas concentraciones determinan la aparición del flagelo y el abandono de la biopelícula, regulando así la adaptación al medio. La síntesis y degradación de di-GMPc se lleva a cabo por proteínas que tienen actividades enzimáticas diguanilato ciclasa y fosfodiesterasa (Figura 6). Estas actividades están presentes en los dominios GGDEF y EAL respectivamente (la nomenclatura se basa en la presencia de dichos aminoácidos conservados, esenciales para la actividad catalítica). A veces una misma proteína presenta ambos dominios y es habitual encontrar en una misma especie bacteriana varias proteínas con estas actividades.

Frecuentemente estos dominios se encuentran fusionados a dominios sensores, lo que indica que las variaciones en la concentración de di-GMPc están controladas por factores ambientales.

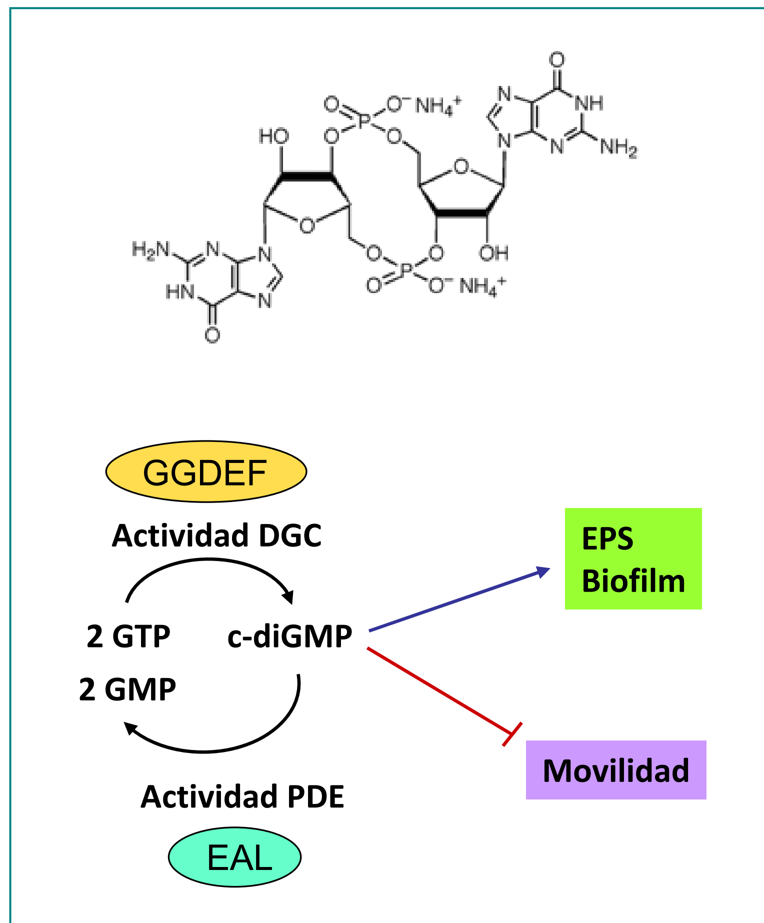

Figura 6. Estructura del segundo mensajero di-GMPc, y su papel en el cambio entre el estilo de vida nómada y sedentario.

En *Pseudomonas fluorescens*, se ha descrito que el di-GMPc participa en el recambio de LapA en la superficie celular. Un trabajo reciente de Newell y colaboradores ha permitido establecer un modelo de regulación de los niveles de LapA en conexión con el di-GMPc (Newell et al., 2011). Cuando existen altos niveles de esta molécula, se produce una asociación del di-GMPc a la proteína LapD, a través de un dominio GGDEF degenerado no catalítico. En esta situación, LapD secuestra a la proteasa LapG en el citoplasma. Dicha proteasa, en ausencia de di-GMPc queda liberada al cambiar la conformación de LapD y se trasloca a la membrana externa,

donde corta a LapA por una secuencia específica. Este corte promovería que la bacteria se despegue del sustrato y pase a estado planctónico.

1.7 Comunicación y biofilms

Entre las bacterias, existen diversos ejemplos de coordinación de respuestas a nivel poblacional. Ejemplos bien conocidos son los de *Myxococcus*, que frente a la privación de nutrientes genera cuerpos fructíferos, la formación de esporas por *Bacillus subtillis,* o la migración de *Serratia liquefaciens* mediante swarming. Las bacterias han evolucionando como organismos interactivos, desarrollando elaborados sitemas de comunicación célula-célula mediados por agentes químicos que permiten estos comportamientos coordinandos, a través del control de la expresión génica. A menudo, esta coordinación de actividades celulares es directamente dependiente de la densidad de la población. Es lo que se define como quórum sensing (Fuqua *et al*., 1994), un sistema de comunicación intercelular basado en la producción, liberación al medio y reconocimiento de moléculas difusibles, llamadas autoinductores (Figura 7), que funcionan por umbrales de concentración. Se han identificado distintas moléculas que funcionan como señales de quorum sensing. Este mecanismo está ampliamente extendido el reino bacteriano. Las señales mejor caracterizadas en bacterias Gram-negativas son las acil-homoserina lactonas (AHL), sintetizadas por proteínas de la familia LuxI y reconocidas por reguladores de la familia LuxR, las cuales pueden variar en cuanto a longitud y grado de insaturación de su cadena lateral, lo que influye en su especificidad. Así, algunos microorganismos son capaces de sintetizar y responder a AHL de distintas longitudes, mientras que en otros casos sólo funcionan como señal AHL de una longitud o estructura específica. También se han descrito otras moléculas señalizadoras como dipéptidos cíclicos, indol, quinolonas, derivados de ácidos grasos o el furanosil borato diéster. En bacterias Gram-positivas, las señales intercelulares descritas corresponden a péptidos de cadena corta modificados.

Aunque hay cierta controversia sobre el papel del quorum sensing en el desarrollo de biofilms, parece evidente que en una situación de elevada densidad de células en poco

espacio, como es el caso de un biofilm, procesos mediados por quorum sensing pueden tener relevancia (Riedel et al., 2001). De hecho, la producción de exopolisacáridos, ciertos tipos de movilidad y mecanismos relacionados con invasividad y virulencia se encuentran bajo este tipo de regulación. También se han descrito procesos de señalización en poblaciones bacterianas asociadas a raíces de plantas (Steidle et al., 2001), aunque no se ha analizado en detalle su posible papel en la colonización.

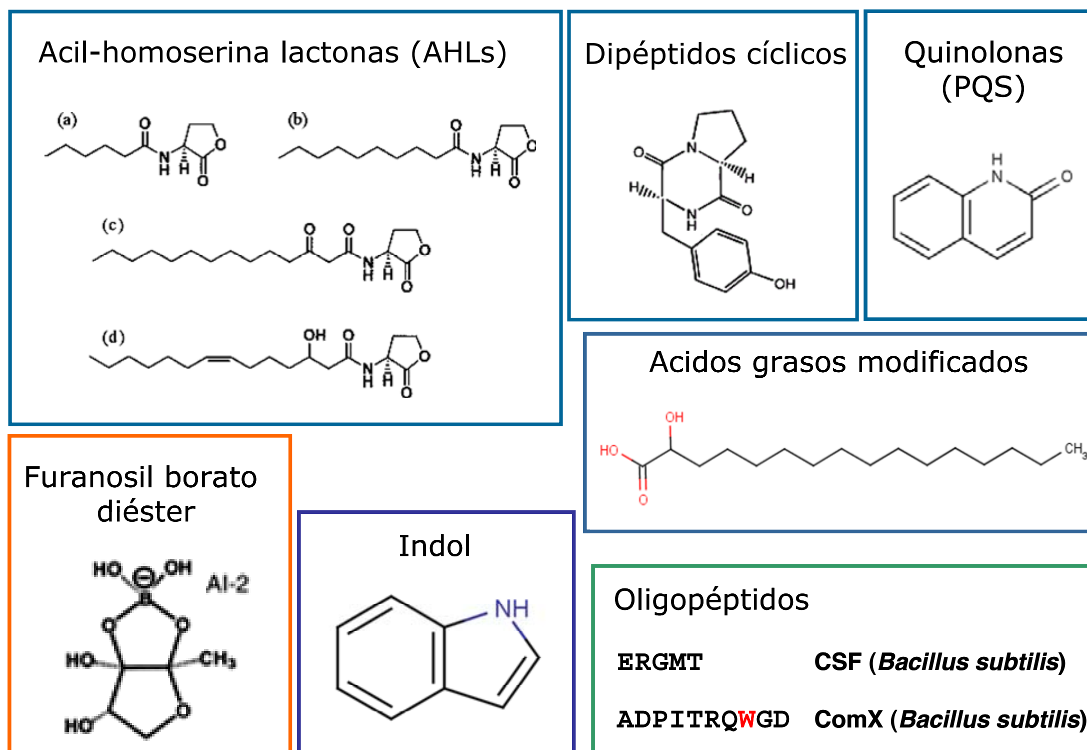

Figura 7. Moleculas involucradas en quorum sensing en distintas bacterias.

Pseudomonas putida KT2440 no produce los autoinductores típicos, pero sí posee un regulador transcripcional perteneciente a la familia de LuxR, llamado PpoR. PpoR no es sensible *in vivo* a la acción de AHLs, pero juega un papel en la supervivencia en sistemas competitivos y la movilidad tipo swarming (Fernández-Piñar et al., 2011). Datos recientes indican la existencia de un sistema de comunicación propio en *P. putida* basado en determinados ácidos grasos (Fernández-Piñar, comunicación personal), así como la capacidad de esta cepa de responder a señales producidas por otras especies de *Pseudomonas*. En concreto KT2440 responde a una quinolona

producida en uno de los dos circuitos de producción de AHLs de *Pseudomonas aeruginosa*, y que a su vez funciona como señal ("*Pseudomonas* quinolone signal", PQS). La producción de PQS por *Pseudomonas aeruginosa* reduce la formación de biopeliculas en *Pseudomonas putida* KT2440, limita la movilidad de tipo swarming y altera el metabolismo del hierro (Fernández-Piñar et al., 2011b).

2. Biopelículas e interacción con plantas

Pseudomonas putida KT2440 se enclava dento del grupo de las rizobacteras promotoras del crecimiento vegetal PGPR (del inglés "Plant Growth Promoting Rhizobacteria") categoria definida por primera vez por Kloepper and Schroth en (XXX). Otros miembros de este grupo incluyen cepas de los géneros *Azoarcus, Azospirillum, Azotobacter, Bacillus, Gluconobacter y Serratia*, entre otros. Estas bacterias colonizan la raíz y sus alrededores, la rizosfera, y ejercen efectos beneficiosos en el desarrollo de las plantas de manera directa o indirecta, tal como se ha detallado más arriba. En contrapartida las bacterias obtienen nutrientes, ya que en torno a la raíz se genera una región nutricionalmente rica a consecuencia de la liberación por la planta de exudados, lisados, etc., que convierten a la rizosfera en una zona capaz de acoger poblaciones bacterianas a densidades de población elevadas.

En el caso de KT2440, se comprobado que activa la respuesta sistémica inducida (ISR) en el sistema modelo *A. thaliana-P. syringae* pv. tomato. En un estudio, que incluyó la variedad silvestre *A. thaliana* Col-0, varios mutantes afectados en rutas de señalización (*jar-1*, *etr1* y *npr1*) y plantas transgénicas NahG, se evidenció que la colonización radicular de plantas Col-0 por KT2440 puede proteger a la parte aérea de las mismas frente a la infección posterior por una bacteria fitopatógena (Matilla et al., 2010). *P. putida* KT2440 presenta la peculiaridad de que, aparentemente, activa dos rutas de señalización, la regulada por etileno/jasmónico (ISR), algo común para otras rizobacterias (Bakker et al., 2007), y la dependiente de ácido salicílico (SAR), de cuya activación son responsables principalmente bacterias fitopatógenas (Vlot et al., 2009; y referencias citadas en éste).

En el proceso de colonización de plantas hay varios puntos clave: la habilidad de sobrevivir a la inoculación en la semilla y adherirse a la misma, la capacidad de multiplicarse en la espermosfera en respuesta a los exudados radiculares, adherirse a la raíz y mantenerse a lo largo de ésta durante su crecimiento. Los trabajos realizados hasta la fecha en este campo se han llevado a cabo fundamentalmente estudiando poblaciones de *Pseudomonas* una vez que éstas se encuentran ya establecidas en la rizosfera (Lugtenberg et al., 2003), mientras que las etapas iniciales de desarrollo de las mismas han recibido muy poca atención. Existían datos con relación al papel que juegan la movilidad flagelar y la quimiotaxis en el inicio del establecimiento de interacciones planta-microorganismo (Turnbull et al., 2001; Bashan, 1986), pero apenas se habían realizado estudios exhaustivos sobre los mecanismos de adhesión bacteriana a superficies vegetales. Únicamente se había descrito el posible papel de flagelos (DeFlaun et al., 1994) o pili tipo IV (Dörr et al., 1998) como adhesinas, y la existencia de procesos de "quorum sensing" en poblaciones asociadas a raíces (Steidle et al., 2001).

Un aspecto poco estudiado, y que sin embargo tiene enorme importancia, es la formación de comunidades sésiles asociadas a semillas. La colonización de semillas es generalmente el primer paso que determina el posterior establecimiento de poblaciones bacterianas epifitas o rizosféricas (Espinosa-Urgel et al., 2002). Además, las semillas constituyen el vehículo principal de dispersión de bacterias asociadas a plantas, tanto patógenas como mutualistas (Hirano & Upper, 2000), y son la mejor vía para introducir microorganismos beneficiosos en aplicaciones agrícolas. La inoculación de semillas puede ser clave en la protección temprana de plantas frente a patógenos (Tombolini et al., 1999).

Nuestro grupo ha sido pionero en este área de investigación, al haber identificado, mediante mutagénesis al azar por transposición, una serie de genes que juegan un papel importante en la adhesión de *Pseudomonas putida* a semillas (Espinosa-Urgel et al., 2000). La publicación de la secuencia completa y anotada del genoma de esta bacteria ha permitido un estudio detallado de las proteínas codificadas por dichos genes. Uno de los aspectos más destacados de estos trabajos fue la caracterización del gen mus-24 (redenominado *lapA*). Como ya se ha mencionado, LapA juega un papel

esencial en adhesión a semillas y formación de biofilms sobre superficies abióticas, constituyendo por tanto una de las primeras adhesinas multifuncionales descritas hasta la fecha en bacterias Gram-negativas (Hinsa et al., 2003).

Tabla 1. Funciones y conservación de genes implicados en adhesión a semillas en *Pseudomonas putida* KT2440. La nomenclatura corresponde a la asignada en www.tigr.org. 2. Existe un gen con cierta similitud pero no homólogo.

Pseudomonas putida KT2440			Presencia en *Pseudomonas*		
mutante	**locus**	**Proteína: características/función**	***P. fluorescens***	***P. aeruginosa***	***P. syringae***
mus-5	PP4615	DdcA. Proteína de membrana, 156 aa. Expresión dependiente de densidad celular (quorum sensing)	+	+	+
mus-9	PP5290	transposasa de *IS*Ppu10, 300 aa	-	-	-
mus-13	PP4641	CstA. Proteína de membrana transporte dipéptidos	+	+	+
mus-20	PP0806	LapF	-	-	-
mus-21	PP0713	bomba de flujo, posiblemente implicada en detoxificación	-	-	-
mus-24	PP0168	LapA	+	-	-
mus-27	PP1449	HlpA. Componente secretado de sistema TPS similar a hemolisinas, 1560 aa	+	+-[1]	-

[1]Existe un gen con cierta similitud pero no homólogo.

Los resultados obtenidos con LapA sugerían un solapamiento entre las funciones necesarias para la formación de biofilms y las requeridas para colonizar raíces. Esto unido a las observaciones mediante microscopía electrónica realizadas en nuestro laboratorio, en las que se aprecian microcolonias de KT2440 sobre la superficie de la raíz embebidas en una matriz (Figura 8), indican la existencia de mecanismos comunes en ambos procesos. El análisis de estos mecanismos, la ubicuidad de proteínas similares a LapA en el mundo bacteriano, y la identificación de elementos que regulan las transiciones entre los modos de vida sésil yplanctónico constituyen el eje de esta Tesis Doctoral.

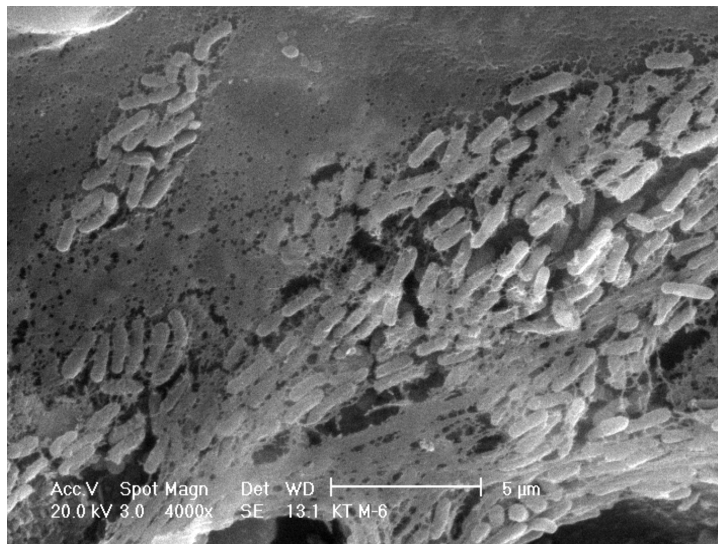

Figura 8. Celulas de *P. putida* KT2440 sobre la raíz de una planta de maíz, observadas mediante microscopía electrónica de barrido.

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OBJETIVOS

Los objetivos planteados en esta Tesis Doctoral han sido los siguientes:

- Analizar la presencia y distribución filogenética de grandes proteínas secretadas, con un posible papel en adhesión, entre bacterias cuyo genoma completo está disponible en las bases de datos. Se pretendía establecer si estas proteínas eran ubicuas, sus características y la similitud entre ellas.

- Identificar nuevos determinantes genéticos implicados en la colonización de superficies vegetales por *Pseudomonas putida* KT2440, estableciendo también su posible papel en la formación de biofilms. El objetivo era completar el análisis genético de la adhesión bacteriana a semillas y raíces y establecer las similitudes y diferencias entre el establecimiento de poblaciones sésiles sobre superficies abióticas y sobre superficies vegetales.

- Identificar y caracterizar mutaciones que favorecieran el establecimiento de biofilms por *P. putida* KT2440, con el fin de explorar posibles mecanismos reguladores de la transición de vida planctónica a vida sésil y viceversa en esta bacteria.

Los trabajos realizados se recogen en las siguientes publicaciones, que constituyen los Capítulos 1, 2 y 3 de esta Tesis:

Yousef, F., and Espinosa-Urgel, M. (2007) *In silico* analysis of large microbial surface proteins. *Res. Microbiol.* **158:** 545-550.

Yousef-Coronado, F., Travieso, M.L., and Espinosa-Urgel, M. (2008). Different, overlapping mechanisms for colonization of abiotic and plant surfaces by *Pseudomonas putida*. *FEMS Microbiol. Lett.* **288:** 118-124.

Yousef-Coronado, F., Yang, L., Molin, S., and Espinosa-Urgel, M. (2011) Selection of hyperadherent mutants in *Pseudomonas putida* biofilms. *Microbiology* (SGM) **157:** 2257-2265, y foto portada.

CAPITULO 1

Análisis *in silico* de grandes proteínas microbianas de superficie

Publicado en: **Yousef, F., and Espinosa-Urgel, M.** (2007) *In silico* analysis of large microbial surface proteins. *Res. Microbiol.* 158: 545-550.

ABSTRACT

We have analyzed the longest predicted proteins encoded in complete microbial genomes. They can be separated in two main classes; non-ribosomal peptide synthetases involved in secondary metabolism, and surface proteins, many of them with a predicted or experimentally observed role in bacterial adhesion and biofilm formation. Such proteins, generally showing a repetitive structure, are widespread among prokaryotes and can be grouped in several different families, based on sequence alignment, characteristics and predicted motifs. This classification may help in the characterization of newly described adhesins. The results of this study indicate that cell-cell interactions and biofilm formation are common events in the microbial world and take place via similar molecular mechanisms.

Keywords: adhesins, biofilm, phylogenetic distribution, calcium

1. Introduction

Bacteria are commonly found forming multicellular communities associated to solid surfaces, known as biofilms. The study of microbial adhesion to solid surfaces and biofilm formation has become an area of increasing interest in microbiology [5,12], and genetic determinants involved in these processes have been identified in different bacteria [7,9,10,14,19]. It is becoming apparent that an important role is played by proteins localized in the bacterial surface that may directly mediate the interaction between bacterial cells and the solid substratum [9,14,15]. These adhesins are often large proteins with a repetitive structure. An example of such proteins is LapA, a 8683 amino acids protein identified in the soil and plant root-colonizing bacteria *Pseudomonas putida* and *Pseudomonas fluorescens*. LapA is secreted through an ABC transporter, remaining loosely associated to the cell surface [9], and plays a key role in biofilm formation on abiotic surfaces, as well as in bacterial colonization of plant seeds [6,9]. Four domains can be distinguished in this protein, two of them (comprising 75% of the protein sequence) showing distinct repetitive structures. It has been postulated that these domains represent different functionalities with respect to bacterial attachment to surfaces, but there is no experimental or homology-based evidence so far. A second large repetitive protein in *P. putida*, which we have named LapF, is also important for bacterial colonization of plant surfaces [6; Espinosa-Urgel, unpublished]. LapF is 6310 amino acids long and presents 63 imperfect repeats of 85-90 amino acids.

Other large adhesins, namely Esp and Bap, have been described in some Gram-positive bacteria (*Enterococcus* and *Staphylococcus*), where they also mediate surface colonization and biofilm formation [15]. Although smaller than LapA or LapF (around 2000 amino acids), these proteins present a similar structural organization.

Maintaining and expressing genes of such length and sequence redundancy must be costly for bacteria, and would imply that a significant selective pressure exists to retain them. This idea made us wonder what were the largest gene sizes supported by small genomes and how extended was the existence of such large proteins among prokaryotes. Taking advantage of the current advances in sequencing data, we have surveyed microbial genomes for large predicted proteins, and selected for a comparative analysis those known to participate or that might be involved in adhesion processes, as well as other predicted surface-associated proteins. Their structural characteristics and domain composition have been analyzed, and several families have been defined.

2. Materials and methods

2.1. Data compilation

Protein size distribution in 351 complete microbial genome sequences in the NCBI database was examined. The size, identity and annotation of the largest protein was recorded for each microorganism, along with taxonomic information and genome size. The percentage of the genome dedicated to the largest protein was calculated, as well as the correlation between genome size and largest protein size. Complete data sets are available from the authors upon request as Excel files with links to protein annotation in the case of adhesins.

2.2. Sequence analysis

BLAST programs [1], available in http://www.ncbi.nlm.nih.gov, were used for sequence comparisons and similarity searches. Analysis of motifs, domains and patterns in protein sequences was done with the ScanProsite (http://www.expasy.org/tools/scanprosite/), InterProScan (http://www.ebi.ac.uk/InterProScan/), FingerPRINTScan (http://www.bioinf.manchester.ac.uk/fingerPRINTScan/) and MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) bioinformatics tools. Multiple alignments, molecular evolution and phylogenetic analyses were performed with the ClustalX [18] and *MEGA* 3.1 [13] programs, freely available at http://bips.u-strasbg.fr and http://www.megasoftware.net/, respectively. Although different methods (Neighbor-Joining, UPMGA, and minimal evolution) were assayed for phylogenetic analyses, the final and most consistent results were obtained using the minimal evolution method, with a Poisson correction model and bootstrap test (2000 replicates; seed=42815), after complete gap deletion.

3. Results and Discussion

The genomes of 351 prokaryotic microorganisms appearing in the NCBI database of complete microbial genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) were analyzed and initially the largest predicted protein present in each genome was picked. These ranged from 1370 amino acids (*Halobacterium* sp. NRC-1) to as much as 36805 residues in *Chlorobium chlorochromatii* CaD3, the average length being calculated in 3557 amino acids. As reflected in Fig. 1, there is no significant correlation between genome size and largest protein size. In fact, proteins significantly over the average length are predicted

in microorganisms with small genome sizes. An example is *Ureaplasma parvum*, with a genome of 0.75 Mbp, where a gene over 15 kb long (which represents 2% of the genome), coding for a putative lipoprotein is present. In 15 microorganisms, more than 1% of the genome is dedicated to one single open reading frame. The most extreme case is again *Chlorobium chlorochromatii* CaD3, where one predicted gene covers 4.3% of the genome. In this organism, six open reading frames represent 10% of the genome.

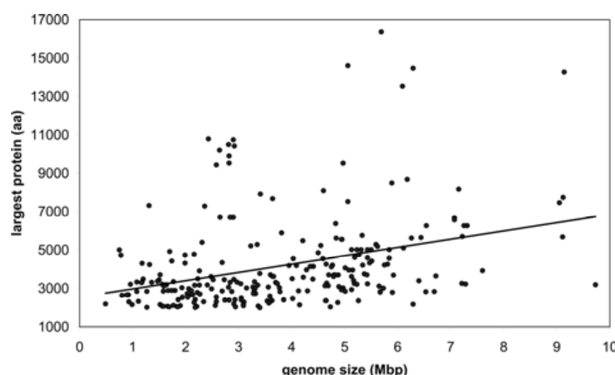

Figure 1. Lack of correlation between prokaryotic genome size (in Mbp) and largest protein size. Data points corresponding to 250 proteins and genomes and trend line are plotted. Pearson's correlation coefficient, calculated for all genomes studied in this work, is 0.31.

In a second stage, genomes were searched not only for their largest polypeptide but for all proteins longer than 3557 amino acids. Their predicted or experimentally observed function, subcellular localization and characteristics were examined, in order to establish what physiological roles would require the maintenance of genes encoding such big polypeptides. When no information was available, a preliminary prediction was done based on sequence similarity after comparison with the databases using BLAST programs. Two broad functional groups could be established among large proteins. One comprises non-ribosomal peptide synthetases, potentially involved in the biosynthesis of antibiotics or other secondary metabolites. The second corresponds to proteins predicted to be secreted and/or associated to the cell surface, including potential adhesins, filamentous haemagglutinins, and others of undefined function.

The group of large surface-associated proteins was analyzed to establish the phylogenetic relationships between them, as well as their common and differentiating features. This analysis encompassed 121 sequences, including three of a smaller size but that

correspond to characterized adhesins; Esp of *Enterococcus faecalis* and Bap of *Staphylococcus aureus* and *S. epidermidis*.

As a first approach, a multiple alignment was performed with all the sequences and used to build a phylogenetic tree. Different phylogeny analysis methods were employed, as described in Materials and Methods. Although the initial results were difficult to interpret due to the length and divergence of the sequences, it became obvious that about half the proteins always clustered in several groups. Thus, a second analysis was done including this collection of 60 proteins, plus two other known adhesins (LapF and Esp). The results produced a consensus tree (after 2000 bootstrap trials) shown in Fig. 2. This allowed us to define seven families of large surface proteins, six of which we have named after their best characterized member (LapA, AidA, Bap, Ebh, FhaL and FhaB). The seventh family we have denominated Bsp for *Bacillus* surface proteins, since most members of the cluster correspond to proteins from this bacterial genus.

The whole collection of sequences was then subjected to a detailed study, including potential motifs or domains that might be present in these proteins and could allow further characterization and classification. The outcome of this analysis is shown in Table 1, and allowed incorporating additional sequences to the above mentioned families. The first noticeable result is the presence, in most of these surface proteins, of one or multiple redundant domains, where a single amino acid is significantly overrepresented.

As indicated in Table 1, the AidA family includes several proteins that are annotated as VCBS. This domain of around 100 residues is found in multiple copies in large proteins from *Vibrio*, *Colwellia*, *Bradyrhizobium*, and *Shewanella* (hence the acronym VCBS), and its annotation suggests a potential role in adhesion, although to our knowledge, no experimental evidence exists.

Most proteins in this group have threonine-rich domains (Table 1), and certain conserved acidic residues. Interestingly, two motifs found in eukaryotic cells appear in some of these proteins; RGD (which has been shown to play a role in adhesion) and a cadherin domain signature. Cadherins are animal glycoproteins that interact with themselves and participate in calcium-dependent cell-cell adhesion. Calcium binding domains are also found in several members of this family.

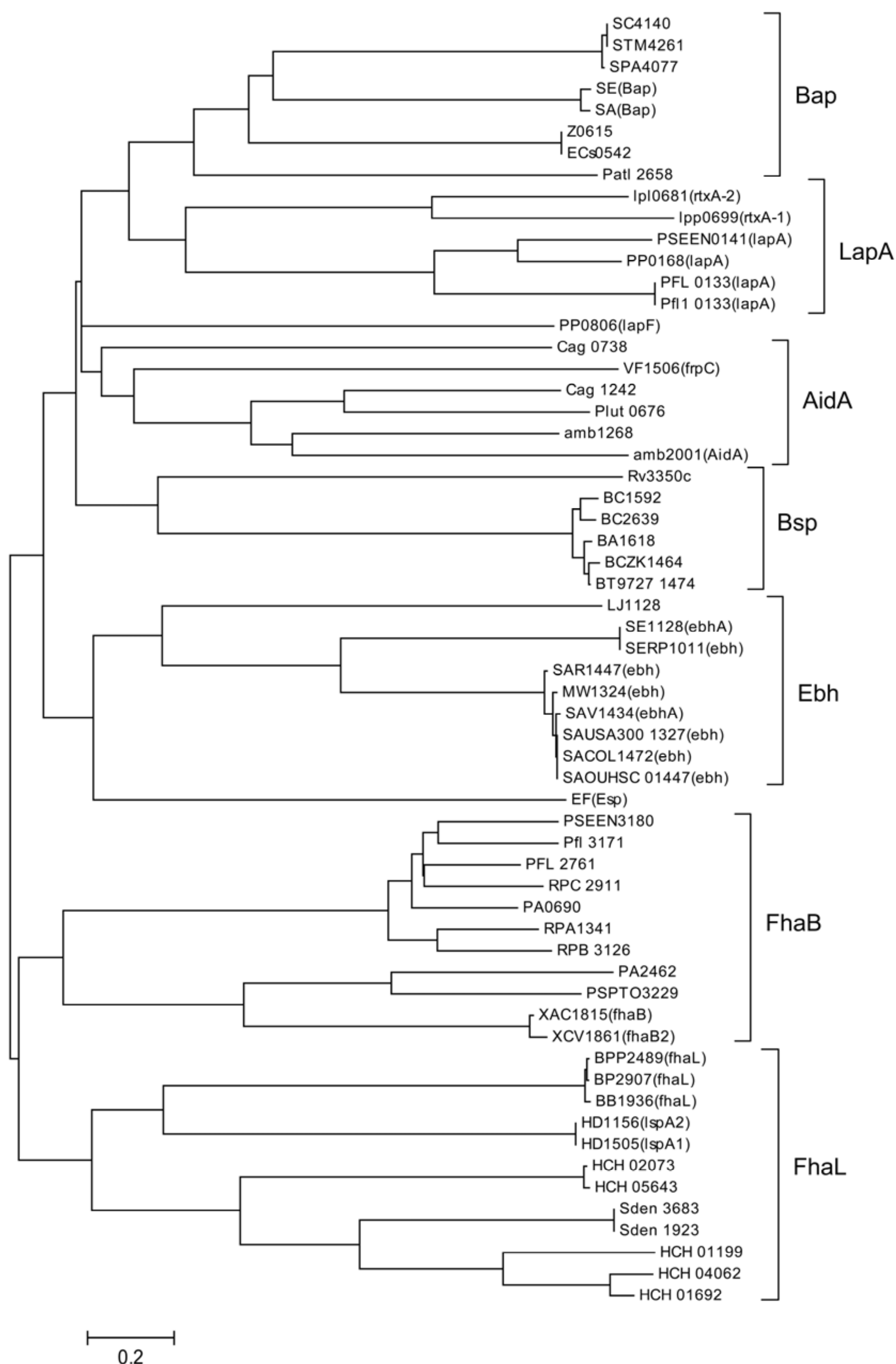

Figure 2. Phylogenetic relationship between large prokaryotic surface proteins. Bootstrap consensus tree obtained by analyzing 62 sequences with the minimal evolution method, as described in Materials and Methods, is shown. Nomenclature corresponds to that in the NCBI database, details can be found in Table 1.

The LapA family includes homologs of this protein found in *Pseudomonas* species and two proteins of *Legionella pneumophila* annotated as Rtx. The role of LapA in biofilm formation has been mentioned above. RTX ("repeats in the structural toxin") toxins are pore-forming protein toxins produced by a broad range of pathogenic Gram-negative bacteria [8], although relatives have been described in non-pathogenic organisms. LapA family proteins show certain conserved acidic residues, and a short signature (GX[N/D]Y[E/N][D/T]A[F/N]), but the main characteristic of all proteins in this family is the presence of hemolysin-type calcium binding motifs, as well as von Willebrand factor A (VWF-A) motifs, which in eukaryotic cells are involved in cell-cell attachment [20], and seem to be unique to the LapA family among prokaryotic adhesins. Based on these two characteristics, a protein of *Dechloromonas aromatica* was also included in this family.

The Bap family includes this adhesin and other proteins, mostly from *Enterobacteriaceae*, showing threonine-rich domains and either calcium binding or putative cadherin domains. The difference between Bap and other members of the family is the presence of aspartic- and asparagine-rich domains in the former. Certain glycine, aspartic and asparagine residues are conserved in all members of this family and a short signature (T[A/V]TDXXGN) can also be recognized, repeated several times in all the sequences.

Overall, these data suggest that large proteins belonging to the LapA, AidA and Bap families are adhesins that may be involved not only in cell-surface interactions but also in cell-cell adhesion, and that calcium plays a significant role in their functioning. Previous evidence has shown that increasing amounts of calcium results in reduced Bap-mediated cell-cell aggregation and adhesion in *Staphylococcus* [3]. In opposition to these results, treatment of bacterial cells with the calcium chelator EGTA prevents attachment of *Pseudomonas putida* to plant seeds (our unpublished observation). It may be that calcium modulates biofilm formation in different ways in a surface-dependent manner. In any case, the role of calcium in the functionality of diverse adhesins deserves further investigation.

As it has been mentioned, most members of the Bsp family correspond to cell surface repeat proteins present in different *Bacillus* species, all of them very similar in length and amino acid composition and showing threonine-rich domains. A protein of *Mycobacterium tuberculosis* has also been included since it clustered with the other members of the family in the different phylogenetic analyses, although it appears to have branched early from the

same root. The same holds true for a protein of *Lactobacillus johnstonii* with respect to the Ebh family. Ebh has been described to bind human fibronectin and is probably important for infection by *Staphylococcus* [4]. This family is characterized by the presence of RGD motifs and glutamine and lysine-rich domains in most members. Curiously, leucine zipper motifs are also present in most proteins of this family, as well as in several other large surface proteins. These motifs have been described in DNA binding proteins and might be involved in dimerization. Whether they may have a similar role in bacterial adhesins is an enticing possibility to be explored.

The remaining two families, FhaL and FhaB, are closely related. Most members of these families show alanine and/or glycine rich domains, as well as RGD motifs. FhaB and similar filamentous hemagglutinins [11] have been mostly characterized in *Bordetella* species, where they mediate bacterial adherence to epithelial cells and macrophages *in vitro* and are required for *in vivo* colonization of host tissues. FhaL is a close relative of FhaB, found in the genomes of different *Bordetella* species [11] and other bacteria. Expression of *fhaL* is controlled by the global virulence two-component regulatory system BvgAS [2], and may therefore be also involved in tissue colonization and invasion processes. From the phylogenetic tree shown in Fig. 2, it seems clear that there was an early divergence in two branches, one corresponding to filamentous haemagglutinins (FhaB and FhaL families) and the other to the rest of adhesins.

Aside from these seven families, three other groups could be clearly established, based on phylogenetic distances (Table 1). Interestingly, two experimentally identified adhesins, LapF and Esp, appeared outside of any of these groups or of the other seven families in every analysis with all or part of the 121 sequences. Esp appears to be phylogenetically near to the Ebh cluster, but its closest relatives are a *Staphylococcus haemolyticus* protein and two mucus binding proteins present in *Lactobacillus* species [17]. All four show threonine-rich domains and a Gram-positive anchoring motif, responsible for protein attachment to the surface of the bacterial cell wall [16]. LapF, although clearly separated in the phylogenetic tree, seems to be placed somewhere in between the LapA and Bap families in terms of its characteristics. In fact, LapF shows regions that might correspond to calcium-binding and von Willebrandt factor domains, altough their similarity to these elements is not as evident as in the case of LapA family members. On the other hand, though somewhat distantly, LapF clusters together with other members of the Bap family when Bap is removed from

phylogenetic analyses, and a short signature (T[A/L]TDAAGN) similar to that of the Bap family is present several times in the sequence.

The remaining proteins could not be placed in any phylogenetic group with sufficent confidence to be regarded as reliable, although some of them show similar characteristics (Table 1). Many present RGD motifs, and several include cadherin or calcium binding domains in their sequences. Future characterization of these proteins should define possible functional and/or phylogenetic relationships among them.

Table 1. Representative motifs in large microbial adhesins

Protein name/id.	Organism	VWF-A	Ca-bind.	aa-rich domains	Cadherin	RGD	other relevant motifs
LapA family							
PSEEN0141	*Pseudomonas entomophila L48*	+	+	T,G	+	-	
PFL_0133(lapA)	*Pseudomonas fluorescens Pf-5*	+	+	T,G	-	-	
Pfl_0133(lapA)	*Pseudomonas fluorescens PfO-1*	+	+	T,G	-	-	
PP0168(lapA)	*Pseudomonas putida KT2440*	+	+	T,G	-	-	
lpl0681(rtxA-2)	*Legionella pneumophila str. Lens*	+	+	-	-	-	
lpp0699(rtxA-1)	*Legionella pneumophila str. Paris*	+	+	-	-	-	
Daro_3199	*Dechloromonas aromatica*	+	+	T	-	-	VCBS
AidA family							
amb2001(aidA)	*Magnetospirillum magneticum AMB-1*	-	+	T,G	+	-	VCBS
amb1268	*Magnetospirillum magneticum AMB-1*	-	+	T,G	+	-	VCBS
VF1506 (frpC)	*Vibrio fischeri ES114*	-	+	T	+	-	VCBS
Cag_1242	*Chlorobium chlorochromatii CaD3*	-	-	T	+	-	VCBS
Plut_0676	*Pelodictyon luteolum DSM 273*	-	-	T	+	-	VCBS
Cag_0738	*Chlorobium chlorochromatii CaD3*	-	-	T	+	-	VCBS
Plut_0367	*Pelodictyon luteolum*	-	-	T	+	-	VCBS
Plut_0379	*Pelodictyon luteolum*	-	+	T	-	+	VCBS
RPB_1638	*Rhodopseudomonas palustris*	-	+	T	-	-	VCBS
Ava_4160	*Anabaena variabilis*	-	-	T	-	+	VCBS
CV0311	*Chromobacterium violaceum*	-	+	-	+	-	VCBS
VC1451	*Vibrio cholerae O1 biovar eltor*	-	+	G	-	+	
Bap family							
AAK38834(Bap)	*Staphylococcus aureus*	-	-	T,D,N	(+)	-	Gram+ anchor
AAY28519(Bap)	*Staphylococcus epidermidis*	-	-	T,D,N,S	(+)	-	Gram+ anchor
Z0615	*Escherichia coli O157:H7 EDL933*	-	+	T	-	-	
ECs0542	*Escherichia coli O157:H7 str. Sakai*	-	+	T	-	-	
SC4140	*Salmonella enterica sv. Choleraesuis*	-	-	T	+	-	
SPA4077	*Salmonella enterica sv. Paratyphi A*	-	-	T	+	-	
STM4261	*Salmonella typhimurium LT2*	-	-	T	+	-	
Patl_2658	*Pseudoalteromonas atlantica T6c*	-	-	T	+	-	
Psyc_1601	*Psychrobacter arcticus 273-4*	-	(+)	T	-	-	
Bsp family							
BA1618	*Bacillus anthracis str. Ames*	-	-	T	-	-	
BC1592	*Bacillus cereus ATCC 14579*	-	-	T	-	-	
BC2639	*Bacillus cereus ATCC 14579*	-	-	T	-	-	
BCZK1464	*Bacillus cereus E33L*	-	-	T	-	-	
BT9727_1474	*Bacillus thuringiensis sv. konkukian*	-	-	T	-	-	
Rv3350c	*Mycobacterium tuberculosis H37Rv*	-	-	A,G,N	-	+	
Ebh family							
SACOL1472	*Staphylococcus aureus COL*	-	-	T,N,Q,K	-	+	Leu zipper
SAR1447(ebh)	*Staphylococcus aureus MRSA252*	-	-	T,NQ,K	-	+	Leu zipper
SAV1434(ebhA)	*Staphylococcus aureus Mu50*	-	-	N,Q,K	-	-	
MW1324(ebh)	*Staphylococcus aureus MW2*	-	-	N,Q,K	-	+	Leu zipper

Protein id.	Organism	VWF-A	Ca-bind.	aa-rich domains	Cadherin	RGD	other relevant motifs
SAOUHSC01447	*Staphylococcus aureus NCTC 8325*	-	-	T,N,Q,K	-	+	Leu zipper
SAUSA3001327	*Staphylococcus aureus USA300*	-	-	T,N,Q,K	-	+	Leu zipper
SE1128(ebhA)	*Staphylococcus epidermidis ATCC 12228*	-	-	Q,K	-	+	Leu zipper
SERP1011(ebh)	*Staphylococcus epidermidis RP62A*	-	-	Q,K	-	+	Leu zipper
LJ1128	*Lactobacillus johnsonii NCC 533*	-	+	A	-	-	gram+ anchor
FhaL family							
BB1936(fhaL)	*Bordetella bronchiseptica RB50*	-	-	A	-	+	Leu zipper
BPP2489(fhaL)	*Bordetella parapertussis 12822*	-	-	A,P	-	+	Leu zipper
BP2907(fhaL)	*Bordetella pertussis Tohama I*	-	-	A	-	+	Leu zipper
HCH_04062	*Hahella chejuensis KCTC 2396*	-	-	A	-	+	
HCH_01692	*Hahella chejuensis KCTC 2396*	-	-	A	-	+	
Sden_1923	*Shewanella denitrificans OS217*	-	-	A,G	-	+	
Sden_3683	*Shewanella denitrificans OS217*	-	-	-	-	+	
HCH_01199	*Hahella chejuensis KCTC 2396*	-	-	-	-	+	
HD1156 (lspA2)	*Haemophilus ducreyi 35000HP*	-	-	N	-	-	Leu zipper
HD1505 (lspA1)	*Haemophilus ducreyi 35000HP*	-	-	N	-	-	Leu zipper
HCH_02073	*Hahella chejuensis KCTC 2396*	-	-	-	(+)	-	Leu zip., intein
HCH_05643	*Hahella chejuensis KCTC 2396*	-	-	-	+	-	Leu zip., cNMP
FhaB family							
XAC1815(fhaB)	*Xanthomonas axonopodis pv. citri*	-	-	A,G	-	+	hemagglutinin
XCV1861	*Xanthomonas campestris pv. vesicatoria*	-	-	A,G	-	+	hemagglutinin
PA2462	*Pseudomonas aeruginosa PAO1*	-	-	A,G	-	+	Leu zipper
PA0690	*Pseudomonas aeruginosa PAO1*	-	-	A,G	-	+	Leu zipper
PSEEN3180	*Pseudomonas entomophila L48*	-	-	A,G	-	+	hemag.,Leu zip.
Pfl_3171	*Pseudomonas fluorescens PfO-1*	-	-	A,G	-	+	Leu zipper
RPC_2911	*Rhodopseudomonas palustris BisB18*	-	-	A,G	-	+	hemagglutinin
RPA1341	*Rhodopseudomonas palustris CGA009*	-	-	A,G	-	+	Leu zipper
PSPTO3229	*Pseudomonas syringae pv. tomato*	-	-	A	-	+	Leu zip., intein
RPB_3126	*Rhodopseudomonas palustris HaA2*	-	-	A	-	+	hemagglutinin
PFL_3688	*Pseudomonas fluorescens Pf-5*	-	-	G	-	+	hemag.,Leu zip.
PSEEN0968	*Pseudomonas entomophila L48*	-	-	G	-	-	haem., autotransp.
PFL_2761	*Pseudomonas fluorescens Pf-5*	-	-	-	-	+	hemagglutinin
PSEEN3946	*Pseudomonas entomophila L48*	-	-	-	-	+	hemagglutinin
RPC_2997	*Rhodopseudomonas palustris BisB18*	-	-	A,T	-	+	hemagglutinin
XC_2160	*Xanthomonas campestris pv. campestris*	-	-	T,G	-	+	hemag., autotransp.
ACIAD0940	*Acinetobacter sp. ADP1*	-	-	A,G	-	+	hemag.,Leu zip.
Other groups & unclassified							
MMOB1040	*Mycoplasma mobile 163K*	-	-	N	-	-	
UU495	*Ureaplasma parvum sv. 3*	-	-	N	-	-	fibronectin
plu1344	*Photorhabdus luminescens ssp. laumondii*	-	+	G	-	+	
PH0954	*Pyrococcus horikoshii OT3*	-	-	-	-	-	PKD, fibronectin
YPN_3592	*Yersinia pestis Nepal516*	-	-	T	-	+	Big-1, intimin
YPTB3789	*Yersinia pseudotuberculosis*	-	-	T	-	+	Big-1, intimin
RB11769	*Rhodopirellula baltica SH 1*	-	-	T	+	-	Calx
GSU1154	*Geobacter sulfurreducens PCA*	-	-	T	-	+	Leu zipper
Cag_0529	*Chlorobium chlorochromatii*	-	+	T	-	-	
slr1028	*Synechocystis sp. PCC 6803*	-	-	S	-	+	lectin, Calx
slr0408	*Synechocystis sp. PCC 6803*	-	-	N	-	+	Calx
AAM21183(Esp)	*Enterococcus faecalis*	-	-	T	-	-	Gram+ anchor
SH1471	*Staphylococcus haemolyticus*	-	-	T	-	-	Gram+ anchor
LJ0484	*Lactobacillus johnsonii NCC 533*	-	-	T	-	-	Gram+ anchor
LBA1392	*Lactobacillus acidophilus NCFM*	-	-	T,P	-	-	Gram+ anchor

Protein id.	Organism	VWF-A	Ca-bind.	aa-rich domains	Cadherin	RGD	other relevant motifs
SYNW0953	*Synechococcus sp. WH 8102*	-	-	T,D,G,S	-	+	VCBS
SAR11_0932	*candidatus Pelagibacter ubique*	-	-	T	-	+	
STY2875	*Salmonella enterica ssp. enterica sv Typhi*	-	-	T	-	-	
amb3790	*Magnetospirillum magneticum*	-	-	T	-	-	
MA1762	*Methanosarcina acetivorans*	-	+	T	-	-	PKD, Big-2
GSU0279	*Geobacter sulfurreducens PCA*	-	-	T	+	+	Calx
SO4149	*Shewanella oneidensis*	-	-	T	+	+	
RB1661	*Rhodopirellula baltica SH 1*	-	-	T,G	+	-	Lectin
RPB_2246	*Rhodopseudomonas palustris HaA2*	-	+	G	+	+	Lectin
nfa8110	*Nocardia farcinica IFM 10152*	-	-	A,G,P,D	-	+	Leu zipper
PM0059	*Pasteurella multocida ssp. multocida*	-	-	E	-	-	Leu zipper
Sde_2049	*Saccharophagus degradans 2-40*	-	+	D	(+)	+	
mll0950	*Mesorhizobium loti*	-	-	G	-	-	Autotransport
amb3422	*Magnetospirillum magneticum AMB-1*	-	+	A,G,Q	-	+	
bll3714	*Bradyrhizobium japonicum*	-	+	-	+	-	
DP1520	*Desulfotalea psychrophila LSv54*	-	-	-	-	+	
RB7341	*Rhodopirellula baltica SH 1*	-	-	-	+	+	ATPase
RB7321	*Rhodopirellula baltica SH 1*	-	-	-	-	+	Leu zip., lectin
MS0748	*Mannheimia succiniciproducens*	-	-	-	-	+	
blr3252	*Bradyrhizobium japonicum USDA 110*	-	-	-	-	+	
SPO2716	*Silicibacter pomeroyi DSS-3*	-	+	-	-	+	
PSPPH_0815	*Pseudomonas syringae*	-	+	-	-	+	
plu0548	*Photorhabdus luminescens ssp. laumondii*	-	-	-	-	+	Leu zipper
PP0806 (LapF)	*Pseudomonas putida KT2440*	(+)	(+)	-	-	-	
RPC2925	*Rhodopseudomonas palustris BisB18*	-	(+)	-	-	-	
bll5471	*Bradyrhizobium japonicum*	-	(+)	-	-	-	

Presence or absence of a motif is indicated by the + and – symbols, respectively. The (+) symbol indicates that a motif might be present, but the E-value is below the significance threshold.

This work represents the first systematic analysis of the largest prokaryotic proteins, with a particular focus on those involved in adhesion processes. The fact that very large adhesins are widespread throughout the microbial world, regardless of genome size, indicates that cell-cell and cell-surface interactions are common events among prokayotes that could take place via similar mechanisms and be often modulated by calcium ions. This study comes to support the increasingly accepted view of biofilm formation as a general strategy in the bacterial life cycle.

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Supplementary information.

Large adhesins & cell surface proteins in complete genomes

Organism	prot. size	Locus	Current annotation
Anabaena variabilis ATCC 29413	6581	Ava_4160	VCBS
Bacillus anthracis str. Ames	5017	BA1618	conserved repeat domain protein
Bacillus cereus ATCC 14579	5017	BC1592	Cell surface protein
Bacillus cereus ATCC 14579	5010	BC2639	Cell surface protein
Bacillus cereus E33L	5017	BCZK1464	conserved hypothetical protein, repeat domains; possible cell surface protein
Bacillus thuringiensis serovar konkukian str. 97-27	5017	BT9727_1474	hypothetical protein BT9727_1474
Bordetella bronchiseptica RB50	4218	BB1936 (fhaL)	adhesin
Bordetella parapertussis 12822	4218	BPP2489 (fhaL)	adhesin
Bordetella pertussis Tohama I	4196	BP2907 (fhaL)	adhesin
Bradyrhizobium japonicum USDA 110	5685	blr3252	hypothetical protein blr3252
Bradyrhizobium japonicum USDA 110	4210	bll5471	hypothetical protein bll5471
Bradyrhizobium japonicum USDA 110	4128	bll3714	hypothetical protein bll3714
Candidatus Pelagibacter ubique HTCC1062	7317	SAR11_0932	hypothetical protein SAR11_0932
Chlorobium chlorochromatii CaD3	16311	Cag_1242	VCBS
Chlorobium chlorochromatii CaD3	8871	Cag_0738	VCBS
Chlorobium chlorochromatii CaD3	4697	Cag_0529	hypothetical protein Cag_0529
Chromobacterium violaceum ATCC 12472	4130	CV0311	probable RTX (repeat in structural toxin)
Dechloromonas aromatica RCB	4854	Daro_3199	VCBS
Ehrlichia canis str. Jake	4245	Ecaj_0387	Ankyrin
Ehrlichia chaffeensis str. Arkansas	4313	ECH_0653	ankyrin repeat protein
Escherichia coli O157:H7 EDL933	5188	Z0615	putative RTX family exoprotein
Escherichia coli O157:H7 str. Sakai	5291	ECs0542	hypothetical protein ECs0542
Geobacter sulfurreducens PCA	5899	GSU0279	cadherin domain/calx-beta domain protein
Geobacter sulfurreducens PCA	4713	GSU1154	surface repeat protein, putative
Haemophilus ducreyi 35000HP	4919	HD1156 (lspA2)	large supernatant protein 2
Haemophilus ducreyi 35000HP	4152	HD1505 (lspA1)	large supernatant protein 1
Hahella chejuensis KCTC 2396	5711	HCH_04062	Rhs family protein
Hahella chejuensis KCTC 2396	5497	HCH_01692	Rhs family protein
Hahella chejuensis KCTC 2396	5389	HCH_01199	Rhs family protein
Lactobacillus acidophilus NCFM	4326	LBA1392	mucus binding protein precursor Mub
Lactobacillus johnsonii NCC 533	4734	LJ1128	hypothetical protein LJ1128
Lactobacillus johnsonii NCC 533	4037	LJ0484	hypothetical protein LJ0484
Legionella pneumophila str. Lens	7919	lpl0681(rtxA-2)	structural toxin protein RtxA
Legionella pneumophila str. Paris	7679	lpp0699(rtxA-1)	structural toxin protein RtxA
Magnetospirillum magneticum AMB-1	9529	amb3422	hypothetical protein amb3422
Magnetospirillum magneticum AMB-1	5299	amb1268	Large exoprotein
Magnetospirillum magneticum AMB-1	4218	amb3790	Large exoprotein involved in heme utilization or adhesion
Mannheimia succiniciproducens MBEL55E	5399	MS0748	hypothetical protein MS0748
Mycoplasma mobile 163K	4727	MMOB1040 (gliB)	Gli521 adhesion and gliding protein
Nocardia farcinica IFM 10152	7192	nfa8110	hypothetical protein nfa8110
Pelodictyon luteolum DSM 273	7284	Plut_0379	VCBS
Pelodictyon luteolum DSM 273	6678	Plut_0367	VCBS
Pelodictyon luteolum DSM 273	4661	Plut_0676	VCBS
Photorhabdus luminescens subsp. laumondii TTO1	4582	plu0548	hypothetical protein plu0548
Photorhabdus luminescens subsp. laumondii TTO1	4070	plu1344	hypothetical protein plu1344
Pseudoalteromonas atlantica T6c	4629	Patl_2658	hypothetical protein Patl_2658
Pseudomonas aeruginosa PAO1	5627	PA2462	hypothetical protein PA2462
Pseudomonas aeruginosa PAO1	4180	PA0690	hypothetical protein PA0690
Pseudomonas entomophila L48	5862	PSEEN0141(lapA)	Surface adhesion protein

Pseudomonas entomophila L48	4658	PSEEN0968	outer membrane autotransporter
Pseudomonas entomophila L48	4197	PSEEN3180	hypothetical protein PSEEN3180
Pseudomonas fluorescens Pf-5	4920	PFL_0133 (lapA)	calcium-binding outer membrane-like protein
Pseudomonas fluorescens Pf-5	4187	PFL_2761	haemagluttinin repeat protein
Pseudomonas fluorescens PfO-1	5218	Pfl_0133 (lapA)	von Willebrand factor, type A
Pseudomonas fluorescens PfO-1	4170	Pfl_3171	Filamentous haemagglutinin-like
Pseudomonas putida KT2440	8682	PP0168 (lapA)	surface adhesion protein, putative
Pseudomonas putida KT2440	6310	PP0806 (lapF)	surface adhesion protein, putative
Pseudomonas syringae pv. phaseolicola 1448A	5107	PSPPH_0815	calcium binding hemolysin protein, putative
Pseudomonas syringae pv. tomato str. DC3000	6274	PSPTO3229	filamentous hemagglutinin, intein-containing, putative
Psychrobacter arcticus 273-4	6715	Psyc_1601	hypothetical protein Psyc_1601
Pyrococcus horikoshii OT3	4436	PH0954	hypothetical protein PH0954
Rhodopirellula baltica SH 1	8173	RB11769	probable aggregation factor core protein MAFp3, isoform C
Rhodopirellula baltica SH 1	7716	RB1661	hypothetical protein RB1661
Rhodopirellula baltica SH 1	7538	RB7341	similar to surface-associated protein cshA precursor
Rhodopirellula baltica SH 1	6157	RB7321	hypothetical protein RB7321
Rhodopirellula baltica SH 1	4630	RB1924	probable fibrinogen-binding protein homolog-possibly involved in cell-cell attachment
Rhodopseudomonas palustris BisB18	4435	RPC_2997	filamentous haemagglutinin-like
Rhodopseudomonas palustris BisB18	4135	RPC_2911	filamentous haemagglutinin-like
Rhodopseudomonas palustris CGA009	4335	RPA1341	hypothetical protein RPA1341
Rhodopseudomonas palustris HaA2	5769	RPB_2246	VCBS
Rhodopseudomonas palustris HaA2	5094	RPB_1638	VCBS
Rhodopseudomonas palustris HaA2	4347	RPB_3126	Filamentous haemagglutinin-like protein
Saccharophagus degradans 2-40	14609	Sde_2049	hypothetical protein Sde_2049
Salmonella enterica subsp. enterica sv. Choleraesuis str. SC-B67	5559	SC4140	putative inner membrane protein
Salmonella enterica subsp. enterica sv. Paratyphi A str. ATCC 9150	4560	SPA4077	putative inner membrane protein
Salmonella typhimurium LT2	5559	STM4261	putative inner membrane protein
Shewanella denitrificans OS217	5236	Sden_3683	Peptidoglycan-binding LysM
Shewanella denitrificans OS217	5189	Sden_1923	Peptidoglycan-binding LysM
Shewanella oneidensis MR-1	5020	SO4149	RTX toxin, putative
Silicibacter pomeroyi DSS-3	8093	SPO2716	type I secretion target repeat protein
Staphylococcus aureus subsp. aureus COL	10498	SACOL1472 (ebh)	Cell wall associated fibronectin-binding protein
Staphylococcus aureus subsp. aureus MRSA252	10746	SAR1447 (ebh)	very large surface anchored protein
Staphylococcus aureus subsp. aureus Mu50	6713	SAV1434 (ebhA)	hypothetical protein ebhA
Staphylococcus aureus subsp. aureus MW2	9904	MW1324 (ebh)	hypothetical protein MW1324
Staphylococcus aureus subsp. aureus NCTC 8325	9535	SAOUHSC_01447 (ebh?)	hypothetical protein SAOUHSC_01447
Staphylococcus aureus subsp. aureus USA300	10421	SAUSA300_1327 (ebh?)	cell surface protein
Staphylococcus epidermidis ATCC 12228	9439	SE1128 (ebhA)	ebhA protein
Staphylococcus epidermidis RP62A	10203	SERP1011 (ebh)	cell wall associated fibronectin-binding protein
Staphylococcus haemolyticus JCSC1435	4354	SH1471	hypothetical protein SH1471
Streptococcus pneumoniae TIGR4	4776	SP1772	cell wall surface anchor family protein
Synechococcus sp. WH 8102	10791	SYNW0953	hypothetical protein SYNW0953
Synechocystis sp. PCC 6803	4199	slr0408	integrin alpha subunit domain-like protein
Ureaplasma parvum serovar 3 str. ATCC 700970	5005	UU495	unique hypothetical membrane lipoprotein

Vibrio cholerae O1 biovar eltor str. N16961	4558	VC1451	RTX toxin RtxA
Xanthomonas axonopodis pv. citri str. 306	4753	XAC1815 (fhaB)	filamentous haemagglutinin
Yersinia pestis Nepal516	4270	YPN_3592	invasin
Yersinia pseudotuberculosis IP 32953	5623	YPTB3789	possible Bacterial Ig-like domain (group 1)
not considered in the alignment			
Acinetobacter sp. ADP1	3711	ACIAD0940	putative hemagglutinin/hemolysin-related protein
Chlorobium chlorochromatii CaD3	3834	Cag_1920	hypothetical protein Cag_1920
Colwellia psychrerythraea 34H	3758	CPS_2430	VCBS repeat protein
Desulfotalea psychrophila LSv54	3628	DP1520	hypothetical protein DP1520
Ehrlichia ruminantium str. Welgevonden	3715	Erum0660	hypothetical protein Erum0660
Hahella chejuensis KCTC 2396	3976	HCH_02073	Rhs family protein
Hahella chejuensis KCTC 2396	3862	HCH_05643	Rhs family protein
Magnetospirillum magneticum AMB-1	3730	amb2001 (aidA)	Type V secretory pathway, adhesin AidA
Mesorhizobium loti MAFF303099	3930	mll4444	hypothetical protein mll4444
Mesorhizobium loti MAFF303099	3659	mll0950	hypothetical protein mll0950
Methanosarcina acetivorans C2A	3988	MA1762	cell surface protein
Mycobacterium tuberculosis H37Rv	3716	Rv3350c(PPE56)	PPE FAMILY PROTEIN
Pasteurella multocida subsp. multocida str. Pm70	3919	PM0059 (phfB2)	PfhB2
Pseudomonas entomophila L48	3863	PSEEN3946	filamentous hemagglutinin
Pseudomonas fluorescens Pf-5	3700	PFL_3688	haemagluttinin repeat protein
Rhodopseudomonas palustris BisB18	3796	RPC_2925	LysM domain protein
Salmonella enterica subsp. enterica serovar Typhi str. CT18	3624	STY2875	large repetitive protein
Staphylococcus haemolyticus JCSC1435	3608	SH0326	hypothetical protein SH0326
Synechocystis sp. PCC 6803	3972	slr1028	integrin alpha-subunit domain-like protein
Vibrio fischeri ES114	3971	VF1506 (frpC)	iron-regulated protein FrpC
Xanthomonas campestris pv. campestris str. 8004	3961	XC_2160 (yapH)	YapH protein
Xanthomonas campestris pv. vesicatoria str. 85-10	3709	XCV1861 (fhaB2)	filamentous hemagglutinin-related protein
Yersinia pestis Antiqua	3705	YPA_3360	putative autotransporter protein
Yersinia pestis biovar Medievalis str. 91001	3710	YP_3415 (yapH)	putative autotransporter protein
Chlorobium chlorochromatii CaD3	36805	Cag_0614	Parallel beta-helix repeat
Chlorobium chlorochromatii CaD3	20646	Cag_0616	Parallel beta-helix repeat

% of the genome dedicated to largest protein

Organism	Genome Size	largest prot aa	% of genome
Chlorobium chlorochromatii CaD3	2.57	36805	**4.30**
Ureaplasma parvum serovar 3 str. ATCC 700970	0.75	5005	**2.00**
Mycoplasma mobile 163K	0.78	4727	**1.82**
Candidatus Pelagibacter ubique HTCC1062	1.31	7317	**1.68**
Nanoarchaeum equitans Kin4-M	0.49	2197	**1.35**
Synechococcus sp. WH 8102	2.43	10791	**1.33**
Staphylococcus epidermidis RP62A	2.64	10203	**1.16**
Staphylococcus aureus subsp. aureus COL	2.81	10498	**1.12**
Staphylococcus aureus subsp. aureus MRSA252	2.9	10746	**1.11**
Staphylococcus epidermidis ATCC 12228	2.58	9439	**1.10**
Ehrlichia chaffeensis str. Arkansas	1.18	4313	**1.10**
Staphylococcus aureus subsp. aureus USA300	2.92	10421	**1.07**
Staphylococcus aureus subsp. aureus MW2	2.82	9904	**1.05**
Staphylococcus aureus subsp. aureus NCTC 8325	2.82	9535	**1.01**
Mycoplasma pulmonis UAB CTIP	0.96	3216	**1.01**

Mycoplasma synoviae 53	0.8	2638	**0.99**
Ehrlichia canis str. Jake	1.32	4245	**0.96**
Mycoplasma genitalium G37	0.58	1805	**0.93**
Chlamydia muridarum Nigg	1.08	3335	**0.93**
Pelodictyon luteolum DSM 273	2.36	7284	**0.93**
Mycoplasma hyopneumoniae 232	0.89	2660	**0.90**
Mycoplasma hyopneumoniae J	0.9	2660	**0.89**
Anaplasma marginale str. St. Maries	1.2	3492	**0.87**
Mycoplasma hyopneumoniae 7448	0.92	2666	**0.87**
Haemophilus ducreyi 35000HP	1.7	4919	**0.87**
Saccharophagus degradans 2-40	5.06	14609	**0.87**
Photorhabdus luminescens subsp. laumondii TTO1	5.69	16367	**0.86**
Chlamydophila caviae GPIC	1.18	3346	**0.85**
Chlamydophila felis Fe/C-56	1.17	3298	**0.85**
Wolbachia endosymbiont strain TRS of Brugia malayi	1.08	2839	**0.79**
Pyrococcus horikoshii OT3	1.74	4436	**0.76**
Psychrobacter arcticus 273-4	2.65	6715	**0.76**
Tropheryma whipplei str. Twist	0.93	2312	**0.75**
Tropheryma whipplei TW08/27	0.93	2308	**0.74**
Ehrlichia ruminantium str. Welgevonden	1.52	3715	**0.73**
Mycoplasma penetrans HF-2	1.36	3317	**0.73**
Lactobacillus johnsonii NCC 533	1.99	4734	**0.71**
Staphylococcus aureus subsp. aureus N315	2.84	6713	**0.71**
Mannheimia succiniciproducens MBEL55E	2.31	5399	**0.70**
Legionella pneumophila str. Lens	3.41	7919	**0.70**
Staphylococcus aureus subsp. aureus Mu50	2.9	6713	**0.69**
Nocardia farcinica IFM 10152	6.29	14474	**0.69**
Ehrlichia ruminantium str. Gardel	1.5	3448	**0.69**
Mesoplasma florum L1	0.79	1814	**0.69**
Mycoplasma pneumoniae M129	0.82	1882	**0.69**
Anaplasma phagocytophilum HZ	1.47	3373	**0.69**
Buchnera aphidicola str. Bp (Baizongia pistaciae)	0.62	1404	**0.68**
Wolbachia endosymbiont of Drosophila melanogaster	1.27	2843	**0.67**
Neorickettsia sennetsu str. Miyayama	0.86	1921	**0.67**
Pseudomonas syringae pv. syringae B728a	6.09	13537	**0.67**
Streptococcus pneumoniae TIGR4	2.16	4776	**0.66**
Buchnera aphidicola str. Sg (Schizaphis graminum)	0.64	1413	**0.66**
Borrelia garinii PBi	0.99	2162	**0.66**
Aster yellows witches'-broom phytoplasma AYWB	0.72	1572	**0.66**
Lactobacillus acidophilus NCFM	1.99	4326	**0.65**
Buchnera aphidicola str. APS (Acyrthosiphon pisum)	0.66	1407	**0.64**
Legionella pneumophila str. Paris	3.64	7679	**0.63**
Rickettsia prowazekii str. Madrid E	1.11	2340	**0.63**
Rickettsia typhi str. Wilmington	1.11	2331	**0.63**
Helicobacter acinonychis str. Sheeba	1.56	3216	**0.62**
Baumannia cicadellinicola str. Hc (Homalodisca coagulata)	0.69	1408	**0.61**
Wigglesworthia glossinidia endosymbiont of Glossina brevipalpis	0.7	1405	**0.60**
Candidatus Blochmannia floridanus	0.71	1420	**0.60**
Helicobacter pylori HPAG1	1.61	3185	**0.59**
Mycoplasma gallisepticum R	1	1976	**0.59**
Rickettsia felis URRWXCal2	1.59	3122	**0.59**
Helicobacter pylori J99	1.64	3194	**0.58**
Mycoplasma capricolum subsp. capricolum ATCC 27343	1.01	1965	**0.58**
Magnetospirillum magneticum AMB-1	4.97	9529	**0.58**
Methanosphaera stadtmanae DSM 3091	1.77	3356	**0.57**
Onion yellows phytoplasma OY-M	0.86	1571	**0.55**
Bartonella quintana str. Toulouse	1.58	2855	**0.54**

Candidatus Blochmannia pennsylvanicus str. BPEN	0.79	1416	**0.54**
Silicibacter pomeroyi DSS-3	4.6	8093	**0.53**
Pasteurella multocida subsp. multocida str. Pm70	2.26	3919	**0.52**
Helicobacter pylori 26695	1.67	2893	**0.52**
Chlamydia trachomatis D/UW-3/CX	1.04	1786	**0.52**
Chlamydia trachomatis A/HAR-13	1.05	1786	**0.51**
Methanocaldococcus jannaschii DSM 2661	1.74	2894	**0.50**
Mycoplasma mycoides subsp. mycoides SC str. PG1	1.21	1972	**0.49**
Staphylococcus haemolyticus JCSC1435	2.69	4354	**0.49**
Nitrosospira multiformis ATCC 25196	3.23	5216	**0.48**
Helicobacter hepaticus ATCC 51449	1.8	2894	**0.48**
Rickettsia conorii str. Malish 7	1.27	2021	**0.48**
Chlamydophila abortus S26/3	1.14	1806	**0.48**
Lactobacillus plantarum WCFS1	3.35	5289	**0.47**
Bartonella henselae str. Houston-1	1.93	3036	**0.47**
Synechococcus sp. CC9902	2.23	3504	**0.47**
Myxococcus xanthus DK 1622	9.14	14274	**0.47**
Geobacter sulfurreducens PCA	3.81	5899	**0.46**
Erwinia carotovora subsp. atroseptica SCRI1043	5.06	7523	**0.45**
Chlamydophila pneumoniae AR39	1.23	1826	**0.45**
Chlamydophila pneumoniae CWL029	1.23	1826	**0.45**
Chlamydophila pneumoniae J138	1.23	1826	**0.45**
Chlamydophila pneumoniae TW-183	1.23	1826	**0.45**
Corynebacterium jeikeium K411	2.48	3618	**0.44**
Fusobacterium nucleatum subsp. nucleatum ATCC 25586	2.17	3165	**0.44**
Zymomonas mobilis subsp. mobilis ZM4	2.06	2984	**0.43**
Pseudomonas entomophila L48	5.89	8493	**0.43**
Borrelia burgdorferi B31	1.52	2166	**0.43**
Pseudomonas putida KT2440	6.18	8682	**0.42**
Bifidobacterium longum NCC2705	2.26	3172	**0.42**
Xylella fastidiosa Temecula1	2.52	3457	**0.41**
Wolinella succinogenes DSM 1740	2.11	2883	**0.41**
Treponema pallidum subsp. pallidum str. Nichols	1.14	1533	**0.40**
Streptococcus mutans UA159	2.03	2724	**0.40**
Thermoplasma acidophilum DSM 1728	1.56	2081	**0.40**
Mycobacterium avium subsp. paratuberculosis K-10	4.83	6384	**0.40**
Candidatus Protochlamydia amoebophila UWE25	2.41	3181	**0.40**
Thermoplasma volcanium GSS1	1.58	2076	**0.39**
Streptococcus pyogenes MGAS10750	1.94	2547	**0.39**
Bacillus subtilis subsp. subtilis str. 168	4.21	5488	**0.39**
Rickettsia bellii RML369-C	1.52	1981	**0.39**
Streptococcus pyogenes MGAS2096	1.86	2416	**0.39**
Xylella fastidiosa 9a5c	2.73	3455	**0.38**
Thermus thermophilus HB8	2.12	2672	**0.38**
Pyrobaculum aerophilum str. IM2	2.22	2785	**0.38**
Thermus thermophilus HB27	2.13	2672	**0.38**
Methanococcus maripaludis S2	1.66	2076	**0.38**
Streptococcus pneumoniae R6	2.04	2551	**0.38**
Dehalococcoides ethenogenes 195	1.47	1834	**0.37**
Methanopyrus kandleri AV19	1.69	2042	**0.36**
Pyrococcus abyssi GE5	1.77	2122	**0.36**
Corynebacterium diphtheriae NCTC 13129	2.49	2977	**0.36**
Thiobacillus denitrificans ATCC 25259	2.91	3472	**0.36**
Neisseria meningitidis MC58	2.27	2703	**0.36**
Treponema denticola ATCC 35405	2.84	3320	**0.35**
Yersinia pseudotuberculosis IP 32953	4.84	5623	**0.35**
Aeropyrum pernix K1	1.67	1933	**0.35**

Shewanella denitrificans OS217	4.55	5236	**0.35**
Rhodopirellula baltica SH 1	7.15	8173	**0.34**
Vibrio cholerae O1 biovar eltor str. N16961	4.03	4558	**0.34**
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	4.94	5559	**0.34**
Salmonella typhimurium LT2	4.95	5559	**0.34**
Methylobacillus flagellatus KT	2.97	3332	**0.34**
Streptococcus pyogenes MGAS5005	1.84	2059	**0.34**
Archaeoglobus fulgidus DSM 4304	2.18	2425	**0.33**
Legionella pneumophila subsp. pneumophila str. Philadelphia 1	3.4	3780	**0.33**
Streptococcus pyogenes MGAS6180	1.9	2106	**0.33**
Streptococcus pyogenes M1 GAS	1.85	2045	**0.33**
Nitrosomonas europaea ATCC 19718	2.81	3064	**0.33**
Rhodopseudomonas palustris HaA2	5.33	5769	**0.32**
Dechloromonas aromatica RCB	4.5	4854	**0.32**
Picrophilus torridus DSM 9790	1.55	1667	**0.32**
Campylobacter jejuni RM1221	1.78	1899	**0.32**
Deinococcus geothermalis DSM 11300	3.04	3243	**0.32**
Thermoanaerobacter tengcongensis MB4	2.69	2862	**0.32**
Synechocystis sp. PCC 6803	3.95	4199	**0.32**
Lactobacillus sakei subsp. sakei 23K	1.88	1987	**0.32**
Streptococcus agalactiae 2603V/R	2.16	2274	**0.32**
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842	1.86	1946	**0.31**
Acinetobacter sp. ADP1	3.6	3711	**0.31**
Bordetella pertussis Tohama I	4.09	4196	**0.31**
Dehalococcoides sp. CBDB1	1.4	1434	**0.31**
Methanothermobacter thermautotrophicus str. Delta H	1.75	1787	**0.31**
Methylococcus capsulatus str. Bath	3.3	3349	**0.30**
Thermobifida fusca YX	3.64	3629	**0.30**
Salmonella enterica subsp. enterica serovar Paratyphi A str. ATCC 9150	4.59	4560	**0.30**
Desulfotalea psychrophila LSv54	3.66	3628	**0.30**
Aquifex aeolicus VF5	1.59	1574	**0.30**
Porphyromonas gingivalis W83	2.34	2316	**0.30**
Prochlorococcus marinus str. NATL2A	1.84	1821	**0.30**
Methanococcoides burtonii DSM 6242	2.58	2552	**0.30**
Clostridium tetani E88	2.87	2838	**0.30**
Shewanella oneidensis MR-1	5.13	5020	**0.29**
Corynebacterium efficiens YS-314	3.15	3022	**0.29**
Pseudomonas syringae pv. tomato str. DC3000	6.54	6274	**0.29**
Bacillus anthracis str. Ames	5.23	5017	**0.29**
Bacillus anthracis str. Sterne	5.23	5017	**0.29**
Mycobacterium bovis AF2122/97	4.35	4151	**0.29**
Streptococcus pyogenes MGAS9429	1.84	1755	**0.29**
Escherichia coli O157:H7 str. Sakai	5.59	5291	**0.28**
Bacillus thuringiensis serovar konkukian str. 97-27	5.31	5017	**0.28**
Enterococcus faecalis V583	3.36	3173	**0.28**
Pseudomonas fluorescens Pf-5	7.07	6675	**0.28**
Mycobacterium tuberculosis CDC1551	4.4	4151	**0.28**
Mycobacterium tuberculosis H37Rv	4.41	4151	**0.28**
Mycobacterium leprae TN	3.27	3076	**0.28**
Haemophilus influenzae 86-028NP	1.91	1794	**0.28**
Streptococcus agalactiae NEM316	2.21	2066	**0.28**
Anabaena variabilis ATCC 29413	7.07	6581	**0.28**
Vibrio fischeri ES114	4.28	3971	**0.28**
Haemophilus influenzae Rd KW20	1.83	1694	**0.28**
Campylobacter jejuni subsp. jejuni NCTC 11168	1.64	1517	**0.28**

Neisseria meningitidis Z2491	2.18	2015	**0.28**
Bacillus cereus ATCC 14579	5.43	5017	**0.28**
Escherichia coli O157:H7 EDL933	5.62	5188	**0.28**
Neisseria gonorrhoeae FA 1090	2.15	1977	**0.28**
Yersinia pestis Nepal516	4.65	4270	**0.28**
Prochlorococcus marinus subsp. pastoris str. CCMP1986	1.66	1521	**0.27**
Bacillus anthracis str. 'Ames Ancestor'	5.5	5017	**0.27**
Pyrococcus furiosus DSM 3638	1.91	1740	**0.27**
Thermotoga maritima MSB8	1.86	1690	**0.27**
Corynebacterium glutamicum ATCC 13032	3.31	2996	**0.27**
Xanthomonas axonopodis pv. citri str. 306	5.27	4753	**0.27**
Pseudomonas aeruginosa PAO1	6.26	5627	**0.27**
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305	2.58	2316	**0.27**
Chromohalobacter salexigens DSM 3043	3.7	3314	**0.27**
Pseudoalteromonas atlantica T6c	5.19	4629	**0.27**
Streptococcus pyogenes MGAS10270	1.93	1715	**0.27**
Bordetella parapertussis 12822	4.77	4218	**0.27**
Pseudomonas fluorescens PfO-1	6.44	5654	**0.26**
Geobacter metallireducens GS-15	4.01	3507	**0.26**
Staphylococcus aureus RF122	2.74	2396	**0.26**
Desulfovibrio desulfuricans G20	3.73	3252	**0.26**
Brucella abortus biovar 1 str. 9-941	3.29	2867	**0.26**
Brucella melitensis 16M	3.29	2867	**0.26**
Prochlorococcus marinus subsp. marinus str. CCMP1375	1.75	1524	**0.26**
Chromobacterium violaceum ATCC 12472	4.75	4130	**0.26**
Streptococcus pyogenes MGAS10394	1.9	1648	**0.26**
Streptococcus pyogenes MGAS8232	1.9	1647	**0.26**
Burkholderia pseudomallei K96243	7.25	6274	**0.26**
Novosphingobium aromaticivorans DSM 12444	3.56	3069	**0.26**
Thermococcus kodakarensis KOD1	2.09	1798	**0.26**
Bacillus cereus E33L	5.84	5017	**0.26**
Streptococcus pyogenes SSI-1	1.89	1623	**0.26**
Prochlorococcus marinus str. MIT 9312	1.71	1468	**0.26**
Burkholderia pseudomallei 1710b	7.31	6274	**0.26**
Coxiella burnetii RSA 493	2.03	1734	**0.26**
Streptococcus pyogenes MGAS315	1.9	1622	**0.26**
Bacillus licheniformis ATCC 14580	4.22	3588	**0.26**
Streptomyces avermitilis MA-4680	9.12	7746	**0.25**
Frankia sp. CcI3	5.43	4606	**0.25**
Staphylococcus aureus subsp. aureus MSSA476	2.82	2391	**0.25**
Thiomicrospira denitrificans ATCC 33889	2.2	1864	**0.25**
Lactococcus lactis subsp. lactis Il1403	2.37	1983	**0.25**
Pseudomonas syringae pv. phaseolicola 1448A	6.11	5107	**0.25**
Brucella melitensis biovar Abortus 2308	3.28	2732	**0.25**
Streptococcus agalactiae A909	2.13	1774	**0.25**
Propionibacterium acnes KPA171202	2.56	2117	**0.25**
Lawsonia intracellularis PHE/MN1-00	1.72	1420	**0.25**
Streptomyces coelicolor A3(2)	9.05	7463	**0.25**
Synechococcus sp. JA-3-3Ab	2.93	2413	**0.25**
Brucella suis 1330	3.32	2732	**0.25**
Synechococcus sp. JA-2-3B'a(2-13)	3.05	2505	**0.25**
Streptococcus thermophilus LMG 18311	1.8	1470	**0.25**
Streptococcus thermophilus CNRZ1066	1.8	1464	**0.24**
Anaeromyxobacter dehalogenans 2CP-C	5.01	4074	**0.24**
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough	3.77	3038	**0.24**
Rhodopseudomonas palustris BisB18	5.51	4435	**0.24**
Rhodopseudomonas palustris CGA009	5.47	4335	**0.24**

Jannaschia sp. CCS1	4.4	3486	**0.24**
Hahella chejuensis KCTC 2396	7.22	5711	**0.24**
Bordetella bronchiseptica RB50	5.34	4218	**0.24**
Erythrobacter litoralis HTCC2594	3.05	2409	**0.24**
Yersinia pestis KIM	4.7	3710	**0.24**
Burkholderia mallei ATCC 23344	5.84	4580	**0.24**
Xanthomonas campestris pv. campestris str. ATCC 33913	5.08	3961	**0.23**
Yersinia pestis biovar Medievalis str. 91001	4.8	3710	**0.23**
Xanthomonas campestris pv. campestris str. 8004	5.15	3961	**0.23**
Leifsonia xyli subsp. xyli str. CTCB07	2.58	1980	**0.23**
Yersinia pestis CO92	4.83	3705	**0.23**
Francisella tularensis subsp. holarctica	1.9	1444	**0.23**
Yersinia pestis Antiqua	4.88	3705	**0.23**
Salmonella enterica subsp. enterica serovar Typhi Ty2	4.79	3624	**0.23**
Listeria monocytogenes str. 4b F2365	2.91	2195	**0.23**
Francisella tularensis subsp. tularensis SCHU S4	1.89	1422	**0.23**
Bdellovibrio bacteriovorus HD100	3.78	2828	**0.22**
Psychrobacter cryohalolentis K5	3.1	2301	**0.22**
Methanosarcina acetivorans C2A	5.75	4226	**0.22**
Ralstonia solanacearum GMI1000	5.81	4268	**0.22**
Prochlorococcus marinus str. MIT 9313	2.41	1765	**0.22**
Natronomonas pharaonis DSM 2160	2.75	1999	**0.22**
Salinibacter ruber DSM 13855	3.59	2597	**0.22**
Salmonella enterica subsp. enterica serovar Typhi str. CT18	5.13	3624	**0.21**
Gloeobacter violaceus PCC 7421	4.66	3277	**0.21**
Chlorobium tepidum TLS	2.15	1510	**0.21**
Listeria innocua Clip11262	3.09	2167	**0.21**
Colwellia psychrerythraea 34H	5.37	3758	**0.21**
Listeria monocytogenes EGD-e	2.94	2044	**0.21**
Clostridium acetobutylicum ATCC 824	4.13	2870	**0.21**
Xanthomonas campestris pv. vesicatoria str. 85-10	5.42	3709	**0.21**
Clostridium perfringens str. 13	3.09	2104	**0.20**
Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130	4.63	3140	**0.20**
Lactobacillus salivarius subsp. salivarius UCC118	2.13	1444	**0.20**
Leptospira interrogans serovar Lai str. 56601	4.69	3141	**0.20**
Bacillus clausii KSM-K16	4.3	2870	**0.20**
Methanospirillum hungatei JF-1	3.54	2353	**0.20**
Vibrio parahaemolyticus RIMD 2210633	5.17	3412	**0.20**
Synechococcus elongatus PCC 7942	2.74	1807	**0.20**
Thermosynechococcus elongatus BP-1	2.59	1706	**0.20**
Thiomicrospira crunogena XCL-2	2.43	1597	**0.20**
Oceanobacillus iheyensis HTE831	3.63	2373	**0.20**
Sulfolobus acidocaldarius DSM 639	2.23	1452	**0.20**
Geobacillus kaustophilus HTA426	3.59	2301	**0.19**
Rhodopseudomonas palustris BisB5	4.89	3094	**0.19**
Escherichia coli UTI89	5.18	3262	**0.19**
Symbiobacterium thermophilum IAM 14863	3.57	2244	**0.19**
Pseudoalteromonas haloplanktis TAC125	3.85	2410	**0.19**
Bradyrhizobium japonicum USDA 110	9.11	5685	**0.19**
Mycobacterium sp. MCS	5.92	3693	**0.19**
Sphingopyxis alaskensis RB2256	3.37	2090	**0.19**
Caulobacter crescentus CB15	4.02	2479	**0.19**
Escherichia coli CFT073	5.23	3216	**0.18**
Synechococcus sp. CC9605	2.51	1533	**0.18**
Rhodoferax ferrireducens T118	4.97	3026	**0.18**
Gluconobacter oxydans 621H	2.92	1750	**0.18**
Bacillus cereus ATCC 10987	5.43	3242	**0.18**

Xanthomonas oryzae pv. oryzae KACC10331	4.94	2941	**0.18**
Xanthomonas oryzae pv. oryzae MAFF 311018	4.94	2927	**0.18**
Deinococcus radiodurans R1	3.28	1940	**0.18**
Nitrobacter winogradskyi Nb-255	3.4	2002	**0.18**
Carboxydothermus hydrogenoformans Z-2901	2.4	1407	**0.18**
Synechococcus elongatus PCC 6301	2.7	1568	**0.17**
Vibrio vulnificus CMCP6	5.13	2937	**0.17**
Idiomarina loihiensis L2TR	2.84	1615	**0.17**
Nitrobacter hamburgensis X14	5.01	2845	**0.17**
Moorella thermoacetica ATCC 39073	2.63	1487	**0.17**
Acidobacteria bacterium Ellin345	5.65	3121	**0.17**
Burkholderia thailandensis E264	6.72	3650	**0.16**
Sulfolobus tokodaii str. 7	2.69	1442	**0.16**
Halobacterium sp. NRC-1	2.57	1370	**0.16**
Photobacterium profundum SS9	6.4	3399	**0.16**
Nitrosococcus oceani ATCC 19707	3.52	1867	**0.16**
Desulfitobacterium hafniense Y51	5.73	3013	**0.16**
Pelobacter carbinolicus DSM 2380	3.67	1925	**0.16**
Silicibacter sp. TM1040	4.15	2150	**0.16**
Mesorhizobium loti MAFF303099	7.6	3930	**0.16**
Escherichia coli K12	4.64	2367	**0.15**
Escherichia coli W3110	4.65	2367	**0.15**
Syntrophus aciditrophicus SB	3.18	1606	**0.15**
Rubrobacter xylanophilus DSM 9941	3.23	1618	**0.15**
Agrobacterium tumefaciens str. C58	5.67	2831	**0.15**
Agrobacterium tumefaciens str. C58	5.67	2802	**0.15**
Sulfolobus solfataricus P2	2.99	1426	**0.14**
Polaromonas sp. JS666	5.9	2793	**0.14**
Methanosarcina barkeri str. fusaro	4.87	2272	**0.14**
Methanosarcina mazei Go1	4.1	1898	**0.14**
Nostoc sp. PCC 7120	7.21	3262	**0.14**
Vibrio vulnificus YJ016	5.26	2365	**0.13**
Rhodospirillum rubrum ATCC 11170	4.41	1981	**0.13**
Burkholderia cenocepacia AU 1054	7.28	3231	**0.13**
Rhizobium etli CFN 42	6.53	2825	**0.13**
Bacillus halodurans C-125	4.2	1816	**0.13**
Azoarcus sp. EbN1	4.73	2043	**0.13**
Sinorhizobium meliloti 1021	6.69	2832	**0.13**
Rhodobacter sphaeroides 2.4.1	4.45	1808	**0.12**
Bacteroides fragilis NCTC 9343	5.24	1957	**0.11**
Bacteroides fragilis YCH46	5.31	1957	**0.11**
Shigella boydii Sb227	4.65	1653	**0.11**
Haloarcula marismortui ATCC 43049	4.27	1515	**0.11**
Shigella dysenteriae Sd197	4.55	1588	**0.10**
Bacteroides thetaiotaomicron VPI-5482	6.29	2183	**0.10**
Sodalis glossinidius str. 'morsitans'	4.29	1484	**0.10**
Shigella flexneri 2a str. 2457T	4.6	1522	**0.10**
Burkholderia xenovorans LB400	9.73	3192	**0.10**
Shigella sonnei Ss046	5.04	1653	**0.10**
Shigella flexneri 2a str. 301	4.83	1522	**0.09**
Ralstonia metallidurans CH34	6.91	1993	**0.09**
Ralstonia eutropha JMP134	7.26	1957	**0.08**
Burkholderia sp. 383	8.68	1967	**0.07**

CAPÍTULO 2

Mecanismos diferentes pero solapantes determinan la colonización de superficies abióticas y vegetales por *Pseudomonas putida*

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Abstract

Mechanisms governing biofilm formation have generated great interest in recent years. Yet, comparative analyses of processes for bacterial establishment on abiotic and biotic surfaces are still limited. In this report we have expanded previous information on genetic determinants required for colonization of plant surfaces by *Pseudomonas putida* populations, and analyzed their correlation with biofilm formation processes on abiotic surfaces. Insertional mutations affecting flagellar genes or the synthesis and transport of the large adhesin LapA lead to decreased adhesion to seeds and biofilm formation on abiotic surfaces. The later also cause reduced fitness in the rhizosphere. Decreased seed adhesion and altered biofilm formation kinetics are observed in mutants affected in heme biosynthesis and a gene that might participate in oxidative stress responses, whereas a mutant in a gene involved in cytochrome oxidase assembly is affected in the bacterium-plant interaction but not in bacterial establishment on abiotic surfaces. Finally, a mutant altered in LPS biosynthesis is impaired in seed and root colonization but seems to initiate attachment to plastic faster than the wild type. This variety of phenotypes reflects the complexity of bacterial adaptation to sessile life, and the partial overlap between mechanisms leading to biofilm formation on abiotic and biotic surfaces.

Introduction

It is now generally accepted that the formation of multicellular, matrix-encased communities associated to solid surfaces (biofilms) is an intrinsic feature of the bacterial life cycle, and a common strategy for microbial persistence in a variety of environments. A number of genetic determinants participating in this process have been described and characterized in different microorganisms (for reviews, see Kierek-Pearson & Karatan, 2005, and Lasa, 2006), and global changes in gene expression in biofilms are being studied (Prüss et al., 2006). However, one of the areas that still remains to be fully explored is the elucidation of the similarities and differences between pathways leading to bacterial colonization of abiotic and biotic surfaces. Aside from its implications in medical microbiology, this information is relevant in the study of plant-bacterial interactions, given the impact that colonization of plant surfaces by pathogenic or mutualistic bacteria can have on plant health, nutritional status or stress responses. The correlation between biofilm formation and bacterial adhesion to plant surfaces may be reflected by the appearance of microcolonies and biofilm-resembling structures of both epiphytic (i.e. colonizers of the aerial parts of the plant) and root-associated bacteria (Monier & Lindow, 2004; Ramos et al., 2000). In the later case, traits that are of relevance for bacterial fitness in the rhizosphere (surface of roots and surrounding soil area) have been studied to a signficant extent in plant-beneficial *Pseudomonas* (Lugtenberg et al., 2001) but there is less information on elements directly involved in the ability of bacterial cells to attach to and establish on the plant surface.

We have previously identified functions required for adhesion of *Pseudomonas putida* to corn seeds (Espinosa-Urgel et al., 2000). Only two of the mutants then analyzed were defective both in seed colonization and in biofilm formation on different abiotic surfaces, suggesting the existence of specific determinants for colonization of the plant surface as well as certain common elements for both processes. These mutants were affected in a 8682 amino acids protein, termed LapA, which was subsequently identified and characterized as a key element for adhesion of *P. putida* to seeds and for the early stages of *P. fluorescens* biofilm formation on abiotic surfaces (Hinsa et al., 2003). That information has now been expanded by isolating and characterizing a new battery of *P. putida* mutants that confirm the importance of flagella and the large adhesin LapA as general adhesion factors, and reveal new functions involved either in both processes or specifically in the establishment and survival of *P. putida* on plant surfaces.

Materials and methods

Strains and culture conditions

Pseudomonas putida KT2440 is a plasmid-free derivative of *P. putida* mt-2, a strain that was isolated from a vegetable-planted field (Nakazawa, 2002). *E. coli* HB101(RK600) and CC118λ*pir*(pUT-Km1) were from our laboratory collection. *P. putida* strains were grown at 30ºC, in LB or M9 minimal medium (Sambrook et al., 1989) with glucose (10 mM) or sodium citrate (15 mM), supplemented with 1 mM SO_4Mg, 0.06% (w/v) Fe-citrate, and 0.25% (v/v) trace elements solution (final concentrations, in g/L: HBO_3, 75; $ZnCl_2$, 12.5; $MnCl_2$, 7.5; $CoCl_2$, 50; $CuCl_2$, 2.5; $NiCl_2$, 5; $NaMoO_4$, 7.5). *E. coli* strains were grown at 37ºC in LB. When appropriate, antibiotics were added at the following concentrations (g/mL): kanamycin (Km), 25; ampicillin (Ap), 100; streptomycin (Sm): 100; chloramphenicol (Cm), 30.

Transposon mutagenesis and selection of seed adhesion deficient mutants

Transposon mutagenesis with mini-Tn*5*[Km1] (de Lorenzo et al., 1990) was performed by triparental mating, with KT2440 as the recipient and *E. coli* CC118λ*pir*(pUT-Km1) and HB101(RK600) as the donor and helper strains, respectively. Selection of mutants with reduced seed colonization capacity was essentially done as described previously (Espinosa-Urgel et al., 2000). Six independent matings were performed, and the mutants obtained in each case were pooled. The pools were grown in LB to mid-exponential phase and diluted in 5 mL of M9 basal medium, and then introduced in syringes filled with hydrated, surface-sterilized corn seeds. After incubation for 1 hour at room temperature, syringes were opened and washed with 5 mL of sterile M9 basal medium. The flow-through was collected and dilutions were plated on selective minimal medium. The resulting colonies were collected and passed once more through seed columns and the clones thus obtained were then assayed individually. Qualitative evaluation of adhesion to seeds was performed as described (Espinosa-Urgel et al., 2000), discarding those clones with obvious growth defects in LB. Clones showing a consistent phenotype through three rounds of assays were kept for further analysis.

Identification of mini-Tn*5* insertion sites

Transposon insertion sites were determined by arbitrary PCR followed by sequencing, in a way similar to that previously described (Espinosa-Urgel et al., 2000). Primer sequences and

PCR conditions are available upon request. Comparison of the obtained sequences with genome databases was done using BLAST programs available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Seed attachment and competitive rhizosphere colonization assays

Quantitative seed attachment assays were done following previously described protocols (Espinosa-Urgel and Ramos, 2004). For rhizosphere colonization experiments, surface-sterilized seeds were allowed to germinate at 30ºC in the dark for 48 h. An otherwise isogenic KT2440 derivative carrying a streptomycin resistance gene in single copy in the chromosome (KT2440-Sm), generated by site specific insertion of miniTn*7*-ΩSm1 (Koch et al., 2001) at an extragenic location near *glmS* was used for these competitive colonization studies. Where indicated, strains labeled in a similar way with miniTn*7* derivatives harboring constitutively expressed *dsred* or *gfp* were used.

Strains were grown in LB and diluted in M9 to an OD_{660} = 1. KT2440-Sm and each mutant were then mixed in a 1:1 proportion (~5x10^{6} cfu of each strain) in 10 ml plant nutrient solution (Ramos-González et al., 2005). This mix was poured on 50 ml Sterilin tubes containing 40 g of sterile washed silica sand, where germinated seeds were then sown. Plants were maintained in a controlled chamber at 24°C and 55-65% humidity 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 Km or Sm, respectively).

Motility, Congo red binding and biofilm formation assays

Swimming motility was tested by spotting 1 L of overnight cultures in the center of LB plates with 0.3% agar and measuring movement halos after 6h at 30ºC. Swarming was assayed at 25ºC as previously described (Matilla et al., 2007b). Congo red binding was assayed on tryptone medium (10 g/L tryptone, 1% agar) with 40 g/mL Congo red. Attachment to abiotic surfaces and biofilm formation were tested in LB in 96-wells microtiter plates, using the crystal violet staining and quantification method described by O’Toole & Kolter (1998), or during growth in borosilicate tubes under orbital shaking at 40 r.p.m.

Sensitivity to UV light and hydrogen peroxide

To test bacterial sensitivity to UV, cultures were grown to mid-exponential phase, centrifuged, resuspended in M9 basal medium and plated on LB after 0, 5, 10, 20 and 30 seconds of exposure to UV light (354 nm). Plates were incubated at 30ºC in the dark and the number of viable counts recorded. Sensitivity to hydrogen peroxide was analyzed by measuring turbidity of overnight cultures grown in LB with different concentrations of H_2O_2.

Results and Discussion

Isolation and characterization of mutants defective in adhesion to corn seeds

In a previous work we had identified eight different mutants that showed reduced capacity to colonize corn seeds (Espinosa-Urgel et al., 2000). Thirty-five new mutants deficient in adhesion to seeds were isolated after transposon mutagenesis and several independent rounds of selection using a similar approach, with the difference that in this case enrichment and selection experiments were done with exponentially growing cells instead of stationary phase cells. In this way we intended to identify functions that might not have been represented in that previous screen due to differential expression under various growth situations. All the selected mutants showed growth rates similar to the wild type in different media (data not shown). The site of insertion of the minitransposon in the chromosome could be determined for all the mutants except two. Data are presented in Table 1, which also includes phenotypic characteristics such as swimming and swarming motility, and Congo red binding.

Most mutants fall in two categories, in terms of functions affected by the transposon insertion. A significant proportion of mutants present insertions at several positions in the genes coding for LapA, as well as the LapB and LapC proteins, which are part of the ABC transporter responsible for its export (Hinsa et al., 2003). No mutant was isolated affecting *lapE*, which in *Pseudomonas fluorescens* forms an operon with *lapB* and *lapC* and encodes the outer membrane component of this ABC transporter. In *P. putida* KT2440, the homolog of *lapE* is located in a different chromosomal region. Three other mutants in this group correspond to the adjacent *lapD* gene (PP_0165), that in *P. fluorescens* has been described to modulate LapA secretion (Hinsa & O'Toole, 2006). As in *P. fluorescens*, *P. putida* LapD shows non-canonical GGDEF and EAL domains. These domains are involved in the turnover

of cyclic di-GMP, modulating the transition between planktonic and sessile lifestyles as well as other multicellular behaviors (Römling et al., 2005).

Table 1. Characteristics of mus mutants

model mutant	num. ≠ hits / total mutants	locus (gene)	function	swim[a]	swarm	Congo red[b]
mus-42	4 / 11	PP_0168 (*lapA*)	large surface adhesin	+	+	red & spread
mus-59	1 / 2	PP_0167 (*lapB*)	LapA secretion	+	+	"
mus-46	2 / 4	PP_0166 (*lapC*)	LapA secretion	+	+	"
mus-49	2 / 3	PP_0165 (*lapD*)	possible LapA regulation	+	+	"
mus-64	1 / 1	PP_4380 (*flgL*)	hook-filament junction	+-	+	as wt
mus-65	1 / 2	PP_4341 (*fliA*)	flagellar sigma factor	-	+	spread
mus-69	1 / 1	PP_4373 (*fleQ*)	flagellar regulator	+-	-	as wt
mus-70	1 / 1	PP_4366 (*fliI*)	flagellar secretion system	-	+-	"
mus-73	1 / 1	PP_4357 (*fliN*)	flagellar motor switch	-	-	"
mus-74	1 / 1	PP_4389 (*flgD*)	flagellar hook scaffolding protein	-	+-	"
mus-72	1 / 1	PP_5101 (*hemN2*)	coproporphyrinogen III oxidase	+	+	as wt
mus-53	1 / 1	PP_0110 (*coxE*)	protoheme farnesyl transferase	+	-	"
mus-40	1 / 1	PP_3821 (*galU*)	UDP-glucose pyrophosphorylase	+-	-	red & rough
mus-63	1 / 1	PP_5311	unknown (oxidative stress)	+	-	as wt

[a]Swimming and swarming patterns relative to the wild type; + similar to KT2440; - no movement observed; +- reduced swimming halos or swarming motility; nd, not determined. [b]Colonies of the wild type (wt) are pink, with a smooth appearance.

A second group corresponds to genes involved in the synthesis, regulation or functionality of the flagellar apparatus. As expected, all these mutants were impaired in swimming motility to different degrees, and some of them also showed defects in swarming motility (detailed below). The remaining mutants were affected in functions such as LPS and heme biosynthesis, cytochrome oxidase assembly, and a hypothetical protein of unknown function. Consistently with previous observations on the role of LPS (Matilla et al., 2007b), mus-40 was deficient in swarming motility. This mutant is affected in *galU*, encoding UDP-glucose pyrophosphorylase, which in *P. aeruginosa* has been shown to be required for the synthesis of intact lipopolysaccharide (Dean & Goldberg, 2002), a role that we could confirm in *P. putida* by electrophoretic analysis of LPS of the wild type and mus-40 (data not shown).

The mutant also showed differences with respect to the wild type on Congo red plates, colonies being darker and less smooth. Defects in swarming were also observed in mutants afected in the flagellar motor switch protein FliN and in the FleQ regulator, as well as in mus-53 (mutated in PP_0110, a gene that we have renamed *coxE*, since it encodes a protoheme IX farnesyl transferase, presumably involved in assembly of the aa_3-type cytochrome oxidase Cox) and mus-63. This mutant is affected in a gene encoding a hypothetical 467 amino acids protein (PP_5311), highly conserved in *Pseudomonas*. Analysis of the genomic region surrounding PP_5311 revealed that this open reading frame is 78 bp downstream of the *oxyR-recG* operon, and transcribed in the same direction. Given the regulatory role of OxyR in oxidative stress response, and the involvement of RecG in DNA replication and repair (Ochsner et al., 2000), we considered the possibility that PP_5311 could also contribute to these processes. Hence, tolerance of KT2440 and mus-63 to H_2O_2 or to UV irradiation was tested. No significant differences in sensitivity to UV could be observed. However, growth of mus-63 (PP_5311) was impaired above 0.35% (v/v) H_2O_2, whereas the minimum inhibitory concentration was 0.7% in the case of the wild type. These data suggest that the protein encoded by PP_5311 may participate in oxidative stress responses.

Mutants representing each class were selected for a quantitative analysis of their seed adhesion capacity. As shown in Figure 1, the ability of all these strains to attach to corn seeds is significantly reduced in comparison with the wild type.

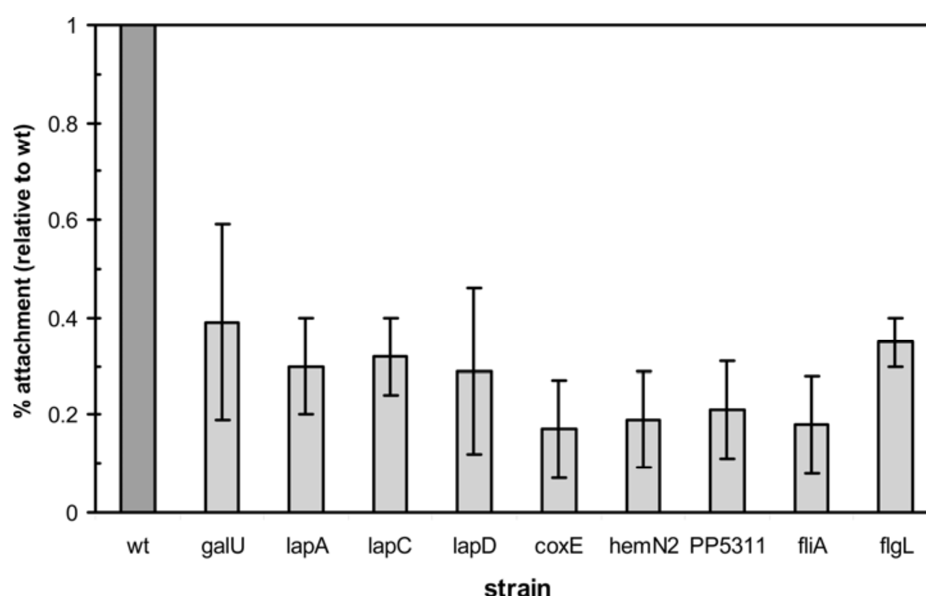

Figure 1. Adhesion of representative mus mutants to corn seeds, relative to the percentage of *P. putida* KT2440 cells attached after 1 h of incubation. Results are averages and standard deviations of at least 3 independent experiments (three seeds per experiment).

Competitive rhizosphere colonization

The potential role played by the identified functions in rhizosphere colonization was also tested. It is worth noting that expression of *coxE* (PP_0110), *lapD* (PP_0165) and several flagellar genes is up-regulated in *P. putida* in the corn rhizosphere (Matilla et al. 2007a). Competitive colonization assays were done with several representative mutants, mixed individually in a 1:1 proportion with the wild type. The mix was directly inoculated in sand tubes before sowing corn seedlings to ensure that the observed results reflected actual differences in rhizosphere fitness and were not only a consequence of the initial seed adhesion defects of the mutants. Bacteria were recovered after one week and the number of wild type and mutant cells was determined. Results are shown in Figure 2.

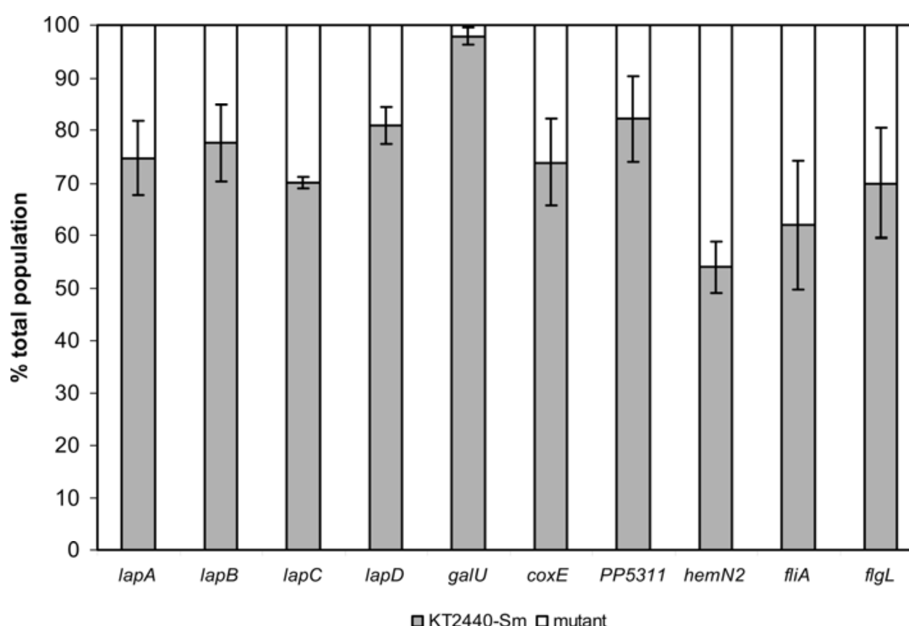

Figure 2. Competitive rhizosphere colonization by KT2440-Sm and different mus mutants. Data are given as the proportion of wild type and mutant strain in the total population and correspond to averages and S.D. of at least 6 plants.

Mutant mus-40 (*galU*) was the most severely affected in competition with the wild type. The number of cells of this mutant recovered from the rhizosphere was two orders of magnitude lower than that of KT2440-Sm cells (4.36×10^5 cfu/g versus 2.95×10^7). This is consistent with the previously reported influence of mutations affecting the O-antigen of LPS in tomato root tip colonization by *P. fluorescens* (Dekkers et al., 1998). Mutants in *lap* genes, as well as mus-53 (*coxE*) and mus-63 (PP_5311), also showed reduced colonization capacity,

but to a lesser extent. No significant reduction in competitive colonization capacity was observed in the case of mus-72, affected in a gene, *hemN-2* (PP_5101), encoding a putative coproporphyrinogen III oxidase, presumed to participate in heme biosynthesis. A second locus in the chromosome of *P. putida* has the same predicted role (*hemN*, PP_4264), hence it is possible that PP_5101 is relevant in the experimental conditions set for seed adhesion but its function is replaced by PP_4264 in the rhizosphere. In fact, a 7.6-fold induction of the expression of this gene has been observed in the rhizosphere in comparison with sessile populations in sand (Matilla et al., 2007), which seems to support this hypothesis. Similarly, the differences in rhizosphere fitness between KT2440 and mus-65, affected in *fliA*, were not significant, whereas a mutant in a structural flagellar gene (mus-64, affected in *flgL*) showed a larger decrease in competitive colonization. Several reports have revealed the importance of flagellar motility for root colonization by other *Pseudomonas* species (Capdevila et al., 2004; Martínez-Granero et al., 2006). It should be noted, however, that the data presented here correspond to the whole root system, while most previously published results refer solely to root tip colonization. One possibility was that although total numbers are similar, the distribution of KT2440 and mus-65 (*fliA*) along the root was different. To test this hypothesis, KT2440 and mus-65 were differentially labeled with *dsRed* and *gfp*, respectively, and root distribution was visualized by fluorescence microscopy. As shown in Figure 3, KT2440(*dsRed*) cells were significantly more abundant in the root tip than mus-65(*gfp*), while this did not happen in older parts of the root.

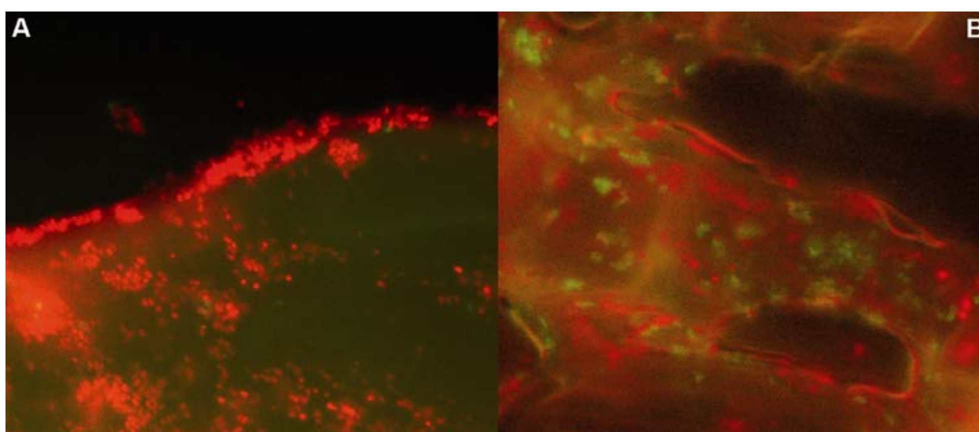

Figure 3. Distribution of KT2440(*dsRed*) and mus-65(*gfp*) on corn roots, close to the root tip (A) or at older parts (B). Each image is a composites of two pictures of the same field taken with specific filter sets for each fluorescent protein. Pictures were taken with a Nikon CCD camera mounted on a Zeiss Axioscope fluorescent microscope. Magnification: ×1000.

Biofilm formation on abiotic surfaces

In order to define which of the identified functions have a general role in adhesion of *P. putida* to solid surfaces and which are specific of the bacterium-plant interaction, the ability of different mutants to attach to abiotic surfaces was tested in static conditions in microtiter plates (Figure 4A), where KT2440 reaches the maximum observed attachment after ~6h of growth, followed by detachment from the surface at later times. All flagellar mutants showed significant reduction in their adhesion capacity with respect to the parental strain, as did the *lapD* mutant, especially at 3 h, but its phenotype is less marked than that previously described for *lapA* mutants (Espinosa-Urgel et al., 2000; Hinsa et al., 2003). Biofilm formation did not progress further in any of these mutants. Mutants in *coxE* and PP_5311 (mus-53 and mus-63), on the other hand, showed attachment kinetics similar to the wild type, although with a slight delay in the second case. The capacity to initiate biofilm formation of the *hemN2* mutant mus-72 was comparable to that of KT2440, but it does not progress further, so that the quantity of biomass attached to the surface is the same after 3 or 6 h. A very different phenotype was observed for the *galU* mutant mus-40. Although data were highly variable, this strain always showed a significant increase in early attachment, followed by a decline in biomass associated to the surface after 6 h. It is possible that LPS hinders initial adhesion, but is required at later stages of biofilm development. This contrasts with the recently reported defect of certain LPS-deficient mutants in the early stages of biofilm formation by *Klebsiella pneumoniae* (Balestrino et al., 2008).

Biofilm formation was also followed during growth in rotating glass tubes (Figure 4B). Under these conditions, the delayed attachment of mus-63 (PP_5311) was more evident, whereas the differences between the wild type and mus-40 (*galU*) were not as significant as in microtiter plates. The most severe defects were observed in the *fleQ* and *lapD* mutants. Intriguingly, in both cases a thick biofilm could be observed after 24 of growth (data not shown), as if the regular kinetics of KT2440 (attachment during exponential growth of the liquid culture and detachment in stationary phase) were reversed in these mutants, a phenotype that was only observed in glass tubes and not in plastic. It seems possible that not only the surface but aeration conditions can explain the differences between the two experiments.

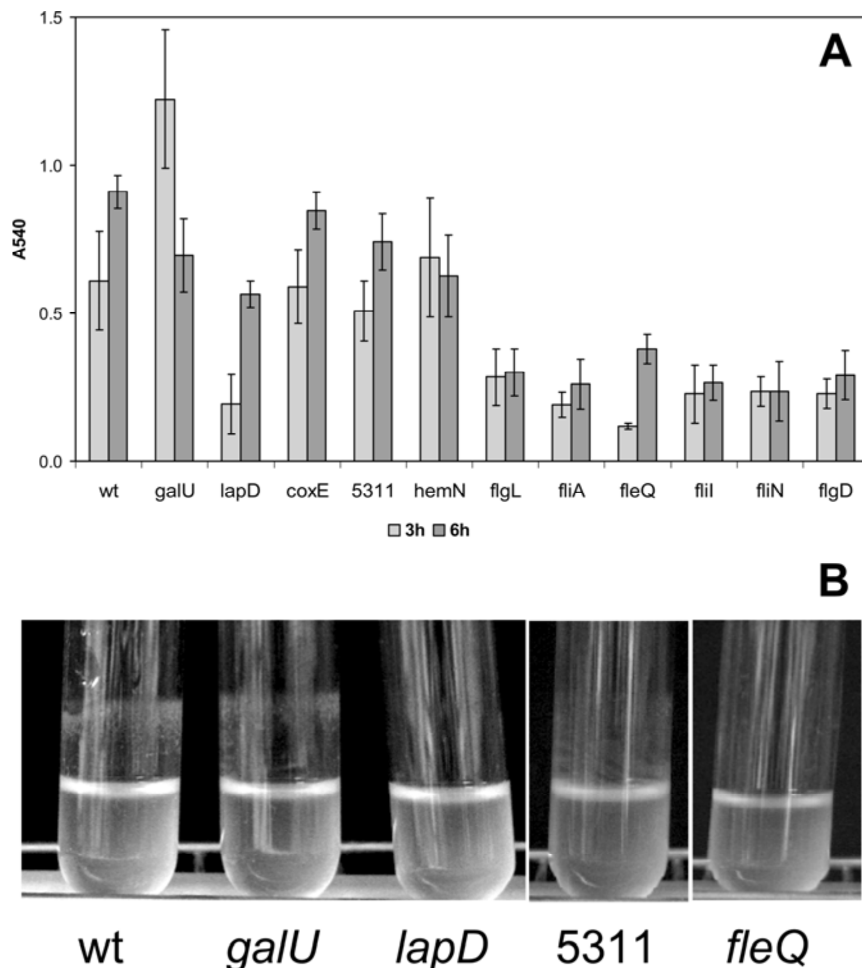

Figure 4. A. Biofilm formation in 96-wells microtiter plates, quantified as absorbance at 540 nm after staining the biofilm with crystal violet and redissolving the stain with ethanol. Measurements were done on a Sunrise microplate reader (Tecan), and are the averages and standard deviations of 3 independent assays, each with 6 replicas per strain. **B.** Biofilm formation in borosilicate glass tubes after 3 h of growth in LB with orbital shaking.

Concluding remarks

The results presented here give an idea of the hierarchy of functions participating in the establishment of sessile communities of *P. putida* in different environments. They support previous data on the role of flagella in attachment to abiotic and seed surfaces (Toutain et al., 2007; DeFlaun et al., 1990) and reveal the importance of the LapA/LapBC/LapD system both in biofilm formation and in bacterial fitness in the plant environment. These elements are common to the pathways for biofilm initiation regardless of the surface, and can be considered global adhesion traits. Large surface proteins with a structure resembling that of LapA are widespread among prokaryotes (Yousef & Espinosa-Urgel, 2007), suggesting the existence of similar molecular mechanisms for biofilm initiation in different bacteria. We

have also identified genes with a differential or surface-dependent role. A specific function in colonization of plant surfaces can be ascribed to *coxE*, while PP_5311 influences also adhesion to hydrophylic abiotic surfaces (glass). HemN2, on the other side, plays a role in seed colonization and biofilm development on hydrophobic abiotic surfaces (plastic), but does not affect rhizosphere fitness or adhesion to glass. The role of LPS appears to be more complex. Intact LPS is key for colonization of plant surfaces, but has a limited influence on attachment to glass and seems to hamper initial attachment to plastic, while being required at later stages. This suggests a time- and surface-dependent participation of LPS in the settlement of sessile *P. putida* populations. It is worth noting that, aside from LapA, no common functions were identified here with respect to our previously published analysis (Espinosa-Urgel et al., 2000). This is consistent with our observation that actively growing *P. putida* show higher seed attachment rates than stationary phase bacteria (Espinosa-Urgel & Ramos, 2004). Thus, the physiological state of the bacteria (stationary phase versus exponentially growing phase) seems also to delimit the requirements for seed colonization by *P. putida*.

Acknowledgements

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CAPÍTULO 3

Selección de mutantes hiperadherentes en biofilms de *Pseudomonas putida*

Publicado en: **Yousef-Coronado, F., Soriano, M.I., Yang, L., Molin, S., and Espinosa-Urgel, M.** (2011). Selection of hyperadherent mutants in *Pseudomonas putida* biofilms. *Microbiology* 157: 2257-2265.

SUMMARY

A number of genetic determinants required for bacterial colonization of solid surfaces and biofilm formation have been identified in different microorganisms. There are fewer accounts of mutations that favor the transition to a sessile mode of life. Here we report the isolation of random transposon *Pseudomonas putida* KT2440 mutants showing increased biofilm formation, and the detailed characterization of one of them. This mutant exhibits a complex phenotype, including altered colony morphology, increased production of extracellular polymeric substances and enhanced swarming motility, along with the formation of denser and more complex biofilms than the parental strain. Sequence analysis revealed that the pleiotropic phenotype exhibited by the mutant resulted from the accumulation of two mutations; the transposon insertion, which disrupted a predicted outer membrane lipoprotein, and a point mutation in *lapG*, a gene involved in the turnover of the large adhesin LapA. The contribution of each alteration to the phenotype and the possibility that prolonged sessile growth results in the selection of hyperadherent mutants are discussed.

INTRODUCTION

The development of multicellular communities associated with a surface and surrounded by an exopolymeric matrix, referred to as biofilms, is common to a variety of bacteria under different environmental conditions. Biofilm formation has received increasing attention due to its importance in medicine, since biofilms are considered relevant for cronic infection and are more resistant to the action of antibiotics and biocidals than planktonic populations (Anderson & O'Toole, 2008; Høiby *et al.*, 2010). Biofilm development is also relevant in industrial settings and in the design of bioreactors (Nicolella *et al.*, 2000; Singh *et al.*, 2006). Genetic determinants playing a role in biofilm formation have been unravelled in different bacterial species, and environmental and cellular signals influencing this process have also been described. These include iron availability, quorum sensing, and the intracellular secondary messenger cyclic di-GMP (Banin *et al.*, 2006; Patriquin *et al.*, 2008; Ueda & Wood, 2009; Coenye, 2010). Changes in the levels of this molecule correlate with phenotypic changes associated with virulence, motility, colony morphology, production of exopolysaccharides, and the transition between planktonic and sessile lifestyles (Hengge, 2009; Römling and Simm, 2009, and references therein). Changes in the expression of different functions are also associated with one or the other; in *Pseudomonas aeruginosa* and *Pseudomonas putida*, for example, differential expression of flagellar components has been observed when comparing planktonic and biofilm populations, and even during the various stages of biofilm development (Sauer & Camper, 2001; Sauer *et al.*, 2002; Toutain *et al.*, 2007). In the former organism, swarming motility and surface attachment are inversely regulated through a pathway that involves BifA, a cyclic-di-GMP phosphodiesterase, and the membrane protein SadC (Merritt *et al.*, 2007; Kuchma *et al.*, 2007).

Biofilm formation under various experimental settings follows a sequence of events that begins with a fraction of the bacterial population attaching to the solid surface and settling on it, while the rest remain as planktonic cells or attach and immediately detach from the surface, in what has been called "reversible attachment" (Monds & O'Toole, 2009). Our own observations of adhesion of the plant-beneficial bacterium *Pseudomonas putida* KT2440 to plant seeds indicate that only a small percentage of the population initiates attachment to the surface (Espinosa-Urgel *et al.*, 2000; Yousef-Coronado *et al.*, 2008). This leaves open the possibility that in the planktonic population a subpopulation specialized in surface colonization arises, and therefore it might be possible to isolate mutants locked in a

biofilm-prone genetic program. Mutations that cause an increase in the intracellular levels of cyclic-di-GMP have been shown to enhance biofilm formation and reduce detachment from the surface in different microorganisms (Kuchma *et al.*, 2010; Gjermansen *et al.*, 2006). In *Escherichia coli*, inactivation of *cysE*, the gene encoding serine acetyltransferase, which converts serine into *o*-acetyl-L-serine, a molecule that may act as an extracellular signal, results in accelerated biofilm formation (Sturgill *et al.*, 2004). In *P. putida*, *lapG*, a gene that causes reduced detachment when disrupted, has been described (Gjermansen *et al.*, 2010). Recently (Newell *et al*., 2011) it has been proven that LapG has proteolytic activity on LapA, the major adhesin of *Pseudomonas fluorescens* and *P. putida*, and seems to be a key element in the turnover of LapA on the bacterial surface in response to environmental signals. In this work we report the isolation of *P. putida* mutants showing increased biofilm formation, and the characterization of mutations that alter the normal attachment/detachment dynamics of this bacterium.

MATERIALS & METHODS

Bacterial strains, plasmids and growth conditions.

Pseudomonas putida KT2440, a plasmid-free derivative of *P. putida* mt-2 (Regenhardt *et al.*, 2002) was used in this study. Mutants ibi-626 and EU9 were obtained as described below. *Escherichia coli* JM109 was used as host for cloning experiments, with plasmid pBBR1-MCS5 as cloning vector (Kovach *et al.*, 1995). *E. coli* donor and helper strains used for random transposon mutagenesis with mini-Tn*5*[Km1] have been described elsewhere (de Lorenzo *et al.*, 1990). Derivatives of KT2440 and ibi-626 carrying *gfp* in single copy in the chromosome were obtained using mini-Tn*7*, as described (Koch *et al.*, 2001).

Unless otherwise stated, cultures were grown in liquid LB (Lennox, 1955) or 1:10 LB, and on LB plates with 1.5% agar, at 30°C (*P. putida*) or 37°C (*E. coli*). When appropriate, antibiotics were used at the following concentrations (in $\mu g\ \mu L^{-1}$): ampicillin, 100; chloramphenicol, 30; kanamycin, 50; gentamycin, 10 or 100 for *E. coli* and *P. putida*, respectively.

Random transposon mutagenesis and selection strategy for hyperadherent mutants

Transposon mutagenesis with mini-Tn*5* was done by triparental conjugation using the protocol previously described (Espinosa-Urgel *et al.*, 2000). Kanamycin resistant clones were

selected in minimal M9 medium with citrate as carbon source. For the selection of mutants with increased attachment capacity, the pool of transposon mutants was collected and ~10^8 cells were inoculated in 3 ml of liquid LB medium and grown in borosilicate glass tubes under orbital rotation. After 2 h of growth, the medium was removed to discard planktonic cells, the tubes were washed with M9 and 3 ml of fresh LB were added. The same procedure was repeated three times. In this way, enrichment in mutants having increased attachment was expected. Finally, attached bacteria were allowed to grow for 3 h and removed from the surface by vortexing for 30 sec. in the presence of glass beads (diameter 3 mm) and plating on selective medium.

Cloning of *olpA* and construction of *olpA* deletion mutant EU9

A 1019 bp fragment containing the *olpA* gene, coding for a putative outer membrane lipoprotein (see Results), and its upstream region, was PCR amplified and cloned in pBBR-MCS5, rendering plasmid pFY1. The insert was sequenced to ensure the absence of mutations. Plasmid pFY1 was used to construct an *olpA* mutant by replacement of an internal 500 bp fragment with a kanamycin resistance cassette from p34S-Km3 (Dennis & Zylstra, 1998). The *olpA::Km* fragment was then cloned into pUC19. The resulting plasmid, pFY3, was electroporated into *P. putida* KT2440 and kanamycin resistant clones were selected. Since the vector does not replicate in this organism, these clones were the result of either the integration of the plasmid into the chromosome by a single recombination event, or the replacement of *olpA* by double recombination. The later were selected after screening by PCR amplification. One clone (EU9) was chosen and the corresponding chromosomal region was sequenced to confirm the mutation.

Swarming motility, biofilm formation and biofilm stability assays.

Swarming motility was tested on 0.5% PG agar plates incubated at 25°C as previously described (Matilla *et al.*, 2007). 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 and quantified by staining with crystal violet, followed by solubilization of the dye with 70% ethanol and measuring absorbance at 580 nm (O'Toole & Kolter, 1998). Biofilm stability after enzymatic treatment was tested by incubating biofilms grown in microtiter plates (after 4

hours of growth) with either DNase (0.1 mg ml^{-1}), cellulase (5 mg ml^{-1}), or proteinase K (0.05 mg ml^{-1}) for 15 or 80 minutes, and then staining and quantifying the attached biomass as described above. Data are given as percentage of biomass retained in the biofilm with respect to untreated controls. ANOVA and Student's *t* test were used for statistical analysis.

A modification of the method described by Gjermansen *et al.* (2006) 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). Overnight cultures were diluted to an OD_{600}=0.5 and 300 µl were injected in the flow chamber. The flow was turned off during the first hour to allow initial attachment of the cells. The flow was then turned on and kept at a constant rate of 3 ml/h (laminar flow conditions). Biofilm structures were visualized at different times of development with a Nikon C1 confocal laser scanning microscope. Images were analyzed with Imaris software (Bitplane), and biofilm parameters were calculated using COMSTAT (Heydorn *et al.*, 2000).

Microscopy

Colony morphology was visualized and photographed using an Euromex inverted microscope. For transmission electron microscopy, bacteria were recovered from exponentially growing cultures (OD_{660} = 0.9) and adsorbed on Formvar coated grids, stained with 1% phosphotungstic acid for 30 sec and air-dried. The grids were observed under a JEOL JEM-1011 transmission electron microscope at 80 kV. Images were obtained using SIS Megaview III capture system and AnalySIS DOCU software. High resolution scanning electron microscopy of biofilm-grown bacteria was performed at the Microscopy Service of Granada University with a Zeiss LEO Gemini-1530.

Molecular biology techniques

Plasmid and chromosomal DNA extraction, electrophoresis, and other molecular techniques were performed following standard procedures. For Southern hybridization, the digoxigenin labeling and detection kit (Roche) was used. PCR amplification was done using Expand High-Fidelity PCR system (Roche). Restriction enzymes and DNA ligase were used as indicated by the manufacturers (New England Biolabs and Roche).

Isolation and quantification of extracellular polymeric substances

The method described by Cérantola *et al.* (2000) was used to extract the extracellular matrix components from biofilm-grown cultures. Essentially, biofilms were recovered after growth in LB, homogenized in PBS and biomass was quantified for normalization by measuring turbidity at 660 nm. Cells were then removed by centrifugation at 10,000×g and the supernatant was precipitated by adding 6 volumes of 95% ethanol followed by centrifugation at 12,000×g (30 min, 4°C). The resulting pellet was resuspended in distilled water, dialyzed overnight and lyophilized. The presence of DNA and proteins was analyzed by electrophoresis and quantified using a ND-1000 spectrophotometer (NanoDrop). Total sugars were quantified using the method described by Altman *et al.* (1987).

Whole-genome sequencing and analysis

Sequencing of the complete genome of ibi-626 was done by BaseClear B.V. (Leiden, Netherlands) using a Roche Illumina Genome analyzer II. 20 µg chromosomal DNA were used as template and 13,152,665 reads with an average length of 58 bases were obtained, resulting in a total of 762,854,570 bases (~120-fold coverage for the 6.2 Mbp genome of KT2440). Mapping of the sequence data was performed using the CLCbio Genomic Workbench version 3.7. De novo assemblies were used for comparison with the reference genome sequence of KT2440 in collaboration with Bio-Iliberis R&D. Potential discrepancies were re-assessed by PCR amplification and sequencing of the corresponding region in the wild type and mutant strains.

RESULTS

Selection of *P. putida* transposon mutants with increased biofilm initiation capacity.

A screen was carried out to identify *P. putida* mutants showing increased biofilm formation, with a focus on the early stages of the bacteria-surface interaction. Random transposon mutagenesis was performed on *P. putida* KT2440 using mini-Tn*5*[Km1] (de Lorenzo *et al.*, 1990). The resulting kanamycin resistant clones were pooled and approximately 10^7 cells were inoculated in liquid LB medium. An enrichment method was designed to identify hyperadherent mutants, consisting in successive rounds of growth in liquid LB, with early removal of planktonic cells and addition of fresh medium, leaving only the initial colonizers of the tube surface, as detailed in the Materials and Methods section. After such rounds of

enrichment, bacteria attached to the surface were recovered. Dilutions were plated and 1920 individual clones were subsequently assayed for biofilm formation in microtiter plates. Those clones showing significantly increased attached biomass after 3 h of growth (termed ibi mutants, for increased biofilm initiation) were chosen for further analysis. Insertion of a single copy of mini-Tn*5* was checked by Southern hybridization, using the kanamycin resistance gene as a probe. Clones in which more than one band was observed were discarded. The insertion sites of the minitransposon were determined in the remaining mutants by arbitrarily-primed PCR and sequencing, as described in Methods. Mutant ibi-206 was affected in PP_5007, encoding an ancillary protein involved in accumulation of polyhydroxyalkanoate (PAH) granules. PAH biosynthesis is differentially regulated in biofilm and planktonic cells of *P. aeruginosa* PAO1, and PAH-deficient mutants have been reported to display increased alginate production, associated with changes in biofilm structure and morphology (Pham *et al.*, 2004; Campisano *et al.*, 2008). The gene disrupted in ibi-815 corresponded to *putA*, encoding proline dehydrogenase. This enzyme catalizes the two-step conversion of proline into glutamic acid, and also has a regulatory role on its own expression, and on that of the proline transporter *putP*, playing also a role in redox signaling (Vílchez *et al.*, 2000, Fernández-Piñar *et al.*, 2008).

In the remaining four mutants, arbitrary PCR and DNA sequencing placed the insertion of mini-Tn*5* in the same position in locus PP_0116. This gene encodes a 235 amino acids protein highly conserved in all *Pseudomonas* species sequenced so far. It shows the distinctive features of outer membrane lipoproteins (Yamaguchi *et al.*, 1988; Terada *et al.*, 2001), with a 20 amino acids signal sequence predicted to be cleaved, leaving an N-terminal cysteine, typically involved in lipid binding, followed by a serine residue (MKPFASRYLLVAAFSLILAA/**C**S). We have therefore termed the gene *olpA* (for outer membrane lipoprotein A). One of these mutants (ibi-626) was chosen for further characterization.

ibi-626 forms thicker and structurally more complex biofilms than KT2440.

Biofilm formation by strain ibi-626 was analyzed in different experimental conditions. As shown in Fig. 1, the mutant formed thick biofilms on glass when grown in LB under rotation, leaving the liquid medium practically clear after 8 h, and remaining attached to the surface after 24 h, in contrast to wild type cells, which were for the most part detached after this

period. Quantitative analysis of biofilm development during growth under static conditions was performed in microtiter plates by staining with crystal violet and measuring absorbance. The results confirmed the increased attachment and reduced detachment phenotype of ibi-626 (Fig. 1).

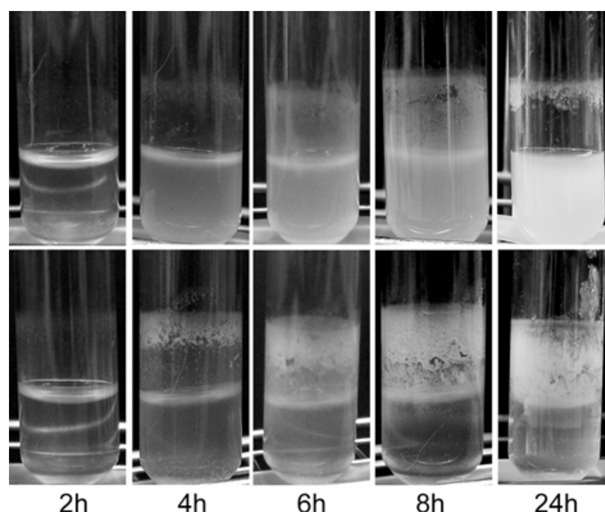

Fig. 1. Attachment of KT2440 (top row) and ibi-626 (bottom row) to glass tubes during growth in LB under orbital aeration.

Biofilm development was then analyzed in flow chambers by confocal laser scanning microscopy (CLSM), with KT2440 and ibi-626 derivatives harboring the *gfp* gene in single copy in the chromosome. While the wild type strain formed relatively flat, unstructured biofilms, biofilms of the mutant were not only significantly thicker but also structurally different, with large protrusions and finger-like extensions visible after 6 h of growth (Fig. 2). Quantitative analysis of biofilm features using the COMSTAT software (Heydorn *et al.*, 2000) showed significant differences in biofilm parameters, including biomass, surface coverage and average thickness at all times tested (Table 1).

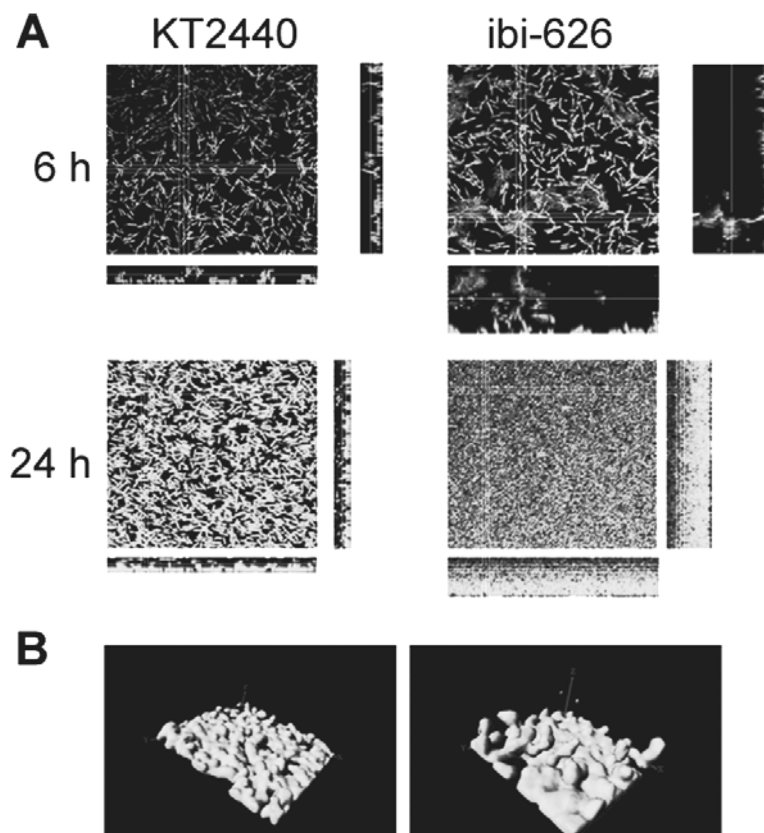

Fig. 2. Top view and sections (A), and three-dimensional reconstruction (B) of biofilm structures formed under flow conditions by KT2440 and ibi-626. Confocal laser scanning microscopy images obtained after 6 and 24 h of growth were processed with Imaris 3.0 software (Bitplane).

Table 1. COMSTAT analysis of biofilm parameters of KT2440 and ibi-626 under flow conditions in 1:10th strength LB.

		KT2440	ibi-626
Biomass ($\mu m^3/\mu m^2$)	3 h	0,670 ± 0,24	1,485 ± 0,76
	6 h	1,093 ± 0,55	2,169 ± 1,26
	24 h	3,719 ± 2,24	20,736 ± 1,59
Average difussion distance (µm)	3 h	0,079 ± 0,02	0,119 ± 0,06
	6 h	0,077 ± 0,03	0,098 ± 0,07
	24 h	0,364 ± 0,17	4,236 ± 1,55
Average thickness (µm)	3 h	0,417 ± 0,18	1,198 ± 0,61
	6 h	0,85 ± 0,52	2,524 ± 1,40
	24 h	3,997 ± 2,79	20,321 ± 1,91
Substratum coverage (µm)	3 h	0,263 ± 0,08	0,384 ± 0,19
	6 h	0,365 ± 0,11	0,423 ± 0,168
	24 h	0,62 ± 0,11	0,96 ± 0,07

The mutation in *olpA* is only partially responsible for the phenotype of ibi-626.

To confirm the role of *olpA*, the wild-type gene was PCR-amplified and cloned in pBBR1-MCS5, as described in Materials and Methods. The resulting plasmid, pFY1, was introduced in ibi-626, and the phenotype of the complemented strain was tested. Intriguingly, the intact *olpA* gene in multicopy did not restore normal biofilm formation kinetics. Thus, ibi-626 harboring pFY1 still formed thicker biofilms than the wild type (not shown). This could indicate either that the mutation in *olpA* had a dominant character or that a secondary, spontaneous mutation was responsible for the increased attachment. The fact that the complemented strain showed an intermediate phenotype in terms of swarming motility (see below), suggested a cumulative effect of the *olpA* mutation and an additional mutation, leading to the pleiotropic effects observed in ibi-626. To test this hypothesis, a null *olpA* mutant was generated by replacement of the gene with a kanamycin resistance cassette, as described in Materials and Methods. The resulting strain, EU9, did show increased attachment, but only at early timepoints; further biofilm development and detachment kinetics were similar to the wild type strain (Fig. 3), indicating that the hyperadherent character of ibi-626 is not solely due to the disruption of *olpA*, and that an additional mutation might be present.

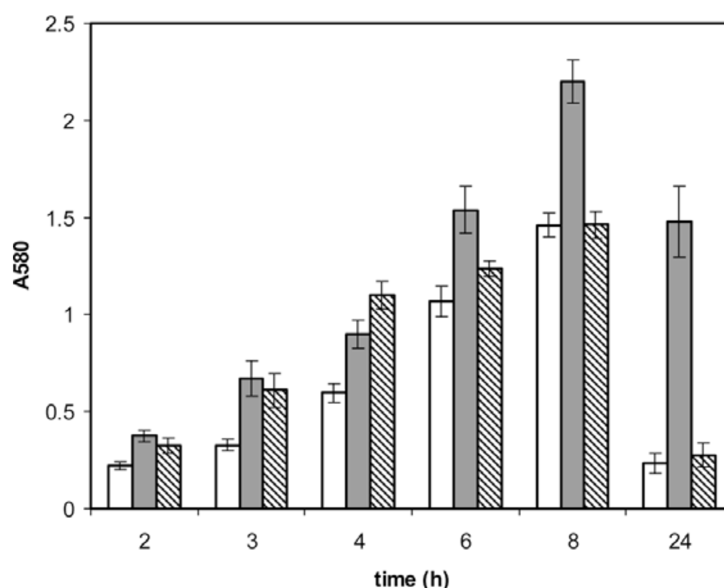

Fig. 3. Quantitative analysis of biofilm formation by KT2440 (white bars), ibi-626 (grey bars), and EU9 (hatched bars) during growth in LB in microtiter plates. Attached biomass was quantified after staining with crystal violet as described in Materials and Methods. Data are averages and S.D. from three independent experiments (4 replicates per experiment). Differences were significant (p=0.05) between EU9 and KT2440 at 3 and 4 h of biofilm growth, and between ibi-626 and KT2440 at all timepoints.

Complete sequencing of ibi-626 reveals a point mutation affecting *lapG*.

The genome of mutant ibi-626 was fully sequenced and compared to the reference genome of KT2440 (Nelson *et al.*, 2002) to define potential mutations that could contribute to the phenotype of this strain. Several apparent discrepancies with the reference sequence were checked by PCR amplification and sequencing of the corresponding region in ibi-626 and our laboratory KT2440 strain. Most were found to be sequencing inaccuracies or assembly gaps. A single-base insertion present in both strains with respect to the reference sequence was detected in locus PP_3932, rendering a protein longer than anotated in the databases but identical in length to homologous proteins of other *Pseudomonas* species (data not shown). However, deletion of a base pair within the coding region corresponding to locus PP_0164, 15 bp downstream the initiation codon, was found in ibi-626, while in KT2440 the coding sequence was identical to the published reference. This change causes a frameshift and early translation stop in the mutant (Fig. S1). In both KT2440 and ibi-626, a single base pair upstream of this locus is missing with respect to the published sequence. PP_0164 corresponds to *lapG*, a gene that had been previously proposed to act as a signal transducer in biofilm formation (Gjermansen *et al.*, 2005), and more recently shown to encode a cysteine protease affecting the function of LapA in *P. fluorescens* (Newell *et al.*, 2011), the main adhesin of this bacterium and of *P. putida* (Hinsa *et al.*, 2003; Yousef-Coronado *et al.*, 2008). Transposon mutants in which *lapG* is disrupted have been shown to be defective in detachment from biofilms, an effect that is compensated when *lapA* is also disrupted (Gjermansen *et al.*, 2005, 2010). Interestingly, the four *olpA* mutants identified in the initial screening for ibi clones had the same point mutation in *lapG*.

The above results indicated that both *olpA* and *lapG* mutations contribute to increased adhesion and reduced detachment of ibi-626. The implication of the *lapG* mutation in the phenotype of ibi-626 was confirmed by cloning the intact gene from KT2440 into vector pBBR1-MCS5, and introducing the resulting plamid, pME164, in KT2440, EU9 and ibi-626. As shown in Fig. 4, the presence of *lapG* in multicopy inhibited adhesion of all strains to glass.

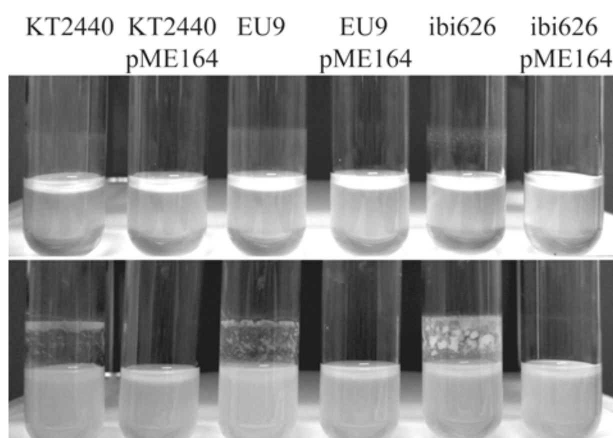

Fig. 4. The presence of *lapG* in multicopy inhibits biofilm formation. Bacterial attachment was followed during growth in glass tubes under orbital rotation. Images correspond to 3 h (top row) and 6 h (bottom row) of growth.

Pleiotropic effects of *lapG* and *olpA* mutations.

In *Pseudomonas aeruginosa*, biofilm formation and swarming motility are divergently regulated (Kuchma *et al.*, 2010), so that conditions promoting transition to the sessile lifestyle are associated with an inhibition of swarming motility (Kuchma *et al.*, 2007; Merritt *et al.*, 2007; Kuchma *et al.*, 2010). However, swarming motility was enhanced in mutant ibi-626 with respect to the wild type strain (Fig. 5A), suggesting that the two processes are not inversely regulated in *P. putida*. Complementation of the olpA mutation with pFY1 resulted in an intermediate phenotype in terms of swarming (Fig. 5A), and a similar phenotype was observed for mutant EU9, indicating that both mutations (*olpA* and *lapG*) contribute to the increase in swarming motility. It has been reported that type IV pili participate in this surface movement in *P. putida* KT2440 (Matilla *et al.*, 2007). Comparison of this strain with ibi-626 by transmission electron microscopy revealed that cells of the ibi-626 mutant showed a larger number of surface appendages (presumably pili) than the wild type (Fig. 5B). This could account for the differences in swarming motility. The mutant also showed altered colony morphology (Fig. 5C) and formed a denser, clumpy pellicle at the air-liquid interface when grown under static conditions (Fig. 5D). Colony morphology and pellicle formation by EU9, however, was indistinguishable from that of KT2440.

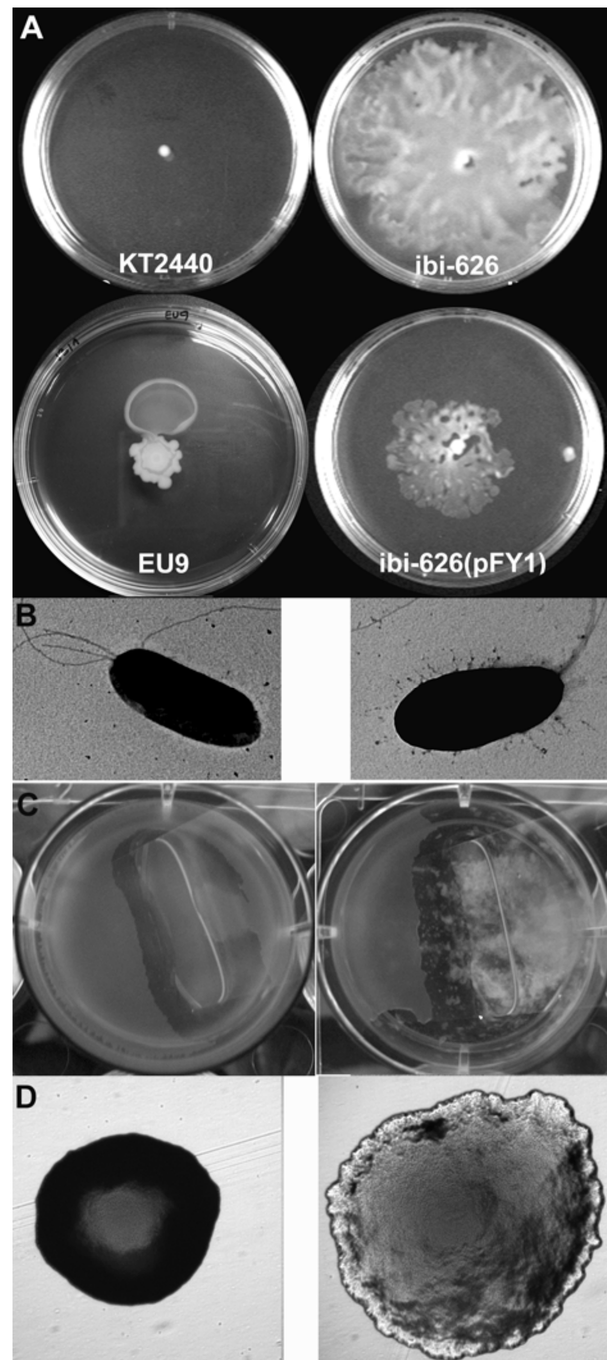

Fig. 5. Phenotype comparison between KT2440 (left) and ibi-626 (right) **A.** Swarming motility (intermediate phenotypes caused by the *olpA* mutation and by *olpA* in multicopy are also shown). **B.** Transmission electron microscopy observation of cells. **C.** Colony morphology. **D.** Pellicle formation.

We considered the possibility that these two characteristics of ibi-626 were due to an increase in EPS production. Scanning electron microscopy was also used to study cells of both strains after 6 h of biofilm development on glass coverslips. Compact aggregates of ibi-626 cells could be observed, with an extracellular matrix clearly distinguishable, whereas in

the case of KT2440 cells at this stage were only beginning to form microcolonies and the extracellular matrix was less evident (Fig. 6). Quantitation of extracellular polymeric substances (EPS) after dialysis and lyophilization confirmed that the amount of EPS was larger in the ibi-626 mutant than in the wild type (data not shown). Analysis of total carbohydrates and DNA did not indicate significant differences between both strains. However, the protein content of the extracellular matrix was 1.7 times higher in the mutant than in the wild type (2.62 and 1.59 mg mL^{-1}, respectively).

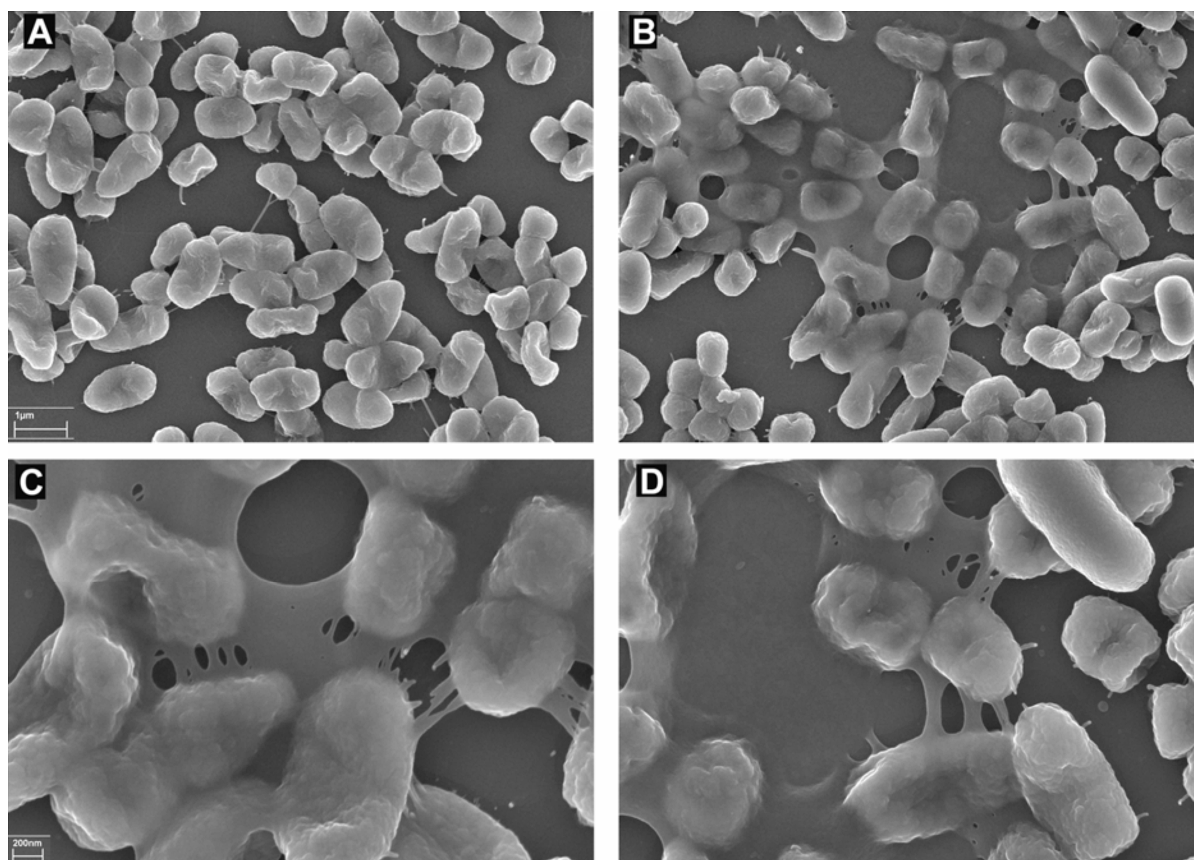

Fig. 6. Scanning electron microscopy images of KT2440 (A) and ibi-626 (B, C, D) cells attached to glass coverslips after 6 h of growth in LB. Panels C and D correspond to details of B at higher magnification.

The robustness of the biofilms formed by KT2440 and ibi-626 was tested by analyzing biofilm dispersal after enzymatic treatments targeting components of the extracellular matrix. As shown in Fig. 7, no significant differences between the two strains were observed after DNase treatment, whereas ibi-626 biofilms were more resistant to protease, requiring prolonged exposure to this enzyme for disgreggation. These and the previous data indicate

that in ibi-626 there is an increase in the amount of extracellular proteins that form part of the biofilm matrix, suggesting that LapG exerts a similar role in *P. putida* as it does in *P. fluorescens*. Addition of cellulase did not have a significant effect on biofilms of either strain (data not shown), which is consistent with indications that cellulose is not the most relevant EPS during biofilm formation by *P. putida* under the conditions used here (Nielsen *et al.*, 2011; Nilsson *et al.*, 2011; Matilla *et al.*, 2011).

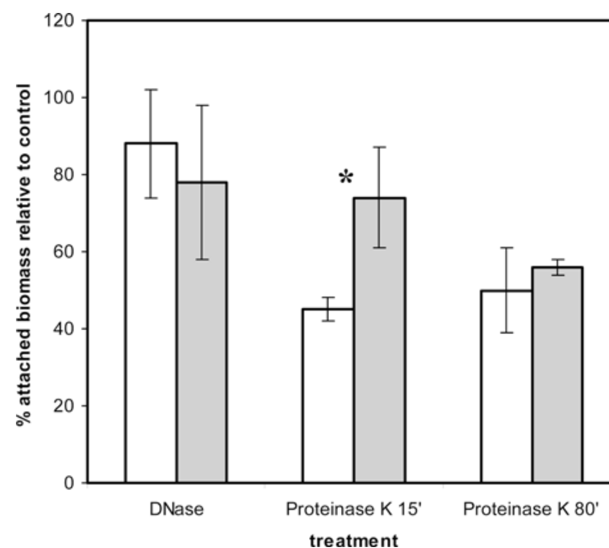

Fig. 7. Dispersal of KT2440 (white bars) and ibi-626 (grey bars) biofilms after treatment with DNase and proteinase K for 15 or 80 min. Data correspond to the mean and S.D. of three experiments. Asterisk indicates significant differences (p=0.01).

DISCUSSION

Genetic analysis of biofilm formation has for the most part been approached with "function loss" strategies, oriented towards the identification of mutations causing defects in bacterial attachment and/or biofilm development. However, our understanding of how bacteria adapt to the biofilm lifestyle, and the selective forces acting on sessile populations can also benefit from the analysis of mutations that give rise to variants with increased biofilm formation capacity. This approach may give indications of the potential specialization of a portion of the bacterial population towards a sessile lifestyle, either as the result of bistability or due to phase variation phenomena. Bistability, i.e. the coexistence of two subpopulations showing different gene expression programs within a genetically identical

population, has been proposed as a mechanism underlying specialization of bacteria in biofilms (Chai *et al.*, 2008). In this work we have obtained transposon mutants showing a hyperadherent phenotype and characterized one of them, ibi-626. The dramatic phenotype exhibited by this mutant turned out to be the consequence of the combined effects of two mutations, the disruption of the putative lipoprotein OlpA by the transposon insertion, and an additional point mutation in *lapG*. The former seems to be responsible for increased early attachment while the later contributes to increased biofilm development and prevents detachment of cells from the established biofilm.

The specific role of OlpA remains to be fully explored. Analysis of its sequence reveals the presence of a tetratricopeptide repeat profile. This profile appears in different proteins, among them a family of lipoproteins (InterPro IPR017689) that includes YfiO, involved in outer membrane assembly in *E. coli* (Gatsos *et al.*, 2008), and ComL, which participates in DNA uptake and transformation in *Neisseria gonorhoeae* (Fussenegger *et al.*, 1997). However, OlpA has little sequence similarity with these two proteins, and homologs of OlpA seem to be restricted to *Pseudomonas* species. Several lipoproteins have previously been described as having an effect on biofilm formation by different bacteria (Vasseur *et al.*, 2007; Uhlich *et al.*, 2009). In *P. putida*, expression of the outer membrane lipoprotein NlpD is downregulated following adhesion to the solid surface (Sauer & Camper, 2001), and recently NlpI, an *Escherichia coli* lipoprotein, has been shown to negatively affect production of extracellular DNA (Sánchez-Torres *et al.*, 2010), which in other microorganisms is an important component of the extracellular matrix in biofilms. In *P. putida* KT2440, however, extracellular DNA seems to have a secondary role, since DNase treatment of biofilms has a limited effect on the biomass associated to the surface, while protease treatment causes significant disruption of the biofilm. The fact that ibi-626 biofilms were more resistant to protease than wild type biofilms is in agreement with the proteolytic effect of LapG upon LapA (Newell *et al.*, 2011). The absence of LapG would prevent LapA, the major adhesin of *P. putida*, from being cleaved from the bacterial surface and hence increase the protein content of the biofilm matrix.

The accumulation of two independent mutations in ibi-626 could be interpreted as indication that the enrichment method used had favored the selection of spontaneous *lapG* mutants present in the planktonic population, while the additional effect of the *olpA* mutation would have contributed to their being chosen as highly hyperadherent in the

analysis of individual clones. Alternatively, the transposon insertion in *olpA* could be responsible for the increased attachment initiation, the point mutation in *lapG* being selected for within the biofilm population, as it might confer an advantage under conditions designed to favor sessile growth.

Preliminary data suggest the second interpretation is correct. An enrichment and selection method identical to that previously used to identify ibi mutants was employed with wild type KT2440 cultures. Under the same conditions and with the same number of biofilm enrichment rounds, no hyperadherent variants were obtained. However, when the enrichment rounds were doubled, three –out of 1920– clones showing increased biofilm formation could be identified. These results are consistent with selection of spontaneous hyperadherent variants taking place under conditions favoring prolonged sessile growth. In the case of ibi-626, these conditions could have been favored by the increased early attachment caused by disruption of *olpA*. Interestingly, sequence analysis of *olpA* and *lapG* in the three spontaneous variants showed that in all cases the two open reading frames were intact, suggesting that multiple genetic traits can be altered to result in a bias towards the sessile mode of life.

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SUPPLEMENTARY FIGURE

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          V  L  L  G  S  L  L  L  G  G  L  H  A  D  W  D  F  S  Q  I
KT2440    GTGCTGCTGGGCAGTCTTCTGCTGGGCGGCTTGCACGCGGATTGGGATTTCTCCCAGATC
ibi-626   GTGCTGCTGGGCAG-CTTCTGCTGGGCGGCTTGCACGCGGATTGGGATTTCTCCCAGATC
          V  L  L  G  S   F  C  W  A  A  C  T  R  I  G  I  S  P  R  S

          S  R  K  S  Q  A  L  Y  G  P  L  G  A  G  Q  G  R  I  D  A …
KT2440    AGCCGCAAGTCGCAGGCTCTGTACGGCCCACTAGGTGCCGGGCAGGGTCGCATCGATGCC
ibi-626   AGCCGCAAGTCGCAGGCTCTGTACGGCCCACTAGGTGCCGGGCAGGGTCGCATCGATGCC
           A  A  S  R  R  L  C  T  A  H  *
```

Sequences of *Pseudomonas putida* strains KT2440 and ibi-626. Deletion of a base pair (boxed) within the coding region corresponding to locus PP_0164, 15 bp downstream from the initiation codon (underlined), was found in ibi-626, while in KT2440 the coding sequence was identical to the published reference sequence. This change causes a frameshift and early translation stop in the mutant (asterisk).

DISCUSIÓN GENERAL

La formación de comunidades multicelulares, encapsuladas en una matriz y asociadas a una superficie sólida, conocidas como biopelículas o biofilms, es una característica intrínseca del ciclo celular bacteriano y una estategia común para la persistencia en una variedad de ambientes. Esto, unido a la importancia de los biofilms en medicina, sistemas industriales, biodeterioro y agrobiología, han hecho que el estudio de la adhesion microbiana a superficies y la formacion de biopelículas se haya convertido en un area de creciente interés en Microbiología. Se han identificado numerosos genes relacionados con este proceso diferentes bacterias, y se han analizado las variaciones fisiologicas dentro del biofilm y cambios en la expression génica.

Esta Tesis Doctoral se ha centrado en los fenomenos de adhesión y la formación de biopeliculas por *Pseudomonas putida* KT2440. Esta bacteria se ha convertido en uno de los organismos preferidos en biotecnología gracias a su versatilidad metabólica y la gran cantidad de información publicada al respecto. Está siendo usada ya, entre otros, para en la produccion de PHA (polihidroxialcanoatos), poliésteres utilizados en la produccion de bioplásticos. Presenta potencial para la descontaminacion; como ejemplo, durante el pretratamieno en biorreactores del material vegetal para la producción de etanol, se liberan ácidos débiles, furanos y compuestos fenólicos, en los que esta bacteria sobrevive, lo que se aprovecha para limpiar el substrato vegetal de estos compuestos para organismos más sensibles como *S. cerevisiae*. Además, *Pseudomonas putida* KT2440 ha demostrado ser capaz de proteger *Arabidopsis thaliana* activando la resistencia sistémica de la planta (Matilla et al., 2010), lo que abre un camino interesante para su explotación a nivel agrícola.

En trabajos previos del grupo y en los desarrollados durante esta Tesis Doctoral, se ha puesto de manifiesto la importancia de proteínas extracelulares de gran tamaño con una estructura repetitiva (LapA y LapF), como adhesinas implicadas en el proceso de formación de biofilms por *Pseudomonas putida* KT2440. Este hecho, unido a la noción de que la formación de biopelículas puede ser una estrategia general en el mundo bacteriano, nos hizo preguntarnos cómo de extendida estaba la existencia de este tipo de proteínas de gran tamano entre los procariotas. En el primer capítulo de esta Tesis, analizamos todos los genomas bacterianos completos disponibles en la base de datos del NCBI en aquel momento (351), en busca de los genes que codifican las mayores proteínas en cada caso. Mediante análisis comparativo, se identificaron aquellas que posiblemente están relacionadas con

procesos de adhesión, asi como otras proteínas asociadas a la superficie celular. Sus características estructurales y composición, y la presencia de motivos conservados fue analizada, lo que nos ha permitido definir siete familias, diferenciadas en función de la presencia o ausencia de determinados dominios conservados. Estas diferencias podrían reflejar distintas especificidades en cuanto a las interacciones que dichas proteínas puedan establecer en la superficie y su modo de acción. Así, por ejemplo, postulamos que las familias LapA, AidA y Bap incluyen adhesinas que pueden jugar un papel no solo en adhesión celula-superficie, sino también en interacciones célula-célula, y que el calcio juega un papel importante en su función.

De los resultados obtenidos se puede inferir que la presencia de grandes proteínas secretadas que podrían funcionar como adhesinas es habitual en la mayoría de las bacterias. Resulta llamativo que no hay una correlación entre tamaño de genoma y tamaño de la mayor proteína codificada; incluso microorganismos con un tamaño de genoma muy pequeño dedican una porción significativa del mismo a la codificación de estas grandes proteínas. Esto sugiere que debe existir una presión selectiva importante para el mantenimiento del tamaño y estructura repetitiva de estas proteínas de superficie, puesto que en caso contrario sería esperable una tendencia a la reducción, facilitada por la mayor probabilidad de pérdida por recombinación homóloga al tratarse de regiones repetidas. En muchas bacterias, además, hay más de un gen que codifica proteínas con estas características. Así por ejemplo, en la bacteria marina *Rhodopirellula baltica* SH1 existen hasta cinco genes que codifican adhesinas de tamaños entre 8173 y 4630 aminoácidos. La ubicuidad y estabilidad de estas proteínas ratifica la idea de que la formación de comunidades multicelulares es algo común entre procariotas. Dado que actualmente hay más de 1800 genomas procarióticos secuenciados (este número incluye distintas cepas de una misma especie), sería interesante comprobar si la clasificación realizada engloba a proteínas de microorganismos cuya secuencia no estaba disponible durante nuestro análisis, o si es posible definir alguna nueva familia. También quedan por establecer las diferencias funcionales entre las distintas familias, para lo que harán falta estudios detallados correlacionando el proceso de formación de biofilms por distintos microorganismos, y la estructura de los mismos con la función de estas proteínas.

En el segundo capítulo hemos ahondado en la identificación de determinantes genéticos requeridos para la colonización de superficies de plantas por poblaciones de *Pseudomonas*

putida KT2440, como continuación de trabajos anteriores del grupo a este respecto (Espinosa-Urgel, 2000) y evaluamos su correlación con procesos de formación de biofilms sobre superficies abióticas. Para ello analizamos 35 nuevos mutantes obtenidos por transposición al azar, deficientes en la adhesion a semillas. La diferencia fundamental con trabajos anteriores fue que en este caso las rondas de enriquecimiento y selección se llevaron a cabo con células en la fase exponencial, en vez de con células en fase estacionaria, para identificar funciones que podrían no estar representadas en el análisis anterior (Espinosa-Urgel, 2000).

Un considerable número de los mutantes aislados estaba afectado en los componentes del sistema de síntesis y transporte de la adhesina de gran tamano LapA (menos en *lapE*, que en el caso de *Pseudomonas putida* no se encuentra en el mismo operon con *lapB* y *lapC*, como pasa en *P. fluorescens*). En todos estos mutantes se observó una disminución en la adhesión a semillas y la capacidad de formación de biofilm en superficies abióticas. Aun así, en los ensayos de colonización competitiva en rizosfera los mutantes no fueron desplazados completamente. Destacamos ademas, la existencia de un segundo grupo de mutantes afectados en adhesión a semillas y a supreficies abióticas, correspondientes a inserciones del transposón en alguno de los genes relacionados con la síntesis, regulación o funcionalidad del aparato flagelar, y que por tanto presentan defectos en movilidad de tipo swimming. Curiosamente, un mutante en *fliA* (que codifica un factor sigma que controla la síntesis de flagelo) no mostró diferencias significativas en colonización competitiva de la rizosfera, lo que contrastaba con datos aportados por otros estudios donde se refleja la importancia del flagelo para la colonización de la raíz por *Pseudomonas fluorescens* (Capdevila et al., 2004; Martínez-Granero et al., 2006), así como la aparición de mutantes hipermóviles en la rizosfera. En estudios recientes realizados en *P. fluorescens* se ha propuesto que la formación de biofilms en superficies abióticas y la colonización de la rizosfera siguen diferentes vias regulatorias (Barahona et al, 2010). En estos trabajos se analizaron mutantes en tres genes reguladores relacionados con la formación de biopelículas y la movilidad, *gacS* (G), *sadB* (S) y *wspR* (W), y una serie de variantes hipermótiles aisladas de la rizosfera, llegando a la conclusión de que mutantes afectados en la formación de biopelículas en superficiens abióticas colonizaban el ápice de la raíz tan eficientemente como la cepa silvestre.

Aunque no podemos descartar la posibilidad de que los mecanismos de colonización sean diferentes para *P. fluorescens* y *P. putida*, es importante indicar que la diferencia entre los trabajos citados y los resultados obtenidos en esta Tesis puede estribar en la metodología: en nuestros ensayos evaluamos la colonización a nivel de la raíz completa, y no sólo del ápice. De hecho, las imágenes de microscopía muestran una localización desigual de la cepa silvestre y el mutante *fliA* a lo largo de la raíz, lo que nos lleva a postular que en *P. putida* la movilidad flagelar determina la distribución de las bacterias a lo largo de la raíz, y la capacidad de ocupar con éxito el ápice, mientras que la adhesión mediada por LapA juega un papel importante en el mantenimiento en la rizosfera a nivel general, que no solo comprende el material biológico (la raíz), sino también el substrato inorgánico circundante. Como expusimos anteriormente, pensamos que debe haber una fuerte razon evolutiva detrás del mantenimiento de proteínas de semejante tamaño, en este caso su papel en el mantenimiento de poblaciones en el nicho natural de *P. putida*.

A nivel de rizosfera, la deficiencia competitiva más marcada fue la de mus-40, un mutante afectado en el gen *galU*, que codifica la UDP-glucosa fosforilasa. En *Pseudomonas aeruginosa* se ha comprobado que es necesaria para la síntesis de lipopolisacárido. El lipopolisacarido complejo es esencial para la colonización se superficies de plantas, pero tiene una influencia limitada en adhesión a cristal y parece dificultar las etapas iniciales de adhesión a plástico, mientras que se require en etapas más tardías. Estos resultados, junto con los que se están obteniendo en otros trabajos del grupo, sugieren una sucesión programada de eventos en el desarrollo de biofilms por *P. putida*, en la que inicialmente se requieren los flagelos y las adhesinas, con posterior participación del lipopolisacárido y finalmente de exopolisacáridos. Sin embargo, este programa podría variar dependiendo de las condiciones y las características de la superficie a colonizar.

En el tercer capítulo abordamos el aislamiento de variantes hiperadherentes. Esta estrategia puede ser una vía para explorar formas de generar biopelículas mas estables, uno de los campos más interesantes por su potencial aplicación biotecnológica (optimización de reactores, mejora en la adhesión). Además, ciertas enfermedades tienen su origen en la bajada en el numero de simbiontes beneficiosos y/o un incremento en el número de patogenos (conocidas como “Microbial shift disease”). La candidiasis recurrente, la infeccion urinaria por *E. coli* y la periodontitis (causa mas común de pérdida de dientes en el mundo) son ejemplos de este tipo de enfermedades, en las que la importancia de la existencia de

poblaciones estables en estos ambientes se ha puesto de manifiesto. Ahora sabemos que la transicion entre una boca sana y una infeccion periodontal va determinada por un cambio de las comunidad bacteriana de "en su mayoría gram-positivas" a "mayoría de bacterias gram-negativas" en la infección. En el tratamiento de algunas enfermedades se explota ya este conocimiento, es el ejemplo del uso de *Lactobacillus* para desplazar infecciones por *Candida* en la zona genital. Se abre así una puerta al tratamiento, mediante consorcios microbianos de diseño, de las perturbaciones locales de la flora que desembocan en patogénesis.

En esta Tesis hemos conseguido aislar mutantes que tienen una capacidad de formación de biopelículas incrementada, y ademas mayor motilidad que el tipo silvestre, como es el mutante ibi-626. El análisis funcional de este mutante reveló un fenotipo pleiotrópico que era resultado de la acumulación de la mutación generada por transposición, localizada en el gen PP_0116, que codifica una lipoproteína que denominamos OlpA, y una mutación secundaria en *lapG*, que codifica una cistein-proteasa. Trabajos recientes han demostrado que en *P. fluorescens* LapG libera LapA de la superficie celular cuando hay bajos niveles de di-GMP cíclico (Navarro et al., 2011; Newell et al., 2011). Nuestros resultados y los de otros autores (Gjermansen et al., 2006) indican que su función es similar en *P. putida* (Figura 1).

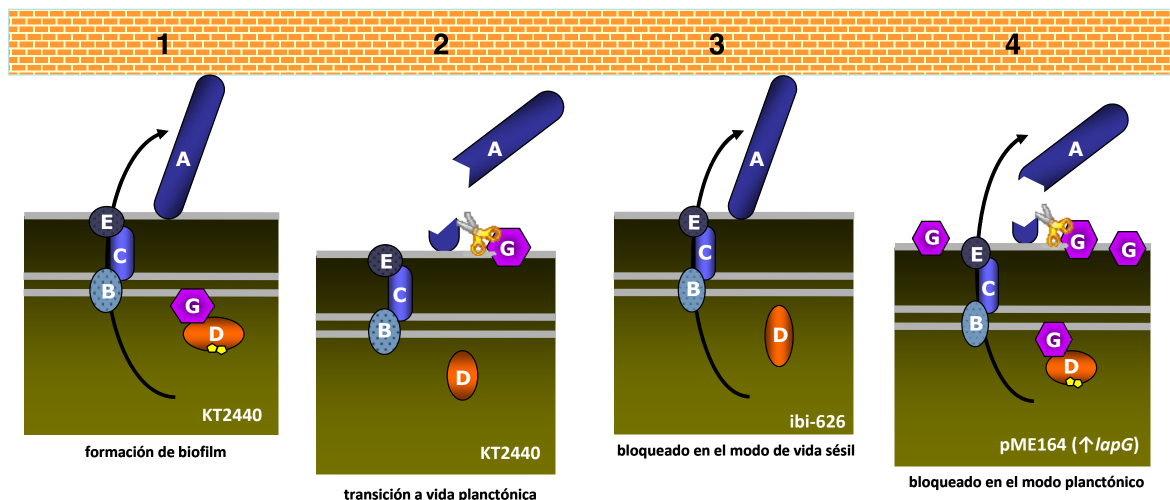

Figura 1. Modelo de recambio de LapA en KT2440 e ibi-626. En condiciones de alto di-GMPc, LapD mantiene secuestrada a LapG en la membrana interna, lo que promueve la adhesión mediada por LapA (1). Bajos niveles del segundo mensajero provocan la liberación de LapG a la superficie celular, donde ejerce su actividad proteasa sobre LapA (2). En ibi-626, la ausencia de LapG deja a las células bloqueadas en el modo de vida en biofilm, independientemente de los niveles de di-GMPc (3). Por el contrario, la sobreexpresión de lapG provoca un escape de la proteína, causando la rotura de LapA en cualquier condición, dejando a las células bloqueadas en el estilo de vida planctónico (4).

Es de destacar que el sistema de selección diseñado en este trabajo, favorecía la aparición de mutaciones espontáneas que alteran el recambio de LapA, algo que no se había descrito hasta la fecha. Esto implicaba además que la presión selectiva hacia el modo de vida en biofilm podría seleccionar variantes espontáneas hiperadherentes. Esta hipótesis se validó empleando un sistema de enriquecimiento similar al descrito para seleccionar ibi-626, pero empleando la cepa silvestre KT2440 en lugar de un pool de mutantes por transposición. Mediante estos ensayos de microevolución, se obtuvieron mutantes espontáneos que no están afectados en *olpA* ni *lapG*, lo que indica que hay más de una ruta evolutiva hacia la hiperadherencia. Uno de estos mutantes, M5, ha sido analizado en más detalle. Muestra un incremento en la formación de biopelículas, una mayor producción de polímeros de tipo celulósico, capaces de unir calcofluor, y una morfología de colonia más compacta. Además, en biofilms mixtos con la cepa parental, presenta una distribución diferencial en la que cubre al tipo silvestre en sistemas de flujo, estabilizando el biofilm y permitiendo que alcance un grosor mayor (Figura 2). Este fenotipo lo hemos denominado GABL ("growth advantage in the biofilm lifestyle"), en analogía al fenotipo GASP ("growth advantage in stationary phase") definido por Zambrano y colaboradores en poblaciones de *Escherichia coli* en situación de hambre prolongada, en la que aparecen subpoblaciones de mutantes con ventaja selectiva en dichas condiciones (Zambrano et al., 1993; Zambrano & Kolter, 1996).

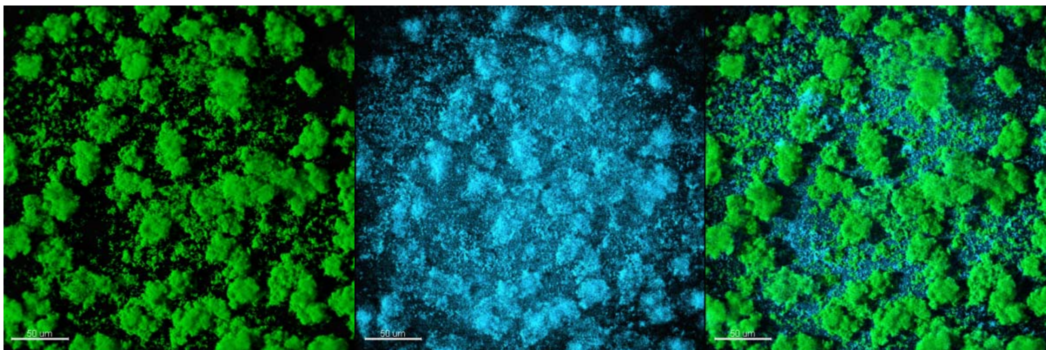

Figura 2. Formación de biopelículas por KT2440-e*cfp* (azul) y y M5-*gfp* (verde) en proporción 1:1, en condiciones de flujo en medio minimo con glucosa como fuente de carbono. La tercera figura es el resultado de la combinacion de las dos anteriores, M5 forma microcolonias sobre KT2440, permitiendo la formación de estructuras de mayor espesor que el tipo silvestre en solitario (que no se muestra en la foto).

Recientemente hemos realizado un estudio de las variantes que aparecen en un biofilm maduro. Aunque el análisis de estos experimentos no se ha podido completar durante esta

Tesis, nuestro objetivo es profundizar en el estudio del equilibrio ecológico que conlleva la existencia de varios genotipos en el mismo sistema.

Tras mantener cinco réplicas en paralelo de biofilms de *P. putida* KT2440 durante 8 días en condiciones de flujo, se recogieron células y se realizó un *screening* masivo comprobando los siguientes fenotipos: adhesión a placas multipocillo, unión de Rojo Congo y Comassie blue y morfología de colonia. Con estos criterios se ha podido definir la aparición de grupos representados en el biofilm con unas determinadas características. Así, se ha comprobado la aparición de variantes con morfología de colonia diferente y distinta capacidad de unión a Rojo Congo (Figura 3), así como de variantes hiperadherentes. Aunque no se han determinado con precisión las proporciones, sí es evidente que la aparición de variantes ocurre en el biofilm a largo plazo y no en biofilms de 1 día. Estamos pendientes de la secuenciación de estas poblaciones y de experimentos de reestablecimiento de equilibrio, en los que queremos ver si introduciendo todas las variantes el sistema se reestablece, ya que en las 5 replicas los grupos bacterianos encontrados presentaban aproximadamente las mismas caracteristicas.

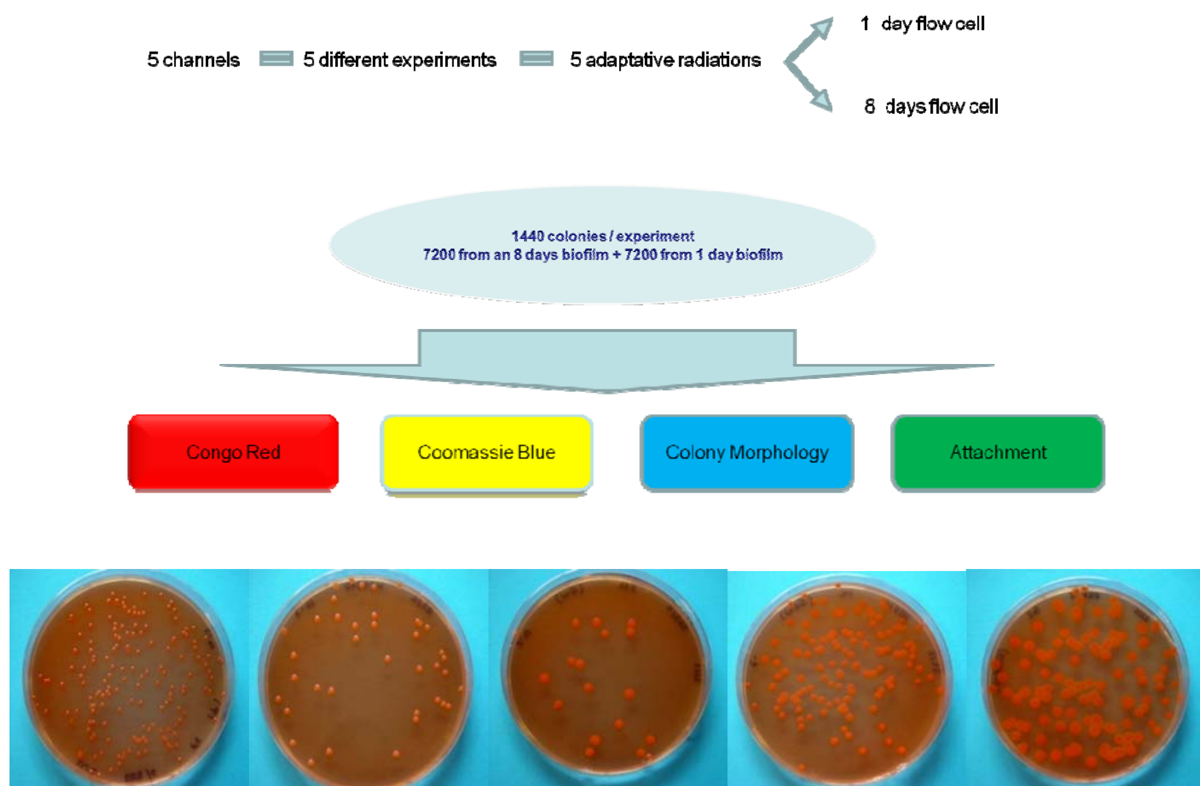

Figura 3. Esquema experimental del sistema microevolutivo y fotos correspondientes a las diferentes categorias de mutantes estables seleccionadas en base al las variaciones en la unión de Coomassie blue, Rojo congo, adhesión a placas multipocillo y morfología de colonia obtenidos en una de las cinco replicas tras 8 días.

En 1987, Naciones Unidas publica el informe Brundtland (tambien conocido como *Our Comon Future*) e introduce el término “sostenibilidad” (“developments that meet the needs of the present without compromising the ability of future generations to meet their own needs”). En el apremia a los diferentes estados miembros a buscar vias para el desarrollo sostenible. Este concepto florece en 1992 en la Conferencia de Rio de Janeiro, con el establecimiento de la Agenda 21 y la “Comission on Sustainable Development” de Naciones Unidas. Asi es como el desarrollo de tecnología responsable desde un punto de vista medio ambiental toma un papel central en los programas de I+D de algunas potencias mundiales. La conservacion del agua, minimizar los vertidos y la reducción de compuestos químicos en la agricultura son algunas de las prioridades establecidad, por ejemplo, en el Programa Marco de la Unión Europea.

En este contexto, y dadas las restricciones de uso de microorganismos modificados genéticamente, los resultados obtenidos no solo son de interés en términos evolutivos sino que sirven como “prueba de concepto” de que es posible diseñar en el laboratorio sistemas de presión selectiva definidos, orientados a obtener mutantes naturales en genes específicos que otorguen ventajas competitivas en ambientes concretos. Esto supone una vía alternativa al diseño de microorganismos modificados genéticamente para la mejora de sus capacidades en cuanto a aplicaciones biotecnológicas, agrícolas o medioambientales.

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CONCLUSIONES

1. La presencia de genes que codifican proteínas de gran tamaño con características de adhesinas y cuya localización se predice extracelular, es un rasgo común a todos los genomas bacterianos, lo que sugiere que la formación de biofilms es una estrategia universal en procariotas.

2. Las grandes proteínas de adhesión se clasifican en siete familias, en función de la relación filogenética entre ellas y los dominios y motivos estructurales que presentan. Postulamos que esta distribución refleja distintas especificidades en cuanto a su modo de acción y las interacciones que pueden establecer.

3. En *Pseudomonas putida*, los procesos de adhesión a superficies abióticas y a semillas presentan elementos comunes y elementos diferenciadores. Así, los flagelos y la adhesina LapA son importantes en ambos procesos, mientras que otras funciones son específicas de la interacción bacteria-planta.

4. Las funciones necesarias para la adhesión de *P. putida* a semillas juegan en general un papel también en la colonización de las raíces, aunque existen excepciones. Así por ejemplo, los flagelos están implicados en adhesión, pero en la rizosfera actúan como determinantes de la distribución de las bacterias a lo largo de la raíz.

5. En *P. putida*, tal como ocurre en *P. fluorescens*, el gen *lapG* codifica una proteína implicada en la transición de vida sésil a vida planctónica. Mutaciones en *lapG* resultan en hiperadherencia, que se compensa al sobreexpresar el gen. Adicionalmente, mutaciones en *olpA*, que codifica una lipoproteína, favorecen las primeras etapas de adhesión de *P. putida* a superficies. La combinación de ambas mutaciones da lugar a un fenotipo pleiotrópico que incluye mayor producción de EPS, hiperpiliación y un sesgo claro hacia el modo de vida sésil.

6. El cultivo de *P. putida* en condiciones que favorecen el crecimiento en superficies da lugar a la selección de mutantes hiperadherentes. Las mutaciones que provocan este fenotipo no siempre implican a *lapG* ni a *olpA*, lo que indica que hay múltiples genes cuya alteración favorece la persistencia en vida sésil.