Molecular basis of chemosensory, biofilm and cell–to–cell signaling in different species of Pseudomonas.

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Memoria presentada para obtener el grado de Doctor en Biología Fundamental y de Sistemas.

Granada, 2016

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Granada, 2016

Director de la Tesis **Doctorando**

Fdo. Dr. Tino Krell Fdo. Q.B.P. Andrés Corral Lugo

Fdo. Dr. Manuel Espinosa Urgel

A mi Madre,

A Mis hermanos y mis Sobrinos,

A Samuel.

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LIST OF ABBREVIATIONS

(ACF) Alternative cellular functions

(AHLs) N-acyl-homoserine lactones

(Apr) Alkaline protease

(BdlA) Biofilm dispersal locus A

(β-gal) Beta-galactosidase

(C4-AHL) N-butanoyl-homoserine lactone

(CACHE) Calcium channels and chemotaxis receptors

(cAMP) Cyclic adenosine monophosphate

(CF) Cystic fibrosis

(CHASE) Cyclases/histidine kinases associated sensory extracellular

(c-di-GMP) Cyclic diguanylate

(DBD) DNA binding domain

(DKPs) Diketopiperazines

(K_D) Dissociation constant

(DNA) Deoxyribonucleic acid

(EPS) Exopolysaccharide

(eDNA) Extracellular DNA

(GABA) Gamma-amino butyrate

(GAF) cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA

(HBM) Helical bimodular

(ITC) Isothermal titration calorimetry

(4-HB) Four-helix bundle

(HK) Histidine kinase

(HTH) Helix–turn–helix

(LBD) Ligand binding domain

(MCP) Methyl-accepting chemotaxis proteins

(NMR) Nuclear magnetic resonance

(OCS) One-component system

(3-Oxo-C12-AHL) N-3-oxododecanoyl-homoserine lactone

(PGP) Plant growth-promoting

(PAS) Period-aryl-single protein

(PON) Paraxonasas

(Pi) Phosphate

(PDC) PhoQ-DcuS-CitA

(Pip) Proline iminopeptidase

(PQS) Pseudomonas quinolone signal

(QS) Quorum sensing

(QSS) Quorum sensing signal

(QSI) Quorum sensing inhibitors

(QQ) Quorum quenching

(QteE) Quorum threshold expression element

(QslA) QS LasR-specific antiactivator

(QteE) Quorum threshold expression element

(RA) Rosmarinic acid

(**RR)** Response regulator

(SAM) S-adenosylmethionine

(TCS) Two-component system

(Tar) Aspartate chemoreceptor

(TCA) Tricarboxylic acid

(ToxA) Exotoxin A

(TP-1) Triphenyl

(*xcp*) General secretory pathway

ABSTRACT

Research described in this dissertation focuses on three important aspects of Pseudomonas signaling: Chemosensory pathways involved in chemotaxis or with alternative cellular functions (ACF), biofilm formation and bacterial communication via quorum sensing (QS).

Bacteria use diverse small molecules for signaling and monitoring their levels provides valuable information on their environment as well as their intracellular status. This capacity in turn enables the bacterium to rapidly adapt to changes. While it is well established that one- and two-component regulatory systems (OCS and TCS) participate in regulating biofilm formation, there also exists evidence suggesting that chemosensory pathways are also involved. However, little information exists about which methyl-accepting proteins (MCP) and signals modulate this process. A major objective of this thesis was to cast light in to this issue. In the first part of this thesis I have generated a significant number of bacterial mutants and have then submitted the full set of all mutants in single Mcp genes to different analyses. These studies have led to the identification four mutants with significantly altered biofilm phenotypes, namely a WspA homologue of *P. aeruginosa*, previously identified to control biofilm formation by regulating cyclic diguanylate (c-di-GMP) levels as well as three uncharacterized MCPs. Subsequent experiments with recombinant ligand binding domains (LBD) have permitted the functional annotation of two of the uncharacterized receptors. The MCP that we termed McpU was found to mediate chemotaxis towards different polyamines. The functional annotation of McpU was initiated by high-throughput thermal shift assays of the receptor LBD. Isothermal titration calorimetry (ITC) showed that McpU-LBD specifically binds the polyamines putrescine, cadaverine and spermidine, which corresponds to a MCP with a novel chemoeffector profile. Moreover, I was able to establish that the MCP that we termed McpA binds specifically 12 different proteinogenic amino acids and mediates chemotaxis towards these compounds. By experimentation with corn plants I was able to show that mutants in McpU and WspA-Pp have a significantly reduced ability to colonize plant roots. Data agree with other reports showing that polyamines are signal molecules involved in the regulation of bacteria-plant communication and biofilm formation.

One major role of bacterial extracellular small-molecule signaling is the cell-cell communication, which involves the production, release, and community-wide detection of molecules called autoinducers. QS systems are essential for bacterial communication and proliferation. There are two principal QS systems in *P. aeruginosa* termed LasI/LasR and RhlI/RhlR. In the second part of the thesis, I overexpressed and purified the RhlR and LasR regulators of *P. aeruginosa* from lysates of *E. coli* grown in the absence of added acyl-homoserine lactone (AHL) signals. The subsequent analysis of both proteins by a series of

techniques provided interesting insight into their function. Microcalorimetric binding studies show that both proteins recognize different AHLs with 1 AHL:1 protein monomer stoichiometry and an affinity of approximately 1 µM. Both proteins did not preferentially bind the AHLs produced by their cognate AHL synthases (RhlI y LasI). LasR and RhlR unfold at T_m values of 63 and 70 °C, respectively and AHL binding did not cause significant T_m shifts. Both proteins are present in a monomer - dimer equilibrium and AHL binding did not alter their oligomeric state. Electrophoretic mobility shift assays demonstrate that purified LasR and RhlR are able to bind to a target promoter. Significant differences in the RhlR mediated stimulation of *in vitro* transcription were observed in the presence of different AHLs. In conclusion, these data thus show that both proteins are active and stable in the absence of bound AHL and that they are able to bind AHL ligands in a reversible manner, revising initial concepts of this protein family. With a view to the future, the availability of AHL-free protein will permit further studies to determine more precisely their mode of action.

To facilitate the establishment of infection, *P. aeruginosa* produces an impressive array of both cell-associated and extracellular virulence factors. Several of these virulence factors have been demonstrated to be regulated by QS. Plants were found to synthesize compounds that interfere with QS either by its stimulation or quenching. However, little information is available as to the molecular identity of these compounds. The interference with the QS system of pathogens represents an alternative strategy to reduce their virulence. In the final part of the thesis, I identified rosmarinic acid (RA) as a plant-derived compound that mimics bacterial AHLs. I have showed that RA bound to RhlR with higher affinity than its cognate N-butanoyl-homoserine lactone (C4-AHL). I have observed that RA causes larger increases in RhlR mediated transcription than C4-AHL. I was able to show that RA stimulates expression from different promoters that were previously shown to by RhlR dependent. Lastly, I demonstrated that the presence of RA causes induction of typical virulence associated and QS dependent phenotypes such as increases in the production of the virulence factors pyocyanin and elastase or an enhancement of biofilm formation. Because *P. aeruginosa* infection induces RA secretion from plant roots, our results indicate that RA secretion is a plant defense mechanism to stimulate a premature QS response. *P. aeruginosa* is a ubiquitous pathogen that infects plants and animals and in this context the identification of RA as an inducer of premature QS responses may be of use to define antibacterial strategies.

RESUMEN

La investigación descrita en esta tesis se centra en tres aspectos importantes de la señalización en Pseudomonas, como son: Las vías quimiosensoras involucradas en quimiotaxis o en funciones celulares alternativas, la formación de biopelículas y la comunicación bacteriana a través de sistemas de *quorum sensing*.

Las bacterias utilizan diversas moléculas de pequeño peso molecular para monitorear su entorno, así como su estado intracelular. Esta capacidad, a su vez permite a las bacterias adaptarse rápidamente a los cambios. Aunque está bien establecido que los sistemas de regulación basados en uno y dos componentes participan en la regulación de la formación de biopelículas, también existe evidencia que sugiere que las vías quimiosensoras están involucradas en dicha regulación. Sin embargo, existe poca información sobre qué quimiorreceptores de la vía quimiosensora y cuales señales modulan este proceso. Por lo tanto, uno de los objetivos principales de esta tesis es investigar el papel que tienen los quimiorreceptores en la formación de biopelículas. Para esto, en la primera parte de esta tesis se generó un importante número de mutantes bacterianos en los quimiorreceptores de *P. putida* KT2440 con el fin de generar una biblioteca de 27 mutantes individuales en cada uno de los genes que codifican para un quimiorreceptor. Esta biblioteca de 27 mutantes individuales fue sometida a diferentes análisis. Los resultados de estos estudios han llevado a la identificación de cuatro mutantes con fenotipos alterados significativamente en la formación de biopelículas, es decir, se identificó un homólogo del quimiorreceptor WspA de *P. aeruginosa*, previamente descrito como un regulador de la formación de biopelículas mediante la modulación de niveles intracelulares de diguanilato cíclico (c-di-GMP), así como de tres quimiorreceptores no caracterizados. Experimentos posteriores con proteínas recombinantes de los dominios de unión de ligando (LBD por sus siglas en ingles) de los quimiorreceptores han permitido la anotación funcional de dos de estos quimiorreceptores no caracterizados. Se determinó que el quimiorreceptor que hemos llamado McpU, regula la quimiotaxis hacia diferentes poliaminas. La anotación funcional de McpU fue llevada a cabo con ensayos de alto rendimiento de tipo “high-throughput thermal shift assays”, usando el LBD del quimiorreceptor para dichos ensayos. Por su parte, experimentos confirmatorios de calorimetría de titulación isotérmica (ITC por sus siglas en inglés) mostraron que McpU-LBD se une específicamente a las poliamidas putrescina, cadaverina y espermidina, lo cual determina que McpU es un nuevo quimiorreceptor con un novedoso perfil de quimioefectores. De igual manera, se tuvo la oportunidad de establecer que el quimiorreceptor que hemos llamado McpA se une específicamente a 12 aminoácidos proteinogénicos diferentes y media la quimiotaxis hacia estos compuestos. Finalmente, en experimentos en los que fueron usadas

plantas de maíz como modelo, se demostró que los mutantes McpU y WspA-Pp tienen una capacidad reducida de colonizar las raíces de las plantas cuando se encuentra en competición. Estos datos concuerdan con otros informes que muestran que las poliaminas son moléculas de señalización implicadas en la regulación de la comunicación entre bacterias y plantas, y la formación de biopelículas.

Una de las principales funciones de la señalización extracelular bacteriana en las que se usa moléculas de pequeño peso molecular, es la comunicación de célula a célula de tipo *quorum sensing*; la cual consiste en la producción, liberación, y la detección de moléculas llamadas autoinductores (acil-homoserina lactona). Los sistemas de *quorum sensing* son esenciales para la comunicación y proliferación bacteriana. Existen dos sistemas principales de *quorum sensing* en *P. aeruginosa* denominados LasI/LasR y RhlI/RhlR. En la segunda parte de la tesis, se sobreexpresaron y purificaron los reguladores RhlR y LasR de *P. aeruginosa* a partir de lisados de *E. coli* cultivadas en ausencia de señales añadidas de tipo acil-homoserina lactona (AHL). El análisis posterior de ambas proteínas por una serie de técnicas biofísicas, proporcionó una interesante visión de su función. Estudios de interacción basados en microcalorimétria muestran que ambas proteínas reconocen diferentes AHLs con una estequiometria 1:1 entre un monómero de proteína y una molécula de AHL con una afinidad de aproximadamente 1 µM. Impactantemente, ambas proteínas no se unen preferentemente a las AHL producidos por sus AHL sintasas (RhlI y LasI) afines. Se determinó que estos reguladores son termoresistentes en ausencia de AHL, observándose que LasR y RhlR se despliegan a una T_m de 63 y 70 °C, respectivamente, y la unión a AHL no causa cambios significativos en la T_m. Ambas proteínas están presentes en un equilibrio monómero-dímero y la adición AHL no altera su estado oligomérico. Ensayos de movilidad electroforética demuestran que las proteínas recombinantes LasR y RhlR son capaces de unirse a un promotor diana (con caja *lux* en su secuencia). Funcionalmente, se observaron diferencias significativas en la estimulación de la transcripción *in vitro* mediada por RhlR en presencia de diferentes AHLs. En conclusión, los datos muestran que ambas proteínas son activas y estables en ausencia de AHL y que son capaces de unirse a las AHL de una manera reversible, lo cual muestra discrepancias con los conceptos iniciales formulados para esta familia de proteínas. Con miras al futuro, la disponibilidad de proteínas (LasR y RhlR) libres de AHL permitirá realizar más estudios, con el fin de determinar con mayor precisión su modo de acción.

Para facilitar su establecimiento durante la infección *P. aeruginosa* produce una impresionante variedad de factores de virulencia extracelulares y asociados a la célula. Varios de estos factores de virulencia se ha demostrado que están regulados por densidad celular a través de

sistemas de *quorum sensing*. Por otra parte, se han identificado plantas que sintetizan compuestos que interfieren con la comunicación bacteriana tipo *quorum sensing*, ya sea por su estimulación o bloqueo. Sin embargo, hay poca información disponible sobre la identidad molecular de estos compuestos. La interferencia con el sistema de *quorum sensing* de algunos patógenos representa una estrategia alternativa para reducir su virulencia. En la parte final de la tesis, se identificó al ácido rosmarínico como un compuesto de origen vegetal que imita a las AHL bacterianas. Se ha mostrado que el ácido rosmarínico se une al regulador RhlR con mayor afinidad que su señal natural la N-butanoil-homoserina lactona (C4-AHL). Se cuantificó que el ácido rosmarínico aumenta en mayor intensidad la transcripción mediada por RhlR que C4-AHL. Se demostró que el ácido rosmarínico estimula la expresión de diferentes promotores dependientes de RhlR. Por último, se observó que la presencia del ácido rosmarínico provoca la inducción de los factores de virulencia y los fenotipos regulados por *quorum sensing* vía RhlR, como son: el aumento en la producción de piocianina y elastasa, o el incremento en la formación de biopelículas. Debido a que la infección por *P. aeruginosa* induce la secreción del ácido rosmarínico a partir de las raíces de las plantas, nuestros resultados indican que la secreción del ácido rosmarínico es un mecanismo de defensa de la planta con el cual estimula la respuesta prematura del sistema de *quorum sensing*. *P. aeruginosa* es un patógeno ubicuo que infecta a las plantas y animales y, en este contexto, la identificación del ácido rosmarínico como un inductor de respuestas prematuras de comunicación bacteriana por *quorum sensing* puede ser de utilidad para definir estrategias antibacterianas.

INTRODUCTION

1. BACTERIAL SENSING

To perceive the external environment our brain uses multiple sources of sensory information derived from several different modalities, including vision, touch and audition (Bourret, 2006). Decision-making in humans is a vital and complex process that involves the coordinated activity of an extended neural network, including several different areas of the brain. Making a decision requires the execution of several subtasks, such as outcome appraisal, cost–benefit analysis, and error perception, before finally selecting and implementing the optimal action (Westerhoff et al, 2014). The overwhelming majority of life on our planet is microbial, virtually every conceivable environmental niche harbors microorganisms capable of growing there (Bourret, 2006). In the microbial world, decisions are made by monitoring the current state of the system, by processing this information and by taking action with the ability to take into account several factors such as recent history, the likely future conditions and the cost and benefit of making a particular decision (Westerhoff et al, 2014). Throughout their lifes, bacteria interact with their environment by exchanging information with other cells, by exploring optimal growth conditions, and by sensing and responding to environmental stress. Thus the signaling network of bacterial is a complex and indispensable aspect of bacterial life (Krämer & Jung, 2010).

Evolutionary success depends on signal transduction, the ability to sense changing environmental conditions and to implement appropriate responses accordingly (Bourret, 2006). For a long time bacteria were regarded as single cell organisms that ensure their survival by adaptation to rapidly changing environmental conditions (Krämer & Jung, 2010). Now it is known that bacteria are able to display a dual lifestyle: either living as single organisms in the absence of communal obligations or displaying complex social interactions when part of a community (Kolter & Greenberg, 2006).

The fact that bacteria were considered simple organisms is a misconception that can be attributed to two main misleading observations: first, an underestimation based on the size of these organisms and second, the apparent absence of communication systems. The term “communication” has largely been associated exclusively with the animal kingdom, whose members are able to transmit messages using verbal or body language (Jimenez et al, 2012), Nevertheless, bacterial cells, like their eukaryotic counterparts, possess elegant signaling pathways that monitor critical parameters of the external environment and internal physiology. The resulting information is used to regulate cellular function as the environment

changes and the cell matures (Falke et al, 1997). The concept of intracellular communication within bacterial populations originates from different discoveries in the 1960s and 1970s proposing the involvement of external factors excreted by bacteria themselves (Krämer & Jung, 2010). Chemistry is unequivocally the most universal cellular language operating in all living organisms (Jimenez et al, 2012). Using chemical signals, bacteria are able to determine population density and diversity, two ecological factors crucial for their survival. Chemical communication between cells ensures coordination of behavior. In prokaryotes, this chemical communication is usually referred to as QS, while eukaryotic cells signal through hormones (Pacheco & Sperandio, 2009).

Bacteria are exposed to constantly varying environmental conditions, like variations in nutrient availability, osmolality, pH, temperature, or cell density. To survive, cells have to monitor and to adapt adequately to these changing conditions. Hence bacteria are equipped with numerous signal transduction systems that mediate the response to varying environmental stimulus (Krämer & Jung, 2010). The cytoplasmic membrane of bacterial cells separates the cytoplasm from the outer world and thus defines the internal from the external compartment. Bacterial signal transduction across to the cell membrane is mediated primarily by the concerted action of several protein families, namely, OCS and TCS, MCP, serine/threonine kinases, adenylate and guanylate cyclase's and phosphodiesterases (Cousin et al, 2013; Jung et al, 2012)

In general, Wuichet and Zhulin have defined three major modes of signal transduction in prokaryotes, namely OCS, TCS and chemosensory pathways (Wuichet & Zhulin, 2010). The simplest signal transduction system consists of a single protein, which is capable of both sensing a signal and directly affecting a cellular response. TCS based signaling is the primary mechanism of transmembrane signaling in bacteria. A TCS is composed of a histidine (sensor) kinase (HK) and a response regulator (RR). The HK, typically integrated into the membrane, - acts in general as a sensor, whereas the RR generates the system output (Krämer & Jung, 2010). Finally, the molecular machinery that controls chemotaxis in bacteria is substantially more complex than any other signal transduction system in prokaryotes. This multiprotein chemosensory pathway is present in most prokaryotic species and evolved from simpler two-component regulatory systems that control prokaryotic transcription (Wuichet & Zhulin, 2010). The number of OCS and TCS per genome positively correlates with the genome size and, in both cases, is roughly proportional to the square of the total number of genes (Ulrich et al, 2005).

A crucial question in signal transduction is: how does an organism integrate multiple signals in the definition of a single output parameter? Most of the present knowledge on bacterial gene regulation is based on studies conducted in laboratory settings, where conditions are carefully controlled and, typically, only one stress is applied at a time (Bijlsma & Groisman, 2003).

1.1. One-component systems

The bacterial cell is surrounded by the envelope, which defines cell shape and its physiological individuality. Signaling can thus be conceptually divided into process that occur outside the cell, across the membrane between the interior and exterior, and within the cytoplasmic compartment (Krämer & Jung, 2010). OCS are evolutionarily older; more widely distributed among bacteria and archaea, and display a greater diversity of domains than TCS (Ulrich et al, 2005).

An OCS is composed of two domains, an input domain and an output domain. Typically, the binding of signal molecules alter the properties of the output domain, which in most cases alters interaction with DNA promoter regions (Bourret, 2006; Ulrich et al, 2005).

1.2. Two-component systems

The most widely distributed type of signal transduction system is the TCS (Krämer & Jung, 2010). In fact, genes encoding TCSs are present in almost all bacteria and typically represent ≤1% of their genomes (Ashby, 2004). Therefore, TCS that link environmental signals to cellular responses are viewed as the primary mode of signal transduction in prokaryotes (Ulrich et al, 2005). Within the family of TCS, prototype and phosphorelay systems can be distinguished (Jung et al, 2012). A prototype system consists of a membrane-integrated HK that senses the stimulus and the cytoplasmatic RR that mediates the output response. This mechanism functions as a result of phosphotransfer between a sensor HK and a RR (Ulrich et al, 2005). The TCS mechanism is based on signal induced modulation of the HK autokinase domain which in turn modulates the transphosphorylation rate/state of the cognate RR. RR phosphorylation alters its properties and RR action is in most of the cases related to transcriptional changes, but a number of alternative RRs have been reported that possess enzymatic activity, or that bind to other targets like RNA, ligands or proteins (Galperin, 2006). The enzymatic activities of RR include: CheB-like methylesterase, GGDEF domain diguanylate cyclase, EAL domain c-di-GMP esterase, c-di-GMP phosphodiesterase, PP2C protein phosphatase and HK (Bourret, 2006).

TCSs are involved in the regulation of virtually all types of processes including virulence, sporulation, metabolism, QS, chemotaxis, transport or nitrogen fixation (Krell et al, 2010). The number of TCSs differs enormously from species to species. It ranges from zero in *Mycoplasma genitalium*, over 30 to 32 in *Escherichia coli* strains and 119 to 132 in *Myxococcus xanthus strains(Stock et al, 2000)*. The varying number of these systems between species seems to be related to the number of different environmental cues and the frequency or environmental changes the corresponding bacteria are exposed to (Krämer & Jung, 2010). Data currently available indicate that RRs form a relatively well-conserved family of proteins (NarL or OmpR families), which are characterized by an N-terminal receiver domain and a C-terminal helix-turn-helix motif-containing DNA binding domain. In contrast, there is extreme functional and structural diversity of sensor kinases. They can be membrane-bound or not, have a large variety of different sensor domains located either in the periplasm or cytosol and recognize signals in many different ways (Mascher et al, 2006).

1.3. Chemosensory pathways

The chemosensory pathways, which are a special case of two-component signal transduction systems, constitute the third mode of bacterial signal transduction (Wuichet & Zhulin, 2010). Most bacterial species are too small to sense concentration gradients along their body lengths (Tindall et al, 2012) and instead use temporal sensing to perform a biased random walk, whereby they alternate periods of smooth swimming with brief direction changes. However, bacterial species differ in the mechanisms that they use for direction changing (Porter et al, 2011). Motile bacteria sense changes in the concentration of chemicals in the environment and exhibit a behavioral response called chemotaxis (Kato et al, 2008). Bacteria use chemotaxis to migrate towards environments that are optimal for growth or move away from toxic compounds. Many bacterial species swim by rotating helical filaments, known as flagella, at up to 1,000 hertz, producing a propulsive force that drives the cell body forward (Porter et al, 2011).

Bacterial chemotaxis can be viewed as an important prelude to metabolism, prey–predator relationships, symbiosis, infections, and other ecological interactions in biological communities (Kato et al, 2008). Chemotaxis is characterized by a high sensitivity and a precise adaptation, properties attributed to an assortment of interactions within the multi-protein signal transduction pathway (He & Bauer, 2014). The major components of the bacterial chemotaxis apparatus include MCPs, the HK kinase CheA, the adaptor protein CheW, the RR CheY as well as the receptor-modification enzymes CheR and CheB. Attractant binding to MCP modulates CheA autophosphorylation activity, and hence the subsequent transfer of this phosphoryl

group to CheY. In many bacterial species a reduction in the CheY-P concentration reduces its binding to the flagellar motor and results in the bacteria swimming towards higher concentrations of the attractant (Wadhams & Armitage, 2004).

The chemosensory pathway that mediate chemotaxis are best understood in *E. coli* and will be described in depth below. Many species contain *che* gene clusters that code for proteins with the simplified standard chemotaxis architecture that is present in *E. coli.* Bioinformatics analyses of 450 prokaryotic genomes identified 416 chemosensory gene clusters within 245 species based on the number of putative CheA proteins (He & Bauer, 2014).

Recent studies have revealed that several Gram-negative species utilize variations of the well-known chemosensory pathways that mediate chemotaxis to switch lifestyles in order to survive environmental stress (alternative functions). Chemosensory pathways with ACF that utilize chemotaxis-like components represent some of the more complex signal transduction pathways in prokaryotes (He & Bauer, 2014). In these systems, the output of the pathway is either at the transcriptional level factor or enzymatic activities. Key features of chemosensory pathways that distinguish them from TCSs are temporal sensing, memory and the ability to adapt to a persistent signal(Porter et al, 2011; Wuichet & Zhulin, 2010).

A chemosensory pathway with ACF has been reported in the soil bacterium *Myxococcus xanthus* (Zusman et al, 2007). *M. xanthus* aggregates upon starvation to form mounds and subsequently fruiting bodies. One of the eight chemosensory pathways in this bacterium (*Che2*), namely *the DiF* pathway, allows exopolysaccharide (EPS) biosynthesis which in turn is essential for fruiting body and spore formation in response to starvation. This ACF pathway consists of homologs of MCP (DifA), CheW (DifC), CheA (DifE), and CheY (DifD)(Black & Yang, 2004; He & Bauer, 2014). In addition, another related cluster (*Che3*) directed myxospore development employing two MCP´s and a CheA-CheY hybrid. (Willett & Kirby, 2011).

Another example of bacteria with multiple chemosensory pathways with ACFs is *Rhodospirillum centenumis* (Berleman & Bauer, 2005*).* This photosynthetic bacterium possess three chemotaxis-like pathways: the *Che1* cascade that controls chemotaxis, the *Che2* signaling cascade that regulates flagella biosynthesis (Lu et al, 2010), and the *Che3* signaling pathway that regulates cyst formation (He et al, 2013). The *Che3* signal transduction cascade represents a unique deviation from the *E. coli* chemotaxis architecture (Berleman & Bauer, 2005).

The best studied chemosensory pathway with ACF is the *wsp* pathway of *P. aeruginosa* (Hickman et al, 2005). The products of the *wsp* operon regulate biofilm formation through

modulation of c-di-GMP levels. Within the *wsp* pathway (Fig. 1), the MCP (WspA) forms dynamic clusters at both polar and lateral subcellular locations, while the RR, WspR, contains a GGDEF domain known to catalyze the formation of a cytoplasmic signaling molecule: c-di-GMP (Hickman et al, 2005; O'Connor et al, 2012). A presently unknown signal that is generated by surface growth of *P. aeruginosa* binds to WspA, which in turn modulates pathway activity and ultimately c-di-GMP levels. This change in c-di-GMP levels stimulates biofilm formation and reduces motility (Tamayo, 2013). In 2014 it was reported that WspA may recognize ethanol produced by *Candida albicans*, which in turn promotes WspR activation (Chen et al, 2014).

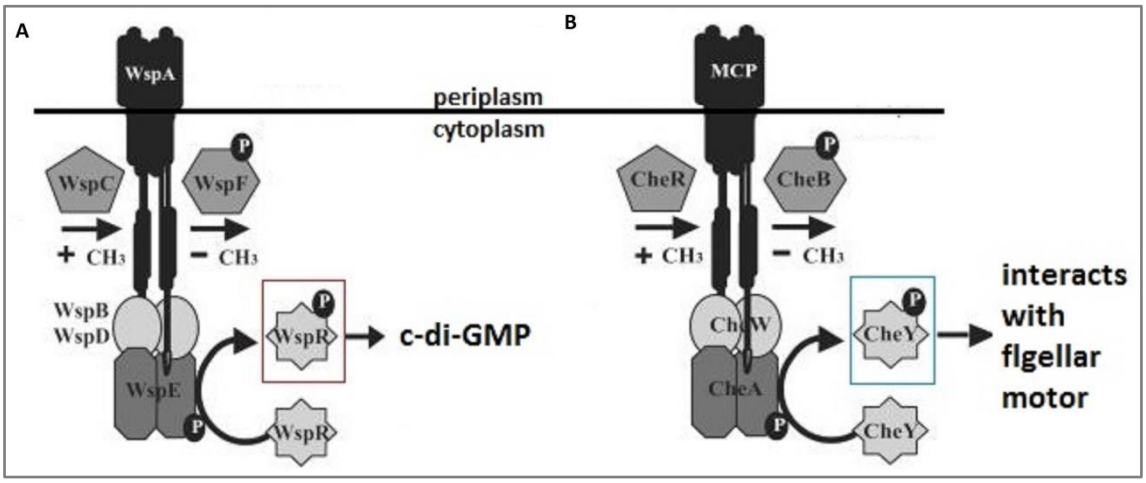


Figure 1 Schematic representation of Wsp chemosensory pathway with ACF (A) and a chemosensory pathway involved in chemotaxis (B). Figures modified from (Guvener & Harwood, 2007).

1.3.1. The prototypal chemotaxis pathway (E. coli)

The molecular mechanism of chemotaxis, including the associated MCPs, has been studied primarily in the Enterobacteriaceae *E. coli* (Wadhams & Armitage, 2004). This well-studied chemotaxis motility pathway can be considered an atypical TCS, comprised of the HK CheA, which does not possess an input domain, and the RR CheY that has a REC domain but lacks the output module (Hazelbauer & Lai, 2010).

The specificity of a chemotactic response is determined by the MCP, which is typically composed of a periplasmic LBD and a cytosolic signaling domain (Hazelbauer et al, 2008). In *E. coli*, five MCPs (Tar, Tsr, Trg, Tap and Aer) serve as sensors for intracellular and extracellular chemical stimuli such as amino acids and sugars (Fig. 2) (Borziak et al, 2013). These MCPs are homodimers that form trimers and higher order clusters in association with CheA and a coupling factor CheW (Fig. 2) (Amin & Hazelbauer, 2010). Association between receptors in

arrays is thought to promote allosteric interactions that result in signal amplification, extended dynamic range, and integration between inputs of different kinds (Sourjik & Berg, 2000).

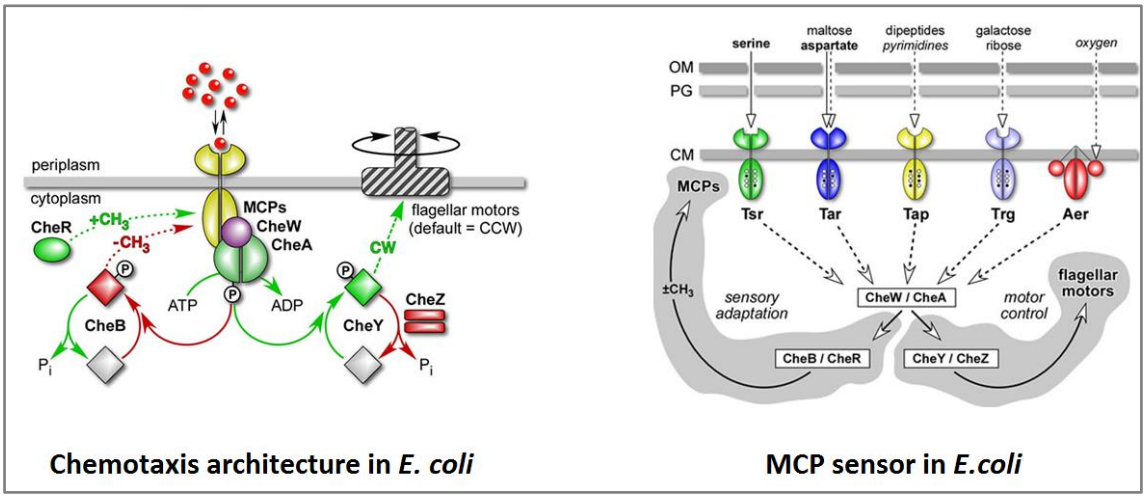


Figure 2 Chemotactic Signaling components (Che) and five MCP-family receptors in *E. coli.* Figures taken from Parkinson Lab (http://chemotaxis.biology.utah.edu/Parkinson_Lab/index.html).

The ligand binding activity of the MCPs is altered by a methyltransferase (CheR) and a methylesterase (CheB) to form an adaptation mechanism. In this process CheR constitutively transfers methyl groups from S-adenosylmethionine (SAM) to specific glutamate residues on MCPs that CheB subsequently removes after being phosphorylated by CheA (Springer & Koshland, 1977). Since ligand binding affinity is controlled by MCP methylation, CheR and CheB thus constitute a feedback mechanism that constantly resets the MCPs to a pre-stimulus state as the bacterium travels through a ligand gradient (Springer et al, 1979).

Finally, the amount of CheY-P in a cell is regulated by its phospho-donor CheA-P as well as a phosphatase CheZ, which accelerates dephosphorylation of CheY-P (He & Bauer, 2014) (Fig. 2).

The addition of MCPs to the TCS scheme allows for signal amplification (hence enhanced sensitivity) and signal adaptation (hence the ability to sense a chemical gradient) through reversible methylation, making the mechanism permitting chemotaxis one of the most intricate sensory pathways in prokaryotes (He & Bauer, 2014). *E. coli* and *Salmonella typhimurium* possess a single Che pathway involved in chemotaxis (Hamer et al, 2010).

1.3.2. More complex chemotaxis pathways: Pseudomonas

In contrast to *E. coli*, the chemotactic behavior of soil and aquatic microorganisms is poorly understood. However, there is increasing genomic evidence that suggests that the chemotactic

apparatus of this group of bacteria is remarkably complex (Lacal et al, 2010a). Genome analysis revealed that a large number of environmental motile bacteria possess a number of genes involved in chemosensing and chemotactic signal transduction, specifically, that soil bacteria generally have a large number of MCPs. This is exemplified by the genome sequence of *Pseudomonas*, *Clostridium* and *Rhizobium* strains, which typically have more than 20, but up to 60 genes coding MCP. From these microorganisms, *P. aeruginosa* PAO1 is best characterized and therefore a model organism (Kato et al, 2008).

P. aeruginosa is a member of the γ-Proteobacteria class of bacteria. *P. aeruginosa* is a Gram-negative, aerobic rod shaped bacterium measuring 0.5–0.8 μm wide and 1.5–3.0 μm long. Almost all strains are motile by means of a single polar flagellum. Its habitat is widespread and it is generally found in soil, water and many other environments (Jayaseelan et al, 2014). As an opportunistic human pathogen, *P. aeruginosa* is a common cause of nosocomial infections and is responsible for the persistent infections in immunocompromised individuals and for the chronic lung infections of patients with cystic fibrosis (CF). *P. aeruginosa* is responsible for nosocomial pneumonia, urinary tract infections, and surgical wound or bloodstream infections (Bottomley et al, 2007). In addition, *P. aeruginosa* is capable of causing serious infections in non-mammalian host species such as insects, nematodes, and plants. The effectiveness of this organism in causing infection is likely due to a number of well-regulated virulence factors and defense mechanisms such as multidrug resistance pumps and biofilm formation(Walker et al, 2004).

In *P. aeruginosa* five gene clusters encoding signaling proteins have been identified that form four chemosensory pathways. Two pathways, termed *che1 and che2,* have a role in chemotaxis, the third pathway (*wsp*) regulates c-di-GMP concentrations (Hickman et al, 2005) and finally, the fourth pathway, *chP* (cluster IV genes), modulates the cyclic adenosine monophosphate (cAMP) level and consequently several other features including type IV pili synthesis and twitching motility (Ferrandez et al, 2002; Fulcher et al, 2010; Hickman et al, 2005).

It has been reported that chemotaxis has important roles in invasive infections of animals by *P. aeruginosa*, infections of plants by *Pseudomonas syringae* and colonization of plant roots by plant growth-promoting (PGP) *P. putida* KT2440 and *Pseudomonas fluorescens* (Cuppels, 1988; de Weert et al, 2002; Kato et al, 2008; Turner et al, 2014). Studies of *P. putida* KT2440, one of the model organisms of this thesis, reveal that there are various paralogues of the cytosolic proteins involved in chemosensory signaling, which form several parallel cytosolic signal

transduction cascades. Based on comparative analysis of the three CheR paralogues of *P. putida* KT2440, Garcia-Fontana et al. conclude that CheR1 forms part of a chemosensory route that controls biofilm formation, CheR2 forms part of a chemosensory pathway involved in chemotaxis, and CheR3 may potentially be involved in type IV pili-based motility (Garcia-Fontana et al, 2013).

Pseudomonads are ubiquitously present in different ecological niches and many strains are characterized by their metabolic diversity (Finnan et al, 2004; Khan et al, 2008; Panagea et al, 2005; Sarkar & Guttman, 2004; Timmis, 2002). Metabolic versatility appears to be reflected in a chemotactic adaptability, and so far 140 compounds have been identified to induce chemotaxis *in* Pseudomonas strains, including organic acids, amino acids, differently substituted aromatic hydrocarbons, biphenols, nucleotide bases, sugars, inorganic Pi, metal ions, or peptides (Sampedro et al, 2015). Ligand are recognized at the LBD of MCP and, considering the diversity of signals, is not surprising that MCP LBDs shows a high degree of sequence and structural diversity, in contrast to the conserved signaling domain. MCP LBDs can be classified according to their size: cluster I sensor domains are of approximately 150 amino acids, whereas cluster II domains harbor approximately 250 amino acids (Lacal et al, 2010b). An inspection of the SMART data base reveals that the large majority of MCP LBDs remains un-annotated. In the case of MCPs with annotated LBDs, these domains belong to different families such as TarH, PAS, GAF, CACHE or CHASE (Lacal et al, 2010a). However, there is a clear need for research to study MCPs with un-annotated LBDs

Bioinformatics analysis using the microbial signal transduction database (MIST 2.2) (Ulrich & Zhulin, 2010), predicted that the *P. puida* KT2440 genome encodes 5,350 proteins of which approximately 10 % are involved in signal transduction by OCS, TCS or chemosensory pathways. Of these, 46 proteins form part of chemosensory pathways, namely 27 MCPs, 6 CheW, 1 CheZ, and three paralogous of each CheA, CheB and CheR are found. Of the 27 MCPs, the most abundant LBD architecture (8 MCPs) are double PhoQ-DcuS-CitA (PDC)(Zhang & Hendrickson, 2010), two groups of four MCPs contain four-helix bundle (TarH) LBD (Ulrich & Zhulin, 2005) and helical bimodular (HBM) domains (Ortega & Krell, 2014), whereas a single MCP has CACHE LBD (Lacal et al, 2010b). Five other MCPs possess PAS type LBDs, whereas two other LBDs are of unknown structure. So far only the McpG, McpH, McpP, McpQ and McpS MCP have been annotated with a function, and were found to mediate chemotaxis toward different organic and amino acids (Garcia et al, 2015; Lacal et al, 2010a; Martin-Mora et al, 2015; Reyes-Darias et al, 2015b).

1.3.3. Information available on Pseudomonas MCP

To date, the best characterized MCP of *P. putida* KT2440, McpS, contains an HBM domain (Ortega & Krell, 2014) and was found to mediate chemotaxis to acetate, butyrate and 6 tricarboxylic acid (TCA) cycle intermediates (Lacal et al, 2010a; Pineda-Molina et al, 2012). The crystal structure of McpS-LBD shows that it is composed of six helices that arrange into two four-helix bundle modules, of which each contains an independent ligand binding region, able to bind different signal molecules. Malate and succinate were found to bind to the membrane-proximal module, whereas acetate binds to the membrane-distal module (Pineda-Molina et al, 2012). Analysis *in vivo* and *in vitro* show that the magnitude of McpS mediated chemotaxis to the above mentioned ligands differs significantly. Recombinant and ligand free McpS-LBD is present in a monomer-dimer equilibrium in solution and ligand binding was shown to promote and stabilize protein dimerization (Lacal et al, 2010a).

McpQ, a McpS paralogue, was recently shown to be implicated in the specific recognition of metal-citrate complexes, which are more abundant in the natural habitat of the bacterium than metal free citrate (Martin-Mora et al, 2015). With this finding it was possible to distinguish two kinds of MCP for Krebs cycle intermediates in *P. putida* KT2440, one with a broad ligand range (McpS) and another specific for citrate and citrate/metal complexes. HBM LBDs, like those in McpS and McpQ, were identified in bacteria and archaea and were found to form part of MCPs and HKs (Ortega & Krell, 2014).

According to MCP classification by Lacal et al. 2010 (Lacal et al, 2010b), a cluster I MCP, McpP, has been identified to recognize non-Krebs cycle organic acids, such as acetate, L-lactate, pyruvate and propionate (Garcia et al, 2015). Ligand recognition occurred with affinities comparable to those for McpS and McpQ (Lacal et al, 2010a; Martin-Mora et al, 2015), with a similar MCP affinity and chemotaxis response.

In addition to McpS, McpQ and McpP from *P. putida* KT2440, (Parales et al, 2013) predict that other orthologous exist in *P. putida* F1 that detect organic acids, McfR from the F1 strain is an orthologous of ORF PP0339 in KT2440 (McpR) and could be responsible for the detection of other organic acids. Similarly to McpP, McpR belongs to cluster I, but in contrast to McpQ and McpP, McpR-LDB is predicted to have a 4-HB-type LBD.

As mentioned above, Pseudomonads are characterized by a metabolic diversity, as evidenced by the fact that some *P. putida* strains can use more than 100 different compounds for growth (Timmis, 2002). Additionally, previous studies have demonstrated that *P. putida* strains are not only capable of growth on a wide range of organic substrates, but also they are chemotactic

towards many of these compounds (Parales et al, 2013). The amino acid gamma-amino butyrate (GABA) is an omnipresent compound in nature, where it exerts multiple functions such as human neurotransmitter, plant signaling or carbon and nitrogen source (Bormann, 2000; Bown & Shelp, 1997). Bacterial chemotaxis to GABA via the PctC MCP, was first described in *P. aeruginosa* (Rico-Jimenez et al, 2013). It was proposed that GABA could play a role in inter-kingdom communication between plants and bacteria(Reyes-Darias et al, 2015b). This notion is supported by the demonstration that McpG, the PctC homologues of *P. putida* KT2440 was found to participate in the attraction of bacteria to and colonization of tomato roots (Reyes-Darias et al, 2015b).

Based on sequence homology with characterized MCPs, the function and cognate ligands for several uncharacterized MCPs of *P. putida* KT2440 could be predicted. The paralogous PctA, PctB and PctC MCPs of *P. aeruginosa* PAO1 mediate chemotaxis to amino acids (Rico-Jimenez et al, 2013). Sequence analysis of uncharacterized *P. putida* KT2440 MCP LBDs have led to the identification of a number of homologues, namely PP2249, PP0584 and PP0320 (33-57% sequence identity with PctA, PctB and PctC) that may also be implicated in amino acids chemotaxis. Sequence alignment of ORF PP0584 with previously identified pyrimidine McpC of *P. putida* F1, shows 99% of identity, suggesting that PP0584 may also be involve in chemotaxis to pyrimidines (cytosine) in KT2440(Liu et al, 2009).

Recently, a specific MCP for purine derivatives has been identified in *P. putida* KT2440 (Fernandez et al, 2015a). The ligands recognized by McpH include purine, adenine, guanine, xanthine, hypoxanthine and uric acid. Chemotaxis to purines and pyrimidines in Pseudomonas has already been shown before (Sampedro et al, 2015); however, the MCP had not been identified. In conclusion, the works of Liu et al. and Fernandez et al. indicate that there exist two independents MCPs for nucleotide baeses in *P. putida*: McpC for pyrimidine bases and McpH for purine bases.

In *P. aeruginosa*, two chemotactic transducers for inorganic phosphate (Pi), designated CtpH and CtpL, have been identified (Wu et al, 2000). In analogy, *P. putida* KT2440 genome encodes two different MCPs involved in chemotaxis to Pi. The ORF PP0562 in *P. putida* KT2440 encodes a CtpL orthologue (57 % sequence identity), that respondes to low Pi concentrations in *P. aeruginosa*. Conversely, *P. putida* KT2440 has a CtpH homologue (ORF PP2120) shown to mediate chemotaxis to high Pi concentrations to in *P. aeruginosa*.

Chemoattractants sensed by the MCPs are frequently carbon or nitrogen sources (sugars, amino acids, Krebs cycle intermediates)(Sampedro et al, 2015) or compounds which serve as

electron acceptors like oxygen, nitrate, fumarate and dimethyl sulfoxide (DMSO) (Borziak et al, 2013; Lacal et al, 2011a). Furthermore, there is evidence that several Pseudomonas strains show chemotaxis towards aromatic hydrocarbons like toluene and naphthalene. The MCPs responsible for this behavior are generally encoded in plasmid-borne sequences and the corresponding MCPs McpT (Lacal et al, 2011b), NbaY (Iwaki et al, 2007) and NahY (Grimm & Harwood, 1999) have been identified in different Pseudomonas strains.

2. BACTERIAL LIFESTYLE

The three most common ways of growing bacteria *in vitro* are as planktonic cultures, colonies on agar plates or as biofilms in continuous-flow systems (Fig. 3) (Mikkelsen et al, 2007). According to the Oxford English Dictionary, planktonic refers to “drifting or floating organic life found at various depths in the ocean or fresh water.” At the microscopic level, a planktonic habitat for prokaryotes can also encompass water films around soil particles, saliva in the mouth, fluids in the intestinal lumen, serum in blood vessels, and urine in the bladder and urinary tract (Marshall, 1976). The transition between planktonic growth and biofilm formation represents a tightly regulated developmental shift that has substantial impact on cell fate (Nagar & Schwarz, 2015). Numerous studies have investigated the relationship between planktonic cells and biofilms, and large differences have been reported between both modes of growth. Most of our understanding of their physiology stems from experiments using liquid cultures of dispersed free-swimming 'planktonic' cells. Planktonic growth may have its advantages under specific conditions. For example, the elevated metabolism characterizing suspended cells compared with cells in a biofilm may support extensive proliferation of the planktonic cells. Additionally, the high motility characterizing the planktonic cells and the ease of relocation once the inhabited growth niche is exhausted may be advantageous. Therefore, switching between a planktonic and biofilm mode of growth is a critical step in bacterial development, which must be tightly regulated and tuned to environmental cues (Nagar & Schwarz, 2015). In a biofilm, cell densities are substantially higher than in planktonic culture. As a consequence, most biofilm cells are likely to encounter nutrient and oxygen limitation as well as higher levels of waste products, secondary metabolites, and secreted factors. Bacteria present in biofilms are known to express genes differently as compared to those in the planktonic state, and biofilm cells are generally believed to closely resemble planktonic cells in stationary phase (Mikkelsen et al, 2007).

Most surfaces on this planet teem with microbial life, creating ecosystems of diverse organisms that flourish in slimy beds of their own making (Kolter & Greenberg, 2006). It is now accepted that naturally, bacteria prefer to live in surface-associated manner. In the biofilm the bacteria are embedded in an extracellular polymer matrix, and are protected against environmental stresses, antimicrobial treatment, and the host immune system (Krämer & Jung, 2010).

Biofilms have also been shown to be implicated in a variety of human persistent infections of tissue-associated or implant-associated nature (Rybtke et al, 2015). In industry, biofilms cause many problems, including fouling of ship hulls, promoting corrosion in pipes, and contaminating food processing equipment (Masak et al, 2014; Van Houdt & Michiels, 2010). In contrast, biofilms can also be exploited biotechnologically, for example, in wastewater treatment plants (Shrout et al, 2011). Bacterial biofilms display a tremendous capacity to resist traditional antimicrobial therapies such antibiotics or biocides (Krämer & Jung, 2010). According to a public announcement of the US National Institutes of Health over 80 % (Davies, 2003) of microbial infections in the human body are caused by biofilms, including lung infections in CF patients, wound infections in burn patients, otitis media, periodontitis, endocarditis, urinary tract infection and infections caused by implants (Bakaletz, 2007; Costerton et al, 2005; Hoiby et al, 2010; Schaber et al, 2007). There are several reasons why the biofilm lifestyle is advantageous to the bacterium. One of these is that biofilms provide protection from a range of stressors, from antibiotics to host immune response and protozoan grazing. They can also facilitate acquisition of nutrients and promote genetic exchange, providing a high local cell density and a stable structured environment for genetic exchange events, such as conjugation and transformation (Shrout et al, 2011).

Evidence has accumulated over the past few years suggesting that biofilm formation proceeds through an ordered series of steps (Fig. 3). For most of the bacteria investigated so far, at least three developmental steps can be distinguished: (i) initial attachment of individual cell to a surface, (ii) aggregation of these cells to microcolonies, and (iii) differentiation of the microcolonies into a mature biofilm (the development of typical three-dimensional biofilm architecture). Biofilm formation follows a genetic program that directs cells from the planktonic into the sessile mode or it could just be a consequence of a series of metabolic adjustments of the cells to rapidly changing microenvironments and thus represents a self-organization process (Krämer & Jung, 2010).

As the surface-associated population grows, the biofilm becomes increasingly sophisticated in its activities, with individual cells taking on specific tasks (Kolter & Greenberg, 2006). Within the biofilm community, bacteria communicate with each other by using chemical signal molecules (Krämer & Jung, 2010).

Natural biofilms nearly always harbor a multitude of microbial species — the region around a single tooth, for example, is often sheathed in a community of several hundred species (Kolter & Greenberg, 2006). Communication between different species occupying the same ecological niche and competing for the common resources includes synergistic as well as antagonistic interactions; moreover interspecies signaling might play an important role in influencing bacterial virulence and response to antimicrobial therapy's (Krämer & Jung, 2010).

Different species build biofilms differently, and many strains have multiple biofilm-formation pathways, but one feature that does seem to be common to all biofilms is that cells secrete a 'matrix' to hold themselves in place and to provide a buffer against the environment. The composition of matrices were found to differ, and, depending on the contributing species and environmental conditions, the matrix can be composed of different polysaccharides, proteins and even nucleic acids (Kolter & Greenberg, 2006).

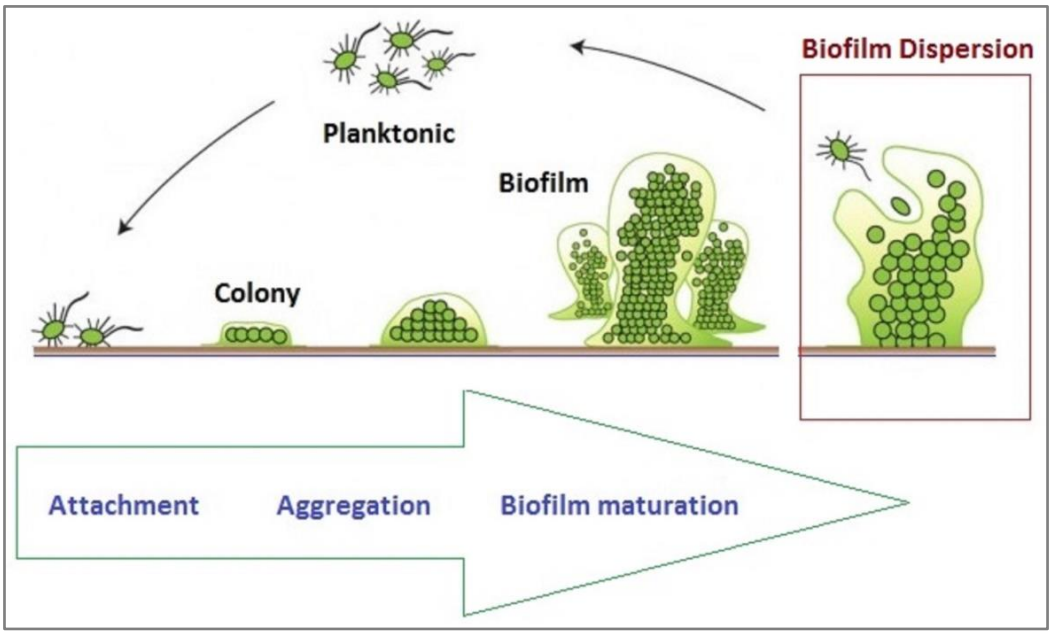


Figure 3 Bacterial life styles. Shown are the stages of biofilm formation and dispersion. Figure modified from web page of Center for Biofilm Engineering at Montana State University.

2.1. Biofilm in Pseudomonas

P. aeruginosa is a versatile organism that can survive in soil, marshes, and marine habitats, on plant and animal tissue, and on nonliving surfaces (Stover et al, 2000). Central to this survival is the ability to adapt and switch between free-living and sessile lifestyles (Waite et al, 2005). The remarkable ability of *P. aeruginosa* to form biofilms in many environments renders antibiotic treatments inefficient and therefore promotes chronic infectious diseases (Rasamiravaka et al, 2015). Several gene encoded functions contribute to biofilm development, including surface structures such as pili and flagella, as well as secreted polysaccharides (Shrout et al, 2011). Depending on the *P. aeruginosa* strain and/or nutritional conditions, different biofilm phenotypes can be observed (Rasamiravaka et al, 2015). The carbon source upon which *P. aeruginosa* is grown dictates the architecture of the biofilm it forms. *P. aeruginosa* is capable of forming undifferentiated "flat" biofilms (growth in succinate) as well as highly structured biofilms containing void spaces and large cell aggregates (growth in 1% tryptic soy broth)(Tsai & Winans, 2010). Other key nutrients such as iron have been shown to influence biofilm formation, under conditions of iron starvation, biofilm formation is inhibited (Banin et al, 2005). In this microscopic world, biofilms are metaphorically called a "city of microbes" with EPS, which represents 85% of total biofilm biomass, as "house of the biofilm cells". EPS is composed mainly of biomolecules, polysaccharides, extracellular DNA (eDNA), and polypeptides that form a highly hydrated polar mixture that contributes to the overall structural scaffold and architecture of the biofilm (Rasamiravaka et al, 2015).

Changes in the levels of c-di-GMP, a ubiquitous intracellular second messenger widely distributed in bacteria, correlate with phenotypic changes associated with virulence, motility, colony morphology, production of EPS, and the transition between planktonic and sessile lifestyles (Yousef-Coronado et al, 2011). C-di-GMP is a determinant that plays a role in biofilm formation, specifically, polysaccharides production is dependent on the intracellular pool of c-di-GMP (Hickman et al, 2005). High levels of c-di-GMP promote the biosynthesis of polysaccharides (alginate and polysaccharide extracellular) as well as adhesines in biofilms and inhibits various forms of motility (Hengge, 2009; Rasamiravaka et al, 2015).

Although the most generalized image of a biofilm is that of a community settled on a submerged abiotic surface, bacteria often attach to and colonize biotic surfaces (Duque et al, 2013). Pseudomonas, which are ubiquitous in the principal terrestrial ecosystems, are frequently found in association with plants, either as mutualists, saprophytes or pathogens (Walker et al, 2004; Zhao et al, 2013). These microorganisms can have a significant impact on agriculture (Perez-Montano et al, 2014; Weller, 2007). *P. fluorescens* and *P. putida*, are

commonly found in association with the plant rhizosphere (Moreno & Rojo, 2013) and their ability to utilize the different nutrients present in leaf leachates and root exudates is one of the keys to the success of Pseudomonas as plant colonizers (Espinosa-Urgel, 2004).

P. putida is a model organism to study the genetic traits playing a role in rhizosphere colonization by plant beneficial bacteria. Biofilm formation and root colonization share mechanistic parallels in *P. putida* (Yousef-Coronado et al, 2008). As for the molecular determinants that mediate attachment of Pseudomonas cells, type IV pili were found to play an important role in biofilm development on abiotic surfaces since they participate in bacterial interactions with other eukaryotic organisms, and are also involved in plant root colonization (Nguyen et al, 2012). In addition, flagella not only play a role in motility and chemotaxis but also in biofilm formation, adhesion and root colonization (Barahona et al, 2010; Fong & Yildiz, 2015; Simpson et al, 1995). There is evidence suggesting that the role of flagella in plant–bacterial interactions is firstly, to mediate motility, but, secondly, flagella may also serve as adhesins permitting cell attachment (Haiko & Westerlund-Wikstrom, 2013). The two surface adhesins that were central in root colonization (Martinez-Gil et al, 2010) are LapA and LapF, which are large proteins (over 8000 and 6000 amino acids, respectively), composed of extended and repetitive domains (Boyd et al, 2014). LapF is involved in microcolony formation and subsequent development of a mature biofilm, while, the larger LapA is necessary for adhesion to seeds and biofilm formation on abiotic surfaces (Barahona et al, 2010). Subcellular localization studies suggest that LapF determines the establishment of cell–cell interactions during sessile growth. Finally, other surface structures such as lipopolysaccharides, the extracellular matrix of bacterial biofilms has at least two key functions in sessile life style: to serve as a structural scaffold for the multicellular community, and to play a protective role against external stress (Martinez-Gil et al, 2013). Other proteins with a role in *P. putida* adhesion include the global regulator RpoN and GacS and the ligand-binding protein PstS (Duque et al, 2013; Espinosa-Urgel et al, 2000).

Biofilms are dynamic structures, and cells may leave the biofilm for several reasons including the search for a better environment when conditions are unfavorable and to spread when conditions are favourable (Fig. 3). The periplasmic cysteine protease LapG is involved in detachment from sessile bacteria, by cleaving LapA (Yousef-Coronado et al, 2011). Also, the biofilm dispersal locus A (BdlA) an MCP, is a key regulator for biofilm dispersal in *P. aeruginosa* (Morgan et al, 2006). The cytoplasmic BdlA controls biofilm dispersal in response to several, yet unidentified nutrient compounds. Additionally, BdlA plays a role in pathogenicity, since

cells lacking *bdlA* are more virulent in chronic infections and less virulent in acute infections (Wood, 2014).

2.2. Chemosensory signaling pathways control biofilm formation and colonization.

Several constituents of the chemosensory and chemotaxis machinery have been demonstrated to be involved in pathogenic and symbiotics processes (Chet & Mitchell, 1976; Kato et al, 2008). The chemotactic movement of bacteria in the rhizosphere has been shown to play key roles in root colonization (Gupta Sood, 2003). A large number of genes are involved in the formation of functional flagella in gram-negative bacteria (Iino, 1977). Motility is a major trait for the competitive colonization and flagella of a plant-growth-stimulating *P. fluorescens* strain were found to be required for efficient colonization of potato roots (De Weger et al, 1987). Moreover, *P. fluorescens cheA* mutants that were defective in flagella-driven chemotaxis but retained motility, showed reduced competitive root colonization, due to the loss of their capacity to move chemotactically towards exudate components (de Weert et al, 2002). The same result was observed in *Ralstonia solanacearum,* where nonchemotactic mutants (*cheA* and *cheW* mutants) had a significantly reduced virulence indistinguishable from that of a nonmotile mutant (flagellar mutants), demonstrating that directed motility, not simply random motion, is required for full virulence (Yao & Allen, 2006). Bacterial motility is also of clinical importance. Motility in *P. aeruginosa* plays a role as a virulence determinant in invasive infections, because nonmotile mutants, although similar to the wild type in their proliferation in burn wounds, did not show the characteristic bacteremia and systemic invasion as observed for the wt (Drake & Montie, 1988).

Finally, motility and chemotaxis play a role in biofilm formation. The initiation of biofilm formation through reversible attachment often requires flagella, and motility on a surface can be crucial for biofilm architecture (Verstraeten et al, 2008). Also, mutations in the sensor HK CheA and the methyltransferases CheR1 strongly affected biofilm formation (Schmidt et al, 2011; Tremaroli et al, 2011). Based on previous findings, Schmidt et al. proposed a model in which motility and chemotaxis impact on initial attachment processes, dispersion and reattachment and increase the efficiency and frequency of surface sampling in *P. aeruginosa* (Schmidt et al, 2011).

Although the connection between chemotaxis, motility (swimming, swarming and twitching) and biofilm formation has been established (Pratt & Kolter, 1998; Yaryura et al, 2008), these studies were based on mutants of the signaling pathway (Garcia-Fontana et al, 2013; Klausen

et al, 2003; Merritt et al, 2007). Therefore only little information is available as to the nature of the chemotactic signals that modulate biofilm formation. To address this issue in this thesis the complete set of the single mutants of all 27 *P. putida* KT2440 MCP was generated and their impact on biofilm formation assessed. This study corresponds to the first comprehensive assessment of the contribution of MCPs to biofilm formation in a bacterial species.

3. CELL TO CELL COMMUNICATION IN BACTERIA

3.1. Quorum sensing

Bacteria are highly social organisms capable to communicate with each other using chemical signal molecules (Williams & Camara, 2009). The chemical communication process involves producing, releasing, detecting and responding to small hormone-like molecules termed autoinducers. Bacteria produce, during their growth, various autoinducers which activate QS mechanisms when a threshold concentration is reached (Waters & Bassler, 2005) and promote the communication of bacteria with each other in order to coordinate the expression of specific genes in a cell density-dependent fashion (Whiteley et al, 1999). This process, allows some bacteria to monitor the environment for other bacteria and to alter behavior of the entire population. In addition, using these signal-response systems, bacteria synchronize particular behaviors on a population-wide scale and thus function as multicellular organisms (Waters & Bassler, 2005). Although various QS signal (QSS) molecules have been described, AHLs are mainly used by Gram-negative bacteria while secreted cyclic oligopeptides are preferred by Gram-positive bacteria (Krämer & Jung, 2010). AHL mediated signaling is primarily based on two proteins, namely the AHL synthase, usually belong LuxI family of proteins, and an AHL receptor protein belonging to the LuxR family of a transcriptional regulators (Fig. 4) (Ng & Bassler, 2009).

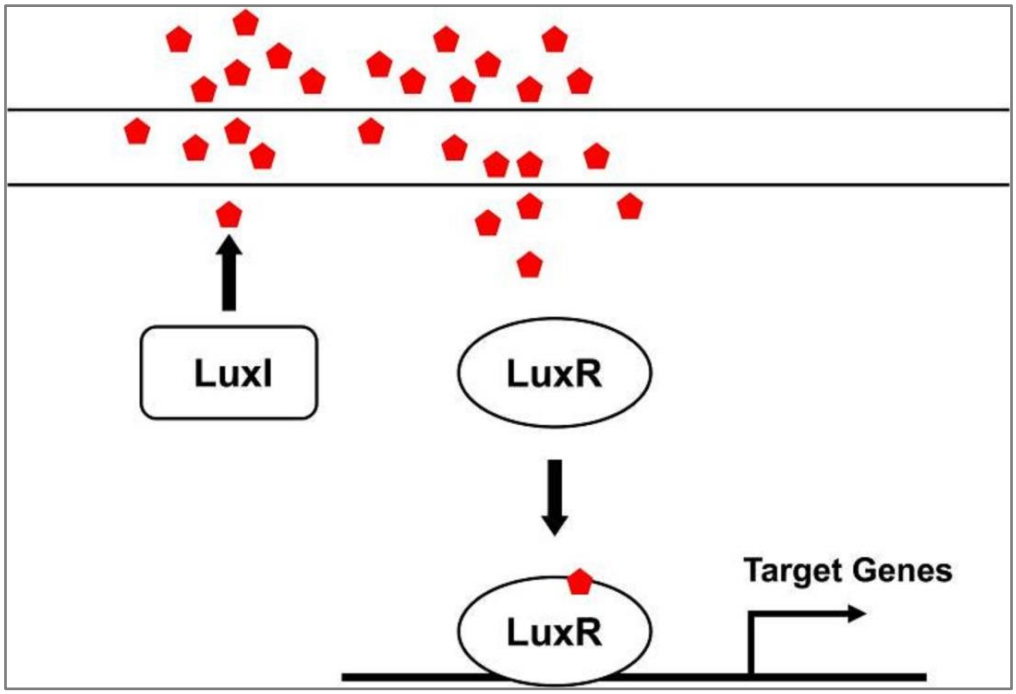


Figure 4 A canonical Gram-negative LuxI/LuxR-type QS system. AHLs are represented as red pentagons. Schema taken from (Ng & Bassler, 2009).

AHL-dependent QS is conserved in a broad range of micro-organisms living in various environments and regulates very diverse physiological functions (Uroz et al, 2005).

LuxI-type AHL synthetases link and lactonize the methionine moiety from SAM to particular fatty acyl chains carried on acyl-acyl carrier proteins (Schaefer et al, 1996). A diverse set of fatty acyl side chains of varying length, backbone saturation, and side-chain substitutions are incorporated into AHL molecules (Fig. 5); these differences are crucial for the signaling specificity (Churchill & Chen, 2011). The acyl groups of the naturally occurring AHLs identified to date range from 4 to 18 carbons in length (Uroz et al, 2005). Structural studies of LuxI-type proteins indicate that each enzyme possesses an acyl-binding pocket that precisely fits a particular side-chain moiety. Thus, most LuxI proteins produce predominantly a given AHL (Churchill & Chen, 2011). Although, there are some LuxI-type proteins that produce multiple AHLs, until now it is not clear if all of them are biologically relevant (Waters & Bassler, 2005). A study of *P. aeruginosa* using a series of structural analogs of N-3-oxododecanoyl-homoserine lactone (3-Oxo-C12-AHL) suggests that the length of the acyl side chain of the AHL molecule is the most critical factor for LasR mediated activity , while a replacement of the oxygen by a sulfur atom in the AHL moiety can be tolerated (Passador et al, 1996).

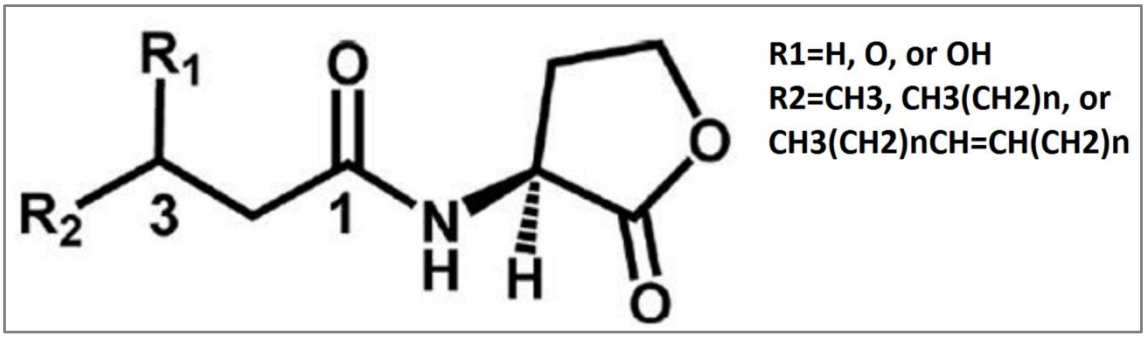


Figure 5 Chemical structure of AHL QSS. The AHLs vary by substitution at the C_3 position (R_1) and the length and saturation state at the C_1 position indicated by R_2. Figure taken from (Churchill & Chen, 2011).

The first described QS system was that of the bioluminescent marine bacterium *Vibrio fischeri*, which colonizes the light organ of the Hawaiian squid *Euprymna scolopes*n involving two proteins, LuxI and LuxR, which control the expression of the luciferase operon (*luxICDABE*) required for light production (Foran & Brown, 1988). LuxI exhibits synthase activity and produces the autoinducer 3-Oxo-C6-AHL (Urbanowski et al, 2004).

To date, AHL-dependent QS circuits have been identified in more than 80 species of Gram-negative bacteria (Krämer & Jung, 2010), in which they regulate several pathogenicity-related functions in a population-density-dependent fashion in bacterial species pathogenic for plants and animals (Uroz et al, 2005). A representative example maybe the synthesis of antibiotics and extracellular hydrolytic enzymes, motility and production of virulence factors in *P. aeruginosa* (Castillo-Juarez et al, 2015; Rutherford & Bassler, 2012). QS also controls functions responsible for the interaction of the microbe with both its physical and biological environments, including bioluminescence, swimming, swarming, biofilm maturation, plasmid conjugative transfer and symbiosis (Callahan & Dunlap, 2000; Li & Tian, 2012; Uroz et al, 2005). More recently, evidence has emerged that AHL QSS are also recognized by eukaryotes, where they induce specific responses often affecting the immune status of the organism (Krämer & Jung, 2010).

3.1.1. QS in Pseudomonas aeruginosa

QS in *P. aeruginosa*, has been studied in depth (Whiteley et al, 1999). *P. aeruginosa* is a highly environmental adaptable pathogen with a large dynamic genome of which approximately 10 % is devoted to regulatory elements including a sophisticated multi-signal QS system (Venturi, 2006) that plays a key role in controlling the production of virulence factors, biofilm maturation, swarming, swimming motility and the expression of antibiotic efflux pumps

(Schuster & Greenberg, 2006). In addition, the QSS produced by *P. aeruginosa* contribute directly to host–pathogen interactions (Williams & Camara, 2009). Current anti- Pseudomonas therapies lack efficacy, partly because *P. aeruginosa* creates and inhabits surface-associated biofilms conferring increased resistance to antibiotics and host immune responses (Bottomley et al, 2007). Hence, QS is essential for chronic *P. aeruginosa* respiratory infection because it controls virulence factor expression, all of which allow persistence in the lung and are required for disease progression (Waters & Bassler, 2005). This presents opportunities for the design of quorum sensing inhibitors (QSI), which reduce virulence, pathogenicity, and resistance rather than directly inhibiting growth, with the important ramifications that they would be unlikely to exert selective pressures leading to the emergence of drug-resistant bacteria and would not kill the beneficial gut flora (Bottomley et al, 2007).

The *P. aeruginosa* QS network consists of two LuxI/LuxR circuits, termed LasI/LasR and RhlI/RhlR (Waters & Bassler, 2005)(Fig. 6). In the Las QS system, the *lasI* gene product directs the formation of the diffusible extracellular QSS, 3-Oxo-C12-AHL, which interacts with LasR to activate the expression of a various genes. The Las system controls the production of multiple virulence factors involved in acute infection and host cell damage, including the LasA and LasB elastases (Toder et al, 1994), exotoxin A (*toxA*), and alkaline protease (*apr*) (Gambello & Iglewski, 1991), synthesis of the siderophore pyoverdine (Jayaseelan et al, 2014), some components of type II secretion system, as well as *lasI* itself (Jimenez et al, 2012; Whiteley & Greenberg, 2001; Whiteley et al, 1999). AHLs can diffuse out and into of bacterial cells, although 3-Oxo-C12-AHL is concentrated inside the cell, possibly due to the partition in bacterial membranes (Yates et al, 2002). In addition, the outflow is dependent on efflux pumps (Waters & Bassler, 2005). The Las system is regulated by the quorum threshold expression element (QteE) that prevents LasR accumulation by reducing its stability (Seet & Zhang, 2011). In addition, the QS LasR-specific antiactivator (QslA) prevents early activation of QS-regulated virulence by forming complexes with LasR that prevent its binding to the target DNA (Fan et al, 2013). These newly discovered regulators provide a logic explanation on how a threshold for activation can be created in bacteria and how early activation of QS-dependent virulence is avoided during the initial growth phase (Jimenez et al, 2012).

The LasR-autoinducer complex also activates the expression of *rhlI* and *rhlR* (Pesci et al, 1997) which encode the second QS circuit identified in 1994 (Ochsner et al, 1994). RhlI produces the C4-AHL. This diffusible QSS in conjunction with RhlR activates expression of the *rhlAB* rhamnolipid synthesis genes, *rhlI*, and to some extent *lasB* (Ochsner et al, 1994; Waters & Bassler, 2005). Other virulence factors and secondary metabolites, including pyocyanin

(Jayaseelan et al, 2014), cyanide (Pessi & Haas, 2000), and chitinase (Winson et al, 1995), are positively regulated by the *rhl* system (Whiteley & Greenberg, 2001; Whiteley et al, 1999). Moreover, RhlR is able to induce LasR-regulated genes (including specific ones such as *lasI*) in the absence of *lasR*. This unveils a new mechanism to bypass a defect in their QS regulation, allowing RhlR to induce the *las* system in situations where LasR is non-functional (Dekimpe & Deziel, 2009). The transcription of the *rhlR* is controlled by four promoters, two of them are regulated by the QS system (LasR/3-Oxo-C12-AHL and RhlR/C4-AHL respectively), another one by RpoN and the latter one by Vfr (Croda-Garcia et al, 2011). RhlR as well as LasR are expressed in a growth-dependent manner, with genes being activated during the second half of log-phase growth (Pesci et al, 1997).

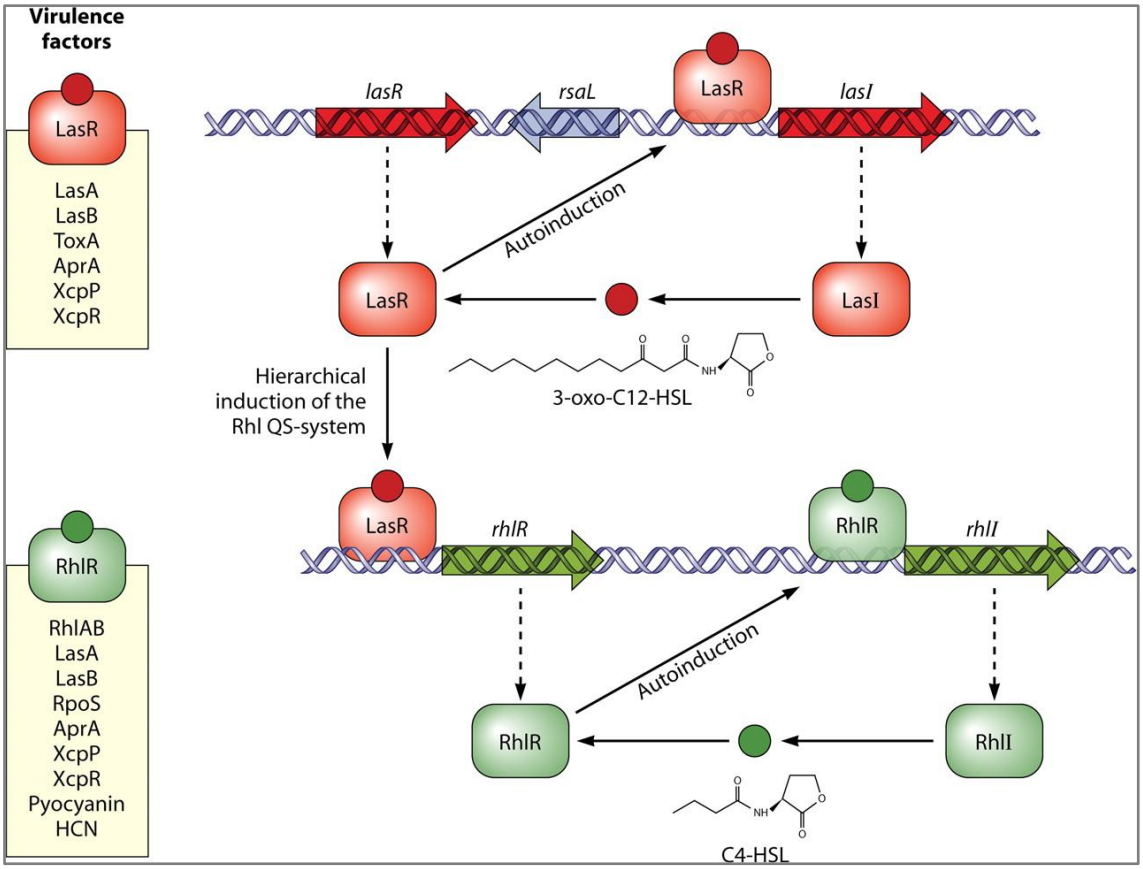


Figure 6 Regulation of *P. aeruginosa* virulence factors by the Las (red) and Rhl (green) QS systems. Figure adapted from (Jimenez et al, 2012) .

The *P. aeruginosa* PAO1 sequence revealed the existence of multiple LuxR-type proteins, beyond LasR and RhlR (Fuqua, 2006). Amongst them, QscR (QS control repressor) exhibits high degree of sequence conservation with AHL-responsive LuxR homologues. The *qscR* gene is not adjacent to an AHL synthase gene but is immediately upstream of the genes of the phenazine

pigment biosynthetic enzymes (Chugani et al, 2001). The QscR genomic organization suggested that this protein is involved in the activity modulation of the Las and Rhl system, although its mechanism (s) remains unclear *(Chugani et al, 2001).* Otherwise, co-expression of QscR with either LasR or RhlR in the absence of AHL, followed by *in vivo* chemical cross-linking, identified apparent QscR-LasR and QscR-RhlR heterodimers, suggesting that QscR might be a Las-Rhl antagonist (Fuqua, 2006). Lequette et al. demonstrate that QscR responds strongly to 3-Oxo-C12-AHL synthesized by *lasI* (Lequette et al, 2006). Nevertheless, another study demonstrates that QscR may be even more sensitive to 3-Oxo-C10-AHL than to 3-Oxo-C12-AHL (Lee et al, 2006) . It seems plausible that the function of QscR may be associated with a response to cohabiting microbes that produce C10-AHL derivatives, which in turn permits the integration of QSS from others species (Fuqua, 2006).

Although the first AHL identified in culture supernatants of *P. aeruginosa* PAO1 was 3-Oxo-C6-AHL (Winson et al, 1995), the primary AHLs were reported to be C4-AHL and 3-Oxo-C12-AHL, and other AHLs present at low concentrations, namely, 3-Oxo-C14-AHL and 3-Oxo-C10-AHL, (Jimenez et al, 2012). Moreover, the RhlI synthase produces C4- and C6-AHL in a molar ratio of 15:1 as detected in the culture supernatants of *P. aeruginosa.* Functionally, C6-AHL as well as C4-AHL could activate the synthesis of elastase and chitinase production (Winson et al, 1995). Furthermore, LasR is soluble and stable in the presence of its cognate AHL, 3-Oxo-C8-AHL, 3-Oxo-C6-AHL, and C4-AHL (Bottomley et al, 2007; Fuqua et al, 1995).

Chugani and Greenberg have revealed an even higher level of complexity in *P. aeruginosa* AHL signaling, reporting a set of 37 genes whose expression was controlled by AHLs in the absence of LasR, RhlR, and QscR (Chugani & Greenberg, 2010). Also, genes indirectly involved in virulence, including the stationary phase sigma factor *rpoS* , and genes coding for components of the general secretory pathway (*xcp*), have been reported to be controlled by QS in *P. aeruginosa* (Whiteley et al, 1999).

3.2. LuxR-type proteins

A large number of gram-negative proteobacteria possess LuxIR-type proteins and recognize AHL QSS (Waters & Bassler, 2005). LuxR type transcription factors genes, are often adjacent to those of the corresponding LuxI-type AHL synthases (Fuqua, 2006). LuxR-type receptors can be reorganized into two domains, a likely C-terminal domain with an HTH (helix turn helix) motif containing DNA binding domain (DBD) and an autoinducer binding domain at the N-terminal end (Fig. 7) (Vannini et al, 2002).

The LuxR-transcriptional regulatory protein bind to palindromic operators, referred to as lux box, located in the promoters region of target genes (Pessi & Haas, 2000; Vannini et al, 2002). The initially described *lux* box is a 20-bp inverted repeat centered at −41.5 bp from the start of the *V. fischeri lux* operon (Whiteley & Greenberg, 2001). LuxR homologues binds to the *lux* box DNA and stimulate the binding of RNA polymerase to an adjacent promotor for promoting gene transcription (Tsai & Winans, 2010).

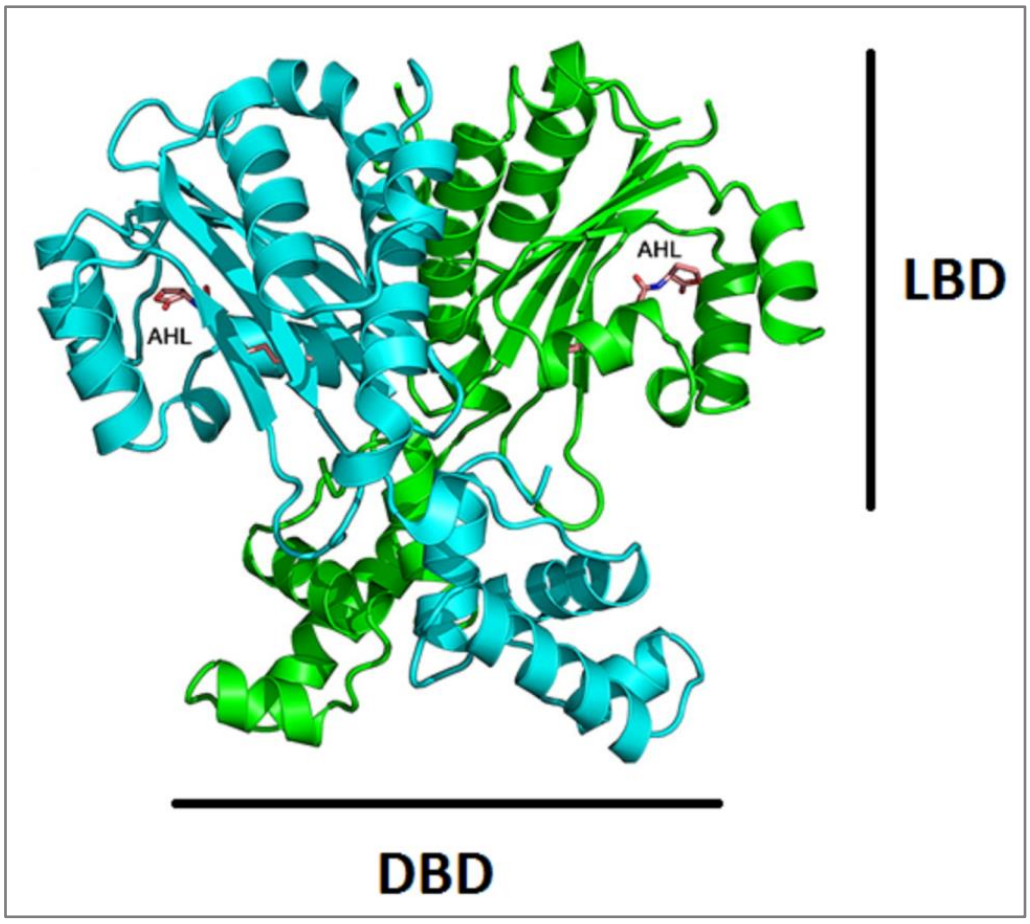


Figure 7 Structure of QscR bound to 3-Oxo-C12-AHL. Ribbon representation shows the LBD and DBD. Chain A (cyan), chain B (green), 3-Oxo-C12-AHL is shown in stick mode. Figure taken from (Lintz et al, 2011).

Surprisingly, proteins that are members of QS LuxR-family show a low degree of sequence similarity (Zhang et al, 2002b). However, 95% of them share nine highly conserved amino acid residues (Whitehead et al, 2001). Six of these residues form the cavity of the AHL-binding domain whereas the remaining three residues are in the DNA-binding domain (Venturi & Fuqua, 2013).

An analysis of 265 proteobacterial genomes showed that 68 had a canonical, paired LuxI/LuxR system, and of these, 45 contained more LuxRs than LuxIs. Another set of 45 genomes

contained only LuxR-type of proteins. These QS LuxR-type proteins that lack a genetically linked LuxI have been termed orphans and, more recently, solos (Venturi & Fuqua, 2013). Some of these orphans are substantially different in size from characterized LuxR homologues, with large truncations or additional sequences (Fuqua & Greenberg, 2002). These atypical LuxR homologues might recognize AHLs by an alternate mechanism, provide ligand-independent activity, act as dominant-negative QSI, or even detect alternative small molecules (Fuqua, 2006). Nevertheless, there are other LuxR-type proteins that are independent of known AHL, such of PpoR from *P. putida* KT2440, which regulates swarming motility and plays a role in the survival of this strain in the presence of potential competitors (Fernandez-Pinar et al, 2011).

The current paradigm for the activation of gene expression in QS suggests that the protein-AHL complex interacts with a specific DNA element and, through contacts with the RNA polymerase stimulates target gene expression (Egland & Greenberg, 2000; Stevens et al, 1994). Most LuxR-family proteins require their cognate AHLs for activity, and at least some of them require AHLs for folding and protease resistance (Tsai & Winans, 2010). Since *E. coli* does not synthesize AHLs, protein purified from *E. coli* was insoluble and inactive (Schuster et al, 2004). Considerable amounts of soluble active LuxR-type protein complex with AHL can be obtained from *E.coli* cells grown in the presence of an appropriate AHL (Schuster et al, 2004; Urbanowski et al, 2004; Zhu & Winans, 2001).

3.2.1. Classes of LuxR-type proteins

QS regulators have also been studied in a number of different organisms. Based on their biochemical properties this family can be divided into two groups:

1. The first group is comprised of AHL-dependent transcription factors that are apparently insoluble in the absence of AHL, of which representative examples are LasR, LuxR, SidA, and TraR are four representative (Yao et al, 2006). TraR has a half-life of a few minutes in the absence of autoinducer, however, in the presence of AHL, the half-life of TraR increases to over 30 minutes (Waters & Bassler, 2005; Zhu & Winans, 2001). It was proposed that AHL binding is required for folding of the nascent polypeptide, and indeed radiolabeled TraR was stabilized only when its cognate AHL was added prior to labeling of the protein (Zhu & Winans, 2001). In analogy to TraR, LuxR was purified in a soluble, active form after high-level production of the protein in the presence of AHL (Urbanowski et al, 2004). Purified LuxR binds specifically and with high affinity to DNA containing a lux box. This binding requires addition of 3-Oxo-C6-AHL. An important property of LuxR that distinguishes it from a TraR and LasR is that binding of 3-Oxo-C6-AHL is reversible

(Urbanowski et al, 2004; Whiteley et al, 1999). Meanwhile, *E.coli* K-12 LuxR homologue SdiA, which is associated with multiple signals, acts as negative regulator of the expression of virulence factors EspD and the *fts*QAZ operon (involved in cell division)(Kanamaru et al, 2000). In the presence of C8-AHL, a significant proportion of the SdiA protein is produced in a folded and soluble form in an *E. coli* expression system, whereas in the absence of AHL, the protein is expressed as insoluble inclusion bodies (Yao et al, 2006). In the same way, LasR was nonfunctional when expressed in the absence of its cognate QSS (Schuster et al, 2004); therefore, LasR was purified in a soluble form by producing it in the presence of 3-Oxo-C12-AHL. The fact that LasR may be inactive and insoluble in the absence of AHL was contradicted in 2011 by Sappington et al. who showed that purified protein LasR is active in the AHL-free *E. coli*. In addition, they showed the rapid dilution of the culture leading to a rapid drop in the environmental AHL concentration caused an immediate drop in transcriptional activity, which is an observation difficult to reconcile with ultralight AHL binding to LasR as proposed earlier. The authors show also that after AHL dissociation LasR could remain in a properly folded conformation capable of reassociating with QSS (Sappington et al, 2011).

2. The second group is able to fold, dimerize and bind AHL and DNA in the absence of AHLs (Tsai & Winans, 2010). It includes few members of the LuxR family. EsaR in *Pantoea stewartii* can fold and bind DNA in the absence of any AHL, in fact, EsaR is inhibited by its cognate AHL (von Bodman et al, 1998). The *Mesorhizobium tianshanense* transcriptional activator MrtR also requires its cognate QSS for dimerization but not for folding. Therefore, MrtR is stable without AHL but biologically inactive (Yang et al, 2009).

3.2.2. LuxR-type proteins oligomeric state

Several studies have indicated that proteins belonging to the LuxR family interact with DNA and the existence of dyad symmetry in the proposed DNA element suggests that members of the LuxR family of proteins might form dimers (Kiratisin et al, 2002). This hypothesis was confirmed in 2002 by Vannini et al. who reported the crystal structure of TraR in complex with its autoinducer and target DNA (Vannini et al, 2002). This structural study reveals an asymmetric homodimer, where one monomer is more elongated than the other and a linker connecting the LBD and DBD. Both domains participate in protein dimerization (Vannini et al, 2002). The dimer structures of LasR and QscR were solved in complex with autoinducers and reveal the structural basis for the molecular recognition of 3-Oxo-C12-AHL (Bottomley et al, 2007; Lintz et al, 2011).

Ligand-induced oligomerization is common among regulatory proteins (Qin et al, 2000; Ventre et al, 2003). Although relatively few biochemical studies have been reported for LuxR-type proteins, there is now considerable evidence that they form dimers and multimers (Ventre et al, 2003). In accordance with crystal structure and gel filtration analyses of purified LasR, this protein forms a dimer in solution and irreversibly bound two molecules of 3-Oxo-C12-AHL (Schuster et al, 2004). Furthermore, protein interaction assays showed that LasR is a monomer and formed multimers (dimers) except in the presence of 3-Oxo-C12-AHL (Kiratisin et al, 2002).

In contrast with other homologues, Oinuma et al. demonstrate that QscR exists as a monomer in solution. This protein could be produced in *E. coli* when supplemented with 3-Oxo-C12-AHL. The resulting purified protein was partially saturated with AHL and the addition of further AHL was necessary for efficient DNA binding (Oinuma & Greenberg, 2011). Using fluorescence anisotropy measurements, it has been shown that QscR could form various complexes depending on the conditions tested: multimers in the absence of any AHL, lower order oligomers complexed either to C4-AHL or to 3-Oxo-C12-AHL and heterodimers with LasR or with RhlR (Ledgham et al, 2003). Related to QscR, the QS receptor SdiA has not been shown to dimerize and recognize multiple noncognate AHLs in the monomeric state (Yao et al, 2006). Fluorescence anisotropy assay have shown that the transcriptional regulator, RhlR forms a homodimer in the absence of AHL (Ventre et al, 2003), and as well in the case of CarR, the QS regulator in *Erwinia carotovora* strain (Welch et al, 2000). In contrast, TraR forms homodimers only in presence of AHL (Vannini et al, 2002; Ventre et al, 2003). Despite the differences between TraR and CarR, in both cases, DNA binding is mediated by protein dimers. To our knowledge, the dimeric state of LuxR-type proteins studied so far studied is a prerequisite for DNA binding.

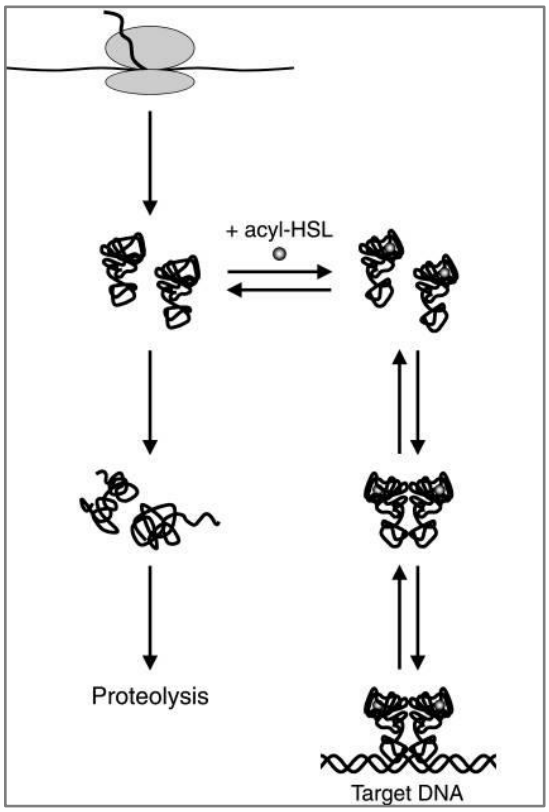
3.2.3. General model for LuxR-type proteins

Based on *in vivo* biochemical evidence, Oinume et al. postulate a new general model for LuxR-type homologs (Oinuma & Greenberg, 2011):

- LuxR-type monomers can be synthesized by Gram negative bacteria even in the absence of an acyl-AHL. As mentioned above LasR is active in the AHL-free *E. coli* (Sappington et al, 2011).
- Nascent LuxR-type protein folds into a functional state without an AHL, but it is unstable and rapidly adopts nonfunctional conformations, which are prone to aggregation and more sensitive to proteolysis. This statement is proposed because

MrtR (Yang et al, 2009), EsaR (Schu et al, 2009) and SdiA (Yao et al, 2006) are active and stable in the absence of bound AHL.

- In the presence of an appropriate AHL, the LuxR-type protein is more stable and functional. This is supported by a study showing the QSS to TraR increases protein stability (Zhu & Winans, 2001).



- LuxR-type proteins exist as a monomer at low concentrations, but upon an increase in concentration, dimers capable of high-affinity binding to target promoters are formed. (Oinuma & Greenberg, 2011).
- At the same time dimer formation may also increase the stability of LuxR-type protein.
- Finally LuxR-type protein seems to have very little selectivity for the different long-chain AHL. Expression studies indicate a broad effector range in the case of QscR (Oinuma & Greenberg, 2011), CarR (Welch et al, 2000) and LasR (Winson et al, 1998).

Figure 8 General model for LuxR-type proteins states *in vivo*. Figure taken from (Oinuma & Greenberg, 2011).

Another aim of this thesis was to verify experimentally the functional model proposed for QS regulators using an array of biophysical approaches. To this end we have chosen LasR and RhlR as model proteins.

3.3. Quorum quenching

In niches in which bacterial populations compete for limited resources, the ability to disrupt QS may give one bacterial species an advantage over another (Waters & Bassler, 2005). Thus, it is not surprising that mechanisms have evolved to interfere with bacterial cell-cell communication in processes termed QQ (Grandclement et al, 2015). Both plants and humans have inducible defense mechanisms. QQ can be engineered in plants and might be used as a strategy to control bacterial pathogens and to build up a proactive defense barrier (Zhang, 2003). Analogous mechanisms presumably exist for promoting QS-controlled behaviors when

such behaviors provide benefits to organism's cohabitating with QS bacteria. These latter processes are not yet well defined (Waters & Bassler, 2005).

The ability of a host to interfere with bacterial cell-cell communication may be crucial in preventing colonization by pathogenic bacteria that use QS to coordinate virulence (Waters & Bassler, 2005). In recent years, a promising new strategy has been developed that targets QS as a tool to fight bacterial infections: instead of attempting to inhibit bacterial growth –with the concomitant induction of bacterial resistance against such agents – attempts have been made to design or screen for specific QSI (Rabin et al, 2013). Additional approaches towards the development of QSI have included the characterization of natural compounds and the design, synthesis, and biological evaluation of new analogs of AHL QSS (Rabin et al, 2013; Zou & Nair, 2009). Some QSIs are related to the AHL structure (Rabin et al, 2013), whereas others lack any appreciable chemical similarities to the AHL QSS (Annapoorani et al, 2012; Zou & Nair, 2009).

3.3.1. Degrading bacterial QSS

Another strategy to interfere with QS is represented by mechanisms that lead to the degradation of QSS. Three types of AHL-degrading enzymes have been reported to date: lactonases, acylases, and oxidoreductases. The enzymes were initially identified in *Bacillus sp*., *Ralstonia sp*. and *Rhodococcus erythropolis* W2 (Jimenez et al, 2012; Lin et al, 2003; Liu et al, 2005; Uroz et al, 2005). Many *Bacillus* and *Vibrio* species secrete a lactonase that cleaves the lactone ring from the acyl moiety of AHLs and the reaction product is used as carbon and nitrogen source (Leadbetter & Greenberg, 2000; Liu et al, 2005). In some cases, bacteria degrade their own autoinducers (Zhang et al, 2002a). *P. aeruginosa* use QQ to degrade long chain AHLs through an AiiD-type acylase named PvdQ, whereas it has no activity toward short chain AHLs. This translates to the fact that of the two primary AHLs of this bacterium 3-Oxo-C12-AHL is susceptible to be degraded whereas the C4-AHL levels remain unchanged (Sio et al, 2006). Interestingly, *pvdQ* is a member of the LasI/LasR regulon and it is thus under control of 3-Oxo-C12-AHL (Wahjudi et al, 2011). In addition, C4-AHL is also resistant to degradation by mammalian paraxonasas (PON) and chemically stable, while 3-Oxo-C12-AHL is base labile and degraded by PONs, which may be considered as QS mediated eukaryote to prokaryote QQ communication (Muh et al, 2006). In agriculture the use of bacteria, that are naturally capable of degrading AHL to control plant pathogens, is considered as a suitable approach to protect plants (Molina et al, 2003).

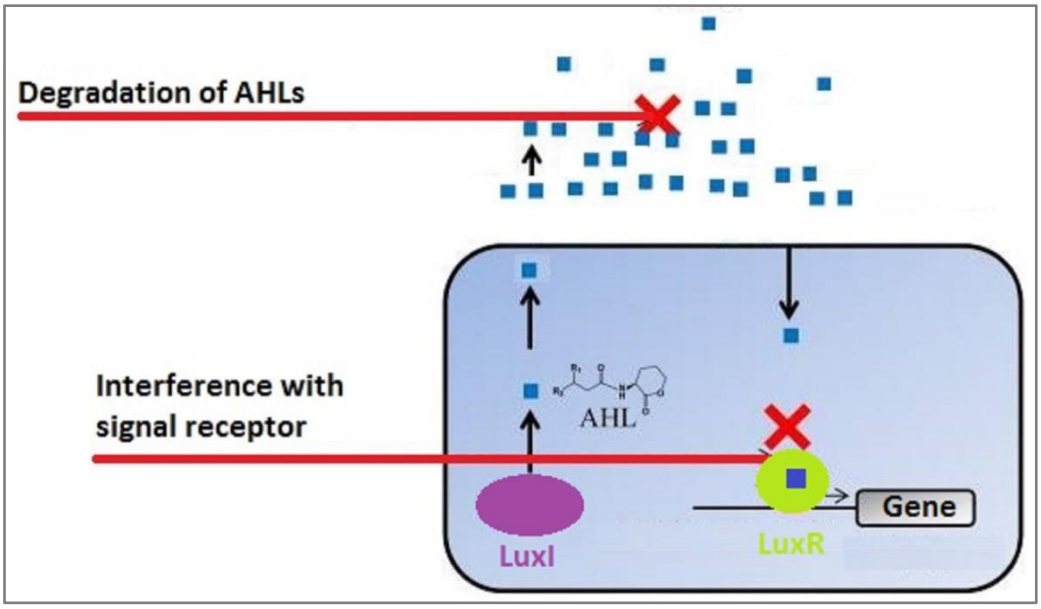


Figure 9 Inhibition of QS in Gram-negative bacteria by various mechanisms. Figure taken form (Lade et al, 2014).

3.3.2. Interference with QSS receptor

Diketopiperazines (DKPs) are used as inter-species QQ molecules. DKPs interference is carried out by binding to the LuxR family of receptors (Fig. 9), either activating or antagonizing AHL QSS, which can be exploited for biotechnological purposes (Gonzalez & Keshavan, 2006). DKPs, also referred to as cyclic dipeptides, are produced by many different bacteria. For example, cyclo (L-Ala-L-Val) and cyclo (L-Pro-L-Tyr) are DKPs that were identified in the supernatants of *P. aeruginosa*, *Proteus mirabilis*, and *Citrobacter freundii* (Holden et al, 1999). The best studied example may be the triphenyl (TP-1) agonist that was found to interact with LasR at the AHL-binding site causing transcriptional activation (Zou & Nair, 2009)(Fig. 9).

A compound similar in structure to TP-1 was also found to bind LasR, but causing antagonistic effects (Muh et al, 2006). In the same way, based on the crystal structure of LasR bound to 3-Oxo-C12-AHL, Rabin, et al. designed *in silico* and synthesized two piperidine analogs by computational methods and both compounds revealed interesting agonist profiles (Rabin et al, 2013). In conclusion, QS agonists could be used to potentially induce the premature expression of immunogenic molecules and therefore 'expose' the bacteria to the host-immune system at an early stage of infection (Rabin et al, 2013). Besides of inhibitory molecules produced by bacteria, there exist QSI of eukaryotic origin(Hughes & Sperandio, 2008).

3.4. Inter-kingdom signaling

Considering the enormous biological diversity present in an ecological niche, it would be naïve to assume that bacterial communication is limited to intraspecies or interspecies signaling. It seems logical that bacteria would produce or receive QSS enabling communication with fungi, plants, and animals (Jimenez et al, 2012). Chemical communication between plant or mammal and bacteria is called inter-kingdom signaling (Venturi & Fuqua, 2013). The human body lives in a permanent stage of communication and interaction with its indigenous microbiota that consists predominantly of bacteria. Symbiotic association with their intestinal microbial flora, is crucial for nutrient assimilation and development of the innate immune system(Hughes & Sperandio, 2008). The host immune system and the microbiota communicate by using different types of language. The major aims of the host are: (i) to keep away the microorganisms from the mucosal surfaces, and (ii) to control growth and composition of the beneficial microbiota (Krämer & Jung, 2010). Generations of chemists and biologists have conducted research on natural products and other metabolites produced by bacteria and other microorganisms that participate in this cross-kingdom signaling (Michiels et al, 2001; Williams, 2007). This has led to an explosion of the knowledge concerning the mechanism by which such natural products are made, ultimately allowing custom redesign of many of these molecules for increased potency and selectivity as therapeutic drugs (Lyon & Muir, 2003).

Inter-kingdom signaling can occur at two levels: either bacterial QSS modulate processes in species of a different kingdom or, alternatively, compounds produced by eukaryotes interfere with bacterial QS (Venturi & Fuqua, 2013). One interesting example of an inter-kingdom signal interaction was discovered between *P. aeruginosa* and the opportunistic fungal pathogen *Candida albicans* (Jimenez et al, 2012). Production of 3-Oxo-C12-AHL by *P. aeruginos*a inhibits *C. albicans* filamentation, a crucial adaptation for the development of opportunistic infections, while production of the fungal metabolite farnesol reduces Pseudomonas quinolone signal (PQS) and pyocyanin levels as well as the swarming motility in *P. aeruginosa* (Cugini et al, 2007).

In addition, co-evolution of prokaryotic species and their respective eukaryotic hosts has exposed bacteria to host hormones and eukaryotic cells to the bacterial AHLs during host colonization. Therefore, it is not surprising that some pathogenic species have highjacked these signaling systems to promote disease (Fig. 10). Pathogenic bacteria are able to recognize host signaling molecules like epinephrine and norepinephrine to activate production of virulence factors (Pacheco & Sperandio, 2009). Particularly, norepinephrine plays a role in the pathogenesis of Salmonella, enhancing its motility and consequently its colonization efficiency

(Bearson & Bearson, 2008). On the other hand, *P. aeruginosa* forms a biofilm in the lungs of CF patients, and 3-Oxo-C12-AHL was detected in patient's sputum samples. It has been shown that 3-Oxo-C12-AHL triggers IL-8, IFN- and Cyclooxygenase 2 production (Smith et al, 2001)

Plants release large amounts of compounds that combat pathogenic microorganisms and attract beneficial ones (Venturi & Fuqua, 2013). The rhizosphere is a nutrient rich environment due to the presence of plant root exudates (Dakora et al, 1993). Many compounds present in root exudates can be used for growth, which in turn attracts many bacterial species including symbionts and pathogens. Root exudates contains flavonoids (Luteolin and Genistein), which are signlas that attract chemotactically to rhizobacteria (Lang et al, 2008; Peters & Long, 1988). In turn, the presence of these symbionts in the rhizosphere was propose to increase the synthesis and releases of flavonoids from the plant (Krämer & Jung, 2010).

Plants have also evolved the ability to influence bacterial AHL-QS systems by producing low-molecular weight compounds that interfere by acting as agonists or antagonists of AHLs in bacteria. The activity of OryR, a LuxR solo receptors from *Xanthomonas oryzae,* was shown to be influenced by compounds present in macerates of rice. OryR was also found to regulate the expression of the proline iminopeptidase gene (*pip*), encoding a virulence factor, in response to rice signals (Venturi & Fuqua, 2013). In another study, OryR was found to up- and down-regulate the expression of 220 and 110 genes, respectively (Gonzalez et al, 2013).

Many phytopathogenic bacteria employ QS in order to spatially and temporally express virulence-associated factors in the plant (Von Bodman et al, 2003b). Plants can respond to AHL QSS, but the molecular mechanism remains unknown. For example, exposure of *M. truncatula* to 3-Oxo-C12-AHL (from plant pathogen-bacteria) and 3-Oxo-C16-AHL (from plant symbiont-bacteria) changed the levels of 154 proteins, of which 23 % had functions possibly related to defense and stress response, 14 % to protein degradation or processing, 5 % to flavonoid biosynthesis, 5 % to a plant hormone responses or synthesis, 10 % to regulatory functions, 6% to cytoskeletal elements and 37% to primary metabolism (Veliz-Vallejos et al, 2014). C6-AHL was found to induce plant hormone response in *Arabidopsis thaliana,* especially genes implicated in auxin and cytokine production (Venturi & Fuqua, 2013).

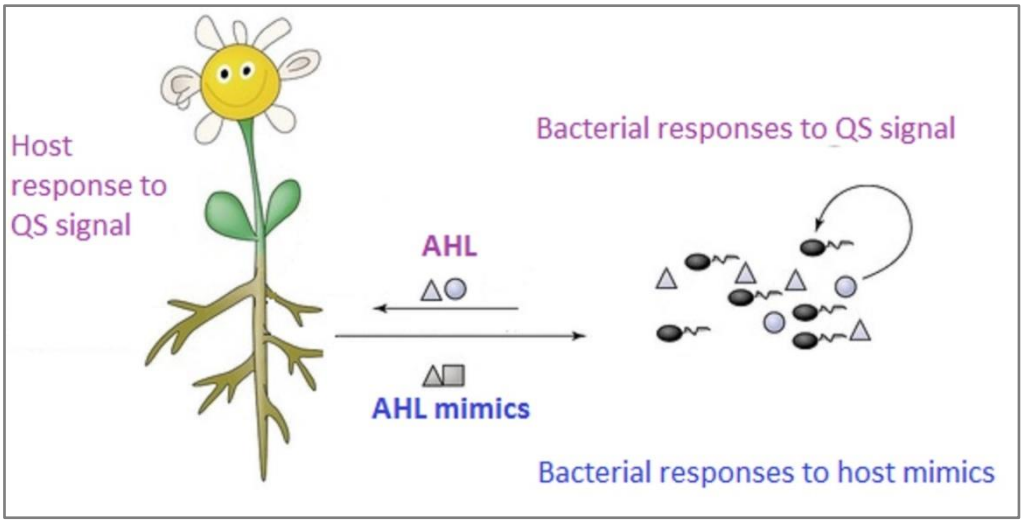


Figure 10 Schematic model of QS-related interactions between plants and bacteria. QSS from bacteria are represented by triangles/circles and plant QSS mimic compounds have shown as triangle/square. Figure modified form (Bauer & Mathesius, 2004).

As mentioned above, several studies have demonstrated that plants are able to synthesize compounds that mimic AHL compounds and interfere with bacterial QS (Fig. 9) This has been observed using plants and plant-extracts that appear to stimulate or inhibit AHL-mediated gene expression; however, none of these plant compounds have yet been chemically identified. One example is the exudate from pea seedlings (*Pisum sativum*) that contain molecules that mimic AHL QSS, either stimulating AHL-regulated behaviors in some strains while inhibiting such behaviors in others (Teplitski et al, 2000). On the other hand, the Australian macroalga *D. pulchra*, is capable of interfering with QS system in *P. aeruginosa* by furanone compounds, which reduce the production of important virulence factors (related with LasR system), and blocks cell signaling and QS in *P. aeruginosa* biofilms (Hentzer et al, 2002). Another study showed that the leguminous plants produce L-canavanine implicated in enhancing the proteolytic degradation of QS LuxR-type proteins (Venturi & Fuqua, 2013).

There is evidence that plant derived compounds interfere with QS in *P. aeruginosa* (Annapoorani et al, 2012; Walker et al, 2004). However, the molecular mechanism by which these compounds act are largely unknown. As stated above LasR and RhlR are the primary QS responsive regulators in *P. aeruginosa*. We have initiated our search for QS active plant compounds using *in silico* screening approaches complemented by experimentation using active and recombinant LasR and RhlR proteins as well as gene expression and phenotypic analysis. This study resulted in the first identification of a plant produced compound that stimulated QS responses.

AIM OF THE THESIS AND SPECIFIC OBJECTIVES

Chemosensory pathways, biofilm formation and bacterial communication via QS have been extensively studied over the last decades, using different microorganisms as model. This thesis concerns the importance of chemosensory pathways in the process of biofilm formation, and novel aspects of cell–to-cell signaling in Pseudomonas species.

Different OCS and TCS are known to regulate the stages of biofilm development. Also, mutation of genes within chemosensory pathways impacts biofilm formation. *P. putida* KT2440 has 27 MCP involved in flagellum-based chemotaxis and type IV pili-based motility. However, the role of 27 MCPs from *P. putida* KT2440 in ACF, such as biofilm formation, has never been studied.

P. aeruginosa has two principal QS system: Las and Rhl, each with QS receptor. Previous works have shown the existence of two kinds of QS receptors, one apparently insoluble in the absence of AHL and another able to fold, dimerize and bind ligands in the absence of AHLs. Several studies have suggested that LasR is non-functional and insoluble in the absence of AHL and that it binds AHLs ultra-tightly. In this thesis we made an unbiased approach to the biophysical and biochemical characterization of the two principal QS receptors (LasR and RhlR) of *P. aeruginosa* in absence and presence of AHL. As part of this work, we have also searched for molecules that can bind and alter the functionality of QS regulators.

The specific objectives of this thesis are:

- Chapter 1: Assessment of the contribution of MCPs mediated signaling processes to biofilm formation in *P. putida* KT2440.
- Chapter 2: Exploration of the possibility that QS responsive regulators LasR and RhlR can be obtained as active and stable proteins *in vitro*.
- Chapter 3: Identification of potential agonists or antagonists that interfere with *P. aeruginosa* QS.

METHODOLOGY

Experimental Procedures

Microbiologic and molecular biology techniques

Materials: C4-AHL, C6-AHL, 3-Oxo-C10-AHL, C12-AHL and 3-Oxo-C12-AHL were purchased from Sigma-Aldrich, whereas 3-OH-C4-AHL, 3-Oxo-C4-AHL, 3-OH-C6-AHL and 3-OH-C10-AHL were purchased form Nottingham University.

Bacterial strains and growth conditions: Bacterial strains and plasmids used in this study are listed in Chapter 1 (Tables 1 and supp. Table 1 and 2), Chapter 2 (supp. Table 1) and Chapter 3 C. *P. putida* KT2440R and *P. aeruginosa* PAO1 and its derivative strains were routinely grown at 30 ºC and 37 ºC respectively, in Luria Broth (LB), minimal saline medium (MS; 10 mM Na_2HPO_4, 20.6 mM KH_2PO_4, 25 mM NH_4NO_3, 0.8 mM $MgSO_4$) supplied with a trace element solution (63 µM $FeCl_3$, 3.3 µM $CoCl_2$, 4 µM $CaCl_2$, 1.2 µM Na_2MoO_4, 1.6 µM H_3BO_3, 0.7 µM $ZnSO_4$, 0.7 µM $CuSO_4$, 0.7 µM $MnSO_4$) or basal M9 medium supplied with 1 mM MgSO4, 6 mg l^{-1} Fe-citrate as described previously (Abril et al, 1989) and glucose (27 mM) as a carbon source. *E. coli* strains were grown at 37 °C in LB. *E. coli* DH5α was used for gene cloning. When appropriate, antibiotics were used at the following final concentrations, unless otherwise stated: chloramphenicol, 25 µg ml^{-1}; gentamicin, 10 µg ml^{-1} (*E. coli* strains) and 100 µg ml^{-1} (*P. putida* strains); kanamycin, 25 µg ml^{-1} (*E. coli* strains) and 50 µg ml^{-1} (Pseudomonas strains); rifampin, 10 µg ml^{-1}, piperacillin, 50 µg ml^{-1}.

Phenotypic assessment of carbon and nitrogen sources of P. putida KT2440R: The strain was grown overnight in M8 minimal medium (Kohler et al, 2000) supplemented with glucose (0.1 % w/v) as carbon source and ammonium chloride (0.1 g/l) as nitrogen source. Cultures were diluted 100-fold into M8 medium supplemented either with glucose and any of the 20 proteinogenic amino acids (5 mM) or ammonium chloride and any of the amino acids. Cultures were grown in 100-well polystyrene plates and incubated at 30 °C in a Bioscreen Microbiological Growth Analyzer. Data shown are means and standard deviation from 3 experiments after 24 hours growth.

Growth experiments of P. aeruginosa in minimal medium supplemented with RA: To assess the potential of the bacterium to use RA as sole growth substrate, sterile honeycomb plates (Bioscreen C) containing 200 µl of M9, supplemented with 1 to 10 mM RA, were inoculated with an overnight culture of *P. aeruginosa* PAO1 grown in M9 minimal medium (Abril et al, 1989) containing 5 mM citrate at 37 °C. Cultures were grown in a Bioscreen C (ThermoFisher

Scientific) instrument under constant shaking at 37 ºC during which time the OD_{660} was measured in 1 h intervals. To assess the effect of different RA concentrations on *P. aeruginosa* growth, honeycomb plates were filled with LB medium containing 1 to 100 µM C4-AHL or RA and cultures were carried out as described above.

Minimal inhibitory concentration assay: These assays were performed in 96-well plates using a modified version of the protocol reported in (Mah, 2014). Wells of a 96-well plate were filled with 200 µL of LB containing different amounts of RA (added using a 250 mM stock solution in DMSO). Control experiments contained the corresponding amounts of DMSO. Wells were inoculated with 10 µl of an overnight culture of *P. aeruginosa* PAO1 in LB medium. Plate was incubated at 37°C for 24 h at which point the viable cell amount was determined by plating out cells on LB-agar medium and counting.

DNA techniques: Genomic DNA was collected using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer´s protocol. Plasmid DNA was isolated using the Bio-Rad Plasmid miniprep kit. Phusion® high fidelity DNA polymerase (New England Biolabs) was used in the amplification of PCR fragments for cloning. DNA fragments were recovered from agarose gels using the QIAprep gel extraction kit (Qiagen).

Construction of P. putida KT2440R strains and plasmids: Sixteen KT2440R mutants defective in MCP encoding genes were obtained from the Pseudomonas Reference Culture Collection (http://www.gbif.es/ic_colecciones.php?ID_Coleccion=9817) (Duque et al, 2007). Mutants in the remaining 11 MCP encoding genes (*pp0779, pp1488, pp1940, pp2111, pp2823, pp2861, pp3414, pp3557, pp4888, pp5020* and *pp5021*) were constructed using derivate plasmids of pCHESIΩKm and pCHESIΩGm. These plasmids, listed in supp. table 1, were generated by amplifying a 0.5-1.1 Kb region of the gene to be mutated using the primers listed in Supp. Table 2. The PCR products were then cloned into pCHESI-derivate vectors in the same transcriptional direction as the P_{lac} promoter. The resulting plasmids were transferred to *P. putida* KT2440R by electroporation or triparental conjugation (using *E. coli* HB101 (pRK600) as helper strain). All mutations were confirmed by southern blot. For the construction of the complementing plasmids, the entire genes (*pp2249*, *pp1488*, *pp1228* and *pp3210*), besides their corresponding complete promoter regions, were amplified using the primers described in Supp. Table 2 and cloned into pBBR1MCS-5. Ligation mixtures were used to electroporate *E. coli* DH5α, and transformants were selected in LB with Gm. These resulting plasmids (pBBR1MCS-5-PP2249, pBBR1MCS-5-PP1288, pBBR1MCS-5-PP1228 and pBBR1MCS-5-PP2310) were verified by DNA sequencing and transformed into Pseudomonas strains by

electroporation. Plasmids for the expression and purification of the LBDs of McpA (PP2249) and McpU (PP1228), were constructed by amplifying the corresponding DNA fragments using primers listed in Supp. Table 2 and subsequent cloning into pET28b (+). All of the inserts were confirmed by sequencing.

Cloning of McpA-LBD and McpU-LBD in pET28b: The DNA fragments encoding McpA-LBD (pp2249) and McpU-LBD (pp1228) were amplified using the primers 5´-CTGTTCACGCATATGAACGACTACCTGCAACGCAATAC -3´ and 5´-ATCAGCATGGCAGGATCCTACGAGGTGCGGAACTG -3´ (McpA-LBD) and 5´-CTTGCTGGTCGCCACTCATATGACCCAGGCCCATC -3´, and 5´-CAAGGCCTATCAGCAGGGATCCTCAGTTGGCGTTC -3´ (McpU-LBD), respectively. These primers contained restriction sites for NdeI and BamHI (LasR). PCR products were digested with these enzymes and cloned into the expression plasmid pET28b(+) (Novagen) linearized with the corresponding enzymes. The resulting plasmids were termed pET28b-McpA-LBD and pET28b-McpU-LBD and were verified by DNA sequencing of the insert and flanking regions.

Cloning of LasR and RhlR in pET28b: The DNA fragments encoding LasR (PA1430) and RhlR (PA4377) were amplified using the primers 5´- GTTTAAGAAGAACGTGCTAGCATGGCCTTG-3´ and 5´-CTGAGAGGGATCCTCAGAGAGTAATAAGAC-3´ (LasR) and 5´-TATCGAGCTAGCCTTACTGCAATGAGGAATGAC-3´, and 5´-CGAGCTCTGCGCTTCAGATGAGACC-3´ (RhlR), respectively. These primers contained restriction sites for NheI and BamHI (LasR) and NheI and SacI (RhlR). PCR products were digested with these enzymes and cloned into the expression plasmid pET28b(+) (Novagen) linearized with the corresponding enzymes. The resulting plasmids were termed pET28b-LasR and pET28b-RhlR and were verified by DNA sequencing of the insert and flanking regions. In both cases the peptide MGSSHHHHHHSSGLVPRGSHM, containing the His-tag, is fused to the protein N-terminus.

Bioinformatics analysis

Molecular docking, homology modelling and structural alignment of LasR and RhlR: The atomic structure LasR was obtained from the Protein Data Bank (www.pdb.org, pdb ID 3IX3). The structure was refined and optimized with the Protein Preparation Wizard of the Schrödinger Suite (Schrödinger Suite 2012 Protein Preparation Wizard; Epik version 2.3, Schrödinger, LLC, New York, NY, 2012; Impact version 5.8, Schrödinger, LLC, New York, NY, 2012; Prime version 3.1, Schrödinger, LLC, New York, NY, 2012.). Ligands were obtained from the Natural Compounds (Metabolites) subset of the Zinc database (Irwin et al, 2012), optimized by LigPrep (LigPrep, version 2.5, Schrödinger, LLC, New York, NY, 2012.) and then submitted to virtual

screening docking experiments to LasR using the Glide dock SP mode (Glide, version 5.8, Schrödinger, LLC, New York, NY, 2012) (Friesner et al, 2004). The best hits were subsequently docked using the Glide dock XP mode. A homology model of the autoinducer domain of RhlR was generated using Swiss-Model (Arnold et al, 2006) and the structure of QscR (pdb ID 3szt) as template. RA was docked onto this structure in the Glide dock XP mode. The structural alignment of the autoinducer domains of LasR and RhlR was generated by Pymol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

Protein purification

McpA-LBD and McpU-LBD overexpression and purification: E. coli BL21(DE3) was transformed with plasmids pET28b-McpU-LBD or pET28b-McpA-LBD. *E. coli* BL21(DE3) pET28b-McpU-LBD cultures were grown in 2 l Erlenmeyer flasks containing 500 ml of LB medium supplemented with 50 µg ml^{-1} kanamycin at 30 °C until the culture reached an OD_{660} of 0.6. For protein induction isopropyl 1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.1 mM and the culture was maintained at 30 °C overnight with shaking. Cells were harvested by centrifugation at 10,000 × g at 4 ºC for 20 min. Similarly, *E. coli* BL21(DE3) containing pET28b-McpA-LBD was grown at 37 °C until the culture reached an OD_{600} of 0.4. The temperature was then lowered to 18 ºC and growth continued until an OD_{660} of 0.6-0.8 at which point protein expression was induced by the addition of 0.1 mM IPTG. Growth was continued at 18 ºC overnight and cells were harvested by centrifugation. Pellets were frozen using liquid nitrogen and stored at −80 °C. Cells derived from 750 ml of *E. coli* culture were resuspended in 30 ml of buffer A (20 mM Tris/HCl, 0.1 mM EDTA, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 5 % (v/v) glycerol, 1 mM DTT, pH 7.8) containing Complete TM protease inhibitor (Roche) and benzonase (Sigma). Cells were broken by French press treatment at 1,000 psi and centrifuged at 20,000 × g for 1 hour at 4 ºC. The supernatant was loaded onto a 5 ml HisTrap column (Amersham Bioscience) previously equilibrated with buffer A. The column was then washed with buffer A containing 45 mM imidazole prior to protein elution using a linear gradient (30 minutes) of 45-500 mM imidazole in buffer A at a flow of 1 ml/min. Protein containing fractions were analysed by SDS-PAGE, pooled and dialyzed into polybuffer (5 mM Tris, 5 mM Pipes, 5 mM Mes, 10 % glycerol (v/v), 150 mM NaCl, pH 7.0).

LasR and RhlR overexpression and purification: E. coli BL21 (DE3) was transformed with either pET28-LasR or pET28-RhlR. Cultures were grown in 2 l Erlenmeyer flasks containing 500 ml LB medium supplemented with 50 µg ml-1 kanamycin at 37 ºC until an OD660 of 0.4 (note: no AHL was added to the culture media). The temperature was then lowered to 18 ºC and growth

continued until an OD660 of 0.6-0.8, at which point protein expression was induced by the addition of 0.1 mM IPTG. Growth was continued at 18 ºC overnight and cells were harvested by centrifugation at 10 000 x g for 30 min. Cell pellets were resuspended in buffer A (20 mM Tris/HCl, 0.1 mM EDTA, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 5% (vol/vol) glycerol, 1 mM DTT, pH 7.8) and broken by French press at 1000 psi. After centrifugation at 20 000 x g for 1 hour, the supernatant was loaded onto a 5 ml HisTrap column (Amersham Bioscience) previously equilibrated with buffer A. The column was then washed with buffer A containing 45 mM imidazole prior to protein elution by applying a linear gradient (20 minutes) of 45-500 mM imidazole in buffer A at a flow of 1 ml/min. Protein containing fractions were pooled and dialyzed into buffer B (50 mM Tris/HCl, 500 mM NaCl, 1 mM DTT pH 7.8) and applied to a HiPrep26/60 Sephacryl S-200 High resolution gel filtration column previously equilibrated with the same buffer. Protein was eluted by a constant flow (1 mL/min) of buffer B at 4°C. Protein containing fractions were analyzed by SDS-PAGE. To this end sample buffer was added to the protein to a final SDS concentration of 0.5 % (w/v) and a β-mercaptoethanol concentration of 5 mM. Samples were then incubated at 95 ºC for 5 minutes, placed on ice, briefly centrifuged and loaded onto 10 % (w/v) polyacrylamide containing SDS gels. Multiple attempts to identify conditions under which the protein could be frozen without loss of activity have failed.

Biophysical analysis

McpA and McpU isothermal titration calorimetry: Titrations were carried out in a VP microcalorimeter (MicroCal, Northampton, MA, USA) at 25 ºC for McpU-LBD and 30 °C for McpA-LBD. Prior to experiments, proteins were thoroughly dialyzed in analysis buffer. The protein concentration was determined by the Bradford assay. Following filtration with 0.45 µm cut-off filters, the protein was placed into the sample cell of the instrument. Ligand solutions were made in the dialysis buffer. Control experiments involved the titration of dialysis buffer with ligand solutions. Typically, 30-35 µM of protein was titrated with 0.5-10 mM ligand solutions. The mean enthalpies measured from injection of the ligands into the buffer were subtracted from raw titration data prior to data fitting using the “One binding site model” of the MicroCal version of the ORIGIN software.

McpU thermal Shift Assay: Thermal shift assays were performed using a BioRad MyIQ2 Real-Time PCR instrument. For high-throughput screening, ligands were prepared by dissolving Biolog Phenotype Microarray compounds in 50 µl of MilliQ water to obtain a final concentration of around 10-20 mM (as indicated by the manufacturer). Screening was performed with compounds from plates PM1 and PM2A, that each contain 95 compounds

shown previously to be bacterial carbon sources and a control. Compounds in this plate are provided in a list at http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf. Each 25 µl standard assay contained 10 µM McpU-LBD in 5 mM polybuffer, 5 x SYPRO orange (Life Technologies) and 2.5 µl of the resuspended Biolog compounds. Samples were heated from 23 °C to 80 °C at a scan rate of 1 ºC/min. The protein unfolding curves were monitored by detecting changes in SYPRO Orange fluorescence. Melting temperatures were determined using the first derivative values from the raw fluorescence data.

LasR and RhlR isothermal titration calorimetry: Experiments were conducted on a VP-microcalorimeter (MicroCal, Amherst, MA) at 25 ºC. Proteins were dialyzed into analysis buffer (50 mM K2HPO4/KH2PO4, 150 mM NaCl, 1 mM DTT, pH 7.8). AHL ligands were made at a concentration of 1 mM in DMSO and subsequently diluted 1:10 with analysis buffer. The corresponding amount of DMSO (10 %, (v/v)) was added to the dialyzed protein sample. Typically, 6-10 µM of protein was titrated with 0.1 mM effector AHL solutions. Control experiments involved the titration of dialysis buffer containing 10 % (v/v) DMSO with AHL ligands. The mean enthalpies measured from the injection of effectors into the buffer were subtracted from raw titration data prior to data analysis with the MicroCal version of ORIGIN. Data were fitted with the "One binding site model" of ORIGIN.

RhlR differential scanning calorimetry (DSC): Differential scanning calorimetry experiments were carried out with a VP-DSC (Valerian-Plotnikov differential scanning calorimeter), capillary-cell microcalorimeter from MicroCal (Northampton, MA) at a scan rate of 90°C/h from 5ºC to 80ºC. Proteins were dialyzed against analysis buffer or into analysis buffer supplemented with 30 µM AHL (C4-AHL or 3-Oxo-C12-AHL). The final protein concentrations were between 16-19 µM. The corresponding dialysis buffer was used as a reference for measurements. Calorimetric cells (operating volume 0.133 ml) were kept under an excess pressure of 60 psi bar to prevent degassing during the scan. Before each experiment several buffer-buffer baselines were obtained to equilibrate the instrument. The experimental thermograms were baseline-subtracted, corrected from the instrument's response and normalized by the protein concentration. The calorimetric enthalpies were estimated by integration of the transition peaks.

LasR and RhlR circular dichroism (CD) spectroscopy: Experiments were performed at 25ºC on a Jasco J-715 spectropolarimeter equipped with a thermostatised cell holder. RhlR was at a concentration of 16-19 µM, whereas LasR at a concentration of 2-3 µM. Both proteins were in analysis buffer. Measurements in the far-UV CD region were made using 0.1 cm (RhlR) and a

0.2 cm (LasR) path length cuvettes. The spectra shown represent averages of 5 scans recorded at 100 nm/min, using 1 nm step resolution, 1 second response and 1 nm bandwidth. The CD spectra were subsequently deconvoluted in order to determine the relative abundance of secondary structure elements using the CDNN software package (Bohm et al, 1992).

LasR and RhlR thermal unfolding followed by circular dichroism: LasR and RhlR were dialyzed against analysis buffer or into analysis buffer supplemented with 30 µM AHL (C4-AHL or 3-Oxo-C12-AHL). Temperature scans were conducted at 90ºC/h after equilibration of the measuring cell within the cell holder at 20ºC for 10 minutes. The CD signal at 222 nm, being one of the negative bands characteristic of α-helix spectrum, was monitored as a function of temperature from 20ºC to 90ºC using a Jasco J-715 spectropolarimeter. Data were averaged for 20 seconds using a band width of 1 nm and a response time of 4 seconds. Baselines obtained from samples containing only buffer were subtracted from raw spectra. Thermal denaturation curves were smoothed using ORIGIN by applying a FFT filter and a window size of 5 points.

LasR and RhlR analytical ultracentrifugation: AUC experiments were performed in a Beckman Coulter Optima XL-I analytical ultracentrifuge (Beckman-Coulter, Palo Alto, CA, USA) equipped with UV-visible absorbance as well as interference optics detection systems, using an An50Ti 8-hole rotor, 12 mm path-length charcoal-filled epon double-sector centrepieces. The experiments were carried out at 10 °C using samples dialyzed against analysis buffer supplemented with 100 µM C4-AHL or 3-Oxo-C12-AHL in the cases needed. Proteins concentrations were 15 µM for RhlR and 8 µM for LasR. AUC experiments were repeated with interference (655 nm wavelength) and absorbance optics (280 nm wavelength). Laser delay was adjusted prior to the runs to obtain high-quality interference fringes.

Sedimentation velocity (SV) runs were carried out at a rotor speed of 48,000 rpm using 400 µL samples with analysis buffer as reference. A series of 40 scans without time intervals between successive scans were acquired for each sample. A least squares boundary modelling of the SV data was used to calculate sedimentation coefficient distributions with the size-distribution c(s) method (Schuck, 2000) implemented in the SEDFIT v11.71 software. Buffer density (= 1.012 g/mL) and viscosity (= 0.0134 Poise) at 10 ºC were calculated from the buffer composition and the partial specific volume of RhlR using SEDNTERP software (Laue et al, 1992). The partial specific volumes used were 0.728 mL/g for RhlR and 0.73 mL/g for LasR as calculated from its amino acid sequence. The molecular masses for RhlR and LasR (calculated from the amino acid sequence) were 30315.6 Da and 29071.2 respectively.

We evaluated ssphere, that is the theoretical sedimentation coefficient of a sphere with the same molecular weight (M) and partial specific volume ($\bar{v}$) as the particle (and would correspond to the maximum possible s-value for a particle of a given molecular mass). It was obtained using:

$$S_{sph} = \frac{M(1-\bar{v}\rho)}{N_A 6\pi\eta\left(\frac{3M\bar{v}}{4\pi N_A}\right)^{1/3}}$$

Where η is the viscosity, NA the Avogadro's number and s20,w is the experimental s-value corrected to a standard state of water at 20 °C and infinite dilution determined using SEDNTERP. δ is the hydration in grams of water per gram of macromolecule that is commonly set to the consensus value of 0.3 g/g for proteins (Lebowitz et al, 2002).

Genotypic studies

Electrophoretic mobility shift assays: The *hcnABC* promoter region (490 bp) of *P. aeruginosa* (Pessi & Haas, 2000) was amplified by PCR using the primers hcn_f 5´-GCACTGAGTCGGACATGACGGAA-3´ and hcn_r 5´-CGTGTTGACGTTCAAGAAGGTGCATTGC-3´. The amplified fragment was isolated from agarose gels and end-labelled with [γ-^{32}P] deoxy-ATP using the T4 polynucleotide kinase. For RhlR, freshly purified protein was dialyzed into analysis buffer with and without C_4-AHL or 3-Oxo-C12-AHL (100 µM). For LasR, 3-Oxo-C12-AHL was added to the protein. A 10 µl sample containing 2 nM of labelled DNA (1.5 × 10^4 cpm) was incubated with increasing concentrations of purified protein for 30 minutes in 10 µl EMSA buffer (20 mM Tris–HCl, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% (vol/vol) glycerol, pH 7.8) containing 20 mg/ml of poly-d(IC) and 200 mg/ml bovine serum albumin. The DNA–protein complexes were resolved by electrophoresis in 4 % (wt/vol) non-denaturing polyacrylamide gels in 1×TBE using BioRad electrophoresis equipment. For RhlR, control DNA was a 339 bp DNA fragment comprising the *oprB* promoter (Wylie & Worobec, 1995), which was amplified using primers oprB_f and oprB_r (Supp. Table 2). For LasR control DNA was a 451 bp DNA fragment of *Serratia plymuthica* A153 (Supp. Table 2).

In vitro transcription assay: Transcription reactions (20 µl) were performed in EMSA buffer containing 50 nM *E. coli* RNA polymerase holoenzyme saturated with σ70 (Epicentre Technologies, Madison, USA), 5 nM linear *hcnABC* DNA (see EMSA section), RhlR or LasR and the respective cognate AHL (0.5-50 µM). The mixtures were incubated for 30 min at 30 °C prior to the addition of 0.1 mM of each ATP, CTP and GTP, 0.05 mM UTP and 3.6 µCi of [$^{\alpha\text{-}32}$P]UTP (10 µCi/µl). After incubation for 50 min, the reactions were stopped by an exposure to 95 ºC and then chilled to 4°C. After the addition of 4 µl formamide sequencing dye samples were

separated on 6.5% (w/v) PAGE gels over a 2 h period. Gels were exposed on a phosphoimager; the resulting images were processed using the Quantity One software 4.6.2 (Bio-Rad laboratories) and the intensity of bands was determined with Scion Image software subtracting individual background levels measured below each band.

Gene expression studies

Effect of AHL in P. aeruginosa. To quantify gene expression β-galactosidase enzyme activity was measured in *P. aeruginosa* PAO1 *las*I$^-$/*las*R$^-$ harboring pMULTIAHLPROM. The strain was grown in LB containing 40 μg ml^{-1} tetracycline and 20 μg ml^{-1} gentamycin at 37 ºC overnight. Fresh medium was then inoculated with the resulting culture (1:100 dilution), grown for 1 h and then diluted two times two-fold at 30 minute intervals to ensure proper dilution of accumulated β-galactosidase after overnight growth. The resulting culture was then grown for another hour prior to induction with different effector molecules (AHL at 100 μM). Ligands were added as 0.14 to 0.52 % (v/v) solutions (depending on their solubility) in DMSO. Growth was continued at 37°C and samples were taken at different time points for the determination of β-galactosidase in permeabilized whole cells as described in (Espinosa-Urgel & Ramos, 2004). At least three independent assays were performed, and activity was expressed in Miller units.

Effect of RA in E.coli and P. aeruginosa. Gene expression experiments were conducted with *E. coli* BL21 harboring pET28b-RhlR (expression plasmid for RhlR) or pET28b (empty plasmid as a control) and pMULTIAHLPROM containing an *rhlI*::*lacZ* transcriptional fusion (Steindler et al, 2008) as well as with *P. aeruginosa* PAO1 *las*I$^-$/*las*R harboring pMULTIAHLPROM. *E. coli* BL21 were grown in LB containing 10 μg ml^{-1} tetracycline and 50 μg ml^{-1} kanamycin and *P. aeruginosa* PAO1 *las*I$^-$/*las*R in LB containing 40 μg ml^{-1} tetracycline and 20 μg ml^{-1} gentamycin at 37 ºC overnight. Stock solutions of RA and chlorogenic acid were prepared in DMSO (100%) and diluted in water to the desired concentration, whereas C4-AHL solutions were prepared in 10 % (v/v) DMSO. Fresh LB medium was then inoculated with the resulting cultures (1:100 dilution), grown for 1 h and then diluted two times two-fold at 30 minute intervals to ensure proper dilution of accumulated β-galactosidase after overnight growth. The resulting culture was then grown for another hour prior to induction with different ligands. To rule out non-specific effects of DMSO (present in the stock solution) control experiments were carried out in which the amount of DMSO corresponding to that present in C4-AHL, RA or chlorogenic acid containing cultures was added. Growth was continued at 37°C and samples were taken at different time points for the determination of β-galactosidase in permeabilised whole cells as described in (Espinosa-Urgel & Ramos, 2004). For dose-response experiments, the β-

galactosidase activity was measured 4 h after induction. Data shown are means and standard deviations from at least three independent experiments. To explore the effect of RA on additional RhlR regulated promoters, plasmids containing *lacZ* fusions were transferred to the wt strain by electroporation. These plasmids were: pß01 (*lasB*::*lacZ*), pß02 (*rhlA*::*lacZ*), pME3823 (*hcnA*::*lacZ*) as well as the insert free pQF50, which has served to construct the former two plasmids (Supp. Table 2). Experimental conditions were as those described above except that cultures were grown for 6 hours following dilution prior to the induction with different ligands.

Phenotypic studies

Assessment of P. putida KT2440R motility: Overnight liquid cultures of *P. putida* KT2440R and its derivative mutant strains were used to inoculate MS medium (Darias et al, 2014) to an OD_{660} of 1. Ten microliters of this mixture was deposited in the centre of a 60 mm diameter petri dish containing MS-glucose agar (MS medium containing 10 mM glucose and 0.25 % (w/v) agar). After 16 hours of incubation at 30 ºC the radius of the bacterial spread was measured. Experiments were performed in duplicate.

P. putida KT2440R Biofilm assays: Biofilm formation in multiwell plates: A modified version of a previously described high-throughput assay was used (Barahona et al, 2010). Overnight cultures of *P. putida* KT2440R and its 27 MCP mutants grown in LB medium were diluted in the same medium to an OD_{600} of 0.01. Subsequently 150 µl of each culture was placed into a well of a sterile, polystyrene multi-well plate. For each experiment five identical plates containing triplicates of each culture were prepared. Plates were incubated at 30 ºC without shaking. At 2, 4, 6, 8 and 24 h the cell suspension was removed from one plate with a multichannel pipette. After washing of the wells with 150 µl sterile water, the same volume of a crystal violet solution (0.4 %, w/v) was added to each well and incubated for 15 min to allow staining of adhered cells. Excess stain was eliminated by rinsing with water. Plates were air dried and 200 µl of 30 % acetic acid (v/v) was added to each well in order to extract crystal violet from cells. Destaining was performed overnight in a microplate shaker (40 r.p.m.); after which, the colour in the wells was measured at OD_{540} on a microplate reader. Experiments were repeated three separate times.

Biofilm formation in borosilicate glass tubes: Biofilm formation was examined as previously described (Martinez-Gil et al, 2014), during growth in LB medium without medium supplementation. Overnight cultures of *P. putida* KT2440R and its 27 mutants were grown at 30 ºC and used to inoculate borosilicate glass tubes containing 2 ml LB medium to an initial

OD_{600} of 0.05. Cultures were incubated in a Stuart SB3 tube rotator for 2, 4, 6, 8 and 24 h at 30 °C, with an angle of 45 degrees at 40 r.p.m. Biomass attached to the surface was visually inspected following the addition of 4 ml of crystal violet (0.4 %; w/v) to each tube and quantified by solubilizing the dye with 30 % acetic acid (v/v) and measuring the absorbance at 540 nm (Martinez-Gil et al, 2014). Data shown are means and standard deviations from three experiments conducted in duplicate.

Chemotaxis assays: Qualitative soft agar plate gradient assays: *P. putida* KT2440R as well as PP1228 and PP2249 mutant strains were grown overnight in M9 minimal medium (basal M9 medium supplemented with Fe-citrate, $MgSO_4$, and trace metals (Espinosa-Urgel et al, 2000)) with glucose (0.1 %, w/v) as a carbon source. Cultures were diluted to an $O.D._{660nm}$ = 1 with fresh minimal medium. Cells were then washed twice with M9 medium by consecutive resuspension and centrifugation at 3 300 × g for 3 min. The cell pellet was resuspended in 1 mL of M9 medium. Square (120 x 120 x 17 mm) polystyrene petri dishes (Greiner Bio-One) were filled with 50 ml of semisolid agar containing minimal medium, 2.5 mM glucose, and 0.25 % (w/v) agar. Plates were cooled at room temperature for 30 min. At the vertical central line of the plate, 10-µl aliquots of chemoattractant solution (amino acids or polyamines) at 3 different concentrations 500, 50 and 5 mM dissolved in sterilized water were placed at equivalent distances. After overnight incubation for gradient formation, two-microliter aliquots of bacterial suspensions were placed horizontally to each of the chemoattractant spots with varying distances to the chemoattractants. Plates were incubated at 30 °C for 16–20 h and then inspected for chemotaxis. Chemotaxis indices were calculated as described in (Pham & Parkinson, 2011).

Quantitative capillary assays: Overnight cultures of *P. putida* KT2440R as well as the PP1228 and PP2249 mutant were diluted to an OD_{600} of 0.05-0.07 in MS medium supplemented with 10 mM succinate. Bacteria were grown to early stationary phase (OD_{660} of 0.3–0.4) at 30 °C. Ten milliliters of culture were centrifuged at 3.300 × g at 4 ºC for 5 minutes and the resulting pellet was resuspended in 20 ml of drop assay medium (30 mM K_2HPO_4, 20 mM KH_2PO_4, 20 mM EDTA, 0.5 % (v/v) glycerol). Polystyrene multi-well plates were filled with 230 µl of bacterial suspension (OD_{660}=0.08). For filling with chemoeffector solutions, capillaries (Microcaps, Drummond Scientific, USA) were heat-sealed at one end, warmed over the flame and the open end inserted into the chemoattractant solution. The capillary was immersed into the cell suspension at its open end. After incubation for 30 min, the capillary was removed from the cell suspension, rinsed with water and emptied into an Eppendorf tube containing 1 ml M9 medium. Serial dilutions were made and 20 µl aliquots of the resulting cell suspension

were plated onto agar plates containing M9 minimal medium supplemented with 15 mM succinate and incubated at 30 ºC. Colonies were counted after growth for 24 hours.

Biofilm formation: Overnight cultures of *P. aeruginosa* PAO1 were grown at 37 ºC and used to inoculate borosilicate glass tubes containing 2 ml of LB medium (supplemented with either 2 mM of RA or chlorogenic acid) to an initial OD_{660}=0.05. Both compounds were added as 143 mM solutions in DMSO and the corresponding control experiments were conducted to assess the effect of the equivalent amount of DMSO on biofilm formation. Cultures were incubated in a Stuart SB3 tube rotator for 2, 4, 6, 8 and 24 hours at 30 °C, with an angle of 45 degrees at 40 rpm. Biofilms formed were visualized by crystal violet (0.4%) staining and quantified by solubilizing the dye with 30% acetic acid and measuring the absorbance at 540 nm (Martinez-Gil et al, 2012). Data shown are means and standard deviations from three experiments conducted in duplicates.

Quantification of pyocyanin production: Cultures of *P. aeruginosa* PAO1 were grown in LB at 37 °C overnight and used to inoculate glass tubes containing 2 ml of LB medium to an initial OD_{660}=0.05. Stock solutions of 143 mM of RA and chlorogenic acid were prepared in DMSO and aliquots were then added to the tubes to final concentrations of 0.5 to 2 mM. The amount of DMSO corresponding to the experiment at 2 mM, was added to the control tube. Growth was continued and pyocyanin production was determined after 8 h. The OD_{660} of cultures was determined prior to centrifugation of cultures at 13 000 rpm for 5 min. The OD_{520} (indicative of pyocyanin production) was measured and values were normalized with the cell density (OD_{660}). LB containing either RA or chlorogenic acid were used as blanks.

Elastolysis assay: Elastase activity in *P. aeruginosa* PAO1 cultures was determined using a modified version of the Elastin Congo Red (ECR) assay (Ishida et al, 2007). Cells were grown with shaking in LB medium at 37 ºC overnight and used to inoculate glass tubes containing 3 ml of LB medium to an initial OD_{660}=0.05. Growth was continued for another 6 h and cultures were induced with 500 µM RA. The equivalent amount of DMSO was added to control tubes. Growth was continued until 24 h and 1 ml of cell suspension was centrifuged at 13 000 rpm for 15 min. The resulting supernatant was added to tubes containing 10 mg of elastin-Congo Red (Sigma) and 1 ml of buffer (0.1 M Tris-HCl, 1 mM $CaCl_2$, pH 7.0). Tubes were incubated at 37°C with shaking (150 rpm) for 24 h. The reaction was stopped by the addition of 1 ml of sodium phosphate buffer (0.7 M, pH 6.0). Residual, solid ECR was removed by centrifugation and the OD_{492} of the supernatant was measured. Shown are means and standard deviations from three replicates conducted in triplicate.

Abril MA, Michan C, Timmis KN, Ramos JL (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. *J Bacteriol* 171: 6782-6790

Plant studies

Competitive root colonization assay: Corn seeds (*Zea mays* L.) were surface-sterilized by rinsing with sterile deionized water, washing for 10 min with 70 % (v/v) ethanol, and 15 minutes with 20 % (v/v) bleach, followed by thorough rinsing with sterile deionized water. Surface sterilized seeds were pregerminated on MS medium (Darias et al, 2014) containing 0.2 % (w/v) phytagel (Sigma, reference: P8169, St. Louis, MO, USA) and 0.5 % (w/v) glucose, at 30 °C in the dark for 48 h. Strains grown overnight in LB were diluted in M9 to an OD_{660} = 1. The wild type strain (KT2440R) was mixed with the individual mutant strains at a 1:1 ratio. CFUs of each strain were counted in the mix using selective LB media supplied with Rif (both strains) or Km (mutant strains). For competitive root colonization assays, six independent seedlings were inoculated with this mixture. Plants were maintained in a controlled chamber at 24 °C with a daily light period of 16 h. To recover bacteria from the rhizosphere, roots of one-week old plants were collected and placed into tubes containing 20 ml of M9 basal medium and 4 g of glass beads (diameter, 3 mm). Tubes were vortexed for 2 min and serial dilutions were then plated onto selective media (LB with Rif or Km) (Martinez-Gil et al, 2013). Data are shown as the index of colonization fitness (ICF) that is measured as 1/[(percentage of recovered wt KT2440R vs mutant per plant 7 days post inoculation)/(percentage of wt KT2440R vs mutant in the initial inocula)] (Martinez-Gil et al, 2013).

Colony-based c-di-GMP reporter assays: Fluorescence intensity analyses using the c-di-GMP biosensor pCdrA::*gfp*^{S} were carried out to determine the cellular levels of the second messenger. Briefly, the reporter plasmid pCdrA::*gfp*^{S} was transformed into KT2440R, and derivative strains, by electroporation. Subsequently, overnight bacterial cultures of the strains to be tested were adjusted to an OD_{660} of 0.05 and 20 µL drops were spotted on LB-agar plates containing the appropriate antibiotics. Following incubation for 48 h at 30 ºC, colony morphology and fluorescence intensity were analysed in a Leica M165 FC stereomicroscope. Fluorescence was visualized employing a GFP filter set (emission/excitation filter 470/525 nm). Pictures were taken using Leica Application Suite software using different exposure times.

RESULTS AND DISCUSSION

CHAPTER 1

Assessment of the contribution of MCP-based signaling to biofilm formation

CHAPTER 1 Assessment of the contribution of MCP-based signaling to biofilm formation

Brief introduction

Many bacteria are present in their natural habitats as biofilms. Biofilm formation is a cooperative group behaviour that involves bacterial populations living embedded in a self-produced extracellular matrix. This process is of enormous clinical relevance (Cos et al, 2010; Sun et al, 2013) because it influences the mechanisms that cause resistance to antibiotics and evasion of host immune defences. In addition, biofilm formation is a phenomenon that is of importance to a diverse range of biotechnological applications (Wang & Chen, 2009). The current model of biofilm formation purports that, within a number of species, the process comprises different life stages (Hobley et al, 2015). The formation of a biofilm is initiated by the reversible attachment of planktonic cells to surfaces, which is an event induced by environmental stimuli. This is followed by an irreversible attachment stage, and then growth and maturation of the biofilm. These stages are characterized by the synthesis of the biofilm matrix and the development of antimicrobial resistance. Finally, environmental detachment signals trigger the bacteria to transition to a planktonic lifestyle and to be released from the biofilm. The transition between the different stages during biofilm development and dispersal are subject to a variety of regulatory mechanisms in response to stimuli that are not yet fully understood.

The regulatory mechanisms that are known to mediate bacterial signal transduction include OCS and TCS as well as chemosensory pathways. Pseudomonads are well-studied model organisms in terms of biofilm formation and there exists a significant body of knowledge regarding the involvement of different one- and two-component regulatory systems (Fazli et al, 2014; Mikkelsen et al, 2011). Cell-to-cell communication is essential for biofilm development; as such, the OCS RhlR and LasR of *P. aeruginosa* have been found to play central roles in the expression of genes related to biofilm formation in response to QSS (O'Loughlin et al, 2013). The TCS formed by the GacS/RetS/LadS sensor kinases and the GacA RR were found to be essential in biofilm regulation. These systems control the transcription of sRNA involved in biofilm formation and respond to yet unidentified environmental cues (Ventre et al, 2006), which in turn induce different phenotypic alterations.

On one hand, these TCS mediate the production of compounds necessary for attachment and biofilm formation, including exopolysaccharides (Friedman & Kolter, 2004); large adhesion proteins (Martinez-Gil et al, 2010); extracellular DNA (Whitchurch et al, 2002); and different surfactants, such as lipopeptides (Kuiper et al, 2004) and rhamnolipids (Boles et al, 2005). On the other hand, they modulate the levels of the bacterial second messenger c-di-GMP - a mode of action that is relevant because increased levels of c-di-GMP promote biofilm formation and reduce bacterial motility (Romling et al, 2013).

Chemosensory pathways represent the third group of major signal transduction systems in bacteria (Wuichet & Zhulin, 2010). The core of a chemosensory pathway is the ternary complex composed of a MCP, a CheA HK and a CheW coupling protein. Signal recognition at the MCP-LBD modulates CheA autophosphorylation and consequently transphosphorylation of the CheY response regulator, which can then bind to the flagellar motor, ultimately leading to chemotaxis (Hazelbauer et al, 2008). Existing genetic and biochemical evidence suggest that chemosensory pathways can modulate various functions, such as flagellum-based chemotaxis, type IV pili-based motility and ACF (Wuichet & Zhulin, 2010). *P. aeruginosa* PAO1 has five gene clusters that encode signaling proteins involved in four different chemosensory pathways (Sampedro et al, 2014). Two of these pathways (*che* and *che2*) are involved in flagellum-mediated taxis (Ferrandez et al, 2002); another pathway (*wsp*) modulates c-di-GMP levels (Hickman et al, 2005); and the fourth pathway (*chp*) is responsible for type IV pili-mediated motility (Whitchurch et al, 2004) and the regulation of cAMP levels (Fulcher et al, 2010).

There is a significant body of evidence demonstrating that chemosensory pathway mediated signaling participates in the formation and dispersal of biofilms in *P. aeruginosa*. The *wsp* pathway is a clear example of a chemosensory pathway that has an alternate cellular function (Hickman et al, 2005). The CheY-homologue of this pathway, WspR, is a fusion of a receiver and a GGDEF domain. It was shown that phosphorylation of WspR enhances the activity of the c-di-GMP-synthesizing GGDEF domain, which in turn enhances biofilm formation (Guvener & Harwood, 2007; Hickman et al, 2005). It was suggested that the *wsp* pathway controls WspR activity via binding of an unidentified signal molecule to a MCP. However, in-frame deletion of the gene encoding the WspA MCP did not significantly alter biofilm formation (Hickman et al, 2005). Another MCP in *P. aeruginosa*, BdlA, was shown to be involved because the corresponding mutant was deficient in biofilm dispersal and showed increased c-di-GMP levels, suggesting that the molecular mechanism for BdlA action is based on alterations in c-di-GMP levels rather than chemotaxis (Morgan et al, 2006). A number of studies also found that inactivation of proteins within chemosensory pathways impacts biofilm formation. Thus,

mutation of the genes encoding the methyltransferase CheR (Schmidt et al, 2011) and the RR CheY (Barken et al, 2008) of the *che* pathway abolished chemotaxis, which effected bacterial surface sampling and biofilm formation. In addition, other studies show that inactivation of chemosensory pathways severely inhibit plant root colonization (de Weert et al, 2002; Yao & Allen, 2007). Most of these studies were based on the inactivation of signaling proteins. However, there is little known about which specific MCPs modulate biofilm formation.

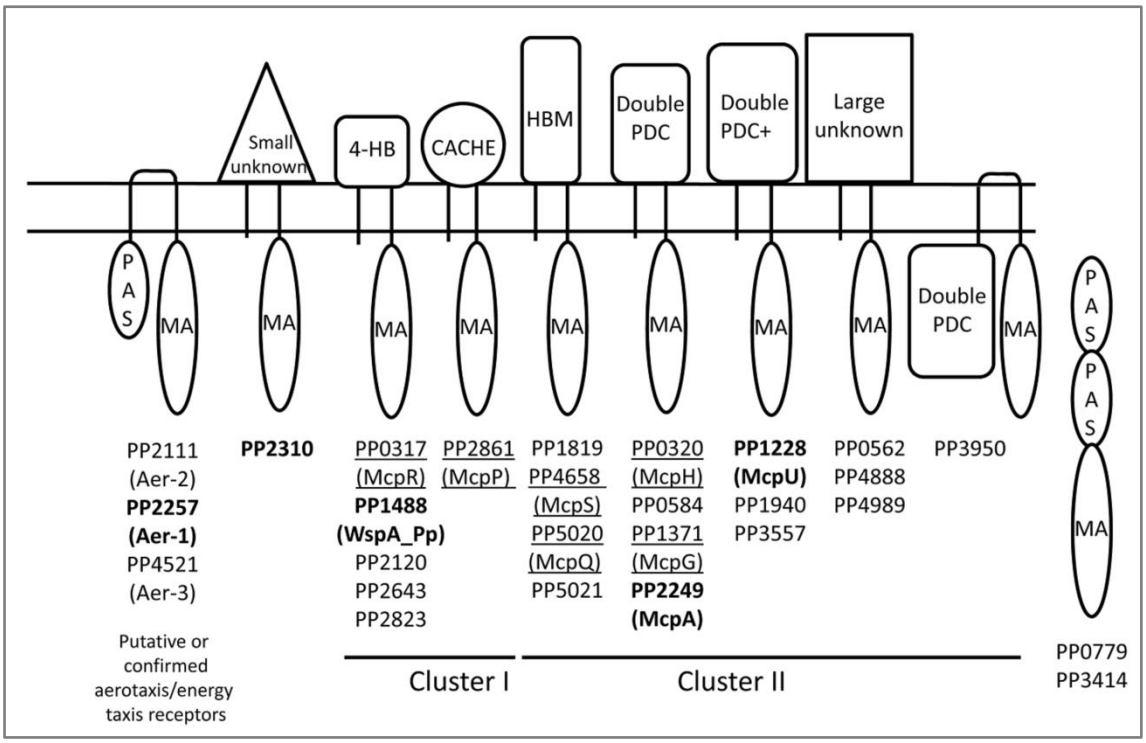


Figure 1) Topology and diversity of MCPs from *P. putida* KT2440. Transmembrane regions were predicted using the DAS algorithm (Cserzo et al., 1997). The prediction of LBD type is based on the fold recognition analyses using the Phyre2 algorithm (Kelley and Sternberg, 2009) and consensus secondary structure predictions (Deleage et al., 1997). Receptors that were annotated previously with a function are underlined and those that modulate biofilm formation are shown in bold. MA, methylaccepting domain; 4-HB, 4-helix bundle domain; HBM, helical bimodular domain; double PDC, repeat of PhoQ/DcuS/CitA–domain (Zhang and Hendrickson, 2010); double PDC+, double PDC domain containing a 40-50 amino acid insert into double PDC domains. HAMP domains have been omitted and classification is done based on receptor topology and LBD type.

In this study, we have addressed this question using *P. putida* KT2440 as model organism. This strain is a metabolically versatile saprophytic soil bacterium that is able to form biofilms and to colonize plant roots efficiently (Espinosa-Urgel et al, 2002; Regenhardt et al, 2002). *P. putida* KT2440 has 27 MCPs that differ in topology and LBD (Fig. 1). Of these receptors, several have been annotated with a function (Fig. 1) and were found to mediate taxis to different organic acids (McpS, McpQ, McpR, McpP) (Garcia et al, 2015; Lacal et al, 2010a; Lacal et al, 2011a; Martín-Mora et al, 2015; Parales et al, 2013; Pineda-Molina et al, 2012), gamma-

aminobutyrate (McpG) (Reyes-Darias et al, 2015a) or purine compounds (McpH) (Fernandez et al, 2015b). In addition, three of the receptors are paralogues to Aer-1, Aer-2 and Aer-3, which have been shown or predicted to mediate aero- and energy taxis (Sarand et al, 2008); and two of the receptors share a high degree of sequence identity with BdlA (Supp. Fig. 1). The remaining MCPs have not yet been annotated and their putative functions are still unknown. Using this model system, we carried out a systematic study of the contribution of each of the MCPs to biofilm formation via the analyses of single mutants for each of the 27 MCPs.

Results

Construction of bacterial MCP mutants and their analysis

Sixteen mutants of *P. putida* KT2440R (Table 1), each deficient in one *mcp* gene, were extracted from the Pseudomonas Reference Culture Collection (Duque et al, 2007). Additionally, we constructed the remaining 11 MCP mutants as described in ANEX (experimental procedures) to achieve the complete set of MCP single mutants. Growth experiments in LB medium were conducted with the 27 mutants (Table 1) and the wt strain. As shown in Supp. Fig. 2, the growth kinetics of all strains were similar, indicating that, under the conditions tested, the MCPs did not significantly impact growth. The motility of these strains was also assessed using swim plate assays on soft agar MS medium supplemented with glucose and the majority of mutants showed motility comparable to the wt strain (Supp. Fig. 3). A very slight reduction in motility ($P<0.5$) was observed for mutants PP1488, PP2257 and PP2643. Subsequently, the capacity of these strains to form biofilms was assessed.

Biofilm formation of *P. putida* KT2440 on abiotic surfaces has been studied previously. Without media replacement, this strain was found to form mature biofilms after 6 to 8 hours, followed by dispersion that was almost complete after 24 hours (Martinez-Gil et al, 2012). Initially, assays were conducted in polystyrene multiwell plates. Four mutant strains, namely PP1228, PP1488, PP2249 and PP2310, showed significant differences in biofilm formation as compared to the wt. These strains were selected and submitted to biofilm formation assays on borosilicate glass surfaces, which confirmed the data obtained on multiwell plates (Fig. 2).Thus, mutant PP1228 showed increased biofilm formation as compared to the wt. In contrast, mutant PP1488 had a reduced capacity to form biofilm. This receptor is the homologue of *P. aeruginosa* WspA and is therefore referred to as WspA-Pp (Supp. Fig 4). Interestingly, a mutant in *pp2249* showed different biofilm kinetics, reaching maximal biofilm

formation earlier than wt (after 6 hours as compared to 8 hours for the wt). Biofilm dispersion in the strain PP2249 also occurred earlier than wt (Fig. 2). Finally, a mutant strain deficient in *pp2310* also showed an increased amount of biofilm formation.

Table 1) Strains used in this study.

Strain	Characteristics	Reference
Escherichia coli BL21(DE3)	F–, *ompI, hsdS*$_B$ (r_{-B} m_{-B}) *gal*, *dam*, *met*	(Jeong et al, 2009)
E. coli DH5α	*supE44 lacU169*(*Ø80lacZΔ M15*) *hsdR17* ($r_K^-m_K^-$) *recA1 endA1 gyrA96 thi-1 relA1*	(Woodcock et al, 1989)
E. coli HB101	F^- Δ*(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1* Δ*(mcrC-mrr) rpsL20* (Sm^r) *xyl-5 mtl-1 recA13 thi-1*	(Boyer & Roulland-Dussoix, 1969)
Pseudomonas putida KT2440R	Rifampicin-resistant derivative of KT2440	(Espinosa-Urgel & Ramos, 2004)
P. putida KT2440R PP0317	*pp0317*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP0320	*pp0320*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP0562	*pp0562*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP0584	*pp0584*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP0779	*pp0779*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP1228	*pp1228*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP1371	*pp1371*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP1488	*pp1488*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP1819	*pp1819*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP1940	*pp1940*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP2111	*pp2111*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP2120	*pp2120*::mini-tn5-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP2249	*pp2249*::mini-tn5-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP2257	*pp2257*::mini-tn5-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP2310	*pp2310*::mini-tn5-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP2643	*pp2643*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP2823	*pp2823*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP2861	*pp2861*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP3414	*pp3414*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP3557	*pp3557*::pCHESIΩGm;Rif^R, Gm^R,	This study
P. putida KT2440R PP3950	*pp3950*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP4521	*pp4521*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP4658	*pp4658*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP4888	*pp4888*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP4989	*pp4989*::mini-tn*5*-Km; Rif^R, Km^R	(Duque et al, 2007)
P. putida KT2440R PP5020	*pp5020*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP5021	*pp5021*::pCHESIΩKm;Rif^R, Km^R,	This study

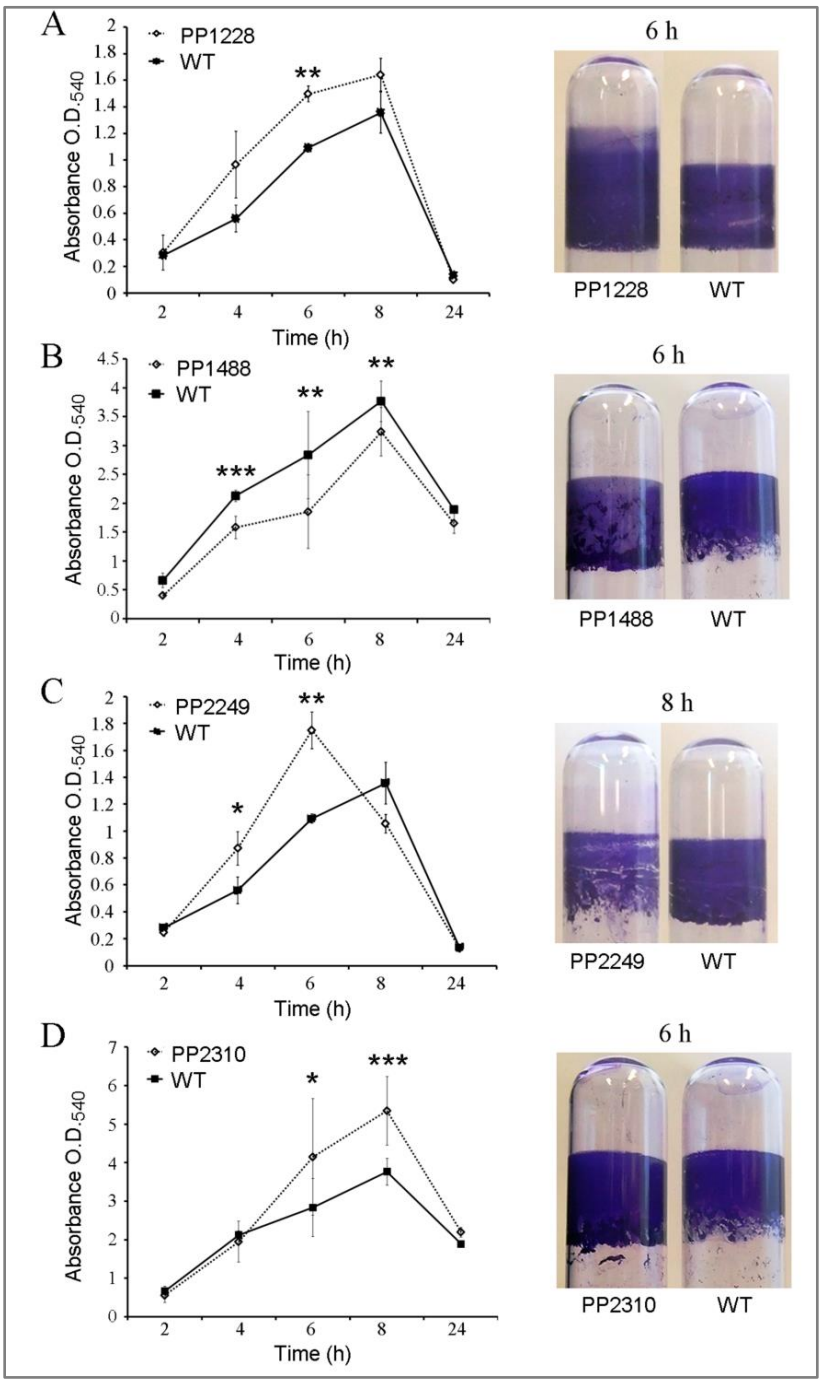


Figure 2) MCP mutants of *P. putida* KT2440R with significantly altered biofilm formation pattern. A) mutant PP1228, **B)** mutant PP1488, **C)** mutant PP2249, **D)** mutant PP2310. Biofilm formation was quantified from cultures grown in borosilicate glass tubes (see Materials and Methods). These data are the result of an analysis of the wt and all 27 mutants deficient in a single MCP (Table 1). Data are means and standard deviations from three independent experiments. Representative borosilicate glass tubes stained with crystal violet are shown. *P<0.05, **P<0.01, ***P<0.001 in Student's t-test indicate statistical significance.

In analogy to the data reported above, statistically relevant differences in biofilm formation between plasmid containing wt and four mutant strains were observed (Fig. 3). For mutant complementation studies, genes *pp1228*, *pp1488*, *pp2249* and *pp2310* were cloned into plasmid pBBR1MCS-5.

Initial experiments involved measurements of biofilm formation of the wt and the four mutant strains harbouring the empty plasmid pBBR1MCS-5. Complementation of the four mutants induced in all cases a reversal of the mutant phenotype, which for three mutants was of statistical relevance (Fig. 3).

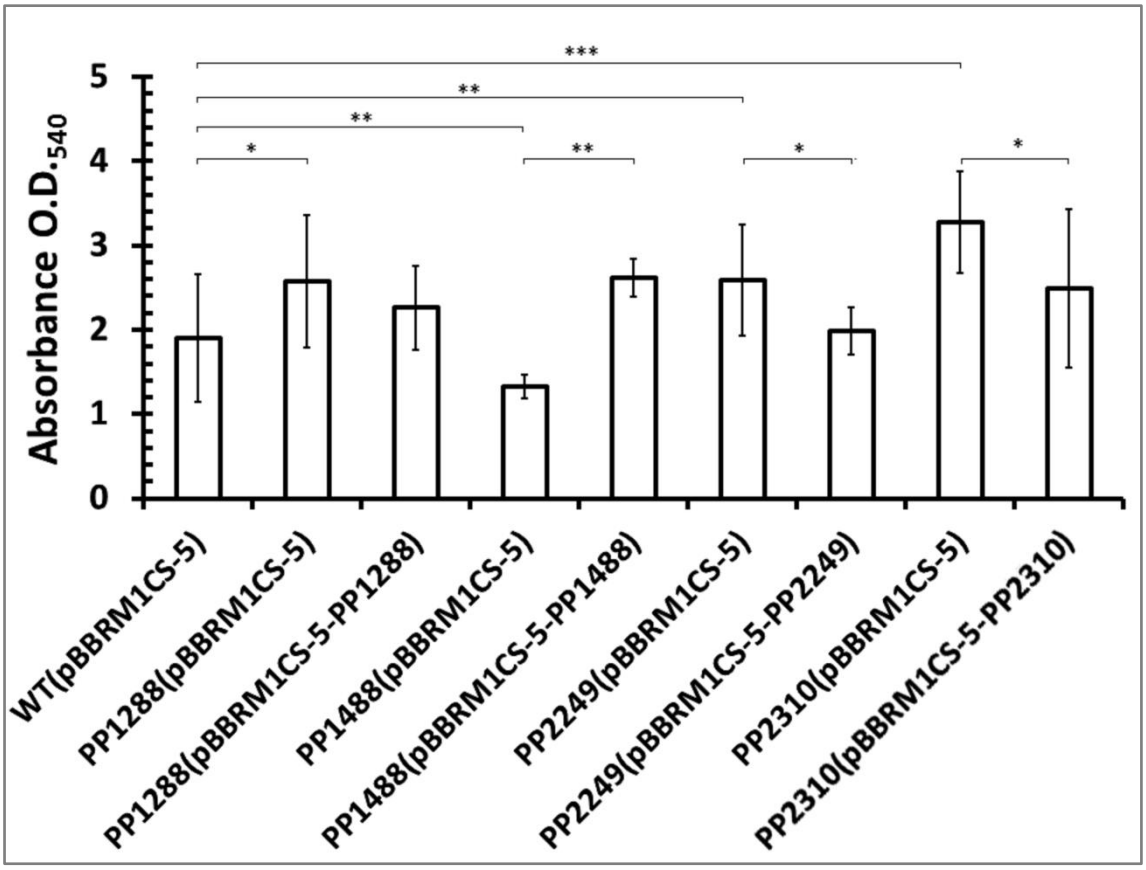


Figure 3) Biofilm assays of mutant and complemented strains. Biofilm formation at 6 hours of the wt strains and mutants PP1228, PP2249, PP1488 and PP2310 harbouring either the empty pBBR1MCS-5 and, in the case of the mutant strains, harbouring a pBBR1MCS-5 derivative containing the corresponding MCP gene. Shown are means and standard deviations from at least three independent experiments. * P ≤0.05, **P ≤0.01 and ***P≤0.001 in Student's t-test indicate statistical significance.

WspA of *P. aeruginosa* feeds into the *wsp* pathway, which ultimately alters c-di-GMP levels (Hickman et al, 2005). In order to establish a correlation between biofilm formation and c-di-GMP levels for WspA-Pp, we decided to quantify the cellular levels of the second messenger. To this end we introduced a plasmid (pCdrA::*gfp*^{S}) into different strains harbouring a c-di-GMP biosensor that corresponds to a transcriptional fusion of the *cdrA* promoter from *P. aeruginosa* PAO1 to the green fluorescent protein encoding gene *gfp*^{S} (Rybtke et al, 2012). As a positive control we introduced plasmid pMAMV1 into the wt strain, which confers high cellular levels of c-di-GMP due to the overexpression of the diguanylate cyclase Rup4959 (Matilla et al, 2011). Colonies of the resulting strains were inspected by fluorescence microscopy. As shown in Fig. 4, the wt strain harbouring pMAMV1 produced a fluorescence signal that was well above that of the wt strain, confirming the presence of elevated c-di-GMP levels in the pMAMV1 containing strain (note the differences in the exposure times).

In contrast, a decrease in the fluorescence signal was observed in the strain PP1488, indicating that the mutation of the WspA-Pp encoding gene resulted in lower c-di-GMP levels which is consistent with the lower amount of biofilm formed by PP1488 (Fig. 2 B). The underlying mechanisms of the remaining three receptors (PP1228, PP2249 and PP2310) in biofilm formation are still unknown. To dissect their implications in the biofilm formation capacity of KT2440 further research was carried out, with special attention to the identification of the ligand(s) which bind to the MCP(s) LBD(s).

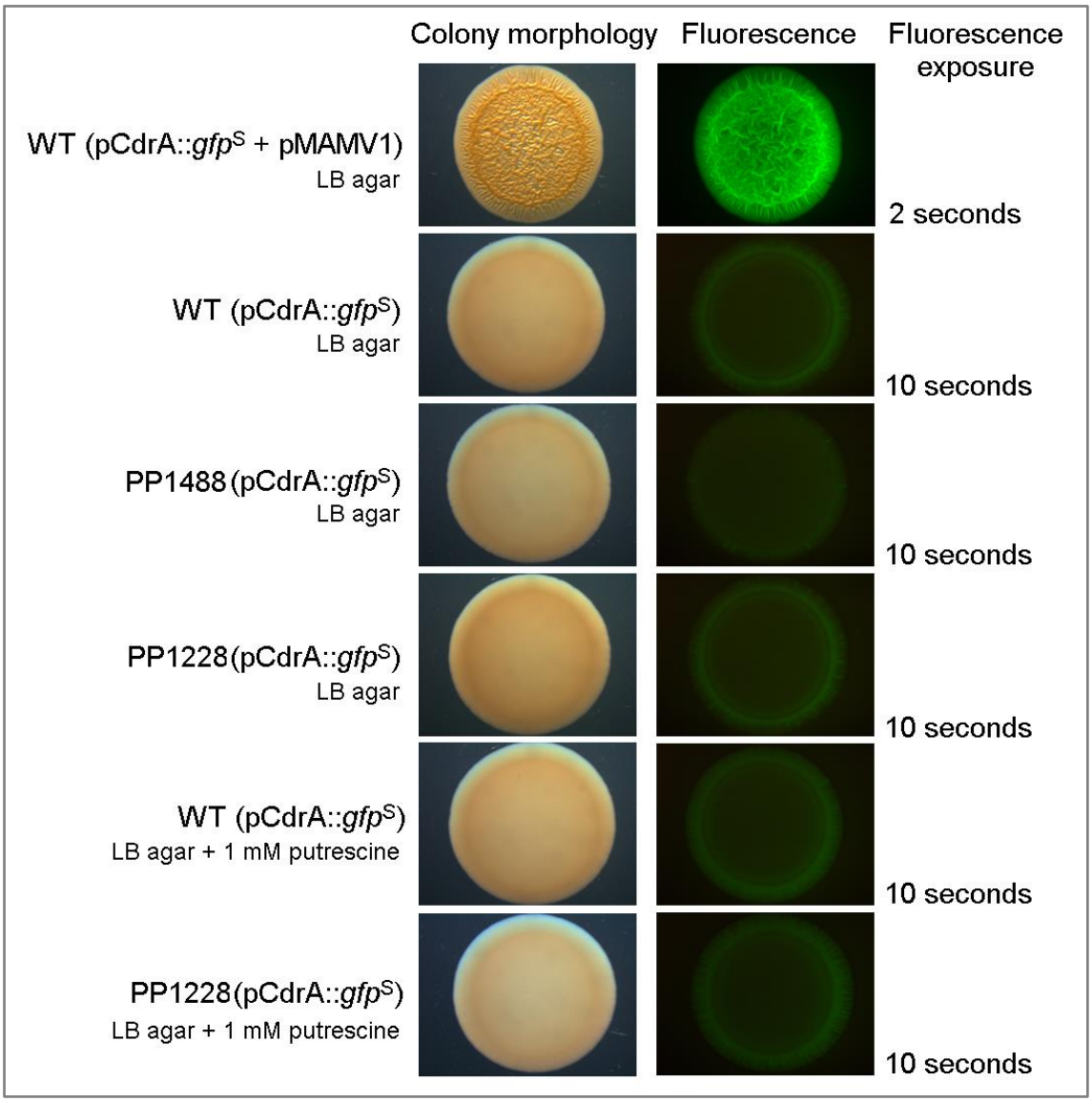


Figure 4) Colony morphology and fluorescence intensity of *P. putida* strains harbouring the c-di-GMP biosensor plasmid pCdrA::*gfp*^{S}. Plasmid pMAMV1 confers high levels of c-di-GMP (Matilla *et al*., 2011). The exposure in fluorescence measurements is indicated. Experiments were conducted on LB agar plates with or without 1 mM putrescine. The assays were repeated three times, and representative results are shown.

Identification of PP2249 as a chemotaxis receptor for L- amino acids

MCP PP2249 has a double PDC (PhoQ/DcuS/CitA) LBD (Zhang & Hendrickson, 2010). PP1228 also has a double PDC domain; however, the domain is distinct in that it contains an insert of approximately 50 amino acids (termed double PDC+ in Fig. 1) (Supp. Fig. 7). In *P. aeruginosa* PAO1, three paralogous receptors with a double PDC domain, PctA, PctB and PctB, were identified and found to mediate chemotaxis towards gamma-aminobutyrate (GABA) and most of the proteinogenic amino acids (Rico-Jimenez et al, 2013; Taguchi et al, 1997). We have shown previously that the recombinant LBDs of these receptors recognize their ligands directly (Rico-Jimenez et al, 2013). We therefore hypothesized that PP1228 and PP2249 function similarly.

To verify this hypothesis, we cloned DNA fragments encoding the periplasmic LBDs of both receptors into an expression plasmid and purified the recombinant proteins from the soluble extract of *E. coli* lysates. Subsequently microcalorimetric binding studies (Krell, 2008) were carried out for both proteins against GABA and all 20 L-amino acids. We were able to show that PP2249-LBD binds to 12 different L-amino acids with affinities that vary between 0.6 and 373 µM (Table 2). Representative titrations for three of these amino acids are shown in Fig. 5A. No binding was observed to dipeptides, D-amino acids, GABA or the other ligands listed in the legend of Table 2. In contrast to the results for PP2249-LBD, no ligand binding was observed for PP1228-LBD.

Table 2) Magnitude of chemotaxis and thermodynamic binding parameters of McpA and McpU ligands. Shown are chemotactic responses of *P. putida* KT2440R to different ligands as determined by the swim plate assay as well as the binding parameters derived from microcalorimetric titration of McpA-LBD and McpU-LBD with different ligands. Data are means and standard deviations from three independent experiments.

Ligand	Chemotaxis[a]	Binding to McpA-LBD[b]	
		K_D (µM)	ΔH (kcal mol^{-1})
Gly	+	35 ± 2	-8.4 ± 0.7
L-Ala	+++	13 ± 0.6	-11.5 ± 0.4
L-Cys	+	0.6 ± 0.1	-38 ± 0.4
L-Ser	+++	43 ± 2	-6.5 ± 1.1
L-Thr	-	No binding[c]	
L-Asn	+	4.3 ± 0.2	-13 ± 0.4
L-Gln	+	5.5 ± 0.2	-8.9 ± 0.1
L-Asp	-	No binding[c]	
L-Glu	-	No binding[c]	
L-Phe	++	2.3 ± 0.1	-1 ± 0.1
L-Tyr	+++	12.1 ± 0.9	-4.4 ± 0.8
L-Trp	-	No binding[c]	

L-Val	+	373 ± 42	0.9 ± 0.7
L-Leu	+	No binding[c]	
L-Ile	+	85 ± 5	0.2 ± 0.05
L-Met	+++	5.8 ± 0.6	-9.7 ± 0.6
L-Arg	+	1.2 ± 0.1	-4 ± 0.1
L-Lys	+	No binding[c]	
L-His	-	No binding[c]	
L-Pro	+++	No binding[c]	
		Binding to McpU-LBD[d]	
Putrescine	+++	2 ± 0.1	-15 ± 0.1
Cadaverine	++	22 ± 2	-15.5 ± 0.5
Spermidine	+++	4.5 ± 0.4	-4.3 ± 0.1

[a] Chemotaxis was assessed using plate gradient assays.

[b] Compounds that were tested for binding to McpA-LBD but that did not reveal binding were: L-amino acids (L-Thr, L-Trp, L-Leu, L-Lys, L-Asp, L-Glu, L-His and L-Pro), D-amino acids (D-Ala, D-Glu, D-Gln), dipeptides (L-Ala-L-Ala, L-Ala-L-Glu, L-Ala-Gly), putrescine, cadaverine, aminopropyl-cadaverine, spermidine, spermine, L-ornithine, gamma-aminobutyrate, butyrate.

[c] It cannot be excluded that ligands bind with very low affinity to the protein. This is due to the fact that amino acids at a maximal concentration of 5 mM could be placed into the injector syringe of the calorimeter to avoid large dilution heats. Therefore, potential very weak binding events cannot be monitored.

[d] Compounds that were tested for binding at McpU-LBD, but that did not show any binding were: all proteinogenic amino acids, aminopropyl-cadaverine, spermine, L-ornithine, gamma-aminobutyrate, butyrate.

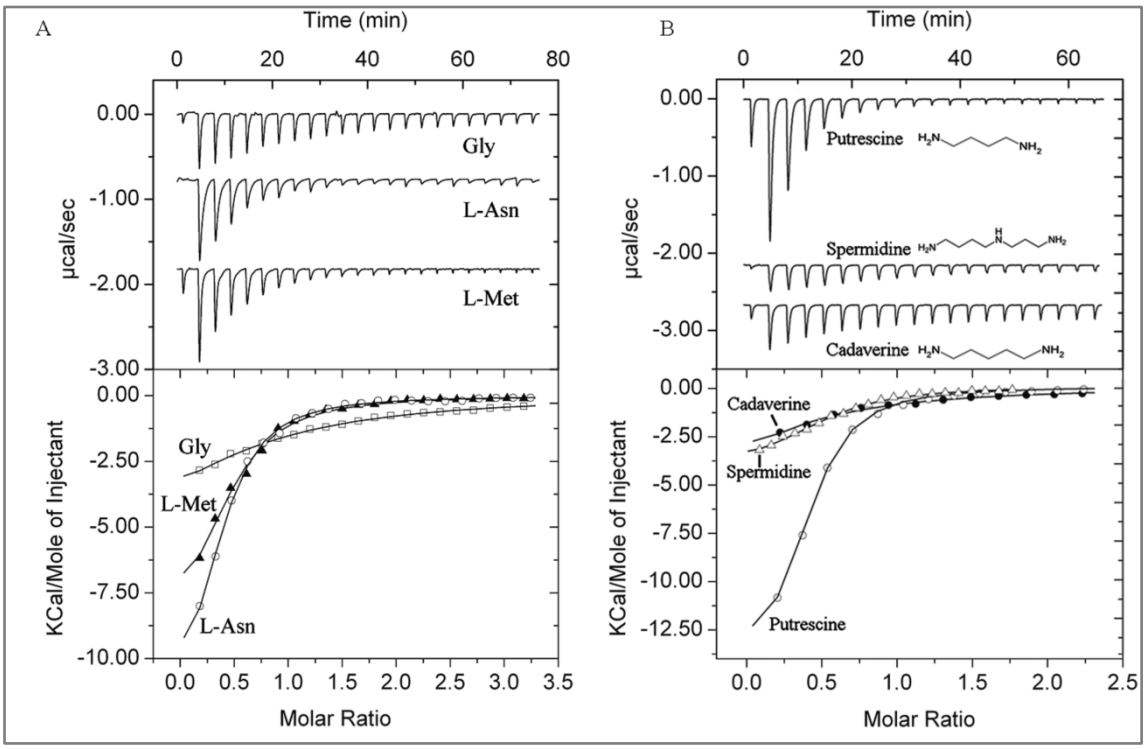


Figure 5) Microcalorimetric studies for the binding of different ligands to the purified recombinant LBDs of MCPs PP2249/McpA (A) and PP1228/McpU (B). Upper panels: raw data for the titration of 25 - 30 µM of protein with 0.25–1 mM of amino acids or polyamines. Typically, a single 1.6 µl injection was followed by a series of 6.4–12.8 µl injections. Lower panels: Dilution-corrected and concentration-normalized integrated peak areas of the raw data. Fitting was done using the "One binding site model" of the MicroCal version of ORIGIN.

To verify whether PP2249 is a chemotaxis receptor, quantitative capillary assays were carried out using wt and PP2249, and taxis towards L-Tyr and L-Val were measured. A maximal chemotactic response was observed for wt at 0.5 mM L-Tyr and 50 mM L-Val (Fig. 6); however, at these concentrations, this response was largely diminished in the PP2249 mutant, indicating that PP2249 does, in fact, mediate chemotaxis towards L-amino acids. The receptor was thus named McpA.

To put these data in a physiological context we semi-quantitatively analysed the chemotactic behaviour of *P. putida* KT2440R towards all proteinogenic amino acids using plate gradient assays. As shown in Table 2, responses were detected for 15 different L-amino acids, including all the 12 amino acids that McpA-LBD was found to bind. We subsequently investigated the amino acid requirement for sustained, efficient bacterial growth and found that 14 amino acids were sufficient as a nitrogen source and 13 amino acids were sufficient as a carbon source (Supp. Fig. 5). There was no significant correlation between these data and the capacity of the strain to respond chemotactically to different amino acids.

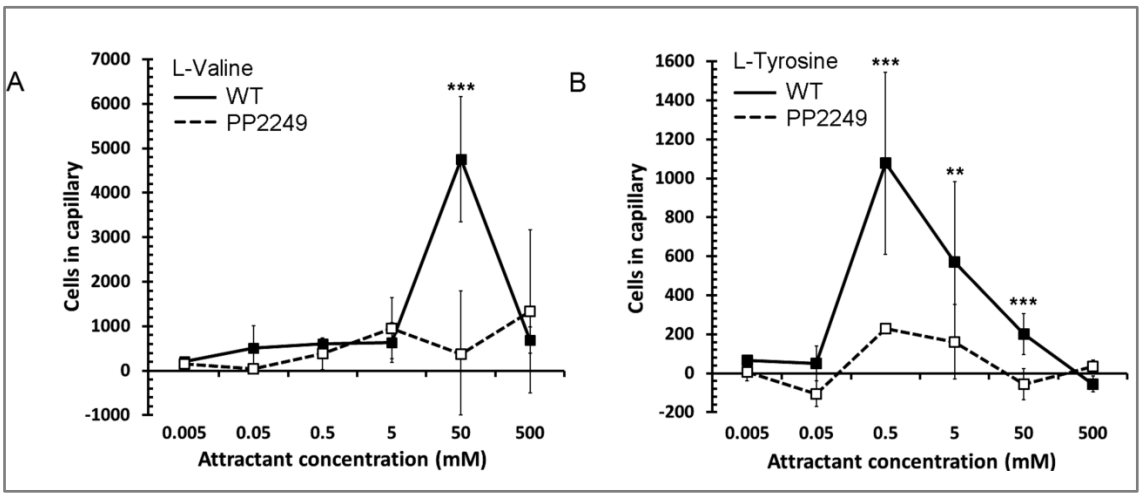


Figure 6) Quantitative capillary chemotaxis assays of *P. putida* KT2440R and its PP2249 toward different concentrations of L-Val (A) and L-Tyr (B). Shown are means and standard deviations from three independent experiments conducted in triplicate. Data were corrected with the number of bacteria that swam into a buffer containing capillary. **P<0.01, ***P<0.001 in Student's t-test indicate statistical significance.

Identification of PP1228 as a polyamine chemotaxis receptor

Based on the failure of PP1228-LBD to recognize any of the ligands tested, we conducted high-throughput thermal shift assay-based ligand screens (Krell, 2015; McKellar et al, 2015). In this assay, a temperature gradient is applied to a mixture of protein and fluorescent dye. Protein

unfolding causes additional dye binding resulting in fluorescence changes, which can be used to calculate the midpoint of protein unfolding (Tm; the temperature at which half the protein is in its native form and the other half is unfolded). Typically, ligand binding to a protein causes an upshift in Tm. We have therefore conducted thermal shift assays of the recombinant PP1228-LBD protein in the presence of compounds from ligand collections. To this end we have used plates PM1 and PM2A from Biolog Inc. that contain 190 different carbon sources. A Tm value of 46 ºC was determined for ligand free PP1228-LBD and Fig. 7 shows the Tm changes for each of the 95 compounds of ligand plate PM2A. Whereas most ligands caused changes from -2 to +1 ºC, putrescine caused an upshift of 11 ºC. To verify this hit, a microcalorimetric titration of the protein with putrescine was carried out (Fig. 5B). Large exothermic heat changes due to binding were recorded and data analysis revealed a dissociation constant (K_D) of 2 µM in a binding process driven by favourable enthalpy changes (Table 2). These results confirm that putrescine is a ligand of PP1228.

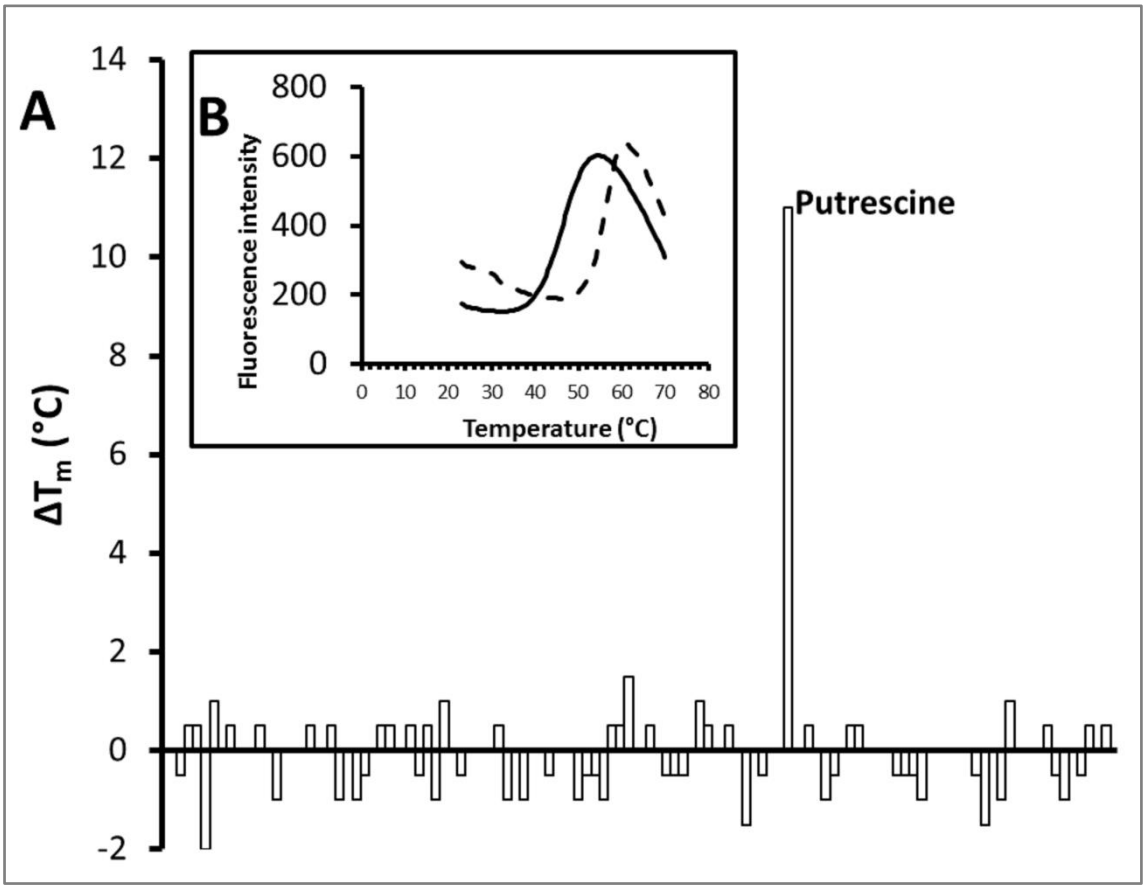


Figure 7) Thermal shift assays for the high throughput identification of PP1228/McpU ligands. **A)** Increases in Tm in the presence of compounds from Biolog plate PM2A (compounds of this plate are listed at http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf. **B)** Fluorescence raw data of the protein without ligand (continuous line) and in the presence of putrescine (dashed line).

We continued to explore the ligand profile of PP1228. Using isothermal titration calorimetry (ITC), we screened a large number of different compounds (see Table 2 legend). Of all these compounds, binding was only observed for spermidine (4.5 µM affinity) and cadaverine (22 µM affinity), as shown in Fig. 5B. No binding was observed for a number of closely related compounds, such as amino acids, aminopropyl-cadaverine, spermine, L-ornithine, gamma-aminobutyrate or butyrate, indicating that this receptor specifically recognizes polyamines.

To elucidate whether PP1228 mediates chemotaxis, plate gradient assays were conducted (Fig. 8A, B). In this assay, the chemoattractant is placed at different points along the central vertical line of a soft agar plate. After overnight incubation for gradient formation, wt and mutant bacteria are placed at defined distances from the site of chemoattractant deposition. The inspection of the plates showed an acentric spread of the wt strains towards immobilized spermidine and putrescine. Chemotaxis indices, calculated according to (Pham & Parkinson, 2011), were found to be 0.58 ± 0.02 (n=5) for spermidine and 0.59 ± 0.03 (n=5) for putrescine—values that are indicative of chemoattraction. For both compounds the spread of the PP1228 strain was minor and only slightly acentric, indicative of a strong reduction in chemotaxis in this mutant strain.

To confirm these results, quantitative capillary chemotaxis experiments were conducted. The response to spermidine was maximal at 5 mM (Fig. 8C), whereas chemotaxis towards putrescine and cadaverine occurred over a wide concentration range (0.1 µM -10 mM) (Fig. 8D, E). In all cases, the PP1228 mutant showed strongly reduced taxis, indicating that PP1228 is a MCP specific to polyamines. Taking these results into consideration, we named the MCP McpU. The residual taxis observed suggests the existence of another receptor that mediates responses to polyamines. We also conducted growth experiments for McpU ligands, the results of which revealed that the three ligands permit growth of the bacterium as sole carbon and energy source (Supp. Fig. 6).

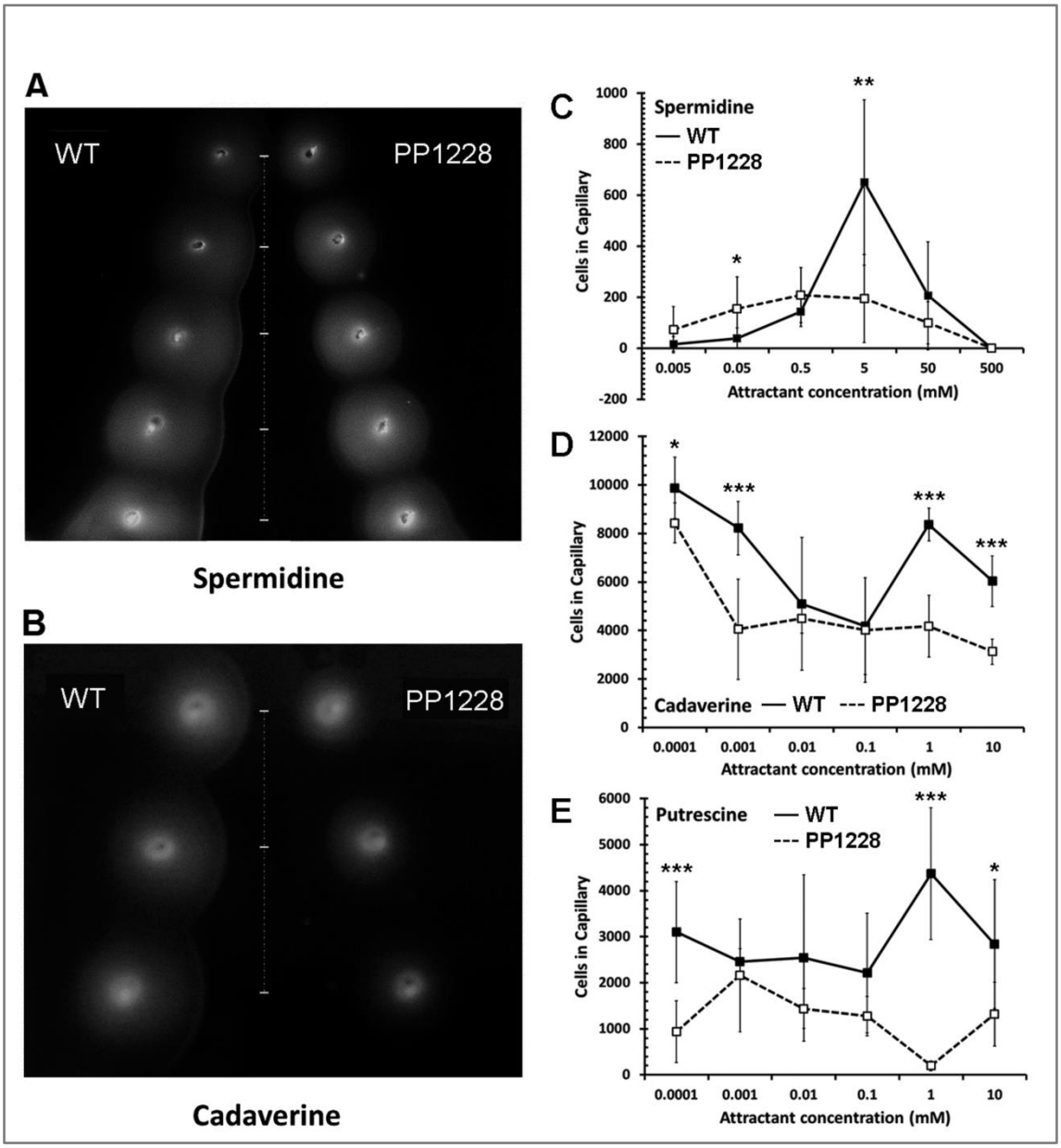


Figure 8) Chemotaxis assays of *P. putida* KT2440R and its mutant PP1228 towards different polyamines. A, B) Plate gradient chemotaxis assays. A 50 mM solution of spermidine or putrescine was deposited at the positions marked along the central vertical line. Aliquots of the wt and mutant strain were deposited at different distances to the site of chemoattractant deposition and plates were inspected the following day. **C-E)** Quantitative capillary chemotaxis assays of *P. putida* KT2440R and its mutant PP1228 to different concentrations of putrescine, cadaverine and spermidine. Shown are means and standard deviations from three independent experiments conducted in triplicate. Data were corrected with the number of bacteria that swam into a buffer containing capillary. *P<0.05, **P<0.01, ***P<0.001 in Student's t-test indicate statistical significance.

Influence of MCP mutations on competitive plant root colonization

We assessed the effect of the mutation of MCPs on the interaction of KT2440R with plants. To this end, the four mutant strains that showed altered biofilm phenotypes were submitted to competitive corn root colonization assays. In this assay seedlings are inoculated with bacterial mixtures containing the same number of wt and mutant cells. After one week of growth, plants were collected and root-associated bacteria were recovered and quantified using the different antibiotic susceptibilities of wt and mutant strains. Fig. 9 shows that mutants PP2249 and PP2310 had approximately the same capacity as the wt to colonize the plant root. In contrast, strains PP1228 and PP1488 were significantly less competitive than wt, indicating that McpU-mediated chemotaxis and WspA-Pp mediated alteration of c-di-GMP levels also modulate root attachment. To verify whether the biofilm and root colonization phenotypes of PP1228 can be attributed to polyamine chemotaxis or potentially to changes in the c-di-GMP level, the PP1228 mutant harbouring the c-di-GMP biosensor has also been analysed by fluorescence microscopy. The fluorescence intensity of the strain, grown on LB agar plates without and with added putrescine, was similar to that of the wild type (Fig. 4). This indicates that the phenotypic changes are not due to alterations in the c-di-GMP level but to chemotaxis to polyamines.

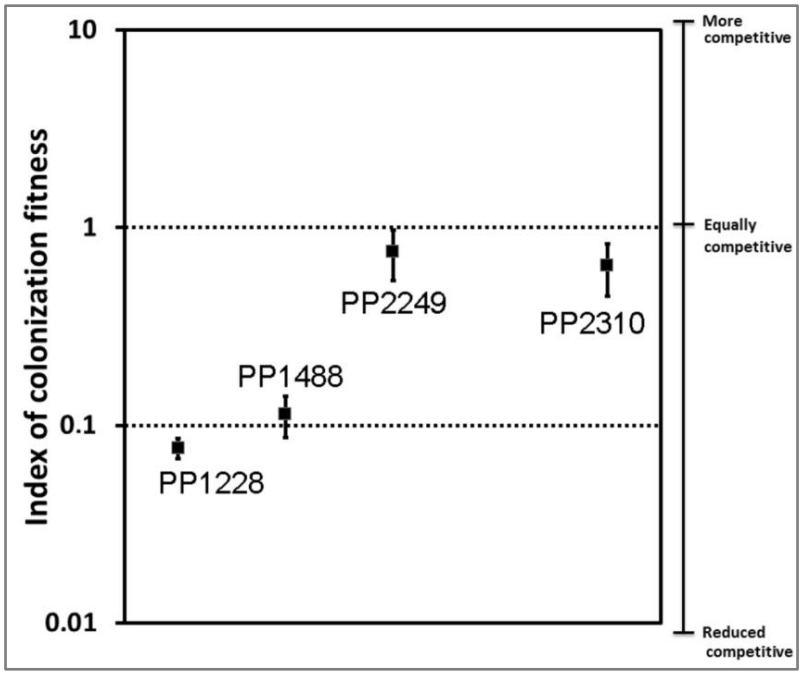


Figure 9) The influence of MCP mutation on plant root colonization. Competitive root colonization assays of *P. putida* KT2440R and each of the four mutant strains with altered biofilm formation capabilities. After 1 week, root associated bacteria were recovered and quantified. Shown is the colonization fitness representing the ratio of recovered wt over mutant strain. Experiments shown are mean and standard deviation of 2 independent experiments conducted on 6 different plants.

Discussion

The *wsp* pathway is the best characterized example of a chemosensory pathway that serves alternative cellular functions (Wuichet & Zhulin, 2010). The gene cluster encoding pathway proteins are highly homologous in *P. aeruginosa* PAO1 and *P. putida* KT2440 (Supp. Fig. 4). Pathway output is mediated by the WspR RR for which phosphorylation was found to stimulate c-di-GMP production and consequently biofilm formation (Hickman et al, 2005). The WspA_Pp mutant PP1488 was the only strain that had significantly reduced biofilm formation (Fig. 2) and mutant complementation resulted in the recovery of a wt-like phenotype (Fig. 3). In addition, the WspA_Pp mutant was significantly less efficient at colonizing plant roots (Fig. 9). These data agree with previous results from our group (Garcia-Fontana et al, 2013), which showed that mutation of the gene encoding the CheR methyltransferase of this pathway (*pp1490*) in *P. putida* KT2440 resulted in a strong reduction in biofilm formation. We show here (Fig. 4) that the mutation of the *wspA-Pp* gene significantly reduced the cellular c-di-GMP levels, indicating that there is a functional *wsp* pathway in *P. putida* KT2440. Interestingly, mutation of WspA in *P. aeruginosa* did not significantly impact biofilm formation (Hickman et al, 2005). These data underline the need for future investigations aimed at establishing the unknown ligand(s) of WspA.

E. coli, the model bacterium used historically for the study of chemotaxis, encodes four well-characterized MCPs and their responses to amino acids, dipeptides and sugars have been established (Hazelbauer et al, 2008). More recent studies in other bacteria have shown that many MCPs respond to two major groups of chemoattractants, namely amino acids (Brennan et al, 2013; Glekas et al, 2012; Hartley-Tassell et al, 2010; Oku et al, 2012; Taguchi et al, 1997; Webb et al, 2014) and different organic acids (Alvarez-Ortega & Harwood, 2007; Lacal et al, 2010a; Luu et al, 2015; Ni et al, 2013; Oku et al, 2014; Parales et al, 2013; Yamamoto & Imae, 1993). The identification of the first polyamine specific MCP, McpU, thus expands the range of known MCP types. Chemotaxis towards cadaverine and putrescine has been observed in *P. aeruginosa* PAO1, but taxis was abrogated in a triple mutant for PctA, PctB and PctC receptors (Taguchi et al, 1997). Because these ligands did not bind to the recombinant LBDs of these receptors, it was proposed that polyamine binding may occur indirectly via a periplasmic binding protein (Rico-Jimenez et al, 2013); however, our thermal shift and ITC binding studies show that McpU function is based on direct ligand recognition.

Polyamines are very abundant in plants, particularly when exposed to different types of stress (Minocha et al, 2014). For example, putrescine was found at a concentration of 275 µM in tobacco leaves (Ioannidis et al, 2012). In addition, significant amounts of putrescine have been detected in different plant exudates (Kuiper et al, 2001). Because *P. putida* KT2440 has a saprophytic lifestyle and is able use polyamines for growth, it is not surprising that we identified a polyamine-specific MCP in this strain. Apart from being a growth substrate, there is also evidence that polyamines act as signaling molecules and were found to play key roles in mediating bacteria-host interactions (reviewed in (Di Martino et al, 2013). In addition, there are several examples demonstrating a link between polyamines and biofilm formation. Specifically, it was shown that the inactivation of polyamine biosynthesis or addition of polyamines to cultures modulated biofilm formation, as reviewed by (Shah & Swiatlo, 2008). A particularly interesting study involved a screen of a mutant library of *Shewanella oneidensis* for strains with enhanced biofilm formation (Ding et al, 2014). A clear phenotype was observed for a mutant with disrupted putrescine biosynthesis. The authors therefore proposed that putrescine is a compound that mediates biofilm matrix disassembly in *S. oneidensis*. A potential molecular mechanism for this effect may be inferred from another study (Cockerell et al, 2014), which found that spermidine reduces biofilm formation of *Vibrio cholerae*. The authors demonstrate that spermidine is sensed by the NspS sensor, which in turn modulates c-di-GMP levels. Based on a comparative genomics analysis, which revealed the presence of NspS-like proteins in a variety of bacteria, the authors proposed that such mechanism may represent a general mechanism. These data are consistent with our finding that abolishing chemotaxis towards putrescine and spermidine (ie, reducing the effective concentration of these compounds) leads to enhanced biofilm formation.

The effect of putrescine on gene expression has been assessed in the highly homologous *P. putida* S12 using the *P. putida* KT2440 micro-array. Amongst the 79 differentially regulated genes was a single MCP encoding gene, namely *pp1228*, which showed a 28-fold upregulation in the presence of putrescine (Bandounas et al, 2011). Here, we show that this gene encodes a MCP for putrescine and data thus indicate strong receptor induction in response to its cognate ligand. The identification of McpU also illustrates the power of the high throughput thermal shift-based method for the identification of signal molecules as previously discussed (Krell, 2015; McKellar et al, 2015). The large Tm shift in the presence of putrescine (11ºC) (Fig. 7) indicate an excellent signal to noise ratio. There is no doubt that this approach will facilitate the functional annotation of other sensor proteins.

Our findings also reveal that McpA is a chemotaxis receptor for L-amino acids. *P. putida* KT2440R shows chemotaxis towards 15 proteinogenic amino acids and twelve of these compounds were found to bind to McpA-LBD (Table 2). For the remaining three amino acids, weak chemotaxis was observed for L-Leu and L-Lys, whereas very strong chemotactic responses were detected towards L-Pro. As detailed in the footnote of Table 2, it cannot be excluded that L-Leu and L-Lys bind to McpA-LBD with a very low affinity. However, chemotaxis towards proline may be mediated by an additional MCP, as a receptor that mediates specific and strong chemotactic responses towards proline has been identified recently in *Sinorhizobium meliloti*, another soil bacterium (Webb et al, 2014). The five amino acids that did not serve as chemoattractants were not found to be McpA ligands, confirming the notion that ligand binding at McpA causes chemotaxis. Similarly to what we observed for McpU, a mutation in McpA led to increased biofilm formation. Similar observations have been made for the multi-ligand binding MCP CcmL of *Campylobacter jejuni* (Rahman et al, 2014). This receptor was shown to mediate chemotaxis towards a wide range of different compounds including organic and amino acids. A CcmL mutant, while showing reduced chemotaxis, exhibited significantly increased biofilm formation.

It has been shown that D-amino acids prevent biofilm formation and promote biofilm dispersal in different bacteria (Kolodkin-Gal et al, 2010). Although our data indicate that McpA is specific for L-amino acids, *P. putida* KT2440 was found to have three different amino acid racemases that can catalyse the racemization of 19 proteinogenic amino acids (Radkov & Moe, 2013). It is possible that chemotaxis towards L-amino acids, which could subsequently be converted to D-isomers, may in part be responsible for the changes in biofilm formation that were observed. In contrast to the well-studied amino acid receptors Tar and Tsr of *E. coli*, which possess a 4-helix bundle LBD (Hazelbauer et al, 2008), McpA has a double PDC LBD (Supp. Fig. 7). Over the last decade a significant number of double PDC-containing amino acid MCPs have been identified in a range of different species (Reyes-Darias et al, 2015c). It had been suggested that this receptor type may be the predominant amino acid receptor in bacteria (Reyes-Darias et al, 2015c)—a notion supported by the identification of McpA. The analysis of the ligand profiles of these receptors has permitted differentiation between receptors with broad and narrow ligand profiles (Reyes-Darias et al, 2015c). Here, we show that McpA binds 12 different amino acids—a trait that is indicative of broad range receptors. PctA of *P. aeruginosa* is a well characterised broad range amino acid MCP (Reyes-Darias et al, 2015c; Rico-Jimenez et al, 2013; Taguchi et al, 1997) and McpA can be regarded as a functional PctA homologue. Also, there remains a possibility that an additional L-Pro specific receptor exists.

P. putida KT2440R responds chemotactically to 15 amino acids and there was no significant correlation between taxis and the use of different amino acids for growth. A lack of correlation between chemotaxis towards particular amino acids and their catabolism has been recently reported for *Bacillus subtilis* (Yang et al, 2015). The authors of that study concluded that amino acids do not attract the bacteria because of their nutritional value, but rather because they serve as environmental cues.

Two MCP mutants showed reduced root colonization capacity. However, the mechanisms for this phenotype differed: whereas McpU is a chemotaxis MCP, WspA-Pp modulates c-di-GMP levels. These observations are consistent with previous studies showing that c-di-GMP levels modulate root colonization capacity (Matilla et al, 2007; Matilla et al, 2011; Perez-Mendoza et al, 2014) and that chemotaxis to root exudates facilitates colonization (de Weert et al, 2002; Oku et al, 2014; Oku et al, 2012; Reyes-Darias et al, 2015a).

In summary, the discovery of a polyamine-specific MCP has broadened the spectrum of known chemotactic receptor types and will facilitate the identification of functional homologues in other species. Further research will show whether the involvement of amino acid and polyamine chemotaxis in biofilm formation is a more general phenomenon.

Supplementary material

Supp. table 1) Strains and plasmids used in this study.

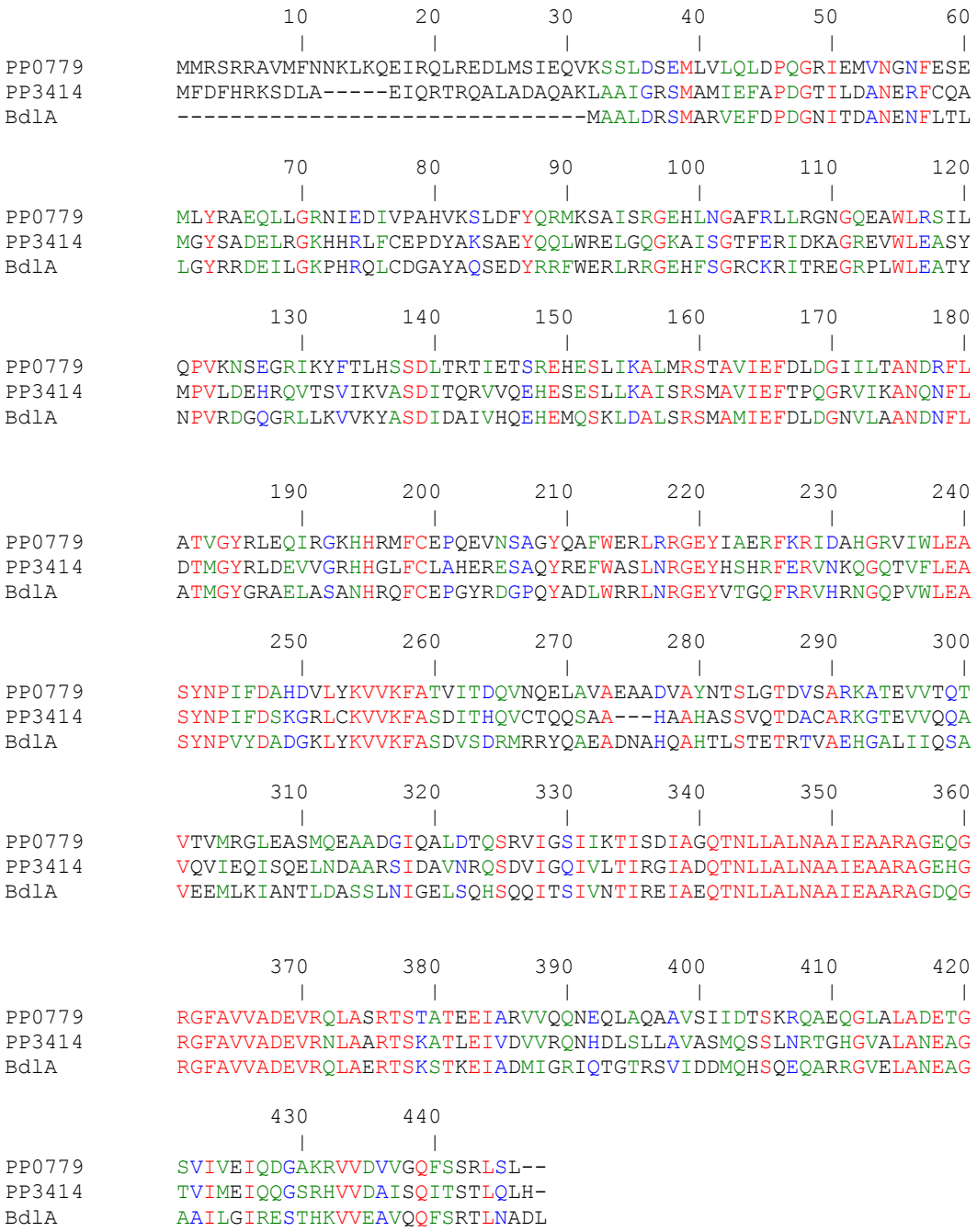
Plasmids	Relevant Characteristics	Reference
pUT-Km	Km^R	(de Lorenzo et al, 1990)
pRK600	Cm^R, *mob tra*	(Finan et al, 1986)
pCHESIΩKm	Km^R	(Kovach et al, 1995)
pCHESIΩGm	Gm^R	Estrella. Duque, personal gift
pCHESI-pp0779	Km^R; PCR product containing a 0.8-Kb region of *pp0779* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp1488	Km^R; PCR product containing a 0.7-Kb region of *pp1488* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp1940	Km^R; PCR product containing a 1.1-Kb region of *pp1940* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp2111	Km^R; PCR product containing a 0.5-Kb region of *pp2111* was inserted into the SacI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp2823	Km^R; PCR product containing a 0.6-Kb region of *pp2823* was inserted into the SacI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp2861	Km^R; PCR product containing a 0.9-Kb region of *pp2861* was inserted into the SacI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp3414	Km^R; PCR product containing a 0.7-Kb region of *pp3414* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp3557	Gm^R; PCR product containing a 0.7-Kb region of *pp3557* was inserted into the EcoRI/KpnI sites of pCHESIΩGm	This study
pCHESI-pp4888	Km^R; PCR product containing a 0.7-Kb region of *pp4888* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp5020	Km^R ; PCR product containing a 0.6-Kb region of *pp5020* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp5021	Km^R; PCR product containing a 0.6-Kb region of *pp5021* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pBBR1MCS-5	Gm^R; *oriRK2*, *mobRK2*	(Kovach et al, 1995)
pET28b	Km^R, protein expression vector	Novagen
pET28b-	Km^R; pET28b derivative used to produce His-tagged	This study

LBDMcpA	LBD of McpA (PP2249)	
pET28b-LBDMcpU	Km^R; pET28b derivative used to produce His-tagged LBD of McpU (PP1228)	This study
pBBR1MCS-5-PP2249	Gm^R; 2.2-Kb PCR product containing *pp2249* inserted into BamHI/SacI sites of pBBRMCS-5; for gene complementation.	This study
pBBR1MCS-5-PP1488	Gm^R; 1.8-Kb PCR product containing *pp1488* inserted into SpeI/SacI sites of pBBRMCS-5; for gene complementation.	This study
pBBR1MCS-5-PP1228	Gm^R; 2.6-Kb PCR product containing *pp1228* inserted into EcoRI/XbaI sites of pBBRMCS-5; for gene complementation.	This study
pBBR1MCS-5-PP2310	Gm^R; 1.8-Kb PCR product containing *pp2310* inserted into EcoRI/XbaI sites of pBBRMCS-5; for gene complementation.	This study
pMAMV1	Gm^R; pBBR1MCS-5 based vector containing *rup4959-4957* genes from KT2440. Confers high levels of c-di-GMP.	(Matilla et al, 2011)
pCdrA::gfpC	Amp^R, Gm^R; pUCP22Not-P*cdrA*-RBS-CDS-RNaseIII-*gfp*(Mut3)-T0-T1. C-di-GMP biosensor.	(Rybtke et al, 2012)

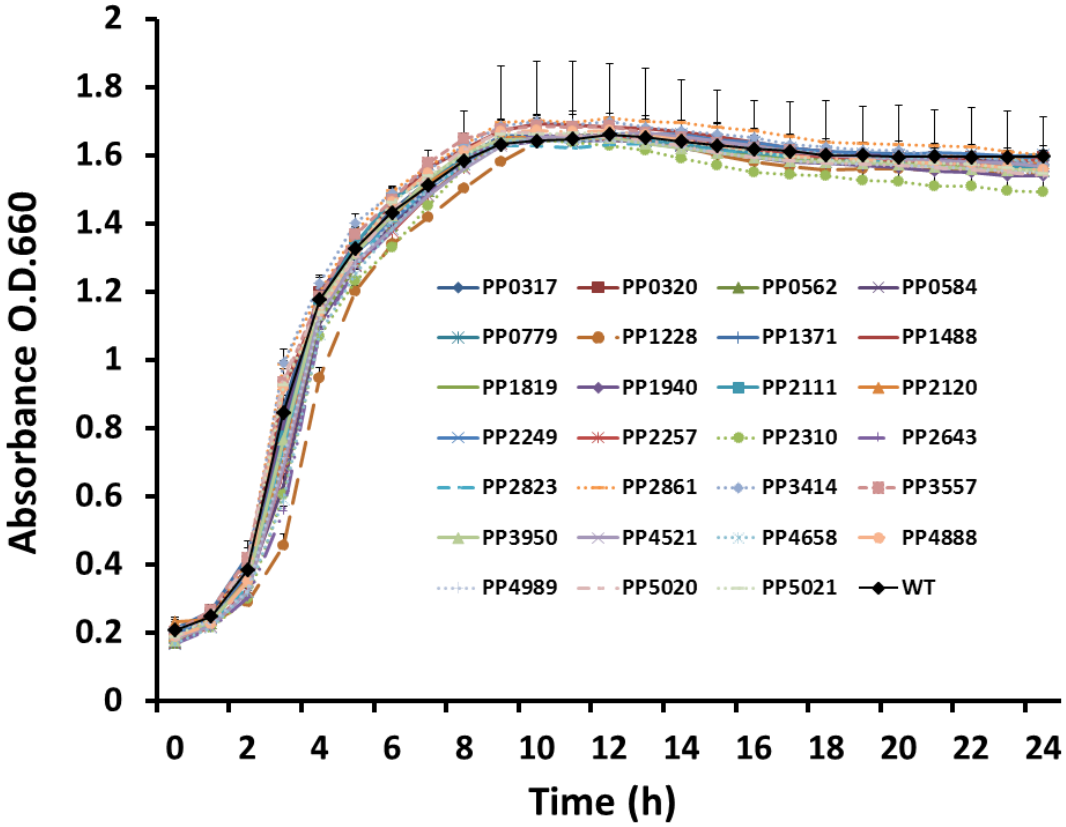
Supp. Table 2) Oligonucleotides used in this study.

Primer	Sequence (5´-3´)	Description
PP1488fw	AAGAATTCGGCAAGTTTCGCCGTGAT	Construction of strain KT2440R TK8
PP1488rev	AAGGATCCGCGATCCAGGCTCAGGCGCA	Construction of strain KT2440R TK8
PP2861-SacI-F	AAGAGCTCGCGCTACGACCATGACGACT	Construction of strain KT2440R TK18
PP2861-BamHI-R	TTGGATCCTCGGCAATGGAACGAATCAC	Construction of strain KT2440R TK18
PP3414-EcoRI-F	AAGAATTCCTGTTCTGTGAGCCGGACTA	Construction of strain KT2440R TK19
PP3414-BamHI-R	AAGGATCCATCACATCCGACTGCCTGTT	Construction of strain KT2440R TK19
PP3557-EcoRI-F	AAGAATTCCTGTCTGGCCGCTATCGTCA	Construction of strain KT2440R TK20
PP3557-KpnI-R	AAGGTACCTGAGAATGCCGACCGTGGTG	Construction of strain KT2440R TK20
PP5020fw	AAGAATTCTGACCGGCTGGCACGGCATG	Construction of strain KT2440R TK26
PP5020rev	AAGGATCCCGCCCATGGCTGTGGCGGCA	Construction of strain KT2440R TK26
PP5021fw	AAGAATTCTCAGCGGCTGGCGTGCCCTG	Construction of strain KT2440R TK27
PP5021rev	AAGGATCCTGATGGCGCGTGGCAAGCCC	Construction of strain KT2440R TK27
PP4888fw	AAGAATTCTCGGCCTGGGCATGGATGTT	Construction of strain KT2440R TK24
PP4888rev	AAGGATCCAGATCGGCGCCATGGCCCAG	Construction of strain KT2440R TK24
pp0779fw	AAGAATTCCGGCAACTGCGCGAAGACCTGATGTCCA	Construction of strain KT2440R TK5
pp0779rev	CGAGCACTGACATCGGATCCCAGCGAGGTGTT	Construction of strain KT2440R TK5
pp2111fw	ACCGCTGGAATTCCGGCGACCATTACTG	Construction of strain KT2440R TK11
pp2111rev	CTGGATCCTCAGGTGTTCGGCACTGTCT	Construction of strain KT2440R TK11
pp2823fw	GCGGTGGAATTCGACGACAACGACAACCGATTG	Construction of strain KT2440R TK17
pp2823rev	CAGGCCGATCATGGATCCGATTTCGTCTGAGGAGT	Construction of strain KT2440R TK17
pp1940fw	CTGCGGAATTCAGCCGTTCTTCAGTCG	Construction of strain KT2440R TK10
pp1940rev	TCACCAGCGCGAGCAGGATCCATAGCA	Construction of strain KT2440R TK10

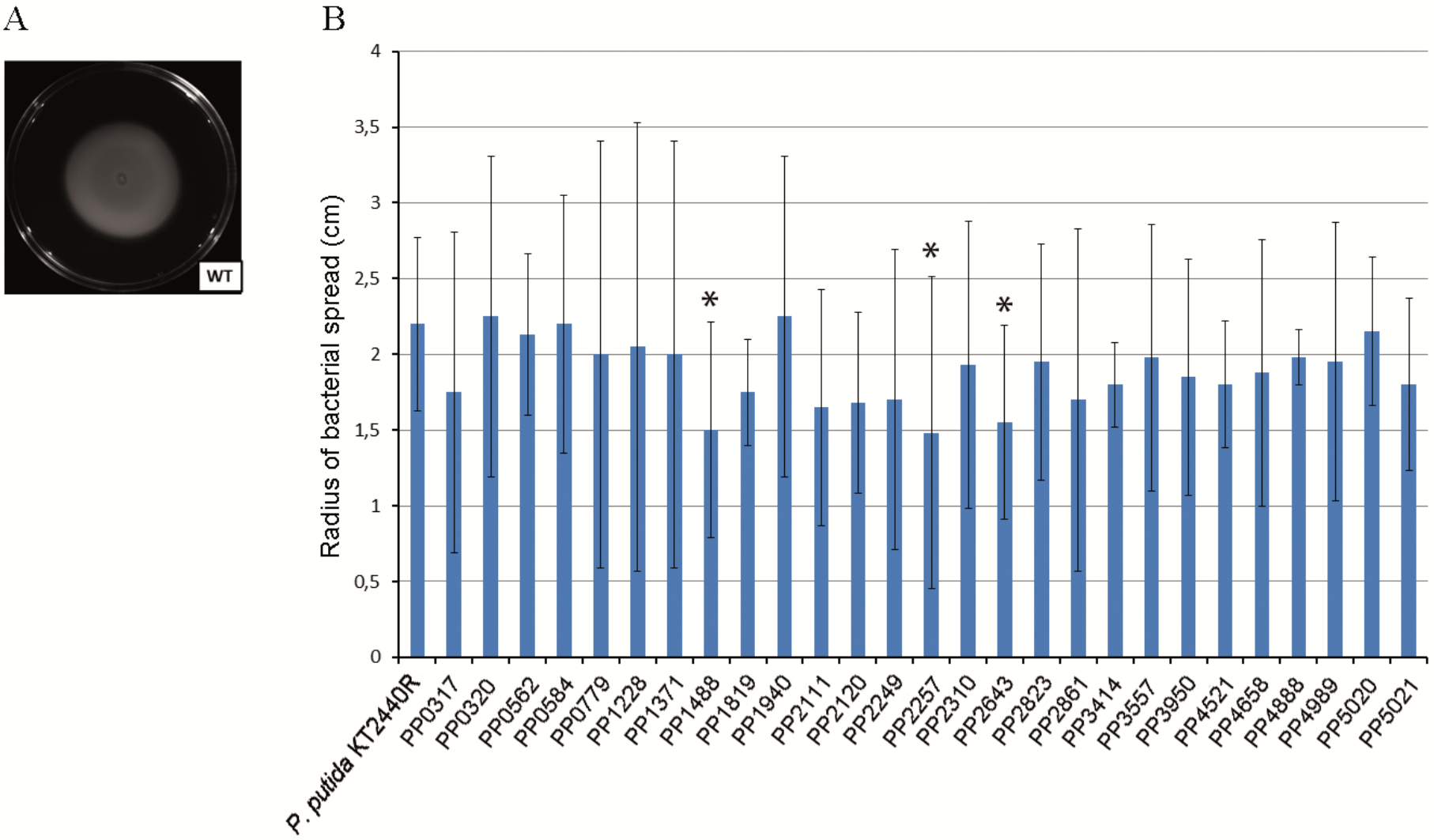
pCHESI Rev17	CAGGAAACAGCTATGAC	Sequencing of pCHESI inserts
Genpp2249fw	TCTGGTGCGCCTGGATCCCCGTAAT	Forward primer to clone PP2249 into pBBR1MCS-5
Genpp2249rv	GAGCTCGTACAGGCGACAACAGGCATAGGC	Reverse primer to clone PP2249 into pBBR1MCS-5
Genpp1488fw	AGACTAGTGGCGGTGCTTGCCACGGCCTATTGA	Forward primer to clone PP1488 into pBBR1MCS-5
Genpp1488rv	AGCGGTGCGCAGTTGGAGCTCGTTCATC	Reverse primer to clone PP1488 into pBBR1MCS-5
Genpp PP1228	AAGAATTCCCTGTCGGTGATCAACCACT	Forward primer to clone PP1228 into pBBR1MCS-5
Genpp PP1228	AATCTAGAGCTCATGCTGGCGTCGACCAG	Reverse primer to clone PP1228 into pBBR1MCS-5
Genpp PP2310	AAGAATTCcaagagcgggcgcaacgagcc	Forward primer to clone PP2310 into pBBR1MCS-5
Genpp PP2310	aaTCTAGAgctcatggggatttcctctcg	Reverse primer to clone PP2310 into pBBR1MCS-5
LBDMcpUfw	CTTGCTGGTCGCCACTCATATGACCCAGGCCCATC	Forward primer to clone LBD of PP1228 into pET28b
LBDMcpUrv	CAAGGCCTATCAGCAGGGATCCTCAGTTGGCGTTC	Reverse primer to clone LBD of PP1228 into pET28b
LBDMcpAfw	CTGTTCACGCATATGAACGACTACCTGCAACGCAATAC	Forward primer to clone LBD of PP2249 into pET28b
LBDMcpArv	ATCAGCATGGCAGGATCCTACGAGGTGCGGAACTG	Reverse primer to clone LBD of PP2249 into pET28b



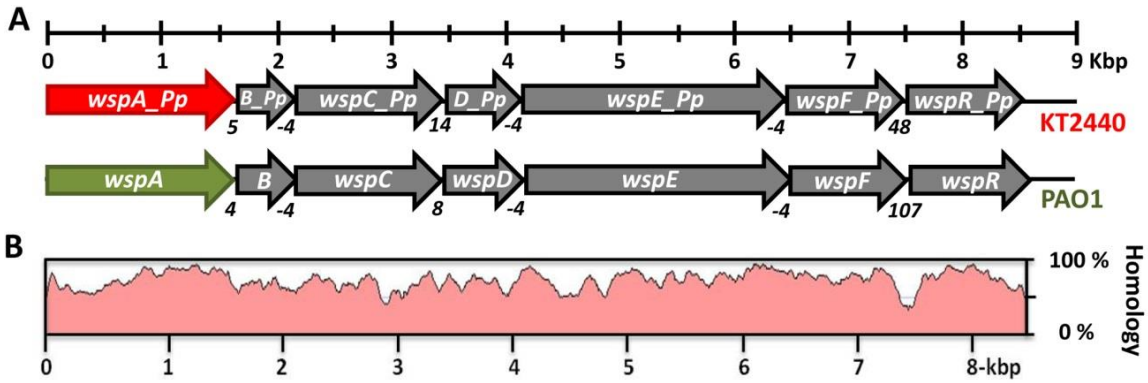
Supp. Fig. 1) Alignment of the sequence of *P. aeruginosa* PAO1 BdlA with its two homologues, PP0779 and PP3414, from *P. putida* KT2440. The overall sequence identity was of 28 %. Pairwise alignments revealed 37 and 45 % sequence identity between BdlA with PP3414 and PP0779, respectively. The alignment was done in the slow mode using the CLUSTALW multiple alignment tool (Thompson et al, 1994) of the NPS@ suite (Combet et al, 2000). The GONNET protein weight matrix was used and gap opening and gap extension penalties of 15 and 0.1 were applied, respectively.



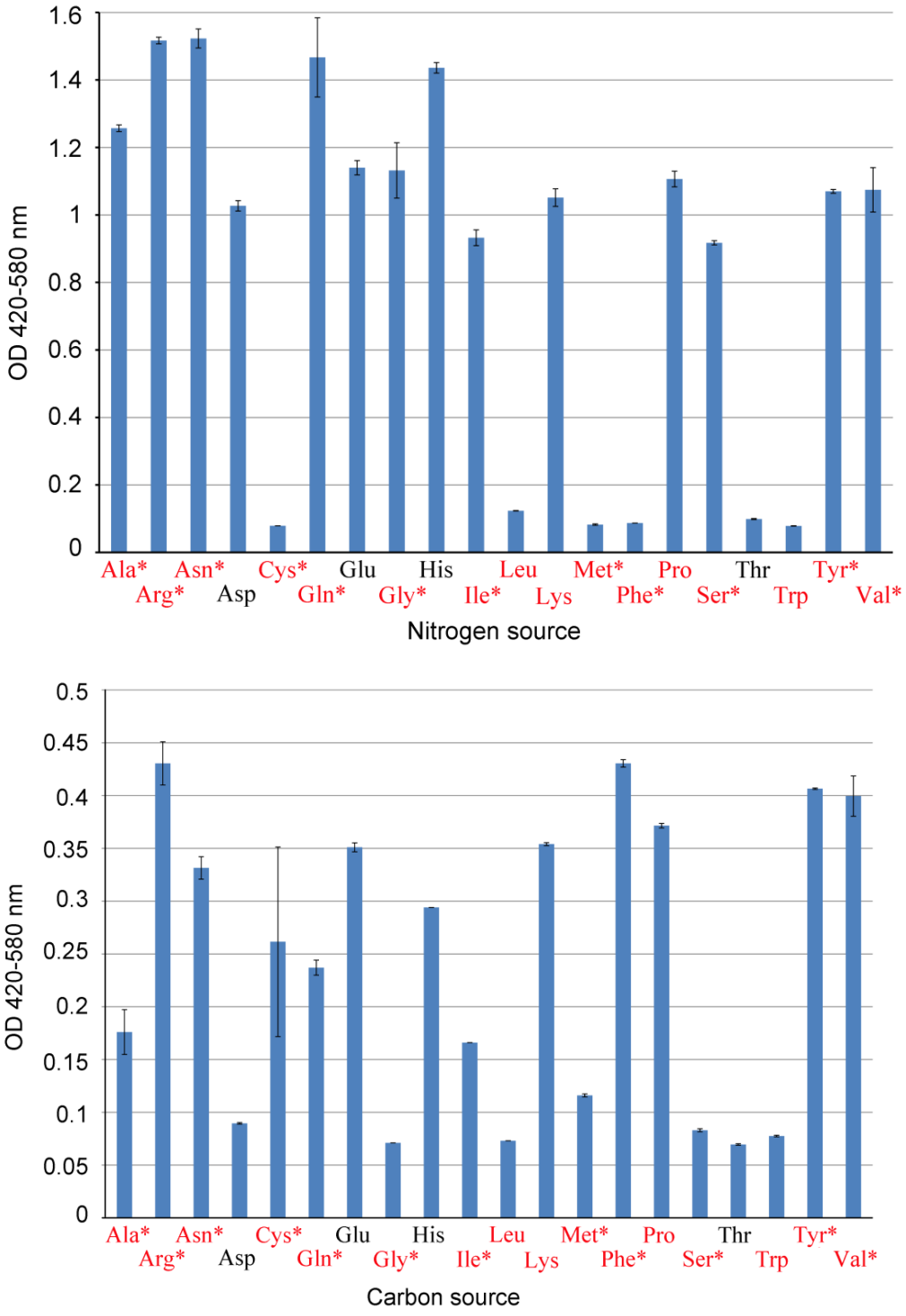
Supp. Fig. 2) Growth curves of *P. putida* KT2440R and its 27 mutants in MCP genes. Overnight cultures of *P. putida* KT2440R and derivative mutant strains were used to inoculate LB cultures to an OD_{660} of 0.01. These cultures were transferred to microwell plates and growth over the time was followed using Bioscreen Microbiological Growth Analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland). Every hour the $OD_{420\text{-}580nm}$ was measured.



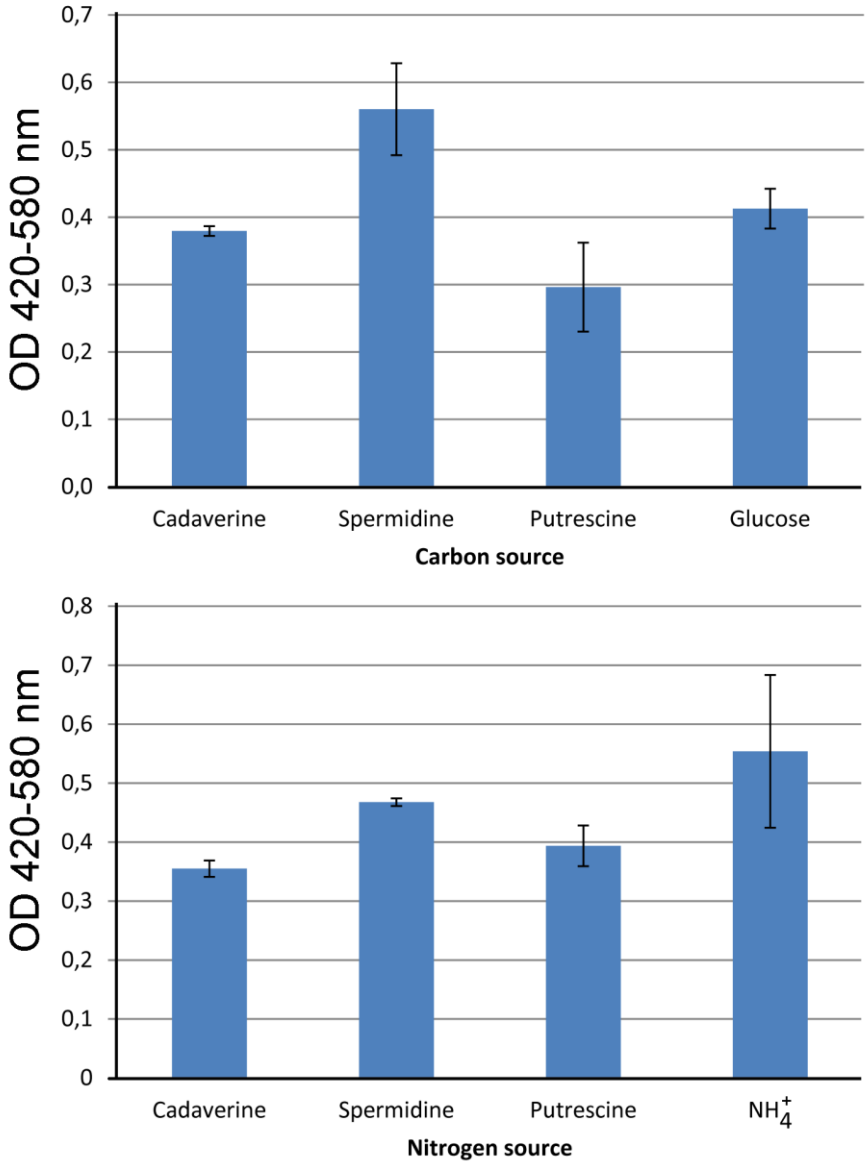
Supp. Fig. 3) Motility of *P. putida* KT2440R and its 27 mutants in MCP genes. **A)** A representative image of the swim plate assay. **B)** Means and standard deviations of the radii of bacterial spread as derived from three independent experiments. * P<0.5 in students' t-test.



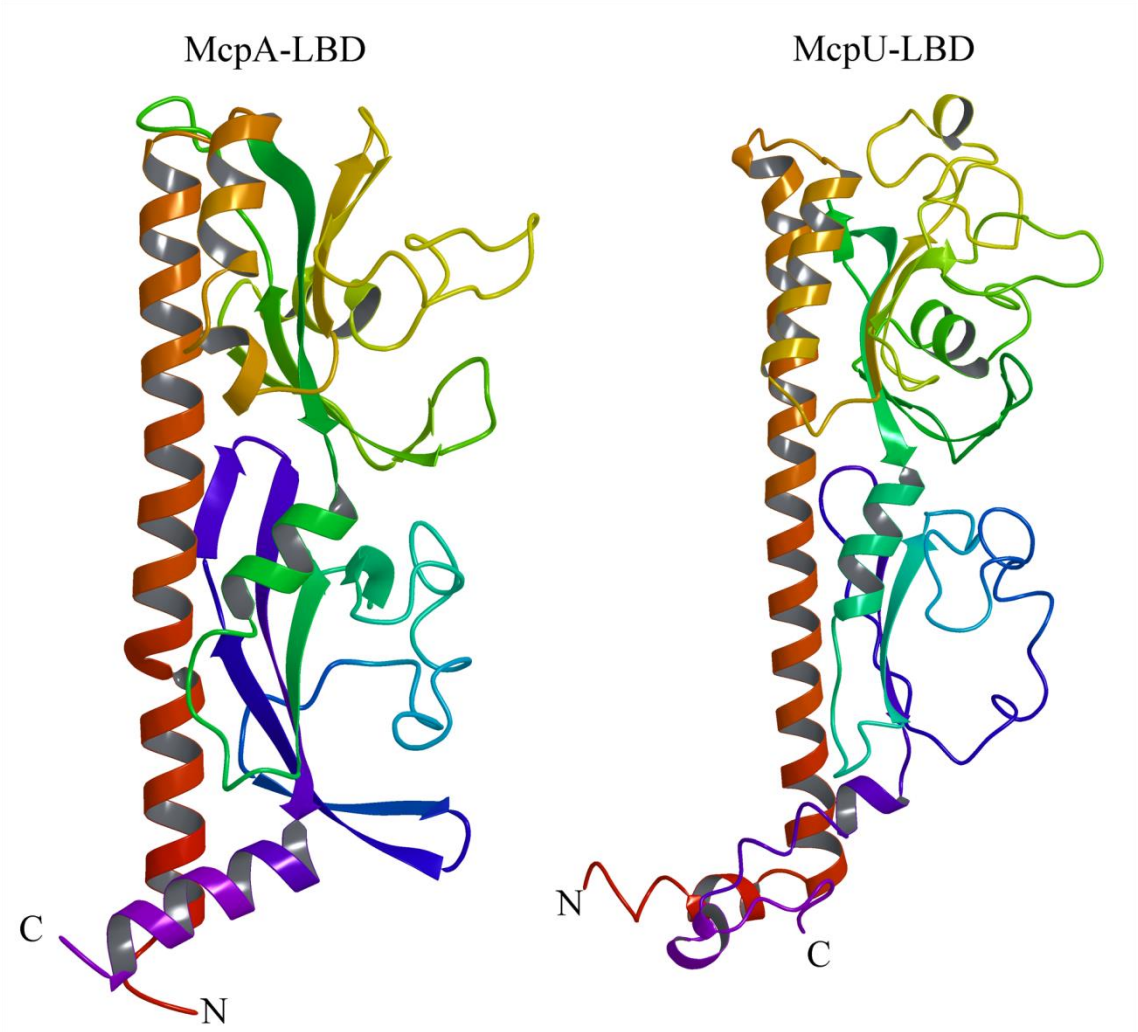
Supp. Fig. 4) The *wsp* gene clusters in *P. putida* KT2440 and *P. aeruginosa* PAO1. A) Genetic organization of the *wsp* gene clusters in KT2440 and PAO1 strains. The MCP genes *wspA_Pp* and *wspA* are shown in red and green, respectively. Numbers below the arrows represent the intergenic distance between contiguous genes, with negative numbers indicating overlapping genes. **B)** DNA homology between the *wsp* gene cluster of KT2440 and PAO1. Alignments were performed using wgVISTA (Frazer et al, 2004).



Supp. Fig. 5) Assessment of the capacity of the 20 proteinogenic amino acids to sustain growth of *P. putida* KT2440R as sole nitrogen or carbon source. Upper panel: Growth in M8 medium (Kohler et al, 2000) supplemented with glucose (0.1 % w/v) as carbon source and each of the 20 proteinogenic amino acids (5 mM) as potential nitrogen sources. Lower panel: Growth in M8 medium supplemented with ammonium chloride (0.1 g/l) as nitrogen source and each of the 20 proteinogenic amino acids (5 mM) as potential carbon sources. Shown are means and standard deviations (n=3) after 24 h growth at 30 ºC. Amino acids that were identified as chemoattractants are shown in red and those that bind to McpA-LBD are marked with an asterisk. Growth was measured using Bioscreen Microbiological Growth Analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland).



Supp. Fig. 6) Assessment of the capacity of polyamines to sustain growth of *P. putida* KT2440R as sole nitrogen or carbon source. Upper panel: Growth in M8 medium supplemented with ammonium chloride (0.1 g/l) as nitrogen source and different carbon sources (5 mM). Lower panel: Growth in M8 medium (Kohler et al, 2000) supplemented with glucose (0.1 % w/v) as carbon source and different nitrogen sources (5 mM). Shown are means and standard deviations (n=3) of absorbance readings after 24 h growth at 30 ºC. Growth was measured using Bioscreen Microbiological Growth Analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland).



Supp. Fig. 7) Homology models of McpA-LBD and McpU-LBD, which were generated using the structures with pdb ID 3C8C and 3libA as templates, respectively. Models were generated using Phyre2 (Kelley & Sternberg, 2009).

CHAPTER 2

Purification of functional recombinant LasR and RhlR of *Pseudomonas aeruginosa* from *Escherichia coli* cultures

CHAPTER 2 Purification of functional recombinant LasR and RhlR of *Pseudomonas aeruginosa* from *Escherichia coli* cultures

Brief Introduction

QS is a mechanism that enables communication amongst bacteria, which is based on the synthesis, detection and response to chemical signals. As cell density increases, these QSS accumulate in the environment and are sensed by receptors, which in turn control the expression of genes that direct activities which are beneficial when performed by groups of bacteria acting in synchrony (Rutherford & Bassler, 2012). The molecular mechanism of QS has been studied in a number of organisms. The opportunistic pathogen *P. aeruginosa* has become a paradigm since it employs a sophisticated multi-signal QS system that is based on the synthesis and detection of two different classes of signal molecules, namely Pseudomonas quinolone signals and AHL. In *P. aeruginosa* PAO1 the two principal AHL quorum-sensing signals, *N*-3-Oxododecanoyl-homoserine lactone (3-Oxo-C12-AHL) and *N*-butanoyl-homoserine lactone (C4-AHL) are produced by the AHL synthases LasI (More et al, 1996; Parsek et al, 1999) and RhlI (Pearson et al, 1995; Winson et al, 1995), respectively. This strain was also shown to produce small quantities of a number of additional AHLs like 3-Oxo-C6-AHL, 3-Oxo-C8-AHL, 3-Oxo-C10-AHL or C6-AHL (Ortori et al, 2011; Shaw et al, 1997; Winson et al, 1995).

The genes encoding the QS receptors LasR and RhlR are found in the vicinity of their cognate synthase genes *lasI* and *rhlI*. The paralogous LasR and RhlR regulators are LuxR type proteins (Brint & Ohman, 1995; Gambello & Iglewski, 1991) and are composed of an N-terminal auto-inducer binding domain and a HTH motif containing DNA binding domain at the C-terminal extension (Lintz et al, 2011). LasR and RhlR recognize AHLs and modulate directly or indirectly the expression of approximately 6 % of the genes (Schuster et al, 2003), amongst which are genes encoding virulence factors like hydrogen cyanide synthase, elastase, proteases and exotoxin A (Gambello et al, 1993; Passador et al, 1993; Pessi & Haas, 2000). Other LasR and RhlR targets are the genes of their cognate AHL synthases, *lasI* and *rhlI*, which triggers an auto-induction mechanism leading to a further increase in AHL concentration (Schuster et al, 2003; Seed et al, 1995). The LasR/LasI system dominates over the RhlR/RhlI system, since the former system is required for the induction of the latter (Latifi et al, 1996; Pearson et al, 1997). In addition to LasR and RhlR, there is a third QS regulator, QscR, that represses several genes that

are under the control of LasR or RhlR (Chugani et al, 2001). In contrast to *lasR* and *rhlR* the *qscR* gene is not found in the vicinity of an AHL-synthase.

QS regulators have also been studied in a number of different organisms. Based on their biochemical properties this family can be divided into two groups. The first group, exemplified by MrtR (Yang et al, 2009), EsaR (Minogue et al, 2002; von Bodman et al, 2003a) or SdiA (Kim et al, 2014), includes proteins that were found to be active and stable in the absence of bound AHL. The second family is composed of proteins that were found to be non-functional and insoluble in the absence of bound AHL, but soluble in its presence. Members of this family are LuxR (Qin et al, 2007; Urbanowski et al, 2004), TraR (Chai & Winans, 2005; Zhu & Winans, 1999; Zhu & Winans, 2001), CepR (Weingart et al, 2005) as well as the above mentioned LasR (Bottomley et al, 2007; Schuster et al, 2004) and QscR (Oinuma & Greenberg, 2011).

To explain this unusual behavior for a transcriptional regulator, an initial model was proposed (Oinuma & Greenberg, 2011; Zhu & Winans, 1999; Zhu & Winans, 2001) in which the regulatory proteins are synthesized as functional but unstable monomers. In the absence of AHLs, the proteins refold into non-functional conformations that aggregate or are tagged for proteolysis. It was proposed that during elongation the nascent polypeptide must fold around the AHL to achieve an active conformation (Zhu & Winans, 2001). The binding of AHL to newly synthesized protein causes protein stabilization leading to its accumulation and dimer assembly. Dimer formation is thought to additionally stabilize the protein. This view was also supported by the observation that AHLs bind with extremely high affinity, virtually irreversibly, to some, TraR (Zhu & Winans, 1999) and LasR (Schuster et al, 2004), but not all members of this group, like LuxR (Urbanowski et al, 2004). These unusual biochemical properties represent a major handicap in the study of these proteins. Since protein purified from lysates of *E. coli*, an organism that does not synthesize AHL, was found to be insoluble, AHL was added to the *E. coli* culture medium (Oinuma & Greenberg, 2011; Qin et al, 2000; Zhu & Winans, 1999). The AHLs were found to co-purify with the protein giving rise to partially saturated AHL receptors (Oinuma & Greenberg, 2011; Qin et al, 2000), which in turn hampers the study of their molecular mechanism.

However, this model is not consistent with several observations from *in vivo* experimentation. Firstly, several studies show that LasR and TraR are active in *E. coli* (Lee et al, 2006; Liu et al, 2011; Passador et al, 1996; Sappington et al, 2011; Winson et al, 1998). Since *E. coli* is unable to synthesize AHLs, these data suggest that both proteins do not require AHL for correct folding. Secondly, other studies showed that a rapid decrease in the environmental AHL

concentration induced rapid decreases in TraR and LasR mediated gene expression, which cannot be reconciled with ultra-tight AHL binding (Luo et al, 2003; Sappington et al, 2011; Su et al, 2008). Further *in vivo* evidence was presented suggesting that LasR remains functional following AHL dissociation (Sappington et al, 2011). The latter authors state that the most plausible explanation for these results is that when the concentration of AHL drops below a threshold, there is a dissociation of LasR and AHL. The authors therefore suggest that LasR may fit the general model for transcription factors rather than the virtually irreversible binding model described above (Sappington et al, 2011). Based on the evidence that LasR is active in AHL-free *E. coli*, we aimed at identifying experimental conditions that permit protein stabilization and purification in the absence of AHL.

Results

LasR and RhlR can be produced as recombinant proteins in E. coli in the absence of added AHL

The *lasR* and *rhlR* genes were cloned into expression vectors and proteins were expressed in *E. coli* in the absence of added AHL. The purification of both proteins was the result of an optimization process in which we tested different conditions for growth and buffers for cell rupture and protein purification. As for cell culture, a critical parameter was to lower growth temperature to 18°C following induction. An essential feature that permitted protein stabilization following cell rupture was the presence of 500 mM NaCl and 5 % glycerol in the lysis buffer and during purification. Since the concentration of 500 mM NaCl is un-physiologically high, we were determined to carry out protein analysis in a physiologically relevant buffer system. To identify a suitable analysis buffer we have then screened different buffers for its stabilizing effect and have identified an analysis buffer with physiologic pH and ionic strength (50 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, 1 mM DTT, pH 7.8). Both proteins were purified from the soluble fraction of the cell lysate and average yields were of 5.5 and 1.5 mg of purified proteins per liter *E. coli* culture for RhlR and LasR, respectively. The SDS-PAGE analysis of purified protein (supp. Fig. 1) showed that their purity is superior to 90 % permitting an analysis by different biophysical analyses that is described below. All analyses were conducted in the same buffer and with freshly purified protein.

LasR and RhlR bind different acyl-homoserine lactones with similar affinity

To establish whether purified protein was functional, microcalorimetric binding studies (Krell, 2008) with different AHLs were carried out. For this series of experiments we selected the two primary AHLs of *P. aeruginosa* (C4- and 3-Oxo-C12-AHL), other AHLs from *P. aeruginosa* such as C6- and 3-Oxo-C10-AHL as well as several AHL that have not been detected in this strain (Table 1).

Control experiments involved the titration of buffer with AHL, as exemplified by the titration of buffer with C6-AHL (Fig. 1, left A) and C4-AHL (Fig. 1, right A). Observed heat changes were small and uniform indicating that these are dilution heats. Initial experiments involved the titration of LasR and RhlR with C4- and 3-Oxo-C12-AHLs. Titrations resulted in exothermic heat changes that diminished as protein saturation advanced (Fig. 1). The K_D values for these 4 interactions were very similar and in the range between 0.86 to 2.17 µM (Table 1), indicating that receptors do not have a preference for the AHL synthesized by their cognate synthase. Assays using other *P. aeruginosa* AHLs, C6- and 3-Oxo-C10-AHL, gave K_D values comparable to those of C4- and 3-Oxo-C12-AHL. We then screened a series of AHLs that are foreign to *P. aeruginosa* (Table 1). The majority of the AHLs showed binding with affinities in the lower micromolar or higher nanomolar range (Table 1), indicating that the proteins bind to foreign AHL in addition to self-AHL. The broad ligand range of both proteins is consistent with *in vivo* studies using *E. coli* showing that both proteins mediate responses to a wide range of different AHLs (Winson et al, 1998).

From sigmoidal titration curves, such as those obtained in our studies, the binding stoichiometry (n) can be determined, which corresponds to the ligand concentration at the point of curve inflection (Fig. 1). Interestingly, n values of approximately 1 were determined for most of the ligands (Table 1), indicating a 1:1 AHL to protein monomer stoichiometry. This also suggests that proteins have not been co-purified with a compound present in the AHL binding site.

Table 1) Thermodynamic binding parameters derived from the microcalorimetric titration of RhlR and LasR with different AHLs.

Ligand	*Synthesized by*	LasR			RhlR		
		n	***K*$_D$ (μM)**	**Δ*H* (kcal/mol)**	***n***	***K*$_D$ (μM)**	**Δ*H* (kcal/mol)**
C4-AHL	*P. aeruginosa PAO1* (Ortori et al, 2011; Winson et al, 1995), *Aeromonas hydrophila* (Swift et al, 1999)	1.18 ± 0.2	0.86 ± 0.2	-13.5 ± 0.2	1.34 ± 0.2	1.66 ± 0.4	-16.1 ± 2
3-OH-C4-AHL	*Vibrio harvey* (Bassler et al, 1993)	No binding[1]			No binding[1]		
3-Oxo-C4-AHL	*Vibrio anguillarum* (Buch et al, 2003)	No binding[1]			1.12 ± 0.1	1.33 ± 0.3	1.12 ± 0.1
C6-AHL	*Chromobacterium violaceum* (McClean et al, 1997), *P. aeruginosa PAO1* (Ortori et al, 2011; Winson et al, 1995)	1.36 ± 0.1	1.87 ± 0.2	-5.6 ± 0.5	1.69 ± 0.5	1.2 ± 0.3	-6.2 ± 0.3
3-OH-C6-AHL	*P. fluorescens* (Shaw et al, 1997), *P. chlororaphis* (Khan et al, 2007)	1.49 ± 0.3	13.1 ± 2	-11.6 ± 2.2	1.11 ± 0.5	1.58 ± 0.2	-4.9 ± 0.5
3-OH-C10-AHL	*P. fluorescens* (Shaw et al, 1997)	0.6 ± 0.2	1.68 ± 0.3	-2.22 ±0.3	1.31 ± 0.3	0.82 ± 0.1	-1.43 ± 0.3
3-Oxo-C10-AHL	*P. aeruginosa 6294* (Charlton et al, 2000), *P. aeruginosa PAO1* (Ortori et al, 2011; Shaw et al, 1997), *Vibrio fluvialis* (Wang et al, 2013)	1.24 ± 0.3	0.97 ± 0.1	-3.0 ± 0.4	1.36 ± 0.4	1.12 ± 0.3	2.96 ± 0.4
3-Oxo-C12-AHL	*P. aeruginosa PAO1* (Ortori et al, 2011; Shaw et al, 1997)	1.44 ± 0.3	1.14 ± 0.2	-9.2 ± 0.4	1.34 ± 0.2	2.17 ± 0.2	-13.8 ± 1

[1] Due to the reduced solubility of AHL, the maximal ligand concentration of these compounds in the ITC injector syringe was 100 μM. For these compounds the injection of 100 μM AHL did not show any significant heat changes. However, it cannot be ruled out that compounds bind to receptor proteins with a reduced affinity and the detection of binding would require AHL concentration beyond 100 μM. n: binding stoichiometry, K_D: dissociation constant, Δ*H:* enthalpy change of binding. Shown are means and standard deviations from three experiments.

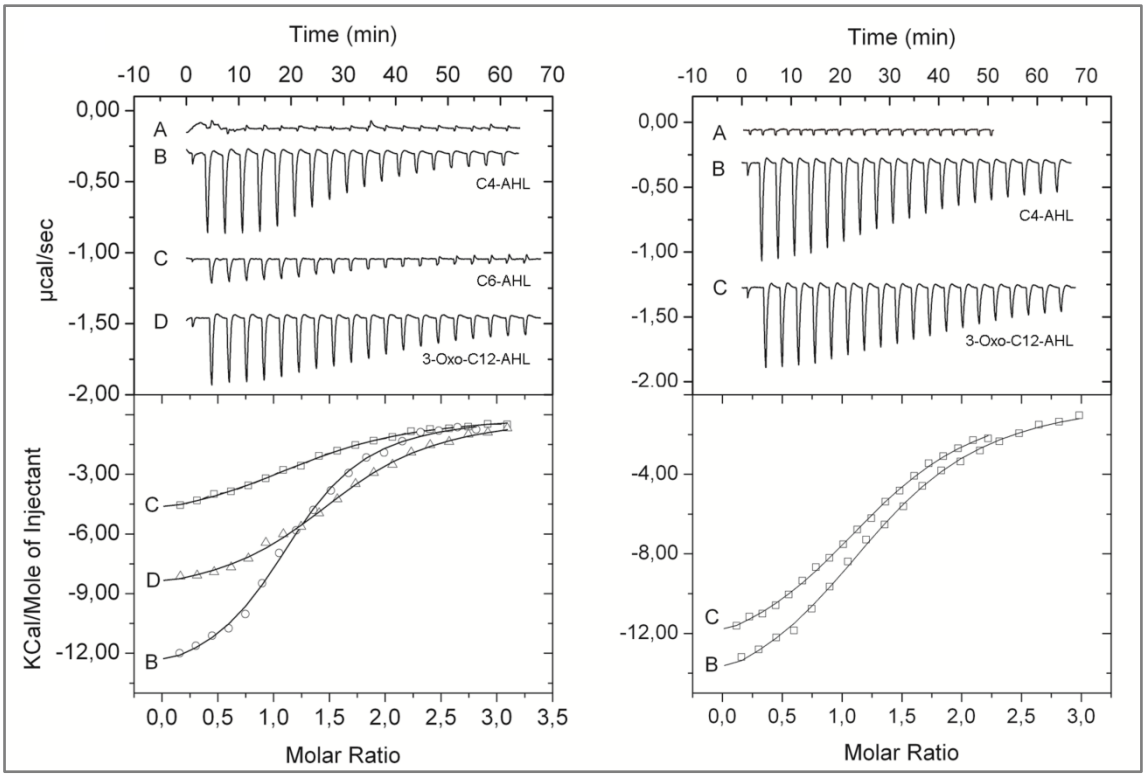


Fig. 1) Microcalorimetric analysis of the binding of different acyl-homoserine lactones to LasR (left) and RhlR (right). The upper panels are raw titration data. Traces labelled with A correspond to the titration of buffer (control) with either 100 µM C6-AHL (left) or 100 µM C4-AHL (right). The remaining curves correspond to the titration of 6-10 µM protein with 100 µM of the AHL indicated. For clarity reasons the traces have been off-set on the y-axis. The lower panels are dilution heat corrected and concentration normalized integrated peak areas of the raw titration data. Data were fitted with the "One Binding site" model of the MicroCal version of ORIGIN. The derived thermodynamic parameters are shown in Table 1.

LasR and RhlR unfold at temperatures beyond 60 °C and AHL binding does not alter protein stability significantly

To assess their structural integrity both proteins were analyzed by far UV circular dichroism (CD) spectroscopy. The corresponding spectra are shown in Fig. 2A and were used to calculate the α-helical content, which was 25 % for RhlR and 27 % for LasR. Both values were very close to the α-helical contents as derived from the corresponding homology models (29 % for RhlR and 32 % for LasR) that were generated (Supp. Fig. 2). The agreement of experimentally determined and structure derived α-helical content is consistent with the notion that purified LasR and RhlR are correctly folded proteins.

It has been proposed that some ligand-free QS regulators show a reduced thermodynamic stability leading to rapid unfolding and proteolysis and AHL binding was proposed to increase protein stability and to induce dimerization (Oinuma & Greenberg, 2011). To assess protein stability, RhlR and LasR were submitted to thermal unfolding experiments that were

monitored by Differential Scanning Calorimetry (DSC) (Krell, 2008) for RhlR and CD spectroscopy (following changes in the molecular ellipticity at 222 nm) for LasR. The choice of CD spectroscopy for LasR was due to the fact that DSC data for LasR were not interpretable. In both techniques a temperature gradient is applied to the protein and the resulting data permit the calculation of the Tm value, which is the temperature at which half the protein is present in its native form and the remaining half unfolded.

The DSC thermograms of RhlR in the absence and the presence of saturating AHL concentrations are shown in Fig. 2B and the derived parameters are provided in Table 2. The protein unfolded in a single sharp event, indicating a cooperative unfolding of both structural domains of this protein. The corresponding Tm of 70 °C indicates significant thermal stability. Importantly, the addition of saturating concentrations of C4-AHL and 3-Oxo-C12 AHL caused only a very marginal increase in Tm, which implies that AHL binding has no significant effect on the thermal stability of RhlR. The thermal unfolding studies of LasR are shown in Fig. 2C. The loss of α-helical content (CD signal at 222 nm) was used as a marker to calculate the fraction of unfolded protein. The steep rise of the signal between 60 and 70 ºC indicates cooperative protein unfolding. For the ligand-free protein a Tm value of 62.8 ºC was derived. In analogy to RhlR, the addition of saturating AHL concentrations did not increase protein stability but resulted in a very minor destabilization (Table 2). Taken together, we conclude that ligand-free RhlR and LasR possess a significant stability and that AHL binding has little or no effect on protein stability.

Table 2) Thermodynamic parameters for the thermal unfolding of RhlR and LasR in the presence and absence of different ligands. Data for RhlR were derived from Differential Scanning Calorimetry studies, whereas LasR data are form CD unfolding studies following changes in the molecular ellipticity at 222 nm. RhlR was at a concentration of 16–19 µM and LasR at a concentration of 2-3. AHL ligands were added at a concentration of 30 µM.

	T_m (ºC)	Δ*H* (kcal/mol)
RhlR	70.02	57
RhlR + C4-AHL	70.15	71
RhlR + 3-Oxo-C12-AHL	70.17	69
LasR	62.82	
LasR + C4-AHL	62.48	
LasR + 3-Oxo-C12-AHL	62.21	

Tm is the midpoint of protein unfolding transition; Δ*H* is the enthalpy change of protein unfolding

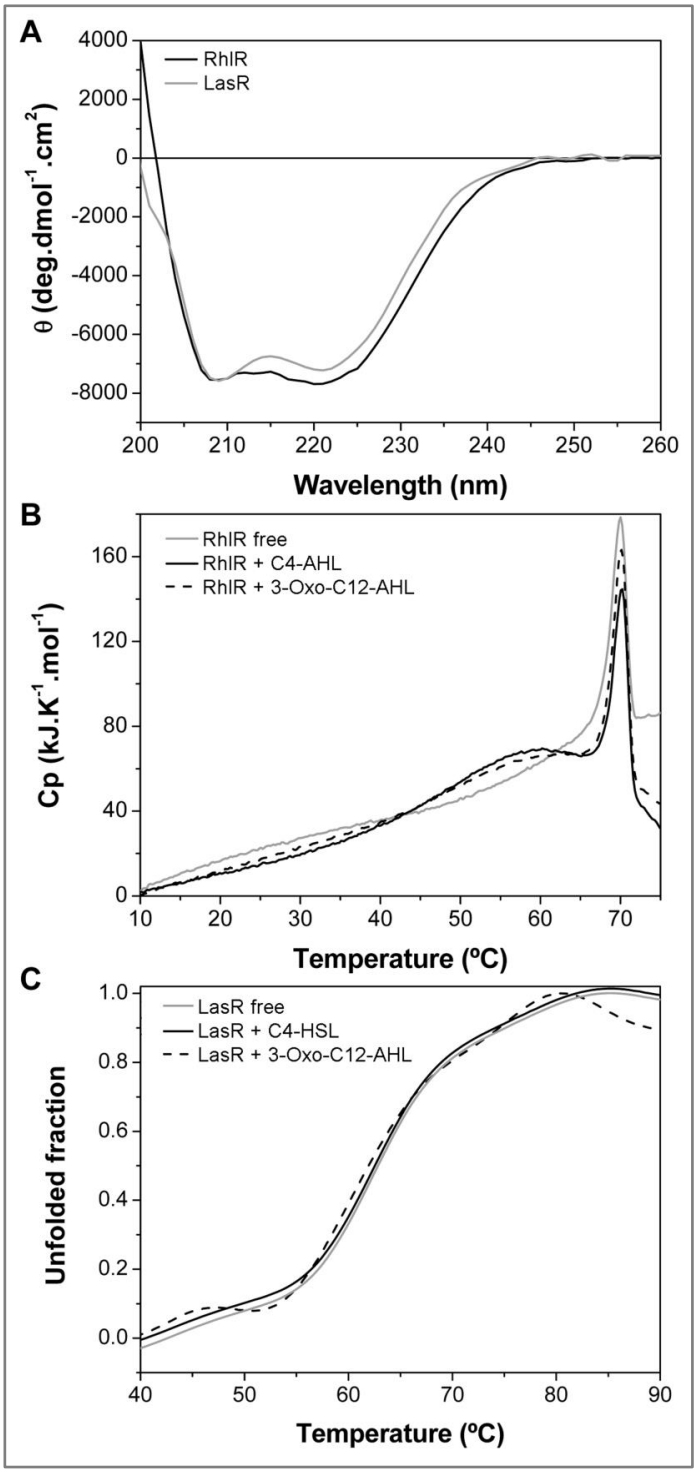


Fig.2) Secondary structure and thermal stability of RhlR and LasR. A) Analysis of RhlR and LasR by far UV CD spectroscopy **B)** Analysis of RhlR thermal unfolding by differential scanning calorimetry **C)** Analysis of LasR thermal unfolding in the absence and presence of different AHLs following changes in the molecular ellipticity at 222 nm as determined by CD spectroscopy. Both proteins were dialyzed into analysis buffer with and without 30 µM of AHL. The protein concentration was 16-19 µM for RhlR and 2-3 µM for LasR.

AHL binding does not alter the oligomeric state of RhlR and LasR

The oligomeric state of RhlR and LasR in the absence and presence of AHLs was studied by sedimentation velocity ultracentrifugation. The sedimentation coefficient distributions observed (Fig. 3) revealed the presence of two main species in both cases. The small RhlR species sedimented with a standard sedimentation coefficient of $s_{w,20}$= 2.8 S, while the larger

species can be observed at 4.2 S in the absence of ligand. LasR showed standard sedimentation coefficients of 2.5 S and 3.9 S. Molecular masses corresponding to both species were then calculated based on the frictional ratios experimentally determined (≈1.3 in both cases). The molecular masses for the two species in the RhlR sample were determined to be 33 kDa and 61 kDa, while those for the two LasR species were of 29.5 kDa and 57 kDa. These experimentally determined values fit very well with the sequence-derived masses of RhlR and LasR monomers (30.31 and 29.07 kDa, respectively) and dimers (60.62 and 58.14 kDa, respectively). These data show that both proteins exist in a monomer-dimer equilibrium at the experimental conditions used.

Using the HYDROPRO software (Ortega et al, 2011) we then calculated the fastest possible sedimentation coefficients for spherical proteins of the size of RhlR and LasR monomers and dimers. Taking into account a protein hydration of □=0.3 g/g, sedimentation coefficients of s_{sph}= 3.0 S and s_{sph}= 4.7 S were predicted for proteins of the size of the RhlR monomer and dimer, respectively. Using the same algorithm sedimentation coefficients of s_{sph}= 2.9 S and s_{sph}= 4.6 S were predicted for proteins of the size of the LasR monomer and dimer. These data are in agreement with the experimental values but slightly higher in all cases, indicative of a small asphericity of both proteins, which is confirmed by the homology models of both proteins (Supp. Fig. 2). When measurements were repeated in the presence of saturating concentrations of C4-AHL or 3-Oxo-C12-AHL, a similar distribution of both oligomeric species was observed (Fig. 3). These data show that AHL binding to RhlR and LasR does not cause significant changes in their oligomeric state.

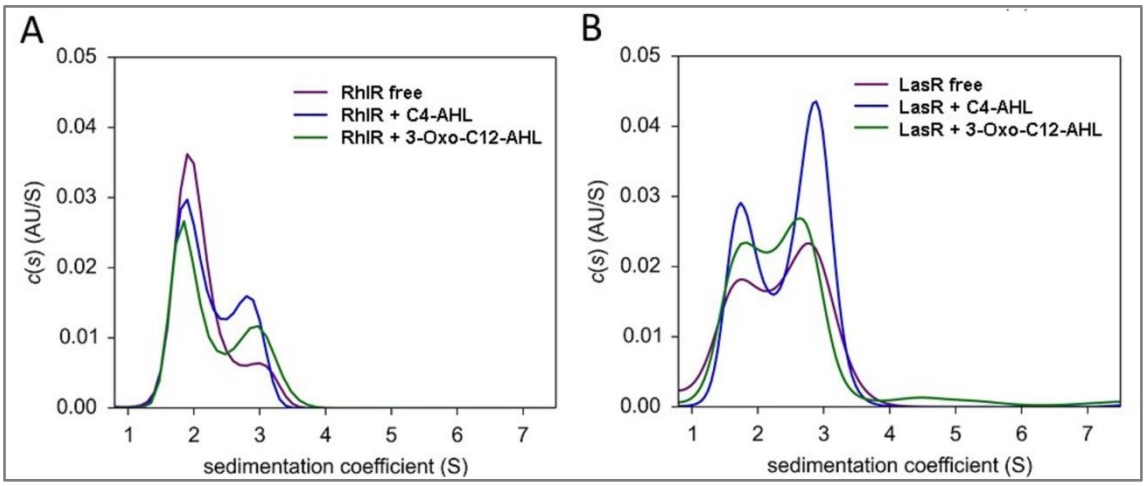


Fig. 3) Analysis of the oligomeric state of RhlR and LasR. Sedimentation velocity analytical ultracentrifugation studies of 15 µM RhlR **(A)** and 8 µM LasR **(B)** in the absence of AHL and in the presence of 100 µM C4-AHL or 3-Oxo-C12-AHL.

RhlR and LasR bind to promoter DNA

To verify whether recombinant RhlR and LasR have DNA binding activity, we carried out electrophoretic mobility shift assays (EMSAs) (Fig. 4). For this we used a 490 bp DNA probe corresponding to the hydrogen cyanide synthase promoter *hcnABC*. This promoter contains two *lux* boxes to which LasR and RhlR bind (Pessi & Haas, 2000). EMSA assays revealed that both proteins were able to bind to the DNA fragment in the presence of 3-Oxo-C12-AHL (Fig. 4A and D) and C4-AHL (Fig. 4B). In all cases two bands representing DNA-protein complexes were observed, which are most likely due to protein binding at each of the *lux* boxes. To verify the specificity of interaction, RhlR and LasR binding to unrelated promoters (Supp. Table 1) were analyzed (lanes 1 in Fig. 4 A-C and Fig. 4E). In all cases an absence of binding was noted. EMSAs were also conducted in the absence of AHL. In the case of LasR data could not be interpreted, due to sample precipitation whereas no DNA complex formation was noted for RhlR (Fig. 4C), indicating that the interaction of RhlR with target DNA depends on the presence of AHL.

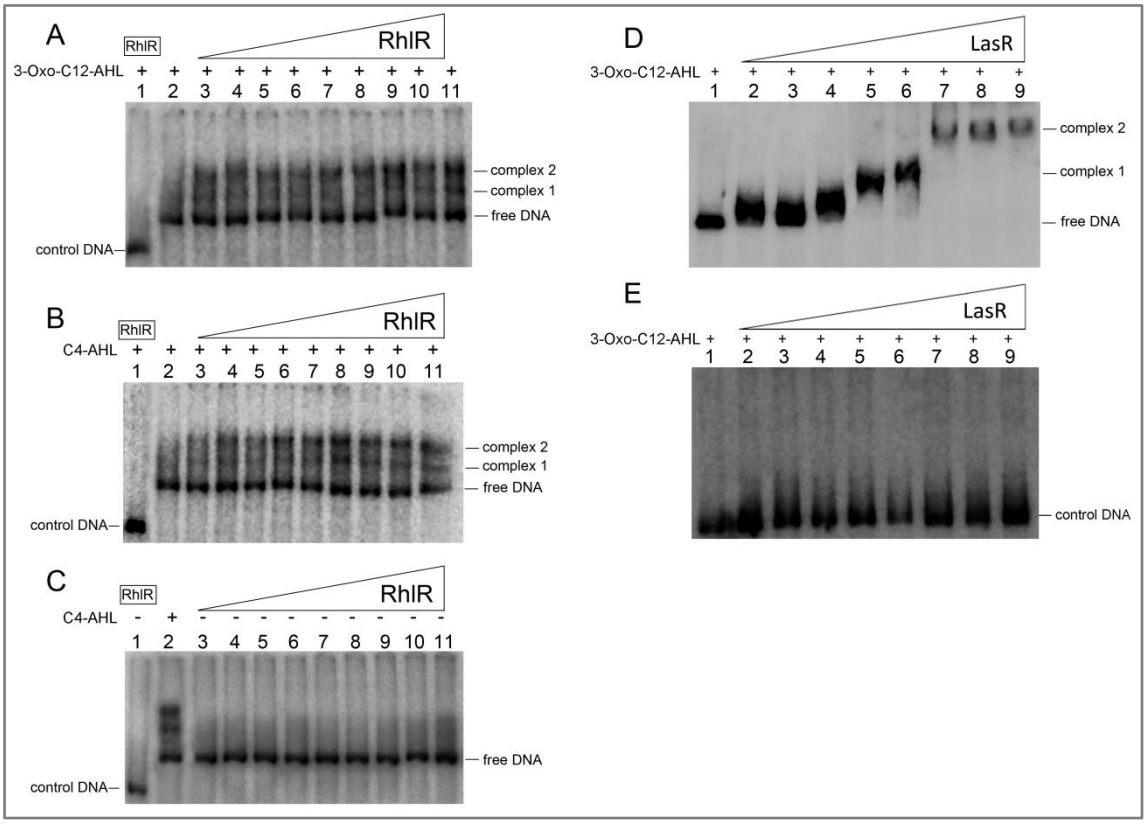


Fig. 4) Electrophoretic mobility shift assays of RhlR and LasR. The DNA used corresponds to the *hcnABC* promoter (490 bp) harboring two *lux* boxes (Pessi & Haas, 2000). **A)** RhlR binding in the presence of 100 µM 3-Oxo-C12-AHL. Lane 1: control DNA (*oprB* promoter, 339 bp (Wylie & Worobec, 1995)) + 4.2 µM of RhlR. Lane 2: control in the absence of protein. Lanes 3-11: RhlR concentrations from 0.8 – 4.2 µM. **B)** RhlR binding in the presence of 100 µM C4-AHL. Lane 1: control DNA + 5.3 µM RhlR. Lane 2: control in the absence of protein. Lanes 3-11: RhlR

concentrations from 1.7 – 5.3 µM. C) RhlR binding in the absence of AHL. Lane 1: Control DNA + 2.7 µM RhlR. Lane 2: positive control: binding in the presence of 2.7 µM RhlR and 100 µM C4-AHL. Lanes 3-11: increasing RhlR concentrations from 0.5 – 2.7 µM. D) LasR binding to the *hcnABC* promoter fragment in the presence of 100 µM 3-Oxo-C12-AHL. Lane 1: no LasR. Lane 2-9: lasR concentration from 1.4 – 13.9 µM. E) Repetition of data shown in D except that control DNA was used (451 bp DNA fragment of *Serratia plymuthica* A153 amplified with the primers listed in Supp. Table 1).

RhlR stimulates transcription in vitro in a protein and AHL concentration dependent manner

Data reported above strongly suggest that purified RhlR and LasR are active proteins. To provide further proof for this statement, *in vitro* transcription assays were conducted using the *hcnABC* promoter. Initial experiments were conducted with RhlR and its cognate C4-AHL. As shown in Fig. 5A no band was observed in the absence of RNA polymerase (lane 1) and weak transcriptional activity was observed in the presence of the RNA polymerase (lane 2) that increased slightly in the presence of RhlR (lane 3). Using saturating concentration of C4-AHL we then tested transcription at different RhlR concentrations (lanes 4-9). A concentration dependent increase in transcription was noted that levelled off at 6 µM. Using saturating RhlR concentrations, transcription was then quantified at different C4-AHL concentrations (lanes 10-13). In analogy to the above data, a dose-dependent increase in transcription was noted. Dose-dependence was also analyzed for other AHLs and Supp. Fig. 3 shows transcription assays at 1 : 1 and 0,5 : 1 AHL/RhlR ratios. These data provide evidence that the protein is able to regulate transcription in an-AHL dependent manner.

We have shown above that different AHLs bind to purified RhlR. The availability of ligand-free RhlR makes it possible to determine the intrinsic capacity of the protein to stimulate transcription in response to the same concentration of different AHLs. To address this we conducted *in vitro* transcription assays using a constant concentration of RhlR and each of the seven AHLs that bound to the purified protein *in vitro* (Table 1). Note that their affinities are very similar (K_D in between 0.82 to 2.17 µM) indicating that the occupancy of protein with AHL during the transcription assay is comparable. Fig. 5B reports the means and standard deviation derived from three independent assays. Six of the seven tested AHLs caused statistically significant increases in transcription. C6-AHL was identified as the most efficient AHL, which was surprising since this AHL is present at low concentration in *P. aeruginosa* (ratio 1:15 with C4-AHL) (Winson et al, 1995). 3-Oxo-C4-AHL was identified as the second most efficient ligand. To our knowledge this AHL has not been detected in *P. aeruginosa*. The cognate C4-AHL and a

group of three other AHLs (3-OH-C6, 3-Oxo-C10 and 3-OH-C10) caused an approximately 2.5 fold stimulation of transcription. *In vitro* transcription assays were also conducted with LasR. In analogy to RhlR, a transcript was visible, which however could not be quantified precisely as in the case of RhlR.

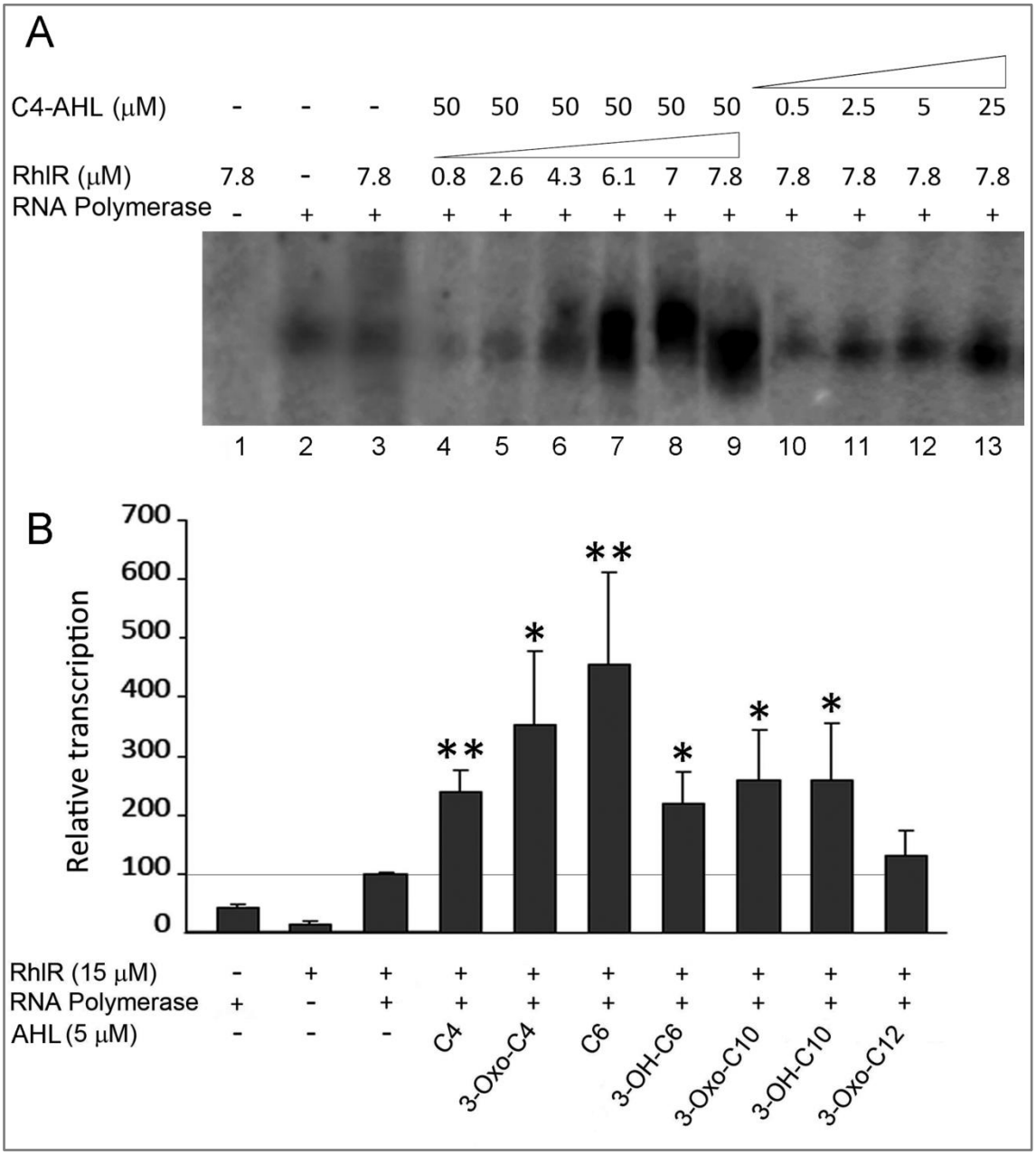


Fig. 5) *In vitro* transcription assays of RhlR in the presence and absence of different AHLs. The DNA fragment used corresponded to the *hcnABC* promoter region (490 bp), which was also used for the EMSA experiments. **A)** *In vitro* transcription activity of RhlR as a function of the concentration of protein and its cognate C4-AHL. Shown is a representative gel. **B)** *In vitro* transcription in the presence of different 15 μM of RhlR and 5 μM for each of the AHLs that were found to bind to the purified protein (Table 1). Shown are means and standard deviations from the densitometric analyses of three different experiments. * P ≤0.05 and **P ≤0.01 in Student's t-test indicate statistical significance.

Differential impact of AHL ligands on the RhlR mediated upregulation of gene expression

We have shown above that multiple AHLs bind with similar affinity to RhlR (Table 1, Fig. 1) and that these ligands differ in their capacity to induce transcription *in vitro* (Fig. 5). We have therefore conducted experiments to assess AHL-mediated gene expression *in vivo*. In an initial series of experiments, pMULTIAHLPROM containing the *rhlI*::*lacZ* transcriptional fusion was introduced into a *las*I$^-$/*las*R$^-$ double mutant. Cells were grown in the presence or absence of C4-AHL (added at the beginning of the growth experiment). In the absence of AHL, a gradual increase in transcriptional activity was observed which was very pronounced at the final time point (Fig. 6A). Similar kinetics were observed in the presence of added AHL only that most of the beta-galactosidase (β-gal) levels were upshifted, indicating that the levels observed are the result of both intrinsically produced and exogenously added AHLs.

This experimental set-up was then used to quantify the effect of nine different AHLs on transcription. AHLs were added to dilute overnight cultures and the resulting measurements were corrected using the control (addition of DMSO to the culture medium). AHLs were added as 19 to 70 mM solutions in DMSO (depending on AHL solubility in DMSO) which translates into DMSO concentrations in the culture of 0.14 to 0.52 % (v/v). Control experiments demonstrated that the DMSO concentration range used did not impact on cell viability (Supp. Fig. 4). After 4 h, five AHLs caused statistically significant increases in gene expression (Fig. 6B). The strongest response was seen for the cognate C4-AHL, but elevated readings were also observed for C6-AHL and 3-Oxo-C10-AHL, molecules which are naturally synthesized by *P. aeruginosa* but in smaller quantities than C4-AHL and under specific environmental conditions. The two AHL that failed to bind to RhlR *in vitro*, 3-OH-C4-AHL and C12-AHL, caused no increase.

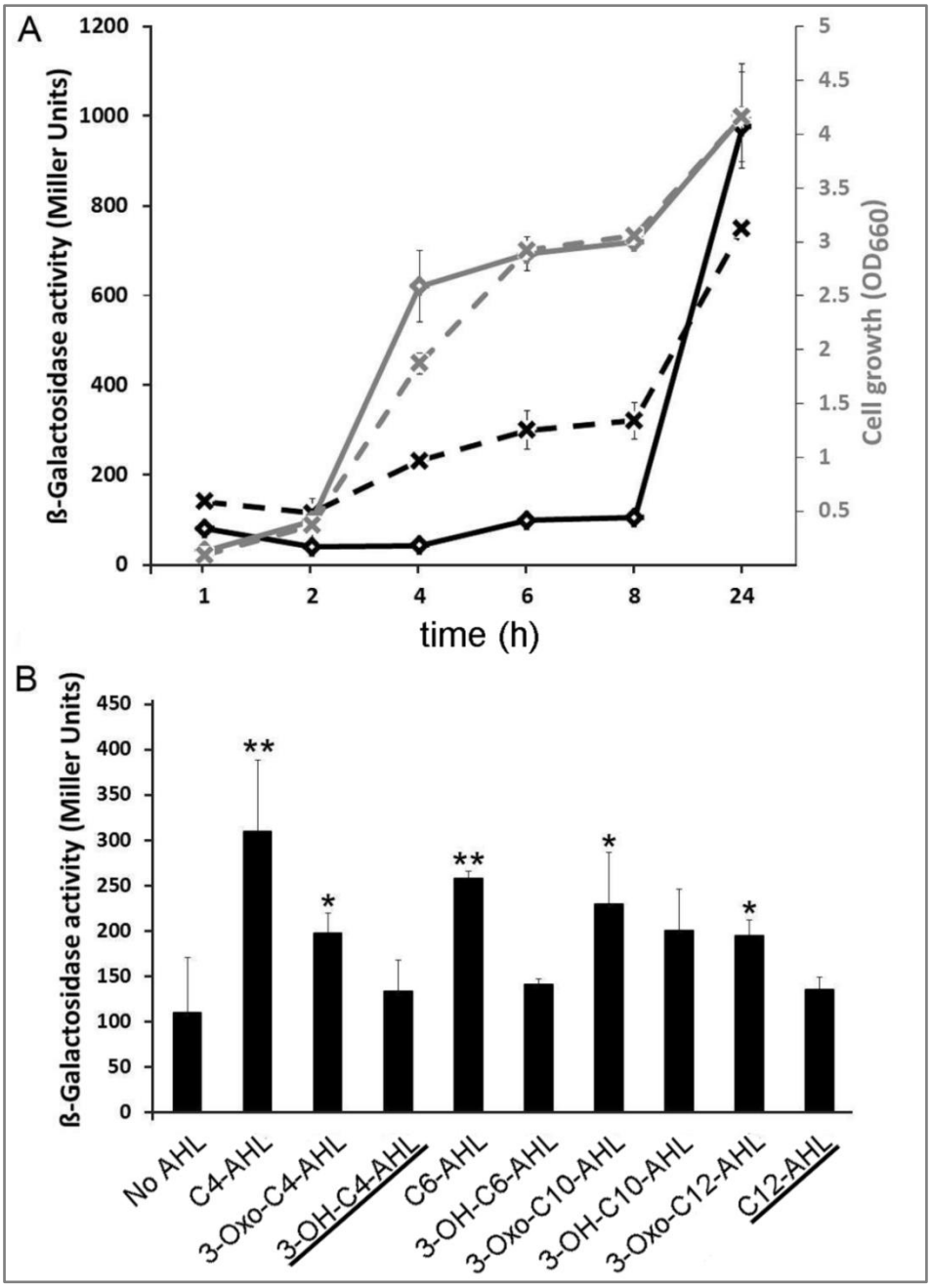


Fig. 6) Gene expression studies. Shown are studies using the plasmid pMULTIAHLPROM containing a *rhlI*::*lacZ* fusion. **A)** Plasmid pMULTIAHLPROM was introduced into a *P. aeruginosa PAO1 las*I$^-$/*las*R$^-$ double mutant**.** Cultures were grown in LB medium in the presence (final concentration 100 µM, dashed line) and absence of C4-AHL (constant line). B-gal activity (black line) and cell growth (grey line) were monitored. **B)** β-gal activity of the *P. aeruginosa las*I$^-$/*las*R$^-$ double mutant in the presence of different AHL (each added at 100 µM). Activity was measured 4 hours after AHL addition and was corrected using the measurements of the DMSO control. Underlined are the AHLs that failed to bind in ITC measurements. Data are the means ± SD of three assays done in triplicates. * P ≤0.05 and **P ≤0.01 in Student's t-test indicate statistical significance.

Discussion

In vivo experimentation suggested that the concept by which some QS regulators are non-functional in the absence of AHL may not be correct (Sappington et al, 2011). Furthermore, the observation that the removal of AHL from the environment leads to a rapid decrease in QS mediated gene expression is not consistent with ultra-tight AHL binding to TraR and LasR (Luo et al, 2003; Sappington et al, 2011; Su et al, 2008). Here we report for the first time the purification of LasR and RhlR from the soluble fraction of *E. coli* grown in the absence of added AHLs.

Our claim that purified proteins are active is based on the following facts: 1) CD spectroscopy studies show that the experimentally determined α-helical content of LasR and RhlR is close to the corresponding values derived from the homology models. 2) DSC and Cd spectroscopy experiments demonstrate that both proteins unfold cooperatively with Tm values above 60 degrees indicative of a significant thermal stability. 3) AUC show that both proteins are present in a monomer-dimer equilibrium, which is a feature frequently observed for transcriptional regulator. 4) ITC experiments provide evidence that LasR and RhlR binds a series of different AHLs amongst which their cognate 3-Oxo-C12-AHL and C4-AHL. 5) EMSAs assays show that LasR and RhlR bind to a promoter previously shown to be regulated by LasR and RhlR. This promoter has two operator sites and for both proteins we observed two DNA-protein complexes with different motility. 6) *In vitro* transcription assays demonstrate that RhlR is able to upregulate transcription in a protein and AHL-concentration dependent fashion.

The microcalorimetric titrations showed that both proteins bind different AHLs. The point of inflection of the sigmoidal ITC binding curves (Fig. 1) corresponds to the n-value that indicates the stoichiometry of interaction. As shown in Table 1, n values obtained were approximately 1 indicating a 1 protein monomer : 1 AHL molecule stoichiometry. This stoichiometry has been determined previously for LasR (Schuster et al, 2004) and a number of other QS regulators (Bottomley et al, 2007; Kim et al, 2014; Kim et al, 2010; Lintz et al, 2011; Minogue et al, 2002) suggesting that this may be a general feature of this protein family. N-values of close to 1 also suggest that the samples contain fully active protein, rather than mixtures of active with inactive protein. The stoichiometry obtained is also consistent with the notion that the AHL binding pocket is free of tightly bound ligand.

LasR and RhlR were found to bind different AHLs with affinities in between 0.82 to 13 µM with most AHL having K_D values of approximately 1 µM. These values do not agree with the ultra-

tight AHL binding observed previously for LasR (Schuster et al, 2004). The conclusion that AHLs bind ultra-tightly to LasR were not derived from binding studies, but from the observation that AHL remained protein-bound following prolonged dialysis (Schuster et al, 2004). Our data thus agree with Sappington *et al*. (2011) who, based on their *in vivo* measurements, proposed that AHLs may be able to rapidly dissociate from LasR and in this way fit the general model for transcription factors. In addition, the affinities we have measured are in a similar range as those for other transcriptional regulators (Daddaoua et al, 2010; Guazzaroni et al, 2005; Teran et al, 2006) or QS regulators. For example, the CarR QS regulator bound 3-Oxo-C8- and 3-Oxo-C6-AHL with K_D values of 0.5 and 1.8 µM, respectively (Welch et al, 2000) and a K_D value of 7 µM was derived from microcalorimetric titrations of SdiA with C4-AHL (Kim et al, 2014). Gel shift assays with different 3-Oxo-C6-AHL concentrations revealed a dissociation constant of approximately 100 nM for LuxR (Urbanowski et al, 2004).

Several studies have assessed the LasR ligand profile *in vivo* (Lee et al, 2006; Passador et al, 1996; Winson et al, 1998). To this end constructions containing the *lasR* and reporter genes as well as a LasR target promoter sequence were introduced into *E. coli* and transcriptional activity in response to different AHLs was measured. Winson *et al*. (1998) analyzed transcription in the presence of 22 different AHLs of which 11 caused a response, suggesting a broad effector range. All three above mentioned studies agree in the fact that highest sensitivity was observed for the cognate 3-Oxo-C12-AHL (Lee et al, 2006; Passador et al, 1996; Winson et al, 1998). We show here that six of the eight AHLs tested bound to LasR *in vitro*. Our data agree well with the data reported by Winson *et al*. (1998) with the exception that low sensitivity responses were noted for 3-Oxo-C4-AHL, which did not cause binding in ITC. However, as we state in the footnote to Table 1, we were unable to detect very low affinity binding events by ITC due to the reduced solubility of many AHLs, which may explain the slight differences observed. As for LasR, RhlR was also found to respond *in vivo* to a wide range of different AHLs (Winson et al, 1998), which agrees with our ITC studies that showed binding for 7 of 8 tested AHLs. Our data also demonstrate that LasR and RhlR have not evolved to preferentially recognize their cognate AHL since their affinities were comparable to that of other AHLs tested.

The quality of *in vitro* transcription assays for RhlR has permitted to quantify the capacity of different AHLs to stimulate transcription. C6-AHL was found most efficient in stimulating RhlR mediated transcription (Fig. 5). These data are in agreement with the study of Winson *et al*. (1998) showing that RhlR was most sensitive to C6-AHL *in vivo*. This AHL is present in *P. aeruginosa* only at a ratio of 1:15 with C4-AHL (Winson et al, 1995), but the functional

relevance of C6-AHL is demonstrated by the fact that addition of either C4-AHL or C6-AHL to a *P. aeruginosa* mutant unable to synthesize either of these autoinducers, restored elastase, chitinase and cyanide production (Winson et al, 1995). In contrast to RhlR, C6-AHL caused only very low sensitivity responses in LasR (Winson et al, 1998) and no responses in QscR harboring *E. coli* (Lee et al, 2006). Contrary to the *in vitro* transcription studies, C4-AHL and not C6-AHL, was the most efficient signal to increase gene expression *in vivo* (Fig. 6). These discrepancies may be due to differences in the bioavailability of the AHLs analyzed.

Protein stability and oligomeric state as well as the effect of AHL binding on both features have been assessed and reveal striking parallels between both proteins. Tm values of 63 and 70 ºC have been obtained for LasR and RhlR, respectively, indicative of significant thermal stabilities, which are similar or superior to stabilities determined for other QS regulators like SmcR (Tm=63 ºC) (Kim et al, 2010), SdiA (Tm=48 ºC) (Kim et al, 2014) or EsaR (Tm~50 ºC) (Minogue et al, 2002). For both proteins AHL binding did not alter Tm values in a significant manner, which is not consistent with the notion that AHL binding causes protein stabilization.

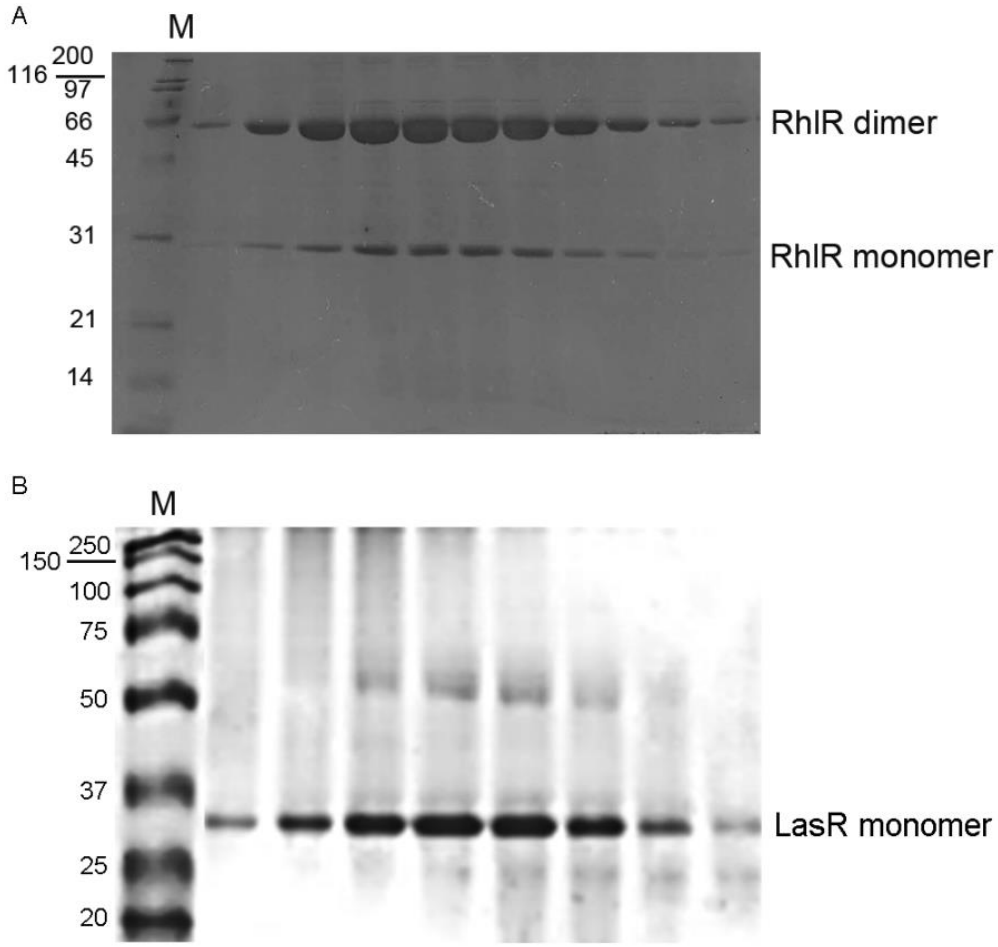
Analytical ultracentrifugation sedimentation profiles of both proteins showed two peaks that were unambiguously identified as monomer and dimer. The addition of saturating AHL concentration did not alter the oligomeric state in a significant manner. LasR was found previously to be a dimer based on gel filtration chromatography (Schuster et al, 2004). However, the mass corresponding to the single peak observed in gel filtration was in between that of the monomer and dimer. The higher resolution of analytical ultracentrifugation techniques may have been the cause for the differences observed. Our studies are not in agreement with an *in vivo* study showing that RhlR-green fluorescent protein fusions are dimeric and that 3-Oxo-C12-AHL addition induces the dissociation into monomers (Ventre et al, 2003).

Supplementary material

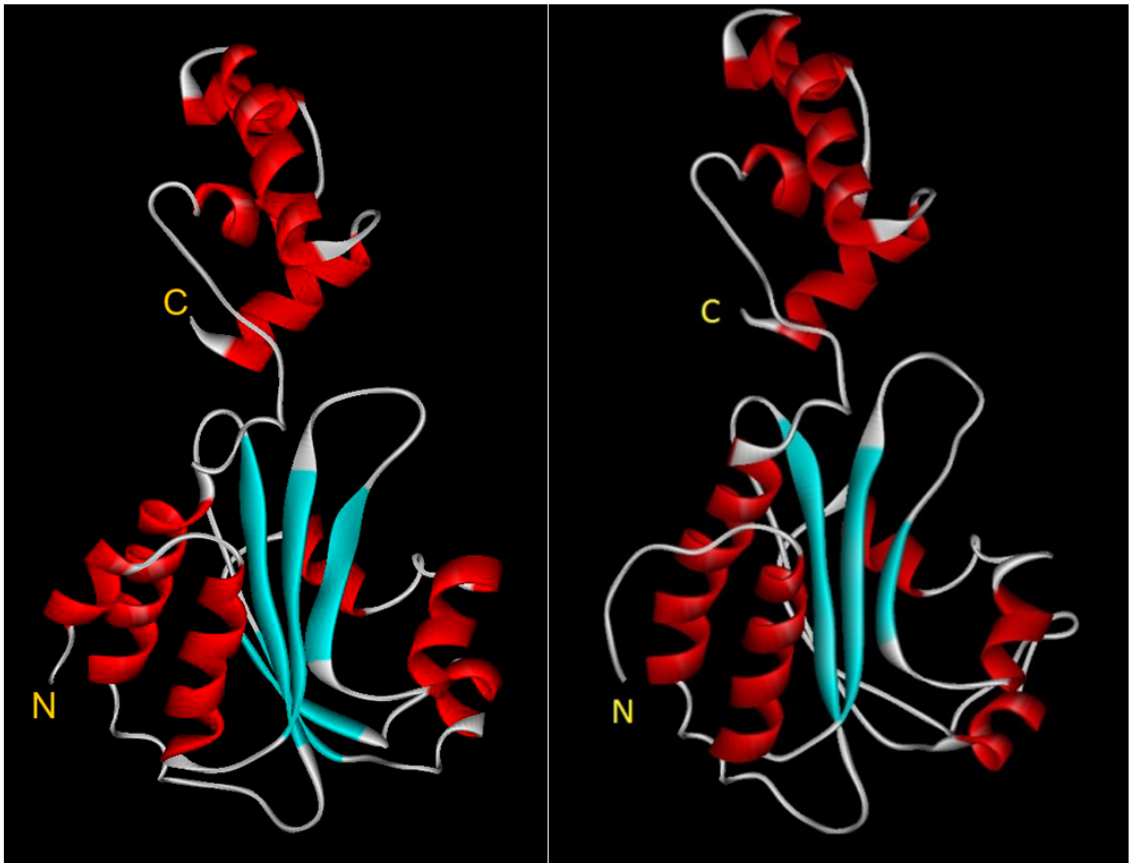
Supp. table 1) Strains and plasmids used in this study.

Strains	**Relevant Characteristics**	**Reference**
P. aeruginosa PAO1	wild type, prototroph; Ap^R	(Stover et al, 2000)
P. aeruginosa PAO1 *lasI*$^-$ */lasR*$^-$	Double mutant in *lasI* and *lasR* genes	Personal gift, M. Cámara, Nottingham
E. coli BL21 (DE3)	F^-, *ompI, hsdS*$_B$ (r^-_B m^-_B)	(Studier & Moffatt, 1986)
E. coli DH5α	*supE44 ΔlacU169* (ϕ80 *lacZΔ*M15) *hsdR1 recA1 endA1 gyrA96 thi1 relA1*	(Hanahan, 1983)
Plasmids	**Features**	**Reference**
pET28b(+)	Km^R, protein expression vector	Novagen
pET28b-LasR	Km^R, pET28b(+) derivative containing *lasR* gene	This work
pET28b-RhlR	Km^R, pET28b(+) derivative containing *rhlR* gene	This work
pMULTIAHLPROM	Tc^R , Broad-host-range plasmid containing 8-*luxI* type promoters fused to a promoterless *lacZ* gene	(Steindler et al, 2008)

Km^R, Ap^R and Tc^R indicate resistance to kanamycin, ampicillin and tetracycline, respectively.

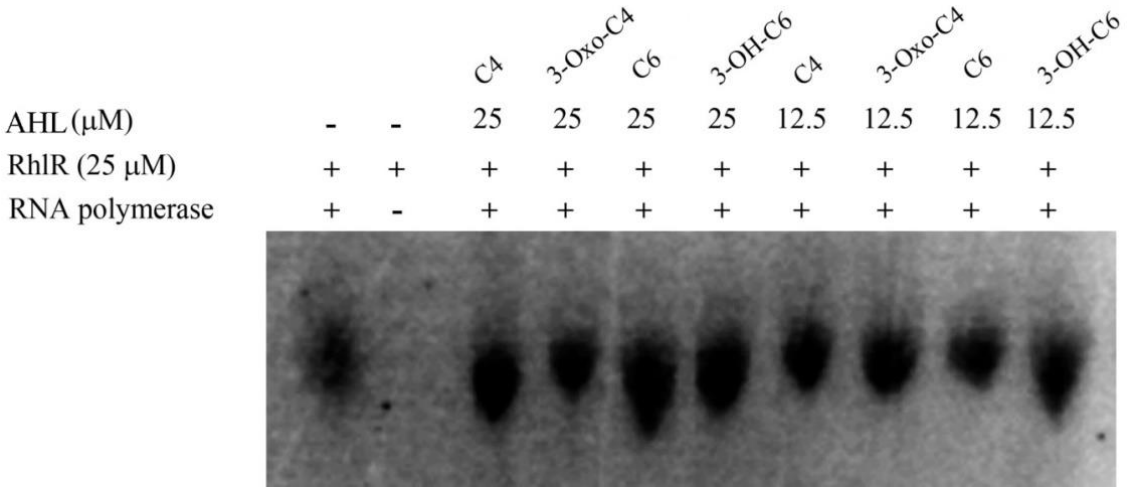


Supp. Fig. 1) Purification of recombinant RhlR and LasR from the soluble fraction of *E. coli* cell lysates. SDS-PAGE gel of fractions of RhlR **(A)** and LasR **(B)**. M: Molecular weight marker. The sequence derived masses of the RhlR and LasR monomers are 30 315 and 29 070 Da, respectively.

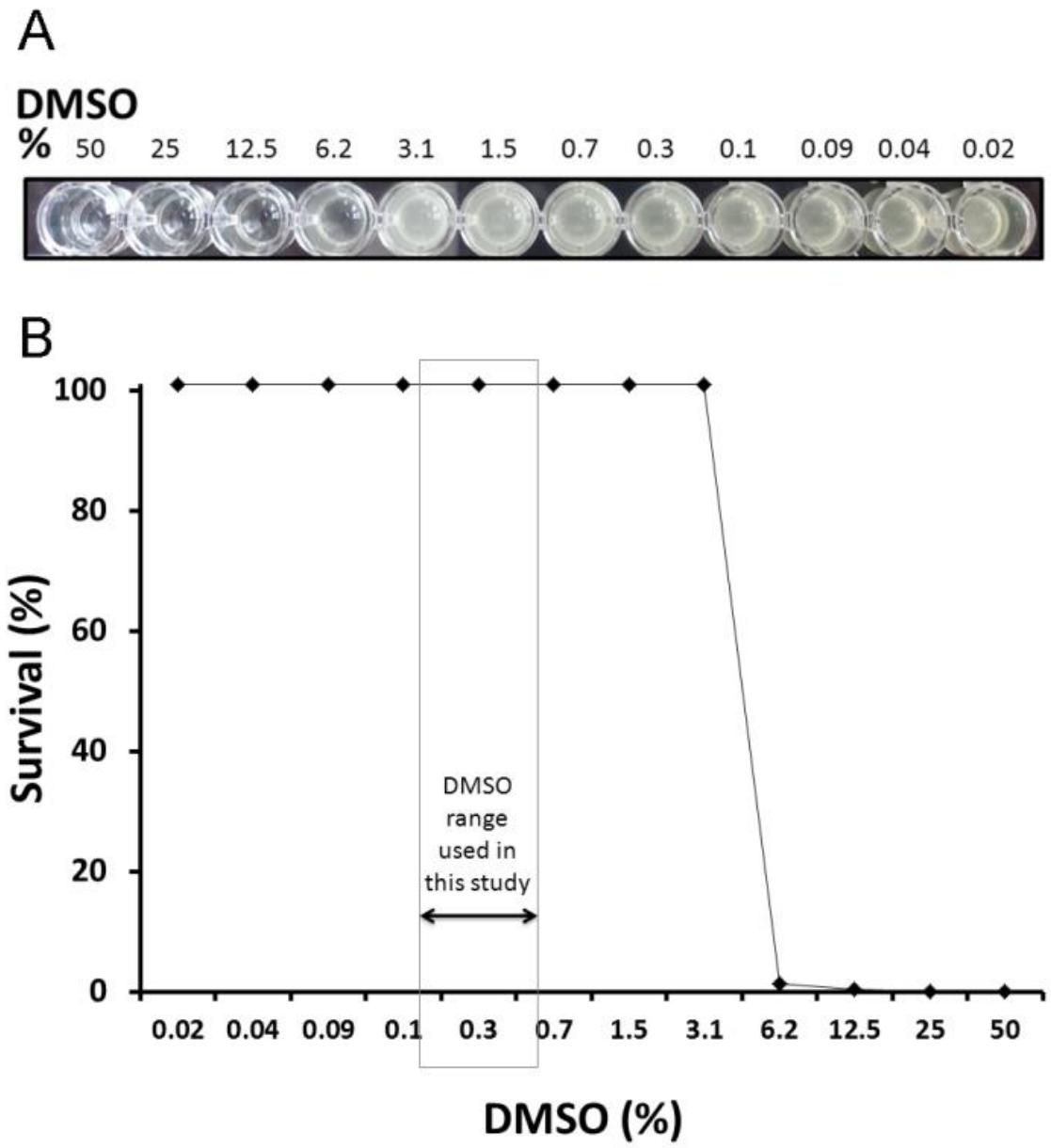


A B

Supp. Fig. 2) Homology models of RhlR (A) and LasR (B) generated using the 3D structure of QscR (pdb ID: 3SZT) as template. The models were generated using the geno3D software (Combet et al, 2002).



Supp. Fig. 3) *In vitro* transcription assays of RhlR in the presence and absence of different AHLs. Experiments are conducted at 1 : 1 and 1 : 0.5 RhlR – AHL ratios. In all cases transcriptional activity depended on the AHL concentration.



Supp. Fig. 4) Effect of DMSO on *P. aeruginosa* PAO1 viability. A) Representative image from the experiment to assess the toxicity of DMSO. These assays were performed in 96-well plates using a modified version of the protocol reported in (Mah, 2014). Wells of a 96-well plate were filled with 200 µL of LB containing different amounts of DMSO. Wells were inoculated with 10 µl of an overnight culture of *P. aeruginosa* PAO1 in LB medium. Plate was incubated at 37°C for 24 h at which point the viable cell amount was determined by plating out cells on LB-agar medium and counting. **B)** Cell counts were normalized using the corresponding values of a culture grown in the absence of DMSO. For values above 100 %, survival is defined as 100 %. Shown are data from a representative experiment. The boxed area indicates the DMSO range used in this study.

CHAPTER 3

Rosmarinic acid is a homoserine lactone mimic produced by plants that activates a bacterial quorum sensing regulator

CHAPTER 3 Rosmarinic acid is a homoserine lactone mimic produced by plants that activates a bacterial quorum sensing regulator

Brief introduction

Plants live in association with fungi and bacteria, and it is believed that plant evolution was influenced by the presence of these associated microorganisms (Venturi & Fuqua, 2013). During this evolution diverse signaling systems emerged that permitted mutual plant-microorganism sensing. QS is a mechanism of communication between bacteria and is based on the synthesis, detection, and response to QSS. As cell density increases, QSS accumulate in the environment and are sensed by bacterial proteins called QS regulators, which in turn control the expression of genes; the products of these genes direct activities that are beneficial when performed by groups of bacteria acting in synchrony (Rutherford & Bassler, 2012). AHLs produced by Gram-negative bacteria are the best studied and possibly the most common group of bacterial QSS (Fuqua et al, 2001). Frequently, pairs of genes encoding the AHL synthase and the AHL-sensing transcriptional regulator are found close to each other in bacterial genomes. In addition, many bacteria have additional paralogs of AHL-sensing regulator genes that are not associated with an AHL synthase gene and that were consequently termed solo or orphan regulators (Gonzalez & Venturi, 2013).

Bacteria-to-plant and plant-to-bacteria signaling are also based on QS systems. A proteomic study showed that AHLs modulate the expression of a large number of genes in the legume *Medicago truncatula* (Mathesius et al, 2003). Similarly, a transcriptomic study revealed that C6-AHL, a bacterial QS molecule produced in the rhizosphere, changed gene expression in *Arabidopsis thaliana* (von Rad et al, 2008). AHL signaling processes are, in part, responsible for the induced systemic resistance of plants toward bacterial pathogens (Schikora et al, 2011); these processes also modulate plant growth (Schenk et al, 2012).

In addition, different plants produce compounds that interfere with the bacterial QS mechanism. Extracts (Degrassi et al, 2007; Tolmacheva et al, 2014) and macerates of different plants, plant parts, and seeds (Ferluga et al, 2007; Ferluga & Venturi, 2009; Gonzalez et al, 2013; Perez-Montano et al, 2013; Subramoni et al, 2011), as well as exudates from seeds (Gao et al, 2003) or seedlings (Teplitski et al, 2000), interfere with bacterial QS mechanisms. Additionally, leaf washings from 17 different plants stimulated or inhibited AHL-dependent

activities in bacteria (Karamanoli & Lindow, 2006). Furthermore, QS-dependent gene expression is altered when pathogenic bacteria grow in their host plants (Zhang et al, 2007). Biofilm formation and AHL-production increase in the presence of different plant-derived phenolic compounds (Plyuta et al, 2013); however, these compounds do not act as AHL mimics. In contrast, most of the experiments using extracts from plants stimulated rather than inhibited QS-dependent gene expression (Venturi & Fuqua, 2013), with the data suggesting that these plant compounds act as AHL mimics and bind to the autoinducer-binding domain of QS regulators. A molecular docking study identified rosmarinic acid (RA), naringin, morin, mangiferin, and chlorogenic acid (Annapoorani et al, 2012) as plant-derived compounds that were predicted to bind to QS regulators. Although in that study each of these compounds inhibited QS-mediated phenotypes, suggesting that they function as QS antagonists, potential toxic effects were not evaluated. Experimental confirmation of the binding of any of these compounds to QS regulators has not been done. The algal compound, lumichrome, which is a riboflavin derivative, stimulates the activity of a QS regulator of *P. aeruginosa* (Rajamani et al, 2008).

Here, we used *P. aeruginosa* PAO1 as a model organism to screen for plant-derived AHL mimics. This bacterium is a ubiquitous pathogen that infects a wide range of species, including humans and different plants such as barley, poplar tree (Attila et al, 2008), and lettuce (Rahme et al, 2000) (Cao et al, 2001). As a model for studying the effect of QS on pathogenic traits, *P. aeruginosa* has a multisignal QS system that is based on the synthesis and detection of signals that belong to two different classes, namely Pseudomonas AHLs and quinolone signals (Pesci et al, 1999; Schuster et al, 2013). The AHL response is mediated by two pairs of synthases and regulators, the synthase LasI and the regulator LasR (LasI/LasR) and the synthase RhlI and the regulator RhlR (RhlI/RhlR), as well as by the orphan regulator QscR. RhlI produces the signaling molecule C4-AHL, and LasI produces 3-Oxo-C12-AHL (Schuster et al, 2013). The *P. aeruginosa* QS system is hierarchically organized with LasR at the top of the signaling cascade: LasR activation stimulates transcription of multiple genes, including *rhlR*, *rhlI*, and *lasI*. The QS cascade then modulates multiple QS phenotypes, including changes in the amounts of elastase, pyocyanin, rhamnolipid, and hydrogen cyanide(Lee & Zhang, 2015).

Here, we used ligand-free LasR and RhlR purified from *E. coli* without added AHL (Corral Lugo et al, 2015) for microcalorimetric binding studies of plant-derived compounds that were selected on the basis of *in silico* docking experiments.

Results

Rosmarinic binds to purified RhlR with high affinity

A major limitation in the study of the AHL-sensing regulators is their instability in the absence of AHL (Bottomley et al, 2007; Oinuma & Greenberg, 2011; Schuster et al, 2004). Although recombinant regulator purified from *E. coli* cultures bindsk AHL added to the culture medium, using most methods the bound AHL copurifies with the protein, resulting in partially saturated regulators, and thus hampering ligand-binding studies. As described earlier, we developed a method that enables the purification of recombinant RhlR and LasR without the addition of AHL, thereby providing a system for performing ligand-binding analysis (Chapter 2) (Corral Lugo et al, 2015). Recombinant RhlR and LasR isolated with this method in the absence of AHL bound C4-AHL and 3-Oxo-C12-AHL, respectively, with K_D values of 1.66 ± 0.4 µM and 1.14 ± 0.2 µM, as determined by ITC (Fig. 1A, B).

To identify potential ligands, we conducted *in silico* docking experiments of ligands present in a database of natural compounds to the structure of LasR and a model of RhlR. We used the structure of the LasR autoinducer-binding domain in complex with 3-Oxo-C12-AHL (PDB ID: 3IX3) to generate a homology model of the analogous domain of RhlR, which could be closely superimposed onto the template (Fig. 1C). We used 3-Oxo-C12-AHL and C4-AHL as controls in the docking experiments (Table 1), and then we screened the Natural Compounds subset of the Zinc compound database (5391 compounds) and selected those of plant origin and with docking scores below -8 for further analysis. Most of the tested compounds had lower docking scores at RhlR or LasR when compared to those of their cognate AHL ligands (Table 1).

Microcalorimetric binding studies with the selected compounds were performed to assess binding to ligand-free LasR and RhlR. We found that RA bound only to RhlR (Fig. 1D) and not to LasR (Fig. 1E). No other selected compound bound to RhlR or LasR (Table 1).

We calculate that RA bound to RhlR with a K_D of 0.49 ± 0.08 µM and had small favorable enthalpy changes (ΔH = -0.4 ± 0.05 kcal/mol). Similar to the AHL ligands, the binding stoichiometry was close to 1:1, which can be observed as the point of inflection of the sigmoidal binding curves in Fig. 1D with respect to the lower x-axis. To assess the specificity of this interaction, we titrated RhlR with chlorogenic acid, a compound structurally similar to RA (Fig. 2), and that had a low docking score (Table 1). Chlorogenic acid did not cause significant heat changes, indicating that this compound did not bind RhlR (Fig. 1D). The RhlR model containing the best fit of docked RA and C4-AHL showed that both ligands overlap (Fig. 2).

Although the docking simulations with LasR predicted that RA could overlap with bound 3-Oxo-C12-AHL (Fig. 2), the ITC studies showed an absence of binding of RA for LasR (Fig. 1E).

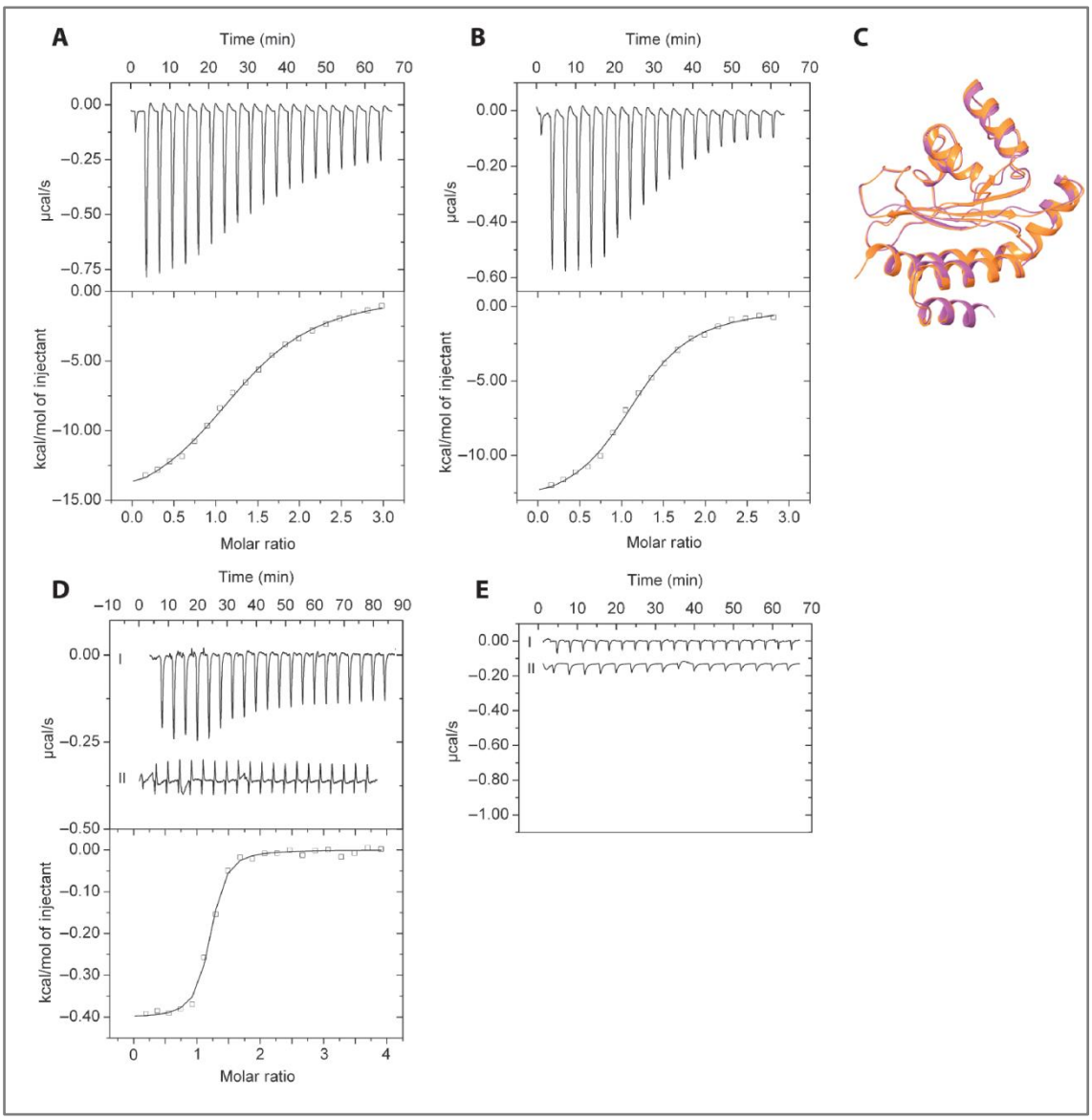


Fig. 1) Microcalorimetric binding studies of LasR and RhlR. (**A**) Titration of 8 µM RhlR with 100 µM C4-AHL. (**B**) Titration of 8 µM LasR with 100 µM 3-Oxo-C12-AHL. **(C)** Structural superimposition of the homology model of the autoinducer binding domain of RhlR (in orange) with the structure of the analogous domain from LasR (in pink, PDB ID: 3IX3). The alignment was done using Subcomb (Kozin & Svergun, 2001). (**D**) Titration of 19 µM RhlR with 0.66 mM RA (I) or chlorogenic acid (II). Lower panel plots the titration data for RA. (**E**) Titration of buffer (I) and 8 µM LasR (II) with 0.66 mM RA. For the titration data (A, B, D, E), the upper panels show the raw titration data and the lower panels are concentration-normalized and dilution heat-corrected integrated peak areas of the titration data fitted with the "One binding site model" of the MicroCal version of ORIGIN.

Table 1) Results from *in silico*-docking and experimental-binding studies of plant-derived compounds to RhlR and LasR. Shown are XP scores for the *in silico* docking of different ligands to a homology model of the RhlR autoinducer domain and to the structure of the analogous domain of LasR. Binding parameters are derived from ITC experiments of purified LasR and RhlR with the ligands listed. Data shown are means and standard deviations from three independent experiments.

Ligand	Docking XP score		Binding parameters			
	RhlR	LasR	RhlR		LasR	
			K_D (μM)	ΔH (kcal/mol)	K_D (μM)	ΔH (kcal/mol^{-})
Isoorientin	-12.33	-14.95	No binding		No binding	
Spiraeoside	-12.69	-14.29	No binding		No binding	
Luteolin-galactoside	-13.01	-13.37	No binding		No binding	
Propanolol	-11.70	-12.36	No binding		No binding	
RA	-8.13	-10.87	0.49 ± 0.1	-0.4 ± 0.05	No binding	
Mangiferin	-8.90	-10.47	No binding		No binding	
Morin	-8.87	-9.57	No binding		No binding	
Chlorogenic Acid	-9.46	-7.42	No binding		No binding	
Naringin	< 4.0	-5.96	No binding		No binding	
3-Oxo-C12-AHL	-7.39	-8.71	Not determined		1.14 ± 0.2	-13.6 ± 0.2
C4-AHL	-4.77	-5.56	1.66 ± 0.4	-16.1 ± 0.2	Not determined	

Rosmarinic acid stimulates RhlR-mediated transcription

To determine whether RA behaved as an agonist or antagonist, we conducted *in vitro* transcription assays with a 490-bp DNA fragment containing the *hcnABC* promoter, which is activated by RhlR (Pessi & Haas, 2000). We performed the experiments with a constant RhlR concentration of 25 μM in the presence of either equimolar or half-equimolar concentrations of C4-AHL and RA. Because the K_D values for both ligands are much less than the protein concentration, we expect that protein saturation at equimolar ligand concentrations is comparable and almost complete. Both C4-AHL and RA stimulated RhlR-dependent transcription (Fig. 3A), but quantification revealed that RA exhibited a significantly greater activity compared to C4-AHL when the response to equal concentrations of ligand was compared to the transcription detected in the control (absence of ligand) condition (Fig. 3B). In the presence of half-equimolar C4-AHL or RA to RhlR concentrations, transcriptional activity

was less than at equimolar concentrations of ligand to regulator, thus indicating dose dependence of the response. Consistent with the lack of binding to RhlR, chlorogenic acid did not increase transcription. These data showed that compared to the bacterial ligand C4-AHL, RA was more effective at activating RhlR-dependent transcription from this promoter.

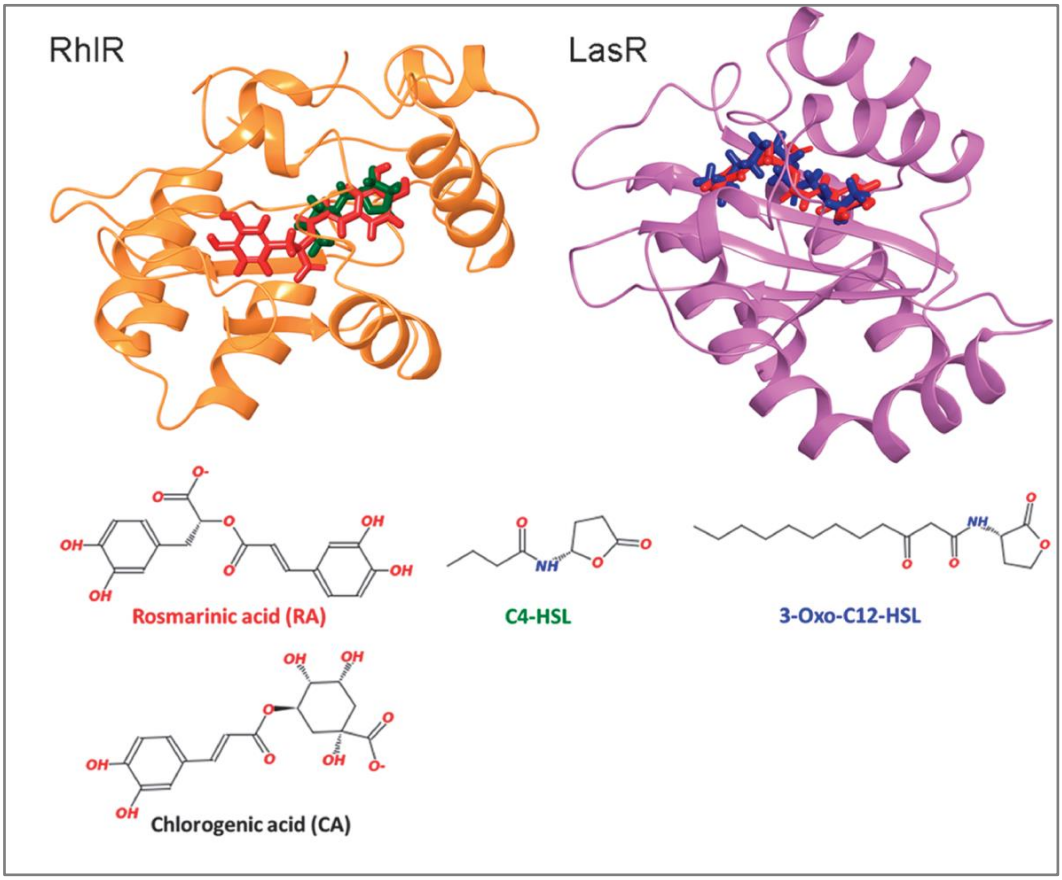


Fig. 2) *In silico* docking of RA to the autoinducer-binding domains of RhlR or LasR. The RhlR autoinducer binding domain is a homology model (see Materials and Methods) and contains docked C4-AHL and the LasR structure (PDB ID: 3IX3) contains bound 3-Oxo-C12-AHL. The best binding position of docked ligands with minimal glide score (XP G score) and glide energy (G energy) are displayed. The docking scores are shown in Table 1. The structures of different ligands are shown in the lower part of the figure.

Rosmarinic acid stimulates QS gene expression in vivo

To assess the capacity of RA to modulate QS-dependent gene expression *in vivo*, we conducted experiments in *E. coli* and *P. aeruginosa*. RhlR controls the expression of the gene encoding its cognate AHL synthase RhlI (Pesci et al, 1997). Therefore, we transformed *E. coli* BL21 with either plasmid pPET28b-RhlR (RhlR expression plasmid) or pPET28b (empty plasmid as control) and pMULTIAHLPROM containing an *rhlI*::*lacZ* transcriptional fusion. B-gal measurements showed statistically significant increases in gene expression the presence of C4-AHL or RA,

whereas chlorogenic acid did not stimulate expression (Fig. 4A). To study gene expression in *P. aeruginosa*, we introduced the *rhlI*::*lacZ* reporter plasmid into the *las*I$^-$/*las*R$^-$ double mutant and measured β-galactosidase activity in samples taken at different time intervals after the addition of either DMSO, C4-AHL, or RA. The data indicated that the bacteria exhibited differential kinetics in response to the two RhlR ligands. Induction of the reporter in the cultures exposed to RA peaked within 1 hour and was significantly greater than that of the control and the C4-AHL-exposed cultures at both 1 and 2 hours (Fig. 4B). At subsequent time points, reporter activity decreased in the RA-containing cultures. We predicted that the reduction in β-galactosidase activity of RA-containing cultures after 2 h indicated that RA was metabolized. The activity of C4-AHL cells was comparable to that of the control after 1 hour but was significantly greater for time points 6 to 8 hours, which may be due to slow uptake.

We determined the dose-response relationships for the increase in gene expression induced by C4-AHL and RA (Fig. 4C). Because of the differences in kinetics, we took measurements for the concentrations of ligand tested at 4 hours; the time point at which C4-AHL and RA induced similar amounts of β-galactosidase activity (Fig. 4B).

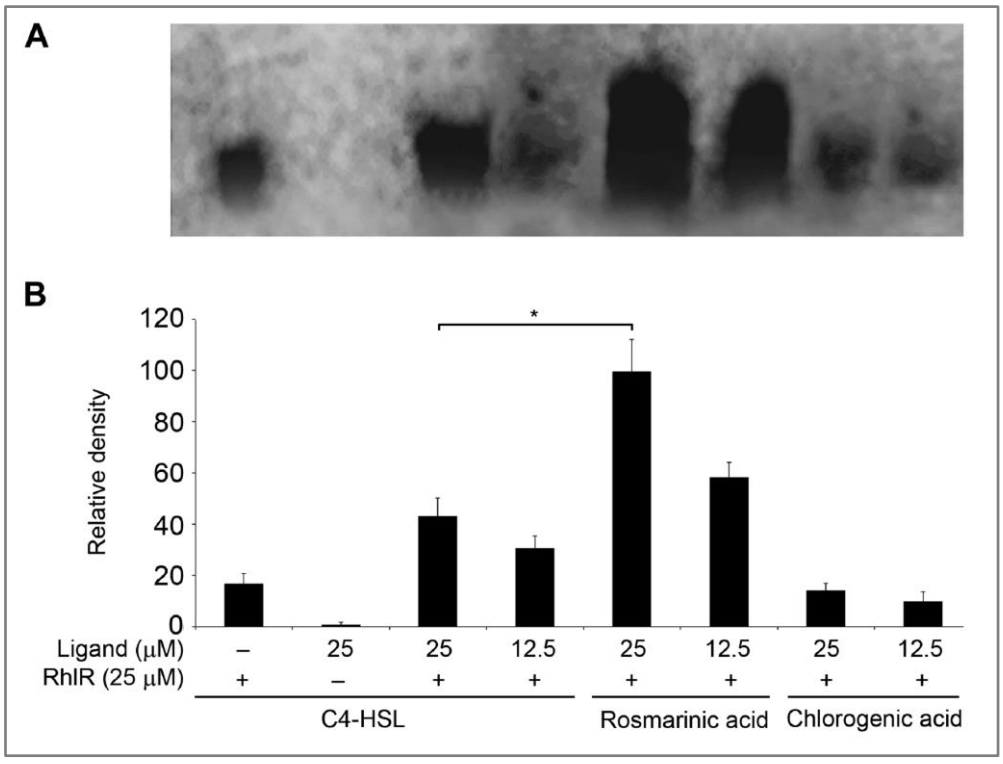


Fig. 3) The capacity of C4-AHL and RA to stimulate RhlR-mediated transcription *in vitro*. (A) Representative acrylamide gel of an in-vitro transcription assay using a DNA fragment containing the *hcnABC* promoter that is induced by RhlR (Pessi & Haas, 2000). Conditions match those listed below the graph in B. Ligands tested included C4-AHL, RA, and chorogenic acid. **(B)** Densitometric analysis of *in vitro* transcription assays. Shown are means and standard deviations from three individual experiments. Student's t-test: * $P < 0.05$.

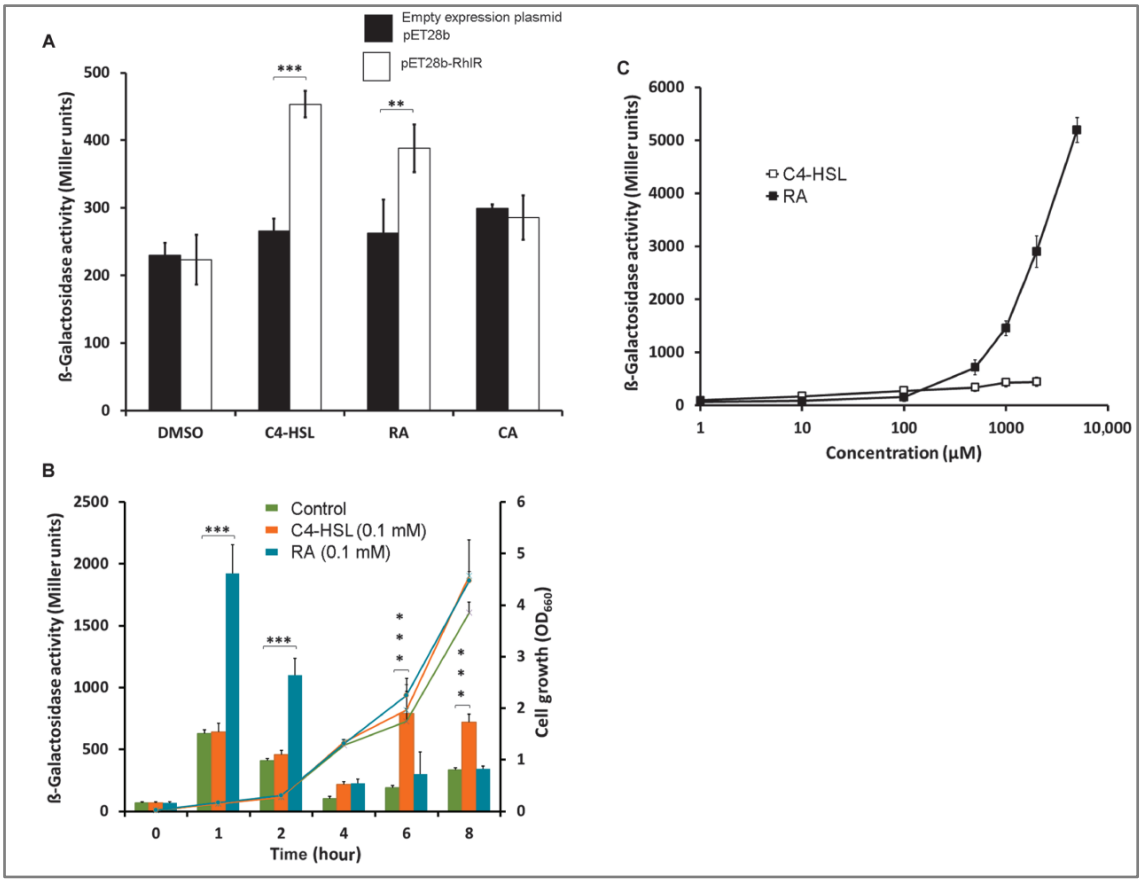


Fig. 4) RA- or C4-AHL-mediated activation of a QS reporter in bacteria. **(A)** Transcriptional activation in *E. coli*. B-gal measurements at 2 h after induction of *E. coli* BL21 containing pET28b-RhlR (expression plasmid for RhlR) or the empty expression plasmid and pMULTIAHLPROM containing a *rhlI*::*lacZ* transcriptional fusion. The ligand concentrations were 100 µM. **(B)** Transcriptional activation over time by the indicated ligands in *P. aeruginosa lasI*$^-$*/lasR*$^-$ containing pMULTIAHLPROM. Bars represent the β-galactosidase measurements at different time intervals after the addition of DMSO (control), C4-AHL, or RA. The line graphs represent growth curves of the corresponding cultures. **(C)** Concentration-dependent transcriptional activation by the indicated ligands in *P. aeruginosa lasI*$^-$*/lasR*$^-$ containing pMULTIAHLPROM. Dose-response curves for each ligand from samples taken 4 hours after the addition of the ligand. Shown are means and standard deviations from three independent experiments conducted in duplicates. Student's t-test: ** $P < 0.01$, *** $P < 0.001$.

At concentrations of 1 to 100 µM, transcriptional activities were comparable, which may be due to metabolization of RA (Fig. 4C). However, at 0.5, 1, and 2 mM the β-galactosidase activity in response to RA was higher than that induced by C4-AHL (Fig. 4C). At 5 mM RA, we observed a further increase in β-galactosidase activity, but we could not perform similar measurements with C4-AHL due to the solubility limit of this compound. These data are consistent with the *in vitro* transcription experiments and support the conclusion that the capacity of RA to stimulate transcription is superior to that of C4-AHL.

To confirm that *P. aeruginosa* could metabolize RA, we analyzed bacteria grown in minimal medium containing 1-10 mM RA as the only carbon source. *P. aeruginosa* grew with 1 to 5 mM RA as the only carbon source (Fig. 5A), consistent with metabolism of this compound and suggesting that metabolism of RA may be responsible for the reduction of its gene induction activity over time. The bacteria did not grow in 10 mM RA. Although our growth data are consistent with those of Annapoorani *et al*. (Annapoorani et al, 2012), they conflict with those of Walker *et al*. (Walker et al, 2004) who reported that RA is toxic at low micromolar concentrations. Therefore, we examined cell viability as a function of RA concentration. We found that viability was not affected by RA concentrations up to 7.8 mM, whereas viability dropped at 15.6 mM (Fig. 5B). Furthermore, we confirmed that the presence of C4-AHL and RA at the concentrations used for gene expression studies did not change growth kinetics (Fig. 5C). These discrepancies in the RA tolerance of *P. aeruginosa* PAO1 may be due to differential evolution of the strain in different laboratories.

The transcriptional reporter studies so far used the *rhlI* promoter. We performed analogous experiments in *P. aeruginosa* PAO1 expressing *lacZ* controlled by *lasB (Pearson et al, 1997)*, *rhlA* (Medina et al, 2003), or *hcnABC* (Pessi & Haas, 2000), which are all induced by RhlR. RA produced a significant increase in β-galactosidase activity for each of the three reporters (Fig. 6, A, B, C); whereas control experiments with bacteria transformed with the empty plasmid did not show an increase in β-galactosidase activity upon RA addition (Fig. 6D).

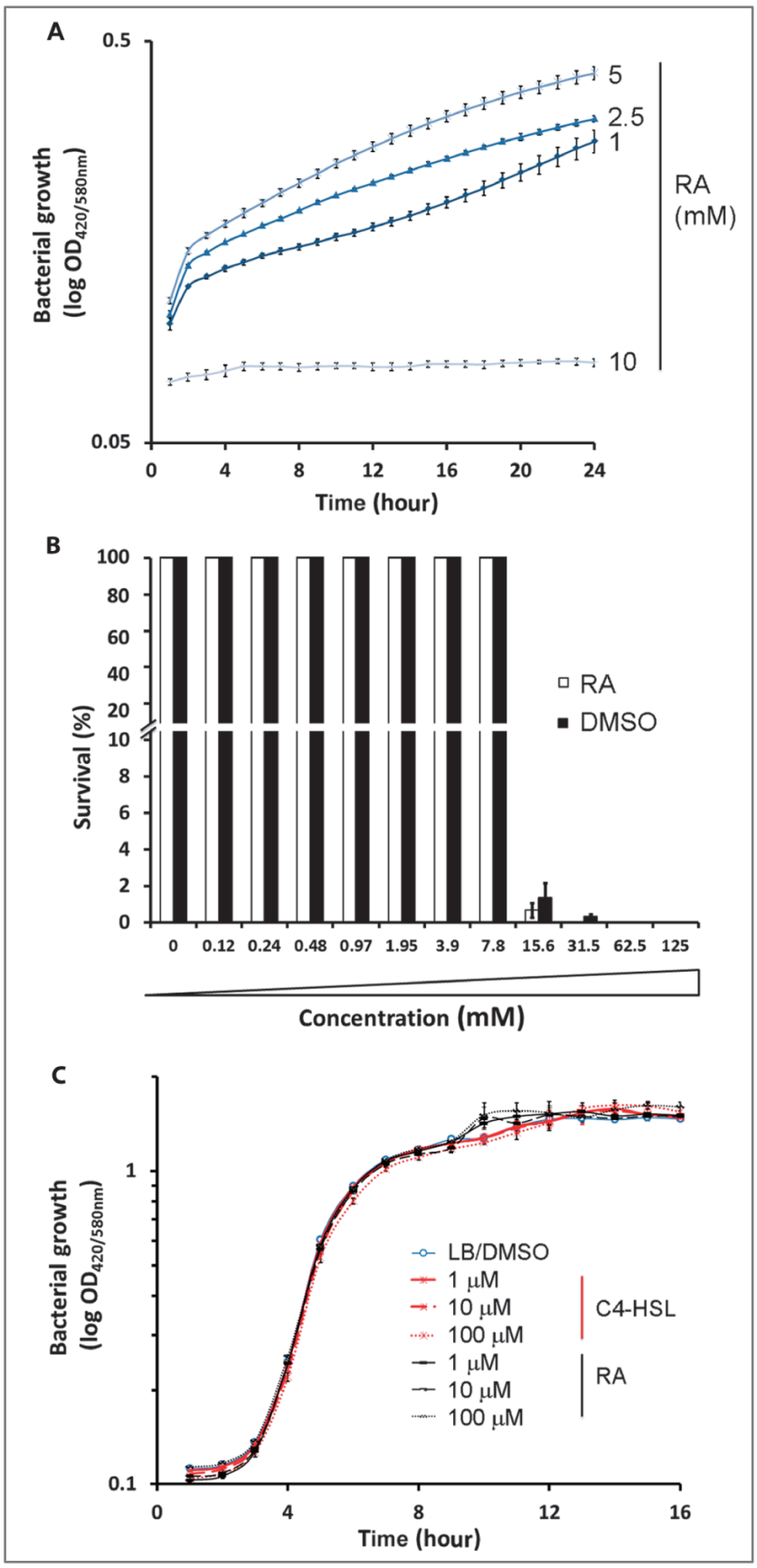


Fig. 5) The effect of RA on *P. aeruginosa* PAO1 survival and growth. (A) Growth curve of *P. aeruginosa* PAO1 in M9 minimal medium supplemented with the indicated concentration of RA. Shown are means and standard deviations from three experiments conducted in triplicate. **(B)** Growth in LB medium supplemented with the indicated concentrations of RA (and the corresponding DMSO-containing controls). Cell survival after 24 hours was determined by plating out on solid medium and cell counting. Shown are means and standard deviations from three independent experiments each conducted in quintuplicate. **(C)** Impact of RA on bacterial growth on rich medium. Shown is a growth curve in LB medium supplemented with different concentrations of C4-AHL or RA. Shown are means and standard deviations from three experiments conducted in triplicate.

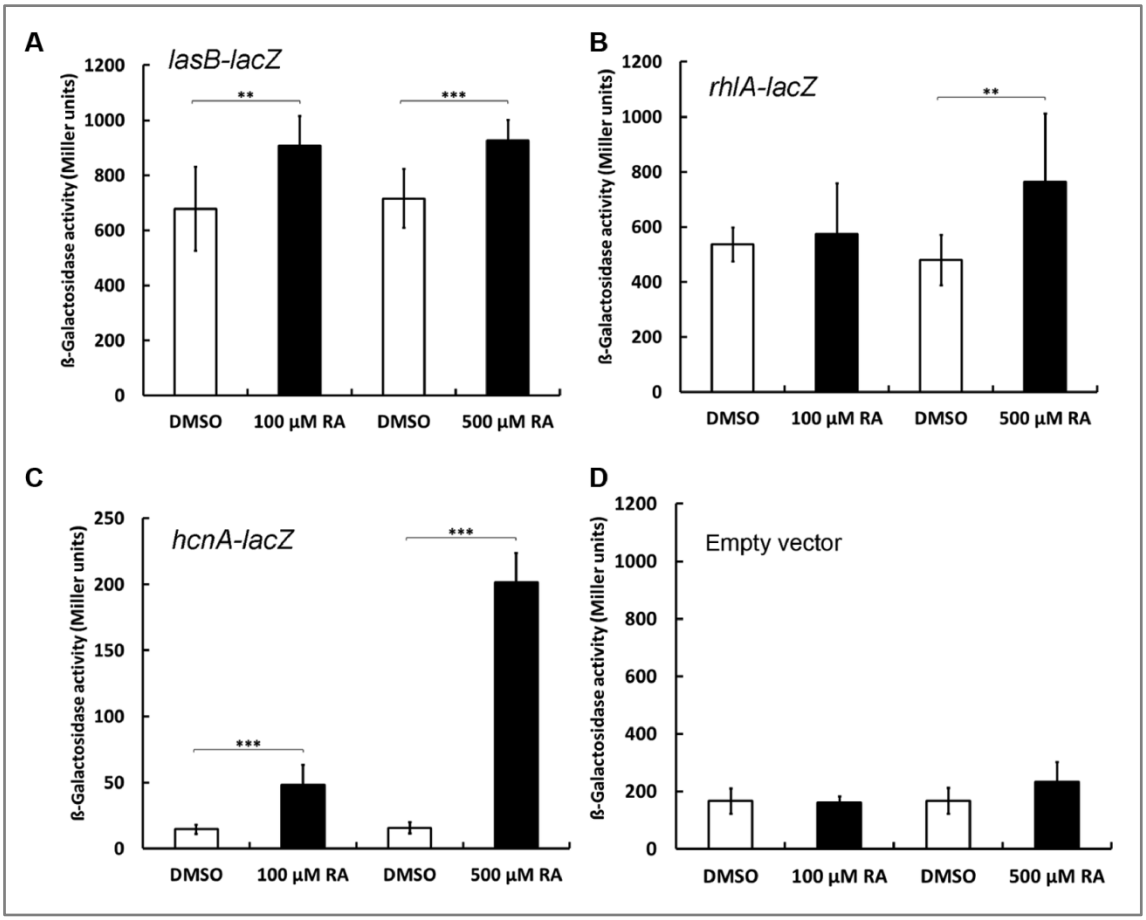


Fig. 6) Effect of RA on the expression from other QS-regulated promoters. Shown are β-galactosidase measurements of *P. aeruginosa* PAO1 containing the plasmids **(A)** pß01 (*lasB-lacZ)*, **(B)** pß02 (*rhlA-lacZ),* **(C)** pME2823 (*hcnA-lacZ)*, or **(D)** pQF50 (empty vector). Measurements were made 2 hours after the addition of RA or the corresponding amount of DMSO. Shown are means and standard deviations from three independent experiments conducted in duplicates. Student's t-test: ** P < 0.01, *** P < 0.001.

Rosmarinic acid increases biofilm formation, pyocyanin production, and elastase synthesis

Increased biofilm formation and the production of the virulence factor pyocyanin are characteristic features of AHL-mediated QS responses (Davies et al, 1998; Sakuragi & Kolter, 2007). We therefore tested these traits in *P. aeruginosa* grown in the presence and absence of different concentrations of RA or chlorogenic acid. We quantified pyocyanin, which is green, by measuring the absorbance at 520 nM and found that RA, but not chlorogenic acid, stimulated a dose-dependent increase the intensity of the green color when corrected for cell density (Fig. 7A).

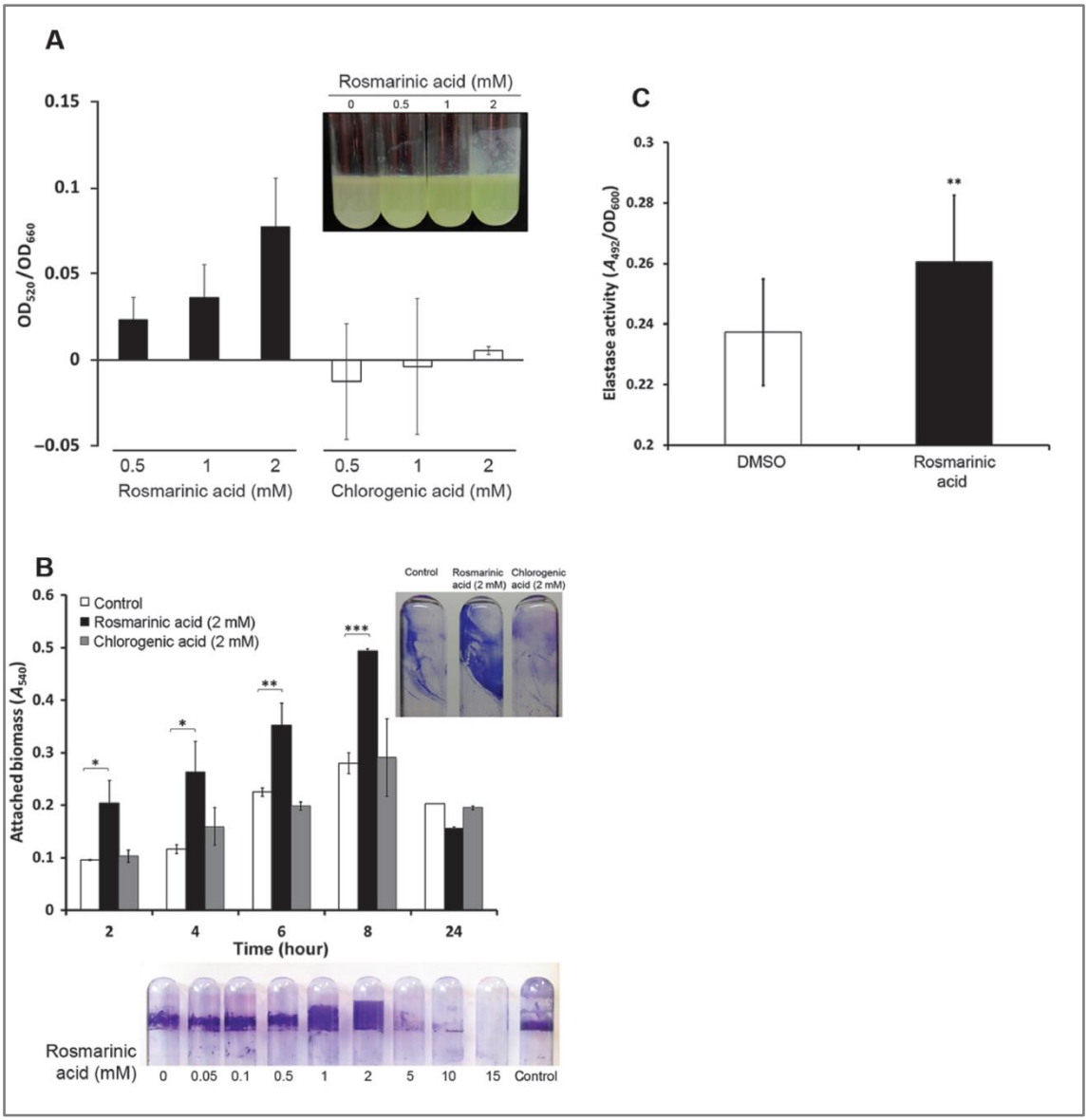


Fig. 7) RA induces QS-regulated phenotypes. (A) Pyocyanin production measured in *P. aeruginosa* PAO1 grown in the presence of different concentrations of RA or chlorogenic acid. Shown are means and standard deviation from three independent experiments of the absorbance at 520 nm (pyocyanin) of *P. aeruginosa* supernatants relative to the absorbance at 660 nm (cell density). The inset shows culture tubes after 8 hours growth. (Note: The amount of DMSO equivalent to that added in the 2 mM RA condition was added as a control to the first tube "0"). **(B)** Biofilm formation quantified in the presence and absence of RA or chlorogenic acid at different time points. Shown are means and standard deviations from three independent experiments. Student's t-test: * P< 0.05, ** P < 0.01, *** P < 0.001. The inset shows representative crystal violet stained tubes containing an 8-hour culture of *P. aeruginosa* in the presence and absence of RA or chlorogenic acid. In the lower part, the tubes show the RA concentration dependence of biofilm formation (after 8 h). **(C)** Elastase synthesis measured as enzymatic activity of *P. aeruginosa* PAO1 cultures grown in the presence or absence of RA. Data are normalized for culture density. Shown are means and standard deviation from three individual experiments conducted in triplicate. Student's t-test: ** P < 0.01.

Visual inspection of culture tubes (inset in Fig. 7A) showed that RA also stimulated biofilm formation. To quantify the effect of RA on biofilm formation, we grew bacterial cultures in borosilicate glass tubes in the presence or absence of either 2 mM RA or chlorogenic acid and quantified biofilm formation at different time points. We found that RA stimulated biofilm formation between 2 to 8 hours of growth, whereas chlorogenic acid had no significant effect (Fig. 7B, upper). After 24 hours the amount of biofilm formed in these three conditions was comparable. The RA-mediated stimulation of biofilm formation contradicted a previous report in which the compound was used at higher concentrations than we used here and inhibited biofilm formation (Annapoorani et al, 2012). To assess this discrepancy, we determined the dose-response relationship of the RA-mediated effect on biofilm (Fig. 7B, lower). RA stimulated biofilm formation at concentrations up to 2 mM, but above this concentration biofilm formation was inhibited by RA.

Elastase synthesis is also stimulated by RhlR-mediated QS activity (Pearson et al, 1997). Our reporter analysis indicated that RA enhanced the activity of the promoter controlling the expression of lasB, encoding elastase (Fig. 6A). To verify whether this results in changes in elastase synthesis, we measured elastase activity in *P. aeruginosa* grown in the absence and presence of RA. RA stimulated the amount of elastase activity in density-normalized cultures (Fig. 7C).

Discussion

Multiple lines of evidence indicate that plants produce AHL mimics, which interfere with the bacterial QS system (Degrassi et al, 2007; Ferluga et al, 2007; Ferluga & Venturi, 2009; Gao et al, 2003; Gonzalez et al, 2013; Perez-Montano et al, 2013; Subramoni et al, 2011; Teplitski et al, 2000; Tolmacheva et al, 2014). However, little information is available regarding the molecular identity of the active compounds that are responsible for this interference (Venturi & Fuqua, 2013).

We report the identification and characterization of a plant compound that directly interacts with a bacterial QS regulator, stimulating its transcriptional activity. RA bound purified RhlR with a higher affinity than C4-AHL, and this translated into a greater stimulatory activity of RA on RhlR-mediated transcription and gene expression, compared with that induced by C4-AHL. Furthermore, RA stimulated biofilm formation and the synthesis of pyocyanin and elastase, which are phenotypic characteristics that are regulated by QS mechanisms in *P. aeruginosa* (Davies et al, 1998; O'Loughlin et al, 2013).

Because interference with bacterial QS mechanisms by plant-derived compounds has been observed for various different plant pathogens, it was proposed that this represents a plant defense strategy (Venturi & Fuqua, 2013). AHL mimics acting as agonists of AHL-mediated sensing may decrease pathogenicity, because these mimics would stimulate premature expression of genes encoding proteins involved in QS-controlled functions (Venturi & Fuqua, 2013). Molecular identification of AHL mimics will enable their application in medicine and agriculture, for example, in the generation of pathogen-resistant plants. However, is this mimicking of a bacterial QSS by RA of physiological relevance? Walker et al. (2004) monitored the consequences of sweet basil root infection by *P. aeruginosa* strains PAO1 and PA14 and determined that infection with either strain induced RA secretion. RA concentration in root exudates gradually increased, reaching a maximum of ~40 μM 6 days after infection (Walker et al, 2004). Thus, plant infection triggers RA release, suggesting that RA release forms part of a plant defense strategy and supporting the model that plant-derived AHL mimics decreased pathogenicity by stimulating premature QS-responsive gene expression (Venturi & Fuqua, 2013). We found that RA was both a growth substrate and a signaling molecule. Bacterial consumption of RA may provide a mechanism to eliminate the signaling effects of this compound, which is consistent with the kinetics of gene induction by RA that we observed.

Reports of the toxic effects of RA to *P. aeruginosa* PAO1 differ. Our data and those reported by Annapoorani et al. (Annapoorani et al, 2012) indicate an effective inhibitory concentration of 2.1 mM with regard to bacterial growth, whereas Walker et al. (Walker et al, 2004) reported minimal inhibitory concentration of 8 μM. These discrepancies may be due to differential strain evolution in different laboratories. Annapoorani et al. showed a reduction of *P. aeruginosa* PAO1 biofilm formation and elastase activity in the presence of RA and a number of related compounds, including chlorogenic acid (Annapoorani et al, 2012); however, our data showed no activity for chlorogenic acid in binding to or stimulating RhlR or in promoting biofilm formation and we found that RA functioned as an AHL mimic.

P. aeruginosa is one of the pathogens that infect a wide range of species, including plants and animals (Cao et al, 2001). Common pathogenic mechanisms enable bacteria to infect phylogenetically different hosts, and there are also parallels in the key features underlying host defense responses in plants, invertebrates, and mammalian hosts (Cao et al, 2001). The production of AHL mimics is considered a plant defense strategy. The detailed knowledge of plant defense mechanisms may enable the development of strategies to protect human from this pathogen. The effect of AHL mimics, including RA on the virulence properties toward mammals is of interest.

RA is synthesized by many plants (Petersen et al, 2009) and can accumulate to high concentrations (Shekarchi et al, 2012). RA has multiple biological activities, including antibacterial (Walker et al, 2004), antiviral (Swarup et al, 2007), antiallergy (Sanbongi et al, 2004), anticarcinogenic (Osakabe et al, 2004), antigenotoxic (Mladenovic et al, 2013), anti-inflammatory (Kang et al, 2003), and antioxidant effects (Liu et al, 1992) activity. In addition, RA was found to be effective against amyloid-β peptide-induced neurotoxicity that is associated with Alzheimer´s disease (Iuvone et al, 2006), reduces atopic dermatitis (Lee et al, 2008), and protects keratinocytes from ultraviolet radiation damage (Psotova et al, 2006). Consequently, plants rich in RA are used as medicinal herbs and by the food industry (Shekarchi et al, 2012).

Solo or orphan QS regulators recognize plant-derived AHL mimics (Venturi & Fuqua, 2013). Solo QS regulators are abundant in plant-associated bacteria, which supports the view that they are involved in interkingdom signaling between plants and bacteria (Venturi & Fuqua, 2013). Our data showed that RhlR has a double function and mediates responses to bacterial AHL and plant-derived RA, thus indicating that recognition of AHL mimics is not limited to solo QS regulators.

Several synthetic AHL and non-AHL ligands have been identified that modulate the activity of *P. aeruginosa* QS regulators (Borlee et al, 2010; Geske et al, 2007; Geske et al, 2005; Mattmann et al, 2008; Rabin et al, 2013; Skovstrup et al, 2013). These compounds behaved either as agonists or antagonists. Structurally the agonists (Borlee et al, 2010; Skovstrup et al, 2013) are similar to RA, because part of these compounds were linear with aromatic moieties at each extension of the molecule.

Supplementary material

Supp. table 1) Strains and plasmids used in this study.

Strains	**Relevant Characteristics**	**Reference**
P. aeruginosa PAO1	wild type, prototroph; Ap^r	(Stover et al, 2000)
P. aeruginosa PAO1 *lasI⁻ /lasR⁻*	Double mutant in *lasI* and *lasR* genes Gm^r	Personal gift, M. Cámara (University of Nottingham)
E. coli BL21 (DE3)	F^-, *ompI, hsdS*$_B$ (r^-_B m^-_B)	(Studier & Moffatt, 1986)
Plasmids	**Features**	**Reference**
pET28b(+)	Km^R, protein expression vector	Novagen
pET28b-LasR	Km^R, pET28b(+) derivative containing *lasR* gene	This work
pET28b-RhlR	Km^R, pET28b(+) derivative containing *rhlR* gene	This work
pMULTIAHLPROM	Tc^R, Broad-host-range plasmid containing 8-*luxI* type promoters fused to a promoter *lacZ* gene	(Steindler et al, 2008)
pQF50	Cb^R, Broad-host-range transcriptional fusion vector	(Ishida et al, 2007)
pß01	Cb^R, pQF50 derivative containing *lasB-lacZ* transcriptional fusion	(Ishida et al, 2007)
pß02	Cb^R, pQF50 derivative containing *rhlA-lacZ* transcriptional fusion	(Ishida et al, 2007)
pME2823	Cb^R, pKT240 derivative containing *hcnA-lacZ* transcriptional fusion	(Pessi & Haas, 2000)

Antibiotics resistance: Ap, ampicillin; Gm, gentamycin Km, kanamycin; Tc, tetracycline; Cb, carbencillin

CONCLUSIONS

1. The analysis of all 27 single mutants in MCP indicated that MCP based sensory mechanisms play important roles in biofilm formation.
2. Two different mechanism can be distinguished by which MCP based signaling modulates biofilm formation, namely by mediating chemotaxis towards amino acids and polyamines or by altering c-di-GMP levels.
3. The McpU MCP identified in this thesis corresponds to a novel type of MCP since it was shown to bind and respond exclusively to polyamines.
4. *P. putida* KT2440 was shown to respond chemotactically to 15 different L-amino acids. In this process the MCP McpA play a key role since it was shown to mediate chemotaxis to 12 different amino acids.
5. Mutation of the WspA_Pp gene reduced cellular c-di-GMP levels indicating that this receptor feeds into the Wsp pathway in *P. putida* KT2440. This contrast with the Wsp pathway in *P. aeruginosa* where the WspA mutation did not alter c-di-GMP levels.
6. Mutants in WspA-Pp and McpU were less efficient in plant root colonization indicating that c-di-GMP levels and polyamine chemotaxis are important factors that influence plant-bacteria interaction.
7. LasR and RhlR are functional and soluble in absence and presence of AHL and may be purified from *E.coli* cultures in the absence of added AHL. The demonstration that both proteins are stable in the absence of bound AHL does not agree with the previously proposed hypothesis that nascent proteins require AHL for correct folding.
8. Binding studies demonstrate that both proteins have not evolved to preferentially recognize their cognate AHL ligands.
9. Both proteins were found to bind AHLs with affinities of approximately 1 µM, which contrast with previous measurements reporting ultra-tight binding. However, our data are in agreement with *in vivo* experiments showing that removal of AHLs causes immediate drop in transcriptional activity, caused by AHL dissociation from their regulators.
10. Contrary to a previously published model AHL binding does neither increase protein stability nor induce dimerization.
11. The plant compound RA binds with high affinity to RhlR and mimics the cognate C4-AHL. RA enhances RhlR dependent transcription *in vitro* and *in vivo* and causes virulence associated and QS mediated phenotypes such as increases in the concentration of the pyocyanin and elastase virulence factors. The ensemble of data represents the first identification of a plant compound that stimulates QS mediated transcription.

12. Since RA is exclusively produced by plants and since plant infection causes RA secretion, we propose that the mechanism elucidated corresponds to plant defence mechanisms against pathogens.
13. The lack of knowledge on the signal molecules that bind to bacterial sensor proteins corresponds to a major limitation in the field of bacterial signal transduction. Data shown in this thesis indicate that high throughput thermal shift assays and *in silico* docking experiments are very potent approaches in the identification of signal molecules.

CONCLUSIONES

1. Él análisis de los 27 mutantes individuales en quimiorreceptores, indicó que los mecanismos de señalización basados en quimiorreceptores desempeñan papeles importantes en la formación de biopelículas.
2. Dos diferentes mecanismos de señalización basados en quimiorreceptores se pueden distinguir por modular la formación de biopelículas, uno en el que la quimiotaxis hacia aminoácidos y poliaminas está involucrado y otro en el que la alteración de los niveles de c-di-GMP determinan la formación de la biopelículas.
3. El quimiorreceptor McpU identificado en esta tesis corresponde a un nuevo tipo de quimiorreceptor, ya que se demostró que se une y responde exclusivamente a poliaminas.
4. Fue demostrado que *P. putida* KT2440 responden quimiotácticamente a 15 L-aminoácidos diferentes. En este proceso, el quimiorreceptor McpA juegan un papel clave, ya que se demostró que es responsable de la quimiotaxis a 12 de estos aminoácidos.
5. La mutación del gen *wspA_Pp* redujo los niveles de c-di-GMP celulares lo cual indica que este receptor pertenece en la vía de Wsp de *P. putida* KT2440. Este contraste con la vía Wsp de *P. aeruginosa* en donde la mutación del quimiorreceptor WspA no alteró los niveles de c-di-GMP.
6. Los mutantes WspA-Pp y McpU son menos eficientes en la colonización de raíces de plantas de maíz, lo que indica que los niveles de c-di-GMP y la quimiotaxis a poliaminas son factores importantes que influyen en la interacción planta-bacteria.
7. LasR y RhlR son solubles y funcionales en ausencia y presencia de AHL, y se pueden purificar a partir de cultivos de *E. coli* en ausencia de AHL. La demostración de que ambas proteínas son estables en ausencia AHL no concuerda con la hipótesis propuesta anteriormente que menciona que la proteína naciente requieren la presencia de una AHL para su correcto plegamiento.
8. Estudios de interacción demuestran que la evolución de ambas proteínas no ha favoreciendo el reconocimiento preferencial de sus ligandos afines.
9. Ambas proteínas unen a las AHL con afinidades de aproximadamente 1 µM, lo cual contrastan con la unión ultra fuerte reportada anteriormente. Sin embargo, nuestros datos están de acuerdo con los experimentos *in vivo* que muestra que la eliminación de AHL provoca la caída inmediata en la actividad transcripcional, causada por la disociación de la AHL de sus reguladores.
10. En contraste con el modelo previamente publicado, la unión a la AHL no aumenta la estabilidad de la proteína, ni induce la dimerización.

11. El ácido rosmarínico, un compuesto sintetizado exclusivamente por plantas se une con alta afinidad al regulador RhlR e imita a su ligando bacteriano C4-AHL. El ácido rosmarínico potencia la transcripción *in vitro* e *in vivo* vía el factor de transcripción RhlR. Así mismo, produce la inducción de factores de virulencia y fenotipos regulados por *quorum sensing,* como son el aumento de la concentración de piocianina y elastasa. El conjunto de datos nos indica que el ácido rosmarínico es el primer compuesto de plantas identificado que estimula la transcripción mediada por *quorum sensing*.
12. Dado que el ácido rosmarínico es producido exclusivamente por las plantas y la infección de plantas provoca la secreción de este compuesto, nosotros proponemos que el mecanismo dilucidado corresponde a un mecanismo de defensa de la planta en contra de los patógenos.
13. La falta de conocimientos sobre las moléculas señalizadoras que se unen a las proteínas sensoras de las bacterias es una limitación importante en el campo de la transducción de señales bacteriana. Los datos mostrados en esta tesis indican las técnicas de *high throughput thermal shift assays* e *in silico docking* son ensayos muy potentes que se pueden usar para la identificación de las moléculas señal.

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Assessment of the contribution of chemoreceptor-based signalling to biofilm formation

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Summary

Although it is well established that one- and two-component regulatory systems participate in regulating biofilm formation, there also exists evidence suggesting that chemosensory pathways are also involved. However, little information exists about which chemoreceptors and signals modulate this process. Here we report the generation of the complete set of chemoreceptor mutants of *Pseudomonas putida* KT2440 and the identification of four mutants with significantly altered biofilm phenotypes. These receptors are a WspA homologue of *Pseudomonas aeruginosa*, previously identified to control biofilm formation by regulating c-di-GMP levels, and three uncharacterized chemoreceptors. One of these receptors, named McpU, was found to mediate chemotaxis towards different polyamines. The functional annotation of McpU was initiated by high-throughput thermal shift assays of the receptor ligand binding domain (LBD). Isothermal titration calorimetry showed that McpU-LBD specifically binds putrescine, cadaverine and spermidine, indicating that McpU represents a novel chemoreceptor type. Another uncharacterized receptor, named McpA, specifically binds 12 different proteinogenic amino acids and mediates chemotaxis towards these compounds. We also show that mutants in McpU and WspA-Pp have a significantly reduced ability to colonize plant roots. Data agree with other reports showing that polyamines are signal molecules involved in the regulation of bacteria–plant communication and biofilm formation.

Received 28 May, 2015; accepted 30 November, 2015. *For correspondence. E-mail tino.krell@eez.csic.es; Tel. (+34) 958 181600 (ext. 294); Fax (+34) 958 135740. Originality-significance statement: This study is the first comprehensive analysis of the contribution of all chemoreceptors of a bacterium to biofilm formation, showing that chemotaxis towards amino acids and polyamines as well as alterations in the c-di-GMP level modulate biofilm formation. This study resulted in the functional annotation of two chemoreceptors. One of them, termed McpU, was shown to bind and respond exclusively to polyamines, which corresponds to a novel chemoreceptor type. This work also illustrates the power of thermal shift assay-based ligand screening in the annotation of chemoreceptors. This approach can also be used to identify ligands for other types of sensor proteins, which currently represents a major bottleneck in signal transduction research.

Introduction

Many bacteria are present in their natural habitats as biofilms. Biofilm formation is a cooperative group behaviour that involves bacterial populations living embedded in a self-produced extracellular matrix. This process is of enormous clinical relevance (Cos *et al.*, 2010; Sun *et al.*, 2013) because it influences the mechanisms that cause resistance to antibiotics and evasion of host immune defences. In addition, biofilm formation is a phenomenon that is of importance to a diverse range of biotechnological applications (Wang and Chen, 2009). The current model of biofilm formation purports that, within a number of species, the process comprises different life stages (Hobley *et al.*, 2015). The formation of a biofilm is initiated by the reversible attachment of planktonic cells to surfaces, which is an event induced by environmental stimuli. This is followed by an irreversible attachment stage, and then growth and maturation of the biofilm. These stages are characterized by the synthesis of the biofilm matrix and the development of antimicrobial resistance. Finally, environmental detachment signals trigger the bacteria to transition to a planktonic lifestyle and to be released from the biofilm. The transition between the different stages during biofilm development and dispersal are subject to a variety of regulatory mechanisms in response to stimuli that are not yet fully understood.

The regulatory mechanisms that are known to mediate bacterial signal transduction include one- and two-component regulatory systems as well as chemosensory pathways. Pseudomonads are well-studied model organisms in terms of biofilm formation and there exists a significant body of knowledge regarding the involvement of different one- and two-component regulatory systems (Mikkelsen *et al.*, 2011; Fazli *et al.*, 2014). Cell-to-cell communication is essential for biofilm development; as

such, the one-component systems RhlR and LasR of *Pseudomonas aeruginosa* have been found to play central roles in the expression of genes related to biofilm formation in response to quorum sensing signals (O'Loughlin *et al*., 2013). The two-component systems formed by the GacS/RetS/LadS sensor kinases and the GacA response regulator were found to be essential in biofilm regulation. These systems control the transcription of sRNA involved in biofilm formation and respond to yet unidentified environmental cues (Ventre *et al*., 2006), which, in turn, induce different phenotypic alterations. On the one hand, these two-component systems mediate the production of compounds necessary for attachment and biofilm formation, including exopolysaccharides (Friedman and Kolter, 2004); large adhesion proteins (Martinez-Gil *et al*., 2010); extracellular DNA (Whitchurch *et al*., 2002); and different surfactants, such as lipopeptides (Kuiper *et al*., 2004) and rhamnolipids (Boles *et al*., 2005). On the other hand, they modulate the levels of the bacterial second messenger c-di-GMP – a mode of action that is relevant because increased levels of c-di-GMP promote biofilm formation and reduce bacterial motility (Romling *et al*., 2013).

Chemosensory pathways represent the third group of major signal transduction systems in bacteria (Wuichet and Zhulin, 2010). The core of a chemosensory pathway is the ternary complex composed of a chemoreceptor, a CheA histidine kinase and a CheW coupling protein. Signal recognition at the chemoreceptor ligand binding domain (LBD) modulates CheA autophosphorylation and consequently transphosphorylation of the CheY response regulator, which can then bind to the flagellar motor, ultimately leading to chemotaxis (Hazelbauer *et al*., 2008). Existing genetic and biochemical evidence suggest that chemosensory pathways can modulate various functions, such as flagellum-based chemotaxis, type IV pili-based motility and non-motility-related cellular functions (Wuichet and Zhulin, 2010). *Pseudomonas aeruginosa* PAO1 has five gene clusters that encode signalling proteins involved in four different chemosensory pathways (Sampedro *et al*., 2014). Two of these pathways (*che* and *che2*) are involved in flagellum-mediated taxis (Ferrandez *et al*., 2002); another pathway (*wsp*) modulates c-di-GMP levels (Hickman *et al*., 2005); and the fourth pathway (*chp*) is responsible for type IV pili-mediated motility (Whitchurch *et al*., 2004) and the regulation of cAMP levels (Fulcher *et al*., 2010).

There is a significant body of evidence demonstrating that chemosensory pathway mediated signalling participates in the formation and dispersal of biofilms in *P. aeruginosa*. The *wsp* pathway is a clear example of a chemosensory pathway that has an alternate cellular function (Hickman *et al*., 2005). The CheY-homologue of this pathway, WspR, is a fusion of a receiver and a GGDEF domain. It was shown that phosphorylation of WspR enhances the activity of the c-di-GMP-synthesizing GGDEF domain, which, in turn, enhances biofilm formation (Hickman *et al*., 2005; Guvener and Harwood, 2007). It was suggested that the *wsp* pathway controls WspR activity via binding of an unidentified signal molecule to a chemoreceptor. However, in-frame deletion of the gene encoding the WspA chemoreceptor did not significantly alter biofilm formation (Hickman *et al*., 2005). Another chemoreceptor in *P. aeruginosa*, BdlA, was shown to be involved because the corresponding mutant was deficient in biofilm dispersal and showed increased c-di-GMP levels, suggesting that the molecular mechanism for BdlA action is based on alterations in c-di-GMP levels rather than chemotaxis (Morgan *et al*., 2006). A number of studies also found that inactivation of proteins within chemotaxis signalling cascades impacts biofilm formation. Thus, mutation of the genes encoding the methyltransferase CheR (Schmidt *et al*., 2011) and the response regulator CheY (Barken *et al*., 2008) of the *che* pathway abolished chemotaxis, which effected bacterial surface sampling and biofilm formation. In addition, other studies show that inactivation of chemotaxis signalling cascades severely inhibits plant root colonization (de Weert *et al*., 2002; Yao and Allen, 2007). Most of these studies were based on the inactivation of signalling proteins. However, there is little known about which specific chemoreceptors modulate biofilm formation.

In this study, we have addressed this question using *Pseudomonas putida* KT2440 as model organism. This strain is a metabolically versatile saprophytic soil bacterium that is able to form biofilms and to colonize plant roots efficiently (Espinosa-Urgel *et al*., 2002; Regenhardt *et al*., 2002). *Pseudomonas putida* KT2440 has 27 chemoreceptors that differ in topology and LBDs (Fig. 1). Of these receptors, several have been annotated with a function (Fig. 1) and were found to mediate taxis to different organic acids (McpS, McpQ, McpR, McpP) (Lacal *et al*., 2010; 2011; Pineda-Molina *et al*., 2012; Parales *et al*., 2013; Garcia *et al*., 2015; Martín-Mora *et al*., 2015), gamma-aminobutyrate (McpG) (Reyes-Darias *et al*., 2015a) or purine compounds (McpH) (Fernandez *et al*., 2015). In addition, three of the receptors are paralogues to Aer-1, Aer-2 and Aer-3, which have been shown or predicted to mediate aero- and energy taxis (Sarand *et al*., 2008); and two of the receptors share a high degree of sequence identity with BdlA (Fig. S1). The remaining chemoreceptors have not yet been annotated and their putative functions are still unknown.

Using this model system, we carried out a systematic study of the contribution of each of the chemoreceptors to biofilm formation via the analyses of single mutants for each of the 27 chemoreceptors. Our data reveal the role of different chemoreceptors in biofilm formation and have led to the identification of a novel type of chemoreceptor.

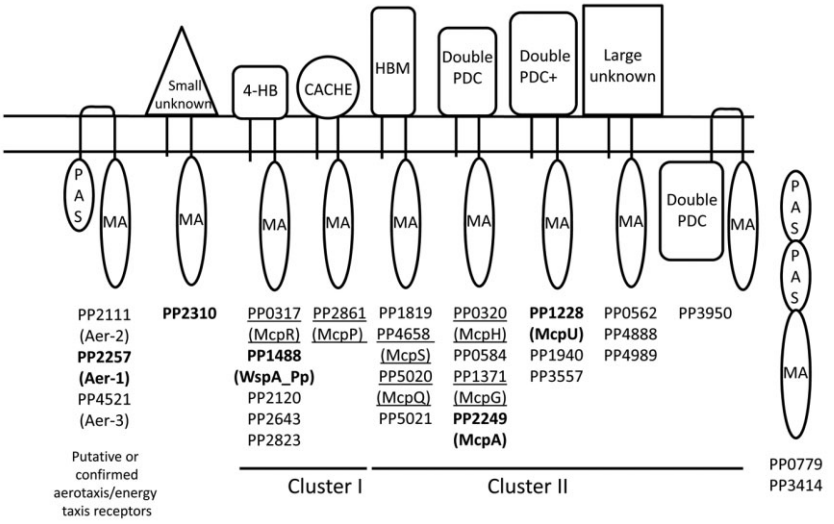

Fig. 1. Topology and diversity of chemoreceptors from *Pseudomonas putida* KT2440. Transmembrane regions were predicted using the DAS algorithm (Cserzo *et al*., 1997). The prediction of LBD type is based on the fold recognition analyses using the Phyre[2] algorithm (Kelley and Sternberg, 2009) and consensus secondary structure predictions (Deleage *et al*., 1997). Receptors that were annotated previously with a function are underlined and those that modulate biofilm formation are shown in bold. MA, methyl-accepting domain; 4-HB, 4-helix bundle domain; HBM, helical bimodular domain; double PDC, repeat of PhoQ/DcuS/CitA-domain (Zhang and Hendrickson, 2010); double PDC+, double PDC domain containing a 40–50 amino acid insert into double PDC domains. HAMP domains have been omitted and classification is done based on receptor topology and LBD type.

Results

Construction of bacterial chemoreceptor mutants and their analysis

Sixteen mutants of *P. putida* KT2440R (Table 1), each deficient in one chemoreceptor gene, were extracted from the *Pseudomonas* Reference Culture Collection (Duque *et al*., 2007). Additionally, we constructed the remaining 11 chemoreceptor mutants as described in the Experimental procedures section to achieve the complete set of chemoreceptor single mutants. Growth experiments in Luria Broth (LB) medium were conducted with the 27 mutants (Table 1) and the wild type (wt) strain. As shown in Fig. S2, the growth kinetics of all strains were similar, indicating that, under the conditions tested, the chemoreceptors did not significantly impact growth. The motility of these strains was also assessed using swim plate assays on soft agar minimal saline (MS) medium supplemented with glucose and the majority of mutants showed motility comparable to the wt strain (Fig. S3). A very slight reduction in motility ($P < 0.5$) was observed for mutants PP1488, PP2257 and PP2643.

Subsequently, the capacity of these strains to form biofilms was assessed. Biofilm formation of *P. putida* KT2440 on abiotic surfaces has been studied previously. Without media replacement, this strain was found to form mature biofilms after 6–8 h, followed by dispersion that was almost complete after 24 h (Martinez-Gil *et al*., 2012). Initially, assays were conducted in polystyrene multiwell plates. Four mutant strains, namely PP1228, PP1488, PP2249 and PP2310, showed significant differences in biofilm formation as compared to the wt. These strains were selected and submitted to biofilm formation assays on borosilicate glass surfaces, which confirmed the data obtained on multiwell plates (Fig. 2). Thus, mutant PP1228 showed increased biofilm formation as compared to the wt. In contrast, mutant PP1488 had a reduced capacity to form biofilm. This receptor is the homologue of *P. aeruginosa* WspA and is therefore referred to as WspA-Pp (Fig. S4). Interestingly, a mutant in *pp2249* showed different biofilm kinetics, reaching maximal biofilm formation earlier than wt (after 6 h as compared to 8 h for the wt). Biofilm dispersion in the strain PP2249 also occurred earlier than wt (Fig. 2). Finally, a mutant strain deficient in *pp2310* also showed an increased amount of biofilm formation.

For mutant complementation studies, genes *pp1228*, *pp1488*, *pp2249* and *pp2310* were cloned into plasmid pBBR1MCS-5. Initial experiments involved measurements of biofilm formation of the wt and the four mutant strains harbouring the empty plasmid pBBR1MCS-5. In analogy to the data reported above, statistically relevant differences in biofilm formation between plasmid containing wt and four mutant strains were observed (Fig. 3). Complementation of the four mutants induced in all cases a reversal of the mutant phenotype, which for three mutants was of statistical relevance (Fig. 3).

WspA of *P. aeruginosa* feeds into the *wsp* pathway, which ultimately alters c-di-GMP levels (Hickman *et al*., 2005). In order to establish a correlation between biofilm formation and c-di-GMP levels for WspA-Pp, we decided to quantify the cellular levels of the second messenger. To this end, we introduced a plasmid (pCdrA::gfp^S) into different strains harbouring a c-di-GMP biosensor that corresponds to a transcriptional fusion of the *cdrA* promoter from *P. aeruginosa* PAO1 to the green fluorescent protein encoding gene gfp^S (Rybtke *et al*., 2012). As a positive control, we introduced plasmid pMAMV1 into the wt strain, which confers high cellular levels of c-di-GMP due to the overexpression of the diguanylate cyclase Rup4959

Table 1. Strains used in this study.

Strain	Characteristics	Reference
Escherichia coli BL21(DE3)	F– , *ompI, hsdS*$_B$ (r_{-B} m_{-B}) *gal*, *dam*, *met*	Jeong *et al.* (2009)
E. coli DH5α	*supE44 lacU169*(*Ø80lacZΔ M15*) *hsdR17* ($r_K^-m_K^-$) *recA1 endA1 gyrA96 thi-1 relA1*	Woodcock *et al.* (1989)
E. coli HB101	F$^-$ *Δ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20* (Sm^r) *xyl-5 mtl-1 recA13 thi-1*	Boyer and Roulland-Dussoix (1969)
Pseudomonas putida KT2440R	Rifampicin-resistant derivative of KT2440	Espinosa-Urgel and Ramos (2004)
P. putida KT2440R PP0317	*pp0317*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP0320	*pp0320*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP0562	*pp0562*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP0584	*pp0584*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP0779	*pp0779*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP1228	*pp1228*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP1371	*pp1371*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP1488	*pp1488*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP1819	*pp1819*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP1940	*pp1940*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP2111	*pp2111*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP2120	*pp2120*::mini-tn5-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP2249	*pp2249*::mini-tn5-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP2257	*pp2257*::mini-tn5-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP2310	*pp2310*::mini-tn5-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP2643	*pp2643*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP2823	*pp2823*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP2861	*pp2861*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP3414	*pp3414*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP3557	*pp3557*::pCHESIΩGm;Rif^R, Gm^R,	This study
P. putida KT2440R PP3950	*pp3950*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP4521	*pp4521*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP4658	*pp4658*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP4888	*pp4888*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP4989	*pp4989*::mini-tn*5*-Km; Rif^R, Km^R	Duque *et al.* (2007)
P. putida KT2440R PP5020	*pp5020*::pCHESIΩKm;Rif^R, Km^R,	This study
P. putida KT2440R PP5021	*pp5021*::pCHESIΩKm;Rif^R, Km^R,	This study

Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Rif, rifampicin.

(Matilla *et al.*, 2011). Colonies of the resulting strains were inspected by fluorescence microscopy. As shown in Fig. 4, the wt strain harbouring pMAMV1 produced a fluorescence signal that was well above that of the wt strain, confirming the presence of elevated c-di-GMP levels in the pMAMV1 containing strain (note the differences in the exposure times). In contrast, a decrease in the fluorescence signal was observed in the strain PP1488, indicating that the mutation of the WspA-Pp encoding gene resulted in lower c-di-GMP levels, which is consistent with the lower amount of biofilm formed by PP1488 (Fig. 2B).

The underlying mechanisms of the remaining three receptors (PP1228, PP2249 and PP2310) in biofilm formation are still unknown. To dissect their implications in the biofilm formation capacity of KT2440, further research was carried out, with special attention to the identification of the ligand(s) which bind to the chemoreceptor(s) LBD(s).

Identification of PP2249 as a chemotaxis receptor for L-amino acids

Chemoreceptor PP2249 has a double PDC PhoQ/DcuS/CitA) LBD (Zhang and Hendrickson, 2010). PP1228 also has a double PDC domain; however, the domain is distinct in that it contains an insert of approximately 50 amino acids (termed double PDC+ in Fig. 1). In *P. aeruginosa* PAO1, three paralogous receptors with a double PDC domain, PctA, PctB and PctB, were identified and found to mediate chemotaxis towards gamma-aminobutyrate (GABA) and most of the proteinogenic amino acids (Taguchi *et al.*, 1997; Rico-Jimenez *et al.*, 2013). We have shown previously that the recombinant LBDs of these receptors recognize their ligands directly (Rico-Jimenez *et al.*, 2013). We therefore hypothesized that PP1228 and PP2249 function similarly.

To verify this hypothesis, we cloned DNA fragments encoding the periplasmic LBDs of both receptors into an expression plasmid and purified the recombinant proteins from the soluble extract of *Escherichia coli* lysates. Subsequently, microcalorimetric binding studies (Krell, 2008) were carried out for both proteins against GABA and all 20 L-amino acids. We were able to show that PP2249-LBD binds to 12 different L-amino acids with affinities that vary between 0.6 and 373 μM (Table 2). Representative titrations for three of these amino acids are shown in Fig. 5A. No binding was observed in dipeptides, D-amino

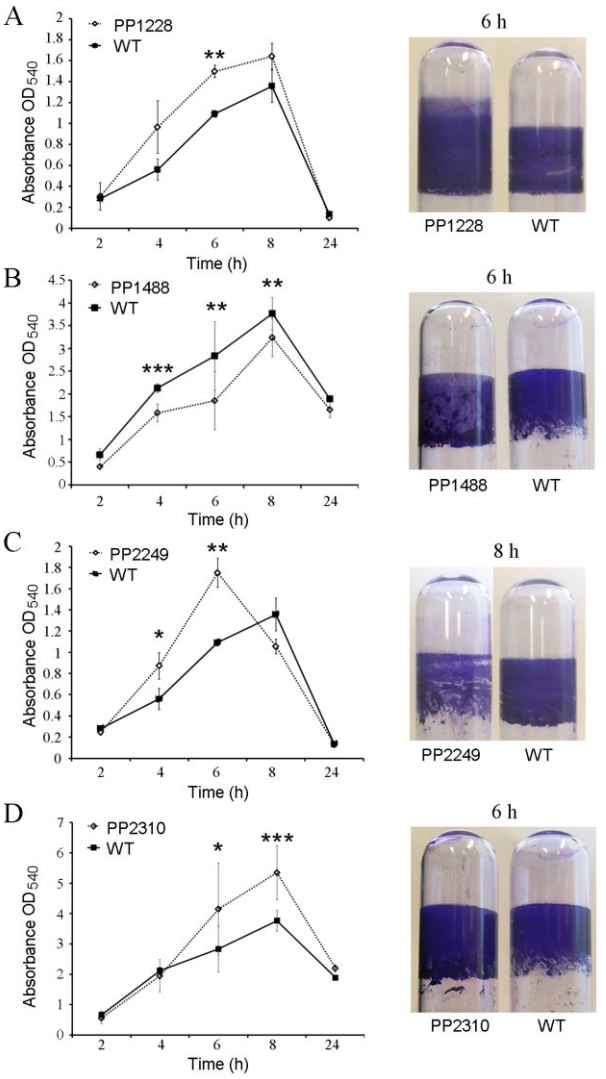

Fig. 2. Chemoreceptor mutants of *Pseudomonas putida* KT2440R with significantly altered biofilm formation pattern. (A) Mutant PP1228, (B) mutant PP1488, (C) mutant PP2249, and (D) mutant PP2310. Biofilm formation was quantified from cultures grown in borosilicate glass tubes (see Experimental procedures section). These data are the result of an analysis of the wt and all 27 mutants deficient in a single chemoreceptor (Table 1). Data are means and standard deviations from three independent experiments. Representative borosilicate glass tubes stained with crystal violet are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in Student's *t*-test indicate statistical significance.

acids, GABA or the other ligands listed in the legend of Table 2. In contrast to the results for PP2249-LBD, no ligand binding was observed for PP1228-LBD. To verify whether PP2249 is a chemotaxis receptor, quantitative capillary assays were carried out using wt and PP2249, and taxis towards L-Tyr and L-Val were measured. A maximal chemotactic response was observed for wt at 0.5 mM L-Tyr and 50 mM L-Val (Fig. 6); however, at these concentrations, this response was largely diminished in the PP2249 mutant, indicating that PP2249 does, in fact, mediate chemotaxis towards L-amino acids. The receptor was thus named McpA.

To put these data in a physiological context, we semi-quantitatively analysed the chemotactic behaviour of *P. putida* KT2440R towards all proteinogenic amino acids using plate gradient assays. As shown in Table 2, responses were detected for 15 different L-amino acids, including all the 12 amino acids that McpA-LBD was found to bind. We subsequently investigated the amino acid requirement for sustained, efficient bacterial growth and found that 14 amino acids were sufficient as a nitrogen source and 13 amino acids were sufficient as a carbon source (Fig. S5). There was no significant correlation between these data and the capacity of the strain to respond chemotactically to different amino acids.

Identification of PP1228 as a polyamine chemotaxis receptor

Based on the failure of PP1228-LBD to recognize any of the ligands tested, we conducted high-throughput thermal shift assay-based ligand screens (Krell, 2015; McKellar *et al.*, 2015). In this assay, a temperature gradient is applied to a mixture of protein and fluorescent dye. Protein unfolding causes additional dye binding, resulting in fluorescence changes, which can be used to calculate the midpoint of protein unfolding (Tm; the temperature at which half of the protein is in its native form and the other half is unfolded). Typically, ligand binding to a protein causes an upshift in Tm. We have therefore conducted

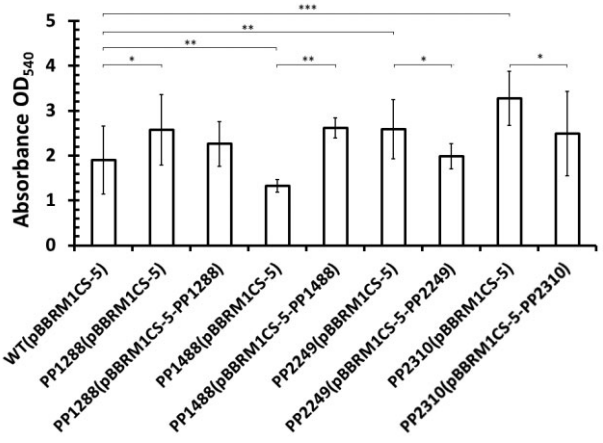

Fig. 3. Biofilm assays of mutant and complemented strains. Biofilm formation at 6 h of the wt strains and mutants PP1228, PP2249, PP1488 and PP2310 harbouring either the empty pBBR1MCS-5 and, in the case of the mutant strains, harbouring a pBBR1MCS-5 derivative containing the corresponding chemoreceptor gene. Shown are means and standard deviations from at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ in Student's *t*-test indicate statistical significance.

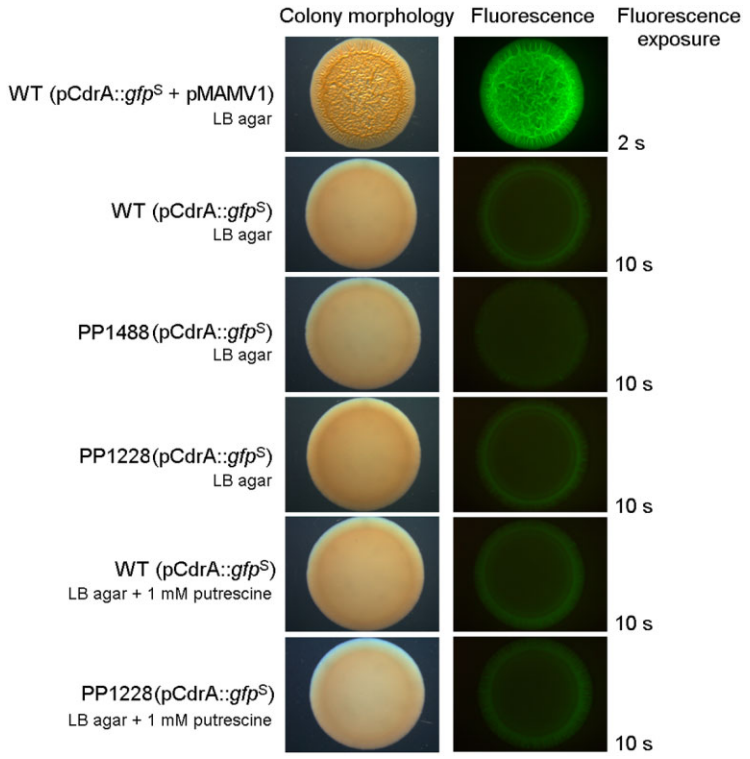

Fig. 4. Colony morphology and fluorescence intensity of *Pseudomonas putida* strains harbouring the c-di-GMP biosensor plasmid pCdrA::*gfp*^{S}. Plasmid pMAMV1 confers high levels of c-di-GMP (Matilla *et al.*, 2011). The exposure in fluorescence measurements is indicated. Experiments were conducted on LB agar plates with or without 1 mM putrescine. The assays were repeated three times, and representative results are shown.

thermal shift assays of the recombinant PP1228-LBD protein in the presence of compounds from ligand collections. To this end, we have used plates PM1 and PM2A from Biolog that contain 190 different carbon sources. A Tm value of 46°C was determined for ligand-free PP1228-LBD, and Fig. 7 shows the Tm changes for each of the 95 compounds of ligand plate PM2A. Whereas most ligands caused changes from –2 to +1°C, putrescine caused an upshift of 11°C. To verify this hit, a microcalorimetric titration of the protein with putrescine was carried out (Fig. 5B). Large exothermic heat changes due to binding were recorded and data analysis revealed a K_D of 2 μM in a binding process driven by favourable enthalpy changes (Table 2). These results confirm that putrescine is a ligand of PP1228.

We continued to explore the ligand profile of PP1228. Using Isothermal Titration Calorimetry (ITC), we screened a large number of different compounds (see Table 2). Of all these compounds, binding was only observed for spermidine (4.5 μM affinity) and cadaverine (22 μM affinity), as shown in Fig. 5B. No binding was observed for a number of closely related compounds, such as amino acids, aminopropyl-cadaverine, spermine, L-ornithine, gamma-aminobutyrate or butyrate, indicating that this receptor specifically recognizes polyamines.

To elucidate whether PP1228 mediates chemotaxis, plate gradient assays were conducted (Fig. 8A and B). In this assay, the chemoattractant is placed at different points along the central vertical line of a soft agar plate. After overnight incubation for gradient formation, wt and mutant bacteria are placed at defined distances from the site of chemoattractant deposition. The inspection of the plates showed an acentric spread of the wt strains towards immobilized spermidine and putrescine. Chemotaxis indices, calculated according to Pham and Parkinson (2011), were found to be 0.58 ± 0.02 ($n = 5$) for spermidine and 0.59 ± 0.03 ($n = 5$) for putrescine – values that are indicative of chemoattraction. For both compounds, the spread of the PP1228 strain was minor and only slightly acentric, indicative of a strong reduction in chemotaxis in this mutant strain.

To confirm these results, quantitative capillary chemotaxis experiments were conducted. The response to spermidine was maximal at 5 mM (Fig. 8C), whereas chemotaxis towards putrescine and cadaverine occurred over a wide concentration range (0.1 μM–10 mM) (Fig. 8D and E). In all cases, the PP1228 mutant showed strongly reduced taxis, indicating that PP1228 is a chemoreceptor specific to polyamines. Taking these results into consideration, we named the chemoreceptor McpU. The residual taxis observed suggests the existence of another receptor that mediates responses to polyamines. We also conducted growth experiments for McpU ligands, the results of which revealed that the three ligands permit growth of the bacterium as sole carbon and energy source (Fig. S6).

Table 2. Magnitude of chemotaxis and thermodynamic binding parameters of McpA and McpU ligands.

		Binding to McpA-LBD[b]	
Ligand	Chemotaxis[a]	K_D (μM)	ΔH (kcal mol^{-1})
Gly	+	35 ± 2	−8.4 ± 0.7
L-Ala	+++	13 ± 0.6	−11.5 ± 0.4
L-Cys	+	0.6 ± 0.1	−38 ± 0.4
L-Ser	+++	43 ± 2	−6.5 ± 1.1
L-Thr	–	No binding[c]	
L-Asn	+	4.3 ± 0.2	−13 ± 0.4
L-Gln	+	5.5 ± 0.2	−8.9 ± 0.1
L-Asp	–	No binding[c]	
L-Glu	–	No binding[c]	
L-Phe	++	2.3 ± 0.1	−1 ± 0.1
L-Tyr	+++	12.1 ± 0.9	−4.4 ± 0.8
L-Trp	–	No binding[c]	
L-Val	+	373 ± 42	0.9 ± 0.7
L-Leu	+	No binding[c]	
L-Ile	+	85 ± 5	0.2 ± 0.05
L-Met	+++	5.8 ± 0.6	−9.7 ± 0.6
L-Arg	+	1.2 ± 0.1	−4 ± 0.1
L-Lys	+	No binding[c]	
L-His	–	No binding[c]	
L-Pro	+++	No binding[c]	
		Binding to McpU-LBD[d]	
Putrescine	+++	2 ± 0.1	−15 ± 0.1
Cadaverine	++	22 ± 2	−15.5 ± 0.5
Spermidine	+++	4.5 ± 0.4	−4.3 ± 0.1

Shown are chemotactic responses of *P. putida* KT2440R to different ligands as determined by the swim plate assay as well as the binding parameters derived from microcalorimetric titration of McpA-LBD and McpU-LBD with different ligands. Data are means and standard deviations from three independent experiments.

a. Chemotaxis was assessed using plate gradient assays.

b. Compounds that were tested for binding to McpA-LBD but that did not reveal binding were: L-amino acids (L-Thr, L-Trp, L-Leu, L-Lys, L-Asp, L-Glu, L-His and L-Pro), D-amino acids (D-Ala, D-Glu, D-Gln), dipeptides (L-Ala-L-Ala, L-Ala-L-Glu, L-Ala-Gly), putrescine, cadaverine, aminopropyl-cadaverine, spermidine, spermine, L-ornithine, gamma-aminobutyrate and butyrate.

c. It cannot be excluded that ligands bind with very low affinity to the protein. This is due to the fact that amino acids at a maximal concentration of 5 mM could be placed into the injector syringe of the calorimeter to avoid large dilution heats. Therefore, potential very weak binding events cannot be monitored.

d. Compounds that were tested for binding at McpU-LBD but that did not show any binding were: all proteinogenic amino acids, aminopropyl-cadaverine, spermine, L-ornithine, gamma-aminobutyrate and butyrate.

Influence of chemoreceptor mutations on competitive plant root colonization

We assessed the effect of the mutation of chemoreceptors on the interaction of KT2440R with plants. To this end, the four mutant strains that showed altered biofilm phenotypes were submitted to competitive corn root colonization assays. In this assay, seedlings are inoculated with bacterial mixtures containing the same number of wt and mutant cells. After 1 week of growth, plants were collected and root-associated bacteria were recovered and quantified using the different antibiotic susceptibilities of wt and mutant strains. Figure 9 shows that mutants PP2249 and PP2310 had approximately the same capacity as the wt to colonize the plant root. In contrast, strains PP1228 and PP1488 were significantly less competitive than wt, indicating that McpU-mediated chemotaxis and WspA-Pp mediated alteration of c-di-GMP levels also modulate root attachment. To verify whether the biofilm and root colonization phenotypes of PP1228 can be attributed to polyamine chemotaxis or potentially to changes in the c-di-GMP level, the PP1228 mutant harbouring the c-di-GMP biosensor has also been analysed by fluorescence microscopy. The fluorescence intensity of the strain, grown on LB agar plates without and with added putrescine, was similar to that of the wt (Fig. 4). This indicates that the phenotypic changes are not due to alterations in the c-di-GMP level but to chemotaxis to polyamines.

Discussion

The *wsp* pathway is the best characterized example of a chemosensory pathway that serves alternative cellular functions (Wuichet and Zhulin, 2010). The gene cluster encoding pathway proteins are highly homologous in *P. aeruginosa* PAO1 and *P. putida* KT2440 (Fig. S4). Pathway output is mediated by the WspR response regulator for which phosphorylation was found to stimulate c-di-GMP production and consequently biofilm formation (Hickman *et al.*, 2005). The WspA_Pp mutant PP1488 was the only strain that had significantly reduced biofilm formation (Fig. 2) and mutant complementation resulted in the recovery of a wt-like phenotype (Fig. 3). In addition, the WspA_Pp mutant was significantly less efficient at colonizing plant roots (Fig. 9). These data agree with previous results from our group (Garcia-Fontana *et al.*, 2013), which showed that mutation of the gene encoding the CheR methyltransferase of this pathway (*pp1490*) in *P. putida* KT2440 resulted in a strong reduction in biofilm formation. We show here (Fig. 4) that the mutation of the *wspA-Pp* gene significantly reduced the cellular c-di-GMP levels, indicating that there is a functional *wsp* pathway in *P. putida* KT2440. Interestingly, mutation of WspA in *P. aeruginosa* did not significantly impact biofilm formation (Hickman *et al.*, 2005). These data underline the need for future investigations aimed at establishing the unknown ligand(s) of WspA.

Escherichia coli, the model bacterium used historically for the study of chemotaxis, encodes four well-characterized chemoreceptors, and their responses to amino acids, dipeptides and sugars have been established (Hazelbauer *et al.*, 2008). More recent studies in other bacteria have shown that many chemoreceptors respond to two major groups of chemoattractants, namely amino acids (Taguchi *et al.*, 1997; Hartley-Tassell *et al.*, 2010; Glekas *et al.*, 2012; Oku *et al.*, 2012; Brennan *et al.*, 2013; Webb *et al.*, 2014) and different organic acids (Yamamoto

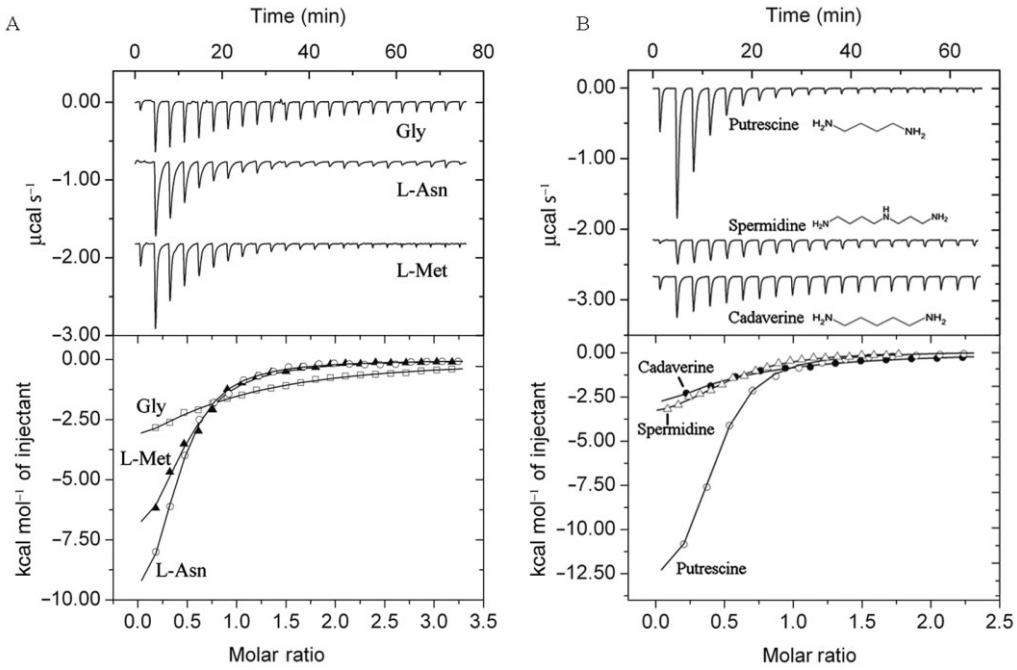

Fig. 5. Microcalorimetric studies for the binding of different ligands to the purified recombinant LBDs of chemoreceptors PP2249/McpA (A) and PP1228/McpU (B). Upper panels: Raw data for the titration of 25–30 μM of protein with 0.25–1 mM of amino acids or polyamines. Typically, a single 1.6 μl injection was followed by a series of 6.4–12.8 μl injections. Lower panels: Dilution-corrected and concentration-normalized integrated peak areas of the raw data. Fitting was done using the 'One binding site model' of the MicroCal version of ORIGIN.

and Imae, 1993; Alvarez-Ortega and Harwood, 2007; Lacal *et al*., 2010; Ni *et al*., 2013; Parales *et al*., 2013; Oku *et al*., 2014; Luu *et al*., 2015). The identification of the first polyamine-specific chemoreceptor, McpU, thus expands the range of known chemoreceptor types. Chemotaxis towards cadaverine and putrescine has been observed in *P. aeruginosa* PAO1, but taxis was abrogated in a triple mutant for PctA, PctB and PctC receptors (Taguchi *et al*., 1997). Because these ligands did not bind to the recombinant LBDs of these receptors, it was proposed that polyamine binding may occur indirectly via a periplasmic binding protein (Rico-Jimenez *et al*., 2013); however, our thermal shift and ITC binding studies show that McpU function is based on direct ligand recognition.

Polyamines are very abundant in plants, particularly when exposed to different types of stress (Minocha *et al*., 2014). For example, putrescine was found at a concentration of 275 μM in tobacco leaves (Ioannidis *et al*., 2012). In addition, significant amounts of putrescine have been detected in different plant exudates (Kuiper *et al*., 2001). Because *P. putida* KT2440 has a saprophytic lifestyle and is able to use polyamines for growth, it is not surprising that we identified a polyamine-specific chemoreceptor in this strain. Apart from being a growth substrate, there is also evidence that polyamines act as signalling molecules and were found to play key roles in mediating bacteria–host interactions (reviewed in Di Martino *et al*., 2013). In addition, there are several examples demonstrating a link between polyamines and biofilm formation. Specifically, it was shown that the inactivation of polyamine biosynthesis or addition of polyamines to cultures modulated biofilm formation, as reviewed by Shah and Swiatlo (2008). A particularly interesting study involved a screen of a mutant library of *Shewanella oneidensis* for strains with enhanced biofilm formation (Ding *et al*., 2014). A clear phenotype was observed for a mutant with disrupted putrescine biosynthesis. The authors therefore proposed that putrescine is a compound that mediates biofilm matrix disassembly in *S. oneidensis*. A potential molecular mechanism for this effect may be inferred from another study (Cockerell *et al*., 2014), which found that spermidine reduces biofilm formation of *Vibrio cholerae*. The authors demonstrate that spermidine is sensed by the NspS sensor, which, in turn, modulates c-di-GMP levels. Based on a comparative genomics analysis, which revealed the presence of NspS-like proteins in a variety of bacteria, the authors proposed that such mechanism may represent a general mechanism. These data are consistent with our finding that abolishing chemotaxis towards putrescine and spermidine (i.e., reducing the effective concentration of these compounds) leads to enhanced biofilm formation.

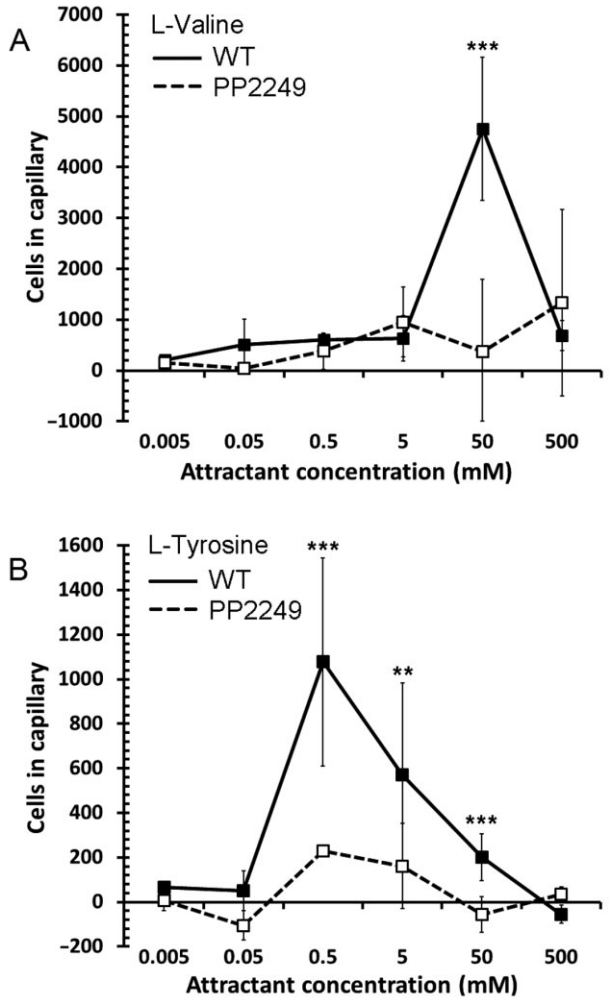

Fig. 6. Quantitative capillary chemotaxis assays of *Pseudomonas putida* KT2440R and its PP2249 towards different concentrations of L-Val (A) and L-Tyr (B). Shown are means and standard deviations from three independent experiments conducted in triplicate. Data were corrected with the number of bacteria that swam into a buffer containing capillary. $^{**}P < 0.01$, $^{***}P < 0.001$ in Student's *t*-test indicate statistical significance.

The effect of putrescine on gene expression has been assessed in the highly homologous *P. putida* S12 using the *P. putida* KT2440 microarray. Among the 79 differentially regulated genes was a single chemoreceptor encoding gene, namely *pp1228*, which showed a 28-fold upregulation in the presence of putrescine (Bandounas *et al.*, 2011). Here, we show that this gene encodes a chemoreceptor for putrescine and data thus indicate strong receptor induction in response to its cognate ligand. The identification of McpU also illustrates the power of the high-throughput thermal shift-based method for the identification of signal molecules as previously discussed (Krell, 2015; McKellar *et al.*, 2015). The large Tm shift in the presence of putrescine (11°C) (Fig. 7) indicates an excellent signal to noise ratio. There is no doubt that this approach will facilitate the functional annotation of other sensor proteins.

Our findings also reveal that McpA is a chemotaxis receptor for L-amino acids. *Pseudomonas putida* KT2440R shows chemotaxis towards 15 proteinogenic amino acids and 12 of these compounds were found to bind to McpA-LBD (Table 2). For the remaining three amino acids, weak chemotaxis was observed for L-Leu and L-Lys, whereas very strong chemotactic responses were detected towards L-Pro. As detailed in the footnote of Table 2, it cannot be excluded that L-Leu and L-Lys bind to McpA-LBD with a very low affinity. However, chemotaxis towards proline may be mediated by an additional chemoreceptor, as a receptor that mediates specific and strong chemotactic responses towards proline has been identified recently in *Sinorhizobium meliloti*, another soil bacterium (Webb *et al.*, 2014). The five amino acids that did not serve as chemoattractants were not found to be McpA ligands, confirming the notion that ligand binding at McpA causes chemotaxis. Similar to what we observed for McpU, a mutation in McpA led to increased biofilm formation. Similar observations have been made for the multi-ligand binding chemoreceptor CcmL of *Campylobacter jejuni* (Rahman *et al.*, 2014). This receptor was shown to mediate chemotaxis towards a wide range of different compounds, including organic and amino acids. A CcmL mutant, while showing reduced chemotaxis, exhibited significantly increased biofilm formation.

It has been shown that D-amino acids prevent biofilm formation and promote biofilm dispersal in different bacteria (Kolodkin-Gal *et al.*, 2010). Although our data indicate that McpA is specific for L-amino acids, *P. putida*

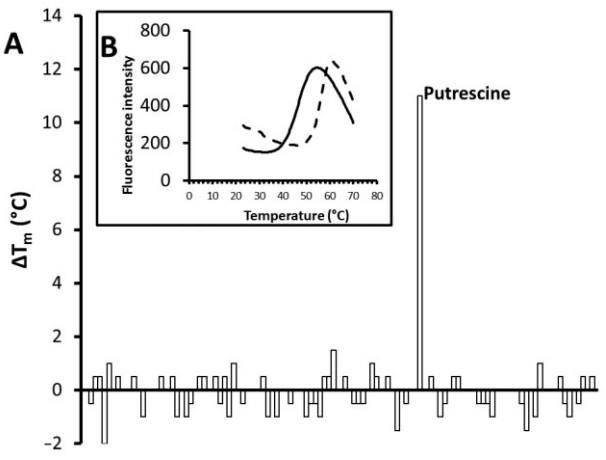

Fig. 7. Thermal shift assays for the high-throughput identification of PP1228/McpU ligands.
A. Increases in Tm in the presence of compounds from Biolog plate PM2A (compounds of this plate are listed at http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf).
B. Fluorescence raw data of the protein without ligand (continuous line) and in the presence of putrescine (dashed line).

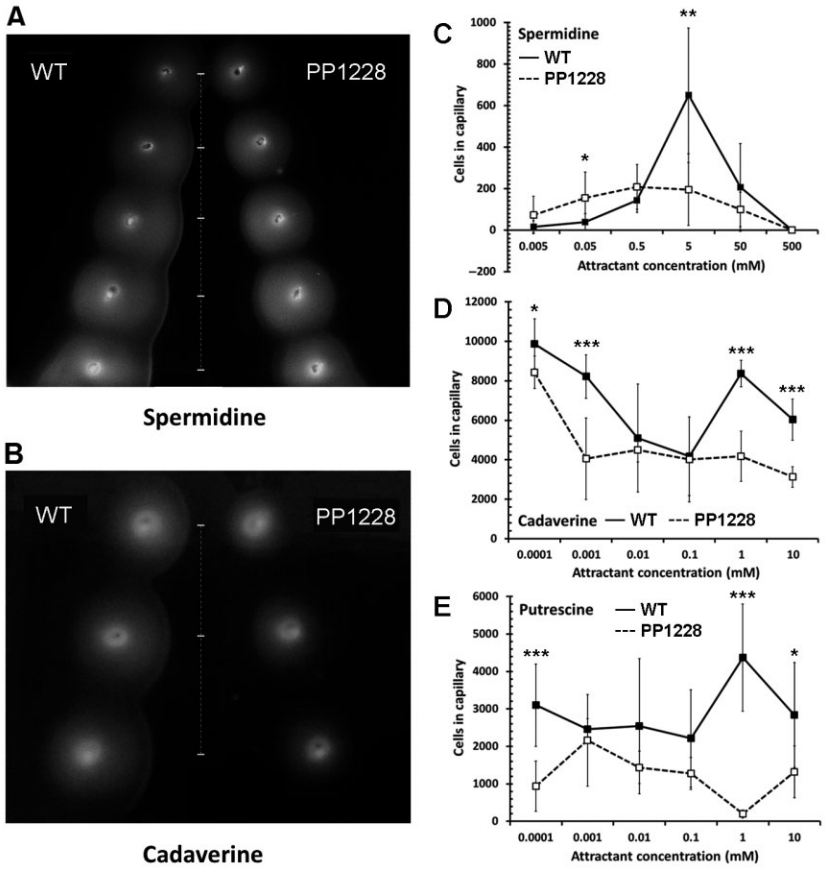

Fig. 8. Chemotaxis assays of *Pseudomonas putida* KT2440R and its mutant PP1228 towards different polyamines.
A, B. Plate gradient chemotaxis assays. A 50 mM solution of spermidine or putrescine was deposited at the positions marked along the central vertical line. Aliquots of the wt and mutant strain were deposited at different distances to the site of chemoattractant deposition and plates were inspected the following day.
C–E. Quantitative capillary chemotaxis assays of *P. putida* KT2440R and its mutant PP1228 to different concentrations of putrescine, cadaverine and spermidine. Shown are means and standard deviations from three independent experiments conducted in triplicate. Data were corrected with the number of bacteria that swam into a buffer containing capillary. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in Student's *t*-test indicate statistical significance.

KT2440 was found to have three different amino acid racemases that can catalyse the racemization of 19 proteinogenic amino acids (Radkov and Moe, 2013). It is possible that chemotaxis towards L-amino acids, which could subsequently be converted to D-isomers, may in part be responsible for the changes in biofilm formation that were observed. In contrast to the well-studied amino acid receptors Tar and Tsr of *E. coli*, which possess a 4-helix bundle LBD (Hazelbauer *et al*., 2008), McpA has a double PDC LBD (Fig. S7). Over the past decade, a significant number of double PDC-containing amino acid chemoreceptors have been identified in a range of different species (Reyes-Darias *et al*., 2015b). It has been suggested that this receptor type may be the predominant amino acid receptor in bacteria (Reyes-Darias *et al*., 2015b) – a notion supported by the identification of McpA. The analysis of the ligand profiles of these receptors has permitted differentiation between receptors with broad and narrow ligand profiles (Reyes-Darias *et al*., 2015b). Here, we show that McpA binds 12 different amino acids – a trait that is indicative of broad range receptors. PctA of *P. aeruginosa* is a well-characterized broad range amino acid chemoreceptor (Taguchi *et al*., 1997; Rico-Jimenez *et al*., 2013; Reyes-Darias *et al*., 2015b) and McpA can be

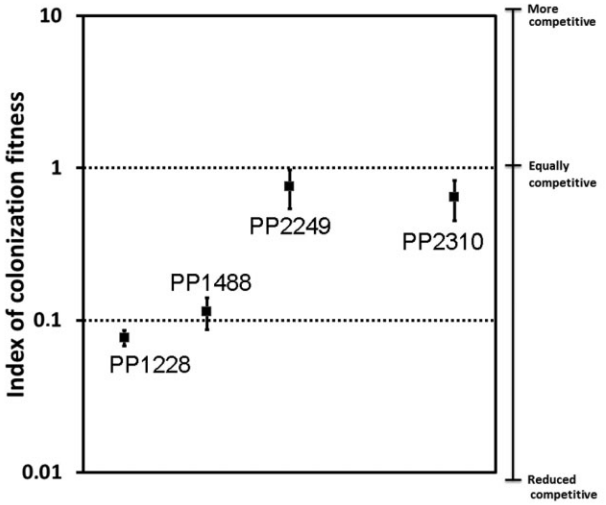

Fig. 9. The influence of chemoreceptor mutation on plant root colonization. Competitive root colonization assays of *Pseudomonas putida* KT2440R and each of the four mutant strains with altered biofilm formation capabilities. After 1 week, root-associated bacteria were recovered and quantified. Shown is the colonization fitness representing the ratio of recovered wt over mutant strain. Experiments shown are mean and standard deviation of two independent experiments conducted on six different plants.

regarded as a functional PctA homologue. Also, there remains a possibility that an additional L-Pro specific receptor exists.

Pseudomonas putida KT2440R responds chemotactically to 15 amino acids and there was no significant correlation between taxis and the use of different amino acids for growth. A lack of correlation between chemotaxis towards particular amino acids and their catabolism has been recently reported for *Bacillus subtilis* (Yang *et al*., 2015). The authors of that study concluded that amino acids do not attract the bacteria because of their nutritional value, but rather because they serve as environmental cues.

Two chemoreceptor mutants showed reduced root colonization capacity. However, the mechanisms for this phenotype differed: whereas McpU is a chemotaxis chemoreceptor, WspA-Pp modulates c-di-GMP levels. These observations are consistent with previous studies showing that c-di-GMP levels modulate root colonization capacity (Matilla *et al*., 2007; 2011; Perez-Mendoza *et al*., 2014) and that chemotaxis to root exudates facilitates colonization (de Weert *et al*., 2002; Oku *et al*., 2012; 2014; Reyes-Darias *et al*., 2015a).

In summary, the discovery of a polyamine-specific chemoreceptor has broadened the spectrum of known chemotactic receptor types and will facilitate the identification of functional homologues in other species. Further research will show whether the involvement of amino acid and polyamine chemotaxis in biofilm formation is a more general phenomenon.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables 1 and 3 respectively. *Pseudomonas putida* KT2440R and its derivative strains were routinely grown at 30°C in LB, minimal saline medium (MS; 10 mM Na_2HPO_4, 20.6 mM KH_2PO_4, 25 mM NH_4NO_3, 0.8 mM $MgSO_4$) supplied with a trace element solution (63 μM $FeCl_3$, 3.3 μM $CoCl_2$, 4 μM $CaCl_2$, 1.2 μM Na_2MoO_4, 1.6 μM H_3BO_3, 0.7 μM $ZnSO_4$, 0.7 μM $CuSO_4$, 0.7 μM $MnSO_4$) or basal M9 medium supplied with 1 mM MgSO4, 6 mg l^{-1} Fe-citrate as described previously (Abril *et al*., 1989) and glucose (27 mM) as a carbon source. *Escherichia coli* strains were grown at 37°C in LB. *Escherichia coli* DH5α was used for gene cloning. When appropriate, antibiotics were used at the following final concentrations, unless otherwise stated: chloramphenicol, 25 μg ml^{-1}; gentamicin, 10 μg ml^{-1} (*E. coli* strains) and 100 μg ml^{-1} (*P. putida* strains); kanamycin, 25 μg ml^{-1} (*E. coli* strains) and 50 μg ml^{-1} (*Pseudomonas* strains); rifampin, 10 μg ml^{-1}, piperacillin, 50 μg ml^{-1}.

DNA techniques

Genomic DNA was collected using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. Plasmid DNA was isolated using the Bio-Rad Plasmid miniprep kit. Phusion® high-fidelity DNA polymerase (New England Biolabs) was used in the amplification of PCR fragments for cloning. DNA fragments were recovered from agarose gels using the QIAprep gel extraction kit (Qiagen).

Construction of bacterial strains and plasmids

Sixteen KT2440R mutants defective in chemoreceptor encoding genes were obtained from the *Pseudomonas* Reference Culture Collection (http://www.gbif.es/ic_colecciones.php?ID_Coleccion=9817) (Duque *et al*., 2007). Mutants in the remaining 11 chemoreceptor encoding genes (*pp0779*, *pp1488*, *pp1940*, *pp2111*, *pp2823*, *pp2861*, *pp3414*, *pp3557*, *pp4888*, *pp5020* and *pp5021*) were constructed using derivate plasmids of pCHESIΩKm and pCHESIΩGm. These plasmids, listed in Table 3, were generated by amplifying a 0.5–1.1 kb region of the gene to be mutated using the primers listed in Table S1. The PCR products were then cloned into pCHESI-derivate vectors in the same transcriptional direction as the P_{lac} promoter. The resulting plasmids were transferred to *P. putida* KT2440R by electroporation or triparental conjugation [using *E. coli* HB101 (pRK600) as helper strain]. All mutations were confirmed by Southern blot. For the construction of the complementing plasmids, the entire genes (*pp2249*, *pp1488*, *pp1228* and *pp3210*), besides their corresponding complete promoter regions, were amplified using the primers described in Table S1 and cloned into pBBR1MCS-5. Ligation mixtures were used to electroporate *E. coli* DH5α, and transformants were selected in LB with Gm. These resulting plasmids (pBBR1MCS-5-PP2249, pBBR1MCS-5-PP1288, pBBR1MCS-5-PP1228 and pBBR1MCS-5-PP2310) were verified by DNA sequencing and transformed into *Pseudomonas* strains by electroporation. Plasmids for the expression and purification of the LBDs of McpA (PP2249) and McpU (PP1228) were constructed by amplifying the corresponding DNA fragments using primers listed in Table S1 and subsequent cloning into pET28b (+). All of the inserts were confirmed by sequencing.

Assessment of bacterial motility

Overnight liquid cultures of *P. putida* KT2440R and its derivative mutant strains were used to inoculate MS medium (Darias *et al*., 2014) to an OD_{660} of 1. Ten microlitres of this mixture was deposited at the centre of a 60 mm diameter Petri dish containing MS-glucose agar [MS medium containing 10 mM glucose and 0.25% (w/v) agar]. After 16 h of incubation at 30°C, the radius of the bacterial spread was measured. Experiments were performed in duplicate.

Phenotypic assessment of carbon and nitrogen sources of P. putida *KT2440R*

The strain was grown overnight in M8 minimal medium (Kohler *et al*., 2000) supplemented with glucose (0.1%, w/v) as carbon source and ammonium chloride (0.1 g l^{-1}) as nitrogen source. Cultures were diluted 100-fold into M8 medium supplemented either with glucose and any of the 20 proteinogenic amino acids (5 mM) or ammonium chloride and

Table 3. Plasmids used in this study.

Plasmids	Relevant characteristics	Reference
pUT-Km	Km^R	de Lorenzo *et al.* (1990)
pRK600	Cm^R, *mob tra*	Finan *et al.* (1986)
pCHESIΩKm	Km^R	Kovach *et al.* (1995)
pCHESIΩGm	Gm^R	Estrella. Duque, personal gift
pCHESI-pp0779	Km^R; PCR product containing a 0.8 kb region of *pp0779* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp1488	Km^R; PCR product containing a 0.7 kb region of *pp1488* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp1940	Km^R ; PCR product containing a 1.1 kb region of *pp1940* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp2111	Km^R; PCR product containing a 0.5 kb region of *pp2111* was inserted into the SacI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp2823	Km^R; PCR product containing a 0.6 kb region of *pp2823* was inserted into the SacI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp2861	Km^R ; PCR product containing a 0.9 kb region of *pp2861* was inserted into the SacI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp3414	Km^R; PCR product containing a 0.7 kb region of *pp3414* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp3557	Gm^R; PCR product containing a 0.7 kb region of *pp3557* was inserted into the EcoRI/KpnI sites of pCHESIΩGm	This study
pCHESI-pp4888	Km^R; PCR product containing a 0.7 kb region of *pp4888* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp5020	Km^R ; PCR product containing a 0.6 kb region of *pp5020* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pCHESI-pp5021	Km^R; PCR product containing a 0.6 kb region of *pp5021* was inserted into the EcoRI/BamHI sites of pCHESIΩKm	This study
pBBR1MCS-5	Gm^R; *oriRK2*, *mobRK2*	Kovach *et al.* (1995)
pET28b	Km^R, protein expression vector	Novagen
pET28b-LBDMcpA	Km^R; pET28b derivative used to produce His-tagged LBD of McpA (PP2249)	This study
pET28b-LBDMcpU	Km^R; pET28b derivative used to produce His-tagged LBD of McpU (PP1228)	This study
pBBR1MCS-5-PP2249	Gm^R; 2.2 kb PCR product containing *pp2249* inserted into BamHI/SacI sites of pBBRMCS-5; for gene complementation	This study
pBBR1MCS-5-PP1488	Gm^R; 1.8 kb PCR product containing *pp1488* inserted into SpeI/SacI sites of pBBRMCS-5; for gene complementation	This study
pBBR1MCS-5-PP1228	Gm^R; 2.6 kb PCR product containing *pp1228* inserted into EcoRI/XbaI sites of pBBRMCS-5; for gene complementation	This study
pBBR1MCS-5- PP2310	Gm^R; 1.8 kb PCR product containing *pp2310* inserted into EcoRI/XbaI sites of pBBRMCS-5; for gene complementation	This study
pMAMV1	Gm^R; pBBR1MCS-5-based vector containing *rup4959-4957* genes from KT2440; confers high levels of c-di-GMP	Matilla *et al.* (2011)
pCdrA::gfpC	Amp^R, Gm^R; pUCP22Not-P*cdrA*-RBS-CDS-RNaseIII-*gfp*(Mut3)-T0-T1; C-di-GMP biosensor	Rybtke *et al.* (2012)

Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Rif, rifampicin.

any of the amino acids. Cultures were grown in 100-well polystyrene plates and incubated at 30°C in a Bioscreen microbiological growth analyser. Data shown are means and standard deviation from three experiments after 24 h growth.

Biofilm assays

Biofilm formation in multiwell plates. A modified version of a previously described high-throughput assay was used (Barahona *et al*., 2010). Overnight cultures of *P. putida* KT2440R and its 27 chemoreceptor mutants grown in LB medium were diluted in the same medium to an OD_{600} of 0.01. Subsequently, 150 μl of each culture was placed into a well of a sterile, polystyrene multi-well plate. For each experiment, five identical plates containing triplicates of each culture were prepared. Plates were incubated at 30°C without shaking. At 2, 4, 6, 8 and 24 h, the cell suspension was removed from one plate with a multichannel pipette. After washing of the wells with 150 μl of sterile water, the same volume of a crystal violet solution (0.4%, w/v) was added to each well and incubated for 15 min to allow staining of adhered cells. Excess stain was eliminated by rinsing with water. Plates were air dried and 200 μl of 30% acetic acid (v/v) was added to each well in order to extract crystal violet from cells. Destaining was performed overnight in a microplate shaker (40 r.p.m.); after which, the colour in the wells was measured at OD_{540} on a microplate reader. Experiments were repeated three separate times.

Biofilm formation in borosilicate glass tubes. Biofilm formation was examined, as previously described (Martinez-Gil *et al*., 2014), during growth in LB medium without medium supplementation. Overnight cultures of *P. putida* KT2440R and its 27 mutants were grown at 30°C and used to inoculate borosilicate glass tubes containing 2 ml of LB medium to an initial OD_{600} of 0.05. Cultures were incubated in a Stuart SB3 tube rotator for 2, 4, 6, 8 and 24 h at 30°C, with an angle of 45 degrees at 40 r.p.m. Biomass attached to the surface was visually inspected following the addition of 4 ml of crystal violet (0.4%, w/v) to each tube and quantified by solubilizing the dye with 30% acetic acid (v/v) and measuring the absorbance at 540 nm (Martinez-Gil *et al*., 2014). Data shown are means and standard deviations from three experiments conducted in duplicate.

Protein overexpression and purification

Escherichia coli BL21(DE3) was transformed with plasmids pET28b-McpU-LBD or pET28b-McpA-LBD. *Escherichia coli* BL21(DE3) pET28b-McpU-LBD cultures were grown in 2 l Erlenmeyer flasks containing 500 ml of LB medium supplemented with 50 μg ml^{-1} kanamycin at 30°C until the culture reached an OD_{660} of 0.6. For protein induction, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.1 mM and the culture was maintained at 30°C overnight with shaking. Cells were harvested by centrifugation at 10,000 × *g* at 4°C for 20 min. Similarly, *E. coli* BL21(DE3) containing pET28b-McpA-LBD was grown at 37°C until the culture reached an OD_{600} of 0.4. The temperature was then lowered to 18°C and growth continued until an OD_{660} of 0.6–0.8 at which point protein expression was induced by the addition of 0.1 mM IPTG. Growth was continued at 18°C overnight and cells were harvested by centrifugation. Pellets were frozen using liquid nitrogen and stored at −80°C. Cells derived from 750 ml of *E. coli* culture were resuspended in 30 ml of buffer A [20 mM Tris/HCl, 0.1 mM EDTA, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 5% (v/v) glycerol, 1 mM DTT, pH 7.8] containing complete TM protease inhibitor (Roche) and benzonase (Sigma). Cells were broken by French press treatment at 1000 psi and centrifuged at 20,000 × *g* for 1 h at 4°C. The supernatant was loaded onto a 5 ml HisTrap column (Amersham Bioscience) previously equilibrated with buffer A. The column was then washed with buffer A containing 45 mM imidazole prior to protein elution using a linear gradient (30 min) of 45–500 mM imidazole in buffer A at a flow of 1 ml min^{-1}. Protein containing fractions were analysed by SDS-PAGE, pooled and dialysed into polybuffer [5 mM Tris, 5 mM PIPES, 5 mM MES, 10% glycerol (v/v), 150 mM NaCl, pH 7.0].

Isothermal titration calorimetry

Titrations were carried out in a VP microcalorimeter (MicroCal, Northampton, MA, USA) at 25°C for McpU-LBD and 30°C for McpA-LBD. Prior to experiments, proteins were thoroughly dialysed in analysis buffer. The protein concentration was determined by the Bradford assay. Following filtration with 0.45 μm cut-off filters, the protein was placed into the sample cell of the instrument. Ligand solutions were made in the dialysis buffer. Control experiments involved the titration of dialysis buffer with ligand solutions. Typically, 30–35 μM of protein was titrated with 0.5–10 mM ligand solutions. The mean enthalpies measured from injection of the ligands into the buffer were subtracted from raw titration data prior to data fitting using the 'One binding site model' of the MicroCal version of the ORIGIN software.

Thermal shift assay

Thermal shift assays were performed using a BioRad MyIQ2 real-time PCR instrument. For high-throughput screening, ligands were prepared by dissolving Biolog Phenotype Microarray compounds in 50 μl of MilliQ water to obtain a final concentration of around 10–20 mM (as indicated by the manufacturer). Screening was performed with compounds from plates PM1 and PM2A, which each contains 95 compounds shown previously to be bacterial carbon sources and a control. Compounds in this plate are provided in a list at http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf. Each 25 μl of standard assay contained 10 μM McpU-LBD in 5 mM polybuffer, 5× SYPRO orange (Life Technologies) and 2.5 μl of the resuspended Biolog compounds. Samples were heated from 23 to 80°C at a scan rate of 1°C min^{-1}. The protein unfolding curves were monitored by detecting changes in SYPRO Orange fluorescence. Melting temperatures were determined using the first derivative values from the raw fluorescence data.

Chemotaxis assays

Qualitative soft agar plate gradient assays. Pseudomonas putida KT2440R as well as PP1228 and PP2249 mutant

strains were grown overnight in M9 minimal medium (basal M9 medium supplemented with Fe-citrate, $MgSO_4$, and trace metals; Espinosa-Urgel *et al*., 2000) with glucose (0.1%, w/v) as a carbon source. Cultures were diluted to an $O.D._{\cdot 660\ nm} = 1$ with fresh minimal medium. Cells were then washed twice with M9 medium by consecutive resuspension and centrifugation at 3300 × *g* for 3 min. The cell pellet was resuspended in 1 ml of M9 medium. Square (120 × 120 × 17 mm) polystyrene Petri dishes (Greiner Bio-One) were filled with 50 ml of semisolid agar containing minimal medium, 2.5 mM glucose and 0.25% (w/v) agar. Plates were cooled at room temperature for 30 min. At the vertical central line of the plate, 10 µl aliquots of chemoattractant solution (amino acids or polyamines) at three different concentrations (500, 50 and 5 mM) dissolved in sterilized water was placed at equivalent distances. After overnight incubation for gradient formation, 2 µl aliquots of bacterial suspensions was placed horizontally to each of the chemoattractant spots with varying distances to the chemoattractants. Plates were incubated at 30°C for 16–20 h and then inspected for chemotaxis. Chemotaxis indices were calculated as described in Pham and Parkinson (2011).

Quantitative capillary assays. Overnight cultures of *P. putida* KT2440R as well as the PP1228 and PP2249 mutant were diluted to an OD_{600} of 0.05–0.07 in MS medium supplemented with 10 mM succinate. Bacteria were grown to early stationary phase (OD_{660} of 0.3–0.4) at 30°C. Ten millilitres of culture was centrifuged at 3.300 × *g* at 4°C for 5 min and the resulting pellet was resuspended in 20 ml of drop assay medium [30 mM K_2HPO_4, 20 mM KH_2PO_4, 20 mM EDTA, 0.5% (v/v) glycerol]. Polystyrene multi-well plates were filled with 230 µl of bacterial suspension ($OD_{660} = 0.08$). For filling with chemoeffector solutions, capillaries (Microcaps, Drummond Scientific, USA) were heat-sealed at one end, warmed over the flame and the open end inserted into the chemoattractant solution. The capillary was immersed into the cell suspension at its open end. After incubation for 30 min, the capillary was removed from the cell suspension, rinsed with water and emptied into an Eppendorf tube containing 1 ml of M9 medium. Serial dilutions were made and 20 µl aliquots of the resulting cell suspension was plated onto agar plates containing M9 minimal medium supplemented with 15 mM succinate and incubated at 30°C. Colonies were counted after growth for 24 h.

Competitive root colonization assay

Corn seeds (*Zea mays* L.) were surface-sterilized by rinsing with sterile deionized water, washing for 10 min with 70% (v/v) ethanol and 15 min with 20% (v/v) bleach, followed by thorough rinsing with sterile deionized water. Surface-sterilized seeds were pregerminated on MS medium (Darias *et al*., 2014) containing 0.2% (w/v) phytagel (Sigma, reference: P8169, St Louis, MO, USA) and 0.5% (w/v) glucose, at 30°C in the dark for 48 h. Strains grown overnight in LB were diluted in M9 to an $OD_{660} = 1$. The wt strain (KT2440R) was mixed with the individual mutant strains at a 1:1 ratio. CFUs of each strain were counted in the mix using selective LB media supplied with Rif (both strains) or Km (mutant strains). For competitive root colonization assays, six independent seedlings were inoculated with this mixture. Plants were maintained in a controlled chamber at 24°C with a daily light period of 16 h. To recover bacteria from the rhizosphere, roots of 1 week old plants were collected and placed into tubes containing 20 ml of M9 basal medium and 4 g of glass beads (diameter, 3 mm). Tubes were vortexed for 2 min and serial dilutions were then plated onto selective media (LB with Rif or Km) (Martinez-Gil *et al*., 2013). Data are shown as the index of colonization fitness (ICF) that is measured as 1/[(percentage of recovered wt KT2440R versus mutant per plant 7 days post-inoculation)/(percentage of wt KT2440R versus mutant in the initial inocula)] (Martinez-Gil *et al*., 2013).

Colony-based c-di-GMP reporter assays

Fluorescence intensity analyses using the c-di-GMP biosensor pCdrA::gfp^S were carried out to determine the cellular levels of the second messenger. Briefly, the reporter plasmid pCdrA::gfp^S was transformed into KT2440R, and derivative strains, by electroporation. Subsequently, overnight bacterial cultures of the strains to be tested were adjusted to an OD_{660} of 0.05 and 20 µl drops were spotted on LB agar plates containing the appropriate antibiotics. Following incubation for 48 h at 30°C, colony morphology and fluorescence intensity were analysed in a Leica M165 FC stereomicroscope. Fluorescence was visualized employing a GFP filter set (emission/excitation filter 470/525 nm). Pictures were taken using Leica Application Suite software using different exposure times.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Alignment of the sequence of *P. aeruginosa* PAO1 BdlA with its two homologues, PP0779 and PP3414, from *P. putida* KT2440.
Fig. S2. Growth curves of *Pseudomonas putida* KT2440R and its 27 mutants in chemoreceptor genes.
Fig. S3. Motility of *Pseudomonas putida* KT2440R and its 27 mutants in chemoreceptor genes.
Fig. S4. The *wsp* gene clusters in *Pseudomonas putida* KT2440 and *P. aeruginosa* PAO1.
Fig. S5. Assessment of the capacity of the 20 proteinogenic amino acids to sustain growth of *P. putida* KT2440R as sole nitrogen or carbon source.
Fig. S6. Assessment of the capacity of polyamines to sustain growth of *P. putida* KT2440R as sole nitrogen or carbon source.
Fig. S7. Homology models of McpA-LBD and McpU-LBD, which were generated using the structures with pdb ID 3C8C and 3libA as templates respectively.
Table S1. Oligonucleotides used in this study.

HOST-PATHOGEN INTERACTIONS

Rosmarinic acid is a homoserine lactone mimic produced by plants that activates a bacterial quorum-sensing regulator

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Quorum sensing is a bacterial communication mechanism that controls genes, enabling bacteria to live as communities, such as biofilms. Homoserine lactone (HSL) molecules function as quorum-sensing signals for Gram-negative bacteria. Plants also produce previously unidentified compounds that affect quorum sensing. We identified rosmarinic acid as a plant-derived compound that functioned as an HSL mimic. In vitro assays showed that rosmarinic acid bound to the quorum-sensing regulator RhlR of *Pseudomonas aeruginosa* PAO1 and competed with the bacterial ligand *N*-butanoyl-homoserine lactone (C4-HSL). Furthermore, rosmarinic acid stimulated a greater increase in RhlR-mediated transcription in vitro than that of C4-HSL. In *P. aeruginosa*, rosmarinic acid induced quorum sensing–dependent gene expression and increased biofilm formation and the production of the virulence factors pyocyanin and elastase. Because *P. aeruginosa* PAO1 infection induces rosmarinic acid secretion from plant roots, our results indicate that rosmarinic acid secretion is a plant defense mechanism to stimulate a premature quorum-sensing response. *P. aeruginosa* is a ubiquitous pathogen that infects plants and animals; therefore, identification of rosmarinic acid as an inducer of premature quorum-sensing responses may be useful in agriculture and inform human therapeutic strategies.

INTRODUCTION

Plants live in association with fungi and bacteria, and it is believed that plant evolution was influenced by the presence of these associated microorganisms (*1*). During this evolution, diverse signaling systems emerged that permitted mutual plant-microorganism sensing. Quorum sensing (QS) is a mechanism of communication between bacteria and is based on the synthesis, detection, and response to QS signals (QSS). As cell density increases, QSS accumulate in the environment and are sensed by bacterial proteins called QS regulators, which in turn control the expression of genes; the products of these genes direct activities that are beneficial when performed by groups of bacteria acting in synchrony (*2*). Homoserine lactones (HSLs) produced by Gram-negative bacteria are the best studied and possibly the most common group of bacterial QSS (*3*). Frequently, pairs of genes encoding the HSL synthase and the HSL-sensing transcriptional regulator are found close to each other in bacterial genomes. In addition, many bacteria have additional paralogs of HSL-sensing regulator genes that are not associated with an HSL synthase gene and that were consequently termed solo or orphan regulators (*4*).

Bacteria-to-plant and plant-to-bacteria signaling are also based on QS systems. A proteomics study showed that HSLs modulate the expression of a large number of genes in the legume *Medicago truncatula* (*5*). Similarly, a transcriptomic study revealed that C6-HSL, a bacterial QS molecule produced in the rhizosphere, changed gene expression in *Arabidopsis thaliana* (*6*). HSL signaling processes are, in part, responsible for the induced systemic resistance of plants toward bacterial pathogens (*7*); these processes also modulate plant growth (*8*).

In addition, different plants produce compounds that interfere with the bacterial QS mechanism. Extracts (*9*, *10*) and macerates of different plants, plant parts, and seeds (*11*–*15*), as well as exudates from seeds (*16*) or seedlings (*17*), interfere with bacterial QS mechanisms. Additionally, leaf washings from 17 different plants stimulated or inhibited HSL-dependent activities in bacteria (*18*). Furthermore, QS-dependent gene expression is altered when pathogenic bacteria grow in their host plants (*19*). Biofilm formation and HSL production increase in the presence of different plant-derived phenolic compounds (*20*); however, these compounds do not act as HSL mimics. In contrast, most of the experiments using extracts from plants stimulated rather than inhibited QS-dependent gene expression (*1*), with the data suggesting that these plant compounds act as HSL mimics and bind to the autoinducer-binding domain of QS regulators. A molecular docking study identified rosmarinic acid (RA), naringin, morin, mangiferin, and chlorogenic acid (*21*) as plant-derived compounds that were predicted to bind to QS regulators. Although in that study each of these compounds inhibited QS-mediated phenotypes, suggesting that they function as QS antagonists, potential toxic effects were not evaluated. Experimental confirmation of the binding of any of these compounds to QS regulators has not been done. The algal compound lumichrome, which is a riboflavin derivative, stimulates the activity of a QS regulator of *Pseudomonas aeruginosa* (*22*).

Here, we used *P. aeruginosa* PAO1 as a model organism to screen for plant-derived HSL mimics. This bacterium is a ubiquitous pathogen that infects a wide range of species, including humans and different plants such as barley, poplar tree (*23*), and lettuce (*24*, *25*). As a model for studying the effect of QS on pathogenic traits, *P. aeruginosa* has a multisignal QS system that is based on the synthesis and detection of signals that belong to two different classes, namely, *Pseudomonas* HSLs and quinolone signals (*26*, *27*). The HSL response is mediated by two pairs of synthases and regulators—the synthase LasI and the regulator LasR (LasI/LasR) and the synthase RhlI and the regulator RhlR (RhlI/RhlR)—as well as by the orphan regulator QscR. RhlI produces the signaling molecule *N*-butanoyl-homoserine lactone (C4-HSL), and LasI produces *N*-3-oxododecanoyl-homoserine lactone (3-Oxo-C12-HSL) (*26*). The *P. aeruginosa* QS system is hierarchically organized with LasR at the top of the signaling cascade: LasR activation stimulates transcription of multiple genes, including *rhlR*, *rhlI*, and *lasI*. The QS cascade then modulates multiple QS phenotypes, including changes in the amounts of elastase, pyocyanin, rhamnolipid, and hydrogen cyanide (*28*).

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Here, we used ligand-free LasR and RhlR purified from *Escherichia coli* without added HSL (*29*) for microcalorimetric binding studies of plant-derived compounds that were selected on the basis of in silico docking experiments. We identified RA as a plant-derived compound that binds with nanomolar affinity to RhlR. In transcription assays with RhlR, RA significantly more effectively stimulated transcription at lower concentrations than C4-HSL. In bioassays, RA, but not the closely related compound chlorogenic acid, stimulated biofilm formation and the production of the virulence factors pyocyanin and elastase. RA is produced exclusively in plants and not in bacteria (*30*, *31*). Thus, these data showed that RA acts as a QS regulator agonist, thereby providing the molecular identification of a plant QSS mimic.

RESULTS

RA binds to purified RhlR with high affinity

A major limitation in the study of the HSL-sensing regulators is their instability in the absence of HSL (*32*–*34*). Because recombinant regulator purified from *E. coli* cultures binds HSL added to the culture medium, using most methods, the bound HSL co-purifies with the protein, resulting in partially saturated regulators and thus hampering ligand-binding studies. We developed a method that enables the purification of recombinant RhlR and LasR without the addition of HSL, thereby providing a system for performing ligand-binding analysis (*29*). Recombinant RhlR and LasR isolated with this method in the absence of HSL bound C4-HSL and 3-Oxo-C12-HSL, respectively, with dissociation constant (K_D) values of 1.66 ± 0.4 μM and 1.14 ± 0.2 μM, as determined by isothermal titration calorimetry (ITC) (Fig. 1, A and B).

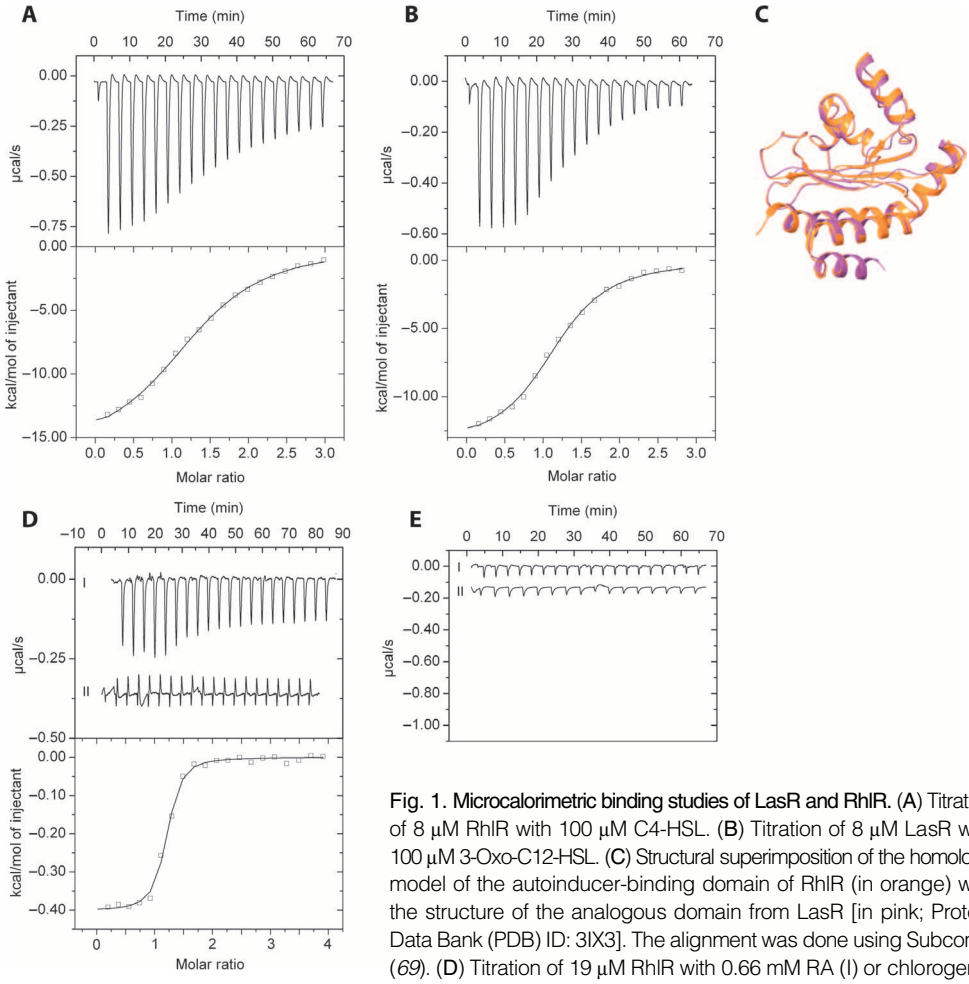

Fig. 1. Microcalorimetric binding studies of LasR and RhlR. (**A**) Titration of 8 μM RhlR with 100 μM C4-HSL. (**B**) Titration of 8 μM LasR with 100 μM 3-Oxo-C12-HSL. (**C**) Structural superimposition of the homology model of the autoinducer-binding domain of RhlR (in orange) with the structure of the analogous domain from LasR [in pink; Protein Data Bank (PDB) ID: 3IX3]. The alignment was done using Subcomb (*69*). (**D**) Titration of 19 μM RhlR with 0.66 mM RA (I) or chlorogenic acid (II). Lower panel plots the titration data for RA. (**E**) Titration of buffer (I) and 8 μM LasR (II) with 0.66 mM RA. For the titration data (A, B, D, and E), the upper panels show the raw titration data and the lower panels are concentration-normalized and dilution heat-corrected integrated peak areas of the titration data fitted with the "One binding site model" of the MicroCal version of Origin.

To identify potential ligands, we conducted in silico docking experiments of ligands present in a database of natural compounds to the structure of LasR and a model of RhlR. We used the structure of the LasR autoinducer-binding domain in complex with 3-Oxo-C12-HSL (PDB ID: 3IX3) to generate a homology model of the analogous domain of RhlR, which could be closely superimposed onto the template (Fig. 1C). We used 3-Oxo-C12-HSL and C4-HSL as controls in the docking experiments (Table 1) and then we screened the Natural Compounds subset of the ZINC compound database (5391 compounds) and selected those of plant origin and with docking scores below −8 for further analysis. Most of the tested compounds had lower docking scores at RhlR or LasR when compared to those of their cognate HSL ligands (Table 1).

Microcalorimetric binding studies with the selected compounds were performed to assess binding to ligand-free LasR and RhlR. We found that RA bound only to RhlR (Fig. 1D) and not to LasR (Fig. 1E). No other selected compound bound to RhlR or LasR (Table 1).

We calculate that RA bound to RhlR with a K_D of 0.49 ± 0.08 μM and had small favorable enthalpy changes ($\Delta H = -0.4 \pm 0.05$ kcal/mol). Similar to the HSL ligands, the binding stoichiometry was close to 1:1, which can be observed as the point of inflection of the sigmoidal binding curves in Fig. 1D with respect to the lower *x* axis. To assess the specificity of this interaction, we titrated RhlR with chlorogenic acid, a compound structurally similar to RA (Fig. 2) and that had a low docking score (Table 1). Chlorogenic acid did not cause significant heat changes, indicating that this compound did not bind RhlR (Fig. 1D).

The RhlR model containing the best fit of docked RA and C4-HSL showed that both ligands overlap (Fig. 2). Although the docking simulations with LasR predicted that RA could overlap with bound 3-Oxo-C12-HSL (Fig. 2), the ITC studies showed an absence of binding of RA for LasR (Fig. 1E).

RA stimulates RhlR-mediated transcription

To determine whether RA behaved as an agonist or antagonist, we conducted in vitro transcription assays with a 490–base pair (bp) DNA fragment

Table 1. Results from in silico docking and experimental binding studies of plant-derived compounds to RhlR and LasR. Shown are XP scores for the in silico docking of different ligands to a homology model of the RhlR autoinducer domain and to the structure of the analogous domain of LasR. Binding parameters are derived from ITC experiments of purified LasR and RhlR with the ligands listed. Data shown are means and SD from three independent experiments.

Ligand	Docking XP score		Binding parameters			
	RhlR	LasR	RhlR		LasR	
			K_D (μM)	ΔH (kcal/mol)	K_D (μM)	ΔH (kcal/mol)
Isoorientin	−12.33	−14.95	No binding		No binding	
Spiraeoside	−12.69	−14.29	No binding		No binding	
Luteolin-galactoside	−13.01	−13.37	No binding		No binding	
Propanolol	−11.70	−12.36	No binding		No binding	
RA	−8.13	−10.87	0.49 ± 0.1	−0.4 ± 0.05	No binding	
Mangiferin	−8.90	−10.47	No binding		No binding	
Morin	−8.87	−9.57	No binding		No binding	
Chlorogenic acid	−9.46	−7.42	No binding		No binding	
Naringin	<4.0	−5.96	No binding		No binding	
3-Oxo-C12-HSL	−7.39	−8.71	Not determined		1.14 ± 0.2	−13.6 ± 0.2
C4-HSL	−4.77	−5.56	1.66 ± 0.4	−16.1 ± 0.2	Not determined	

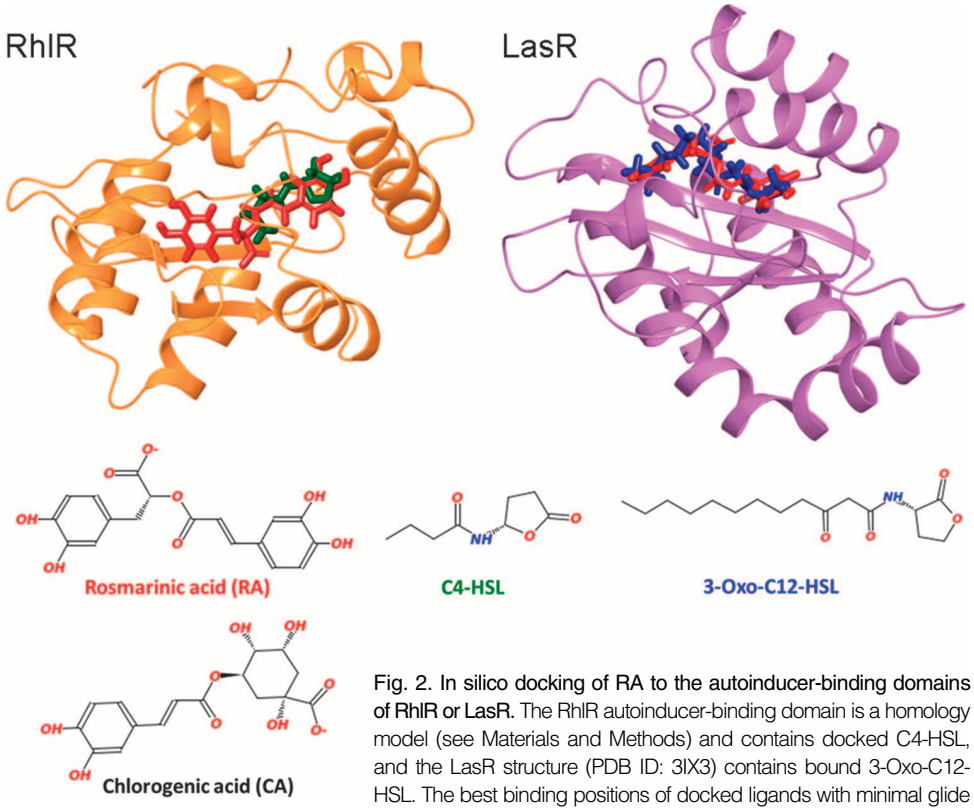

Fig. 2. In silico docking of RA to the autoinducer-binding domains of RhlR or LasR. The RhlR autoinducer-binding domain is a homology model (see Materials and Methods) and contains docked C4-HSL, and the LasR structure (PDB ID: 3IX3) contains bound 3-Oxo-C12-HSL. The best binding positions of docked ligands with minimal glide score (XP G score) and glide energy (G energy) are displayed. The docking scores are shown in Table 1. The structures of different ligands are shown in the lower part of the figure.

containing the *hcnABC* promoter, which is activated by RhlR (*35*). We performed the experiments with a constant RhlR concentration of 25 μM in the presence of either equimolar or half-equimolar concentrations of C4-HSL and RA. Because the K_D values for both ligands are much less than the protein concentration, we expect that protein saturation at equimolar ligand concentrations is comparable and almost complete. Both C4-HSL and RA stimulated RhlR-dependent transcription (Fig. 3A), but quantification revealed that RA exhibited a significantly greater activity compared to C4-HSL when the response to equal concentrations of ligand was compared to the transcription detected in the control (absence of ligand) condition (Fig. 3B). In the presence of half-equimolar C4-HSL or RA to RhlR concentrations, transcriptional activity was less than at equimolar concentrations of ligand to regulator, thus indicating dose dependence of the response. Consistent with the lack of binding to RhlR, chlorogenic acid did not increase transcription. These data showed that compared to the bacterial ligand C4-HSL, RA was more effective at activating RhlR-dependent transcription from this promoter.

RA stimulates QS gene expression in vivo

To assess the capacity of RA to modulate QS-dependent gene expression in vivo, we conducted experiments in *E. coli* and *P. aeruginosa*. RhlR controls the expression of the gene encoding its cognate HSL synthase RhlI (*36*). Therefore, we transformed *E. coli* BL21 with either plasmid pPET28b-RhlR (RhlR expression plasmid) or pPET28b (empty plasmid as control) and pMULTIAHLPROM containing an *rhlI*::*lacZ* transcriptional fusion. β-Galactosidase measurements showed statistically significant increases in gene expression in the presence of C4-HSL or RA, whereas chlorogenic acid did not stimulate expression (Fig. 4A).

To study gene expression in *P. aeruginosa*, we introduced the *rhlI*::*lacZ* reporter plasmid into the *las*I$^-$/*las*R$^-$ double mutant and measured β-galactosidase activity in samples taken at different time intervals after

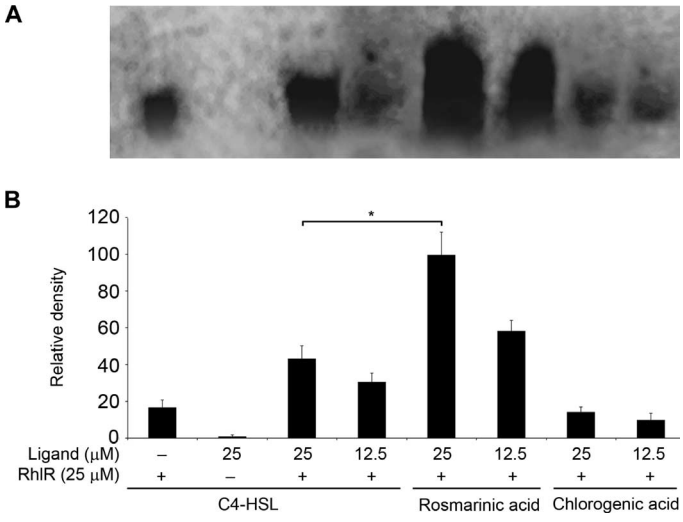

Fig. 3. The capacity of C4-HSL and RA to stimulate RhlR-mediated transcription in vitro. (**A**) Representative acrylamide gel of an in vitro transcription assay using a DNA fragment containing the *hcnABC* promoter that is induced by RhlR (*35*). Conditions match those listed below the graph in (B). Ligands tested included C4-HSL, RA, and chlorogenic acid. (**B**) Densitometric analysis of in vitro transcription assays. Shown are means and SD from three individual experiments. *$P < 0.05$, Student's *t* test.

the addition of either DMSO, C4-HSL, or RA. The data indicated that the bacteria exhibited differential kinetics in response to the two RhlR ligands. Induction of the reporter in the cultures exposed to RA peaked within 1 hour and was significantly greater than that of the control and the C4-HSL–exposed cultures at both 1 and 2 hours (Fig. 4B). At subsequent time points, reporter activity decreased in the RA-containing cultures. We predicted that the reduction in β-galactosidase activity of RA-containing cultures after 2 hours indicated that RA was metabolized. The activity of C4-HSL cells was comparable to that of the control after 1 hour but was significantly greater for time points 6 to 8 hours, which may be due to slow uptake.

We determined the dose-response relationships for the increase in gene expression induced by C4-HSL and RA (Fig. 4C). Because of the differences in kinetics, we took measurements for the concentrations of ligand tested at 4 hours, the time point at which C4-HSL and RA induced similar amounts of β-galactosidase activity (Fig. 4B). At concentrations of 1 to 100 μM, transcriptional activities were comparable, which may be due to metabolization of RA (Fig. 4C). However, at 0.5, 1, and 2 mM, the β-galactosidase activity in response to RA was higher than that induced by C4-HSL (Fig. 4C). At 5 mM RA, we observed a further increase in β-galactosidase activity, but we could not perform similar measurements with C4-HSL due to the solubility limit of this compound. These data are consistent with the in vitro transcription experiments and support the conclusion that the capacity of RA to stimulate transcription is superior to that of C4-HSL.

To confirm that *P. aeruginosa* could metabolize RA, we analyzed bacteria grown in minimal medium containing 1 to 10 mM RA as the only carbon source. *P. aeruginosa* grew with 1 to 5 mM RA as the only carbon source (Fig. 5A), consistent with metabolism of this compound and suggesting that metabolism of RA may be responsible for the reduction of its gene induction activity over time. The bacteria did not grow in 10 mM RA. Although our growth data are consistent with those of Annapoorani *et al.* (*21*), they conflict with those of Walker *et al.* (*31*) who reported that RA is toxic at low micromolar concentrations. Therefore, we examined cell

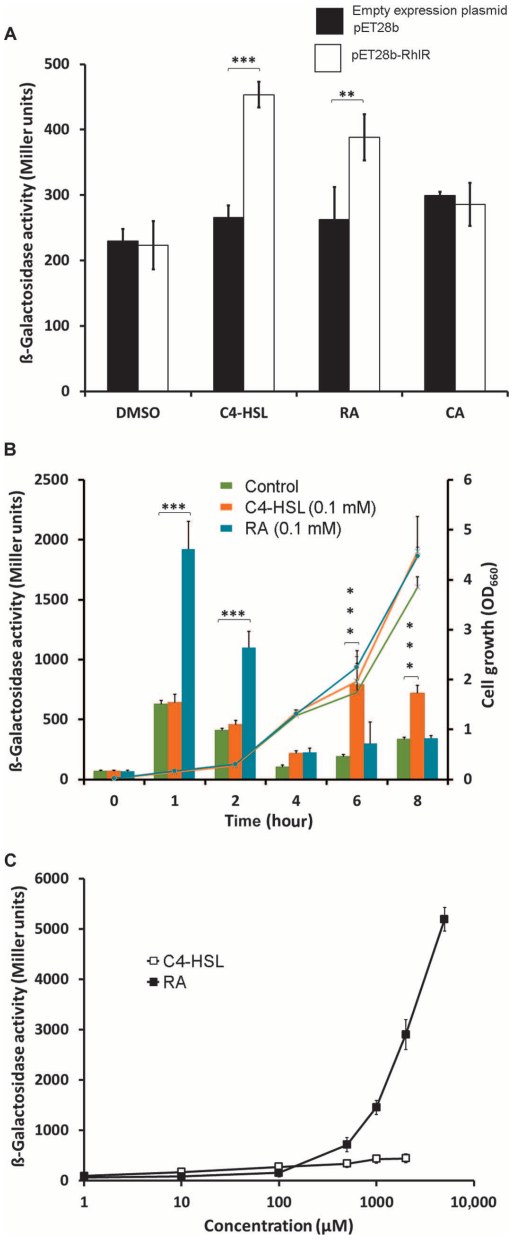

Fig. 4. RA- or C4-HSL–mediated activation of a QS reporter in bacteria. (**A**) Transcriptional activation in *E. coli*. β-Galactosidase measurements at 2 hours after induction of *E. coli* BL21 containing pET28b-RhlR (expression plasmid for RhlR) or the empty expression plasmid and pMULTIAHLPROM containing a *rhlI*::*lacZ* transcriptional fusion. The ligand concentrations were 100 μM. CA, chlorogenic acid. (**B**) Transcriptional activation over time by the indicated ligands in *P. aeruginosa lasI*$^-$/*lasR*$^-$ containing pMULTIAHLPROM. Bars represent the β-galactosidase measurements at different time intervals after the addition of dimethyl sulfoxide (DMSO; control), C4-HSL, or RA. The line graphs represent growth curves of the corresponding cultures. (**C**) Concentration-dependent transcriptional activation by the indicated ligands in *P. aeruginosa lasI*$^-$/*lasR*$^-$ containing pMULTIAHLPROM. Dose-response curves for each ligand from samples taken 4 hours after the addition of the ligand. Shown are means and SD from three independent experiments conducted in duplicate. **$P < 0.01$, ***$P < 0.001$, Student's *t* test.

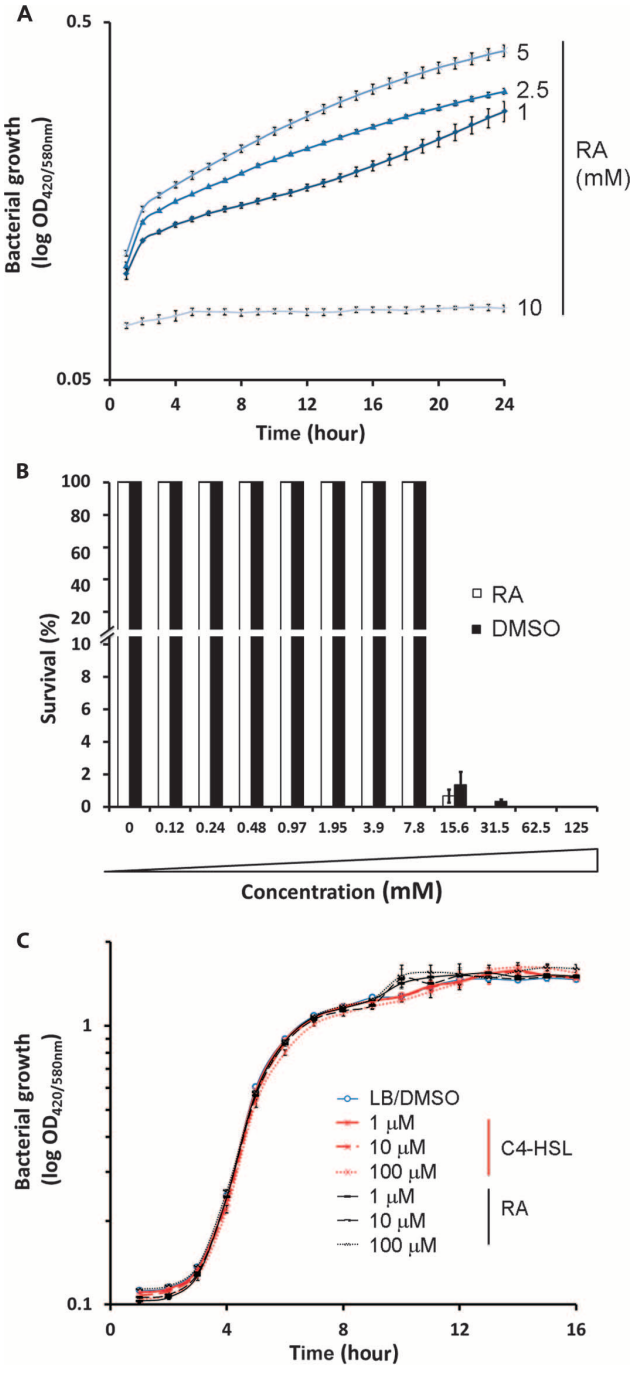

Fig. 5. The effect of RA on *P. aeruginosa* PAO1 survival and growth. (A) Growth curve of *P. aeruginosa* PAO1 in M9 minimal medium supplemented with the indicated concentration of RA. Shown are means and SD from three experiments conducted in triplicate. (B) Growth in LB medium supplemented with the indicated concentrations of RA (and the corresponding DMSO-containing controls). Cell survival after 24 hours was determined by plating out on solid medium and cell counting. Shown are means and SD from three independent experiments each conducted in quintuplicate. (C) Impact of RA on bacterial growth on rich medium. Shown is a growth curve in LB medium supplemented with different concentrations of C4-HSL or RA. Shown are means and SD from three experiments conducted in triplicate.

viability as a function of RA concentration. We found that viability was not affected by RA concentrations up to 7.8 mM, whereas viability dropped at 15.6 mM (Fig. 5B). Furthermore, we confirmed that the presence of C4-HSL and RA at the concentrations used for gene expression studies did not change growth kinetics (Fig. 5C). These discrepancies in the RA tolerance of *P. aeruginosa* PAO1 may be due to differential evolution of the strain in different laboratories.

The transcriptional reporter studies so far used the *rhlI* promoter. We performed analogous experiments in *P. aeruginosa* PAO1 expressing *lacZ* controlled by *lasB* (*37*), *rhlA* (*38*), or *hcnABC* (*35*), which are all induced by RhlR. RA produced a significant increase in β-galactosidase activity for each of the three reporters (Fig. 6, A to C), whereas control experiments with bacteria transformed with the empty plasmid did not show an increase in β-galactosidase activity upon RA addition (Fig. 6D).

RA increases biofilm formation, pyocyanin production, and elastase synthesis

Increased biofilm formation and the production of the virulence factor pyocyanin are characteristic features of HSL-mediated QS responses (*39*, *40*). We therefore tested these traits in *P. aeruginosa* grown in the presence and absence of different concentrations of RA or chlorogenic acid. We quantified pyocyanin, which is green, by measuring the absorbance at 520 nM and found that RA, but not chlorogenic acid, stimulated a dose-dependent increase in the intensity of the green color when corrected for cell density (Fig. 7A).

Visual inspection of culture tubes (inset in Fig. 7A) showed that RA also stimulated biofilm formation. To quantify the effect of RA on biofilm formation, we grew bacterial cultures in borosilicate glass tubes in the presence or absence of either 2 mM RA or chlorogenic acid and quantified biofilm formation at different time points. We found that RA stimulated biofilm formation between 2 to 8 hours of growth, whereas chlorogenic acid had no significant effect (Fig. 7B, upper). After 24 hours, the amount of biofilm formed in these three conditions was comparable. The RA-mediated stimulation of biofilm formation contradicted a previous report in which the compound was used at higher concentrations than what we used here and inhibited biofilm formation (*21*). To assess this discrepancy, we determined the dose-response relationship of the RA-mediated effect on biofilm (Fig. 7B, lower). RA stimulated biofilm formation at concentrations up to 2 mM, but above this concentration, biofilm formation was inhibited by RA.

Elastase synthesis is also stimulated by RhlR-mediated QS activity (*37*). Our reporter analysis indicated that RA enhanced the activity of the promoter controlling the expression of *lasB*, encoding elastase (Fig. 6A). To verify whether this results in changes in elastase synthesis, we measured elastase activity in *P. aeruginosa* grown in the absence and presence of RA. RA stimulated the amount of elastase activity in density-normalized cultures (Fig. 7C).

DISCUSSION

Multiple lines of evidence indicate that plants produce HSL mimics, which interfere with the bacterial QS system (*9*–*17*). However, little information is available regarding the molecular identity of the active compounds that are responsible for this interference (*1*).

We report the identification and characterization of a plant compound that directly interacts with a bacterial QS regulator, stimulating its transcriptional activity. RA bound purified RhlR with a higher affinity than C4-HSL, and this translated into a greater stimulatory activity of RA on RhlR-mediated transcription and gene expression compared with that induced by C4-HSL. Furthermore, RA stimulated biofilm formation and the synthesis of pyocyanin and elastase, which are

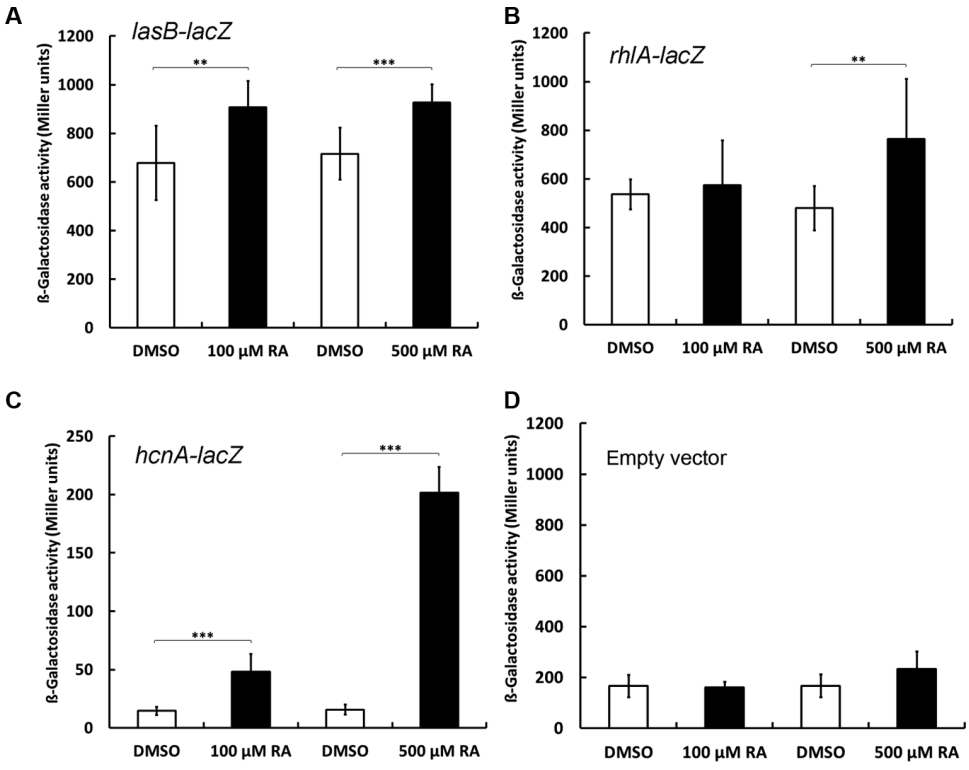

Fig. 6. Effect of RA on the expression from other QS-regulated promoters. (A to D) β-Galactosidase measurements of *P. aeruginosa* PAO1 containing the plasmids pβ01 (*lasB-lacZ*) (A), pβ02 (*rhlA-lacZ*) (B), pME2823 (*hcnA-lacZ*) (C), or pQF50 (empty vector) (D). Measurements were made 2 hours after the addition of RA or the corresponding amount of DMSO. Shown are means and SD from three independent experiments conducted in duplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test.

phenotypic characteristics that are regulated by QS mechanisms in *P. aeruginosa* (*40*, *41*).

Because interference with bacterial QS mechanisms by plant-derived compounds has been observed for various different plant pathogens, it was proposed that this represents a plant defense strategy (*1*). HSL mimics acting as agonists of HSL-mediated sensing may decrease pathogenicity because these mimics would stimulate premature expression of genes encoding proteins involved in QS-controlled functions (*1*). Molecular identification of HSL mimics will enable their application in medicine and agriculture, for example, in the generation of pathogen-resistant plants. However, is this mimicking of a bacterial QSS by RA of physiological relevance? Walker *et al.* (*31*) monitored the consequences of sweet basil root infection by *P. aeruginosa* strains PAO1 and PA14 and determined that infection with either strain induced RA secretion. RA concentration in root exudates gradually increased, reaching a maximum of ~40 µM 6 days after infection (*31*). Thus, plant infection triggers RA release, suggesting that RA release forms part of a plant defense strategy and supporting the model that plant-derived HSL mimics decreased pathogenicity by stimulating premature QS-responsive gene expression (*1*). We found that RA was both a growth substrate and a signaling molecule. Bacterial consumption of RA may provide

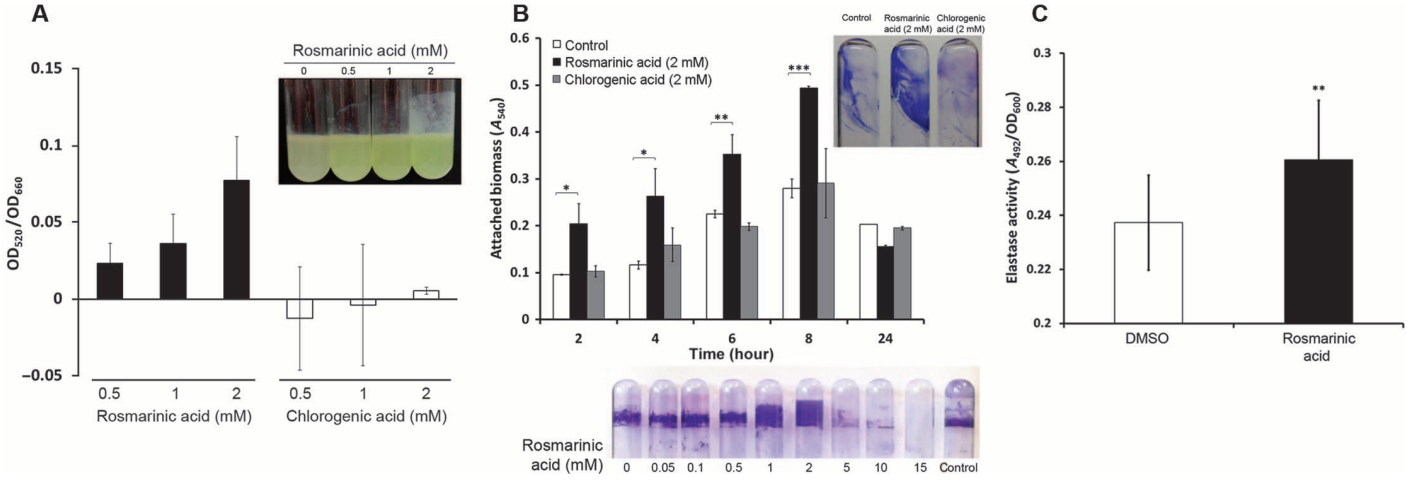

Fig. 7. RA induces QS-regulated phenotypes. (A) Pyocyanin production measured in *P. aeruginosa* PAO1 grown in the presence of different concentrations of RA or chlorogenic acid. Shown are means and SD from three independent experiments of the absorbance at 520 nm (pyocyanin) of *P. aeruginosa* supernatants relative to the absorbance at 660 nm (cell density). The inset shows culture tubes after 8 hours of growth. (The amount of DMSO equivalent to that added in the 2 mM RA condition was added as a control to the first tube "0.") (B) Biofilm formation quantified in the presence and absence of RA or chlorogenic acid at different time points. Shown are means and SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test. The inset shows representative crystal violet–stained tubes containing an 8-hour culture of *P. aeruginosa* in the presence and absence of RA or chlorogenic acid. In the lower part, the tubes show the RA concentration dependence of biofilm formation (after 8 hours). (C) Elastase synthesis measured as enzymatic activity of *P. aeruginosa* PAO1 cultures grown in the presence or absence of RA. Data are normalized for culture density. Shown are means and SD from three individual experiments conducted in triplicate. $^{**}P < 0.01$, Student's *t* test.

a mechanism to eliminate the signaling effects of this compound, which is consistent with the kinetics of gene induction by RA that we observed.

Reports of the toxic effects of RA to *P. aeruginosa* PAO1 differ. Our data and those reported by Annapoorani *et al*. (*21*) indicate an effective inhibitory concentration of 2.1 mM with regard to bacterial growth, whereas Walker *et al*. (*31*) reported minimal inhibitory concentration of 8 μM. These discrepancies may be due to differential strain evolution in different laboratories. Annapoorani *et al*. (*21*) showed a reduction of *P. aeruginosa* PAO1 biofilm formation and elastase activity in the presence of RA and a number of related compounds, including chlorogenic acid; however, our data showed no activity for chlorogenic acid in binding to or stimulating RhlR or in promoting biofilm formation, and we found that RA functioned as an HSL mimic.

P. aeruginosa is one of the pathogens that infect a wide range of species, including plants and animals (*25*). Common pathogenic mechanisms enable bacteria to infect phylogenetically different hosts, and there are also parallels in the key features underlying host defense responses in plants, invertebrates, and mammalian hosts (*25*). The production of HSL mimics is considered a plant defense strategy. The detailed knowledge of plant defense mechanisms may enable the development of strategies to protect human from this pathogen. The effect of HSL mimics, including R, on the virulence properties toward mammals is of interest.

RA is synthesized by many plants (*42*) and can accumulate to high concentrations (*43*). RA has multiple biological activities, including antibacterial (*31*), antiviral (*44*), antiallergy (*45*), anticarcinogenic (*46*), antigenotoxic (*47*), anti-inflammatory (*48*), and antioxidant effects (*49*) activity. In addition, RA was found to be effective against amyloid-β peptide–induced neurotoxicity that is associated with Alzheimer's disease (*50*), reduces atopic dermatitis (*51*), and protects keratinocytes from ultraviolet radiation damage (*52*). Consequently, plants rich in RA are used as medicinal herbs and by the food industry (*43*).

Solo or orphan QS regulators recognize plant-derived HSL mimics (*1*). Solo QS regulators are abundant in plant-associated bacteria, which supports the view that they are involved in interkingdom signaling between plants and bacteria (*1*). Our data showed that RhlR has a double function and mediates responses to bacterial HSL and plant-derived RA, thus indicating that recognition of HSL mimics is not limited to solo QS regulators.

Several synthetic HSL and non-HSL ligands have been identified that modulate the activity of *P. aeruginosa* QS regulators (*53*–*58*). These compounds behaved as either agonists or antagonists. Structurally, the agonists (*53*, *58*) are similar to RA because part of these compounds were linear with aromatic moieties at each extension of the molecule.

Here, we identified RA as an HSL mimic. Indeed, RA mimicked the action of C4-HSL in vitro and in vivo despite being dissimilar structurally. RA is not the only plant-derived HSL mimic. Gao *et al*. (*16*) estimated that there are 15 to 20 separable compounds with the capacity to affect HSL-based QS processes. Although RA can serve as a lead compound, this work demonstrated that mimics can have a structure very different from that of the cognate ligand.

MATERIALS AND METHODS

Materials

The strains and plasmids used in this study are provided in Table 2. HSL and plant-derived compounds (Table 1) were purchased from Sigma-Aldrich.

Purification of LasR and RhlR

The DNA fragments encoding LasR and RhlR were amplified using the primers 5′-GTTTAAGAAGAACGTGCTAGCATGGCCTTG-3′ and 5′-CTGAGAGGGATCCTCAGAGAGTAATAAGAC-3′ (LasR) and 5′-TATCGAGCTAGCCTTACTGCAATGAGGAATGAC-3′ and 5′-CGAGCTCTGCGCTTCAGATGAGACC-3′ (RhlR), respectively. These primers contained restriction sites (underlined) for *Nhe* I and *Bam* HI (LasR) and *Nhe* I and *Sac* I (RhlR). Polymerase chain reaction (PCR) products were digested with these enzymes and cloned into the expression plasmid pET28b(+). *E. coli* BL21 (DE3) was transformed with the resulting plasmids, and cultures were grown in LB medium supplemented with kanamycin (50 μg/ml) at 37°C until an OD_{660} (optical density at 660 nm) of 0.4. The temperature was then lowered to 18°C, and growth continued until an OD_{660} of 0.6 to 0.8, at which point protein production was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. Growth was continued at 18°C overnight, and cells were harvested by centrifugation at 10,000*g* for 30 min. Cell pellets were resuspended in buffer A [20 mM tris-HCl, 0.1 mM EDTA, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 5% (v/v) glycerol, 1 mM dithiothreitol (DTT) (pH 7.8)] and broken by French press at 1000 psi. After centrifugation at 20,000*g* for 1 hour, the supernatant was loaded onto a 5-ml HisTrap column (Amersham Biosciences) previously equilibrated with buffer A. The column was then washed with buffer A containing 45 mM imidazole before protein elution with a linear gradient (20 min) of 45 to 500 mM imidazole in buffer A at a flow of 1 ml/min. Protein-containing fractions were pooled and dialyzed into buffer B [50 mM tris-HCl, 500 mM NaCl, 1 mM DTT (pH 7.8)] and

Table 2. Strains and plasmids used in this study. Antibiotic resistance: Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Tc, tetracycline; Cb, carbenicillin.

Strains or plasmids	Relevant characteristics	Reference
P. aeruginosa PAO1	Wild type, prototroph; Ap^r	(*70*)
P. aeruginosa PAO1 *lasI*$^-$/*lasR*$^-$	Double mutant in *lasI* and *lasR* genes Gm^r	Personal gift, M. Cámara (University of Nottingham)
E. coli BL21 (DE3)	F^-, *ompI*, $hsdS_B$ (r^-_B m^-_B)	(*71*)
pET28b(+)	Km^R, protein expression vector	Novagen
pET28b-LasR	Km^R, pET28b(+) derivative containing *lasR* gene	This work
pET28b-RhlR	Km^R, pET28b(+) derivative containing *rhlR* gene	This work
pMULTIAHLPROM	Tc^R, broad-host-range plasmid containing 8-*luxI* type promoters fused to a promoter *lacZ* gene	(*63*)
pQF50	Cb^R, broad-host-range transcriptional fusion vector	(*68*)
pβ01	Cb^R, pQF50 derivative containing *lasB-lacZ* transcriptional fusion	(*68*)
pβ02	Cb^R, pQF50 derivative containing *rhlA-lacZ* transcriptional fusion	(*68*)
pME2823	Cb^R, pKT240 derivative containing *hcnA-lacZ* transcriptional fusion	(*35*)

applied to a HiPrep26/60 Sephacryl S-200 high resolution gel filtration column previously equilibrated with the same buffer. Protein was eluted by a constant flow (1 ml/min) of buffer B at 4°C.

Isothermal titration calorimetry

Experiments were conducted on a VP-microcalorimeter (MicroCal) at 25° and 30°C. Proteins were dialyzed in analysis buffer [50 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, 1 mM DTT (pH 7.8)]. HSL ligands were prepared at a concentration of 1 mM in DMSO and subsequently diluted 1:10 with analysis buffer. The corresponding amount of DMSO [10% (v/v)] was added to the dialyzed protein sample. Typically, 8 to 15 μM protein was titrated with 0.1 mM HSL solution. Control experiments involved the titration of dialysis buffer containing 10% (v/v) DMSO with HSL ligand. For the non-HSL ligands, a solution of 660 μM was prepared directly in dialysis buffer and used for the titration of the dialyzed protein. The mean enthalpies measured from the injection of ligands into the buffer were subtracted from raw titration data before data analysis with the MicroCal version of Origin.

Molecular docking, homology modeling, and structural alignment

The atomic structure of LasR was obtained from the PDB (www.pdb.org; PDB ID: 3IX3). The structure was refined and optimized with the Protein Preparation Wizard of the Schrödinger Suite (Schrödinger Suite 2012 Protein Preparation Wizard; Epik version 2.3, Schrödinger, LLC, New York, NY, 2012; Impact version 5.8, Schrödinger, LLC, New York, NY, 2012; Prime version 3.1, Schrödinger, LLC, New York, NY, 2012). Ligands were obtained from the Natural Compounds (Metabolites) subset of the ZINC database (*59*), optimized by LigPrep (LigPrep, version 2.5, Schrödinger, LLC, New York, NY, 2012), and then submitted to virtual screening docking experiments to LasR using the Glide dock SP mode (Glide, version 5.8, Schrödinger, LLC, New York, NY, 2012) (*60*). The best hits were subsequently docked using the Glide dock XP mode. A homology model of the autoinducer domain of RhlR was generated using Swiss-Model (*61*) and the structure of QscR (PDB ID: 3SZT) as template. RA was docked onto this structure in the Glide dock XP mode. The structural alignment of the autoinducer domains of LasR and RhlR was generated by PyMOL (PyMOL Molecular Graphics System, version 1.5.0.4, Schrödinger, LLC).

In vitro transcription assay

A 490-bp DNA fragment of the *hcnABC* promoter of *P. aeruginosa* (*35*) was amplified by PCR using the primers 5′-GCACTGAGTCGGACATGACGGAA-3′ and 5′-CGTGTTGACGTTCAAGAAGGTGCATTGC-3′ and used as a template for these assays. Transcription reactions (20 μl) were performed in binding buffer [20 mM tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol (pH 7.8)] containing 50 nM *E. coli* RNA polymerase holoenzyme saturated with σ^{70} sigma factor (Epicentre Technologies), 5 nM linear *hcnABC* DNA, 25 μM RhlR, and different effector molecules (12.5 or 25 μM C4-HSL, RA, and chlorogenic acid). The mixtures were incubated at 30°C for 20 min before the addition of 0.1 mM adenosine triphosphate, cytidine triphosphate, and guanosine triphosphate; 0.05 mM uridine triphosphate (UTP); and 3.6 μCi of [α-^{32}P]UTP (10 μCi/μl) (1 Ci = 37 GBq). After incubation for 50 min, the reactions were stopped by transferring them to a 95°C thermoblock and then subsequently chilled at 4°C, at which point 4 μl of formamide sequencing dye was added. Samples were separated on 6.5% (w/v) polyacrylamide gel electrophoresis gels for 2 hours. Gels were dried and then exposed on a phosphorimager, and the resulting images were processed with the Quantity One software 4.6.2 (Bio-Rad Laboratories). The densitometric analysis was carried out using the program ImageJ (*62*).

Gene expression studies

Gene expression experiments were conducted with *E. coli* BL21 harboring pET28b-RhlR (expression plasmid for RhlR) or pET28b (empty plasmid as a control) and pMULTIAHLPROM containing an *rhlI*::*lacZ* transcriptional fusion (*63*), as well as with *P. aeruginosa* PAO1 *las*I⁻/*las*R harboring pMULTIAHLPROM. *E. coli* BL21 were grown in LB containing tetracycline (10 μg/ml) and kanamycin (50 μg/ml), and *P. aeruginosa* PAO1 *las*I⁻/*las*R in LB containing tetracycline (40 μg/ml) and gentamicin (20 μg/ml) at 37°C overnight. Stock solutions of RA and chlorogenic acid were prepared in DMSO (100%) and diluted in water to the desired concentration, whereas C4-HSL solutions were prepared in 10% (v/v) DMSO. Fresh LB medium was then inoculated with the resulting cultures (1:100 dilution), grown for 1 hour, and then diluted twofold twice at 30-min intervals to ensure proper dilution of accumulated β-galactosidase after overnight growth. The resulting culture was then grown for another hour before induction with different ligands. To rule out nonspecific effects of DMSO (present in the stock solution), control experiments were performed in which the amount of DMSO corresponding to that present in C4-HSL–, RA-, or chlorogenic acid–containing cultures was added. Growth was continued at 37°C, and samples were taken t different time points for the determination of β-galactosidase in permeabilized whole cells as described in (*64*). For dose-response experiments, the β-galactosidase activity was measured 4 hours after induction. Data shown are means and SD from at least three independent experiments.

To explore the effect of RA on other RhlR-regulated promoters, plasmids containing *lacZ* fusions were transferred to the wild-type strain by electroporation. These plasmids were pß01 (*lasB*::*lacZ*), pß02 (*rhlA*::*lacZ*), pME3823 (*hcnA*::*lacZ*), and the insert-free pQF50, which has served to construct the former two plasmids (Table 2). Experimental conditions were as those described above except that cultures were grown for 6 hours after dilution before the induction with different ligands.

Minimal inhibitory concentration assay

These assays were performed in 96-well plates using a modified version of the protocol reported in (*65*). Wells of a 96-well plate were filled with 200 μl of LB containing different amounts of RA (added using a 250 mM stock solution in DMSO). Control experiments contained the corresponding amounts of DMSO. Wells were inoculated with 10 μl of an overnight culture of *P. aeruginosa* PAO1 in LB medium. Plate was incubated at 37°C for 24 hours, at which point the viable cell amount was determined by plating out cells on LB agar medium and counting.

Growth experiments

To assess the potential of the bacterium to use RA as sole growth substrate, sterile honeycomb plates (Bioscreen C) containing 200 μl of M9, supplemented with 1 to 10 mM RA, were inoculated with an overnight culture of *P. aeruginosa* PAO1 grown in M9 minimal medium (*66*) containing 5 mM citrate at 37°C. Cultures were grown in a Bioscreen C (Thermo Fisher Scientific) instrument under constant shaking at 37°C during which time the OD_{660} was measured in 1-hour intervals. To assess the effect of different RA concentrations on *P. aeruginosa* growth, honeycomb plates were filled with LB medium containing 1 to 100 μM C4-HSL or RA, and cultures were carried out as described above.

Biofilm formation

Overnight cultures of *P. aeruginosa* PAO1 were grown at 37°C and used to inoculate borosilicate glass tubes containing 2 ml of LB medium (supplemented with either 2 mM RA or chlorogenic acid) to an initial OD_{660} of 0.05. Both compounds were added as 143 mM solutions in DMSO, and the corresponding control experiments were conducted to assess the effect of the equivalent amount of DMSO on biofilm formation. Cultures were

incubated in a Stuart SB3 tube rotator for 2, 4, 6, 8, and 24 hours at 30°C, with an angle of 45° at 40 rpm. Biofilms formed were visualized by crystal violet (0.4%) staining and quantified by solubilizing the dye with 30% acetic acid and measuring the absorbance at 540 nm (*67*). Data shown are means and SD from three experiments conducted in duplicate.

Quantification of pyocyanin production

Cultures of *P. aeruginosa* PAO1 were grown in LB at 37°C overnight and used to inoculate glass tubes containing 2 ml of LB medium to an initial OD_{660} of 0.05. Stock solutions of 143 mM RA or chlorogenic acid were prepared in DMSO, and aliquots were then added to the tubes to final concentrations of 0.5 to 2 mM. The amount of DMSO corresponding to the experiment at 2 mM was added to the control tube. Growth was continued, and pyocyanin production was determined after 8 hours. The OD_{660} of cultures was determined before centrifugation of cultures at 13,000 rpm for 5 min. The OD_{520} (indicative of pyocyanin production) was measured, and values were normalized with the cell density (OD_{660}). LB media containing either RA or chlorogenic acid were used as blanks.

Elastolysis assay

Elastase activity in *P. aeruginosa* PAO1 cultures was determined using a modified version of the elastin–Congo red (ECR) assay (*68*). Cells were grown with shaking in LB medium at 37°C overnight and used to inoculate glass tubes containing 3 ml of LB medium to an initial OD_{660} of 0.05. Growth was continued for another 6 hours, and cultures were induced with 500 μM RA. The equivalent amount of DMSO was added to control tubes. Growth was continued until 24 hours, and 1 ml of cell suspension was centrifuged at 13,000 rpm for 15 min. The resulting supernatant was added to tubes containing 10 mg of ECR (Sigma) and 1 ml of buffer [0.1 M tris-HCl, 1 mM $CaCl_2$ (pH 7.0)]. Tubes were incubated at 37°C with shaking (150 rpm) for 24 hours. The reaction was stopped by the addition of 1 ml of sodium phosphate buffer (0.7 M; pH 6.0). Residual, solid ECR was removed by centrifugation, and the OD_{492} of the supernatant was measured. Shown are means and SD from three replicates conducted in triplicate.

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