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DEPARTAMENTO DE MICROBIOLOGÍA. FACULTAD DE FARMACIA. INSTITUTO UNIVERSITARIO DE INVESTIGACIÓN DEL AGUA

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Complejidad y diversidad en los sistemas de transducción de señales en *Pseudomonas*

Complexity and diversity in signal transduction systems in *Pseudomonas*

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Complexity and diversity in signal transduction systems in *Pseudomonas*

Memoria presentada para aspirar al Grado de Doctor con mención internacional por la Licenciada en Bioquímica Dña. Cristina García Fontana

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El doctorando **Cristina García Fontana** y los directores de la tesis **Tino Krell y Maximino Manzanera Ruiz** garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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“El fracaso es una gran oportunidad para empezar otra vez con más inteligencia”

Henry Ford

“No mires hacia atrás con ira, ni hacia adelante con miedo, sino alrededor con atención”

J. Thurker

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CONTENIDOS

LISTA DE ABREVIATURAS

AK: AutoKinase

ATP: Adenosine tri-phosphate

CFU: Colony forming units

C-terminal: Carboxi terminal

Da: Dalton

DNA: Deoxyribonucleic acid

DSC: Differential Scanning Calorimetry

EAL: Glu-Ala-Leu Motive

FPLC: Fast Protein Liquid Chromatography

GGDEF: Gly-Gly-Asp-Glu-Phe Motive

HAMP: Domain present in Histidine kinases, Adenylate cyclases, Methyl accepting proteins and Phosphatases

HK: Histidine kinase

HTH: Helix Turn Helix

IPTG: Isopropil-β-D-1-t thiogalactopyranoside

ITC: Isothermal Titration Calorimetry

K_A: Association Constant

K_D: Dissociation Constant

LB: Luria Bertani

LBD: Ligand binding domain

LBR: Ligand binding region

MA: Methyl accepting

MCP: Methyl accepting chemotaxis protein

N-terminal: Amino terminal

O.D.: Optical density

O/N: Over night

OCS: One component system

PAS: Per-Arnt-Sim

Pi: Inorganic phosphate

PIPES: 1,4-Piperazinediethanesulfonic acid

r.p.m.: Revolutions per minute

RNA: Ribonucleic acid

RR: Response Regulator

RRR: Response Regulator Receiver Domain

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SDS: Sodium dodecyl sulfate

SK: Sensor Kinase

TAE: Tris Acetate EDTA

TCS: Two component system

TM: Transmembrane

TPR: Tetratricopeptide repetition

TSA: Triptone Soya Agar

TSB: Triptona Soya Broth

UV: Ultra violet

WT: Wild type

Δ: Delection

ΔH: Enthalpy change

ΔS: Entropy change

RESUMEN/ABSTRACT

El conjunto de mecanismos encargados de captar señales ambientales para desencadenar las respuestas fisiológicas adecuadas, se denomina sistemas de transducción de señales clasificándose estos sistemas mayoritariamente en tres grupos: sistemas de un componente (OCS, del inglés *one component system*), sistemas de dos componentes (TCS, del inglés, *two component system*) y sistemas de señalización química. En esta tesis se han estudiado algunos sistemas de transducción de señales pertenecientes a los grupos TCS y sistemas de señalización química utilizando para ello, tres cepas distintas del género *Pseudomonas*.

Con respecto a los TCS, se estudió el sistema TmoS/TmoT de *P. mendocina* KR1 para determinar el mecanismo de su funcionamiento. En este contexto, se realizó un estudio a nivel de secuencia, comparándolo con el TCS TodS/TodT de *P. putida* DOT-T1E. Este estudio pone de manifiesto que los aminoácidos implicados en la fosforilación y transfosforilación en cada uno de los dos componentes, permanecen muy conservados en ambos sistemas. Igualmente, el estudio de las secuencias promotoras así como de las secuencias implicadas en la unión al ADN del regulador de respuesta, presentan un elevado grado de similitud en ambos sistemas, lo que sugiere que el mecanismo de transducción de señal, es un mecanismo común en este tipo de TCS.

Por otro lado, se llevó a cabo un estudio del perfil de ligandos de la Histidin Kinasa (HK) TmoS, para determinar qué ligandos son capaces de unirse a la HK, cuáles son capaces de inducir su autofosforilación, y cuáles producen la inducción del promotor P_{tmoX}. Para ello, se realizaron estudios de Calorimetría Isoterma de Titulación (ITC, del inglés, *Isotermal Titration Calorimetry*) con un amplio rango de compuestos aromáticos, ensayos de actividad β-Galactosidasa así como ensayos de fosforilación usando ATP marcado con ^{32}P. Los resultados obtenidos revelaron que TmoS es capaz de reconocer y unirse a un elevado rango de hidrocarburos aromáticos con elevada afinidad, sin embargo sólo algunos de estos compuestos son capaces de inducir la fosforilación de la HK y activar la transcripción de los genes implicados en la ruta de degradación, lo que sugiere que el reconocimiento de agonistas y antagonistas, es una característica común en esta familia de proteínas.

Con respecto a los sistemas de señalización química, se emplearon dos cepas bacterianas distintas pertenecientes al género *Pseudomonas*: *P. putida* KT2440 y *P. aeruginosa* PAO1. En *P. putida* KT2440 existen tres parálogos de la metiltransferasa CheR. Para determinar que todos los parálogos son funcionales, y que todos ellos están sujetos a inhibición por S-adenosilhomocisteína (SAH), se realizaron ensayos de ITC. Por otro lado se ha determinado la función de dos de los tres parálogos de CheR descritos en esta cepa. Para ello, se construyeron mutantes en los genes que codifican para cada uno de los parálogos de CheR, y posteriormente, se procedió a la realización de distintos tipos de estudios como ensayos de quimiotaxis, metilación, formación de biopelículas, o ensayos de motilidad mediante *pilus* tipo IV (*twitching motility*). Los resultados revelaron la implicación del parálogo CheR1 en una vía homóloga a la vía WSP, por lo que se concluyó que CheR1 es el único parálogo implicado en la formación de biopelículas. Con respecto al parálogo CheR2, se determinó que está implicado en procesos quimiotácticos. Sin embargo, ensayos realizados con CheR3 y su correspondiente mutante, no mostraron resultados concluyentes, de manera que este parálogo no pudo relacionarse con ninguna vía de señalización.

Para determinar la especificidad de unión entre los distintos parálogos de CheR en *P. aeruginosa* PAO1 con los quimiorreceptores, se procedió al estudio de las secuencias de los 4 parálogos presentes en esta cepa, así como de los quimiorreceptores. Estos análisis revelaron la existencia de un pentapéptido C-terminal en 3 de los 26 quimiorreceptores de esta cepa, y la existencia de un tripéptido en el subdominio β del parálogo CheR2. Mediante estudios de mutagénesis dirigida, y mediante la realización de ensayos de interacción por ITC, y metilación, se determinó que únicamente el parálogo CheR2 es capaz de unir el pentapéptido del quimiorreceptor McpB. Por otro lado, se concluyó que tanto el pentapéptido del quimiorreceptor McpB, como el tripéptido GPN de CheR2, son esenciales para la unión de ambas proteínas y para la metilación eficaz del quimiorreceptor, por parte de la metiltransferasa. La inserción del tripéptido GPN en el subdominio β de la metiltransferasa CheR2, determina la clasificación de las metiltransferasas en dos familias distintas, diferenciadas por su capacidad de unión a pentapéptidos C-terminales, pudiendo utilizar esta característica,

como herramienta predictiva para identificar metiltransferasas del tipo CheR con capacidad de unir pentapéptidos.

Por último, en esta tesis doctoral se desarrollaron distintos ensayos de bioseguridad con organismos tales como *Caenorhabditis elegans*, *Eisenia foetida*, *Adalia bipunctata*, *Chrysoperla carnea* o *Daphia magna*, entre otros, para determinar la posible relación del quimiorreceptor McpB del patógeno *P. aeruginosa* PAO1 con virulencia. Para ello, se construyó un mutante en el gen *mcpB* y se llevaron a cabo los citados bioensayos con la cepa silvestre y con el correspondiente mutante en el quimiorreceptor. Los resultados obtenidos demostraron que, en aquellos ensayos que suponen un contacto directo del organismo con el patógeno, como pueden ser aquellos en los que se suministró la bacteria como parte de la dieta, existía una relación directa entre McpB con procesos de virulencia. Sin embargo, en aquellos ensayos que suponen un contacto con las sustancias expulsadas al medio por ambas cepas, no se observaron diferencias significativas entre la cepa silvestre y el mutante. Estos resultados sugieren que McpB está implicado en alguna vía relacionada con la virulencia de la cepa, aunque los mecanismos moleculares de esta vía, aún no se conocen.

The set of mechanisms to capture an environmental signal to develop the appropriate physiological response is called signal transduction systems. These systems are mainly classified into three groups: one-component systems (OCS), two-component system (TCS), and chemical signaling systems. In this thesis, some signal transduction systems belonging to TCS and chemical signaling systems has been studied, using three different strains of *Pseudomonas*.

With respect to TCS, the TmoS/TmoT system in *Pseudomonas mendocina* KR1 was studied to determine the mechanism of its operation. In this context, a study was conducted at sequence level comparing this system with the TCS TodS/TodT present in *Pseudomonas putida* DOT-T1E. The study shows that the amino acids involved in the phosphorylation and transphosphorylation in each of the two components remain highly conserved in both systems. Similarly, the study of promoter sequences and sequences involved in DNA binding domain of the response regulator have a high degree of homology in both systems, suggesting that the signal transduction mechanism is a common mechanism in this type of TCS.

On the other hand, a study of ligands profile of Histidin Kinase (HK) TmoS was conducted to determine which ligands are able of binding to HK which are able to induce its autophosphorylation and which of them induce the P_{tmoX} promoter expression. Therefor, studies of Isothermal Titration Calorimetry (ITC) with a wide range of aromatic compounds, assays for β-galactosidase activity, and phosphorylation assays using ^{32}P-labeled ATP were performed. The results revealed that TmoS is able to recognize and bind to a high range of aromatic hydrocarbons with high affinity, but only some of these compounds are able to induce phosphorylation of HK and activate transcription of genes involved in the degradation pathway, suggesting these results, the recognition of agonists and antagonists is a common feature of this protein family.

With respect to chemical signaling systems, two different bacterial strains belonging to the genus *Pseudomonas* were used: *P. putida* KT2440 and *P. aeruginosa* PAO1. *P. putida* KT2440 presents three paralogs of CheR methyltransferase. To determine that all paralogs are functional and all of them are subjected to inhibition by S-adenosyl

homocysteine (SAH), ITC assays were performed. Furthermore, the function of two out of three paralogs of CheR described in the strain was determined. Therefor, mutants were constructed in the genes encoding each CheR paralog and then proceeded to carry out different types of tests as chemotaxis assays, methylation, biofilm formation, motility assays or twitching motility assays by type IV pilus. Results revealed the involvement of CheR1 paralog in a homologous WSP pathway, leading to the conclusion that CheR1 is the only paralog involved in biofilm formation. The CheR2 paralog was determined to be involved in chemotactic processes. However, studies with CheR3 and its corresponding mutant showed no conclusive results, so this paralog can not be linked to any signaling pathway.

To determine the specificity of binding between the different paralogs in *P. aeruginosa* PAO1 CheR with chemoreceptors, we proceeded to study the sequences of the 4 paralogs present in this strain, as well as the chemoreceptors sequences. These analyses revealed the existence of a C-terminal pentapeptide in 3 of the 26 chemoreceptors of this strain and the existence of a tripeptide in the β subdomain CheR2 paralog. By directed mutagenesis studies, ITC, and methylation assays it was determined that only CheR2 paralog is able to bind to the pentapeptide present in McpB chemoreceptor. Furthermore, it was concluded that the pentapeptide of McpB chemoreceptor and the tripeptide GPN of CheR2 both are essential for the binding of both proteins and for effective chemoreceptor methylation by methyltransferase. GPN tripeptide insertion in the β subdomain of CheR2 methyltransferase determines the classification of methyltransferases in two distinct families, distinguished by their ability to bind to C-terminal pentapeptides. This feature can be used as a predictive tool to identify methyltransferases CheR that are able to bind pentapeptides.

Finally, in this thesis several biosafety tests with different organism models such as *Caenorhabditis elegans*, *Eisenia foetida*, *Adalia bipunctata*, *Chrysoperla carnea,* and *Daphia magna* were developed to determine the possible relationship between McpB chemoreceptor in *P. aeruginosa* PAO1 and virulence. Therefor, a mutant lacking the *mcpB* gene was constructed and bioassays were conducted using the wild-type strain

and the corresponding mutant in the McpB chemoreceptor. The results showed that in tests involving a direct contact of the organism with the pathogen, there is a direct link between McpB chemoreceptor and virulence. However, in tests involving a contact with secondary metabolites exported to extracellular media by both strains, no significant differences between wild-type and mutant were observed. These results suggest that McpB is involved in some pathway related to the virulence of the strain, although the molecular mechanism of this route has to be studied in more detail.

INTRODUCCIÓN

1. Sistemas de transducción de señales en bacterias

El medio ambiente está sometido a constantes cambios en numerosos factores, como temperatura, pH, disponibilidad de nutrientes, presencia de patógenos y contaminantes entre otros. Estos cambios, provocan por una parte, la extinción de aquellas especies que no son capaces de tolerar dichos cambios, y por otra, la evolución y perpetuación de aquellas especies que son capaces de adaptarse al entorno cambiante que las rodea (Sokolova *et al*., 2012).

En este contexto, el desarrollo de sistemas que permitan una mejor adaptación a cambios en el entorno, son fundamentales para asegurar la supervivencia y la perpetuación de la especie. Cuanto mayor sea la complejidad en estos mecanismos de adaptación, mayor será el abanico de respuestas que podrán desarrollar los organismos para sobrevivir. A lo largo de la evolución, los microorganismos han ido desarrollando y perfeccionando estrategias a nivel transcriptómico, proteómico y metabolómico para conseguir una mejor adaptación a estos cambios ambientales (Brunke and Hube, 2014).

La mayoría de datos disponibles sobre estos mecanismos reguladores en bacterias, provienen de estudios realizados en enterobacterias como organismos modelo (Webre *et al*., 2003), que se caracterizan por encontrarse en hábitats ricos en nutrientes y en condiciones poco cambiantes. Sin embargo, al estudiar otros sistemas en especies pertenecientes a otros géneros como *Pseudomonas*, presentes en entornos cambiantes, se observa la complejidad y sofisticación de estos sistemas, en comparación con los organismos normalmente caracterizados.

Los mecanismos encargados de captar una señal para desencadenar la respuesta fisiológica adecuada, se denominan sistemas de transducción de señales que afectan a una secuencia de reacciones bioquímicas en las que intervienen enzimas y segundos mensajeros.

En los procesos de transducción de señales, pueden intervenir desde una sola proteína, hasta un número elevado de enzimas y sustancias desde la percepción del estímulo, hasta el final del proceso (Stock *et al*., 2000; Ulrich *et al*., 2005; Wuichet and Zhulin, 2010).

Estos procesos comienzan con la adhesión de un ligando al receptor (normalmente de membrana), el cual se activa de manera que el estímulo se transforma en respuesta, a través de una cadena de reacciones (cascada de señalización) cuyo resultado se traduce en la amplificación de la señal. Este mecanismo provoca que un pequeño estímulo dé lugar a una gran respuesta celular (Falke *et al*., 1997).

En bacterias, los procesos de transducción de señales les permiten responder a las fluctuaciones del medio ambiente que les rodea. La gran variedad de señales fisicoquímicas a las que los microorganismos pueden responder, haría pensar en una amplia diversidad de mecanismos de transducción de señales, sin embargo, la mayoría de estos sistemas se pueden clasificar en tres grupos: sistemas de un componente (OCS , del inglés *one component system*), sistemas de dos componentes (TCS , del inglés *two component system*) y sistemas de señalización química (Stock *et al*., 2000; Ulrich *et al*., 2005; Wuichet and Zhulin, 2010). Estos sistemas se detallan a continuación.

1.1 Sistemas de un componente (OCS)

Este grupo de sistemas de transducción de señales, engloba a los sistemas más sencillos, consistentes en una única proteína que es capaz tanto de percibir un estímulo, como de llevar a cabo una respuesta ante dicho estímulo. En este grupo, se pueden englobar por ejemplo, algunos reguladores transcripcionales de unión a ligando. Estas proteínas normalmente se denominan sistemas de un componente (Ulrich *et al*., 2005), y habitualmente están constituidas por dos dominios diferentes: dominio sensor o *input* encargado de captar la señal, y dominio regulador o *output*, que es el encargado de producir la respuesta adecuada (Figura 1). Dentro de los dominios *input*, existe una elevada diversidad, encontrando por ejemplo, dominios PAS (PER-ARNT-SIM), sensores relacionados con cambios de luz, potencial redox, niveles de oxígeno, pequeños ligandos, detección del nivel energético celular (Taylor and Zhulin, 1999), dominios GAF (Aravind and Ponting, 1997) dominios sensores de nitrato NIT (Shu *et al*., 2003), dominios CACHE (Anantharaman and Aravind, 2000) y dominios CHASE entre otros (Mougel and Zhulin, 2001).

Sin embargo, los dominios *output* están mucho más conservados, ya que el 84 % de estos dominios son dominios de unión al DNA, que presentan un motivo HTH (del inglés *helix turn helix*) (Ulrich *et al*., 2005).

Estos sistemas se encargan mayoritariamente de la detección de señales intracelulares, y están principalmente asociados a la regulación de la expresión génica (Ulrich *et al*., 2005).

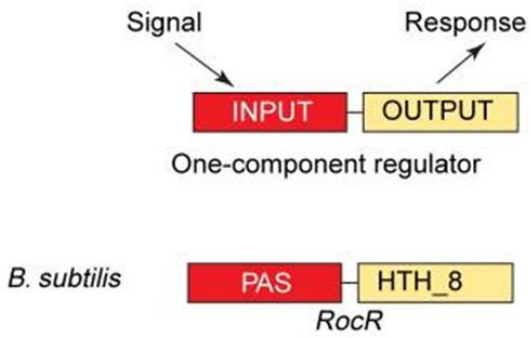

Figura 1. Mecanismo de acción de un sistema de un componente. En rojo, se muestra el dominio *input* para la recepción de señales y en amarillo, el dominio *output*, encargado de la emisión de respuesta. El mismo sistema de colores se utiliza para el caso concreto del sistema *OCS* RocR de *Bacillus subtilis* (Ulrich *et al*., 2005).

1.2 Sistemas de dos componentes (TCS)

Este grupo de sistemas de transducción de señales engloba sistemas más complejos, constituidos por dos proteínas: una proteína sensora que es una Histidin Kinasa (HK), y un regulador de respuesta (RR) que en la mayoría de los casos es un regulador transcripcional, cuya actividad está controlada por la HK (Laub and Goulian, 2007) (Figura 2). Ambas proteínas constituyen un sistema de dos componentes (Stock *et al*., 2000). La unión del efector a la HK, modula su actividad de autofosforilación a partir de ATP. La HK una vez fosforilada en un residuo específico de histidina, transfiere el grupo fosforilo a un determinado residuo de aspártico del RR produciéndose un cambio conformacional del mismo. Este cambio en la conformación, normalmente culmina con la activación del dominio *output* de dicho regulador, desencadenando la respuesta

adecuada, que en la mayoría de los casos, consiste en la modulación de la transcripción de determinados genes (Stock *et al*., 2000, 1989).

Los TCS median distintas respuestas entre las que podemos incluir aquellas involucradas en adquisición de nutrientes, metabolismo energético, virulencia, transferencia de plásmidos, producción de toxinas, adaptación a condiciones físico-químicas del ambiente como cambios de pH, osmolaridad, luz, etc; producción de factores de adherencia, quimiotaxis o esporulación, entre otras (Calva and Oropeza, 2006; Foussard *et al*., 2001; Skerker *et al*., 2005; Stock *et al*., 2000).

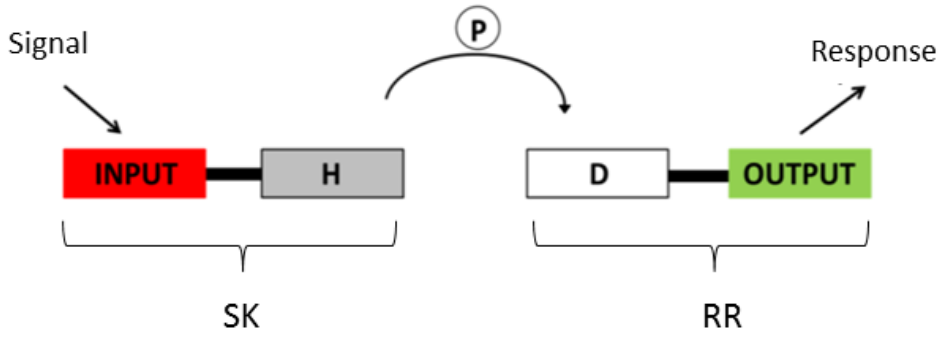

Figura 2. Mecanismo de acción de un sistema de dos componentes prototipo. Se muestra la sensor Kinasa (SK) que recibe la señal, y el regulador de respuesta (RR), que produce la respuesta adecuada. H: residuo de histidina del de la HK, D: residuo de aspártico del RR del grupo fosforilo proveniente de la HK, P: Grupo fosforilo. (Ulrich *et al*., 2005).

Las HK presentan una enorme variabilidad en cuanto a longitud, topología y secuencia, probablemente, para abarcar la gran cantidad de señales ambientales o intracelulares que tienen que detectar (Krell *et al*., 2010). La mayoría de HK, presentan dos regiones transmembrana en el dominio N-terminal, originando una región periplásmica encargada de detectar señales extracelulares, aunque existen otros tipos de HK que aunque no tienen dominios periplásmicos, presentan una región unida a la membrana (Krell *et al*., 2010). Otras, presentan varios pasos transmembrana, quedando el dominio de unión a ligando en el citoplasma. En otros casos, las HK carecen de dominios transmembrana y periplásmicos, como ocurre con CheA y NtrB en

Escherichia coli o existen aquellas HK que presentan varios dominios funcionales (Krell *et al*., 2010) (Figura 3).

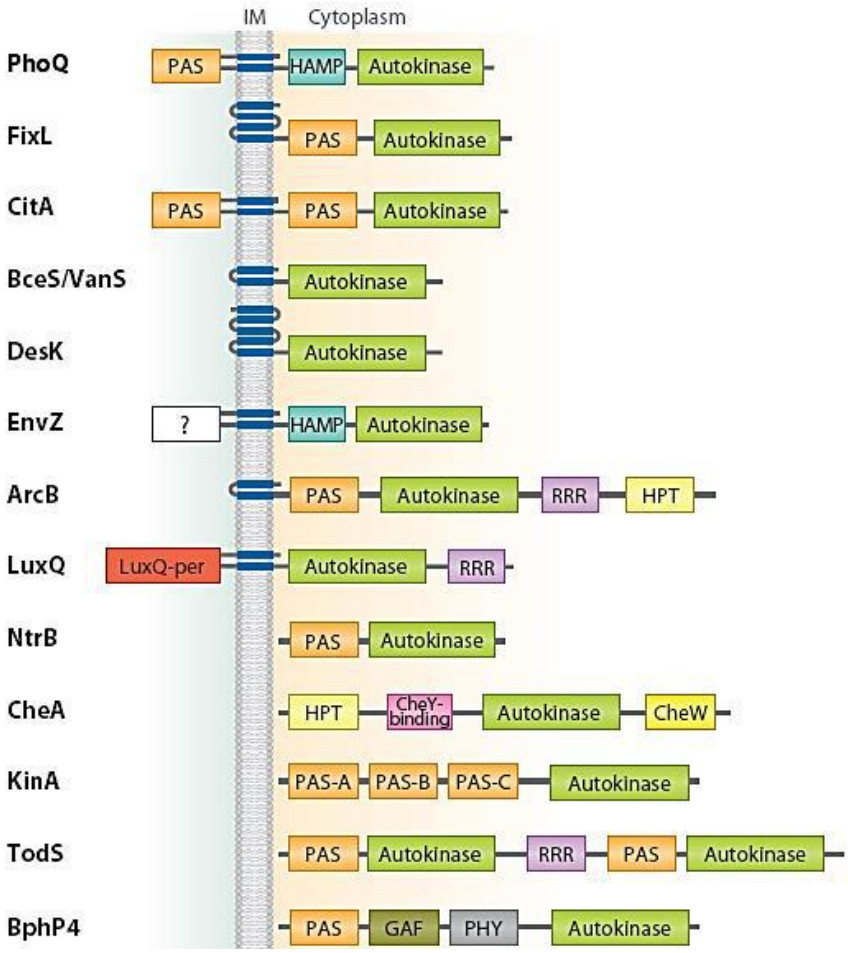

Figura 3. Variabilidad en la topología y organización de HKs. Abreviaciones: PAS: PER-ARNT-SIM; HAMP: dominio encontrado en HKs, adenil ciclasas, quimiorreceptores, y fosfatasas; RRR: *response regulator receiver*; HPT: *histidine containing phosphotransfer*; IM: membrana interna (*inner membrane*); GAF: dominios GAF; PHY: fitocromo (Krell *et al*., 2010).

En los TCS, el RR prototipo se encuentra en el citosol (Krell *et al*., 2010). Este RR está compuesto por un dominio receptor en el extremo N-terminal, que contiene un residuo de aspártico aceptor de grupos fosforilo, y por un dominio regulador en el extremo C-terminal, encargado de llevar a cabo la respuesta. Dado que la mayoría de los RR contienen un dominio regulador de unión a DNA con un motivo HTH, la

regulación ocurre principalmente a nivel transcripcional (Galperin, 2010) tal y como sucede en los OCS. Sin embargo, existen otros tipos de RR, que presentan un dominio REC capaz de recibir el grupo fosforilo y unirse al motor flagelar, como es el caso de CheY en *E. coli* (Jenal and Galperin, 2009; Wadhams and Armitage, 2004). Algunos tipos de RR contienen dominios ANTAR o CsrA que interactúan con RNA, otros presentan actividad enzimática como los dominios GGDEF, EAL, y HD-GYP o son capaces de unirse a pequeños ligandos o proteínas como es el caso de los dominios PAS, GAF, TPR (Galperin, 2006; Galperin *et al*., 2001).

En estos casos, la regulación final se produce a nivel transcripcional, post-transcripcional o post-traduccional. Debido a la gran variedad de moléculas señal reconocidas, los TCS se caracterizan por una gran diversidad en sus mecanismos de percepción y de acción (Krell *et al*., 2010) de manera que se pueden encontrar subfamilias de HK con múltiples dominios sensores y reguladores (Galperin, 2006; Zhulin *et al*., 2003); además pueden existir proteínas fosfo-donadoras y fosfo-aceptoras adicionales, que pueden dar lugar a un sistema de fosforilación mucho más complejo (Appleby *et al*., 1996).

Se ha propuesto que los OCS podrían preceder evolutivamente a los TCS (Ulrich *et al*., 2005). Al tratarse los TCS de sistemas más complejos a nivel estructural, su mantenimiento supone una carga energética y metabólica mayor, por lo que podemos suponer, que representan una ventaja evolutiva funcional para las especies que los presentan. Esta ventaja, puede radicar en el hecho de que la mayoría de ellos presentan una HK con un dominio sensor en el espacio extracitoplasmático (Cock and Whitworth, 2007; Ulrich *et al*., 2005) de manera que pueden recibir señales externas, lo que supone una ventaja importante respecto de los OCS que son proteínas citosólicas (Ulrich *et al*., 2005).

Como ejemplo del TCS prototipo, podemos mencionar el sistema EnvZ/OmpR de *E. coli*, que regula la expresión de los genes *ompF* y *ompC*, que codifican las principales porinas de la membrana externa. Estas porinas funcionan como canales acuosos para el transporte de pequeñas moléculas hidrófilas, a través de la barrera hidrófoba de las membranas (Pratt *et al*., 1996). EnvZ funciona como un sensor para condiciones ambientales específicas y señales tales como pH, osmolaridad, temperatura, o

exposición a ciertas toxinas y nutrientes (Forst and Inouye, 1988; Pratt *et al*., 1996; Wang *et al*., 2012). EnvZ se autofosforila en su residuo de histidina 243, (Forst *et al*., 1989; Igo and Silhavy, 1988), y cede entonces, el grupo fosforilo al residuo de aspártico 55 del RR OmpR, que se activa. Esta activación produce la interacción del dominio de unión al DNA del RR con la región promotora de los genes *ompF* y *ompC* modulando la transcripción de dichos genes (Delgado *et al*., 1993; Forst *et al*., 1989; Rampersaud *et al*., 1994).

Sin embargo, existen otros TCS en los cuales la HK sensora no presenta dominios transmembrana, como ocurre en el sistema TodS/TodT de *Pseudomonas putida* DOT-T1E.

1.2.1. Sistema de dos componentes TodS/TodT

Este TCS, regula la transcripción de genes implicados en la degradación de compuestos aromáticos a través de la ruta tolueno dioxigenasa (ruta TOD) (Figura 4) (Mosqueda *et al*., 1999).

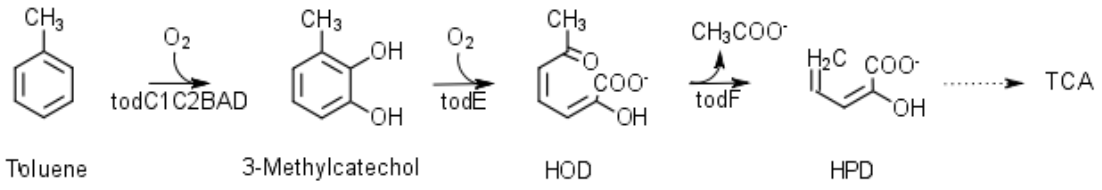

Figura 4. Ruta TOD de degradación de tolueno. Se indican los genes de *P. putida* DOT-T1E que codifican para las enzimas necesarias en cada paso de la ruta. HOD: 2-hidroxi-6-oxohepta-2,4-dienoato;HPD:2-hidroxipenta-2,4-dienoato. http://2015.igem.org/Team:Toronto/Parts.

El sistema TodS/TodT está compuesto por dos proteínas, una HK (TodS) y un RR (TodT). Este último, se une al promotor P_{todX}, activando la transcripción de los genes que codifican para las enzimas implicadas en la degradación de tolueno, benceno y etilbenceno. En presencia de algunos hidrocarburos aromáticos, se ha observado una

inducción en la expresión del promotor, siendo el mayor inductor, el tolueno (Busch *et al*., 2007; Lacal *et al*., 2006).

La HK de este TCS (TodS) es una proteína de 108 KDa. Presenta 7 dominios: el primer dominio, es un dominio PAS, capaz de unir tolueno con una elevada afinidad (Lacal *et al*., 2006). Adicionalmente, presenta dos módulos transmisores AutoKinasa (AK1 y AK2). Estos módulos están compuestos por dos dominios cada uno (un dominio de dimerización/fosfotransferencia y un dominio catalítico que cataliza la autofosforilación del dominio usando ATP como fosfodonador). Los módulos AK1 y AK2 están separados por un dominio RR interno o dominio receptor de regulador de respuesta (RRR) y un dominio PAS 2 (Figura 5).

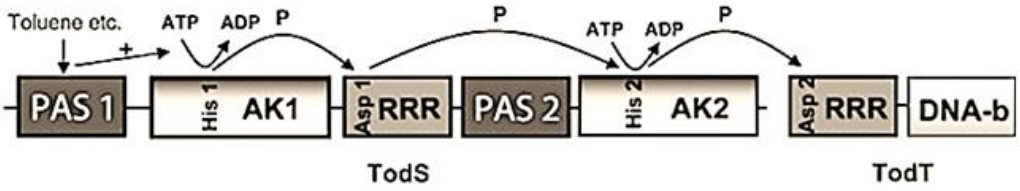

Figura 5. Mecanismo de fosfotransferencia en el sistema de dos componentes TodS/TodT. Se representa la transferencia del grupo fosforilo a través de los distintos dominios de la Sensor Kinasa (TodS), hasta el regulador de respuesta (TodT). AK1: Dominio transmisor autokinasa 1; AK2: Dominio transmisor autokinasa 2; RRR: Dominio receptor del regulador de respuesta; DNA-b: Dominio de unión al DNA. En cada dominio se muestran los aminoácidos implicados en la recepción del grupo fosforilo (Krell *et al*., 2012).

La presencia en el medio de hidrocarburos aromáticos como el tolueno, aumenta la autofosforilación de la histidina en posición 190 (His-190) del dominio AK1, ocurriendo seguidamente, la transfosforilación al residuo de aspártico en posición 500 del RR interno en primer lugar, y al residuo de histidina en posición 760 del módulo AK2 en segundo lugar. Posteriormente ocurre la transfosforilación del segundo componente de este TCS, TodT en el residuo de aspártico en posición 57 (Busch *et al*., 2007). Este tipo de composición en cuanto a dominios en los TCS se clasifica como TRTR según la clasificación propuesta por Williams y Whitworth (2010), que se diferencia de los TCS

de tipo TRPR en que en vez de presentar un único módulo AK, presenta dos módulos de este tipo (Williams and Whitworth, 2010).

El RR de este TCS (la proteína TodT), tiene un tamaño de 23 KDa, y presenta dos dominios: un dominio receptor del regulador de respuesta (RRR) que es el que recibe el grupo fosforilo de TodS, y un dominio de unión al DNA, que es el que interacciona con el promotor P_{todX} en tres sitios específicos, siendo capaz de unirse en las posiciones -57, -85, y -106 con respecto al sitio de inicio de la transcripción (Lacal *et al*., 2008).

Este TCS, presenta la peculiaridad de reconocer e interaccionar con dos tipos de compuestos clasificados como agonistas y antagonistas (Busch *et al*., 2007). Los agonistas se definen como aquellos hidrocarburos aromáticos capaces de unirse a la HK TodS, e inducir su actividad de autofosforilación y posterior transfosforilación del RR TodT. Sin embargo, los antagonistas, aunque son capaces de unirse de igual modo a la HK, no son capaces de modular la actividad basal de TodS (Busch *et al*., 2007).

1.2.2. Sentido biológico de la existencia de TCS citosólicos

Como se ha mencionado anteriormente, la ventaja principal de los TCS con respecto a los OCS, puede ser la detección de señales extracelulares (Ulrich *et al*., 2005), pero en el caso de los TCS citosólicos, como es el caso del sistema TodS/TodT, no se sabe qué sentido biológico podría tener la producción y mantenimiento de dichos sistemas, que no presentan dominios periplásmicos para detección de señales extracelulares. La explicación podría estar relacionada con en el hecho de que estos TCS citosólicos, podrían estar implicados en la percepción de varios tipos de señales, y no sólo un tipo, como ocurre en los OCS (Krell *et al*., 2009). Esta hipótesis, se ve reforzada por el hecho de que la mayoría de los TCS citosólicos, suelen presentar varios dominios funcionales en sus HK (Mascher *et al*., 2006), lo que podría estar relacionado con la percepción de distintos tipos de señales a través de estos dominios adicionales (Krell *et al*., 2009) (Figura 3).

Por otro lado, se ha observado que el RR de los TCS, puede ser fosforilado adicionalmente por compuestos de bajo peso molecular como acetilfosfato (Wolfe, 2005). Esta fosforilación del RR a partir de acetilfosfato podría responder a la

necesidad de generar una respuesta específica ante una señal global, ya se ha visto que los niveles de acetilfosfato varían dependiendo de la fase de crecimiento en la que se encuentre la comunidad (Prüss and Wolfe, 1994). La fosforilación de los RR por moléculas de pequeño tamaño podría dar lugar a regulación de procesos celulares que van desde la biosíntesis de orgánulos, hasta la regulación del ciclo celular o formación de biopelículas y patogénesis (Wolfe, 2005). En este contexto, se ha observado que el 21 % de HK bacterianas contienen un dominio regulador de respuesta interno y por tanto, son susceptibles a la fosforilación por acetilfosfato. La presencia de este dominio receptor susceptible de ser fosforilado por moléculas pequeñas, es más frecuente en HK citosólicas que HK de membrana (Krell *et al*., 2009). Esta fosforilación de los dominios RRR en HK citosólicas, podría considerarse como otro modo para la detección de señales adicionales (Krell *et al*., 2009). Esto refuerza la idea de que estos TCS citosólicos podrían intervenir en la percepción de distintos tipos de señales a distintos niveles.

Como ejemplo, podemos mencionar el TCS TodS/TodT, en el cual TodS, contiene dos dominios sensores de tipo PAS, separados por un dominio RRR y no presenta ningún dominio transmembrana. Se ha observado que el primer dominio sensor tipo PAS es capaz de reconocer un amplio espectro de compuestos aromáticos monocíclicos de manera que se produce un cambio conformacional de la proteína dando lugar a la autofosforilación del dominio HK1 (Busch *et al*., 2007; Lacal *et al*., 2006). El segundo dominio PAS podría estar implicado en la detección de oxígeno (Lau *et al*., 1997).

Este TCS presenta por tanto una serie de características particulares, como son la presencia de varios dominios funcionales, localización citosólica, participación en procesos de degradación de hidrocarburos aromáticos, reconocimiento de agonistas y antagonistas, entre otras.

En esta tesis doctoral, se plantea la pregunta de si las características particulares de este tipo de TCS se dan sólo en la especie *P. putida,* o si se trata de características generales del género *Pseudomonas.* Para responder a estas preguntas, en este trabajo se ha llevado a cabo la caracterización del sistema TmoS/TmoT, homólogo al descrito en *P. putida*, que regula la expresión de genes implicados en la degradación del

tolueno (Ramos-González *et al*., 2003). Como organismo modelo de este estudio, se utilizó la cepa *Pseudomonas mendocina* KR1.

1.2.3. *Pseudomonas mendocina*

La especie bacteriana *P. mendocina*, pertenece al grupo de las Gamma-Proteobacterias, y al género *Pseudomonas*, un género de bacilos rectos o ligeramente curvados, Gram negativos, oxidasa-positivos, aeróbicos estrictos aunque en algunos casos pueden utilizar el nitrato como aceptor de electrones (Koike and Hattori, 1975).
Los miembros de este género generalmente son móviles gracias a uno o más flagelos polares, presentan actividad catalasa y no forman esporas (Kobayashi *et al*., 1998). Algunas especies sintetizan una cápsula de exopolisacáridos que facilita la adhesión celular, la formación de biopelículas (Davies *et al*., 1993) y las protege de la fagocitosis, de los anticuerpos o del complemento, aumentando así su patogenicidad (Jensen *et al*., 1993). El género presenta una gran diversidad metabólica, y consecuentemente son capaces de colonizar un amplio rango de nichos ecológicos. Se han aislado bacterias de este género tanto en suelos contaminados por productos biogénicos y xenobióticos como en suelos libres de contaminantes. Las bacterias de este género, también son microbiota predominante en la rizosfera y en la filosfera de plantas; del mismo modo, se han aislado de ambientes acuáticos, tanto de agua dulce como de aguas marinas (Römling *et al*., 1994). En general, los miembros de este género, son inocuas para el hombre, aunque también existen patógenos oportunistas como *Pseudomonas aeruginosa* (Tümmler *et al*., 1997); patógenos de animales (Mahajan-Miklos *et al*., 1999) y patógenos de plantas como *Pseudomonas syringae* (Salch and Shaw, 1988). Este género es uno de los más relacionados con la degradación de compuestos orgánicos, en muchos casos, debido a la presencia de determinantes plasmídicos y transposones autotransmisibles (Fernández et al., 2012; Fillet et al., 2012; Marqués et al., 1999; Molina et al., 2011). Dentro de las cepas con mayor potencial catabólico, se han descrito especialmente cepas pertenecientes a la especie *P. putida* (Udaondo *et al*., 2012, 2013) y *P. mendocina* (Ramos-González *et al*., 2003), objeto de estudio de esta tesis doctoral.

En *P. mendocina* KR1, la vía metabólica para la degradación del tolueno está codificada a nivel cromosómico (Wright and Olsen, 1994). La tolueno 4-monooxigenasa (T4MO), codificada por los genes *tmo,* es responsable de la primera etapa del catabolismo del tolueno (Whited and Gibson, 1991). Por la actividad de T4MO, el tolueno se hidroxila en p-cresol, que es oxidado por los productos de los genes *pcu* hasta 4-hidroxibenzoato (4-HBA) (Wright and Olsen, 1994). Este compuesto es hidroxilado a 3,4-dihidroxibenzoato por la p-hidroxibenzoato hidroxilasa codificada por el gen *pobA* hasta protocatecuato, que es catabolizado por la vía beta-cetoadipato (Harwood and Parales, 1996) (Figura 6).

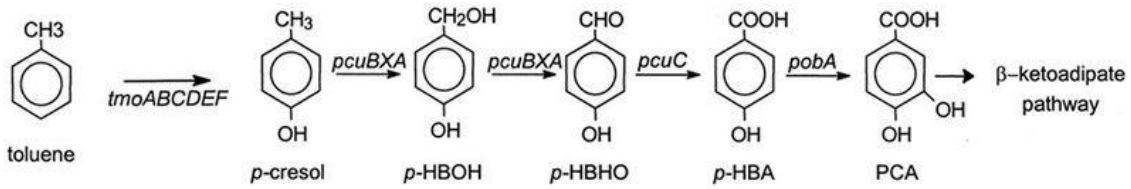

Figura 6. Vía de degradación de tolueno en *P. mendocina* KR1. Se indican los genes de *P. mendocina* KR1 que codifican para las enzimas necesarias en cada paso de la ruta. p-HBOH: p-hidroxibencilalcohol; p-HBHO: p-hidroxibencilaldehido; p-HBA: p-hidroxibenzoato; PCA: protocatecuato. *tmoABCDEF* codifica para la T4MO; *pcuBXA y pcuC* son genes de utilización del p-cresol; *pobA* codifica para la p-hidroxibenzoato hidroxilasa. (Ramos-González *et al*., 2003).

Aguas arriba del operón *tmoABCDEF*, se encuentra el promotor P_{tmoX}, que controla la expresión del operón a través del TCS TmoS/TmoT, que comparte un 85 % de identidad a nivel de secuencia proteica con el sistema TodS/TodT de *P. putida* DOT-T1E (Ramos-González *et al*., 2002).

El sistema TodS/TodT está ampliamente estudiado, sin embargo, otros sistemas como TmoS/TmoT, son poco conocidos, por lo que la caracterización del TCS TmoS/TmoT presente en *P. mendocina* KR1, es uno de los objetivos de estudio de esta tesis doctoral.

1.3. Sistemas de señalización química

Los sistemas de señalización química constituyen un grupo de transducción de señales más complejo que los OCS y los TCS. Se trata de un caso especial de transducción de señal mediante sistema de dos componentes, en el que participan otras proteínas adicionales que regulan el proceso (Wuichet and Zhulin, 2010).

El inicio del proceso comienza con la detección de un estímulo ambiental, que activa a un quimiorreceptor (normalmente de membrana), activándose una cadena de reacciones de fosforilaciones / defosforilaciones, y metilaciones / desmetilaciones. El resultado de esta cascada de señalización da lugar a una respuesta final que puede clasificarse en tres grupos: i) taxis (Szurmant and Ordal, 2004), ii) motilidad mediada por pilus tipo IV (Bhaya *et al*., 2001; Zusman *et al*., 2007), e iii) funciones celulares alternativas donde se incluyen procesos tales como el desarrollo (Berleman and Bauer, 2005; Kirby and Zusman, 2003), la formación de biopelículas (Hickman *et al*., 2005), la morfología celular, interacciones célula-célula (Bible *et al*., 2008), o biosíntesis de flagelos (Berleman and Bauer, 2005).

Aunque el resultado de cada una de estas vías de señalización química sea distinto, en todas ellas intervienen una serie de proteínas principales, llamadas “proteínas núcleo”, y otras secundarias llamadas “proteínas auxiliares”, que en función de las condiciones ambientales, estímulos, concentración de segundos mensajeros, etc, darán lugar a una u otra respuesta (Wuichet and Zhulin, 2010).

La familia más estudiada dentro del grupo de sistemas de señalización química, es el sistema de quimiotaxis. Muchas bacterias se desplazan en gradientes químicos mediante regulación de su motor flagelar (Wadhams and Armitage, 2004), comportamiento conocido como quimiotaxis. Este proceso se caracteriza por una elevada sensibilidad y una precisa adaptación, propiedades atribuidas a una gran variedad de interacciones dentro del sistema de transducción de señales donde intervienen múltiples proteínas (Hazelbauer *et al*., 2008; Wuichet and Zhulin, 2010).

El mecanismo molecular en que se basa la quimiotaxis bacteriana ha sido extensamente estudiado en las bacterias entéricas *Escherichia coli* y *Salmonella enterica serovar Typhimurium* (Armitage, 1999; Bren and Eisenbach, 2000).

A continuación se describe de manera detallada el sistema de quimiotaxis en *E. coli.*

1.3.1. Quimiotaxis en *E. coli*

Cuando *E. coli* se encuentra en un ambiente uniforme se mueve de forma aleatoria alternando los movimientos de sus flagelos en sentido contrario a las agujas del reloj, generando un movimiento hacia adelante conocido como carrera (Wadhams and Armitage, 2004; Weis and Koshland, 1990), y movimientos en sentido de las agujas del reloj, produciéndose un giro conocido como voltereta.

Estos movimientos son aleatorios, sin embargo, cuando la bacteria se expone a un gradiente de determinadas señales químicas, se produce un control selectivo de estos movimientos flagelares, de manera que la bacteria es capaz de dirigir su movimiento acercándose hacia el estímulo en caso de ser positivo o alejándose del mismo, si por el contrario se tratase de un estímulo negativo o perjudicial (Bardy *et al*., 2003). Esta respuesta fisiológica se lleva a cabo por un complejo sistema de transducción de señales, englobado en el grupo de mecanismos de señalización química mencionado anteriormente.

Al inicio de la cascada de señalización se encuentran los quimiorreceptores que son los encargados de detectar los ligandos que producen este movimiento dirigido o quimiotaxis (Chelsky and Dahlquist, 1980). Estos quimiorreceptores normalmente contienen una región N-terminal de unión a ligando LBD (del inglés, *ligand binding domain*), y una región de señalización en el extremo C-terminal que contiene un dominio MA (del inglés, *methyl accepting*), donde se encuentran los sitios de metilación (Chelsky and Dahlquist, 1980) (Figura 7).

Las secuencias de los dominios N-terminal de los quimiorreceptores varían significativamente entre ellos (Lacal *et al*., 2010a). Esta alta diversidad no es sorprendente ya que existen numerosos estímulos que deben ser reconocidos por estos dominios, que son distintos entre sí dependiendo de la señal que reconozcan. Los dominios C-terminales de los quimiorreceptores son citoplásmicos y sus secuencias están altamente conservadas (Briegel *et al*., 2009), ya que estos dominios, siempre interactúan con las mismas proteínas dentro de la cascada de señalización.

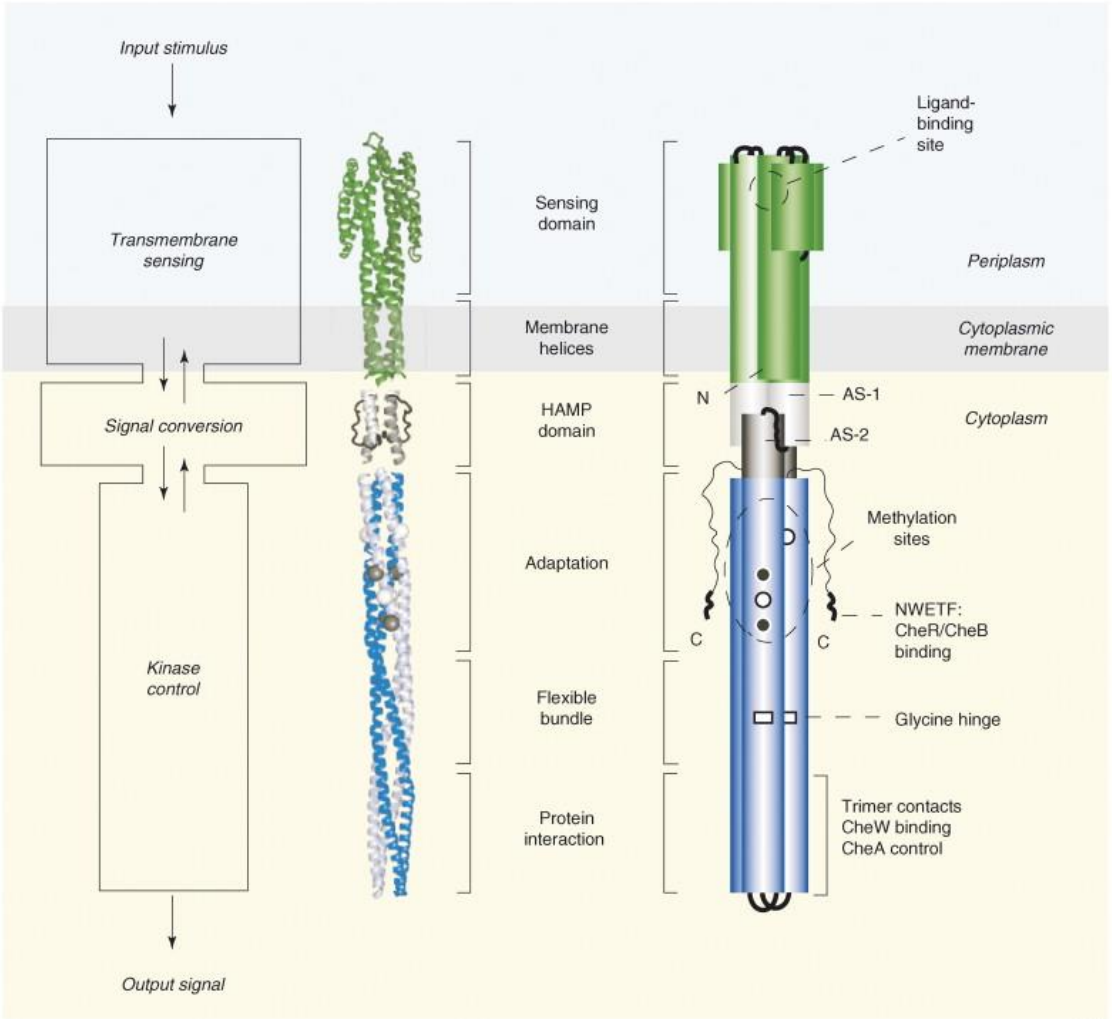

Figura 7. Estructura representativa de un quimiorreceptor. En verde se muestran las regiones perisplámica y transmembrana, y en azul se muestra la región citoplasmática (Hazelbauer *et al*., 2008).

E. coli presenta 4 quimiorreceptores (Tar, Tsr, Trg, Tap) anclados a la membrana, que presentan dominios periplásmicos LBD y un aerorreceptor, (Hazelbauer *et al*., 2008; Vladimirov and Sourjik, 2009) que presenta un LBD citosólico capaz de interaccionar con FAD como cofactor (Levit *et al*., 1998).

El receptor Tar es capaz de detectar aspartato y maltosa, Tsr interacciona con serina, Trg es el receptor para ribosa y galactosa y Tap reconoce dipéptidos (Falke and Hazelbauer, 2001; Hazelbauer *et al*., 2008; Liu and Parales, 2008). Los LBD de los distintos quimiorreceptores de *E. coli* son muy similares en tamaño (154–160

aminoácidos) y consisten en un haz de 4 hélices-α antiparalelas (Lacal *et al*., 2010a; Wadhams and Armitage, 2004).

Los quimiorreceptores se disponen formando dímeros los cuales pueden interactuar entre sí para formar trímeros de dímeros (Boldog *et al*., 2006; Kim *et al*., 1999; Studdert and Parkinson, 2004). El dominio C-terminal de los quimiorreceptores, interactúa con la HK CheA y con la proteína adaptadora CheW, formando así un complejo de señalización (Wadhams and Armitage, 2004) altamente estable (Erbse and Falke, 2009). El papel de CheW, no está muy bien caracterizado, pero se ha demostrado que juega un papel fundamental en la quimiotaxis, ya que un mutante nulo en el gen que codifica para CheW, no presenta actividad quimiotáctica (Szurmant and Ordal, 2004).

Cuando el dominio LBD de los quimiorreceptores reconoce el estímulo, se crea una cascada molecular que se transmite al dominio citoplásmático de señalización, lo que produce una disminución de la autofosforilación de CheA. CheA es capaz de transferir el grupo fosforilo hasta los RR CheY y CheB (Borkovich *et al*., 1989; Garrity and Ordal, 1997) por lo que en presencia del estímulo, los estados fosforilados de ambos reguladores de respuesta disminuyen. CheY fosforilado interactúa con el motor flagelar (Bren and Eisenbach, 1998), basándose el movimiento quimiotáctico, en una modulación diferencial de la actividad motora provocada por los estados CheY y CheY-P (Szurmant y Ordal, 2004). La fosfatasa CheZ actúa aumentando la tasa de autodefosforilación espontanea de CheY-P. La proteína CheB cuando se encuentra fosforilada compite por la unión al quimiorreceptor con CheR controlando el grado de metilación de los quimiorreceptores y permitiendo esta modulación, la adaptación del sistema (Wadhams and Armitage, 2004) (Figura 8).

CheA, a diferencia de los HK de los TCS prototipo, no tiene dominio sensor, y CheY, carece de un dominio regulador (Kofoid and Parkinson, 1988; Wuichet *et al*., 2007).

En este complejo sistema de transducción de señal, los quimiorreceptores junto con CheA y CheY constituyen una vía de excitación en la quimiotaxis, mientras que la fosfatasa CheZ, la metiltransferasa CheR y la metilesterasa CheB (que modifican covalentemente los quimiorreceptores mediante metilación y desmetilación respectivamente), constituyen una vía de adaptación.

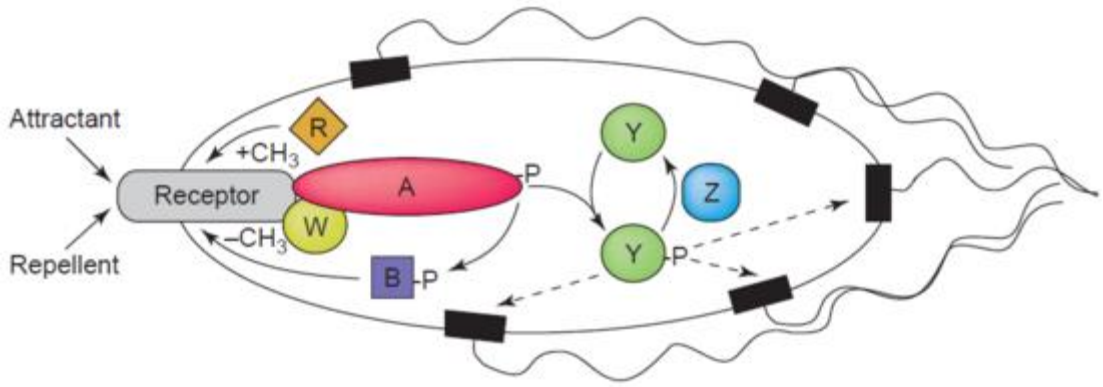

Figura 8. Vía de señalización química de *E. coli*. W: CheW, proteína adaptadora; A: CheA, autokinasa; Y: CheY, regulador de respuesta; Z: CheZ, fosfatasa; R: CheR, metiltransferasa, proteína adaptadora; B: CheB, metilesterasa. (Sourjik, 2004).

Por lo tanto, el sistema de quimiotaxis modelo, incluye siete tipos diferentes de proteínas, por lo que es el sistema de transducción de señales en procariotas más complejo descrito hasta ahora. Se han descrito sistemas similares en docenas de especies distintas a *E. coli* (Szurmant and Ordal, 2004; Wadhams and Armitage, 2004).

1.3.2. Importancia del mecanismo de adaptación en quimiotaxis

Los mecanismos adaptativos son necesarios para que se lleve a cabo la taxis y éstos corresponden a la restauración del comportamiento original cuando no hay estímulo, y a un cambio organizado y direccional en presencia del estímulo (Krell *et al*., 2011; Porter *et al*., 2008).

La adaptación se produce fundamentalmente por metilación y desmetilación del quimiorreceptor. La metilación se lleva a cabo de forma constitutiva por la proteína CheR. Esta metilación en el quimiorreceptor reduce la sensibilidad de los mecanismos de excitación frente a las señales causando la adaptación del sistema a la concentración de la señal actual (Clausznitzer *et al*., 2010; Engström and Hazelbauer, 1980; Wadhams and Armitage, 2004). Como se ha mencionado anteriormente, CheA también fosforila a CheB incrementando su actividad metilesterasa sobre los quimiorreceptores. Cuando se une la señal al quimiorreceptor, disminuye la concentración de CheB-P predominando por tanto la actividad constitutiva de CheR.

De este modo, el quimiorreceptor se encuentra metilado y por tanto, es menos sensible a las señales externas. Cuando disminuye la concentración de señal en el medio, aumenta la concentración de CheB-P que actúa sobre los quimiorreceptores desmetilándolos y permitiendo que regresen al estado previo al estímulo. Este mecanismo permite que un incremento de la concentración de señal pueda causar la excitación de la vía. Ésto confiere al sistema la capacidad de reconocer gradientes del compuesto (Vladimirov *et al*., 2008).

1.3.3. Anotación de genomas y sistemas de señalización química

Este es el esquema general de quimiotaxis, ampliamente estudiado en *E. coli* (Armitage, 1999; Bren and Eisenbach, 2000), sin embargo, gracias a la secuenciación y anotación de genomas de otras especies bacterianas se ha observado una significativa diversidad en los mecanismos de quimiotaxis que se detallan en la revisión de Krell y colaboradores (Krell *et al*., 2011). Las diferencias en estos sistemas de señalización química con respecto a enterobacterias incluyen abundancia genómica, tamaño y topología de quimiorreceptores, el modo de señal vinculante, la presencia de proteínas citoplasmáticas de transducción de señal adicionales o el mecanismo del motor flagelar. Esta diversidad de mecanismos quimiotácticos se debe en parte, a la diversa naturaleza de las señales producidas por los estímulos.

Gracias al estudio de los genomas de distintas especies bacterianas, hemos podido observar ciertas características en microorganismos que se encuentran en distintos ecosistemas que nos indican la complejidad y sofisticación que estos sistemas de transducción de señal pueden llegar a tener. A continuación se detallan algunas de estas características relacionadas con una mayor complejidad:

A) Elevada diversidad de quimiorreceptores

Existe una gran variedad en el número de quimiorreceptores por bacteria variando también el tamaño de los mismos. Se ha observado que la presencia de un mayor o menor número de quimiorreceptores, no está tan relacionada con el tamaño del genoma de la bacteria, como con el estilo de vida y la diversidad metabólica de la

bacteria. Debido a ésto, se observa un mayor número de quimiorreceptores en bacterias que están sometidas a algún tipo de estrés causado por los cambios en su hábitat, en comparación con bacterias que se encuentran en ambientes estables, que presentan un número más reducido de quimiorreceptores (Alexandre *et al*., 2004). En general, en bacterias que codifican genes del flagelo, se encuentra una media de 14 quimiorreceptores por genoma, aunque los límites son bastante amplios, oscilando entre los 64 quimiorreceptores que presenta *Magnetospirillum magnetotacticum* (Alexandre *et al*., 2004) hasta un único quimiorreceptor en distintas cepas, como ocurre en el caso de *Bacillus anthracis* (Lacal *et al*., 2010a).

Por otro lado, los quimiorreceptores también presentan una elevada diversidad en las secuencias de los dominios N-terminal que codifican para los LBD. Estos dominios se pueden clasificar según su tamaño (Lacal *et al*., 2010a) en dos grupos:

- Clúster I: comprende aquellos quimiorreceptores con un LBD entre 120 y 210 aminoácidos. Este grupo comprende el 60 % de los quimiorreceptores.
- Clúster II: comprende aquellos quimiorreceptores con LBD entre 220 y 299 aminoácidos. El 40 % de los quimiorreceptores se engloban en este grupo.

En relación a su topología, los quimiorreceptores se pueden clasificar en seis topologías diferentes (Lacal *et al*., 2010a) (Figura 9).

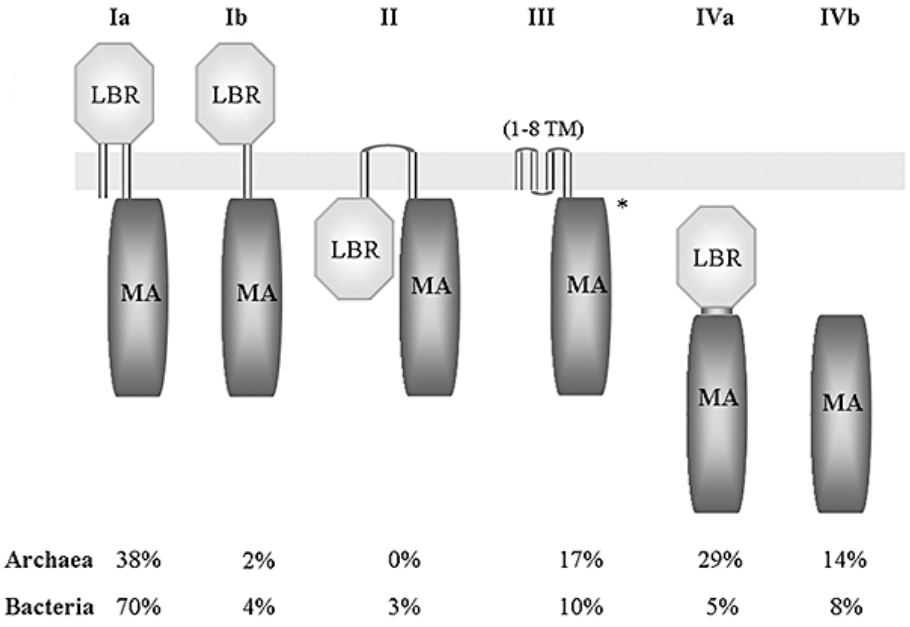

Figura 9. Clasificación de los quimiorreceptores en 6 diferentes topologías. En la parte inferior de la figura se muestra la abundancia relativa de los receptores con una topología dada en arqueas y bacterias. MA: Dominio aceptor de grupo metilo (*methyl-accepting domain*); LBR: Región de unión a ligando (*ligand binding region*); TM: Transmembrana. El asterisco indica que se trata de casos poco frecuentes. (Lacal *et al*., 2010a).

El LBD puede ser tanto periplásmico (cuando el quimiorreceptor presenta dos o más pasos transmembrana), como citosólico.

La topología más abundante en bacterias y arqueas es la topología de clase I, que se caracteriza por la presencia de un LBD extracitoplasmático y un dominio de señalización citosólico.

B) Elevado número de parálogos de una misma proteína dentro de los sistemas de señalización química

Uno de los ejemplos mejor estudiados a este nivel, lo encontramos en el patógeno *P. aeruginosa*. Esta bacteria es quimiotáctica para la mayoría de los compuestos orgánicos en los que puede crecer (Hamilton and Sheeley, 1971; Kearns *et al*., 2001). Presenta 26 quimiorreceptores y múltiples copias de genes que codifican las proteínas

de señalización que se agrupan en cinco Clusters distintos (Ferrández *et al*., 2002; Hong *et al*., 2004; Stover *et al*., 2000) (Figura 10).

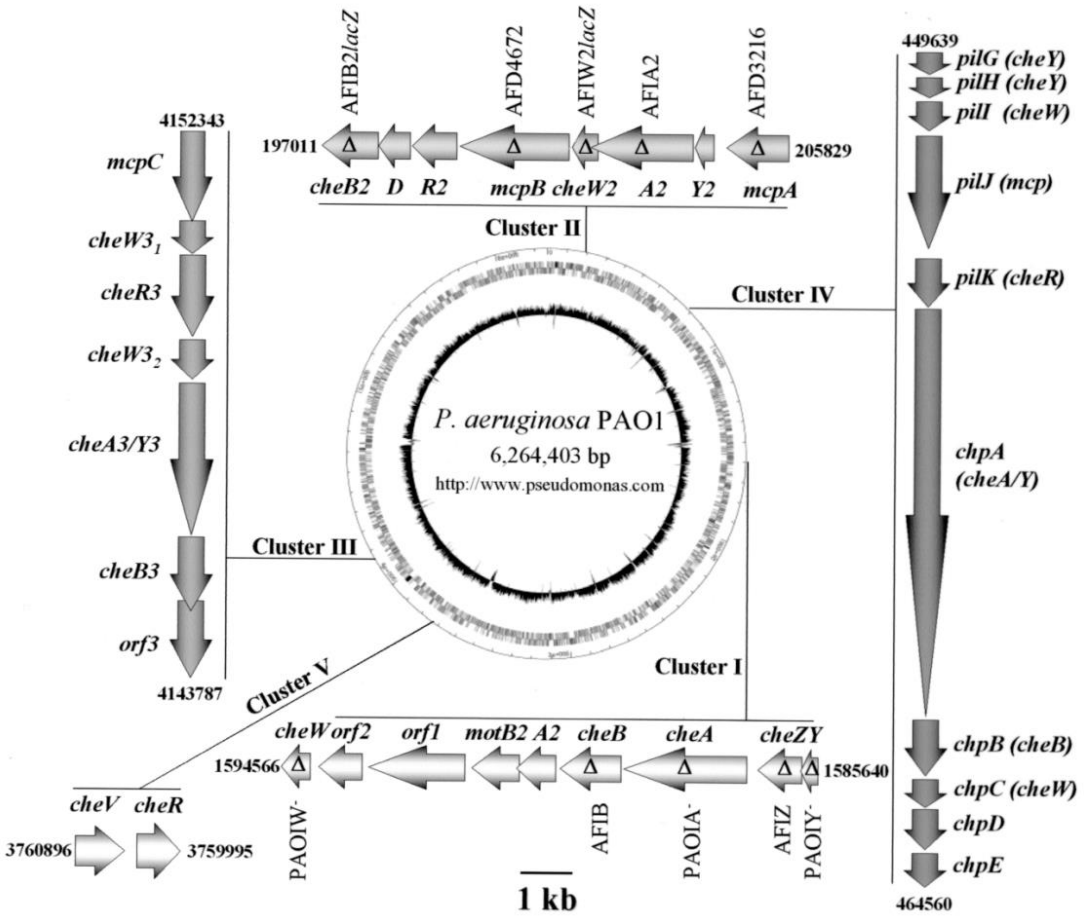

Figura 10. Genoma de *Pseudomonas aeruginosa* PAO1, y organización génica de los genes implicados en rutas de señalización química, en 5 clusters diferentes (Ferrández *et al*., 2002). Los símbolos (Δ), en el interior de las flechas, muestran los mutantes construidos en el estudio de Ferrández y colaboradores (2002).

Los Cluster I, II y V, se ha demostrado que son esenciales para quimiotaxis (Ferrández *et al*., 2002; Kato *et al*., 1999; Masduki *et al*., 1995) (Figura 11).

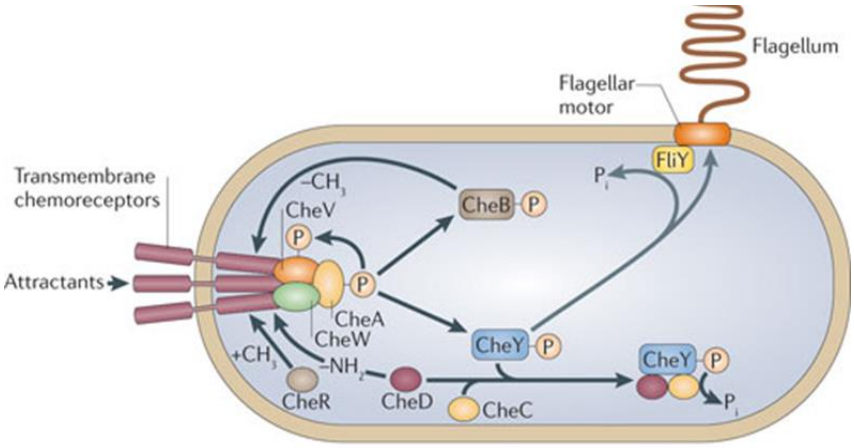

Figura 11. Ruta de quimiotaxis en *Bacillus subtilis* equivalente a la cascada de señalización que se produce en *P. aeruginosa*. Se muestran las proteínas implicadas en la cascada de señalización que culmina con el movimiento flagelar (Porter *et al*., 2011).

El Cluster III, se ha demostrado que está implicado en la formación de biopelículas, a través de la vía WSP. Determinadas señales causan cambios en la actividad de dicha vía, resultando en cambios de los niveles de diguanilato cíclico (diGMPc) (Hickman *et al*., 2005) (Figura 12).

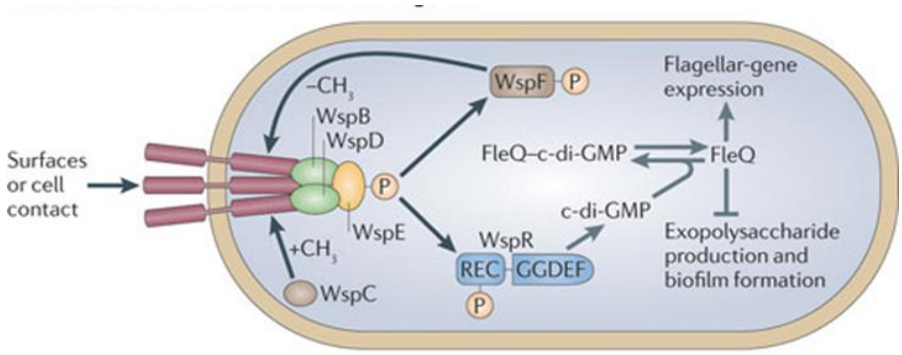

Figura 12. Funcionamiento de la vía WSP que participa en procesos de formación de biopelículas en *P. aeruginosa.* Se muestran las proteínas implicadas en la cascada de señalización que culmina con la formación de biopelículas (Porter *et al*., 2011).

El Cluster VI está relacionado con la motilidad mediante pilus tipo IV (*twitching motility*) (Darzins, 1994; Kearns *et al*., 2001) así como con la expresión de sistemas de

virulencia múltiple que están regulados por la concentración del segundo mensajero AMP cíclico (AMPc) (Fulcher *et al*., 2010) (Figura 13).

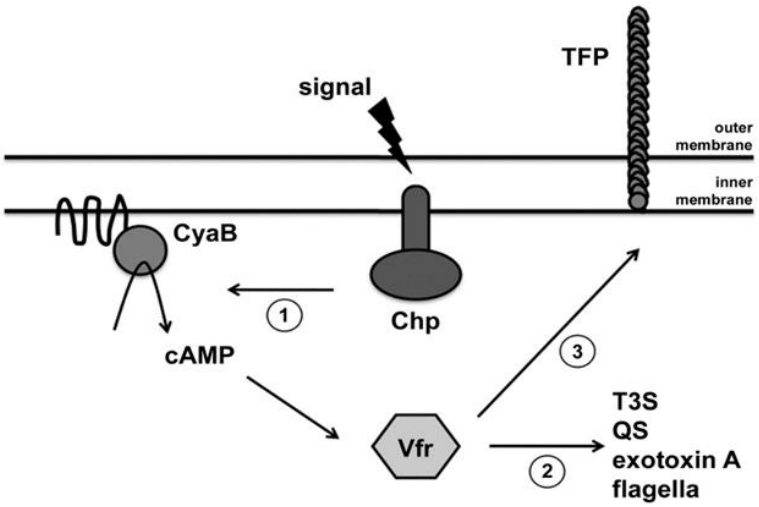

Figura 13. Modelo de acoplamiento de detección de señales ambientales del sistema de señalización química Chp con la expresión de factores de virulencia en *P. aeruginosa.* (Fulcher *et al*., 2010). (1) En respuesta a una señal desconocida, el sistema de cogeneración de AMPc por la actividad de la enzima CyaB se activa. El factor de transcripción Vfr, dependiente de AMPc activa múltiples sistemas de virulencia, incluyendo (2) T3S, QS, biosíntesis flagelar, múltiples toxinas y enzimas de degradación y (3) factores estructurales y reguladores responsables de la expresión y biosíntesis de pilus tipo IV.

Como se puede observar, en este patógeno, existen distintas respuestas fisiológicas relacionadas con la actividad de los distintos parálogos de las proteínas núcleo implicadas en estas vías de señalización química.

En este trabajo, se llevó a cabo el estudio de los distintos parálogos de algunas de estas proteínas usando como modelo de estudio la cepa *P. putida* KT2440, para tratar de caracterizar su función y su relación con las distintas vías de señalización.

Pseudomonas putida

P. putida es una de las especies de mayor interés industrial entre las bacterias del género *Pseudomonas*, ya que unido a su potencial de degradación de compuestos aromáticos y xenobióticos (Krell *et al*., 2012), presenta la capacidad de colonizar el sistema radicular de plantas (Planchamp *et al*., 2015), formar biopelículas (Jiménez-Fernández *et al*., 2015) y presenta un elevado potencial desde el punto de vista genético. Desde hace varias décadas, especies de este género se utilizan en procesos de biocontrol, aplicándolas a semillas de cereales y a suelos para prevenir el crecimiento o colonización de patógenos, en lo que ha venido a llamarse biocontrol (Berg *et al*., 2001; Keane *et al*., 2008; Renault *et al*., 2007). Una de las cepas mejor estudiadas en este sentido es *P. putida* KT2440. El genoma completo de esta cepa se encuentra disponible en bases de datos, lo que constituye una herramienta muy valiosa para el análisis funcional de la información genética del microorganismo (Nelson *et al*., 2002).

Muchos estudios realizados sobre tolerancia a hidrocarburos en *Pseudomonas* se han llevado a cabo en la cepa *P. putida* DOT-T1E (Ramos *et al*., 1995). Se ha observado que *P. putida* es quimiotáctica hacia varios compuestos derivados del ciclo de Krebs (Lacal *et al*., 2010b), así como a numerosos hidrocarburos aromáticos (Krell *et al*., 2012; Luu *et al*., 2015; Wang *et al*., 2015).

C) Existencia de pentapéptidos presentes en los extremos C-terminales de algunos quimiorreceptores

En *E. coli*, existen 5 quimiorreceptores clasificados en dos grupos según su abundancia: quimioreceptores de alta abundancia (Tsr y Tar), y de baja abundancia (Tgr, Tap y Aer). Los quimiorreceptores de alta abundancia están presentes en concentraciones celulares aproximadamente 10 veces superiores a las de los receptores de baja abundancia (Hazelbauer and Engström, 1981; Hazelbauer *et al*., 1981).

Estas dos clases exhiben diferencias muy significativas en su actividad. Los receptores de alta abundancia son efectivos en su capacidad de aceptar los grupos metilo a partir de la metiltransferasa CheR, usando como sustrato de la reacción la molécula S-

adenosilmetionina (SAM) y produciendo S-adenosilhomocisteína (SAH), existiendo una inhibición por producto final en esta reacción (Simms and Subbaramaiah, 1991) (Figura 14). Estos quimiorreceptores de alta abundancia son capaces de llevar a cabo una quimiotaxis eficiente (Feng *et al*., 1999; Weerasuriya *et al*., 1998).

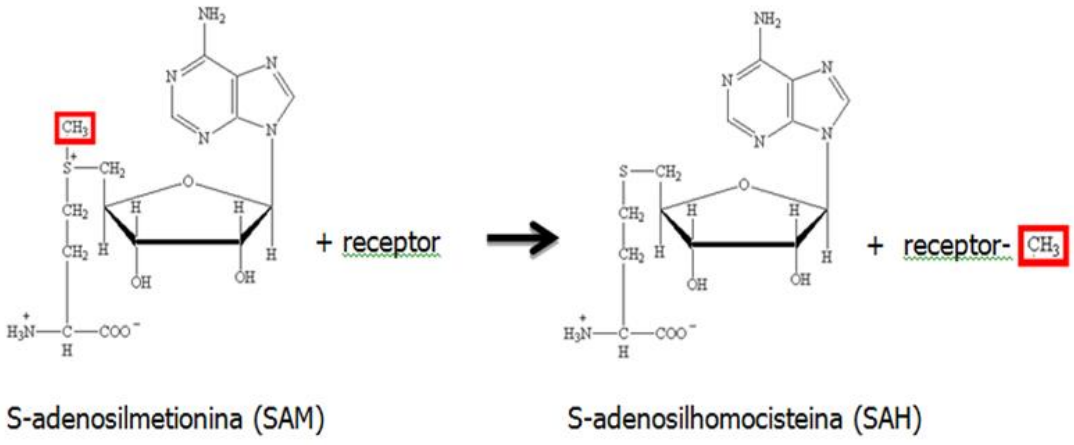

Figura 14. Reacción catalizada por la metiltranferasa CheR. Se destaca mediante un recuadro rojo la posición del grupo metilo que pasa de SAM al quimiorreceptor.

Los receptores de baja abundancia, producen pocos giros y sus tiempos de adaptación son demasiado extensos. Su capacidad de mediar migración dirigida en gradientes espaciales está bastante afectada (Feng *et al*., 1997; Hazelbauer and Engström, 1980; Hazelbauer *et al*., 1989; Okumura *et al*., 1998; Wu *et al*., 1996; Yamamoto *et al*., 1990). Se ha observado que estas diferencias en la función y en la actividad, están relacionadas con la presencia de una determinada secuencia en el extremo C-terminal de los quimiorreceptores de alta abundancia Tsr y Tar. Esta secuencia se compone de un brazo flexible o *linker* de unos 25-30 aminoácidos, seguida de un pentapéptido con una secuencia conservada (NWETF) que representa un sitio de unión específico a la metiltransferasa CheR. Esta secuencia no se localiza en los receptores de baja abundancia Trg, Tap y Aer (Figura 15) (Wu *et al*., 1996).

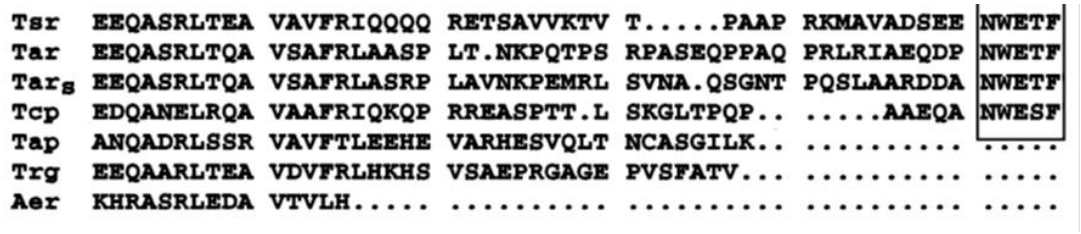

Figura 15. Alineamiento de las secuencias de aminoácidos C-terminales de los receptores de *E. coli* Tsr, Tar, Trg, Tap, y Aer y de *Salmonella typhimurium* Tar y Tcp. En el recuadro se muestra la secuencia C-terminal conservada en receptores de alta abundancia (Feng *et al*., 1999).

Feng y colaboradores, consiguieron demostrar, que añadiendo la extensión de 19 aminoácidos presentes en el extremo C-terminal del receptor Tsr, a los receptores de baja abundancia, conseguían transformar dichos receptores, en receptores con actividad óptima característica de los receptores de alta abundancia (Feng *et al*., 1999). Con este trabajo se concluyó que la diferencia funcional entre los receptores de baja abundancia y los de alta abundancia, se debe al nivel de metilación conferido por el sitio de reconocimiento quimiorreceptor-pentapéptido a través de la metiltransferasa CheR. Este sitio de unión al pentapéptido, consiste en un sitio de unión distinto e independiente al sitio de unión a SAM y SAH en la metiltransferasa, sin existir entre ambos sitios un efecto de unión cooperativo (Yi and Weis, 2002).

En 2005, Lai y colaboradores, observaron que el pentapéptido del extremo C-terminal de *E. coli*, no sólo era capaz de interaccionar con la metiltransferasa CheR, sino también con la correspondiente metilesterasa CheB, ambas proteínas adaptadoras en el sistema de quimiotaxis en *E. coli* (Figura 16) (Lai and Hazelbauer, 2005; Lai *et al*., 2006).

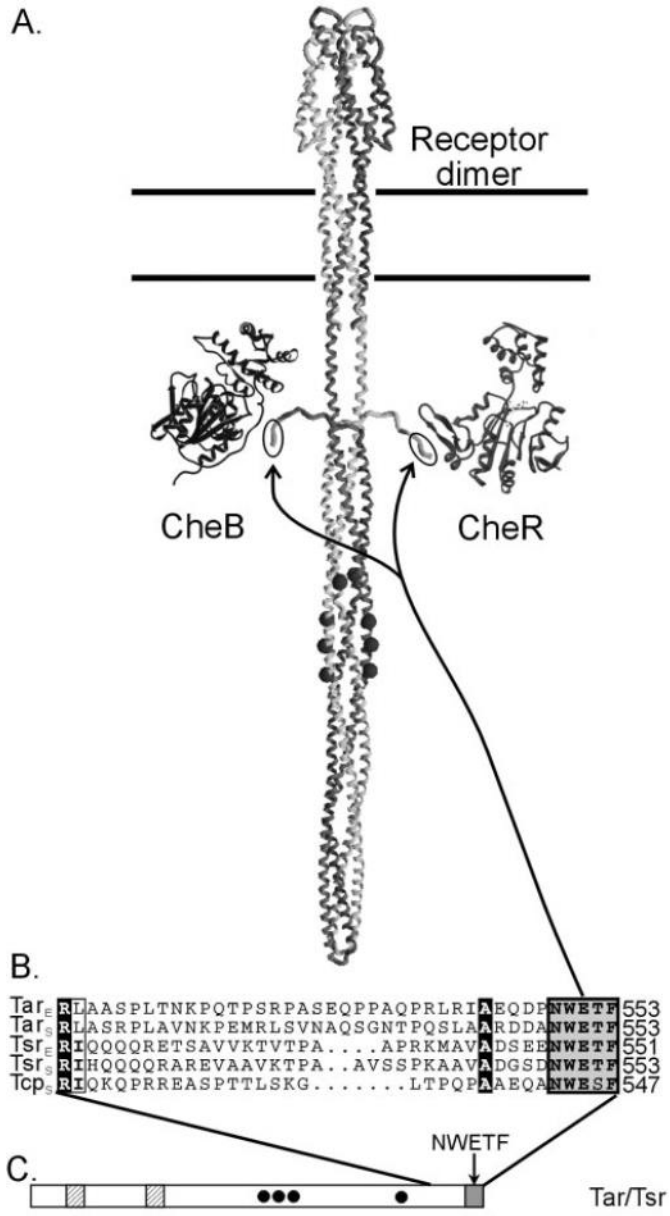

Figura 16. Modelo de la estructura de un dímero de un quimiorreceptor, una metiltransferasa (CheR), y una metilesterasa (CheB). En el panel A se muestra la estructura tridimensional de CheR (Djordjevic and Stock, 1997) y CheB (Djordjevic *et al*., 1998) y un modelo de un quimiorreceptor basado en estructuras tridimensionales obtenidas a partir de fragmentos de un quimiorreceptor (Kim *et al*., 1999). La secuencia del pentapéptido conservado en los receptores de *E. coli* (E), y *Salmonella* (S) se muestran en un recuadro en el panel B. En el panel C, se representan los dominios de quimiorreceptor: las dos líneas horizontales sombreadas indican los dominios transmembrana. Los sitios de metilación en los quimiorreceptores están marcadas por puntos. La secuencia del pentapéptido C-terminal se representa en el último recuadro sombreado (Lai and Hazelbauer, 2005).

Varios estudios demostraron que el pentapéptido de *E. coli*, y de *S. typhimurium* (que tiene un sistema de quimiotaxis homólogo al de *E. coli*), es totalmente imprescindible para las actividades de metilación, desmetilación y quimiotaxis en ambas cepas. El reconocimiento e interacción de este pentapéptido de los quimiorreceptores de alta abundancia con las proteínas CheR y CheB, tiene por tanto, un papel fundamental para un correcto desarrollo de las rutas de quimiotaxis (Bartelli and Hazelbauer, 2011).

Como teoría sobre la existencia de receptores de baja abundancia que no interaccionan con este pentapéptido, varios estudios proponen, que la ineficiencia de estos receptores de baja abundancia, se ve necesitada de la asistencia de los receptores de alta abundancia. Estos últimos, reclutarían metiltransferasas y metilesterasas que interaccionarían con su pentapéptido, aumentando la concentración de estas proteínas en las inmediaciones de los receptores de baja abundancia. A través del brazo flexible o *linker*, los receptores de alta abundancia acercarían las metiltransferasas y metilesterasas a los receptores de baja abundancia aumentando su metilación y desmetilación respectivamente (Le Moual *et al*., 1997; Li and Hazelbauer, 2005; Li *et al*., 1997).

Los receptores que presentan pentapéptidos C-terminales se encuentran en muchos tipos de bacterias (Perez and Stock, 2007), y la abundancia relativa de tales quimiorreceptores varía considerablemente. Mientras que algunas bacterias carecen de receptores que contienen pentapéptidos, en otras especies, tales receptores representan aproximadamente la mitad del número total de receptores (Perez and Stock, 2007). Una de las cepas que contiene pentapéptidos C-terminales en sus quimiorreceptores, es *P. aeruginosa*, por lo que uno de los objetivos de esta tesis doctoral es el estudio en detalle de la presencia de estos pentapéptidos en la cepa *P. aeruginosa* PAO1, y su función.

Pseudomonas aeruginosa

P. aeruginosa es una bacteria perteneciente al grupo de Gram negativas, patógeno oportunista presente en varios hábitats tales como suelo, agua y plantas (Cornelis, 2008; Pereira *et al*., 2014) y es capaz de infectar diferentes organismos incluyendo

plantas, animales y humanos (Breidenstein *et al*., 2011; Lister *et al*., 2009; Pereira *et al*., 2014). En humanos, *P. aeruginosa* es capaz de infectar el tracto pulmonar, el urinario, tejidos, heridas, y también causa otras infecciones de sangre (Custovic *et al*., 2014).

La fibrosis quística pulmonar es otra enfermedad predispuesta a la infección con *P. aeruginosa* (Tümmler *et al*., 1997). Es un poderoso patógeno en *Arabidopsis* (Walker *et al*., 2004) y en varias especies animales como *Caenorhabditis elegans* (Mahajan-Miklos *et al*., 1999), *Drosophila* (D'Argenio *et al*., 2001) y *Galleria mellonella* (Miyata *et al*., 2003). Para llevar a cabo su patogénesis, *P. aeruginosa* dispone de un arsenal de elementos de virulencia necesarios para invadir las células huésped y eludir las defensas del huésped (Ballok and O'Toole, 2013; Cornelis, 2008). Estos mecanismos de virulencia incluyen factores secretados, tales como proteasas, elastasa, piocianina, exotoxina A, fosfolipasa C, y exoenzima S, así como factores asociados a células tales como lipopolisacáridos, flagelos y *pili* (Cornelis, 2008; Strateva and Mitov, 2011). *P. aeruginosa* también tiene capacidad de adherirse a superficies, tales como las de los dispositivos médicos o las células epiteliales, y de formar biopelículas que consisten en comunidades microbianas embebidas en una matriz de sustancias poliméricas extracelulares o exoplisacáridos (Anderson *et al*., 2008; Drenkard and Ausubel, 2002; Garcia-Medina *et al*., 2005; Moreau-Marquis *et al*., 2008; Watnick and Kolter, 2000). Las biopelículas se asocian frecuentemente con infecciones crónicas, ya que su estructura confiere a las células bacterianas, protección frente agentes antimicrobianos (Costerton *et al*., 1999).

D) Existencia de escasos quimiorreceptores solubles en bacterias

En arqueas, el 43 % de los quimiorreceptores carecen de regiones transmembrana y son por lo tanto, receptores solubles ubicados en el citosol (topología IV, Figura 9). Sin embargo, en bacterias, la presencia de quimiorreceptores con esta topología es significativamente menor, ya que sólo un 14 % de ellos son citosólicos (Collins et al., 2014; Lacal et al., 2010a). Ésto es debido al alto porcentaje de detección de señales externas que se da frecuentemente en bacterias. Los dominios sensores más

frecuentes encontrados en este tipo de quimiorreceptores, son del tipo PAS, aunque se pueden encontrar otros tipos de dominios sensores menos frecuentes en este tipo de quimiorreceptores (Figura 17) ((Collins et al., 2014).

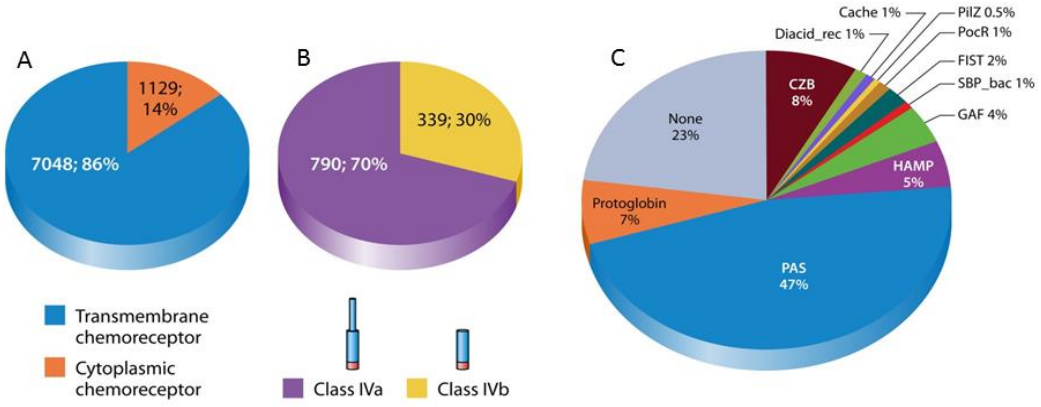

Figura 17. Abundancia de quimiorreceptores solubles en bacterias (A), topología de los mismos (B), y organización de dominios sensores (C). Las clases IVa y IVb se corresponden con las topologías mostradas en la figura 9. (Collins et al., 2014).

Actualmente, existe muy poca información acerca de la función de estos quimiorreceptores solubles, y en muchos casos, los estudios realizados sobre algunos de ellos, es bastante confusa, como es el caso del quimiorreceptor McpB de *P. aeruginosa* PAO1.

El quimiorreceptor soluble McpB

Como se ha indicado anteriormente, *P. aeruginosa* presenta 26 quimiorreceptores. Dos quimiorreceptores, codificados por los genes *mcpA* y *mcpB*, se encuentran en el Cluster de genes *che2* (o Cluster II) que codifica para la vía Che2 (Figura 18).

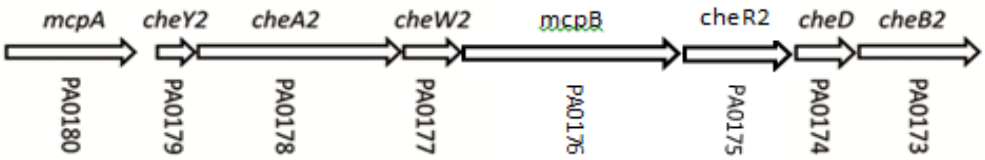

Figura 18. Organización génica del Cluster II en *Pseudomonas aeruginosa*. (www.pseudomonas.com).

El quimiorreceptor McpB tiene un dominio sensor de tipo PAS, capaz de unir grupos hemo (Watts *et al*., 2011); posee además cinco dominios tipo HAMP, cuya estructura ha sido recientemente resuelta por cristalografía de rayos X (Airola *et al*., 2013); carece de regiones transmembrana y se prevé que sea de localización citosólica (Güvener *et al*., 2006). Presenta un pentapéptido en su extremo C-terminal, homólogo al encontrado en los receptores de *E. coli* (Figura 19) (Watts *et al*., 2011).

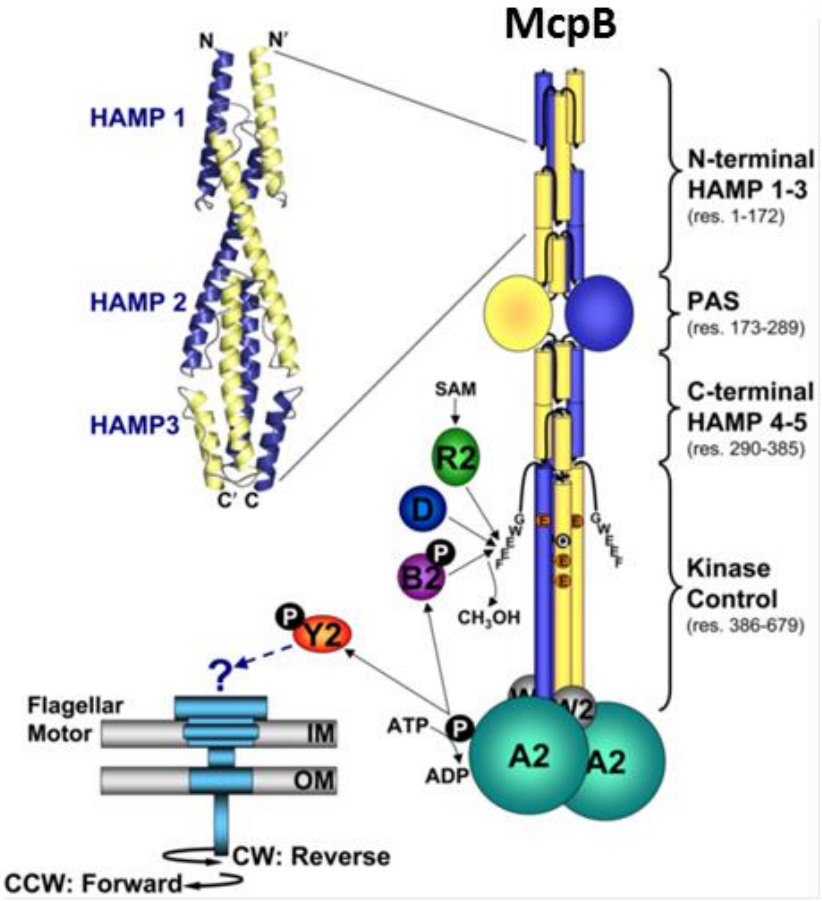

Figura 19. Estructura del quimiorreceptor McpB. Se muestra la interacción con las distintas proteínas involucradas en la cascada de señalización. R2: CheR2; B2: CheB2; D: CheD; W2: CheW2; A2: CheA2, Y2: CheY2 (Watts *et al*., 2011).

Los intentos de identificar la función de McpB no han sido concluyentes. Estudios anteriores, relacionaron la proteína McpB perteneciente al Cluster II, con aerotaxis (Hong *et al*., 2004). Por otro lado, se le asignó un papel central en la quimiotaxis, ya

que una mutación en el gen que codifica para este receptor, desencadenaba la pérdida de la quimiotaxis hacia más de 60 compuestos ensayados, indicando este dato, que este receptor jugaba un papel esencial en la quimiotaxis del patógeno (Ferrández *et al*., 2002). Sin embargo, estos resultados no fueron reproducibles en posteriores estudios (Güvener *et al*., 2006).

Como se ha indicado anteriormente, el gen que codifica para este quimiorreceptor, se encuentra localizado en el Cluster II, que ha sido recientemente relacionado con virulencia (Garvis *et al*., 2009). Garvis y colaboradores observaron que el Cluster II, además de estar implicado en quimiotaxis, está implicado en virulencia, ya que un mutante en la metilesterasa CheB2, además de verse afectado en la motilidad, reducía considerablemente la virulencia en *C. elegans* y en modelos murinos, recuperándose el fenotipo de virulencia cuando se hacía la complementación de dicho mutante (Garvis *et al*., 2009).

Por otra parte, el mutante en la metilesterasa CheB1 perteneciente al Cluster I, no mostró ningún efecto sobre la virulencia en los modelos animales previamente descritos, sin embargo, se observó una reducción significativa en la motilidad. Estos datos sugieren que el papel de la ruta codificada por el Cluster II está relacionada con la respuesta quimiotáctica durante la infección debido a una molécula señal desconocida que desencadenaría el proceso (Garvis *et al*., 2009), mientras que el Cluster I juega un papel dominante en la quimiotaxis de *P. aeruginosa*.

Por otro lado, Burrowes y colaboradores describieron que RsmA controla la expresión de *cheB2* ya que en un mutante de *RsmA*, se observó una reducción de 10 veces en la expresión de *cheB2* (Burrowes *et al*., 2006). Estudios previos, demostraron que *RsmA* trabaja en conjunto con RNAs pequeños no codificantes para regular la expresión de múltiples genes implicados en virulencia, incluyendo los genes de *quorum sensing, lasI* y *rhlI* (Pessi *et al*., 2001), por lo que podría sugerirse que el Cluster II, podría estar implicado en procesos de virulencia y podría ser regulado por *RsmA*. En esta tesis doctoral se han llevado a cabo varios bioensayos para intentar esclarecer la función del quimiorreceptor McpB e investigar su posible relación con virulencia.

Todas estas características derivadas del análisis de genomas, están relacionadas con la complejidad en los sistemas de señalización química, lo que nos lleva a la necesidad de estudiar dichos sistemas de señalización en otras bacterias en las que se vean reflejadas las observaciones derivadas de estos análisis.

Por este motivo, en esta tesis doctoral, además de la caracterización del sistema de dos componentes TmoS/TmoT presente en *P. mendocina* KR1, se intentaron responder las siguientes preguntas relacionadas con los sistemas de señalización química:

1. ¿La existencia de distintos parálogos de proteínas implicadas en señalización química está relacionada con la existencia de diferentes vías de señalización paralelas o independientes?
2. En *E. coli* se ha descrito una inhibición por SAH, el producto final de la metiltransferasa CheR. ¿Es esta inhibición un fenómeno exclusivo de *E. coli*, o se trata de un fenómeno general?
3. ¿Cuál es el sentido de la existencia de pentapéptidos C-terminales en algunos quimiorreceptores bacterianos? ¿Cuál es la función de estos pentapéptidos? ¿Interaccionan con todos los parálogos de CheR?
4. ¿Cuál es la relevancia de quimiorreceptores solubles en bacterias como el quimiorreceptor McpB? ¿Cuál es su función? ¿Con qué ruta está relacionado? ¿Está implicado en fenómenos de virulencia?

OBJETIVOS

Los mecanismos de transducción de señales son esenciales para la detección de estímulos, permitiendo la adaptación de los microorganismos al medio en el que se encuentran. Debido a ésto, la caracterización de los mecanismos moleculares implicados en estos sistemas, es de suma importancia para el desarrollo de numerosas aplicaciones en distintas áreas, como pueden ser el tratamiento de enfermedades o biorremediación entre otras.

El objetivo principal de esta tesis consiste en la caracterización de distintos sistemas de transducción de señales utilizando distintas cepas de *Pseudomonas* como modelo. Los objetivos concretos de esta tesis son:

1. Estudiar el sistema de dos componentes TmoS/TmoT de *P. mendocina* KR1, y relacionarlo con el sistema TodS/TodT de *P. putida* DOT-T1E, para determinar si las características observadas en este último son exclusivas de esta especie o características comunes del género.

2. Determinar la relevancia de la existencia de distintos parálogos de proteínas en sistemas de señalización química en *Pseudomonas* utilizando para ello, los tres parálogos de la metiltransferasa CheR de *P. putida* KT2440.

3. Determinar la función de pentapéptidos presentes en algunos quimiorreceptores de *P. aeruginosa* PAO1.

4. Determinar el papel en virulencia del quimiorreceptor McpB en *P. aeruginosa* PAO1 mediante un panel de bioensayos de virulencia y ecotoxicidad.

RESULTADOS/ RESULTS

CHAPTER I

Study of the TmoS/TmoT two-component system: towards the functional characterization of the family of TodS/TodT like systems.

Adapted from: Study of the TmoS/TmoT two-component system: towards the functional characterization of the family of TodS/TodT like systems.

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1. Abstract

The two-component system TmoS/TmoT controls the expression of the toluene-4-monooxygenase pathway in *Pseudomonas mendocina* KR1 via modulation of P_{tmoX} activity. The TmoS/TmoT system belongs to the family of TodS/TodT like proteins. The sensor kinase TmoS is a 108 kDa protein composed of 7 different domains. Using isothermal titration calorimetry we show that purified TmoS binds a wide range of aromatic compounds with high affinities. Tightest ligand binding was observed for toluene (K_D=150 nM), which corresponds to the highest affinity measured between an effector and a sensor kinase. Other compounds with affinities in the nanomolar range include benzene, the 3 xylene isomers, styrene, nitrobenzene or *p*-chlorotoluene. We demonstrate that only part of the ligands which bind to TmoS increase protein autophosphorylation *in vitro* and consequently pathway expression *in vivo.* These compounds are referred to as agonists. Other TmoS ligands, termed antagonists, failed to increase TmoS autophosphorylation which resulted in their incapacity to stimulate gene expression *in vivo*. We also show that TmoS saturated with different agonists differs in their autokinase activities. The effector screening of gene expression showed that promoter activity of P_{tmoX} and P_{todX} (controlled by the TodS/TodT system) is mediated by the same set of 22 compounds. The common structural feature of these compounds is the presence of a single aromatic ring. Among these ligands, toluene was the most potent inducer of both promoter activities. Information on the TmoS/TmoT and TodS/TodT system combined with a sequence analysis of family members permits to identify distinct features which define this protein family.

2. Introduction

An important mechanism by which environmental signals are sensed and their changes translated into modulatory responses is based on the action of two-component systems (TCS) (Galperin, 2005; Krell *et al*., 2010). Bacteria contain a large number of TCSs and were found to harbour on average 52 TCS genes (Cock and Whitworth, 2007). TCSs are involved in the regulation of virtually all types of processes including virulence, sporulation, metabolism, quorum sensing, chemotaxis, transport or nitrogen fixation which has been reviewed in Krell *et al*. (2010) and Mascher *et al*. (2006). A prototypal TCS contains a sensor kinase (SK) and a response regulator (RR) and its mechanism is based on a simple His to Asp transphosphorylation. Signal recognition by the SK leads to a modulation of its autokinase activity and/or its phosphatase activity towards its cognate RR (Krell *et al*., 2010). The resulting changes in the SK phosphorylation state reflect on the rate of phosphoryltransfer towards the receiver domain of the RR, which in turn alters the properties of its output domain. This domain has in most cases DNA binding properties and modulates gene expression by binding to promoter regions (Galperin, 2006).

During evolution genes of prototypal TCSs have fused to more complex systems referred to as phosphorelay TCS (Whitworth and Cock, 2009) which employ a His1-Asp1-His2-Asp2 phosphorylation cascade. Phosphorelay systems so far studied include for example the ArcBA, TorSR and EvgSA systems of *E. coli* (Malpica *et al*., 2006; Perraud *et al*., 1999), the BvgST system of *Bordetella* sp. (Beier and Gross, 2008) or the sporulation phosphorelay of *Bacillus subtilis* (Burbulys *et al*., 1991). All of these systems were classified as TRPR systems (Figure 20), implying that the relay involves consecutive phosphorylation of a **t**ransmitter module, followed by a first **r**eceiver domain, a histidine containing **p**hosphotransfer domain and a second **r**eceiver domain (Williams and Whitworth, 2010). A transmitter module is composed of a dimerization and histidine phosphotransfer domain and a catalytic domain that catalyses transmitter module autophosphorylation using ATP as phosphoryldonor. However, genome analyses indicated that there is a different type of phosphorelay system, termed TRTR, which contain two transmitter modules and two receiver domains (Figure 20). At the genetic level it was shown that TRTR systems are as abundant as the

TRPR systems (Williams and Whitworth, 2010). However, at the functional level there is little data available on this type of system.

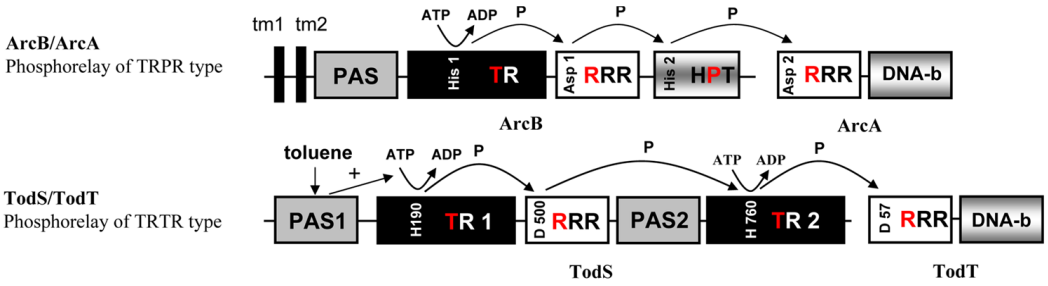

Figure 20. Schematic representation of domain organization and mode of action of phosphorelay two-component systems. A) The ArcB/ArcA phosphorelay. This system belongs to the TRPR type of phosphorelay according to the classification proposed by Williams and Whitworth (2010). B) The TodS/TodT system which belongs to the TRTR type of phosphorelays. The sequence of phosphorylgroup transfer is indicated. tm: transmembrane region; PAS, Per-Arnt-Sim type of sensor domain; TR: transmitter module comprised of a dimerization/histidine phosphotransfer domain and a catalytic domain; RRR: response regulator receiver domain; HPT: histidine containing phosphotransfer domain; DNA-b: DNA-binding domain.

The TodS/TodT TCS is the first TRTR system which has been functionally characterised (Figure 20) (Busch *et al*., 2009; Lacal *et al*., 2006, 2008a, 2008b). This system modulates the expression of the *tod* (toluene dioxygenase) operon encoding the enzymes of the TOD pathway which permits the mineralization of benzene, toluene and ethylbenzene in strains of *P. putida* (Lau *et al*., 1997; Mosqueda *et al*., 1999; Zylstra and Gibson, 1989). Although the TOD pathway uses toluene, benzene and ethylbenzene as substrates, our group was previously able to demonstrate that TodS/TodT mediates regulation of P_{todX} expression in response to a wide range of different aromatic compounds, including different methyl-, amino-, nitro- and halogen-substituted benzene and toluene derivatives (Lacal *et al*., 2006). The TodS SK is unusually large (108 kDa), lacks transmembrane regions and is entirely located in the cytosol (Lacal *et al*., 2006). It is composed of two transmitter modules, two PAS domains and an internal RR receiver domain (Figure 20). Both transmitter modules were found to

possess autokinase activity (Busch *et al*., 2009). Effector molecules, such as toluene, bind to the PAS 1 domain with high affinity (Lacal *et al*., 2006). Efector binding was found to stimulate exclusively the activity of the N-terminal transmitter domain (Busch *et al*., 2009). The phosphoryl group is then transferred to Asp500 of the internal receiver domain and subsequently to the C-terminal transmitter module prior to the phosphorylation of Asp57 at the TodT RR (Figure 20) (Busch *et al*., 2009). It has been shown that TodT, monomeric in solution, binds to 5 different sites at the P_{todX} promoter in a sequential and cooperative manner (Lacal *et al*., 2008a, 2008b). In addition, it has been demonstrated that the activity of TodS/TodT is mediated by agonists and antagonists (Busch *et al*., 2007). Agonists (for example toluene or *m*-xylene) are structural very similar to antagonists (*o*- xylene o *o*-chlorotoluene). Both classes of compounds compete for binding at the PAS sensor 1 domain. However, only agonists cause an increase in TodS autophosphorylation activity and consequently induce gene expression. Although antagonists bind to TodS with comparable affinities, these compounds fail to increase TodS autophosphorylation and consequently gene expression (Busch *et al*., 2007). The physiological relevance of these observations is not understood.

There are a number of other TCSs which share significant similarities in sequence and domain arrangement with TodS/TodT. Members of this family are for example StyS/StyR, involved in styrene degradation (Leoni *et al*., 2003, 2005; O'Leary *et al*., 2001; Rampioni *et al*., 2008; Velasco *et al*., 1998) or the control of phenylacetyl-coenzyme A ligase expression (del Peso-Santos *et al*., 2008), TutC/TutB for anaerobic toluene degradation in *Thauera aromatica* (Coschigano and Young, 1997) or TmoS/TmoT involved in toluene degradation in *P. mendocina* (Ramos-González *et al*., 2002). Furthermore, genome sequencing projects have revealed that TodS/TodT like proteins are found in other bacteria such as *Dechloromonas aromatica* (Coates *et al*., 2001; Salinero *et al*., 2009) and *Methylibium petroleiphilum* (Kane *et al*., 2007) or in nonobligate predator of soil bacteria *Cupriavidus necator* N-1 (Poehlein *et al*., 2011).

These systems form thus the family of TodS/TodT like TCS (Figure 21) and the particular domain arrangement of TodS appears to be a specific feature of this family.

```
                  10        20        30        40        50        60        70        80
                   |         |         |         |         |         |         |    ↓    |
TodS      ----------------------------------MSSLDRKKPQNRSKNNYYNICLKEKGSEELTCEEHARIIFDGLY
TmoS      ----------------------------------MSSLDKRKTQNRSKKNSYSICLKEKASAELKREELARIIFDGLY
NodV      MVYNTSEISGNLQRLQRSVNAGRFLRMIPQRHRNRTMQTINPQLFSHGQTLRVGGVTLRATDGLRTHREKLARIVLDQMY
A2SDY5    ----------------------------------MPNINRQLFAASDSLCVRDVTLRATDDVQTHREKLARIVLDELY
TutC      ----------------------------------MTSNNSSVSDISAVLRVRDVTLRAVDDLQTYREKLARVVLDGLY
Q479E0    ----------------------------------MTSEN-AMPNKEMQASVKGVTLVPDDATEVRRQKLARIILDAMY
StyS Ps   --------------------------------MPGAWNVMSATDLPGDSVRSVGNVILNPDDSPQTHSEKMARIILDRMY
StyS Pf   --------------------------------MPGAWNVMSATDLSGGSVRSVGNVILNPDDSPQTHSEKMARIILDRMY

                  90       100       110       120       130       140       150       160
                   |         |         ↓    ↓    |         |         |         ↓         |
TodS      EFVGLLDAHGNVLEVNQVALEGAGITLEEIRGKPFWKARWWQISKKTEATQKRLVETASSGEFVRCDVEILGKSGGREVI
TmoS      EFVGLLDAQGNVLEVNQAALNGAGVTLEEIRGKPFWKARWWQISKESVANQKRLVEAASSGEFVRCDIEILGKSGGREVI
NodV      QFVGLLDADGLTLEINEAALAGAGIRLDDIQGKPFWEARWWCVSKQTQECAQDAIERACRGEFVRFDVEVYGRTGGEETI
A2SDY5    EFVGLLDAHGTTLEINRAALEGAGIALDDIQGRPFWEARWWATSPEVRREQREVIRRAGEGEFVRRDFEIYGQQGGQETI
TutC      EFVGLLDAKGNTLEINQAALDGAGTRLEDIRDKPFWEARWWQVSRETQEEQRKLIARASAGEFVRCDVEIYGRASGEETI
Q479E0    QFLGLLDVDGTVLEINRAALEGAGICLDEVIGKPFWEARWWAISEEARNRVRSMVEQARNGEFVRCDIEIFGDLQGKKSI
StySPs    HFAGLLDRDGTILEINLPALEGAGVRIEDIRGTPFWEARWLAVSEESKELQHQLVQRAAAGEFIRCDLEVYGEGSGEQTI
StySPf    HFAGLLDRDGTILEINLPALEGAGLRIEDIRGTPFWEARWFAVSQESKALQHQLVQRAAAGEFIRCDLEVYGEGSGEQTI

                 170       180       190       200       210       220       230       240
                   |         |         |         |         |         |    ↓    |         |
TodS      AVDFSLLPICNEEGSIVYLLAEGRNITEKKKAEAMLALKNQELEQSVECIRKLDNAKSDFFAKVSHELRTPLSLILGPLE
TmoS      AVDFSLLPIRDEQENIVFLLAEGRNITDKKKAEAMLALKNHELEQLVERIRKLDNAKSDFFAKVSHELRTPLSLILGPLE
NodV      IVDFSLLPVKDRRNEVMFLLAEARNITEKKRSEAEIVRKNEELQQLLDKIRQIDALKSDFFAKVSHELRTPLALILGPAE
A2SDY5    LIDYSLLPIRDNSGKIVFLLPEGRNITDKKRAEAEIARKNRELQRLLDKIQRLDDAKSDFFANVSHELRTPLALILGPSE
TutC      VVDYSILPIRDCNGKVVFLLPEGRNITDKKLAEAELARKNEELQHLLEKIRQLDEAKNEFFANLSHELRTPLSLILGSVE
Q479E0    FVDFSLTPIRDDAGRVAFLLPEGRNITEKIAIEAELTRKNGELQLALEKLREIDGFKTKFFANVSHELRTPLALILGPVD
StySPs    VVDYSLTPLRDNHGEVAFLLAEGRNITSKKKYEQEIARKNAELEKLVEQIRMLDEQKNRFFSNLSHELRTPLSLILGPVD
StySPf    VTDYSLTPLRDNHGEVAFLLAEGRNITSKKKYEQEIARKNSELEKLVEQIRKLDEQKSRFFSNLSHELRTPLSLILGPVD

                 250       260       270       280       290       300       310       320
                   |         |         |         |         |         |         |         |
TodS      AVMAAEAGRESPYWKQFEVIQRNAMTLLKQVNTLLDLAKMDARQMGLSYRRANLSQLTRTISSNFEGIAQQKSITFDTKL
TmoS      TIMEAESGRGSPYWKKFEVIQRNAMTLLKQVNTLLDLAKMDAQQMGLSYRRADLSQLTRVISSNFDGIAQQKSITLDAEL
NodV      SLIAGSDNLNEQQRRDLTVIRRNATTLLKHVNDLLDLAKLDAGKISLDYARIDVAHTVRAVAAHFDTLAPQRSYSYVVAL
A2SDY5    SLLATSEGLSDAQRRDLRVIQRNAAMLMKHVNDLLDLAKFDAGKMALRYTRVDLAAEVRTLAAHFEAVAAERSLSYVVQA
TutC      SLLADSGDYSGVQRVDLDVIQRNAITLLKYVNDLLDLAKLQAEKLQLHYSRVDLAAVTRMICAHFEALAEYKCLSYVIDA
Q479E0    QMLRESEQLGERERFRLTTIKRNAQSLHQQVNDLLDLARIDAQQMPLAYVCVNVVALLREVAAGFAAAAEERAISLIIEG
StySPs    EMLVSS-EFSEHQHTNLASIRRNAVTLLRHVNELLDLAKIDAGKLQLAYELIDITGLVKEITAHFEAHAKQRRIHCAVLS
StySPf    EMLVSS-EFSERHDTNLASIRRNAVTLLRHVNELLDLAKVDAGKLQLAYERIDIKGLVEDIAAHFEAHAKQRRIRCAVLS

                 330       340       350       360       370       380       390       400
                   |         |         |         |         |         |         |         |
TodS      PVQMVAEVDCEKYERIILNLLSNAFKFTPDGGLIRCCLSLSRPNYALVTVSDSGPGIPPALRKEIFERFHQLSQEGQQAT
TmoS      PPHLIAEVDCEKYERIILNLLSNAFKFTPDGGLIRCHLSLSQPAHALITVSDSGPGIPQNLRKEIFERFHQLNQEGQQAN
NodV      PEACEAEVDPQKFERIVLNLLSNAFKFTPPGGRIRCGLEPSGNSRFLVTVQDSGPGVAPEMRTVLFEPFCQGRAGMAGEF
A2SDY5    PAALEVEVDQQMFERILLNLLSNAFKFTPDFGRIRCSLEANPDHSIQLVVEDSGCGVRADLREEIFERFHQAQSGTTRSF
TutC      PAFMEAEVDVEKYERIVLNLLSNAFKFSPDGGRIRCSLSATGTGRILLSIQDSGPGIPADQQSEIFGRFRQGGDIKSRQF
Q479E0    ADELQADVDRAKFARVLANLLSNAFKFTPAGGRICCSITRVANDRFLLSVQDNGPGVPPPMKQQIFDRFAQGQGGLSG--
StySPs    PGPILLEADPEKISHVVFNLVANAFNATPDGGRISCHVEIGEGNRCLLTVSDTGPGVPPDMRQRIFERFQQGVEEHGEAR
StySPf    PGPILVEADPERIGHVVFNLMANAFNATPDGGRISCRVEIGRGNRCLLTVSDSGPGIPPEMRQRIFERFQQGLEDHGQAR

                 410       420       430       440       450       460       470       480
                   |         |         |         |         |         |         |         |
TodS      RGTGLGLSIVKEFVELHRGTISVSDAPGGGALFQVKLPLNAPEGAYVASNTAPRRDNPQVVDTDEYLLLAPNAENEAEVL
TmoS      QGTGLGLSIVKEFVELHHGTISVSDAPGGGALFQVKLPLNAPEGAYVANNAMSRSDNPQTVNPDEYLLPIPTAGSGAELP
NodV      GGTGLGLAIVKEFVDLHGGTVSMSEAPGGGALFQVELPLLAPKGTYVRQDVALPQRVVPAVNVDVNEALLATELPYADSR
A2SDY5    SGTGLGLAIAKEFVDLHTGTISVSDAIGGGAQFRVELPSRAPLGAYIRSVDSPLGNRNRGQIVGTIEELQRAEFDAVSDL
TutC      GGTGLGLTIVKDFVCLHGGVVVVSDAPGGGALFQIELPRNAPSGVYVNAVAKAGELSPTSFDISAWGLEGRSEWTSAEG-
Q479E0    IGSGLGLNIVKEFVELHFGTVVVLDAPGGGAIFQVEMPKRAPNGVFVRESGEGIG----LVTPQDIDFLEPSSHPASAY-
StySPs    AGSGLGLAIVKEFIELHGGTVTVGEAPSSGAIFQVELPAAAPPQVLVRKGSVREQTFSPELPSGGDVSLLPGRGLVSDG-
StySPf    AGSGLGLAIVKEFIELHGGTVTVGEAPGSGAIFQVEIPAFAPPQAVVRSGSTGEQAFSHDMSLEADIDIRPGRRLVSDT-
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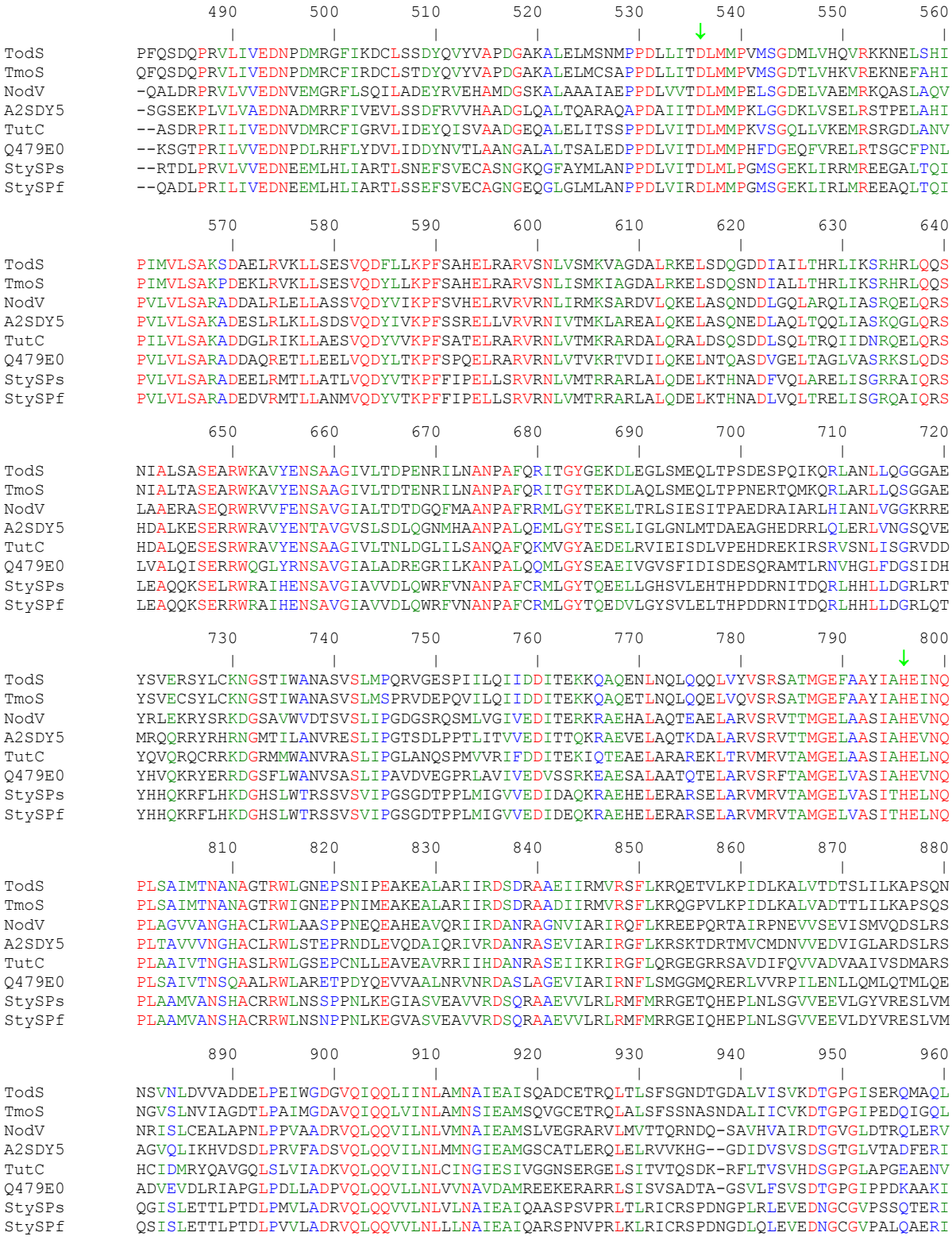

490 500 510 520 530 540 550 560
TodS PFQSDQPRVLIVEDNPDMRGFIKDCLSSDYQVYVAPDGAKALELMSNMPPDLLITDLMMPVMSGDMLVHQVRKKNELSHI
TmoS QFQSDQPRVLIVEDNPDMRCFIRDCLSTDYQVYVAPDGAKALELMCSAPPDLLITDLMMPVMSGDTLVHKVREKNEFAHI
NodV -QALDRPRVLVVEDNVEMGRFLSQILADEYRVEHAMDGSKALAAAIAEPPDLVVTDLMMPELSGDELVAEMRKQASLAQV
A2SDY5 -SGSEKPLVLVAEDNADMRRFIVEVLSSDFRVVHAADGLQALTQARAQAPDAIITDLMMPKLGGDKLVSELRSTPELAHI
TutC --ASDRPRILIVEDNVDMRCFIGRVLIDEYQISVAADGEQALELITSSPPDLVITDLMMPKVSGQLLVKEMRSRGDLANV
Q479E0 --KSGTPRILVVEDNPDLRHFLYDVLIDDYNVTLAANGALALTSALEDPPDLVITDLMMPHFDGEQFVRELRTSGCFPNL
StySPs --RTDLPRVLVVEDNEEMLHLIARTLSNEFSVECASNGKQGFAYMLANPPDLVITDLMLPGMSGEKLIRRMREEGALTQI
StySPf --QADLPRILIVEDNEEMLHLIARTLSSEFSVECAGNGEQGLGLMLANPPDLVIRDLMMPGMSGEKLIRLMREEAQLTQI
570 580 590 600 610 620 630 640
TodS PIMVLSAKSDAELRVKLLSESVQDFLLKPFSAHELRARVSNLVSMKVAGDALRKELSDQGDDIAILTHRLIKSRHRLQQS
TmoS PIMVLSAKPDEKLRVKLLSESVQDYLLKPFSAHELRARVSNLISMKIAGDALRKELSDQSNDIALLTHRLIKSRHRLQQS
NodV PVLVLSARADDALRLELLASSVQDYVIKPFSVHELRVRVRNLIRMKSARDVLQKELASQNDDLGQLARQLIASRQELQRS
A2SDY5 PVLVLSAKADESLRLKLLSDSVQDYIVKPFSSRELLVRVRNIVTMKLAREALQKELASQNEDLAQLTQQLIASKQGLQRS
TutC PILVLSAKADDGLRIKLLAESVQDYVVKPFSATELRARVRNLVTMKRARDALQRALDSQSDDLSQLTRQIIDNRQELQRS
Q479E0 PVLVLSARADDAQRETLLEELVQDYLTKPFSPQELRARVRNLVTVKRTVDILQKELNTQASDVGELTAGLVASRKSLQDS
StySPs PVLVLSARADEELRMTLLATLVQDYVTKPFFIPELLSRVRNLVMTRRARLALQDELKTHNADFVQLARELISGRRAIQRS
StySPf PVLVLSARADEDVRMTLLANMVQDYVTKPFFIPELLSRVRNLVMTRRARLALQDELKTHNADLVQLTRELISGRQAIQRS
650 660 670 680 690 700 710 720
TodS NIALSASEARWKAVYENSAAGIVLTDPENRILNANPAFQRITGYGEKDLEGLSMEQLTPSDESPQIKQRLANLLQGGGAE
TmoS NIALTASEARWKAVYENSAAGIVLTDTENRILNANPAFQRITGYTEKDLAQLSMEQLTPPNERTQMKQRLARLLQSGGAE
NodV LAAERASEQRWRVVFENSAVGIALTDTDGQFMAANPAFRRMLGYTEKELTRLSIESITPAEDRAIARLHIANLVGGKRRE
A2SDY5 HDALKESERRWRAVYENTAVGVSLSDLQGNMHAANPALQEMLGYTESELIGLGNLMTDAEAGHEDRRLQLERLVNGSQVE
TutC HDALQESESRWRAVYENSAAGIVLTNLDGLILSANQAFQKMVGYAEDELRVIEISDLVPEHDREKIRSRVSNLISGRVDD
Q479E0 LVALQISERRWQGLYRNSAVGIALADREGRILKANPALQQMLGYSEAEIVGVSFIDISDESQRAMTLRNVHGLFDGSIDH
StySPs LEAQQKSELRWRAIHENSAVGIAVVDLQWRFVNANPAFCRMLGYTQEELLGHSVLEHTHPDDRNITDQRLHHLLDGRLRT
StySPf LEAQQKSERRWRAIHENSAVGIAVVDLQWRFVNANPAFCRMLGYTQEDVLGYSVLELTHPDDRNITDQRLHHLLDGRLQT
730 740 750 760 770 780 790 800
TodS YSVERSYLCKNGSTIWANASVSLMPQRVGESPIILQIIDDITEKKQAQENLNQLQQQLVYVSRSATMGEFAAYIAHEINQ
TmoS YSVECSYLCKNGSTIWANASVSLMSPRVDEPQVILQIIDDITEKKQAQETLNQLQQELVQVSRSATMGEFAAYIAHEINQ
NodV YRLEKRYSRKDGSAVWVDTSVSLIPGDGSRQSMLVGIVEDITERKRAEHALAQTEAELARVSRVTTMGELAASIAHEVNQ
A2SDY5 MRQQRRYRHRNGMTILANVRESLIPGTSDLPPTLITVVEDITTQKRAEVELAQTKDALARVSRVTTMGELAASIAHEVNQ
TutC YQVQRQCRRKDGRMMWANVRASLIPGLANQSPMVVRIFDDITEKIQTEAELARAREKLTRVMRVTAMGELAASIAHELNQ
Q479E0 YHVQKRYERRDGSFLWANVSASLIPAVDVEGPRLAVIVEDVSSRKEAESALAATQTELARVSRFTAMGELVASIAHEVNQ
StySPs YHHQKRFLHKDGHSLWTRSSVSVIPGSGDTPPLMIGVVEDIDAQKRAEHELERARSELARVMRVTAMGELVASITHELNQ
StySPf YHHQKRFLHKDGHSLWTRSSVSVIPGSGDTPPLMIGVVEDIDEQKRAEHELERARSELARVMRVTAMGELVASITHELNQ
810 820 830 840 850 860 870 880
TodS PLSAIMTNANAGTRWLGNEPSNIPEAKEALARIIRDSDRAAEIIRMVRSFLKRQETVLKPIDLKALVTDTSLILKAPSQN
TmoS PLSAIMTNANAGTRWIGNEPPNIMEAKEALARIIRDSDRAADIIRMVRSFLKRQGPVLKPIDLKALVADTTLILKAPSQS
NodV PLAGVVANGHACLRWLAASPPNEQEAHEAVQRIIRDANRAGNVIARIRQFLKREEPQRTAIRPNEVVSEVISMVQDSLRS
A2SDY5 PLTAVVVNGHACLRWLSTEPRNDLEVQDAIQRIVRDANRASEVIARIRGFLKRSKTDRTMVCMDNVVEDVIGLARDSLRS
TutC PLAAIVTNGHASLRWLGSEPCNLLEAVEAVRRIIHDANRASEIIKRIRGFLQRGEGRRSAVDIFQVVADVAAIVSDMARS
Q479E0 PLSAIVTNSQAALRWLARETPDYQEVVAALNRVNRDASLAGEVIARIRNFLSMGGMQRERLVVRPILENLLQMLQTMLQE
StySPs PLAAMVANSHACRRWLNSSPPNLKEGIASVEAVVRDSQRAAEVVLRLRMFMRRGETQHEPLNLSGVVEEVLGYVRESLVM
StySPf PLAAMVANSHACRRWLNSNPPNLKEGVASVEAVVRDSQRAAEVVLRLRMFMRRGEIQHEPLNLSGVVEEVLDYVRESLVM
890 900 910 920 930 940 950 960
TodS NSVNLDVVADDELPEIWGDGVQIQQLIINLAMNAIEAISQADCETRQLTLSFSGNDTGDALVISVKDTGPGISERQMAQL
TmoS NGVSLNVIAGDTLPAIMGDAVQIQQLVINLAMNSIEAMSQVGCETRQLALSFSSNASNDALIICVKDTGPGIPEDQIGQL
NodV NRISLCEALAPNLPPVAADRVQLQQVILNLVMNAIEAMSLVEGRARVLMVTTQRNDQ-SAVHVAIRDTGVGLDTRQLERV
A2SDY5 AGVQLIKHVDSDLPRVFADSVQLQQVILNLMMNGIEAMGSCATLERQLELRVVKHG--GDIDVSVSDSGTGLVTADFERI
TutC HCIDMRYQAVGQLSLVIADKVQLQQVILNLCINGIESIVGGNSERGELSITVTQSDK-RFLTVSVHDSGPGLAPGEAENV
Q479E0 ADVEVDLRIAPGLPDLLADPVQLQQVLLNLVVNAVDAMREEKERARRLSISVSADTA-GSVLFSVSDTGPGIPPDKAAKI
StySPs QGISLETTLPTDLPMVLADRVQLQQVVLNLVLNAIEAIQAASPSVPRLTLRICRSPDNGPLRLEVEDNGCGVPSSQTERI
StySPf QSISLETTLPTDLPVVLADRVQLQQVVLNLLLNAIEAIQARSPNVPRLKLRICRSPDNGDLQLEVEDNGCGVPALQAERI

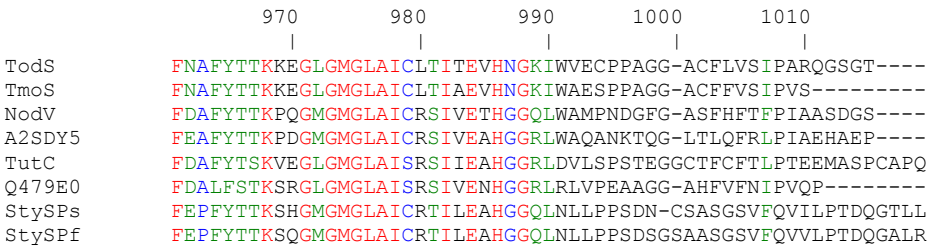


Figure 21. Sequence alignment of TodS like sensor kinases. The following sequences were used for this alignment: TodS of *P. putida* DOT-T1E; TmoS of *P. mendocina* KR1, NodV of *C. necator* N-1; A2SDY5 of *M. petroleiphilum* PM1; TutC of *T. aromatica*; Q479E0 of *D. aromatica* RCB; StyS of *P. fluorescens* (StyS Pf) and StyS of *Pseudomonas* sp. Y2 (StyS Ps). The amino acids which form part of the intra-TodS phosphorelay (Busch *et al*., 2009) are highlighted by green arrows. The amino acids which are involved in effector recognition (Busch *et al*., 2007) are marked with red arrows. Sequences were aligned using the Clustal W multiple sequence alignment algorithm (Thompson *et al*., 1994) of the NPS@ server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). Fully conserved amino acids are shown in red, highly conserved residues in green and weakly conserved amino acids in blue.

The information available on the remaining TCS of the TodS/TodT family is primarily of genetic or microbiological nature. However, the functional characterization of the TodS/TodT system has revealed a number of intriguing features like (1) the TodS effector range is much larger than the substrate range (2) contrary to most SKs, TodS lacks transmembrane regions and is located in the cytosol, (3) ligand binding occurs with very high affinity, (4) effectors can be classified into agonists or antagonists, or (5) only agonists and not antagonists increase SK autokinase activity.

To elucidate whether these properties are specific to the TodS/TodT system or correspond to feature common to the protein family, we report here the analysis of a second member of this protein family, the TmoS/TmoT TCS (Ramos-González *et al*., 2002). This system regulates the expression of the toluene-4-monooxygenase (T4MO) pathway in *P. mendocina* KR1 (Whited and Gibson, 1991a, 1991b). The toluene-4-monooxygenase of this strain, the initial pathway enzyme, has been studied extensively over the last decades and is now considered a paradigm for

monooxygenases (Bailey *et al*., 2008; Mitchell *et al*., 2002, 2003). We would like to note that *P. putida* DOT-T1E, which harbours *todST*, was isolated in Spain (Ramos *et al*., 1995), whereas *P. mendocina* KR1 was isolated in Texas (Whited and Gibson, 1991a).

The functional analysis of the TmoS/TmoT TCS combined with sequence analysis of family members permit the proposition of common functional features.

3. Material and Methods

3.1 Strains and plasmids used in this study

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference
strains		
E. coli BL21 (DE3)	F^-, *ompI, hsdS*$_B$ (r^-_B m^-_B)	Studier and Moffatt, 1986
P. putida DOT-T1E	Prototroph, Tol+ (*tod* pathway)	Ramos *et al*., 1995
P. mendocina KR1	Prototroph, Tol+ (*tmo* pathway)	Whited and Gibson, 1991a
plasmids		
pMIR77	Tc^R, P_{todX} ::'*lacZ*	Ramos-González *et al*., 2002
pMIR38	Tc^R, P_{tmoX}::'*lacZ*	Ramos-González *et al*., 2002
pMAX-47-2	Gm^R, derivative of pBBR1MCS-5 containing *tmoST* genes	Ramos-González *et al*., 2002
pET28b	Protein expression plasmid	Novagen
pET28b-TmoS	pET28b containing *tmoS*	This work
pET28b-TmoT	pET28b containing *tmoT*	This work

3.2 Construction of TmoS and TmoT expression plasmid

The *tmoS* and *tmoT* genes were cloned into the expression vector pET28b+ (Novagen). The DNA fragment of *tmoS* was amplified by PCR using the following primers:

Forward: 5´-CTTGTGAGTCATTCCATATGAGCTCCTTGG-3´

Reverse: 5´-ATTCATGTGCTGGGATCCTAACTGACCGG-3´ and plasmid pMAX-47-2 as template (Ramos-González *et al*., 2002). The *tmoT* fragment was amplified using the primers:

Forward: 5'-TTAGGACGCCAGCATATGAATGATCAAGAG-3'

Reverse: 5'-GCCAAAAGCAGGATCCTATTTCAGACTATCCT-3' and genomic DNA of *Pseudomonas mendocina* KR1 as template.

The resulting PCR products were digested with *Nde*I and *Bam*HI and cloned into pET28b(+) (Novagen) linearized with the same enzymes. The plasmids pET28b-TmoS and pET28b-TmoT were verified by sequencing the inserts and flanking regions.

3.3 Overexpression and purification of TmoS

E. coli BL21 (DE3) was transformed with plasmid pET28b-TmoS. Cultures were grown in 2 l Erlenmeyer flasks containing 500 ml of LB medium supplemented with 50 μg/ml kanamycin at 30^{o}C. At an OD_{660} of 0.6, protein expression was induced by the addition of 0.1 mM IPTG. Growth was continued at 16^{o}C overnight prior to cell harvest by centrifugation at 10,000 x g for 30 min. Cell pellets were resuspended in buffer A [20 mM Tris, 0.1 mM EDTA, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, and 5 % (v/v) glycerol, pH 8.0] and broken using a French press at 1,000 psi. After centrifugation at 20,000 x g for 1 hour, the supernatant was loaded onto a 5 ml HisTrap column (Amersham Bioscience), washed with 10 column volumes of buffer A and eluted with an imidazole gradient of 45-500 mM in buffer A. Fractions containing TmoS were dialyzed against analysis buffer 50 mM Tris, 200 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, and 10 % (v/v) glycerol, pH 7.5 for immediate analysis. Purified protein had to be analyzed within 2 days of this dialysis. Protein freezing led to complete loss of activity.

3.4 Isothermal Titration Calorimetry

ITC experiments were conducted using freshly purified protein and a VP-microcalorimeter (Microcal, Amherst, MA). Protein was dialyzed into analysis buffer (50 mM Tris-HCl, 200 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 10 % glycerol, pH 7.5) and placed into the sample cell. Typically, 10 μM TmoS was titrated with 500 μM effector solutions. Since these compounds are volatile and hydrophobic, these solutions were made up in glass vessels immediately before use. 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”.

3.5 Phosphorylation assay of TmoS

Assays were done in 50 mM Tris-HCl, 200 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 % (v/v) glycerol, 2 mM DTT, pH 7.5. The autophosphorylation assay was performed at 4^{o}C with 10 µM purified TmoS in a final reaction volume of 100 µl in the presence or absence of different effectors at a concentration of 100 µM. Reactions were initiated by adding radiolabeled ATP (200 µM ATP containing 4 µCi [γ^{32}P]ATP), and samples were removed at different times. The reaction was stopped by adding 4 X SDS sample buffer. Samples were then stored on ice prior to analysis by SDS-PAGE using 7.5 % (w/v) gels.

3.6 Beta galactosidase measurements

P. putida DOT-T1E bearing pMIR77 (Ramos-Gonzalez *et al.*, 2002, containing a P_{todX}::*lacZ* fusion) and *P. mendocina* KR1 containing pMIR38 (Ramos-González *et al*., 2002) were used. Cells were grown overnight on LB medium supplemented with 10 µg/ml tetracycline. Cultures were diluted 100 times with the same medium and the different compounds were added at a concentration of 1.5 mM to *P. putida* cultures and a concentration of 0.5 mM to *P. mendocina* cultures. When the cultures reached an OD_{600} of 0.8 ± 0.05, β-galactosidase activity was determined in permeabilized cells as described in Ramos-González *et al*. (2002).

4. Results

4.1 Sequence analysis of family members

The sequence alignment of representative members of the TodS/TodT like family is shown in Figure 21. In this alignment the 4 amino acids which participate in the TodS/TodT phosphorelay (H190, D500, H760 of TodS, and D57 of TodT, Busch *et al*., 2009) are strictly conserved. It has been also identified phenylalanine 79 of TodS (located in the PAS 1 domain) as essential for effector binding. Mutation of this residue abolished recognition of agonists and antagonists (Busch *et al*., 2007). In the alignment this residue is also fully conserved. In addition, three other amino acids (F46, I74 and I114) were found to be involved in TodS effector recognition. As shown in Figure 21, the hydrophobic nature of these amino acids is also conserved. These data are consistent with the notion that other family members also employ a phosphorelay mechanism and that similar effector molecules are recognized by the respective SKs.

Most SKs are either transmembrane (TM) proteins or are membrane anchored via TM regions (Krell *et al*., 2010). In this respect TodS was found to differ since it had no predicted TM regions, which was then confirmed by the soluble nature of purified TodS (Lacal *et al*., 2006). Sequences of the remaining family members were submitted to the TM prediction algorithm DAS (Cserzö *et al*., 1997). This analysis predicted all family members to be devoid of TM regions and thus to be soluble proteins, consistent with a cytosolic location.

The 3 D structure of StyR RR from *P. fluorececens* has been solved (Milani *et al*., 2005). StyR is composed of an N-terminal RR receiver domain that is connected via a long helical linker to the DNA-binding domain harbouring a winged helix-turn-helix (HTH) motif. Milani *et al*. (2005) propose a model for the StyR-DNA interaction in which the recognition helix α 8 of the HTH inserts into the major groove of the DNA. According to this model protein-DNA contact is primarily mediated by contacts with helix α 8. The sequence alignment of RRs of this family (Figure 22) revealed a significant conservation of this helix for which the following consensus sequence can be derived: E-x-T-[I/V]-KVHRH-[N/R]-x-[M/T]-K.

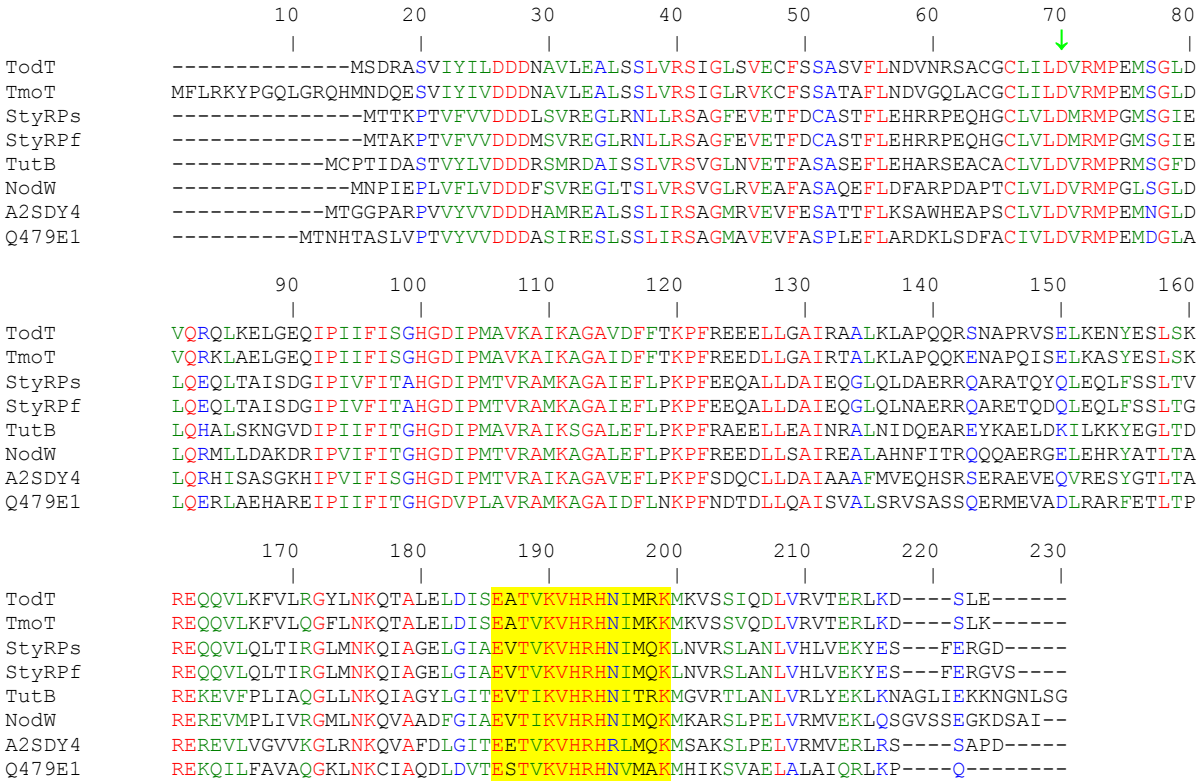


Figure 22. Sequence alignment of TodT like response regulators. The following sequences were used for this alignment: TodT of *P.s putida* DOT-T1E; TmoT of *P. mendocina* KR1, StyR of *Pseudomonas* sp. Y2 (StyR Ps); StyR of *P. fluoresens* (StyR Pf); TutB of *T. aromatica*; NodW of *C. necator* N-1; A2SDY4 of *M. petroleiphilum* PM1 and Q479E1 of *D. aromatica* RCB. The same alignment procedure as detailed in Figure 21 was used. The phosphoryl group accepting aspartate in TodS is highlighted by a green arrow. The region shaded in yellow corresponds to the recognition helix of the helix-turn-helix DNA binding motif of the StyR structure (Milani *et al*., 2005).

The alignment of the promoters P_{todX} and P_{tmoX} (Figure 23) show 68 % of sequence identity. The binding of TodT to P_{todX} has been studied extensively for *P. putida* strains F1 (Lau *et al*., 1997) and DOT-T1E (Lacal *et al*., 2006, 2008a, 2008b). In total 5 monomers were found to bind to boxes 1-3, of which boxes 1 and 2 are pseudopalindromes whereas box 3 corresponds to a half-palindrome. In the alignment of promoters P_{todX} and P_{tmoX} both these binding sites are well conserved and are thus proposed to form TmoT operator sites.

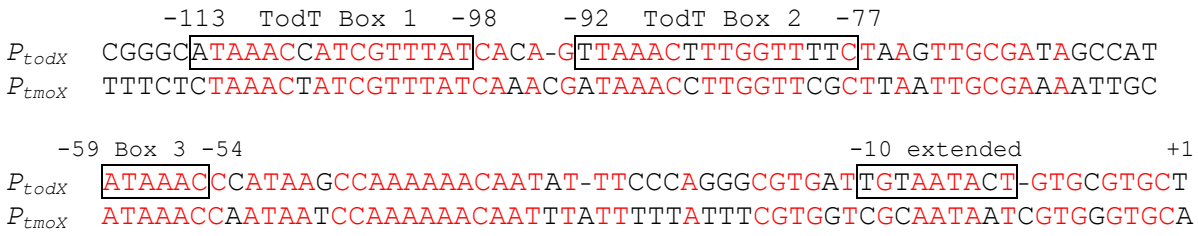


Figure 23. Alignment of the promoters P_{todX} and P_{tmoX}. The three TodT binding sites and the -10 extended region are boxed (Lacal *et al.*, 2008a, 2008b). Transcription start is marked.

In addition, the StyR operator has been experimentally identified for the protein from *P. fluorescens* (Leoni *et al.*, 2005; Rampioni *et al.*, 2008) and operator sites were predicted for the for *Pseudomonas* sp. strain Y2 (Velasco *et al.*, 1998). The alignment of either experimentally determined or predicted operator sites of family members is shown in Figure 24. From this alignment the following consensus can be derived: AAA-x_2-[AT]-x_2-GT-[TA]-[TC].

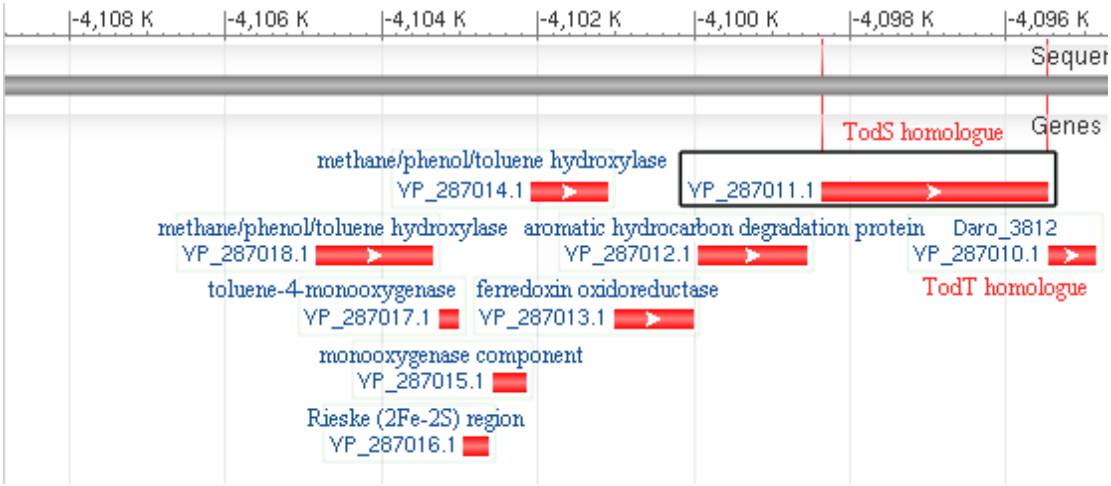
TodT – box 1[1]	-113	ATAAACCATCGTTTAT -98
TodT – box 2[1]	-92	TTAAACTTTGGTTTTC -77
StyR – box 1 (Pf)[2]	-109	GTAAATATAAGTTTAT -94
StyR – box 2 (Pf)[2]	-49	ATAAACCACGGTTTAT -34
StyR – box 3 (Pf)[2]	+16	CAAAAACAAGGTATAA +31
StyR – box 1 (PY2)[3]	-112	ATAAATATAAGTTTCT -97
StyR – box 2 (PY2)[3]	-49	ATAAACCATGGTTTAT -34
StyR – box 3 (PY2)[3]	+15	ACAAAAAAAGGTATAA +30
TmoT – box 1[4]	-115	CTAAACTATCGTTTAT -100
TmoT – box 2[4]	-93	ATAAACCTTGGTTCGC -78
Consensus:		AAA-x_2-[AT]-x_2-GT-[TA]-[TC]

Figure 24. Alignment of operator sites for members of the TodT family.

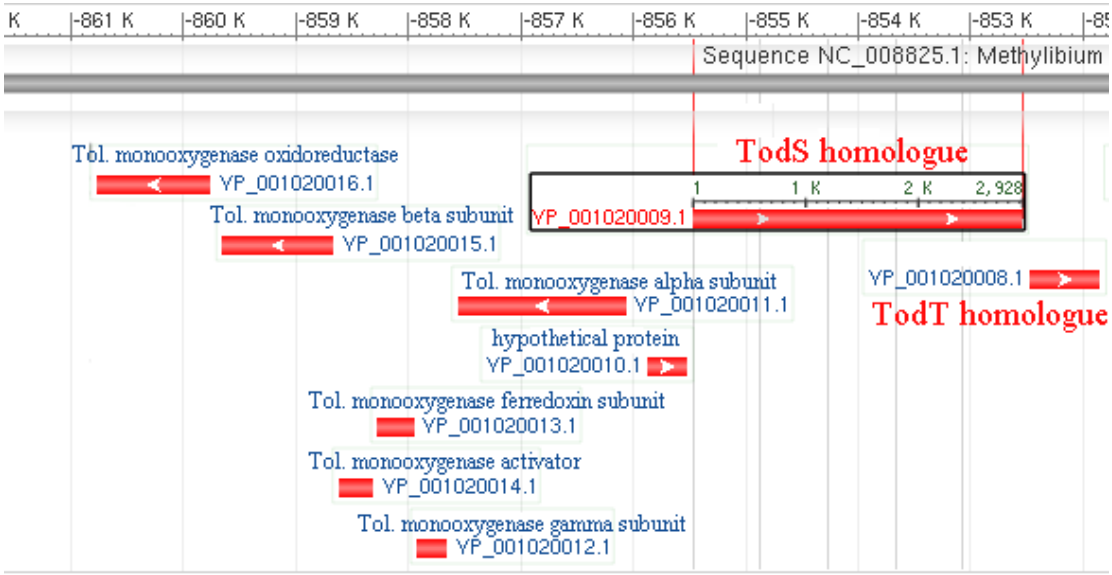
[1] Experimentally determined TodT binding sites for *P. putida* F1 (Lau *et al.*, 1997) and *P. putida* DOT-T1E (Lacal *et al.*, 2006). [2] Experimentally determined StyR sites for *P. fluorescens* (Leoni *et al.*, 2005; Rampioni *et al.*, 2008). [3] Proposed StyR binding sites for *Pseudomonas* sp. strain Y2 (Velasco *et al.*, 1998). [4] Proposed TmoT binding site. The consensus sequence is indicated.

It has been shown that the systems TodS/TodT, TmoS/TmoT, TutC/TutB and StyS/StyR are involved in the regulation of aromatic hydrocarbon degradation routes. Close TodS and TodT homologues have also been detected in *Dechloromonas aromatica* (Salinero *et al*., 2009) and *Methylibium petroleiphilum* (Kane *et al*., 2007) or in the nonobligate predator of soil bacteria *Cupriavidus necator* N-1 (Poehlein *et al*., 2011). Figure 25 shows the genetic environment of the *todS/todT* genes in these microorganisms.

Dechloromonas aromatica



Methylibium petroleiphilum



Cupriavidus necator

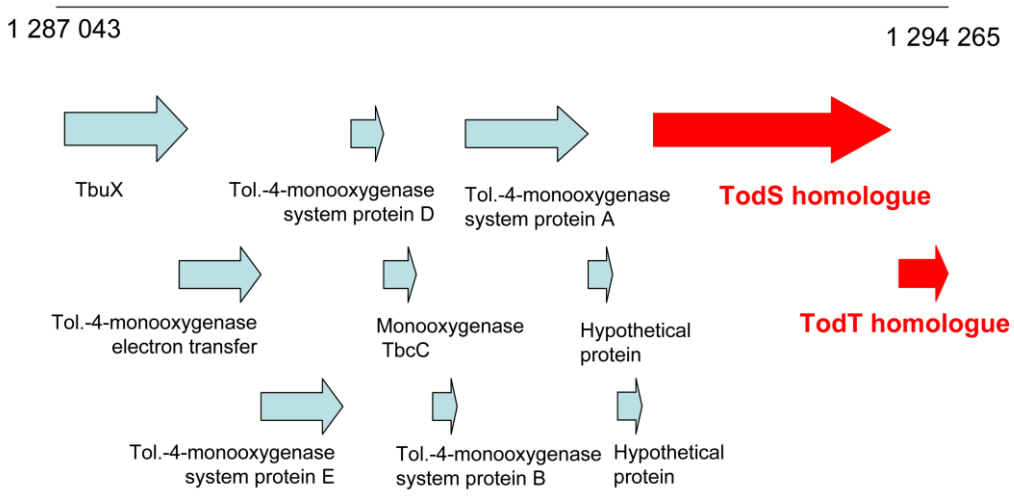


Figure 25. Genetic environment of TodS/TodT homologues in *Dechloromonas aromatica*, *Methylibium petroleiphilum* and *Cupriavidus necator*. Figures were prepared using the genome data bank in ncbi (http://www.ncbi.nlm.nih.gov/genome). In *D. aromatica* and *M. petroleiphilum* genes were present on the chromosome. In the case of *Cupriavidus necator* genes were present on the plasmid pBB1p.

Interestingly, in all three cases these genes are associated with gene clusters encoding different subunits of either toluene-4-monooxygenase or methane/phenol/toluene hydroxylase as well as TodX homologues. To obtain initial information as to whether the TodS/TodT like proteins are involved in the regulation of the expression of these catabolic genes, the DNA segment prior to the initial gene of the catabolic gene were aligned with the well-studied promoter P_{todX} of *P. putida* DOT-T1E. These segments were in detail (see also Figure 25) the segments upstream of YP_287018.1 (methane/phenol/tolune hydroxylase of *D. aromatica*), YP_001020011.1 (toluene monooxygenase α-subunit of *M. petroleiphilum*) and *tbuX* (encoding a TodX homologue) in *C. necator.*

The alignment (Figure 26) shows significant sequence conservation in *D. aromatica* and *C. necator.* In both cases a TodT operator and the IHF binding site appear to be conserved. In *D. aromatica* the presumed TodT binding site matched the consensus sequence described above. In *M. petroleiphilum* sequence similarities are less obvious. Taken together, these observations are consistent with the notion that the family of TodS/TodT like proteins controls the expression of aromatic hydrocarbon degradation pathways.

A) *Dechloromonas aromatica*

```
          4 103 176                                                                  4 103 257
             |                                                                          |
Dechlorom.   CTAAACCAAAGTCTA--ATAGCCGCCTTTGGGCTTTCAGAATAACTTTGTCTCCAGGCGAATTATCGAGAAAAAACGATTATT
P. put.      ATAAACCATCGTTTATCACAGTTAAACTTTGGTTTTCTAAGTTGCGATAGCCATATA-AACCCATAAGCCAAAAAACAATATT

          4 103 258                                                                  4 103 332
             |                                                                          |
P. put.      TCCCAGGGCGTGATTG--TAATACTGTGCGTGCTGTAAGGCGGTGTTTGCCTACTTCACTTTATAAAAAAAATA---AGATG
Dechlorom.   TCGCAGATTTCGGGCGAACAAGATCGCTTTGATTCGGGAATGGCGGGCCCCCAAGTCCTGCTGTAAAGAGGAGACATAGATG
```

The ATG corresponds to the start codon of the methane/phenol/toluene hydroxylase with YP_287018.1 (see Figure25).

TodT boxes 1 and 2 in *P. putida* are shaded in yellow. The IHF binding site is boxed.

B) *Methylibium petroleiphilum*

```
          855 875                                                                855 938
             |                                                                      |
Methylib     CGTGACCTTCGGCA--GTTCGACACCTCACGCATTGAAAGCCGAGGTCGTCTGCGCCGCTCGAGCC
P. put       -ATCAGAAGCGGCATAAACCATCGTTTATCACAGTTAAA-CTTTGGTTTTCTAAGTTGCGATAGCC

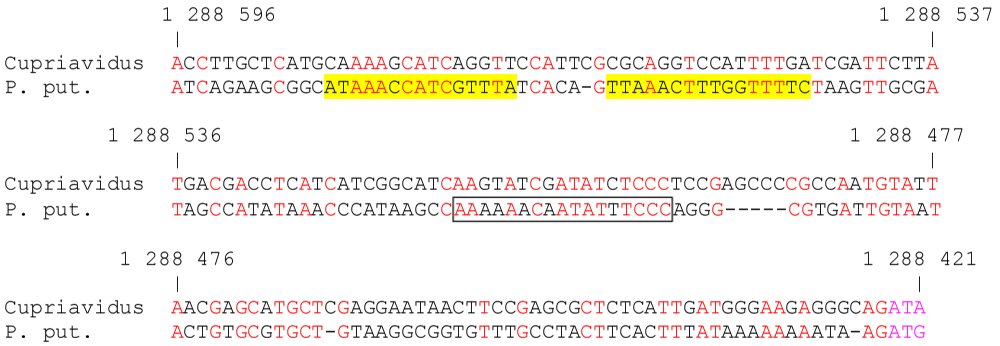
          855 938                                                                856 003
             |                                                                      |
Methylib     GGTGTTGACGCTCCGCGATGAACGCCTCCGTCGATTCGATGCGGTGACTTGGATCGAACGTGTCTG
P. put.      A-TATAAAC-CCATAAGCCAAAAAACAATATT--TCCCAGGGCGTGATTGTAAT---AC-TGTGCG

          856 004                                                            856 061
             |                                                                  |
Methylib     TGTATCAAAGCCCGAGCGCGTTGCCGCTCGGATCTGTCAGGTCTAACGGAGAGAACGATG
P. put.      TGCTGTAAGGC--G-GTGTTTGCCTACTTCACTTTATAA--------AAAAAATAAGATG
```

The ATG corresponds to the start codon of the toluene monooxygenase alpha subunit (YP_001020011.1, see Figure 25)

TodT boxes 1 and 2 in *P. putida* are shaded in yellow.

C) *Cupriavidus necator*



ATA is the initial codon of the TbuX protein (see Figure 25).

TbuX shares 40 % sequence identity with the TodX protein of *P. putida*.

The *todX* gene is the first gene of the *tod* operon in *P. putida*.

The IHF binding site is boxed.

Figure 26. Alignments of the P_{todX} promoter of *P. putida* DOT-T1E with DNA regions preceding the toluene monooxygenase clusters in *Dechloromonas aromatica, Methylibium petroleiphilum and Cupriavidus necator*

4.2 TmoS recognizes a wide range of effectors with very high affinity

To initiate the functional analysis of the TmoS/TmoT TCS, the corresponding genes were cloned into the expression plasmid pET28. Both proteins were expressed in *E. coli* and then purified by affinity chromatography from the soluble fraction of the cell lysate. Despite significant solvent engineering attempts, purified TmoT could not be stabilized in an active conformation. In contrast active TmoS could be obtained but was found to have a reduced stability. All biochemical studies of this protein had to be conducted on the two days following of its purification. Reduced protein stability was also a characteristic for TodS/TodT and might account for the scarceness of functional studies of TRTR type phosphorelay systems as noted by Williams and Withworth (2010). All subsequent analyses were conducted using the experimental conditions used for the study of TodS.

Isothermal titration calorimetry experiments (Krell, 2008) were conducted to study the interaction of purified TmoS with effector molecules. For these experiments 14 compounds were chosen which bind with different affinities to TodS and which had either agonistic or antagonistic effects on its activity (Busch *et al*., 2007; Table 2). For these experiments purified TmoS was titrated with aliquots of effector molecules and resulting heat changes are measured.

Table 2. Thermodynamic parameters derived from the microcalorimetric titrations of TmoS with effectors.

Ligand	Effect on TodS	K_A (M^{-1})	K_D (μM)	ΔH kcal/mol	$T\Delta S$ kcal/mol	[1] $\frac{K_{D\ TodS}}{K_{D\ TmoS}}$
Benzene	Agonist	$(5.96 \pm 0.4)\ 10^6$	0.17 ± 0.01	-5.3 ± 0.1	3.6 ± 0.1	4.5
Toluene	Agonist	$(6.41 \pm 0.5)\ 10^6$	0.15 ± 0.01	-6.5 ± 0.1	2.4 ± 0.1	4.6
Ethylbenzene	Agonist	$(6.39 \pm 0.4)\ 10^5$	1.56 ± 0.1	-3.5 ± 0.1	4.1 ± 0.1	2.0
m-xylene	Agonist	$(1.41 \pm 0.1)\ 10^6$	0.71 ± 0.09	-7.0 ± 0.8	1.1 ± 0.6	1.7
p-xylene	Agonist	$(2.17 \pm 0.3)\ 10^6$	0.46 ± 0.05	-3.4 ± 0.3	4.9 ± 0.29	1.7
m-Chlorotoluene	Agonist	$(4.82 \pm 0.1)\ 10^5$	2.07 ± 0.6	-5.9 ± 0.9	1.5 ± 0.8	4.0
p-Chlorotoluene	Agonist	$(1.24 \pm 0.1)\ 10^6$	0.80 ± 0.09	-2.7 ± 0.1	5.3 ± 0.1	0.4
Chlorobenzene	Agonist	$(7.98 \pm 0.4)\ 10^5$	1.25 ± 0.07	-6.9 ± 0.3	0.9 ± 0.3	1.0
Nitrobenzene	Agonist	$(4.32 \pm 0.5)\ 10^6$	0.23 ± 0.03	-4.1 ± 0.1	4.5 ± 0.1	29
Styrene	Agonist	$(1.24 \pm 0.2)\ 10^6$	0.81 ± 0.1	-4.8 ± 0.3	3.3 ± 0.3	0.7
o-xylene	Antagonist	$(3.48 \pm 0.1)\ 10^6$	0.29 ± 0.01	-8.9 ± 0.1	-0.3 ± 0.1	2.0
o-Chlorotoluene	Antagonist	$(4.68 \pm 0.9)\ 10^5$	2.14 ± 0.4	-5.1 ± 0.9	2.4 ± 0.9	0.3
1,2,4 Trimethylbenzene	Antagonist	$(2.89 \pm 0.4)\ 10^5$	3.46 ± 0.5	-1.5 ± 0.2	5.6 ± 0.2	0.5
cyclohexane	No binding	No binding				

[1] The K_D values for the binding of effectors to TodS are taken from Busch *et al*. (2007).

Figure 27 shows the titration of TmoS with benzene, toluene and ethylbenzene and the derived thermodynamic parameters are given in Table 2. In all cases downwards going peaks are observed indicative of exothermic heat changes implying favourable enthalpy changes. Binding curves for benzene and toluene were very similar and K_D values of 170 and 150 nM were determined, respectively. These affinities are around 4.5 times higher than the corresponding values obtained for TodS. To our knowledge, these values correspond to the highest affinities observed for an interaction of a SK with effector molecules. Ethylbenzene bound with a weaker affinity of 1.5 μM, which however was also tighter than its binding at TodS.

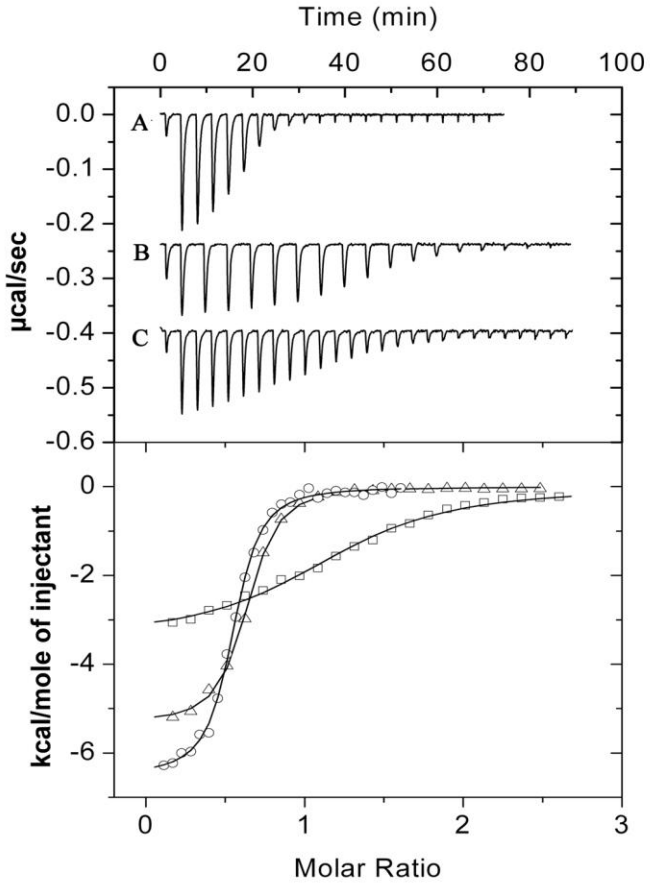


Figure 27. Binding of effector molecules to the purified TmoS. Shown are microcalorimetric titrations of 10 μM TmoS with 500 μM benzene (A), toluene (B) and ethylbenzene (C). Injection volumes were 1.6 μl for (B) and 3.2 μl for (A) and (C). Upper panel: titration raw data. Lower panel: Integrated and dilution-corrected peak areas of raw data. Data were fitted with the "One binding site model" of the MicroCal version of ORIGIN. Δ: benzene; ο: toluene; □ ethylbenzene. The derived thermodynamic parameters are given in Table 2.

Subsequently the binding of methyl- and chloro- substituted toluene isomers were studied. The three xylene isomers bound with affinities in the range of 290-710 nM, which were again higher than the TodS affinities (Table 2). The three chlorotoluene isomers also bound with high affinity. As in the case of TodS, the *para*-substituted isomer bound with highest affinity. The affinities of chlorobenzene and styrene were comparable to the values determined for TodS, whereas nitrobenzene binding to TmoS was 29 times tighter than to TodS. Finally, the triply substituted benzene derivative 1,2,4-trimethylbenzene also showed tight binding. The predominant binding mode was characterised by favourable enthalpy changes supported by equally favourable entropy changes, which is a binding mode frequently observed for hydrophobic interactions. In analogy to TodS, cyclohexane, the only ligand which lacks an aromatic ring, failed to bind to TmoS. It can be concluded that TmoS recognizes a very wide range of different aromatic effector molecules.

4.3 TmoS ligands can be classified into agonists and antagonists

A feature of TodS which remains poorly understood is its differential response to agonists and antagonists (Busch *et al*., 2007). Agonists, which stimulate kinase activity, increase also gene expression, whereas antagonists, which bind but which do not stimulate kinase activity, fail to upregulate gene expression. The presence of antagonists was shown to reduce the magnitude of agonist-mediated upregulation of gene expression (Busch *et al*., 2007).

Subsequent experiments were aimed at identifying whether such behaviour is also observed for TmoS. To this end purified TmoS was subjected to autophosphorylation assays with 4 TodS agonists (toluene, benzene, chlorobenzene and ethylbenzene) and 3 antagonists (*o*-xylene, *o*-chlorotoluene, 1,2,4-trimethylbenzene). All of these compounds have been found to bind to with affinitites between 0.15-3.5 μM to TmoS (Table 2). Agonists were chosen to cover the magnitudes of regulatory responses as observed in beta galactosidase measurements and include the most efficient agonist (toluene), the weakest agonist (ethylbenzene) and two effectors with an intermediate activity (benzene and chlorobenzene) (Busch *et al*., 2007). TmoS was incubated during 30 minutes with [γ^{32}P]ATP in the absence and presence of saturating concentrations of

effector molecules prior to SDS-PAGE. The first lane in figure 28A shows the phosphorylation state of TmoS in the absence of effector. Interestingly, the 4 TodS agonists increased phosphorylation of TmoS. In contrast, the TmoS phosphorylation state in the presence of the 3 TodS antagonists was very similar to the control. These data indicate that the existence of agonists/antagonists is a feature common to TodS and TmoS.

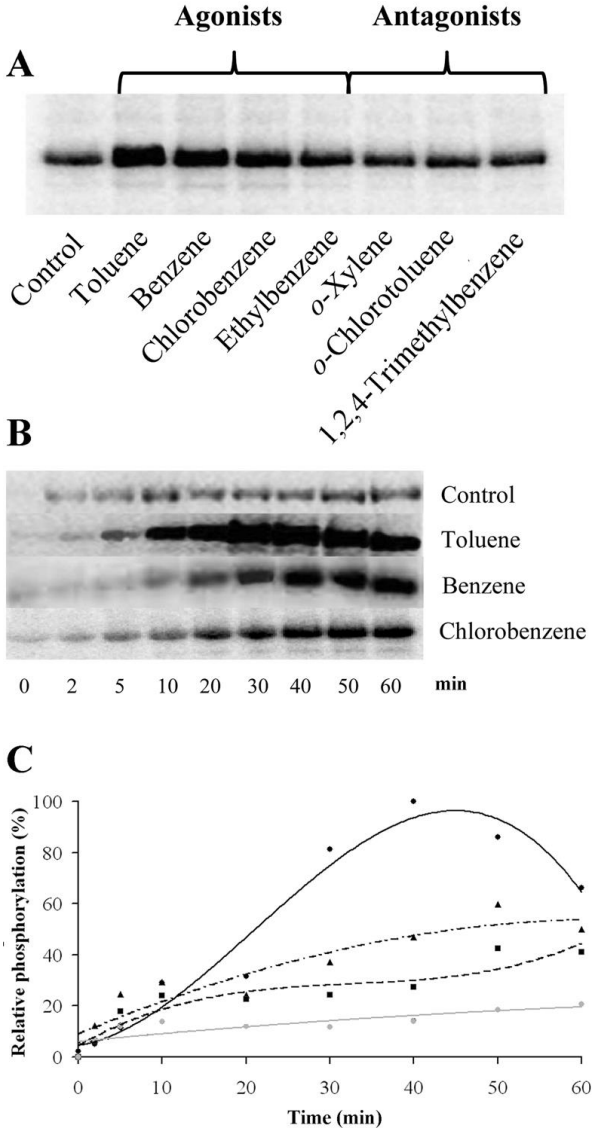


Figure 28. Autophosphorylation of TmoS in the absence and presence of agonists and antagonists. Assays were conducted as described in Materials and Methods. A) Autophosphorylation of TmoS in the absence and presence of 100 μM effector molecules. Autophophorylation reactions were stopped after 30 minutes. B) Kinetics of autophosphorylation of TmoS in the presence of 100 μM of the effector molecules indicated. C) Densitometric analysis of data presented in B:●, toluene; ▲, benzene; ■, chlorobenzene; grey line: buffer control.

4.4 Differential impact of agonists on the stimulation of TmoS autophosphorylation

A feature which has not been assessed for TodS concerns the question whether sensor protein saturated with different agonists differs in its capacity to stimulate autokinase activity. Figure 28A also reveals that the amount of autokinase stimulation in the presence of the 4 agonists differs significantly. Toluene caused the most pronounced increase followed by benzene, chlorobenzene and ethylbenzene. Under the experimental conditions used TmoS is entirely saturated with effector molecules which were added at a concentration of 100 μM which is largely superior to the respective K_D values. To study the effect of toluene, benzene and chlorobenzene on TmoS in more detail, the kinetics of its autophosphorylation was analysed (Figure 28B, C). In the presence of toluene maximal phosphorylation was observed after 40 minutes and the reduction in autophosphorylation is due to the depletion of ATP in the reaction mixture and the intrinsic dephosphorylation activity. The stimulation of autokinase activity in the presence of benzene and chlorotoluene was significantly inferior to the experiments conducted with toluene. In both cases a steady increase in TmoS autophosphorylation was noted, which was slightly more pronounced for benzene.

4.5 The TmoS/TmoT system activates transcription from P_{tmoX} in response to a wide range of aromatic compounds

To compare the capacities of the TodS/TodT and the TmoS/TmoT systems to induce gene expression in response to a wide range of different aromatic compounds, beta-galactosidase measurements were conducted. To this end, fusions of promoters P_{todX} and P_{tmoX} with the *lacZ* gene were introduced into *P. putida* DOT-T1E and *P. mendocina* KR1, respectively. In total 54 mono- and biaromatic compounds were selected for screening. The initial enzymes of both pathways, toluene 2,3-dioxygenase and toluene-4-monooxygenase, are characterised by a wide substrate range and most compounds selected are substrate of one or the other enzyme (Boyd *et al*., 2006; Feingersch *et al*., 2008; Gibson and Parales, 2000; Tao *et al*., 2004a, 2004b).

The data initially reported on the P_{todX} expression were obtained using a protocol in which effector at a final concentration of 1.5 mM is added to the bacterial cultures

(Lacal *et al*., 2006). Initial experiments with *P. mendocina* KR1 revealed that this effector concentration is toxic for this strain. Therefore, the effector concentration was reduced to 0.5 mM for experiments with *P. mendocina* KR1, whereas all experiments with *P. putida* DOT-T1E were conducted with 1.5 mM effector. It was shown previously that the solvent resistance of strain DOT-T1E is largely due to the action of the plasmid-encoded efflux pump TtgGHI (Rojas *et al*., 2001).

The basal activities of promoters P_{todX} and P_{tmoX} was of 2 $\pm$ 1 and 15 $\pm$ 4 Miller units, respectively. Effectors which caused activities larger than two times the basal activities were considered as compounds which induce gene expression. From the 54 compounds analyzed, 22 compounds induced gene expression in both systems. A plot of the activities of both promoters in response to this set of 22 compounds is shown in Figure 29.

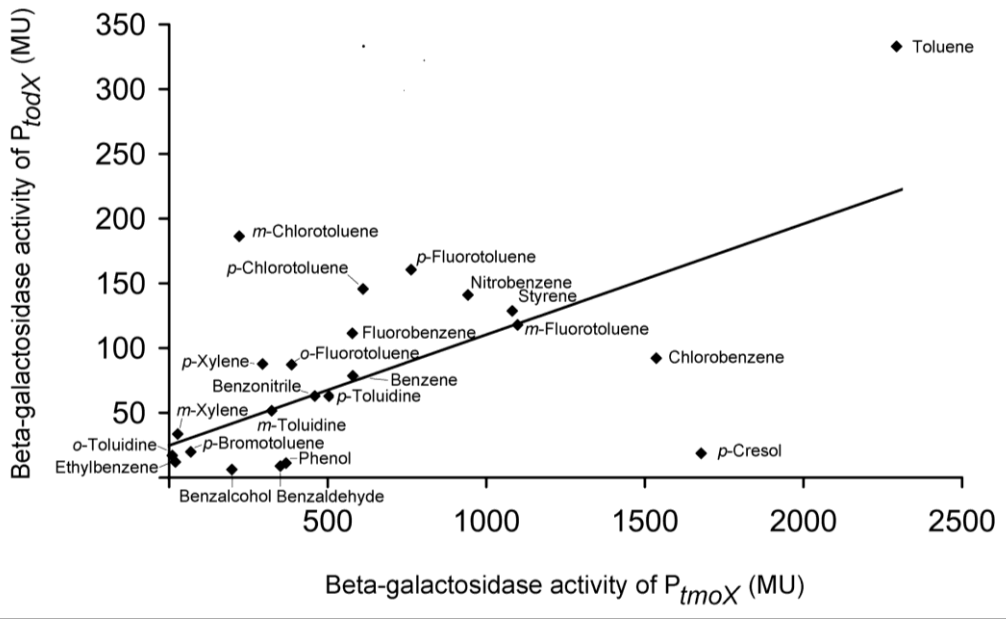


Figure 29. Expression from promoters P_{todX} and P_{tmoX}. Shown is a plot of beta galactosidase activity of P_{todX} (y-axis) against the corresponding value of P_{tmoX} (x-axis) for different compounds. Beta galactosidase measurements were carried our as described in Materials and Methods. Note that 1.5 mM of each compound were added to *P. putida* DOT-T1E harbouring pMIR77 (P_{todX} *::'lacZ*), whereas 0.5 mM of each compound were added to *P. mendocina* harbouring pMIR38 (P_{tmoX} ::'*lacZ*).
Expression in the absence of any added compound was found to be 2 $\pm$ 1 and 15 $\pm$ 4 MU, respectively, for P_{todX} and P_{tmoX}. Shown in this graph are the compounds for

which an activity of at least twice the basal rate is observed for both promoters. Compounds which were analysed but which did not induce any of the promoters are: 1-hexanol, cyclohexane, propyl-, butyl- and isopropylbenzene, the 3 trimethylbenzene isomers, 1,2,4 trihydroxybenzene, 1,2,4 trichlorobenzene, *o*-xylene, *o*-chlorotoluene, the 3 iodotoluene isomers, the 3 nitrotoluene isomers, *m*-ethyltoluene, benzenesulfonic acid, benzamide, *p*-hydroxybenzaldehyde, *p*-toluylaldehyde, 2,3 dimethylphenol, resorcinol, hydroquinone, benzoate, p-hydroxybenzoate, naphthalene, 1,2,3,4 tetrahydroxynaphthalene. Experiments were means of at least three independent experiments conducted in triplicates. The derived standard deviations are in all cases below 25 % of the mean. Part of the measurements of P_{todX} activity have been reported in Busch *et al*. (2007).

The effector profile of P_{todX} was only marginally larger than that of P_{tmoX} since only two compounds which were weak inducers of P_{todX}, namely catechol (13 $\pm$ 2 MU) and *m*-bromotoluene (9 $\pm$ 2 MU), failed to induce P_{tmoX}. The remaining 30 compounds, which are listed in the legend to Figure 29, neither induced P_{todX} nor for P_{tmoX}. Among these 30 compounds were *o*-xylene, *o*-chlorotoluene and 1,2,4-trimethylbenzene, which were shown to bind tightly to purified TmoS (Table 2) but which failed to increase its phosphorylation state (Figure 28). To elucidate whether compounds that bind but do not induce gene expression reduce the toluene-mediated upregulation in gene expression, beta galactosidase measurements with mixtures of toluene with *o*-xylene or *o*-chlorotoluene were conducted. Cultures of *P. mendocina* KR1 harboring pMIR38 were grown in LB to an OD_{600} of 0.2, at which point 0.25 mM *o*-chlorotoluene, *o*-xylene or the corresponding volume of buffer was added to three cultures. At an OD_{600} of 0.5 0.25 mM toluene was added to these cultures and β-galactosidase activity was measured after another 2 hours of growth. Similarly to the analogous experiments reported for the TodS/TodT system (Busch *et al*., 2007) the presence of *o*-xylene and *o*-chlorotoluene reduced the toluene mediated gene expression to 55 $\pm$ 7 and 32 $\pm$ 6 %, respectively, of the value obtained for the culture containing toluene only.

Apart from the observation that the set of effectors which induce the expression from both promoters *in vivo* is almost identical, several other conclusions can be drawn. Firstly, toluene was the compound which had in both cases the most pronounced effect of gene expression. Toluene is one of the 3 pathway substrates for the TOD pathway and also the substrate of the T4MO pathway, but it is not known whether the

T4MO pathway mineralizes further compounds. However, the case of toluene demonstrates a clear link between the potency in pathway expression and degradability by the corresponding pathway. Secondly, despite the fact that the effector concentration added to *P. mendocina* was only a third of the concentration used for *P. putida*, the induction in gene expression of P_{tmoX} was largely superior to that of P_{todX}. The data shown in Figure 29 were fitted by a linear regression model, resulting in the dependency y=0.085x + 28.0 (r^2=0.42).

This implies that the regulatory response of the P_{tmoX} system is on average around 12 times stronger than that of P_{todX}. Thirdly, the linear regression shown in Figure 29 is characterized by an r^2=0.42, indicative of a modest correlation between both sets of measurements. This is consistent with the idea that the capacity of different compounds to induce gene expression has been conserved during evolution to a certain degree. Fourthly, the minimal structural requirement of an effector capable of inducing both promoters appears to be the presence of a single aromatic ring since experiments in the presence of 1-hexanol, cyclohexane and the biaromatic naphthalene and 1,2,3,4 tetrahydroxynaphthalene showed no promoter activity. Lastly, benzene was found to be more efficient than chlorobenzene to increase TmoS autophosphorylation (Figure 28). This, however, was not reflected in the gene expression studies (Figure 29) where a superior activity was observed for chlorobenzene. We are currently unable to provide an explanation for this observation. To characterise the dependency of binding affinity of agonists to purified TodS and the induced increase in gene expression, the K_A values (Table 2) were plotted against the beta galactosidase measurements (Figure 30).

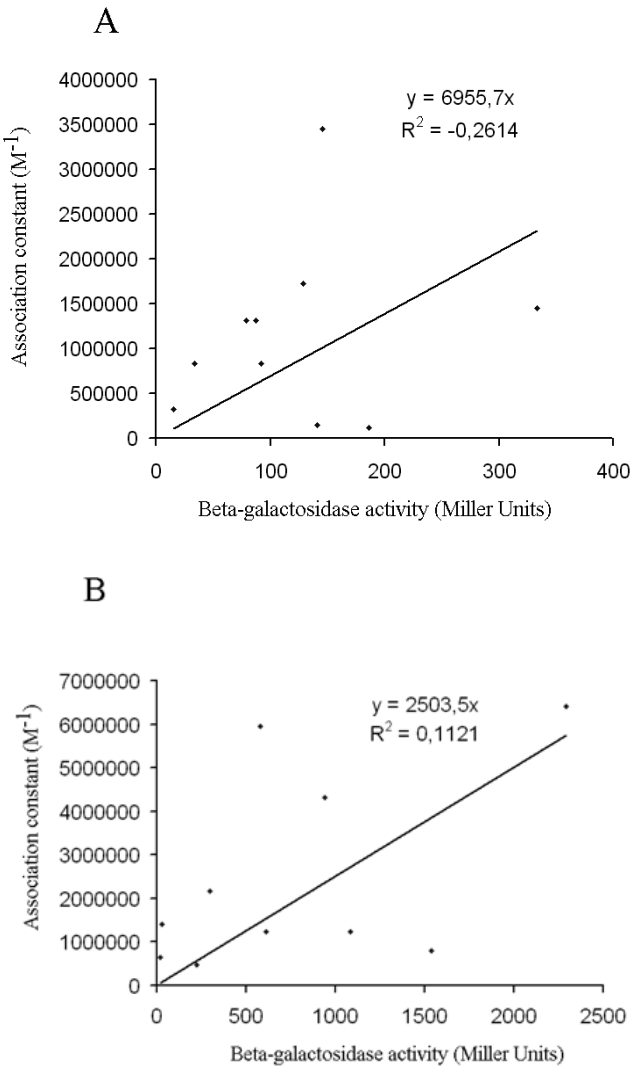


Figure 30. Plot of beta-galactosidase measurements for the promoter P_{todX} (A) and P_{tmoX} (B) against the association constants determined for the binding of different agonists to purified TodS (A) or TmoS (B). Values for TodS were taken from Busch *et al*. (2007) and values for TmoS were taken from Table 2 of this work.

The resulting r^2 values of the corresponding linear fits were -0.26 (for the TodS/T system) and 0.11 (for the TmoS/T system). This indicates that there is only a weak correlation between the binding affinity and the response observed *in vivo*.

5. Discussion

The combined interpretation of data available on the TmoS/TmoT and the TodS/TodT system can be used to propose features which might be common to the protein family. The majority of SKs possess either one or several TM regions (Krell *et al*., 2010; Mascher *et al*., 2006). Purified TodS and TmoS are soluble proteins in the absence of detergents. In addition the prediction of TM regions for the TodS like SKs (Figure 21) resulted in all cases in an absence of such regions. Taken together it appears that the family of TodS like SKs forms part of the small group of soluble SKs with cytosolic location (Mascher *et al*., 2006).

TodS like proteins are involved in the regulation of degradation pathways of toxic compounds. It has been well documented that the major bacterial resistance mechanism towards organic solvents consists in its expulsion into the medium (Ramos *et al*., 2009). This implies that there are significant differences between the cytosolic and extracytoplasmic concentration of these compounds. In this context the detection of signal molecules in the periplasm might not reflect well the availability of cytosolic pathway substrates and the regulatory events triggered may be of little precision. In contrast, cytosolic substrate sensing reflects well the concentration of available substrates and the resulting responses are more precise. Many soluble cytosolic SKs possess a complex domain arrangement with frequently multiple sensor domains (Krell *et al*., 2010). All TodS like SK sequences harbour two PAS domains of which the PAS 1 domain was found to sense aromatic effectors in the case of TodS (Lacal *et al*., 2006). Based on sequence similarities with FixL, Lau *et al*. (1997) proposed that the PAS 2 is involved in oxygen sensing. The sensing of a secondary signal molecule present in the cytosol might thus result in a fine-tuning of the regulatory response.

Another striking feature is the high affinity by which signal molecules are recognized by TmoS and TodS. Since toluene is toxic to the cell at higher concentrations, the degradation pathways have evolved to respond to low substrate concentrations. The kinetic characterization of the initial enzyme of the T4MO pathway, the toluene-4-monooxygenase, resulted in a K_M of 4 μM for toluene (Mitchell *et al*., 2002).

TmoS and TodS bind many agonists with nanomolar affinity. Busch *et al*. (2007) have shown that tight toluene binding translates into an increase in protein autophosphorylation at low toluene concentrations, as evidenced by a half-maximal autophosphorylation at 10 μM. The discrepancy between the affinity of TodS for toluene (0.7 μM) and the concentration at which half maximal autokinase stimulation is achieved (10 μM) shows that initial ligand recognition must not trigger subsequent molecular events in the signalling cascade in an immediate manner. However, data show that transcriptional activation sets in at low toluene concentrations, which in turn permits toluene utilization at sub-lethal concentrations. The toluene degradation mechanism has co-evolved with toluene resistance mechanism. The action of the TtgGHI efflux pump was found to be the primary determinant for toluene resistance (Rojas *et al*., 2001). Pump expression is under the control of the TtgV repressor, which recognizes toluene and de-represses pump expression (Guazzaroni *et al*., 2004). However, TtgV was found to bind toluene with a relatively weak affinity of 118 μM (Guazzaroni *et al*., 2005), which is far above the concentration at which transcriptional activation occurs (Busch *et al*., 2007).

For most TCSs the cognate signals are unknown (Krell *et al*., 2010). For some TCS with known signal molecule the corresponding binding constants have been determined. For example citrate binds to the CitA SK with a K_D of 5.5 μM (Kaspar *et al*., 1999, also determined by ITC), nitrate to NarX with a K_D of 35 μM (Lee *et al*., 1999, as determined by autophosphorylation stimulation), oxygen to FixL with a K_D of 50 μM (Gilles-Gonzalez *et al*., 1994, determined by spectroscopic techniques) or Mg^{2+} to PhoQ with a K_D of 300 μM (Lesley and Waldburger, 2001, determined by circular dichroism and fluorescence spectroscopy). In this context the affinities of toluene for TmoS (0.15 μM) and TodS (0.69 μM) are the highest affinities reported. We suggest that the toxic nature of these effectors is one reason for the high affinity.

Gene expression studies of promoters P_{todX} and P_{tmoX} in the presence of 54 different effectors showed that the same set of 22 compounds (agonists) activated transcription in both cases (Figure 29), whereas 30 other compounds did not induce any of the two promoters.

The magnitude of transcriptional activation of these 22 compounds appeared to correlate for both promoters since the linear regression showed a r^2 of 0.42. In both cases toluene was the most potent inducer. The common structural feature of these 22 activating compounds was the presence of a single aromatic ring. We have shown previously that phenylalanine 79 is essential for effector binding since its mutation abolished effector recognition (Busch *et al*., 2007). Effector molecules are likely to interact via pi stacking with this residue. Interestingly, this phenylalanine is conserved among all family members (Figure 21). However, many of the 22 agonists share striking structural similarities with the 30 compounds that did not induce transcription. In both cases the *m*- and *p*- isomers of xylene and chlorotoluene induced transcription whereas both *o*-isomers were inactive. We show that *o*-xylene and *o*-chlorotoluene bind to TmoS with affinities comparable to the other isomers (Table 2). However, the binding of these compounds did not trigger an increase in TmoS autophosphorylation. The data presented thus suggest that the existence of agonists and antagonists is not a feature specific to TodS but might correspond to a characteristic of the entire family. Environmental pollutants can be understood as a mixture of agonists and antagonists. Bacteria exposed to complex mixtures were found to express degradation pathways inefficiently (Cases and de Lorenzo, 2005), which might be partly due to the combined action of agonists and antagonists.

We have shown that effector binding to TodS and TmoS can either cause an agonistic or antagonistic effect. In this work we have also compared the magnitudes of the effects of the agonists toluene, benzene and chlorobenzene on the TmoS phosphorylation state (Figure 28). The measurement of protein autophosphorylation under conditions guaranteeing complete saturation with effectors showed significant differences (Figure 28). Exposure to toluene caused the most pronounced increase, followed by benzene and chlorobenzene. Therefore, TmoS effector molecules can be classified into agonists and antagonists and, in addition, agonists differ in their capacity to increase TmoS gene expression. This might be due to different binding modes of agonists to the effector binding pocket which has multidrug binding properties.

Similar observations have been made for the binding of aromatic compounds to TtgV (Guazzaroni *et al.*, 2007). In summary, data presented here permit to initiate the definition of a family of TCS and advances the understanding of the so far poorly characterised phosphorelay systems of the type TRTR. The work provides also important insight into entirely cytosolic TCS, for which only very scarce information is available.

6. References

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CHAPTER II

High specificity in CheR methyltransferase function CheR2 of Pseudomonas putida is essential for chemotaxis, whereas CheR1 is involved in biofilm formation

Adapted from: High specificity in CheR methyltransferase function CheR2 of Pseudomonas putida is essential for chemotaxis, whereas CheR1 is involved in biofilm formation

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1. Abstract

Chemosensory pathways are a major signal transduction mechanism in bacteria. CheR methyltransferases catalyze the methylation of the cytosolic signaling domain of chemoreceptors and are amongst the core proteins of chemosensory cascades. These enzymes have primarily been studied *Escherichia coli* and *Salmonella typhimurium* that possess a single CheR involved in chemotaxis. Many other bacteria possess multiple *cheR* genes. Since the sequences of chemoreceptor signaling domains are highly conserved, it remains to be established with what degree of specificity CheR paralogues exert their activity. We report here a comparative analysis of the three CheR paralogues of *Pseudomonas putida*. Isothermal titration calorimetry studies show that these paralogues bind the product of the methylation reaction, S-adenosylhomocysteine (SAH), with much higher affinity (K_D of 0.14–2.2 μM) than the substrate S-adenosylmethionine (SAM, K_D of 22-43 μM), which indicates product-feedback inhibition. Product binding was particularly tight for CheR2. Analytical ultracentrifugation experiments demonstrate that CheR2 is monomeric in the absence and presence of SAM or SAH. Methylation assays show that CheR2, but not the other paralogues, methylates the McpS and McpT chemotaxis receptors. The mutant in CheR2 was deficient in chemotaxis whereas mutation of CheR1 and CheR3 had either no or little effect on chemotaxis. In contrast, biofilm formation of the CheR1 mutant was largely impaired but not affected in the other mutants. We conclude that CheR2 forms part of a chemotaxis pathway and CheR1 of a chemosensory route that controls biofilm formation. Data suggest that CheR methyltransferases act with high specificity on their cognate chemoreceptors.

2. Introduction

Bacteria need to constantly sense and adapt to changing environmental conditions to assure survival. This important function is primarily mediated by one-component systems, two-component systems and chemosensory pathways (Galperin, 2005; Sourjik and Wingreen, 2012; Ulrich *et al*., 2005; Wuichet and Zhulin, 2010). The latter pathways have initially been described since they mediate flagellum mediated taxis but more recent studies have shown that these routes are also involved in type IV pili based taxis or may carry out alternative cellular functions (Wuichet and Zhulin, 2010). A bioinformatic study has led to the identification of core proteins that are present with high frequency in chemosensory pathways and a series of auxiliary proteins that are found with lower frequency (Wuichet and Zhulin, 2010). Core proteins are the chemoreceptors, the CheA histidine kinase, the CheW coupling protein, the CheY response regulator as well as the CheR methyltransferase and the CheB methylesterase. The central feature of chemosensory pathways is the ternary complex formed by the chemoreceptor, the CheA histidine kinase and the CheW coupling protein. The function of chemosensory pathways is based on the concerted action of excitatory and adaptational mechanisms. Canonical pathway excitation is triggered by the recognition of signal molecules at the chemoreceptor sensor domain that causes a molecular stimulus that is transduced across the membrane where it modulates CheA autophosphorylation and in turn transphosphorylation activity towards the response regulator CheY. When phosphorylated, CheY undergoes a conformational change, which, in the case of chemotaxis pathways, permits an interaction with the flagellar motor causing a modulation of its activity (Hazelbauer *et al*., 2008).

Adaptation mechanisms have evolved to assure a restoration of the pre-stimulus behavior in the presence of the stimulus (Lazova *et al*., 2011; Min *et al*., 2012; Vladimirov and Sourjik, 2009; Yuan *et al*., 2012). The canonical adaptation mechanism consists in the methylation and demethylation of chemoreceptors by the CheR methyltransferase and CheB methylesterase, respectively. The fact that CheR is amongst the core proteins of chemosensory pathways is also supported by studies that show that *cheR* mutation either abolishes or impairs aerotactic or chemotactic behavior in many different species (Ely *et al*., 1986; Kanungpean *et al*., 2011; Stephens

et al., 2006; Stock *et al*., 1981; Wong *et al*., 1995). The CheR methyltransferases from *E. coli* and *S. typhimurium* have been studied in the past. It was shown that ligand binding at the chemoreceptor increases CheR-mediated methylation of 4-6 glutamate residues at the chemoreceptor signaling domain, which in turn modulates the capacity of the receptor to alter CheA autophosphorylation (Borkovich *et al*., 1992; Terwilliger and Koshland, 1984). Receptor methylation was found to significantly alter the affinity of signal molecules for the MCP-CheA-CheW ternary complex (Li and Weis, 2000).

CheR uses S-adenosylmethionine (SAM) as substrate and the methylation reaction gives rise to S-adenosylhomocysteine (SAH) (Simms and Subbaramaiah, 1991). Interestingly, SAM and SAH compete for binding to the same site at CheR. Since SAH was found to bind tighter than SAM, CheR is thus subject to product feedback inhibition (Simms and Subbaramaiah, 1991; Simms *et al*., 1987; Yi and Weis, 2002). As a consequence biological methylation processes were found to be inhibited by SAH. The three-dimensional structure of CheR from *S. typhimurium* has been solved in complex with SAH and the pentapeptide NWETF (Djordjevic and Stock, 1998). This pentapeptide is present at the C-terminal extension of the enterobacterial high-abundance chemoreceptors and represents a site for CheR tethering to the chemoreceptor (Muppirala *et al*., 2009).

Much of what we know on chemosensory pathways is due to the study of flagellum mediated taxis in *E. coli* and *S. typhimurium* (Krell *et al*., 2011; Sourjik and Wingreen, 2012). Both species contain a single chemosensory pathway, a single *cheR* gene and a limited number of chemoreceptors. Many free living bacteria contain multiple copies of *cheR* genes (Hamer *et al*., 2010) and significantly more chemoreceptor genes than *E. coli* or *S. typhimurium* (Lacal *et al*., 2010a). The site of CheR methylation lies within the cytoplasmic signaling domain of chemoreceptors which is the most conserved domain of all proteins that participate in chemosensory pathways (Le Moual and Koshland, 1996; Zhulin, 2001). This hence raises the question of the specificity of the CheR – chemoreceptor interaction.

We have addressed this question using *Pseudomonas* as model organism. In *Pseudomonas aeruginosa* 5 gene Clusters encoding signaling proteins have been identified that form 4 chemosensory pathways (Ferrández *et al*., 2002). Two pathways,

termed *che* (encoded by Clusters I and V) (Kato *et al*., 1999; Masduki *et al*., 1995) and *che2* (encoded by Cluster II) (Ferrández *et al*., 2002) have a role in chemotaxis. The third pathway (*wsp)* (Hickman *et al*., 2005) regulates cyclic diguanylate concentrations (formed by the Cluster III gene products) which in turn was found to modulate biofilm formation. The fourth pathway, *chP* (Cluster IV genes), modulates the cAMP level (Fulcher *et al*., 2010) and consequently several other features including type IV pili synthesis and twitching motility (Darzins, 1994; Kearns *et al*., 2001). Since the signaling proteins of these pathways are paralogous, there exists thus the possibility of cross-talk between pathways.

Many strains of *P. putida* show an elevated resistance to stress factors and due to their metabolic versatility are able to degrade a series of toxic compounds, which offers the possibility of using these strains for the biodegradation of pollutants and biotransformation purposes (Ramos *et al*., 2009; Timmis, 2002). A series of reports show that chemotaxis towards pollutants increases biodegradation efficiency (reviewed in Lacal *et al*., 2013) and a pollutant chemoreceptor, McpT has recently been identified (Lacal *et al*., 2011a). Despite the biotechnological importance of *P. putida* strains no information as to the existence of individual chemosensory routes is available. Both, *P. aeruginosa* and *P. putida,* contain 26 chemoreceptors genes but the organization of genes encoding the cytosolic signaling proteins is different in these two species (Figure 31, Ferrández *et al*., 2002).

To close this gap of knowledge and to assess the specificity of multiple CheR paralogues, we report here a study of the three CheR paralogues of *P. putida* KT2440. In the first part of this research, the three recombinant proteins were analyzed by different biophysical techniques. We then demonstrate that exclusively CheR2 methylates the McpS (Lacal *et al*., 2010b, 2011b; Pineda-Molina *et al*., 2012) and McpT (Lacal *et al*., 2011a) chemotaxis chemoreceptors.

The analysis of the three *cheR* mutants revealed that CheR2 is essential for chemotaxis, confirming the methylation assays, whereas mutation of CheR1 caused a dramatic reduction in biofilm formation. Data show a high specificity of action of the CheR paralogues and point to the existence of two signaling pathways that control either chemotaxis or biofilm formation.

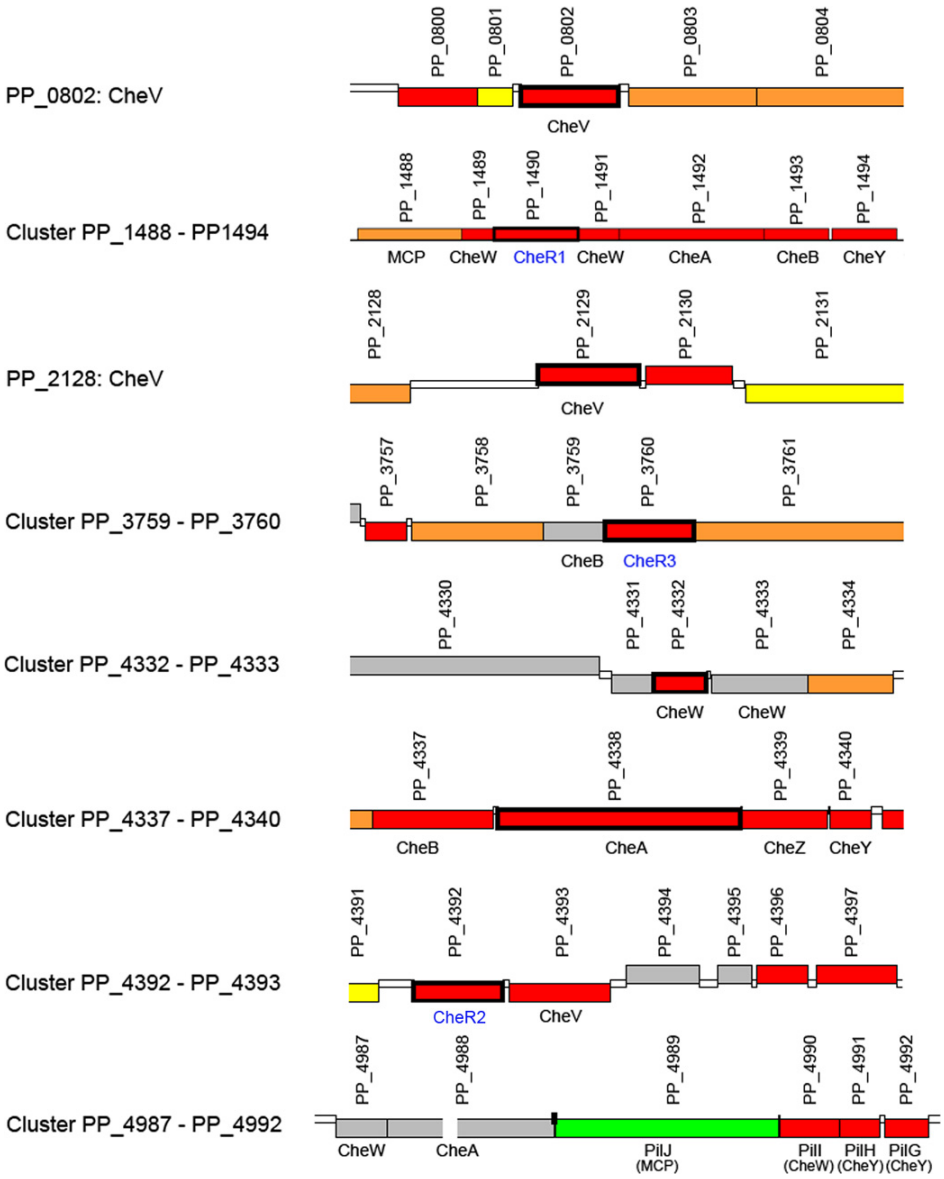


Figure 31. Predicted genes for chemosensory signaling proteins in *P. putida* KT2440. Gene annotation is according to *Pseudomonas* Genome Database. The three CheR paralogues analyzed in this article are shown in blue.

3. Material and Methods

3.1 Strains and Plasmids

Table 3. Strains and plasmids used in this study.

	Features	Reference
Strains		
P. putida KT2440	mt-2 pWW0 cured, TolS$^-$	Nelson *et al*., 2002
P. putida DOT-T1E	Tol$^+$, wild type	Ramos *et al*., 1995
P. putida KT2440R	Rif^r, derivative of *P. putida* KT2440	Espinosa-Urgel and Ramos, 2004
P. putida KT2440R-TK1038	Rif^r, *cheR1*::mini-Tn*5* Km^r	Duque *et al*., 2004
P. putida KT2440R-TK1116	KT2440R with plasmid pCHESI-3760 inserted into the *cheR3* gene, Km^r (KT2440R *PP3760*::ΩKm, Km^r)	This study
P. putida KT2440R-TK1117	KT2440R with plasmid pCHESI-4392 inserted into the *cheR2* gene, Km^r (KT2440R *PP4392*:: ΩKm, Km^r)	This study
E. coli BL21 (DE3)	F$^-$, *ompI, hsdS$_B$* (r^-_B m^-_B)	Studier and Moffatt, 1986
E. coli C41 (DE3)	F – *ompT hsdSB* (*rB- mB-*) *gal dcm* (DE3)	Miroux and Walker, 1996
E.coli BL21 Star^{TM} (DE3)	F- *ompT hsdSB (rB-mB-) gal dcm rne131* (D3)	Invitrogen
E. coli DH5α	*supE44 ΔlacU169* (ϕ80 *lacZΔ*M15) *hsdR1 recA1 endA1 gyrA96 thi1 relA1*	Hanahan, 1983
E. coli DH5α-TK1043	DH5α with pGEM-T-3760, Ap^r	This study
E. coli DH5α-TK1044	DH5α with pGEM-T -4392, Ap^r	This study
E. coli DH5α-TK1048	DH5α with pChesi-3760, Km^r	This study
E. coli DH5α-TK1049	DH5α with pChesi-4390, Km^r	This study
Plasmids		
pET200/DTOPO	Km^r, protein expression vector	Invitrogen
pET28b(+)	Km^r, protein expression vector	Novagen
pET28b-WspC-Pp	Km^r, pET28b(+) derivative	Muñoz-Martínez *et al*., 2012
pET28b-CheR2	Km^r, pET28b(+) derivative	This work
pET28b-CheR3	Km^r, pET28b(+) derivative	This work
pET200/D-TOPO	Km^r, protein expression vector	Invitrogen
pET200/D-TOPO-mcpT	*mcpT* gene inserted in pET200/D-TOPO, Km^R	This work
pET28b-mcpS	*mcpS* gene inserted in pET28b(+), Km^R	This work
pGRT1	large self-transmissible plasmid present in *P. putida* DOT-T1E	Rodríguez-Herva *et al*., 2007
pGEM-T	Cloning vector with polyA, amp^r	Promega
pGEM-T-3760	pGEM-T carrying between *Eco*RI and and *Bam*HI sites, a 400-bp chromosomal fragment from the *P. putida* KT2440R c*heR3* gene obtained by PCR as an *Eco*RI-*Bam*HI fragment, Ap^r	This study
pGEM-T -4392	pGEM-T carrying between *Eco*RI and *Bam*HI sites, a 400-bp chromosomal fragment from the *P. putida* KT2440R *cheR2* gene obtained by PCR as an *Eco*RI-*Bam*HI fragment ,Ap^r	This study
pCHESIΩKm	Ap^r Km^r, pUNϕ18 with the *Hin*dIII insert from pHP45ϕKm (Ω-Km interposon) at the *Hin*dIII site, *oriT* RP4	Llamas *et al*., 2003
pChesi-3760	pCHESIΩKm carrying between *Eco*RI and *Bam*HI sites, a 400-bp chromosomal fragment from the *P. putida* KT2440R *cheR3* gene obtained by PCR as an *Eco*RI-*Bam*HI fragment	This study
pChesi-4392	pCHESIΩKm carrying between *Eco*RI and *Bam*HI sites, a 400-bp chromosomal fragment from the *P. putida* KT2440R c*heR2* gene obtained by PCR as an *Eco*RI-*Bam*HI fragment	This study

Table 4. Oligonucleotides used in this study.

Name	Sequence	Construction of
CheR2-f	5´-AGAGGCGGCACATATGTCTACGGGTAATTTGG-3´	pET28b-CheR2
CheR2-r	5´-CATCTCTCGATACCGGCCTGGATCCTTACTTG-3´	pET28b-CheR2
CheR3-f	5´-CAACTGGAGCGCATATGACTAGCGAACGCA-3´	pET28b-CheR3
CheR3-f	5´-ACTGCGCGTAGGATCCTCATGATTTACGGA-3´	pET28b-CheR3
mcpT-f	5'-CACATTGAATTCTTAGATAATGAGGTGACAC-3'	pET200/D-TOPO-mcpT
mcpT-r	5'-TTCAGAGGATCCCTAAAGACGGAACATG-3'	pET200/D-TOPO-mcpT
mcpS-f	5'-AGAGCGCATATGAACAGCTGGTTCGCCAACATC-3'	pET28b-mcpS
mcpS-r	5'-TGCGGATCCTCAGACGCGGAACTGGCTGACCAG-3'	pET28b-mcpS
PP3760F	5'- AAGAATTC TTGACTAGCGAACGCAACAC -3'	pChesi-3760
PP3760R	5'- AAGGATCC AGATGATGGTGCGTTCAAGC -3'	pChesi-3760
PP4392F	5'- AAGAATTCGTGTCTACGGGTAATTTGGATTTC -3'	pChesi-4392
PP4390R	5'- AAGGATCC GGCCGAGGTTGCTGCGCTCGAA-3'	pChesi-4392

3.2 Cloning, expression, and purification of the 3 CheR paralogues

The cloning, expression and purification of CheR1 (WspC-*Pp*) has been reported in Muñoz-Martínez *et al*. (2012). DNA sequences encoding CheR2 (PP4392) and CheR3 (PP3760) were amplified by PCR using the oligonucleotides indicated in Table 4 and genomic DNA of *P. putida* KT2440 as template. The resulting products were digested with *Nde*I and *Bam*HI and cloned into pET28b(+) (Novagen) linearized with the same enzymes. The resulting plasmids, pET28b-CheR2 and pET28b-CheR3, were verified by sequencing the insert and flanking regions.

For protein overexpression *E. coli* BL21 (DE3) was transformed with pET28b-CheR2 and *E. coli* C41 (DE3) was transformed with pET28b-CheR3 (note: CheR3 did not express in *E. coli* BL21 (DE3)). The resulting strains were grown in 2 l Erlenmeyer flasks containing 500 ml LB medium supplemented with 50 μg/ml of kanamycin at 30°C to an OD_{660} of 0.6. Protein production was induced by the addition of 0.1 mM IPTG and growth was continued at 16°C overnight prior to cell harvest by centrifugation at 10,000 g for 30 min. Cell pellets were resuspended in buffer A (20 mM Tris, 0.1 mM EDTA, 500 mM NaCl, 10 mM Imidazole, 5 mM β-mercaptoetanol, 5 % (v/v) glycerol, pH 8.0) and

broken by French press treatment at 1,000 p.s.i. After centrifugation at 20,000 x g for 1 hour, the supernatant was passed through 0.22 μM filters (Millipore) and then loaded onto 5 ml HisTrap HP columns (Amersham Bioscience) equilibrated with buffer A and eluted with an imidazole gradient of 45-500 mM in buffer A. The proteins produced had the N-terminal sequence fusion MGSSHHHHHHSSGLVPRGSH containing the histidine tag for protein purification.

3.3 Isothermal Titration Calorimetry

Measurements were done on a VP-microcalorimeter (MicroCal, Northampton, MA, USA) at 25^{o}C. Protein was dialyzed into analysis buffer (20 mM Tris/HCl, 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, pH 7.5) and placed into the sample cell of the instrument. The ligand solutions were made up in the dialysis buffer and placed into the injector syringe. For the study of the interaction with SAM and SAH, typically 16-28 μM of protein was titrated with 3.2-6.4 μl of SAM (1 mM) or SAH (0.5-1 mM). To study the interaction of the 3 CheR paralogues with the pentapeptide NWETF (synthesized by Biomedal, Sevilla, Spain), 30 μM of protein was titrated with 12.8 μl aliquots of 1 mM peptide. In all cases, heat changes resulting from the titration of buffer with the respective ligands were subtracted from the titration data. Integrated, corrected and concentration-normalized peak areas of raw data were fitted with the “One binding site model” of the MicroCal version of ORIGIN. The algorithm for data analysis is detailed in (Wiseman *et al*., 1989).

3.4 Analytical Ultracentrifugation Studies

An Optima XL-I analytical ultracentrifuge (Beckman-Coulter) was used to perform the analytical ultracentrifugation experiments of CheR2 (0.25-1.0 mg/ml) in the absence and presence of ligands (1 mM). The detection was carried out by means of a UV-visible absorbance detection system. Experiments were conducted at 20^{o}C using an AnTi50 eight-hole rotor and Epon-charcoal standard double sector centerpieces (12 mm optical path). Absorbance scans were taken at the appropriate wavelength (280-295 nm). Sedimentation velocity experiments were performed at 48,000 rpm using

400 µl samples in 50 mM Tris/HCl, 300 mM NaCl, 1 mM DTT, pH 8.0. Differential sedimentation coefficient distributions, *c(s)*, were calculated by least squares boundary modeling of sedimentation velocity data using the program SEDFIT (Schuck, 2000). From this analysis, the experimental sedimentation coefficients of the proteins were corrected for solvent composition and temperature with the program SEDNTERP (Jones *et al*., 1992) to obtain the corresponding standard *s* values.

3.5 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry experiments were carried out on a VP-DSC instrument (MicroCal, Northampton, MA) at a scan rate of 60^{o}C/h. Protein was dialyzed against 20 mM PIPES, 150 mM NaCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 5 mM TCEP, pH 7.5. The dialysis buffer was placed into the reference cell of the instrument. Calorimetric cells were kept under a pressure of 60 p.s.i. Several buffer-buffer baselines were obtained before each run with protein solutions in order to ascertain proper equilibration of the instrument. Reheating runs were carried out to determine the calorimetric reversibility of the denaturation process. The DSC experiments were carried out at a concentration of 1 mg/ml for CheR1 and CheR2 and 0.5 mg/ml for CheR3. For the binding studies, ligands were added at a concentration of 1 mM.

3.6 Cloning and overexpression of McpS and McpT chemoreceptors

The *mcpT* gene was amplified by PCR from *P. putida* DOT-T1E containing the megaplasmid pGRT1 using mcpT-f and mcpT-r primers (Table 4), which contain *Eco*RI and *Bam*HI sites, respectively. The resulting PCR product was cloned into pET200/D-TOPO. The *mcpS* gene (PP4658) was amplified by PCR from *P. putida* KT2440 using primers mcpS-f and mcpS-r (Table 4), containing *Nde*I and *BamH*I sites, respectively, and the resulting PCR product was cloned in pET28b(+).

The resulting pET200/D-TOPO-*mcpT* and pET28b-*mcpS* plasmids were transformed into One Shot® *E. coli* BL21 Star™ (DE3) and *E. coli* C41 (DE3), respectively. The resulting strains were grown in 2 l Erlenmeyer flasks containing 500 ml LB medium, supplemented with 50 µg/ml kanamycin, at 30^{o}C to an OD_{660} of 0.6. Protein production

was induced by adding IPTG (0.1 mM for McpT and 1 mM for McpS) and growth was continued at 18^{o}C overnight prior to cell harvest by centrifugation at 4,000 g for 20 min at 4^{o}C. Cells were then frozen at -80^{o}C. All subsequent manipulations were done at 4^{o}C. Crude McpT- or McpS-enriched membranes were prepared by thawing cell pellets on ice, resuspending them with 30 ml of ice-cold 30 mM HEPES buffer, 100 mM NaCl, pH 7.0 containing EDTA-free protease inhibitor cocktail (Roche) and 100 U benzonase (Roche). Cells were broken by 3 passages through a French Press at 1100 p.s.i. The homogenized cells were then centrifuged at 4,000 x g for 15 min, the pellet discarded and the supernatant centrifuged at 100,000 x g for 1h. The resulting pellet of receptor-enriched membranes was resuspended in 30 mM HEPES, 100 mM NaCl, pH 7.0, containing 10 % (w/v) sucrose, homogenized by passing 30 times through a 25-gauge needle, flash frozen in liquid nitrogen and stored at -80^{o}C. Aliquots of McpT- or McpS-enriched membranes were thawed just before each methylation experiment. To generate mock membranes that do not contain McpT or McpS, this procedure was applied to cells containing the empty expression plasmid.

3.7 Methyltransferase assay

The methyltransferase activity was determined using the protocol described by Stock *et al*. (Stock *et al*., 1984). Briefly, McpT- or McpS-enriched membranes (1.5 mg of total membrane proteins/sample) were incubated either in the absence or in the presence of 4 µM of each purified CheR protein with 100 µM S-adenosyl-[methyl-^{3}H]-methionine (^{3}H-SAM: 0.83 µCi/sample; Perkin Elmer, ref. NET155250UC) and an aliquot of crude cytosolic extract from *P. putida* KT2440 (final protein concentration 5 mg/ml). This extract contains the enzymes necessary for the degradation of SAH, avoiding thus its accumulation and feedback-inhibition. The final volume of the sample mix was adjusted to 100 µl using 10 mM Tris/HCl, 100 mM NaCl, pH 7.4. The resulting mixtures were incubated at 30^{o}C for 20 min and the reaction was stopped by adding 500 µl ice-cold 10 % (v/v) acetic acid. To quantify the amount of methyl ester groups transferred to McpS and McpT, the vapor-phase equilibrium procedure described by Campillo and Ashcroft (Campillo and Ashcroft, 1982) was used. Samples were washed

three times with ice-cold 10 % (v/v) acetic acid to remove the ^{3}H-SAM that was not consumed during the reaction prior to a re-suspension of the pellet in 200 µl 1N NaOH. The open tubes were then placed into a 7 ml liquid scintillation vial containing 2.4 ml scintillation fluid. The vial was closed and incubated overnight at 37^{o}C without shaking. The recovery of ^{3}H-methanol was quantified the following day in a scintillation counter.

3.8 Construction of *P. putida* CheR mutants

The mutant in c*heR1* (*P. putida* KT2440R-TK1038) was retrieved from the *Pseudomonas* Reference Culture Collection (http://artemisa.eez.csic.es/prcc/). These mutants were isolated after random mini-Tn*5*–Km mutagenesis as described in (Duque *et al*., 2004). The mini-Tn*5* insertion was located at the c*heR1* gene (PP1490). Mutants in c*heR2* (PP4392) and c*heR3* (PP3760) were constructed as follows: Plasmid pCHESIΩKm is a pUC18 derivative containing the *oriT* origin of transfer of RP4 and the Ω-Km interposon of plasmid pHP45ΩKm (Fellay *et al*., 1987). To generate *cheR2* and *cheR3* mutants, 400-bp fragments of the corresponding genes from *P. putida* KT2440R were amplified by PCR using primers PP3760F and PP760R as well as PP4392F and PP4390R, respectively (Table 4). The forward and reverse primers contained restriction sites for *EcoR*I and *BamH*I, respectively. PCR products were cloned into the *EcoR*I and *BamH*I sites of pCHESIΩKm in the same transcriptional direction as the P_{lac} promoter. Resulting plasmids, pCHESI-3760 and pCHESI-4392, were mobilized from *E. coli* DH5α into *P. putida* KT2440 by electroporation. *P. putida* KT2440 bearing pCHESI-3760 and pCHESI-4392 plasmids in the host chromosome were selected on M9 minimal medium supplemented with 10 mM benzoic acid as the sole carbon source and 50 µg/ml kanamycin. A few Km^{r} clones were chosen for Southern blot and PCR analyses to confirm that pCHESI-3760 and pCHESI-4392 plasmids disrupted the desired genes. All of the clones analyzed contained an inactivated *cheR2* or *cheR3* gene and the resulting strains *P. putida* KT2440R-*ΔcheR2* and *P. putida* KT2440R-*ΔcheR3* were used for further analyses.

3.9 Chemotaxis assays and swim plate motility assays

Bacteria from single colonies grown overnight on LB agar plates were transferred with a toothpick to the center of swim agar plates (10 % LB and 0.25 % (w/v) agar). Plates were incubated overnight at 30^{o}C and taxis was monitored the following day.

Plate gradient assays: Bacteria were grown overnight in MS medium supplemented with 10 mM succinate and diluted to an OD_{600} of 0.8-1 with fresh MS medium. Cells are then washed twice with MS medium by consecutive resuspension and centrifugation at 6,000 rpm for 3 minutes. Square Petri dishes were filled with 50 ml of semisolid agar containing minimal MS medium, 10 mM glucose and 0.25 % (w/v) agar. Plates were cooled at room temperature for at least 0.5 h. At the vertical central line of the plate, 10 μl aliquots of chemoattractant solution dissolved in MS were placed at regular distances. Plates were incubated for 12–16 h at 4^{o}C to create a chemoattractant concentration gradient. Two microliter aliquots of bacterial suspension were then placed horizontally to each of the chemoattractant spots with a distance of 3 cm to the vertical line. Plates were incubated at 30^{o}C for 16-20 h.

3.10 Biofilm assays and quantification of crystal violet-stained attached cells

Biofilm formation was examined using a borosilicate glass tube (hydrophilic surface) screening assay adapted from the method described by O'Toole & Kolter (O'Toole *et al*., 1999). Overnight *P. putida* cultures were diluted with fresh LB broth medium to an OD_{600} of 0.05. Two ml aliquots of each diluted culture were dispensed into three borosilicate glass tubes and incubated at room temperature under orbital shaking at 40 rpm. for 4 hours. Unattached cells were removed by rinsing the borosilicate glass tubes thoroughly with water, and attached cells were subsequently stained by incubation with 0.1 % (w/v) crystal violet at room temperature for 15 min. Subsequently, tubes were washed twice with water to remove any unbound stain. Crystal violet was then solubilized by the addition of 5 ml of 10 % (v/v) glacial acetic acid and the OD was measured at 600 nm. Prior to staining, the OD_{600} of bacterial cultures ($CVOD_{600}$) was determined to quantify planktonic growth. Biofilm formation was then normalized with the corresponding cell density.

4. Results

4.1 *P. putida* KT2440 has 3 CheR paralogues

It has been recently reported a study of CheR1 that is encoded by the ORF PP1490. This protein was found to correspond to a fusion of a methyltransferase with a tetratricopeptide repeat containing binding domain (Muñoz-Martínez *et al*., 2012). To identify further CheR paralogues a BLAST search within the translated ORFs of *P. putida* KT2440 was conducted using the *E. coli* CheR sequence (P07364). Two other ORFs, PP4392 (renamed CheR2) and PP3760 (renamed CheR3), were detected which shared 23 and 27 % protein sequence identity with the *E. coli* enzyme, respectively. Structural studies and site-directed mutagenesis data of the *S. typhimurium* CheR have suggested that R98 and Y235 are essential catalytic residues (Figure 32) (Djordjevic and Stock, 1997; Shiomi *et al*., 2002; Simms and Subbaramaiah, 1991).

The sequence alignment of the three *P. putida* paralogues with their homologues from *E. coli* and *S. typhimurium* (Figure 33) revealed a sequence identity of only 8 %. However, R98 and Y235 were amongst the conserved residues, which is consistent with the notion that these 3 paralogues are functional proteins.

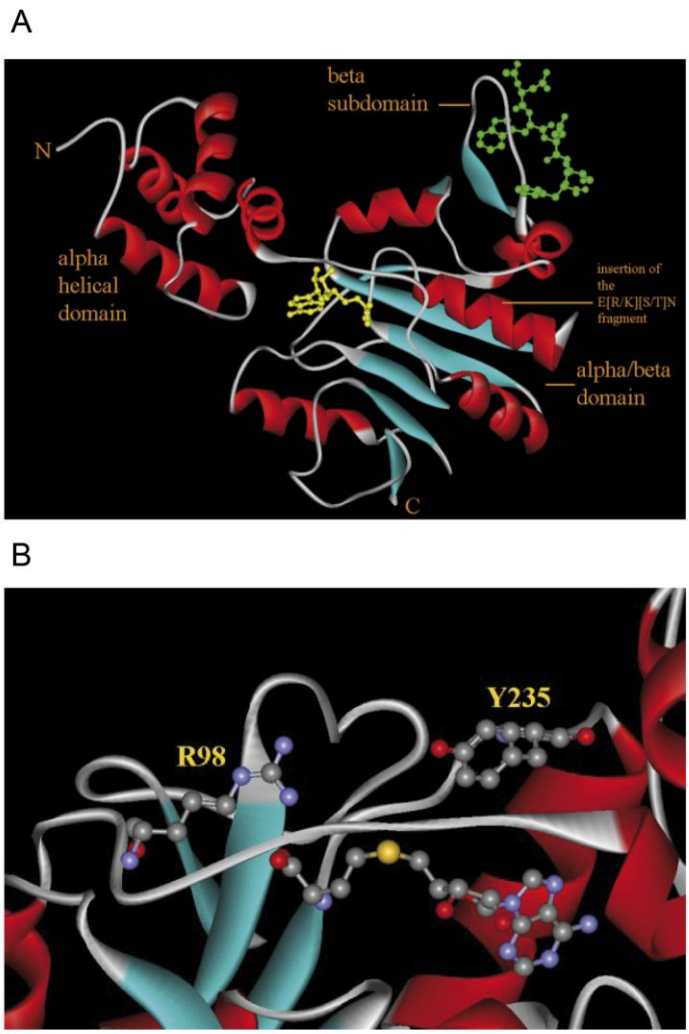


Figure 32. The three-dimensional structure of CheR from *S. Typhimurium* (PDB code 1af7 (Djordjevic and Stock, 1997)). Panel A: Shown is an overall structure. Bound SAH is shown in yellow, and the bound pentapeptide NWETF is in green. The three domains are indicated. The site of insertion of the E(R/K)T(S/T)N motif is highlighted. Panel B: Shown is a zoom of the active site. Bound SAH as well as catalytic residues Arg-98 and Tyr-235 are shown in ball-and-stick mode.

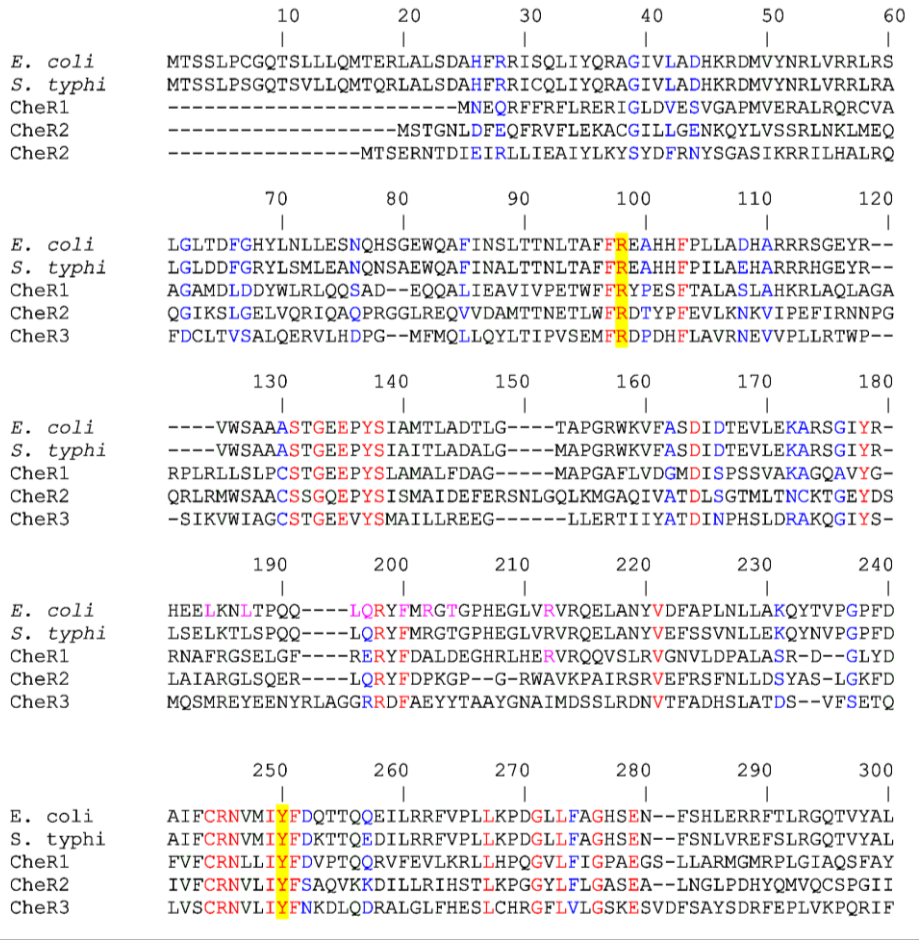


Figure 33. Segment of sequence alignment of the 3 CheR paralogues from *P. putida* KT2440 with the CheR sequences form *E. coli* (P07364) and *Salmonella typhimurium* (P07801). Sequences were aligned using CLUSTALW of the Network Protein Sequence Analysis (NPSA) server at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html. Amino acids in red indicate identity, in blue high similarity and in green low similarity. The amino acids which are involved in catalysis are shaded in yellow. The amino acids corresponding to the amino acids R98 and Y235 in *S. typhimurium* CheR are shaded in yellow.

4.2 Large differences of the three CheR paralogues in the ratio of affinities for SAH and SAM

The *cheR* genes were cloned into an expression vector, proteins expressed in *E. coli* and purified from the soluble fraction of cell lysates. The methyltransferase proteins were subsequently submitted to isothermal titration calorimetry (Krell, 2008) studies with SAM and SAH. The resulting thermograms are shown in Figure 34 and the derived thermodynamic data are provided in Table 5. Dissociation constants of approximately 22 μM were obtained for the SAM binding to CheR2 and CheR3. In contrast, SAM bound with significantly weaker affinity (K_D=43 μM) to CheR3. The n values determined (Table 5) were in the range between 0.95 – 1.61 but are little reliable due to the reduced c-value of these experiments (Turnbull and Daranas, 2003). In contrast to the hyperbolic titration curves for SAM, sigmoidal curves were obtained for SAH titrations indicating higher affinities. In all cases SAH bound with much higher affinity to the CheRs than the substrate SAM. Very tight binding was observed for CheR2 for which a K_D of 140 nM was determined. SAH was found to bind with similar affinities to CheR1 (K_D= 1.7 μM) and CheR3 (K_D=2.2 μM). Due to more elevated c-values, the enthalpic and entropic contributions to binding could be determined precisely. In all three cases binding was driven by very favorable enthalpy changes and counterbalanced by unfavorable entropy changes. The n-values were between 0.72 and 0.78 and are in all cases inferior to the expected value of 1 indicative of a 1:1 binding stoichiometry. All proteins analyzed had a purity superior to 95 % and these differences in stoichiometry may have been caused by the presence of unfolded protein. Data show thus that SAH binds much tighter to the three paralogues than SAM, indicative of product feedback inhibition. However, significant differences were observed in the magnitudes of feedback inhibition. The ratios of K_DSAM:K_DSAH are provided in Table 5. In the case of CheR2 this value is of 165, whereas it is only 25 and 10 for CheR1 and CheR3, respectively. This implies that there is a window in which alterations of the SAH concentration modulate the activity of CheR2 without affecting in a significant manner CheR1 and CheR3 activity.

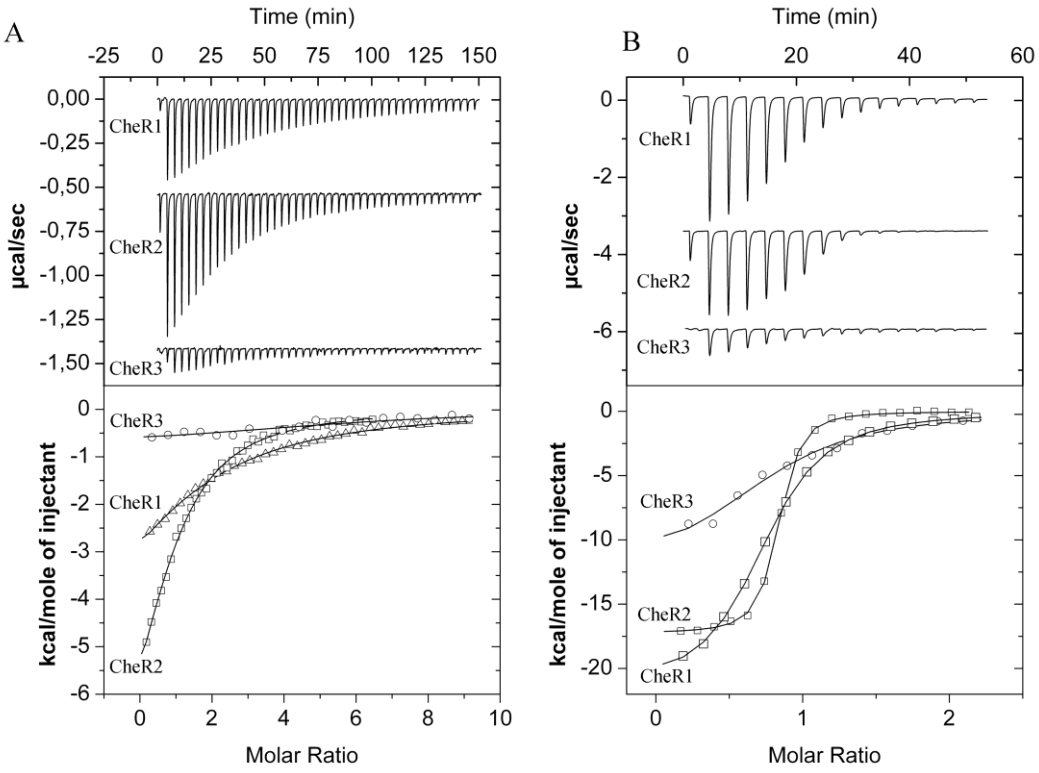


Figure 34. Microcalorimetric titrations of the 3 CheR paralogues with of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). A) Titration of the 3 CheR proteins with SAM. B) Titration of the 3 CheR paralogues with SAH. The upper panels show titration raw data. The protein concentration used was between 16-28 µM and the ligand concentration was either 0.5 or 1 mM. A titration consisted of an initial injection of 1.6 µl followed by a series of 3.2 or 6.4 µl injections. Lower panel: Integrated, dilution-corrected and concentration-normalized titration raw data, which were fitted using the "One binding site model" of the ORIGIN version from MicroCal (Northampton, MA, USA).

Table 5. Thermodynamic parameters for the binding of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) to the three CheR paralogues of *P. putida* KT2440. The microcalorimetric titration data are shown in Figure 34. Data for CheR1 have been reported previously (Muñoz-Martínez *et al*., 2012) and are shown as a reference.

protein	ligand	n[1]	K_D (mM)	K_A (M^{-1})	ΔH[1] kcal/mol	$T\Delta S$[1] kcal/mol	Ligand	n	K_D (mM)	K_A (M^{-1})	ΔH kcal/mol	$T\Delta S$ kcal/mol	$K_{D\,SAM}/K_{D\,SAH}$
CheR1	SAM	1.58	43 ± 2	$(2.3 \pm 0.1)\ 10^4$	-7.4 ± 0.6	-1.5 ± 0.6	SAH	0.72	1.7 ± 0.1	$(5.9 \pm 0.1)\ 10^5$	-21.7 ± 0.1	-13.8 ± 0.1	25
CheR2	SAM	0.95	22.8 ± 1	$(4.38 \pm 0.2)\ 10^4$	-10.2 ± 0.5	-3.9 ± 0.5	SAH	0.78	0.14 ± 0.01	$(7.1 \pm 0.3)\ 10^6$	-17.3 ± 0.1	-7.5 ± 0.1	165
CheR3	SAM	1.61	22.3 ± 1	$(4.48 \pm 0.1)\ 10^4$	-0.8 ± 0.1	5.5 ± 0.2	SAH	0.75	2.2 ± 0.2	$(4.6 \pm 0.5)\ 10^5$	-12.7 ± 0.6	-4.9 ± 0.6	10

a Caution in the interpretation of the n values as well as the enthalpy and entropy changes for SAM binding is advised as the products of protein concentration and KA (c value) are low (in the range of 0.37–1.25; (Turnbull and Daranas, 2003)).

4.3 The three CheR paralogues do not bind the NWETF Pentapeptide

The CheRs of *E. coli* and *S. typhimurium* recognize the NWETF pentapeptide, which form the C-terminal extensions of the high abundance chemoreceptors in these species (Djordjevic and Stock, 1998; Shiomi *et al*., 2002). Although *P. putida* lacks chemoreceptors with C-terminal pentapeptides we wanted to establish whether the three CheR paralogues of *P. putida* have maintained the capacity to recognize this pentapeptide. The three proteins at a concentration of 30 µM were titrated with aliquots of 1 mM NWETF pentapeptide. Experiments were conducted at 25 and 15^{o}C, but in all cases an absence of binding was noted, which indicates that this pentapeptide is not recognized by any of the 3 CheRs.

4.4 The 3 CheR paralogues unfold cooperatively in a single event

The CheR of *S. typhimurium* is composed of three structural domains (Djordjevic and Stock, 1998). CheR2 and CheR3, which are similar in size, are likely to possess the same domain architecture. CheR1 contains an additional 150 amino acid C-terminal extension harboring a tetratricopeptide binding domain (Muñoz-Martínez *et al*., 2012). Differential Scanning Calorimetry (DSC) studies can provide insight into domain interaction. Using this technique a temperature gradient is applied to the purified protein and heats resulting from the thermal protein unfolding (endothermic) are recorded. The DSC thermograms of these three paralogues (Figure 35) reveal a single peak indicative that all proteins unfold cooperatively in a single event, which may suggest functional inter-domain communication. Unfolding occurs over a similar temperature range as shown by Tm values in between 44 to 49°C (Table 6). The enthalpy change associated with the CheR3 unfolding was significantly below that of the remaining two proteins, which may be potentially due to an amorphous aggregation. The addition of 1 mM SAM to the proteins resulted in a modest increase in thermal stability of 2.4-3.1°C. The SAH mediated stabilization was much more pronounced as shown by Tm increases of 5.7-8.4°C (Figure 35, Table 6).

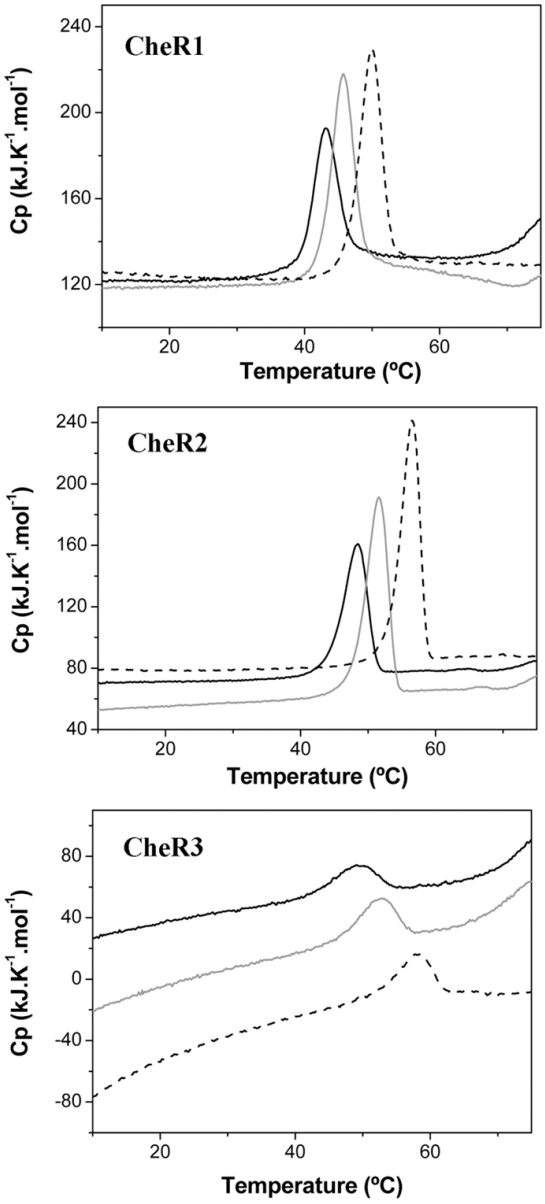


Figure 35. Analysis of the three CheR paralogues by Differential Scanning Calorimetry (DSC). CheR1 and CheR2 were at a concentration of 1 mg/ml, whereas CheR3 was at 0.5 mg/ml. SAM and SAH were added at a concentration of 1 mM. The derived parameters ΔH (enthalpy change of protein unfolding) and T_m (midpoint of protein unfolding transition) are given in Table 6. Black line: protein without ligand, grey line: protein with SAM, dotted line: protein with SAH.

Table 6. Thermodynamic parameters for the thermal unfolding of the three CheR paralogues of *P. putida* KT2440 as determined by differential scanning calorimetry (see Figure 35).

Protein	T_m (ºC)	D*H* (kcal/mol)
CheR1	44.03	57,8
CheR1 + SAM	46.46	107,1
CheR1 + SAH	49.74	74,0
CheR2	48.27	94,2
CheR2 + SAM	51.04	94,5
CheR2 + SAH	56.02	104,2
CheR3	49.01	22,8
CheR3 + SAM	52.13	90,4
CheR3 + SAH	57.42	54,7

4.5 The McpS and McpT chemoreceptors are exclusively methylated by CheR2

The McpS and McpT chemoreceptors of *P. putida* mediate chemotaxis towards Krebs cycle intermediates (Lacal *et al*., 2010b, 2011b; Pineda-Molina *et al*., 2012) and aromatic hydrocarbons (Lacal *et al*., 2011a), respectively. To determine which of the three CheR methylates these chemoreceptors, the coding sequence of both chemoreceptor genes were cloned into an expression vector. Both chemoreceptors were expressed in *E. coli* and membranes enriched in each of these proteins were prepared. In parallel, mock membranes were prepared using the same experimental procedure except that *E. coli* cells were transformed with the empty expression plasmid. A SDS-PAGE analysis of these membranes showed the overexpression of both receptors. The methylation assay mix contained also an extract of soluble proteins from *P. putida* KT2440, that contain the enzymes necessary to degrade SAH, since above results have shown that SAH binds preferentially to the CheR proteins.

The initial experiments consisted in the analysis of methylation activity in mock membranes in the absence and presence of added CheR. As shown in the first 4 columns of Figure 36 the methylation activity of mock membranes in the absence and presence of added CheR was similar. Subsequently, methylation of McpS and McpT containing membranes in the absence of CheR were measured (lanes 5 and 9 of Figure 36).

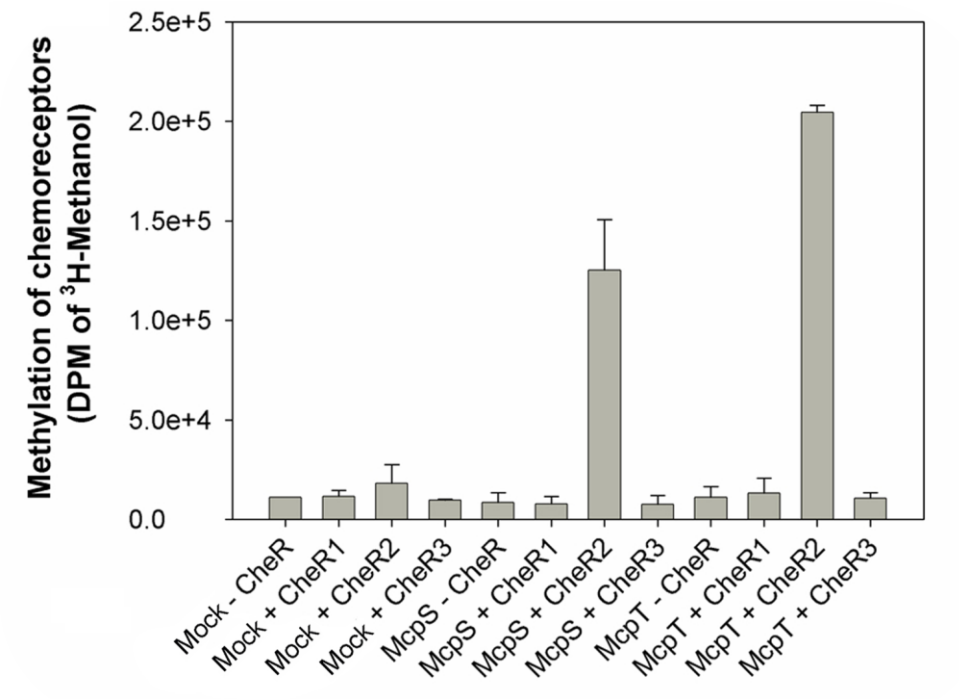


Figure 36. Methylation of the McpS and McpT chemoreceptors using the three purified CheR paralogues. *E. coli* membranes enriched in McpS and McpT were prepared as described in Materials and Methods. Mock corresponds to experiments where the McpS and McpT containing membranes are replaced by membranes generated from *E. coli* transformed with the empty expression plasmid. The assay mix contained also an extract of soluble proteins of *P. putida* KT2440, which contains the enzymes necessary to assure the metabolization of SAH. Purified CheR paralogues were added at a final concentration of 4 μM.

These activities were comparable to the experiments with mock membranes indicating that methylation of both chemoreceptors by proteins present in the soluble extract of *Pseudomonas* proteins is negligible. The third series of experiments involved the evaluation of McpS and McpT methylation by the purified CheR paralogues. A significant methylation of both chemoreceptors was observed by CheR2 whereas the corresponding measurements in the presence of CheR1 and CheR3 were comparable to the control experiments (Figure 36). These experiments indicate that exclusively CheR2 methylates both chemoreceptors. These data also show that CheR2 can methylate a chemoreceptor derived from the same bacterial strain, McpS, but can also methylate a chemoreceptor, McpT, that is derived from a different strain (*P. putida* DOT-T1E).

4.6 CheR2 is monomeric in solution

Based on the central role of CheR2 in the methylation of McpS and McpT chemoreceptors, we used analytical ultracentrifugation techniques to assess its oligomeric state. Sedimentation velocity studies of CheR2 over the concentration range of 0.25-1.0 mg/ml showed a single peak at 2.5 S in the sedimentation coefficient distribution (Figure 37), indicative of a monomeric state. Subsequently sedimentation experiments of CheR2 at 1 mg/ml were repeated in the presence of 1 mM SAM or SAH. As shown in Figure 37 the addition of both ligands did not result in any significant increase in the sedimentation coefficient indicating that the addition of both compounds does not alter its oligomeric state. CheR2 at a concentration of 0.5 mg/ml was then analyzed by sedimentation equilibrium ultracentrifugation. Data analyses revealed a mass of 38,600 ± 600 Da, which is close to the sequence-derived masses of 32,247 Da, confirming the monomeric state of the protein.

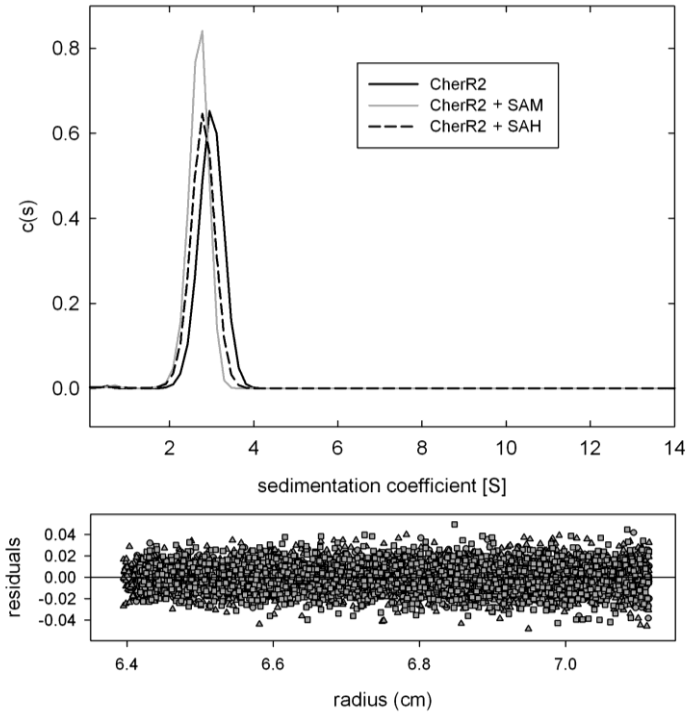


Figure 37. Analyses of CheR2 by sedimentation velocity ultracentrifugation. Protein concentration was of 1 mg/ml. SAM and SAH were added at a concentrations 1 mM. The upper panel shows the sedimentation coefficient distributions c(s), and the lower panel shows the residuals of curve-fitting.

4.7 Only CheR2 is essential for Chemotaxis

To study the contribution of the CheR paralogues in mediating chemotaxis, the corresponding bacterial mutant strains were constructed and analyzed. Microscopic inspection of wild type and mutant strains indicated a similar degree of motility. Initial experiments were carried out to study the bacterial growth of mutant and wild types strains. As shown in figure 38 the growth kinetics of the CheR2 and CheR3 mutants were comparable to that of the wild type strain, whereas mutant CheR1 grew somewhat slower.

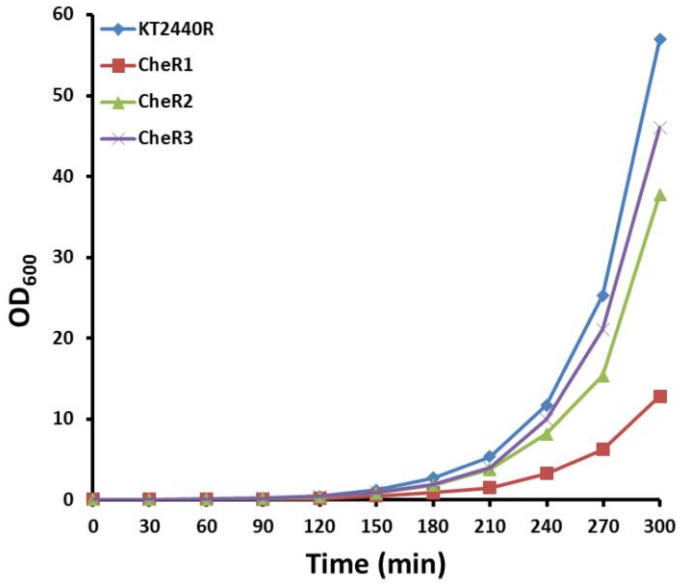


Figure 38. Growth properties of wild type and mutant *P. putida* KT2440R. Cultures of KT2440R (wild-type), TK1038 (Δ*cheR1*), TK1117 (Δ*cheR2*), and TK1116 (Δ*cheR3*) were grown at 30°C with agitation (200 rpm.) in Luria Bertani medium. Every 30 min, the OD_{600} values were measured and cultures were diluted with fresh prewarmed medium and the culture was continued. This procedure enabled measurements in the linear range of 0.1–0.5 OD_{600}. All OD_{600} values were corrected with the appropriate dilution factor.

The primary physiological reason for chemotaxis resides in the capacity to approach compounds that serve as carbon or energy source. To assess the general contribution of the three CheR paralogues in chemotaxis towards growth substrates, soft agar swim plate chemotaxis assays were carried out. In this assay cells are placed into the center of a soft agar LB plate and the size of halo formation is a measure of chemotaxis. After overnight incubation a large halo that reaches almost the border of the petri dish is observed for the wild type strain (Figure 39) and a similar halo is observed for the CheR3 mutant.

The halo of the CheR1 mutant was slightly reduced in size, whereas that of the CheR2 mutant was dramatically reduced.

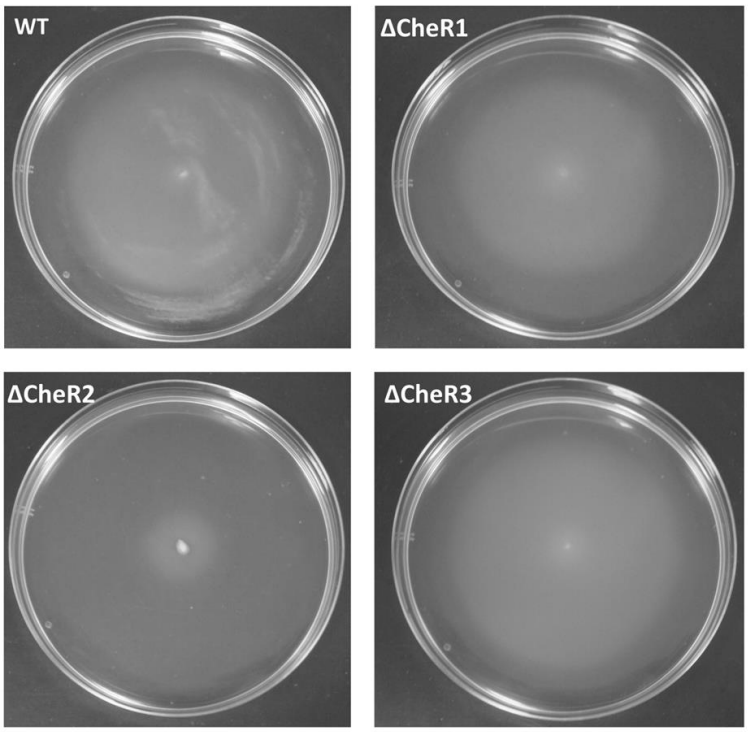


Figure 39. Chemotaxis of wild type and mutant *P. putida* KT2440R cells towards LB medium. Soft agar swim plate chemotaxis assays of wild type and mutant strains of *P. putida* KT2440. The center of soft agar swim plates containing LB medium is inoculated with bacteria. Plates were incubated at 30°C overnight and inspected the following day.

Subsequently, chemotaxis towards specific chemoattractants, casamino acids and malate, was measured. We have identified previously McpS as the sole malate receptor of this strain (Lacal *et al*., 2010b). Plate gradient assays were conducted in which chemoattractant aliquots are placed on a vertical line in the middle of the plate (Figure 40) followed by an overnight incubation to permit gradient formation. Aliquots of bacterial cultures are then deposited on each side of the chemoattractant. The CheR2 mutant was found to be deficient in chemotaxis towards malate and casamino acids, whereas taxis of the CheR3 mutant was comparable to the wild type (Figure 40). For both chemoattractants, taxis of the CheR1 mutant was slightly reduced, which may be a consequence of its delayed growth. These results agree with the swim plate assays reported above.

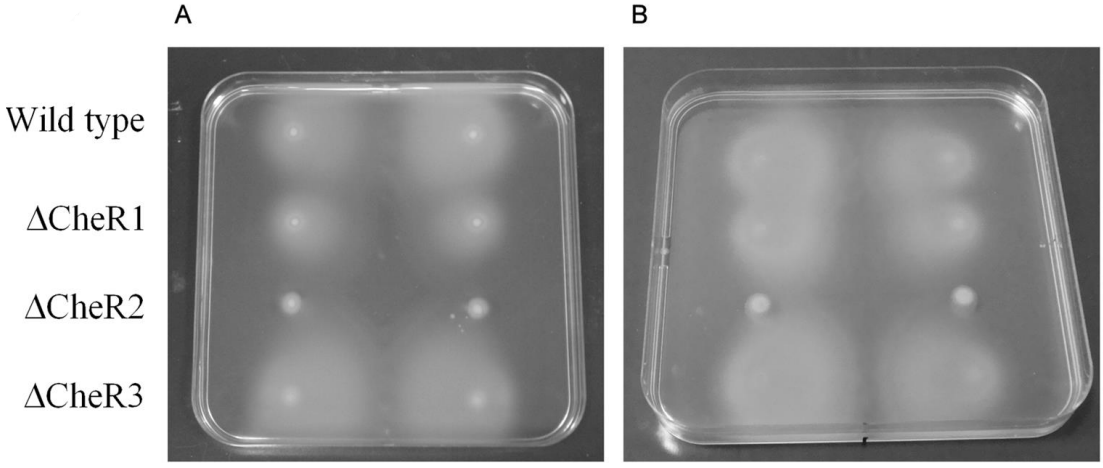


Figure 40. Chemotaxis wild type and mutant *P. putida* KT2440R towards casamino acids (A) and malate (B). Shown are plate gradient assays. Ten µl aliquots of a 10 % (w/v) of casamino acids (A) or 10 mM malate (B) were placed on the vertical line in the middle of the plate. Plates were incubated at 4°C for 12–16 h for the generation of a concentration gradient. Two microliter aliquots of bacterial suspension were then placed on both sides of the central vertical line. Images were taken after incubation at 30°C for 16-20 h. Shown are duplicate experiments.

4.8 CheR1 is essential for efficient biofilm formation

The capacity of wild type and mutant strains to form biofilms was analyzed. As shown in Figure 41A biofilm formation on borosilicate tubes of the CheR2 and CheR3 mutants

was comparable to the wild type strain, whereas that of the CheR1 mutant was significantly reduced. Since growth of the CheR1 was slightly slower than the wild type (Figure 38), biofilm formation was quantified and normalized using the cell density of the bacterial supernatant. The resulting values are presented in Figure 41B and show that mutation of CheR1 reduces biofilm formation to around half of that observed for the wild type and the CheR2 and CheR3 mutants.

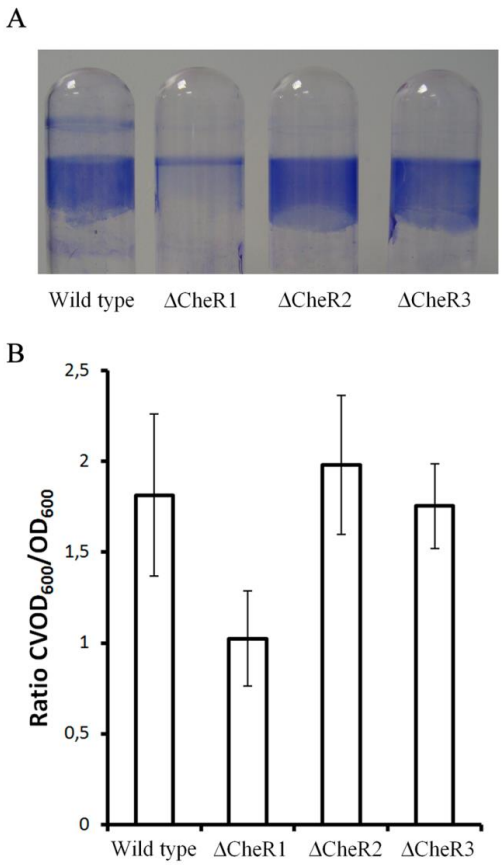


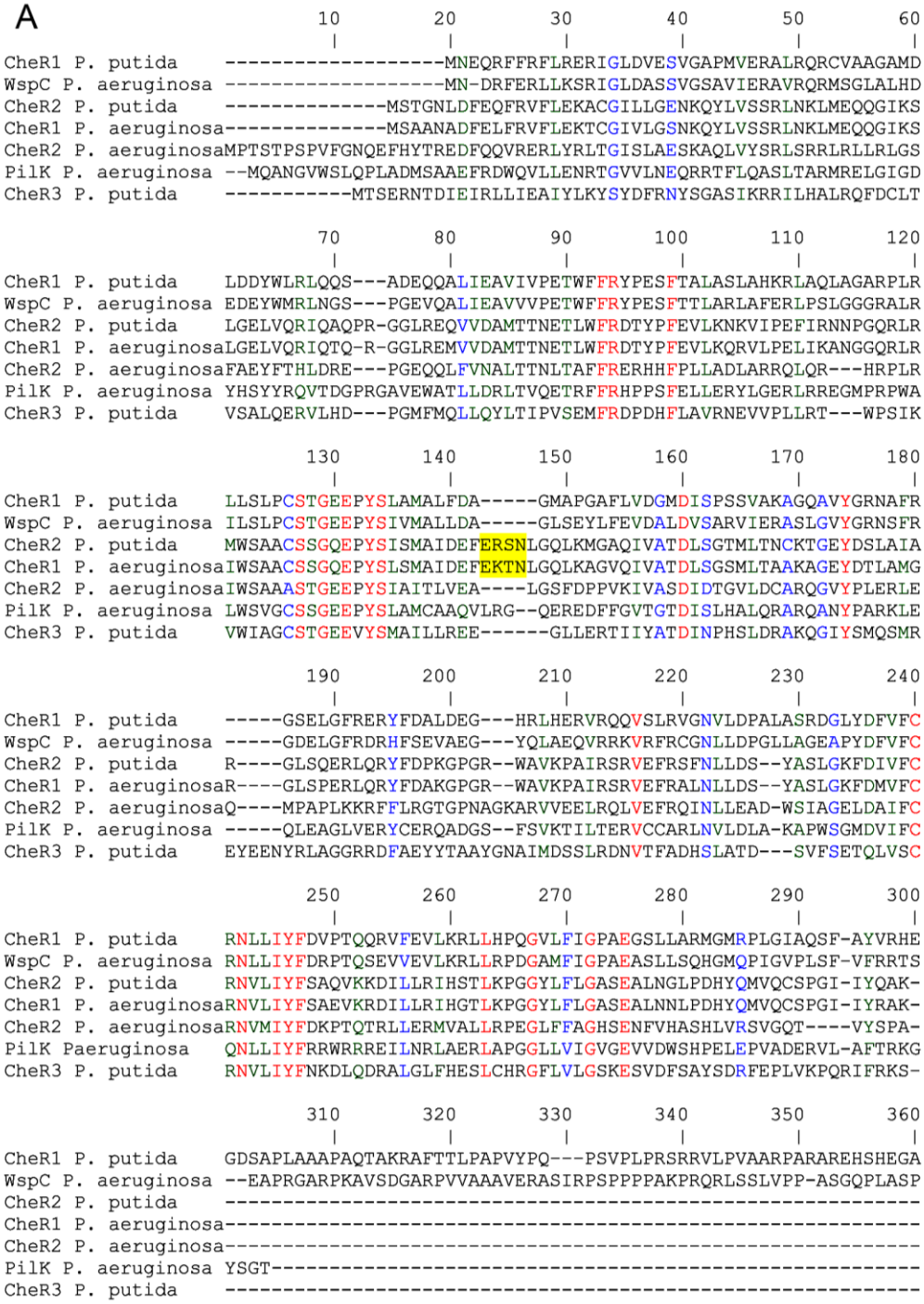
Figure 41. Influence of the mutation of CheR paralogues on the capacity of *P. putida* KT2440R to form biofilms. A, shown is visualization of biofilm formation of wild type and mutant strains of *P. putida* KT2440. Shown are stained borosilicate tubes after 4 h of growth. B, shown is quantification of biofilm formation of the above strains on borosilicate tubes after 4 h of growth using the crystal violet test. Shown is the ratio of A600, obtained after crystal violet staining of attached cells, to culture viable A600, representing the optical density of the cultures after 4 h growth. Data are the means and S.E., derived from three independent experiments with triplicate tubes incubated per experiment (the means of triplicate experiments were calculated, and the data presented are averages of the resulting three means derived from three independent experiments).

5. Discussion

CheR methyltransferases are among the core proteins of chemosensory pathways and therefore of general importance for their proper functioning (Wuichet and Zhulin, 2010). Many bacteria have multiple sets of chemosensory signaling proteins that arrange into different pathways. Here we present a comparative study of the three CheR paralogues from *P. putida* KT2440. A major finding of this work resides in the demonstration that exclusively CheR2 methylates two chemotaxis receptors, whereas the remaining two paralogues have no methylation activity on these receptors. These results are underlined by the general chemotaxis defect of the CheR2 mutant whereas the remaining mutants showed chemotaxis comparable to the wild type. The presence of three CheR paralogues suggests the existence of three different chemosensory pathways and signal recognition for each pathway is achieved by dedicated chemoreceptors. Our data suggest that CheR methyltransferases have evolved to recognize and methylate their cognate chemoreceptors with high specificity.

The specificity of action of CheR paralogues is also illustrated by the reduction of biofilm formation in the CheR1 mutant, whereas the other two mutants showed a biofilm phenotype comparable to that of the wild type strain. The gene Cluster containing *cheR1* is homologous to the *wsp* Cluster in *P. aeruginosa* (Hickman *et al*., 2005). The output of the *wsp* pathway consists in the modulation of cyclic diguanylate concentration which in turn regulates biofilm formation. CheR1 is thus a homologue of the WspC methyltransferase of the *wsp* pathway. It was shown previously that mutation of WspC abolishes pathway signal transduction (O'Connor *et al*., 2012) and its overexpression causes changes in cell morphology (Bantinaki *et al*., 2007). Here we show that the mutation of the WspC homologue in *P. putida* reduces biofilm formation which is consistent with the above findings. The *chP* chemosensory pathway of *P. aeruginosa* was found to mediate type IV pili based motility (Darzins, 1994, 1994; Kearns *et al*., 2001). To assess whether CheR3 may potentially be involved in mediating this type of motility, we have thus studied the twitching motility of *P. putida* KT2440. Despite numerous attempts and the exploration of many different experimental condition only a minor and little reproducible twitching motility was observed, which did not permit to analyze the effect of CheR mutation on this phenotype.

P. aeruginosa and *P. putida* have thus in total 7 CheR proteins. To establish their relationship a sequence alignment and Clustering analysis made (Figure 42).



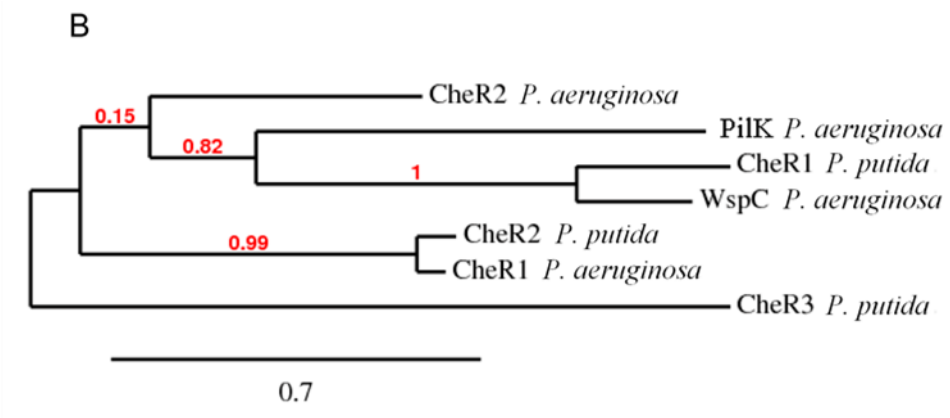


Figure 42. Sequence similarities between CheR paralogues from *P. putida* KT2440 and *P. aeruginosa* PAO1. A) Segment of sequence alignment of CheR proteins from *P. putida* and *P. aeruginosa*. The accession codes of these proteins are: CheR1 *P. putida*: PP1490; WspC *P. aeruginosa*: PA3706; CheR2 *P. putida*: PP4392; CheR1 *P. aeruginosa*: PA3348; CheR2 *P. aeruginosa*: PA0175; PilK *P. aeruginosa*: PA0412; CheR3 *P. putida*: PP3760. The sequence insert specific to CheR1 of *P. aeruginosa* and CheR2 of *P. putida* is shaded in yellow. B) Sequence Clustering of CheR paralogues of *P. aeruginosa* and *P. putida*. Tree constructed using the Phylogeny.fr server (Dereeper *et al*., 2008) using default settings.

Interestingly, WspC of *P. aeruginosa* pairs up with *P. putida* CheR1 and CheR2 of *P. putida* is closely related to CheR1 of *P. aeruginosa*. As discussed above the former two proteins are part of pathways that modulate biofilm formation, whereas the latter two proteins are both involved in chemotaxis pathways since CheR1 of *P. aeruginosa* forms part of the *che* chemotaxis pathway (Ferrández *et al*., 2002; Kato *et al*., 1999; Masduki *et al*., 1995) and CheR2 of *P. putida* is here shown to be essential for chemotaxis. The data thus demonstrate that similarities in CheR function of related bacterial species are reflected in sequence similarities, which may be useful for the annotation of homologues from other strains. In the Clustering analysis (Figure 42) CheR3 of *P. putida* as well as CheR2 and PilK of *P. aeruginosa* do not have equivalents. A difference in the chemosensory pathways of both species may be the presence of two chemotaxis pathways in *P. aeruginosa* whereas there appears to be a single chemotaxis pathway in *P. putida*. The sequence alignment of these 7 sequences (Figure 42) shows that CheR1 of *P. aeruginosa* and CheR2 of *P. putida,* both involved in chemotaxis*,* have a sequence insert with the consensus sequence E[R/K][S/T]N. This

sequence insert is located on a helix close to the pentapeptide binding site (Figure 32A) and may therefore be part of the interaction interface with the chemoreceptor. It may be plausible that this motif plays a role in mediating the specificity in the CheR-chemoreceptor interaction. This cluster analysis thus shows that it is possible to predict CheR function by sequence alignments with characterized proteins.

The CheR of *S. typhimurium* is characterized by product feedback inhibition, since SAH binds tighter to CheR than the substrate SAM (Simms and Subbaramaiah, 1991). Using ITC we have determined SAM/SAH binding affinities and data show that all three proteins recognize SAH with higher affinity than SAM, suggesting that product-feedback inhibition is a general feature of this protein family. The affinities of SAM for the *Pseudomonas* proteins were between 22 to 43 μM, which are similar to the analogous values for the *S. typhimurium* enzyme of 17 μM (Simms *et al*., 1987) and a 11 μM (Simms and Subbaramaiah, 1991).

There were significant differences in the affinities of the three paralogues for SAH since CheR1 and CheR3 showed K_D values of around 2 μM, whereas much tighter binding was observed for CheR2 with a K_D of 0.14 μM. However, this value is very similar to the SAH binding to the *S. typhimurium* CheR with a K_D of 0.22 μM (Yi and Weis, 2002). Both, CheR from *S. typhimurium* and *P. putida* CheR2 are involved in chemotaxis. One could thus hypothesize that methyltransferases involved in chemotaxis show tight SAH binding whereas enzymes involved in other pathways may show lower affinity SAH binding. These differences in affinities amongst the *Pseudomonas* proteins may have functional consequences since it implies that there is a window in which alterations of the SAH concentration modulate the activity of CheR2 but leave that of CheR1 and CheR3 unchanged. Future work will be necessary to verify this hypothesis.

Differential Scanning Calorimetry studies showed a single thermal unfolding event indicative of a cooperative unfolding of all domains of the three CheR paralogues. DSC studies of other proteins have revealed in a number of cases that cooperative domain unfolding reflects functional inter-domain cross-talk (Błaszczyk and Wasylewski, 2003; Fillet *et al*., 2011; Kedracka-Krok and Wasylewski, 2003). Data thus suggest functional inter-domain interaction in CheR. Gel filtration studies have suggested that CheR of *S.*

typhimurium is a monomeric protein (Simms *et al*., 1987). The sedimentation velocity and sedimentation equilibrium studies of CheR2 at different concentrations have clearly shown that this protein is monomeric and the binding of SAM and SAH does not alter the oligomeric state of the protein. These data thus indicate that a monomeric state is a general property of CheR methyltransferases.

Another interesting finding resides in the demonstration that CheR2 methylates the McpS and McpT chemoreceptors with the same efficiency. McpS is encoded by the genome of *P. putida* KT2440 whereas McpT is a plasmid encoded receptor present in the strain *P. putida* DOT-T1E (Molina *et al*., 2011). The McpT receptor is of biotechnological relevance since it is responsible for the hyperchemotaxis phenotype towards aromatic toxic hydrocarbons such as toluene (Lacal *et al*., 2011a). It has been shown that there is a link between the degradation of aromatic hydrocarbons and chemotaxis towards these compounds. There are a number of examples demonstrating that chemotaxis towards aromatic hydrocarbons enhances their degradation rate (reviewed in (Lacal *et al*., 2013)). We have shown previously that the transfer of the *mcpT* gene to other species confers chemotaxis towards aromatic compounds, suggesting the establishment of functional signaling complexes between McpT and host proteins (Lacal *et al*., 2011a). The demonstration of efficient methylation of McpT by a foreign CheR methyltransferase is consistent with this suggestion and offers the possibility of “chemotactic engineering” of related strains by the transfer of chemoreceptor genes.

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CHAPTER III

Specificity of the CheR2 Methyltransferase in Pseudomonas aeruginosa is Directed by a C-Terminal Pentapeptide in the McpB Chemoreceptor

Adapted from: Specificity of the CheR2 Methyltransferase in Pseudomonas aeruginosa Is Directed by a C-Terminal Pentapeptide in the McpB Chemoreceptor

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1. Abstract

Methyltransferases of the CheR family and methylesterases of the CheB family control chemoreceptor methylation, and this dynamic posttranslational modification is necessary for proper chemotaxis of bacteria. Studies with enterobacteria that contain a single CheR or CheB show that, in addition to binding at the methylation site, some chemoreceptors bind CheR or CheB through additional high-affinity sites at distinct pentapeptide sequences in the chemoreceptors. We investigated the recognition of chemoreceptors by CheR proteins in the human pathogen *Pseudomonas aeruginosa* PAO1. Of the four methyltransferases in PAO1, we detected an interaction only between CheR2 and the chemoreceptor methyl-accepting chemotaxis protein B (McpB), which contains the pentapeptide GWEEF at its carboxyl terminus. Furthermore, CheR2 was also the only paralog that methylated McpB in vitro, and deletion of the pentapeptide sequence abolished both the CheR2-McpB interaction and the methylation of McpB. When clustered according to protein sequence, bacterial CheR proteins form two distinct families those that bind pentapeptide-containing chemoreceptors and those that do not. These two families are distinguished by an insertion of three amino acids in the β-subdomain of CheR. Deletion of this insertion in CheR2 prevented its interaction with and methylation of McpB. Pentapeptide-containing chemoreceptors are common to many bacteria species; thus, these short, distinct motifs may enable the specific assembly of signaling complexes that mediate different responses.

2. Introduction

Bacteria constantly sense and adapt to changing environmental conditions to assure survival. This important function is primarily mediated by one component systems, two-component systems, and chemosensory pathways (Galperin, 2005; Ulrich *et al*., 2005; Wuichet and Zhulin, 2010). Chemosensory pathways are involved in mediating flagellum and type IV pili mediated taxis and also carry out alternative cellular functions (Wuichet and Zhulin, 2010). The proteins of chemosensory pathways have been classified as auxiliary proteins and core proteins based on the frequency of their occurrence (Wuichet and Zhulin, 2010). Core proteins are the CheA sensor kinase, CheW coupling protein, CheY response regulator, CheR methyltransferase, CheB methylesterase, and chemoreceptors (Wuichet and Zhulin, 2010). Pathway function involves the concerted action of the excitatory pathway and adaptational mechanism(s). The canonical excitatory pathway is initiated by signal recognition at the chemoreceptor, which in turn modulates CheA autophosphorylation and, subsequently, the transphosphorylation of CheY. When phosphorylated, CheY undergoes a conformational change, triggering an alteration of its activity (Hazelbauer *et al*., 2008).

A number of adaptation mechanisms have evolved to restore the prestimulus behavior in the presence of the signal (Vladimirov and Sourjik, 2009; Yuan *et al*., 2012). The canonical adaptation mechanism consists of the methylation and demethylation of chemoreceptors catalyzed by the CheR methyltransferase and CheB methylesterase, respectively (Hazelbauer *et al*., 2008).

Much of what we know in regard to chemosensory pathways is the result of studies of flagellum-mediated taxis in *Escherichia coli* and *Salmonella typhimurium* (reviewed in Hazelbauer *et al*., 2008). *E. coli* has five chemoreceptors that feed stimuli into a single chemosensory pathway. However, genome analyses have shown that other bacteria have an elevated number of chemoreceptors and multiple copies of chemosensory signaling proteins that form different chemosensory pathways (Hamer *et al*., 2010; Wuichet and Zhulin, 2010). For such bacteria, the human pathogen *Pseudomonas aeruginosa* has become a model organism (Kato *et al*., 2008). This specie has five gene

Clusters that encode chemosensory signaling proteins that assemble into four chemosensory pathways, termed Che, Che2, Wsp, and Chp. Two of the pathways mediate chemotaxis; whereas the Che pathway is essential for chemotaxis (Kato *et al*., 1999), the role of the Che2 pathway in chemotaxis is less clear (Güvener *et al*., 2006). The Wsp and Chp pathways modulate diverse celular processes by altering the abundance of cyclic diguanosine monophosphate (c-di-GMP) and cyclic adenosine monophosphate (cAMP), respectively (Fulcher *et al*., 2010; Hickman *et al*., 2005). *P. aeruginosa* has 26 chemoreceptor genes, of which only 4 are found in these gene Clusters; the remaining chemoreceptor genes are scattered through out the genome (Kato *et al*., 2008). Two chemoreceptors, encoded by *mcpA* and *mcpB*, are located in the Cluster II (Figure 44).

McpB (methyl accepting chemotaxis protein B) has a PAS (Per-Arnt-Sim)–type sensor domain, lacks transmembrane regions, and is predicted to be of cytosolic location (Güvener *et al*., 2006). Attempts to identify the function of McpB have been inconclusive. Initially, a study indicated that McpB was involved in aerotaxis (Hong *et al*., 2004), but a subsequent study showed that this was not the case (Güvener *et al*., 2006). It was demonstrated that CheB2 of this pathway was essential for *P. aeruginosa* infection in a murine lung infection model, from which the authors concluded that the CheB2 and the Cluster II are involved in a specific chemotactic response triggered during infection by a yet unknown signal (Garvis *et al*., 2009).

As mentioned, the CheR methyltransferases are among the core proteins of chemosensory pathways. CheR methylates glutamyl residues at the chemoreceptor signaling domain. The extent of methylation, in turn, modulates the capacity of the receptor to control CheA autophosphorylation (Li and Weis, 2000). This mechanism adapts CheA autophosphorylation activity to a given signal concentration. CheR uses S-adenosylmethionine (SAM) as a substrate, and the methylation reaction gives rise to S-adenosylhomocysteine (SAH) (Simms and Subbaramaiah, 1991). CheR and CheB bind to the methylation site of the chemoreceptor. However, the high-abundance chemoreceptors in *E. coli* and *S. typhimurium* have an additional binding site for CheR and CheB formed by a pentapeptide tethered through a flexible linker to the C-terminal end of the signaling domain (Barnakov *et al*., 1999; Djordjevic and Stock,

1998; Li and Hazelbauer, 2006; Okumura *et al*., 1998; Wu *et al*., 1996). Truncation of this pentapeptide abolishes receptor binding (Le Moual *et al*., 1997; Wu *et al*., 1996), markedly decreases methylation and demethylation amounts (Lai and Hazelbauer, 2005; Le Moual *et al*., 1997; Wu *et al*., 1996), and impairs tactic responses (Okumura *et al*., 1998), whereas its addition greatly improves methylation (Barnakov *et al*., 1998). In contrast, the low abundance receptors of *E. coli* do not have these pentapeptides. *E. coli* has a single CheR that acts on receptors with and without a pentapeptide.

The physiological relevance of this pentapeptide binding site is not fully understood but may reside in an increase in the concentration of CheR at the methylation site, thereby increasing the effective enzyme concentration (Windisch *et al*., 2006). *P. aeruginosa*, the model organism of this study, has four CheR methyltransferases (CheR1, CheR2, CheR3, and WspC) that are encoded by four different chemosensory gene Clusters. Receptors with a C-terminal pentapeptide are found in many different bacteria (Perez and Stock, 2007), and the relative abundance of such chemoreceptors varies considerably: whereas some bacteria lack pentapeptide-containing receptors, in other species, such receptors account for about half of the total number of receptors (Perez and Stock, 2007).

In general, the interaction between two proteins is mediated by a single interface. In this context, the dual binding sites of CheR and CheB on pentapeptide-containing chemoreceptors are remarkable exceptions. This phenomenon raises the question of what forces prompted their evolution.The interaction of CheR with pentapeptides has been studied in species that contain a single CheR. However, many bacterial genomes contain multiple copies of CheR (Hamer *et al*., 2010), raising the question of whether all or only a subset of the CheR paralogs interact with the pentapeptide. We have addressed these questions using *P. aeruginosa* as a model organism.

3. Material and Methods

3.1 Strains and plasmids

Table 7. Strains and plasmids used in this study.

Strains	**Use**	**Reference**
*P. aeruginosa*PAO1	Strain from which proteins analyzed in this study are derived from, used for PCR amplification of genes	Stover *et al*., 2000
E. coli BL21 (DE3)	Strain used for protein expression	Studier and Moffatt, 1986
*E. coli*DH5α	Strain used forcloning procedures	Hanahan, 1983
Plasmids	**Use**	**Reference**
pET28b(+)	Km^R, protein expression vector	Novagen
pET28b-CheR1	Protein expression, Km^R, pET28b(+) derivative containing *cheR1* gene	This work
pET28b-CheR2	Protein expression, Km^R, pET28b(+) derivative containing *cheR2* gene	This work
pET28b-CheR3	Protein expression, Km^R, pET28b(+) derivative containing *cheR3* gene	This work
pET28b-WspC	Protein expression, Km^R, pET28b(+) derivative containing *wspC* gene	This work
pET28b-McpB	Protein expression, Km^R, pET28b(+)derivative containing *mcpB* gene	This work
pET28b-McpBΔGWEEF	Protein expression, Km^R, pET28b(+) derivative containing *mcpB* lacking sequence for the final pentapeptide	This work
pET28b-CheR2ΔGPN	Protein expression, Km^R, pET28b(+) derivative containing *cheR2* lacking sequence of GPN tripeptide (amino acids 186-188)	This work
pET28b-CheR1+GPN	Protein expression, Km^R, pET28b(+) derivative containing *cheR1* with GPN insertion following amino acid 180	This work

3.2 Generation of recombinant proteins

The DNA fragments encoding CheR1 (PA3348), CheR2 (PA0175), CheR3 (PA0412), WspC (PA3706), McpB (PA0176), and mutant in McpB receptor lacking the C-terminal pentapeptide called McpBΔGWEEF were amplified by polymerase chain reaction (PCR) (Table 8) and genomic DNA of *P. aeruginosa* PAO1. For *wspC*, PCR products were digested with *Nde*I and *Sac*I; for the *cheR* constructions, with *Nde*I and *EcoR*I; and for *mcpB* and *mcpB*ΔGWEEF, with *Nde*I and *BamH*I. The resulting products were cloned into pET28b(+) (Novagen) linearized with the corresponding enzymes. To generate the

CheR2 derivative lacking amino acids 186 to 188 (CheR2ΔGPN) and a CheR1 derivative with a GPN insertion after amino acid 180 (CheR1+GPN), a modified version of the Hemsley method (Hemsley *et al*., 1989) was used. Pairs of overlapping mutagenic primers (Table 8) were used to amplify the entire plasmid with *Pfu*Turbo DNA polymerase (Agilent Technologies) followed by the elimination of template DNA by digestión with *Dpn*I. The resulting PCR products were transformed into *E. coli* DH5α, and colonies were selected on LB agar-coated plates supplemented with kanamycin (50 mg/ml). Inserts of all plasmids and flanking regions were sequenced. *E. coli* BL21 (DE3) was transformed with the expression plasmids, and cells were grown at 30°C in LB suspension supplemented with kanamycin (50 µg/ml). At an OD_{660} (optical density at 660 nm) 0.4, the temperature was decreased to 18°C, and protein expression was induced at an OD_{660}=0.8 by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight growth, cells were harvested by centrifugation at 10,000 x g. 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 (pH 8.0)] and broken by French Press. After centrifugation at 20,000 x g, supernatants were loaded onto a HisTrap HP column (Amersham Biosciences). Proteins were eluted by applying a linear gradient (for 30min) to 500 mM imidazole in buffer A.

3.3 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed on a VP microcalorimeter (MicroCal) at 25°C. Proteins were dialyzed into 20 mM tris-HCl, 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) (pH 7.5) for titrations of CheR paralogs and mutants with small ligands, and into 20 mM PIPES, 150 mM NaCl, and 1 mM DTT (pH 7.5) for titrations of McpB and McpBΔGWEEF. For protein-protein interaction, both ligands were dialyzed into the latter buffer. The solutions of small molecules were prepared in the dialysis buffer and placed into the injector syringe. In all cases, control experiments involved the injection of syringe ligand into buffer. Raw data were

integrated, corrected for dilution effects, and concentration-normalized before curve fitting using the “one binding site model” of ORIGIN.

3.4 Methylation assays

For the assay using tritium-labeled SAM, a modified version of the assays described by Stock *et al*. (Stock *et al*., 1984) was used. Reactions were carried out in 20 mM Pipes, 150 mM NaCl (pH 7.5). Purified McpB or McpBΔGWEEF (1.7 µM) was incubated in the presence or absence of equimolar concentrations of CheR with 100 µM [^{3}H] SAM (0.83 mCi per sample; PerkinElmer) and a crude extract of *P. aeruginosa* PAO1 (final protein concentration, 5mg/ml). The resulting mixtures were incubated at 30°C for 30 min, and the reaction was stopped by adding 25 µl of ice-cold 10 % (v/v) acetic acid. The amount of methyl ester groups transferred to McpB was quantified as described in Campillo and Ashcroft (1982). For the SDS-PAGE assay, the reaction mixtures in the above buffer contained 1.7 µM McpB or McpBΔGWEEF, 1.6 µM CheR2 or CheR2ΔGPN, and 100 µM SAM. Samples were incubated at 30°C, and 18 µl of aliquots was removed at intervals. A volume of 6 µl of 4X sample buffer was added to each aliquot, which was resolved on 10 % (w/v) SDS-PAGE gels.

4. Results

4.1 *P. aeruginosa* PAO1 has three chemoreceptors with a C-terminal extension

To identify chemoreceptors with a C-terminal extension that may harbor pentapeptides for CheR binding, we aligned the sequences of the cytosolic fragments of the 26 chemoreceptors from *P. aeruginosa*. The C-terminal section of this alignment (Figure 43) shows that there are two receptors, McpA and McpB, that have a C-terminal extension of 30 amino acids, which is comparable to the length of enterobacterial receptors (Li and Hazelbauer, 2006).

```
PA0411   MASEMRNSVSGFKLPEGVEQA
WspA     VANGLRNGVSRFKV
PA1930
PA2573   VAATMRSSVERFKI
BdlA
PA1646   LGRELQGMVRHFRL
PA5072   LGTQLQAQVGRFRL
PA1608   LAIDLNNMVTRFV
PA1251   LANDLKGMIGRFV
PA1561   TAQSQYSLVERFNR
CtpH     LSAQLGDALQRLRA
PA4633   LAEQQRRLVNQFRV
PA2654   LANHQQGLMEQFKA
PctB     QAGRLRQLVDSFKI
PctC     QAGRLRHLVDSFKI
PctA     QAGRLRQLVDSFKI
PA2652   LGQGLGRLVGQFRI
PA4520   QVTQLKRLIGAFRV
PA2920   LSSELARLVGRFRV
PA2867   LAGDLTRAIGQLRL
PA4915   LANGLEQQIQRFT
PA2788   HMAGLRVVLGRFRT
CtpL     LGGDLRTTVQAFRL
McpB     DTPPSVVQLASARPSAPRPSAPAPLARSGMARASKARKEDGWEEF
McpA     GLGALEARPRQEWPERVAREVEELRRPLPSPVSQNSVNVGEVELF
```

Figure 43. C-terminal segment of an alignment of *P. aeruginosa* PAO1 chemoreceptors.

Potential pentapeptide CheR binding sites are in red, and linker regions are in green. Proline residues are shaded in yellow. Receptors BdlA and PA1930 are not shown in this alignment because their signaling domains are shorter than those of the receptors shown.

Both receptors are encoded by part of the che2 gene Cluster (Figure 44). In addition, the receptor PA0411 contains a six–amino acid extension. We hypothesized that these three extensions could constitute CheR docking sites. Therefore, the terminal pentapeptides GWEEF, EVELF, and GVEQA were synthesized for binding studies.

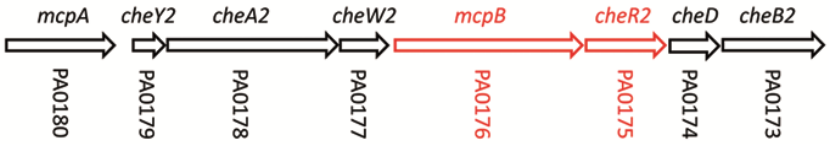

Figure 44. Organization of the Cluster II chemotaxis gene Cluster of *P. aeruginosa* PAO1. In red are shown *mcpB* and *cheR2* genes.

4.2 Affinities of CheR paralogs for SAM and SAH differ largely

The four CheR methyltransferases of *P. aeruginosa* were produced as purified recombinant proteins, and their functionality was validated by microcalorimetric titrations with SAM and SAH (Figure 45 and Table 8). The binding of SAM to CheR1, CheR3, and WspC had dissociation constants (K_Ds) between 15 and 47 mM, values that are similar to that of *S. typhimurium* CheR (Simms and Subbaramaiah, 1991; Simms *et al*., 1987). In contrast, SAM bound with markedly weaker affinity to CheR2 (K_D of almost 200 mM). SAH bound to all four paralogs more tightly than to SAM, with K_D values that ranged from 0.1 to 3.5 mM (Figure 45 and Table 8). That SAH bound more tightly than SAM is also observed for enterobacterial enzymes (Simms and Subbaramaiah, 1991; Yi and Weis, 2002) and is indicative of product feedback inhibition. However, the ratios of the K_D values for SAM and SAH (Table 8), which determine the magnitude of product feedback inhibition, vary greatly among the CheR paralogs: 13 for WspC, indicating modest feedback inhibition, and 361 for CheR2, suggesting strong feedback inhibition.

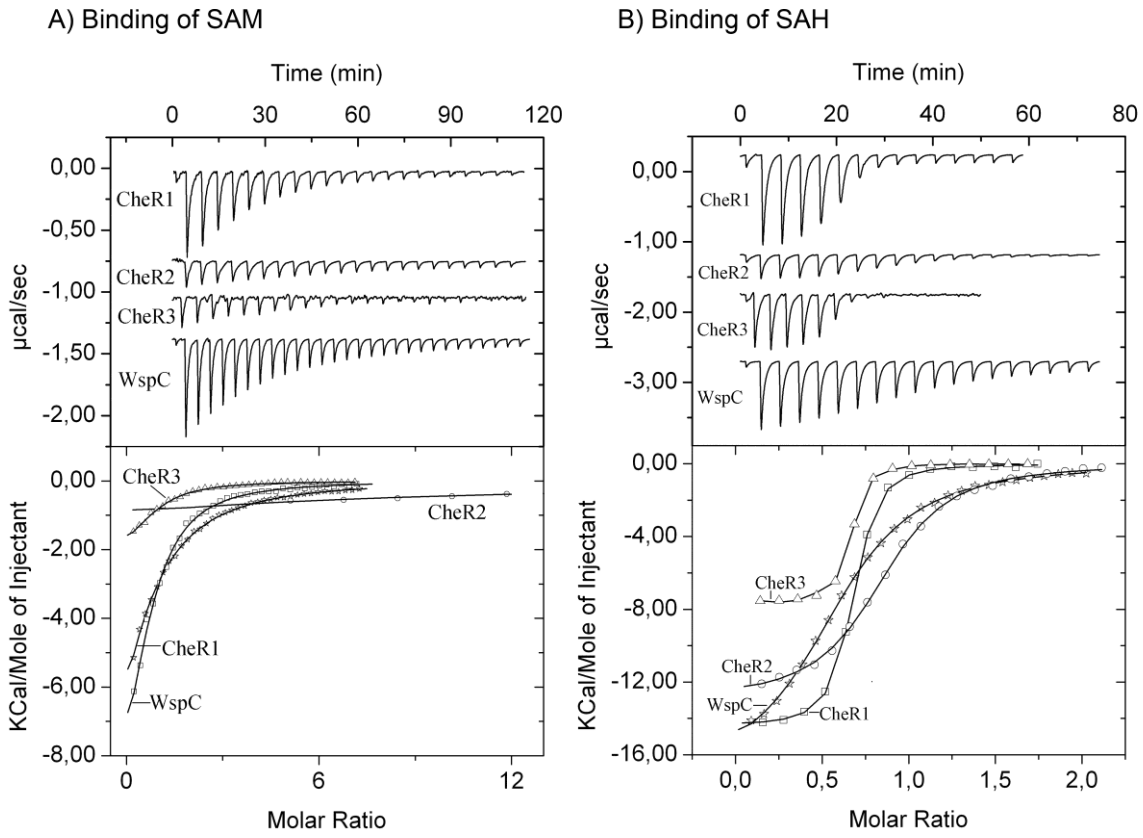

Figure 45. Microcalorimetric titrations of the 4 CheR paralogues of *P. aeruginosa* PAO1 with S-adenosylmetionine (SAM) and S-adenosylhomocysteine (SAH). Upper panels show titrations of the 4 CheR paralogues with 3.2- 6.4 µl aliquots of 0.5-1 mM of SAH and 8.0-12.8 µl aliquots of 1 to 2 mM SAM. Protein concentrations were in the range of 11-31 µM. Lower panel: Integrated, dilution-corrected and concentration normalized peak areas of titration raw data. The derived thermodynamic binding parameters are listed in Table 8. Data are representative of two experiments. Shown are representative experiments.

Table 8. Thermodynamic parameters derived from the microcalorimetric titration of the four CheR paralogs of *P. eruginosa* PAO1 with SAM and SAH.

	SAM		SAH		*average* K_DSAM/ *average* K_DSAH
Protein	K_D (μM)	Δ*H* (kcal/mol)	K_D (μM)	Δ*H* (kcal/mol)	
CheR1	17.2 ± 1	-12.6 ± 0.8	0.21 ± 0.1	-14.4 ± 0.04	72
	15.0 ± 2	-13.0 ± 4	0.24 ± 0.1	-13.9 ± 0.05	
CheR2	199 ± 37	-17.1 ± 6.3	0.56 ± 0.02	-13.0 ± 0.09	361
	181 ± 30	-18.8 ± 6.0	0.49 ± 0.02	-12.2 ± 0.12	
CheR3	18.5 ± 1	-2.7 ± 0.2	0.1 ± 0.01	-7.6 ± 0.04	163
	19.1 ± 3	-3.2 ± 0.7	0.13 ± 0.02	-7.9 ± 0.09	
WspC	47.0 ± 2	-19.2 ± 1.8	3.4 ± 0.1	-17.3 ± 0.12	13
	44.0 ± 2.6	-15.9 ± 1.7	3.5 ± 0.07	-17.5 ± 0.06	

The corresponding data are shown in Figure 45. Data are of two different measurements with the errors of curve fitting indicated.

4.3 Of the four CheR paralogs, only CheR2 binds pentapeptides

To establish whether any of the four CheR paralogs bound to the pentapeptides identified, we titrated the purified CheR proteins with the pentapeptides GWEEF (corresponding to McpB), EVELF (corresponding to McpA), and GVEQA (corresponding to PA0411). As a control, protein was titrated after every experiment with SAH to verify its capacity to recognize the methylation substrate (as marker for the native state of the protein). None of the four CheR enzymes bound either the EVELF or the GVEQA pentapeptides. However, whereas CheR1, CheR3, and WspC did not bind the GWEEF pentapeptide, CheR2 bound this pentapeptide with high affinity (Figure 46 and Table 10).

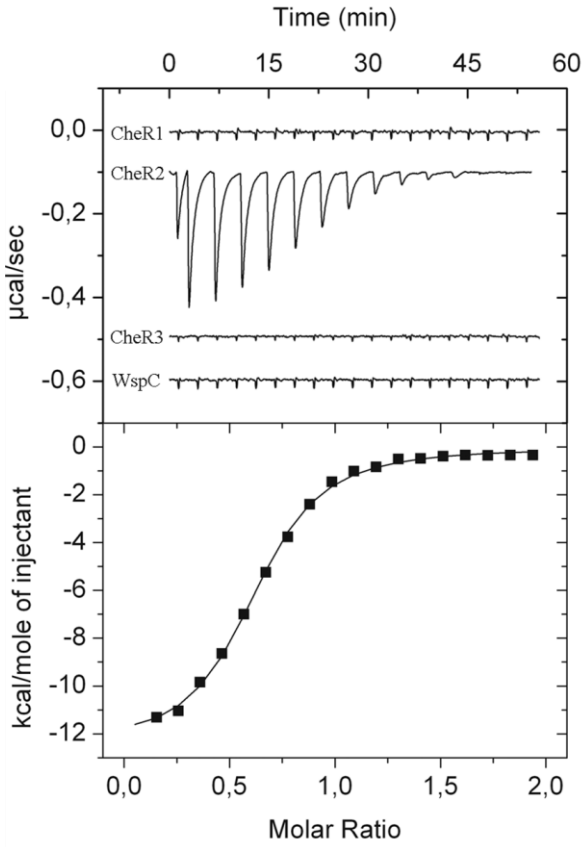

Figure 46. Microcalorimetric titration of CheR paralogs with the peptide GWEEF. Upper panel: Raw data for the titration of 10 to 30 µM protein with 0.5 to 1 mM pentapeptide. Lower panel: Integrated, dilution-corrected, and concentration-normalized data for the titration of CheR2 with the pentapeptide. The resulting binding parameters are given in Table 10. Shown is a representative of two experiments.

We then investigated whether any of the proteins bound the enterobacterial NWETF pentapeptide. Shiomi *et al* (Shiomi *et al*., 2000) reported that the W (Trp) and F (Phe) of this peptide are essential for CheR binding, whereas mutation of the remaining three amino acids had only a slight effect. We found that CheR2, but none of the other paralogs, bound this non cognate pentapeptide (Figure 47). With a K_D of about 1.4 mM (Table 10), the affinity of NWETF for CheR2 was greater than that for its cognate CheR in *E. coli* (reportedly 10 mM) (Yi and Weis, 2002).

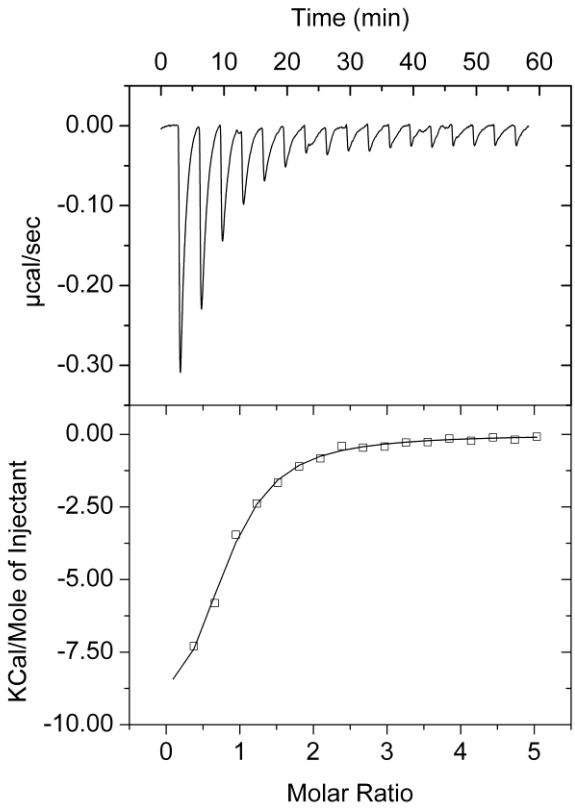

Figure 47. Microcalorimetric titration of CheR2 with the NWETF pentapeptide. Shown is in the upper panel the titration of 6 µM of CheR2 with 0.5 mM of NWETF pentapeptide. The injection volume was 4.8 µl. The lower panel shows integrated, dilution-corrected and concentration normalized peak areas of titration raw data. Derived thermodynamic binding data are given in Table 10. Shown is a representative experiment.

4.4 CheR2 specifically targets the McpB chemoreceptor

The results, thus far, show that, in *P. aeruginosa*, there is a single chemoreceptor-pentapeptide interaction, namely, that between McpB and CheR2. The genes encoding the proteins are located next to each other in the genome, and both form part of the Cluster II (Figure 44) (Ferrández *et al*., 2002). Because McpB lacks transmembrane regions, we produced the full length recombinant protein and performed microcalorimetric binding studies with purified CheR2 (Figure 48A). McpB and CheR2 had a high affinity interaction (K_D of about 0.15 µM), which was about three fold greater than that of the McpB-associated pentapeptide, GWEEF, for CheR2 (Table 10). To determine the contribution of the pentapeptide in CheR2 binding, a shortened

construct of McpB that lacked the GWEEF peptide was titrated with CheR2 (Figure 48B). Deletion of the peptide resulted in complete loss of binding; heat changes observed were similar to those of titrations of CheR2 with buffer.

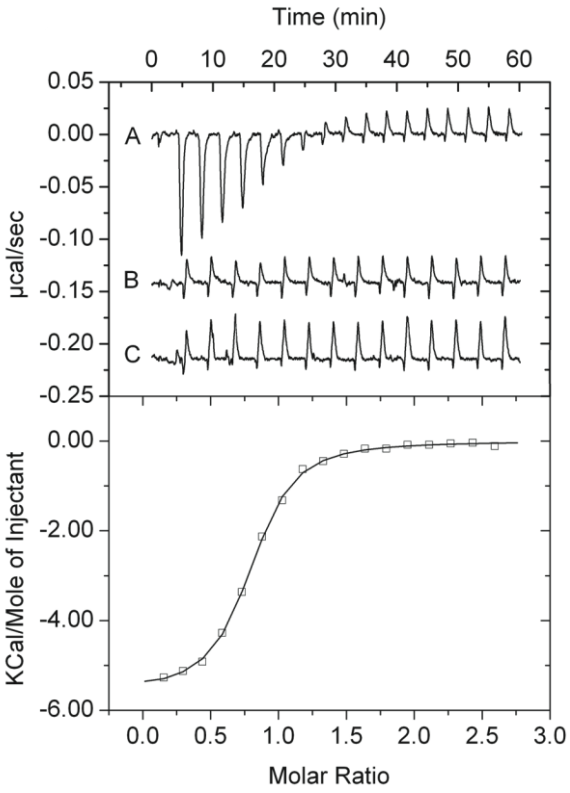

Figure 48. Binding studies of native and mutants McpB and CheR2 (A to C). Upper panel: Microcalorimetric titrations of 4 to 6 µM wild-type or mutant McpB with 50 to 70 mM wild-type or mutant CheR2: (A) titration of McpB with CheR2, (B) titration of McpBΔGWEEF with CheR2, and (C) titration of McpB with CheR2ΔGPN (Section 4.6 of Results). Lower panel: Integrated, dilution-corrected, and concentration-normalized peak areas of the titration of McpB with CheR2. The resulting binding parameters are given in Table 10. Shown are representatives of two experiments each.

4.5 No allostery exists between the SAM or SAH binding site and the pentapeptide binding site

Multiple binding sites on proteins often are indicative of allosteric effects. To explore this possibility for CheR2, a series of microcalorimetric titrations were conducted in which the protein was saturated with one ligand and then titrated with the remaining ligand. We saturated CheR2 with SAM or SAH and tested its titration with GWEEF, or

we saturated CheR2 with GWEEF and tested its titration with SAM or SAH (Figure 49). The derived parameters (Table 9) were then compared to those obtained from controls with ligand free CheR2 (Figure 49). Our data showed that the addition of saturating concentrations of SAM or SAH did not alter binding of the GWEEF pentapeptide to CheR2 and viceversa, suggesting that there are no allosteric effects between the SAH/SAM and pentapeptide binding sites at CheR2.

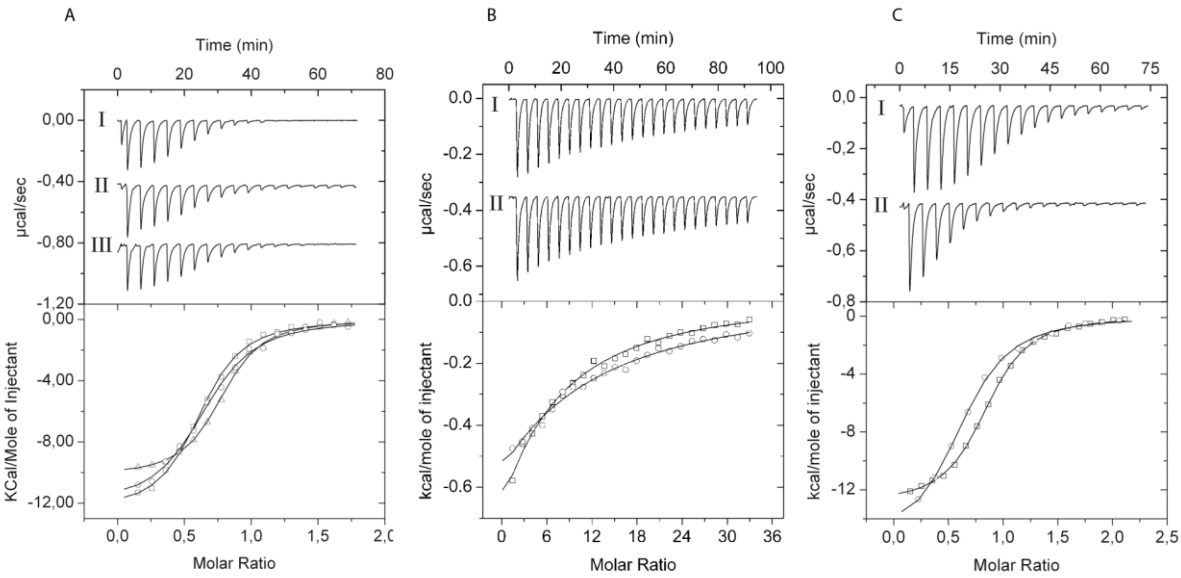

Figure 49. Lack of an allosteric interaction between the pentapeptide-binding site and SAM/SAH-binding site in CheR2. (A) Microcalorimetric titrations of CheR2 in the absence (I) or presence of SAM (II) or SAH (III) with the pentapeptide. Experiment I corresponds to an injection of 500 μM pentapeptide GWEEF into 11 μM CheR2. Experiments II and III are repetitions of experiment I except that 2 mM SAM or 30 μM SAH, respectively, were added to both titrants. □ = absence of ligands, ○ = in the presence of 2 mM SAM, Δ = presence of 30 μM SAH. (B) Microcalorimetric titrations of CheR2 (7 to 11 μM) in the absence (I) and presence (II) of the pentapeptide GWEEF (30 μM) with SAM. (C). Microcalorimetric titrations of CheR2 in the absence (I) and presence (II) of the pentapeptide GWEEF (30 μM) with SAH. □, in the absence of pentapeptide, ○ in the presence of pentapeptide. Shown are representative experiments.

Table 9. Lack of allostery between the pentapeptide-binding site and the SAM/SAH-binding site in CheR2.

Solution 1	Solution 2	K_D (μM)	ΔH (kcal/mol)
CheR2	GWEEF	0.52 ± 0.03	-12.6 ± 0.2
		0.49 ± 0.1	-11.8 ± 0.5
CheR2+SAM	GWEEF + SAM	0.71 ± 0.06	-12.2 ± 0.2
		0.68 ± 0.07	-12.8 ± 0.3
CheR2+SAH	GWEEF + SAH	0.37 ± 0.02	-10.3 ± 0.1
		0.35 ± 0.03	-10.7 ± 0.02
CheR2	SAM	199 ± 37	-17.1 ± 6.3
		181 ± 30	-18.8 ± 6.0
CheR2+GWEEF	SAM + GWEEF	246 ± 77	-11.8 ± 5.6
		273 ± 24	-9.4 ± 0.50
CheR2	SAH	0.56 ± 0.02	-13.0 ± 0.1
		0.49 ± 0.02	-12.2 ± 0.12
CheR2+GWEEF	SAH + GWEEF	0.74 ± 0.04	-16.0 ± 0.3
		0.75 ± 0.06	-15.5 ± 0.4

Thermodynamic parameters for the titration of CheR2 in the absence or presence of different ligands. The experimental data are shown in Figure 49. Data are of two different measurements with the errors of curve-fitting indicated.

4.6 CheR methyltransferases form two distinct protein families on the basis of pentapeptide binding

Only one of the four CheR paralogs in *P. aeruginosa* bound pentapeptides, raising the question whether structural or sequence features determine the binding of a CheR to pentapeptides. To address this question, we aligned the CheR sequences of proteins known to bind or not to bind pentapeptides (Perez and Stock, 2007) (Figure 50).

```
                10        20        30        40        50        60        70        80        90        100       110       120       130
                |         |         |         |         |         |         |         |         |         |         |         |         |
E. coli         ----MTSSLPCGQ---TSLLLQMTERLALSDAHFRRISQLIYQRAGIVLADHKRDMVYNRLVRRLRSLGLTDFGHYLNLLESNQH-SGEWQAFINSLTTNLTAFFR-EAHHFPLLADHAR---RR--SGE
S. typhimurium  ----MTSSLPSGQ---TSVLLQMTQRLALSDAHFRRICQLIYQRAGIVLADHKRDMVYNRLVRRLRALGLDDFGRYLSMLEANQN-SAEWQAFINALTTNLTAFFR-EAHHFPILAEHAR---RR--HGE
E. carotova     ----MKSTPSQNRPESASILTQMVDRLPLSDTHFRRISQLIYQRAGIVLADHKREMYNRLVRRLRLLGINDFGQYLALLESDPN-SAEWQAFVNALTTNLTAFFR-EAHHFPIIAEHAK---KR--PNG
B. mallei       MPARAASRLDAPE--PQERSGDAARDFEFTSADFTKIRALIHRRAGISLSEHKRDMAYSRLARRLRARGLNRFCDYLLELLERENDD-PSEWEAFTNSLTTNLTAFFR-ESHHFPIIAEFVK---RR--EQP
R. solanacearum  ---MAQTLAPVS--PTLAS----REFSFTAEDFARIRSLIYRRVGISLSDRKSEMVYSRLARRLRIVNIASFRDYLDALENGHL-PDEWEAFTNALTTNLTAFFR-EQHHFPIIAEHAK---AK--RTP
P. aerug CheR2  ----MPTSTPSPV---FG-----NQEFHYTREDFQQVRERLYRLTGISLAESKAQLVYSRLSRRLRLLRLGSFAEYFTHLDRE---PGEQQLFVNALTTNLTAFFR-ERHHFPLLADLARRQLQR--HRP
B. subtilis     ----------------------------MDTYSVFTTKWKQLTGVDLTLYKEAQMKRRLTSLYEKKGFQSFKDFAAALEKD---QALLNETLDRMTINVSEFYRNYKRWEVLETAILPLIKT---SRP
T. maritima     ----MQEERSEKKIGPFKFQSN-FEWKEFPQEEFEWFVKEVEKRFGLNLSSYKPQRVKKR-TELLLRKYNVGYREYLNMLIKD---KKYLDEFMDKMTINVTEFFRNPEKWWELRDEIIPLIAKN--SLR
P. aerug CheR1  ----------------------------MSAANADFELFRVFLEKTCGIVLGSNKQYLVSSRLNKLMEQQGIKSLGELVQRIQTQR--GGLREMVVDAMTNETLWFRDTYPFEVLKQRVLPELIKANGGQR
P. aerug CheR3  ----------MQANG-----VWSLQPLADMSAAEFRDWQVLLENNTGVVLNEQRRTFLQASLTARMRELGIGDYHSYYRQVTDGPRGAVEWATLLDRLTVQETRFFRHPPSFELLERYLGERLRREGMPRP
P. aerug WspC    ------------------------------MNDRFERLLKSRIGLDASSVGSAVIERAVRQRMSGLALHDEDEYWMRLNGS---PGEVQALIEAVVPETWFFRYPESFTTLARLAFERLPSLGGGRA

                140       150       160       170       180       190       200       210       220       230       240       250       260
                |         |         |         |         |         |         |         |         |         |         |         |         |
E. coli         YRVWSAAASTGEEPYSIAMTLADTLG----TAPGRWKVFASDIDTEVLEKARSGIYRHEELKN-LTPQQLQRYFMRGTGPHEGLVRVRQELANYVDFAPLNLLAKQYTVPGP-FDAIFCRNVMIYFDQTT
S. typhimurium  YRVWSAAASTGEEPYSIAITLADALG----MAPGRWKVFASDIDTEVLEKARSGIYRLSELKT-LSPQQLQRYFMRGTGPHEGLVRVRQELANYVEFSSVNLLEKQYNVPGP-FDAIFCRNVMIYFDKTT
E. carotova     YTIWSTAASTGEEPYSLAMTLAEVLG----NKASGCQVWASDIDTQVLEKATAGIYRQEELRS-LSPQQLQRFFLRGTGPHSGLVRVRPELASMVHFQQLNLLAPDWSVPAP-FDAIFCRNVMIYFDKET
B. mallei       VSVWCSAASTGEEPYSIAMTLVEALGE---SAARSASVIATDLDTQVLAKADAGVYAFEQVKH-LSPERLKRFFLKGTGTQAGRVKVRPELRAMIRFAQLNLTDADYGLTKP-FDAIFCRNVMIYFDKPT
R. solanacearum  FSVWCSASSTGEEPYSIAITLAETFGS---MSPGQVSVVASDVDTNALARARAGIYPMERVSA-LSADRLKKYFLRGTGKQEGYARVRPELQAMIDFRQINLLDRDWPLTQK-FDVIFCRNVMIYFDKPT
P. aerug CheR2  LRIWSAAASTGEEPYSIAITLVEALG----SPDPFVKIVASDIDTGVLDCARQGVYPLERLEQ-MPAPLKKRFFLRGTGPNAGKARVVEELRQLVEFRQINLLEADWSIAGE-LDAIFCRNVMIYFDKPT
B. subtilis     LKIWSAACSTGEEPYTLAMLLDQQKG------LPGYQILATDIDEKALEKAKKGVYQERSLQE-VPLSVKDRYFTQNA---NRSYEVKTEIKKNITFKKHNLLADRYEQD--FDLIVCRNVFIYFTESA
T. maritima     MKFWSAGCSSGEEPYSLAILVHELNL------SYKTRILATDIDIGVLRRAQEGIYEERAFVS-TPKEYLEKYFEKLP---DGRYRIKDSVKKIVEFKRHDLLKDPFEKN---FDLIVCRNVVIYFEPEA
P. aerug CheR1  LRIWSAACSSGQEPYSLSMAIDEFEKTNLGQLKAGVQIVATDLSGSMLTAAKAGEYDTLAMGRGLSPERLQRYFDAKG---PGRWAVKPAIRSRVEFRALNLLDSYASLG-K-FDMVFCRNVLIYFSAEV
P. aerug CheR3  WALWSVGCCSSGEEPYSLAMCAAQVLRGQ--ERDDFFGVTGTDISLHALQRARQANYPARKLEQ-LEAGLVERYCERQA---DGSFSVKTILTERVCCARLNVLDLAKAPWSG-MDVIFCQNLLIYFRRWR
P. aerug WspC    LRILSLPCSTGEEPYSIVMALLDAGLS-----EYLFEVDALDVSARVIERASLGVVGRNSFRG-DELGFRDRHFSEVA---EGYQLAEQVRRKVRFRCGNLLDPGLLAGEAPYDFVFCRNLLIYFDRPT
```

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                    270       280       290       300       310       320       330       340       350       360       370       380       390
                     |         |         |         |         |         |         |         |         |         |         |         |         |
E. coli          QQEILRRFVPLLKPDGLLFAGHSENFSHLERRFTLRGQTVYALS----------KD----------------------------------------------------------------
S. typhimurium   QEDILRRFVPLLKPDGLLFAGHSENFSNLVREFSLRGQTVYALS----------KDKA--------------------------------------------------------------
E. carotova      QERILRRFVPMLKPGGLLFAGHSENFSQISREFYLRGQTVYGLA----------KER-----------------------------------------------------------------
B. mallei        QSQVLARFEPLMKPGGLLFAGHSENFTYVTQAFRLRGQTVYELTRDGQSAAPRVRARIAAAAAEEELR-------------------------------------------------------
R. solanacearum QAKILEHFVDVMKPDGLLFAGHSESFLQVTKAWSLRGKTVYEI------A-PEIRAKLGAR-------------------------------------------------------------
P. aerug CheR2   QTRLLERMVALLRPEGLFFAGHSENFVHASHLVRSVGQTVYSPA-------------------------------------------------------------------------------
B. subtilis      KEELYLKMAHSLKKNGVLFVGSTEQIFNPEKFGLVPADTFFYQKR-------------------------------------------------------------------------------
T. maritima      KNELYRKFAESLKPGGFLFVGNTERIFNYKELGFEIYKPFIYRKVK-------------------------------------------------------------------------------
P. aerug CheR1   KRDILLRIHGTLKPGGYLFLGASEALNNLPDHYQMVQCSPGIIYRAK-------------------------------------------------------------------------------
P. aerug CheR3   RREILNRLAERLAPGGLLVIGVGEVVDWSHPELEPVADERVLAFTRKGYSGT-------------------------------------------------------------------------------
P. aerug WspC    QSEVVEVLKRLLRPDGAMFIGPAEASLLSQHGMQPIGVPLSFVFRRTSEAPRGARPKAVSDGARPVVAAAVERASIRPSPPPPAKPRQRLSSLVPPASGQPLASPVGEFDEIARLADAGQHREARAACER

                    400       410       420       430       440       450       460
                     |         |         |         |         |         |         |
E. coli          ------------------------------------------------------------------------
S. typhimurium   ------------------------------------------------------------------------
E. carotova      ------------------------------------------------------------------------
B. mallei        ------------------------------------------------------------------------
R. solanacearum ------------------------------------------------------------------------
P. aerug CheR2   ------------------------------------------------------------------------
B. subtilis      ------------------------------------------------------------------------
T. maritima      ------------------------------------------------------------------------
P. aerug CheR1   ------------------------------------------------------------------------
P. aerug CheR33 ------------------------------------------------------------------------
P. aerug WspC    QLAARGPSATVFYWLGLLSDVAGQEQEAQDFYRKALYLEPQHAEALAHLAALLAARGDHAGARRLQQRAARGVNKDG
```

Figure 50. Sequence alignment of different CheR sequences. The accession codes of these sequences are as follows: **P07364** (CHER ECOLI), **P07801** (CHER_SALTY), **Q6D6I8** (Q6D6I8_ERWCT), **Q62G10** (Q62G10_BURMA), **Q8XQ81** (Q8XQ81_RALSO), **P31105** (CHER_BACSU), **Q9WYT5** (CHER_THEMA), **O87131** (CHER1_PSEAE), **Q9I6V7** (CHER2_PSEAE), **G3XCT5** (CHER3_PSEAE), **Q9HXT5** (WspC_PSEAE). The amino acids that were removed in the Clustering analysis shown in Figure 56 are highlighted in yellow.

The selection of sequences was based on the following rationale: The NWETF peptide binds to CheR of *S. typhimurium* (Barnakov *et al*., 1999; Wu *et al*., 1996) and *E. coli* (Barnakov *et al*., 1999; Li and Hazelbauer, 2006). *Erwinia carotovora*, *Burkholderia mallei*, and *Ralstonia solanacearum* each has a single CheR and 19, 3, or 4 pentapeptide containing chemoreceptors, respectively (Perez and Stock, 2007), indicating that the CheR binds pentapeptides. We found here that *P. aeruginosa* CheR2 binds GWEEF pentapeptide, whereas the remaining three paralogs did not. CheR mediated receptor methylation has also been observed in *Bacillus subtilis* (Kirby *et al*., 1999) and *Thermotoga maritima* (Perez and Stock, 2007). However, both bacteria are devoid of chemoreceptors with pentapeptide extensions (Perez and Stock, 2007), which strongly suggests that the CheR does not bind pentapeptides. CheRs that bind pentapeptides and those that do not, cluster into two distinct groups (Figure 51).

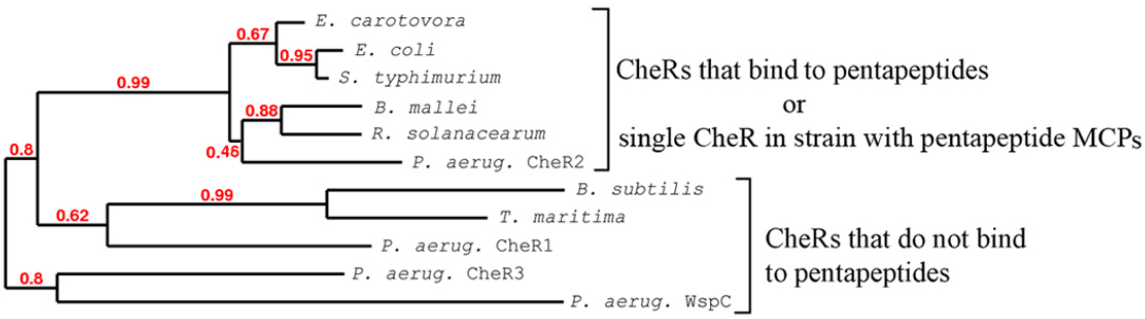

Figure 51. Clustering of sequences that are pentapeptide-dependent or pentapeptide-independent. The corresponding accession codes are provided in the legend of Figure 50. The figure was produced using the phylogeny.fr server.

We noticed that pentapeptide-binding CheR proteins have a three–amino acid insertion (GXX; GPN in *P. aeruginosa*) that is absent in CheR proteins that do not bind pentapeptides (Figure 52).

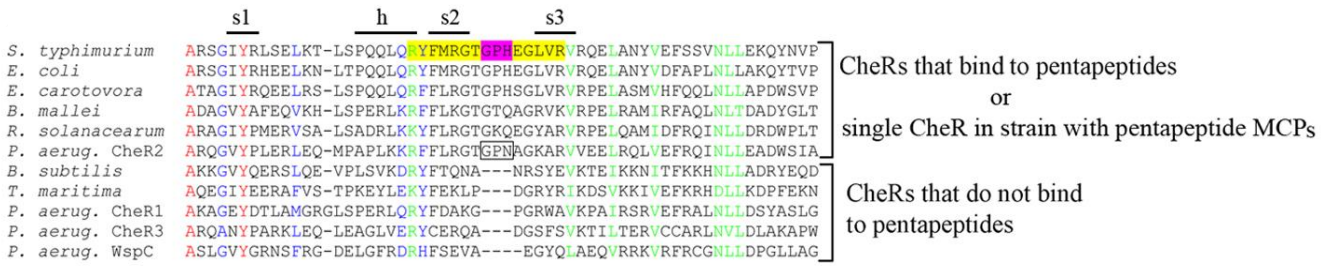

Figure 52. Segment of the sequence alignment comprising the β-subdomain of pentapeptide dependent and pentapeptide independent CheR. The full alignment is shown in Figure 50. Secondary structure elements are indicated: h, helix; s, strand. Residues in yellow and pink interact with the pentapeptide. The tripeptide that is absent in the pentapeptide-independent methyltransferases is shown in pink.

The inspection of the CheR structure revealed that this insertion is part of a loop that links strands 2 and 3 of the CheR β-subdomain that contains the pentapeptide-binding site (Djordjevic and Stock, 1998) (Figure 53).

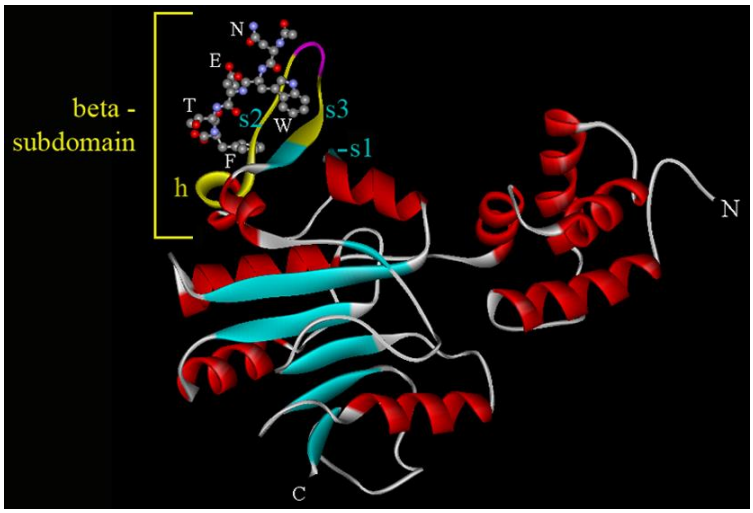

Figure 53. CheR structure of *S. typhimurium* (Djordjevic and Stock, 1998) with bound pentapeptide shown in ball-and-stick mode. The structural elements shown in yellow and pink correspond to the sequence fragments of the same color in Figure 52.

To determine whether this insertion is essential for pentapeptide binding, we generated a CheR2 mutant in which GPN (Figure 52) was deleted. The titration of CheR2ΔGPN with SAH (Figure 54) revealed that the CheR2 mutant had an affinity for SAH that was similar to that of the wild-type protein (Table 10), indicating that this mutation did not substantially alter protein structure. However, CheR2ΔGPN did not bind the GWEEF or NWETF peptides (Figure 54) or full-length McpB (Figure 48C and 55), indicating that this insertion is essential for pentapeptide recognition.

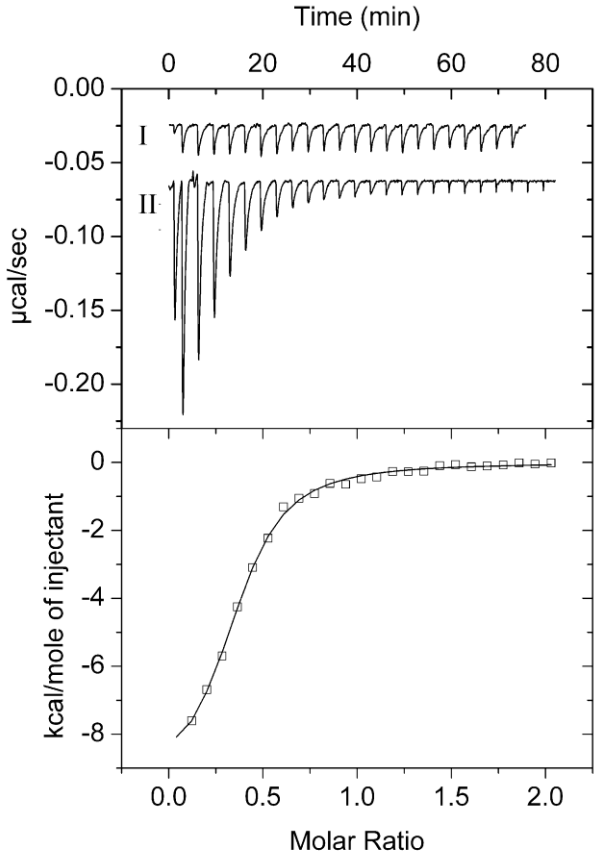

Figure 54. Microcalorimetric titration of CheR2ΔGPN with the GWEEF pentapeptide (I) and SAH (II). The upper panel shows titrations of 7 µM CheR2ΔGPN with 0.5 mM of GWEEF pentapeptide (I) and 0.25 mM of SAH (II) (injection volume of 3.2 µl). Lower panel: Integrated, dilution-corrected and concentration normalized peak areas of SAH titration data. The derived thermodynamic parameters are given in Table 10. Shown is a representative experiment.

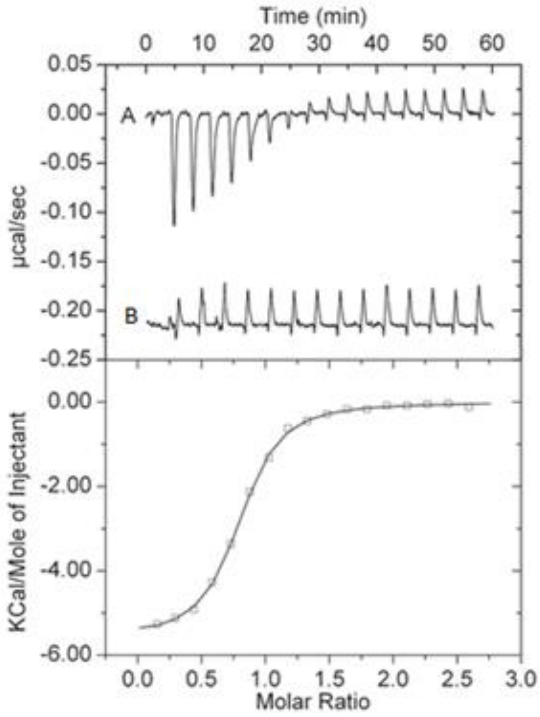

Figure 55. Titration of McpB with CheR2 (A) and CheR2ΔGPN (B). The upper panel shows titrations of 4-6 µM of McpB with 40-60 µM of CheR2 (A) or with 40-60 µM of CheR2ΔGPN (B) (injection volume of 3.2 µl). Lower panel: Integrated, dilution-corrected and concentration normalized peak areas of CheR2 titration data. The derived thermodynamic parameters are given in Table 10. Shown is a representative experiment.

To assess the effect of the tripeptide on the CheR sequence clustering, the cluster analysis shown in Figure 51 was repeated using the sequences of pentapeptide-binding CheR proteins from which the tripeptide was deleted. The resulting sequence clustering (Figure 56) was very similar to that obtained for the native sequences (Figure 51), suggesting that this tripeptide is only one of several sequence characteristics that define these two distinct protein families.

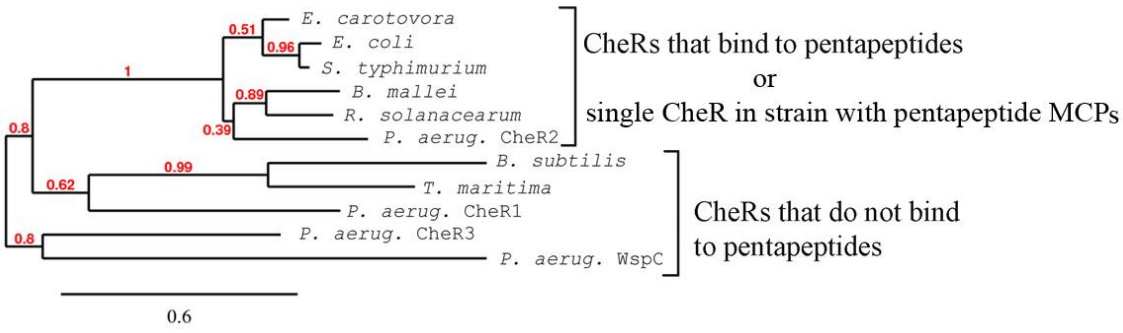

Figure 56. Clustering of CheR sequences after deletion of the GXX tripeptide. The identity of protein sequences is provided in the legend of Figure 50. Similarity scores are provided in red. Shown below is the distance scale bar that represents the genetic distance calculated by the number of amino acid substitutions per site of 0.6.

To verify this conclusion, we constructed a CheR1 mutant into which the GPN sequence was inserted at the appropriate position (Figure 52). The resulting CheR1+GPN protein bound SAH tightly but did not acquire the capacity to bind pentapeptides (Figure 57 and Table 10), confirming that the tripeptide insert is only one of several features that determine pentapeptide binding.

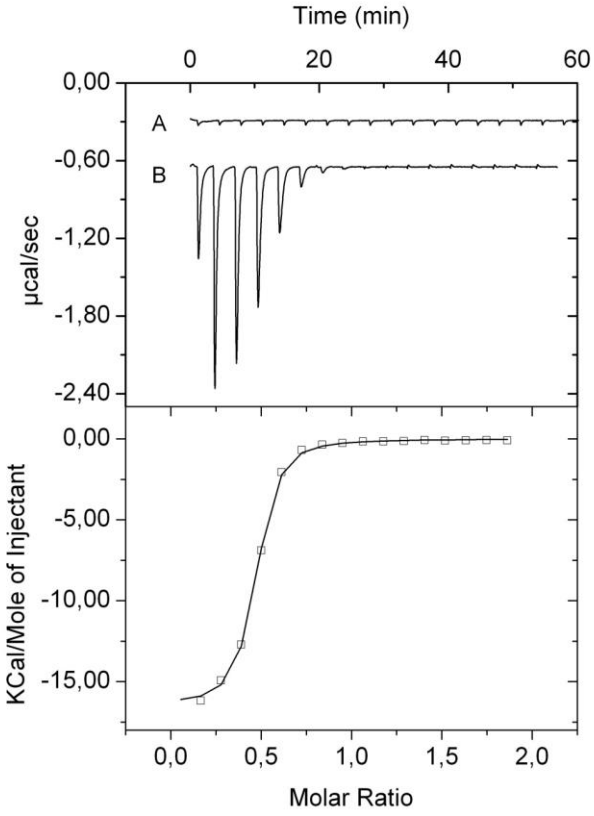

Figure 57. Microcalorimetric titrations of CheR1+GPN with the GWEEF pentapeptide or SAH. Upper panel: Titrations of 20 µM CheR1+GPN with 1 mM GWEEF pentapeptide (A) and 1 mM SAH (B). The injection volume was 4.8 µl and 3.2 µl, respectively. Lower panel: Integrated, dilution-corrected, and concentration-normalized peak areas of raw titration data. The derived thermodynamic data are shown in Table 10. Shown is a representative experiment.

Table 10. Thermodynamic parameters derived from the titration of native/mutant methyltransferases with pentapeptides, SAH or with native/mutant McpB chemoreceptor. The corresponding data are shown in Figs. 46, 47, 48, 54, 55 and 57. Data are of two different measurements with the errors of curve-fitting indicated.

Analyte	Titrant	K_D (μM)	ΔH (kcal/mol)
CheR2	GWEEF	0.52 ± 0.03	-12.6 ± 0.20
		0.49 ± 0.1	-11.8 ± 0.5
CheR2	NWETF	1.42 ± 0.17	-11.4 ± 0.70
		1.34 ± 0.3	-11.8 ± 1.9
CheR2	McpB	0.15 ± 0.01	-5.59 ± 0.04
		0.17 ± 0.04	-5.27 ± 0.1
CheR2	McpBΔGWEEF	No binding	
CheR2ΔGPN	SAH	0.43 ± 0.03	-9.64 ± 0.2
		0.46 ± 0.5	-10.6 ± 1.5
CheR2ΔGPN	GWEEF	No binding	
CheR2ΔGPN	NWETF	No binding	
CheR2ΔGPN	McpB	No binding	
CheR1+GPN	SAH	0.17 ± 0.01	-16.5 ± 0.10
		0.22 ± 0.02	-14.1 ± 0.10
CheR1+GPN	GWEEF	No binding	
CheR1+GPN	NWETF	No binding	
CheR1+GPN	McpB	No binding	

4.7 McpB is exclusively methylated by CheR2

To determine which of the four CheRs methylates McpB, we conducted methylation assays using purified proteins. All samples, including the controls, were composed of an extract of soluble *P. aeruginosa* proteins that contains the proteins necessary to degrade SAH and avoid product feed back inhibition. Thus, the activity in the control samples corresponds to the methylation caused by components of this cellular extract (Figure 58A).

The amount of McpB methylation in the presence of CheR1, CheR3, or WspC was similar to the control, which implies that McpB is not methylated by these enzymes.

In contrast, in the presence of CheR2, the amountof methylated McpB was about 12-fold greater than that in the control sample, demonstrating that CheR2 is the only paralog that methylates McpB. To further characterize the CheR2-mediated methylation, samples were run on SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The methylation of other chemoreceptors was found to increase their electrophoretic mobility on SDS-PAGE gels (Engström and Hazelbauer, 1980; Kehry and Dahlquist, 1982; Levit and Stock, 2002; Shiomi *et al*., 2002; Springer *et al*., 1977). Exposure of McpB to CheR2 resulted in four distinct bands with increased electrophoretic mobility that likely corresponds to different methylated forms of McpB (Figure 58B).

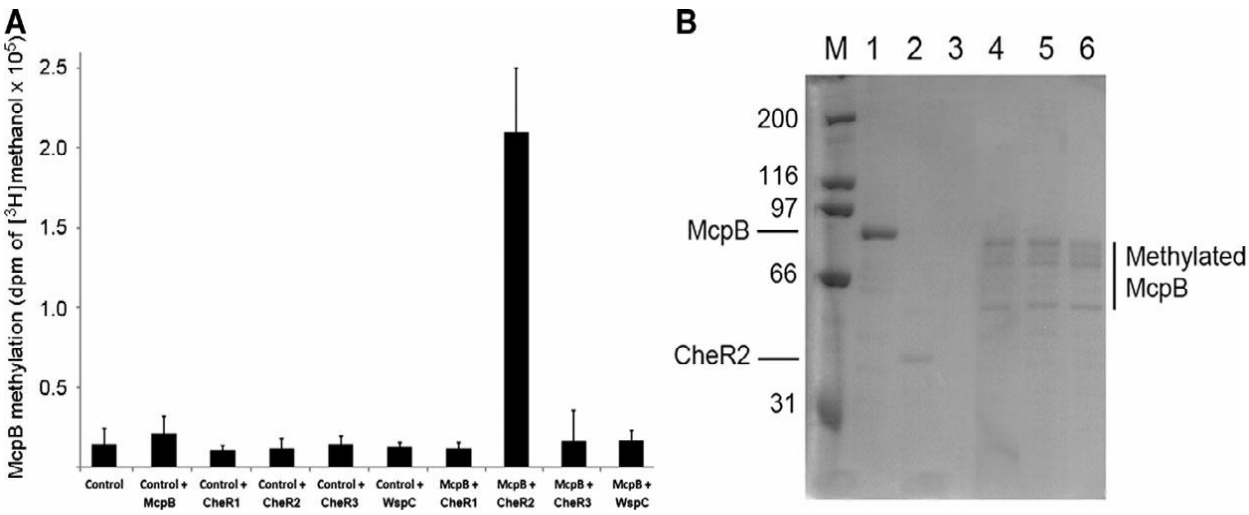

Figure 58. Methylation of McpB by the four CheR paralogs. (A) Methyltransferase assays of purified McpB and CheR paralogs. Data are means ± SD from three experiments. (B) SDS-PAGE gel stained with Coomassie detecting the shift in McpB mobility because of its methylation by CheR2. Lanes 1 and 2: purified McpB and CheR2, respectively; lane 3: soluble *P. aeruginosa* protein extract; lanes 4 to 6: methylation reaction of purified McpB and CheR (in the presence of *P. aeruginosa* protein extract) after 30, 60, and 90 min.

4.8 Disabling the CheR2 pentapeptide interaction abolishes McpB methylation

Having established that McpB was exclusively methylated by CheR2, we investigated the impact of disabling the pentapeptide-McpB interaction on the methylation of McpB by performing methylation assays with McpB, McpBΔGWEEF, CheR2, and CheR2ΔGPN. As previously shown, the interaction was detected exclusively between McpB and CheR2. Deletion of the terminal pentapeptide from McpB or the deletion of the GPN tripeptide, both of which abolished an interaction, also reduced the methylation of McpB to amounts similar to controls (Figure 59). We can therefore conclude that the interaction of CheR2 with the McpB pentapeptide is essential for methylation under normal conditions.

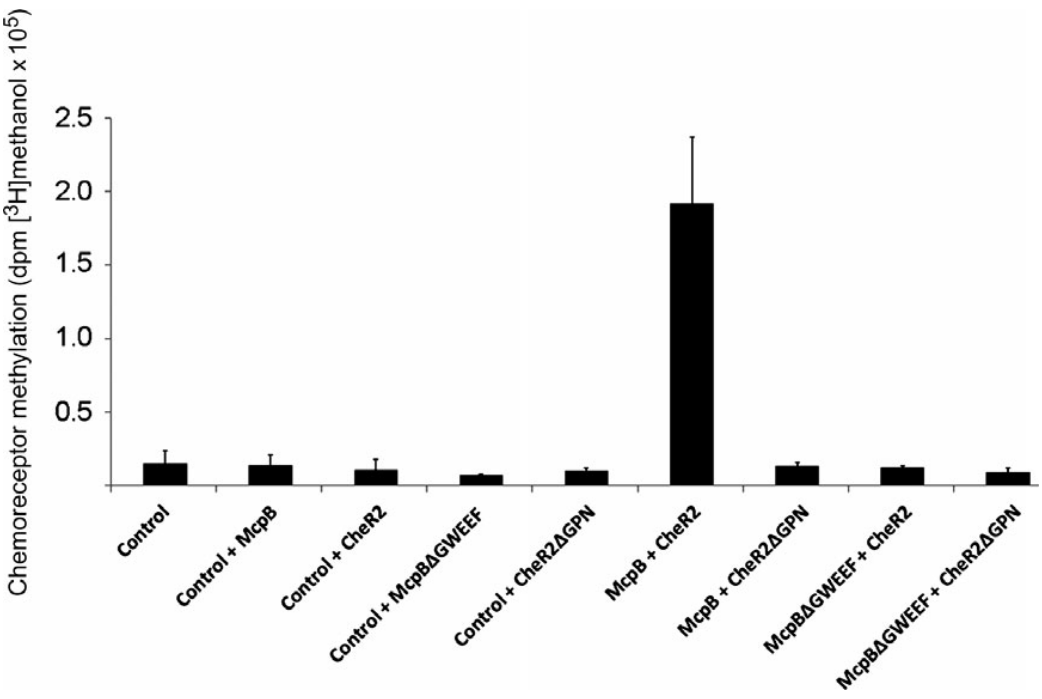

Figure 59. Pentapeptide-mediated CheR2-McpB interaction is essential for protein methylation. Methylation assays using McpB and its mutant lacking the pentapeptide (McpBΔGWEEF), and CheR2 and its mutant lacking the central GPN tripeptide (CheR2ΔGPN). Data are means ± SD from three independent experiments.

5. Discussion

In general, protein-protein interactions are mediated by a single interface. CheR and CheB appear to be exceptions because both proteins bind to two distinct sites. What is the reason for this unusual binding mode? From studies using *E. coli* and *S. typhimurium*, which have a single CheR and CheB and chemoreceptors with and without C-terminal pentapeptides, it was concluded that the physiological reason for the high-affinity bindingof CheR to the pentapeptide is to increase the local concentration of CheR with the flexible linker, enabling methylation of neighboring receptors (Levin *et al*., 2002; Muppirala *et al*., 2009; Wu *et al*., 1996; Yi and Weis, 2002).

Genome analyses indicate that many species have multiple copies of signaling proteins. Here, we investigated this issue using an organism that has multiple CheR proteins. We demonstrate that in *P. aeruginosa*, the only interaction between a chemoreceptor pentapeptide and CheR is that between McpB and CheR2. The genes encoding these two proteins are next to each other and form part of gene Cluster II, which encodes proteins of the Che2 chemotaxis pathway. Therefore, we conclude that the physiological relevance of the pentapeptide docking site is to target a specific chemoreceptor to a specific methyltransferase. Many of the bacteria that have pentapeptide-containing chemoreceptors have multiple CheR paralogs (Perez and Stock, 2007), which suggests that this specific targeting mechanism described here for CheR2 and McpB may also be applicable to other CheR-chemoreceptor interactions.

The physiological role of the Cluster II is still unclear (Güvener *et al*., 2006). However, it has been suggested that it mediates chemotactic responses to an as yet unidentified signal molecule during infection (Garvis *et al*., 2009). This prediction is supported by the demonstration that phosphate starvation increases general virulence-associated phenotypes and induces expression of the genes located in Cluster II (Bains *et al*., 2012). Güvener *et al* (Güvener *et al*., 2006) have shown that proteins of the Cluster II do not colocalize with those of the Cluster I, the principal pathway for chemotaxis. Instead, proteins of the Cluster II form their own signaling complexes. Because the formation protein complexes encoded by Cluster II depended on McpB, Güvener *et al* concluded that among the 26 chemoreceptors, McpB is the only one that participates

in the formation of these signaling complexes. Together, these observations ssuggest that the formation of the Cluster II signaling complexes, which consist of CheW2, McpB, CheA2 and CheY2, is highly specific.

Our results are in full agreement with the conclusions drawn by Güvener *et al*. (2006) because we demonstrated that pentapeptide-mediated CheR binding guarantees specific binding of CheR2 to McpB. Apart from the specificity of assembly of the Cluster II signaling complex, the pentapeptide-mediated interaction enables a specific interaction of this complex with the cognate protein involved in environmental adaptation. We also showed that the pentapeptide of McpA, also encoded by the Cluster II, is not recognized by any of the CheR paralogs, which is in agreement with the demonstration that the McpA receptor forms signaling complexes with proteins of the Cluster I, but not the Cluster II (Güvener *et al*., 2006). Our results therefore reveal one molecular mechanism that enables the specific interaction of proteins of the Cluster II with a specific chemoreceptor of this pathway.

In *P. aeruginosa*, McpB appears to be the only receptor that feeds into the Cluster II (Güvener *et al*., 2006), and CheR2 is the only methyltransferase that methylates McpB.

In *E. coli*, the single CheR acts on chemoreceptors with and without a pentapeptide, and the output of the single pathway reflects the activity of all receptors. Therefore, currently, it is difficult to tell whether the high specificity of McpB in pathway assembly is a more general feature of chemosensory pathways.

A major difference of CheR compared with other SAM-dependent methyltransferases was the insertion of the β-subdomain into the seven-stranded core domain, corresponding to the CheR C-terminal domain (Djordjevic and Stock, 1998; Martin and McMillan, 2002). Because this subdomain contains the pentapeptide binding site, it could be that pentapeptide recognition was the evolutionary driving force leading to insertion of this subdomain. However, all four CheR paralogs of *P. aeruginosa* contain this β-subdomain, whereas only CheR2 binds pentapeptides; thus, the mere presence of the β-subdomain in methyltransferases did not direct receptor-specific recognition.

Sequence clustering identified that CheR methyltransferases form two families distinguished by their ability to bind pentapeptide-containing receptors. We found

that a three–amino acid insertion in the loop linking strands 2 and 3 of the subdomain is a distinct feature of pentapeptide binding CheR proteins.

Using site-directed mutagenesis, we also showed that this tripeptide is essential for pentapeptide binding. Inspection of the CheR pentapeptide structure illustrates that these three amino acids form few interactions with the bound pentapeptides (Djordjevic and Stock, 1998). However, the presence of this tripeptide may be essential to orient strands 2 and 3 of the β-subdomain to interact correctly with the pentapeptide. The presence of this insertion can potentially be used to make sequence-based predictions of pentapeptide binding CheR proteins.

CheR2 bound exclusively to the terminal pentapeptide of McpB with an affinity about 20-fold higher than the affinity of the NWETF peptide to *E. coli* CheR (Yi and Weis, 2002). Although the latter peptide is absent from *P. aeruginosa* receptors, CheR2 also recognized this peptide with high affinity. A bioinformatics analysis of pentapeptides from different species has identified the most frequent amino acids for each of the five positions as [D/N]-[W/F]-[E/Q]-[T/E]-[F] (Perez and Stock, 2007).

This cross-species NWETF pentapeptide–CheR2 binding may be the consequence of the fact that the enterobacterial NWETF pentapeptide contains the most abundant amino acids at each position of the consensus motif.

SAM and SAH compete for binding to CheR. Studies of the enterobacterial enzymes show that SAH binds more tightly to CheR than does SAM (Simms and Subbaramaiah, 1991; Yi and Weis, 2002), which implies that the enzymatic activity of CheR is subject to product feedback inhibition. We made similar observations for the four CheR paralogs of *P. aeruginosa*, indicating that this is a general feature of CheR proteins. However, large differences between the ratios of the SAH/SAM affinities were observed for the different paralogs. The differences were particularly pronounced for CheR2, which bound SAH much more tightly than it did SAM. An increase in the cellular SAH concentration would thus selectively inhibit CheR2. Further studies will be necessary to verify whether this is of physiological relevance.

Methylation assays showed that McpB was exclusively methylated by CheR2. Disrupting the CheR2-pentapeptide interaction, by deleting either the pentapeptide

from McpB or the three amino acid insertion from CheR2, abolished receptor binding and reduced methylation activity to background amounts (Figure 59).

These results contrast with the corresponding data from enterobacterial systems, in which residual methylation activity is observed after removal of the pentapeptide (Le Moual *et al*., 1997; Wu *et al*., 1996). This suggests that the CheR2-McpB interaction is a strict requirement for any methylation activity.

Chemosensory pathways have been primarily studied in enterobacteria. It is now apparent that these systems are comparatively simple compared with those of other species that contain a greater number of receptors and multiple copies of signaling proteins (Hamer *et al*., 2010). To assure that multiple copies of sensory proteins assemble into the correct pathways, molecular mechanisms must exist to guarantee the specificity of protein interactions. Here, we identified one of these mechanisms. Pentapeptide containing chemoreceptors are found in many different species, suggesting that this mechanism is widespread.

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CHAPTER IV

McpB soluble chemoreceptor in *Pseudomonas aeruginosa* PAO1 has an essential role in virulence and pathogenesis

(Manuscript in preparation)

1. Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen that causes infections in a variety of animal and plants hosts. The gene *mcpB* is part of the chemotactic gene Cluster II that encodes for a soluble chemoreceptor, but its function still remains unknown. Recent studies, show that *cheB2* gene, located in Cluster II, where *mcpB* is located as well, is involved in a specific chemotactic response, that takes place during infection and is required for *P. aeruginosa* pathogenicity.

In order to determine the role of McpB chemoreceptor in virulence, we generated a mutant in the *mcpB* gene of *P. aeruginosa* PAO1, and tested its phenotype in virulence and pathogenesis with different virulence models including *Caenorhabditis elegans*, *Escherichia coli* MC4100, *Vibrio fischeri*, *Daphnia magna*, *Eisenia foetida*, *Adalia bipunctata*, *Chrysoperla carnea* and *Capsicum annumm.* Most of these assays show an attenuation of virulence in the McpB mutant compared to wild type *P. aeruginosa* PAO1. Our results suggest that the virulence reduction of the McpB mutant is not linked with a global virulence system through secondary metabolites production, but to the regulation through a new pathway that needs to be studied in more detail. The identification of this pathway opens up other possibilities to identify effectors to fight PAO1 in pathogenesis.

2. Introduction

P. aeruginosa is an opportunistic human pathogen and also is able to infect different animals and plants (Breidenstein *et al*., 2011; Lister *et al*., 2009; Pereira *et al*., 2014). It has a single polar flagellum that allows swimming in liquid media. This strain can also move in solid and semisolid surfaces, using alternative mechanisms including swarming (Köhler *et al.*, 2000) and twitching (Semmler *et al.*, 1999). *P. aeruginosa* presents chemotaxis to most of the organic compounds available as carbon sources, and several repellants have been identified (Hamilton and Sheeley, 1971; Kato *et al.*, 1992; Kelly-Wintenberg and Montie, 1994; Moench and Konetzka, 1978; Moulton and Montie, 1979; Ohga *et al.*, 1993). To adapt efficiently to the changes in its surroundings, *Pseudomonas* has evolved sophisticated regulatory networks. The existence of these complex regulatory systems might explain the large number of genes (~10 % of the genome) coding for proteins with regulatory functions in *P. aeruginosa* (Stover *et al.*, 2000). Chemosensory pathways are involved in mediating flagellum and type IV pili mediated taxis but also carry out alternative cellular functions (Wuichet and Zhulin, 2010). For instance, the regulation of the cellular concentration of the second messengers cyclic di-GMP or cAMP, which in turn impacts on a wide range of cellular processes like biofilm formation, quorum sensing or the secretion of virulence factors (Fulcher *et al.*, 2010; Hickman *et al.*, 2005). *P. aeruginosa* PAO1 has a total of 26 genes encoding for chemoreceptors and has multiple copies of *E. coli*-like chemotaxis genes arranged in five Clusters (Stover *et al.*, 2000). Cluster I and V, have previously been shown to be essential for chemotaxis (Kato *et al.*, 1999; Masduki *et al.*, 1995). Cluster III, has been shown to be involved in biofilm formation (Güvener and Harwood, 2007; Hickman and Harwood, 2008; Hickman *et al.*, 2005), while Cluster IV has been shown to be involved in twitching motility (Darzins, 1994; Kearns *et al.*, 2001). However, the Cluster II has not been studied so far. Here, we investigate the role that Cluster II may play in *P. aeruginosa* PAO1.

The Cluster II is composed by eight genes, two of them encoding for chemoreceptors called McpA (PA0180) and McpB (PA0176). Both genes are expressed in stationary phase (Güvener *et al*., 2006). McpA interacts with the proteins encoded by Cluster I

(Schuster *et al.*, 2004), but not with the proteins belonging to Cluster II in stationary phase cells. However, McpB is required to form complexes by Cluster II encoded proteins (Güvener *et al*., 2006). This implies that these protein complexes have a function that depends on just one chemoreceptor (Güvener *et al.*, 2006).

Analysis of the amino acid sequence using the Simple Modular Architecture Research Tool (SMART), a biological database that is used in the identification and analysis of protein domains within protein sequences (Letunic *et al.*, 2002; Schultz *et al.*, 1998), indicates that McpB has a highly conserved domain (Le Moual and Koshland, 1996), a PAS (for Per, ARNT, Sim) domain typically involved in sensing redox potential, oxygen, or light (Taylor and Zhulin, 1999), and no predicted transmembrane domains.

McpB has one C-terminal pentapeptide (GWEEF) related to that found on the high-abundance receptors of *E. coli* (NWETF) (Okumura *et al.*, 1998). The C-terminal pentapeptide in McpB, is fundamental for the binding and methylation activity by CheR2 and for instance, it is required for the activation of the Cluster II encoded signalling pathway (García-Fontana *et al.*, 2014). This reinforces the concept that McpB may play a more general and central role in chemosensing signal transduction than do typical MCPs.

Previously, Cluster II had been related to chemotaxis (Ferrández *et al.*, 2002); however, these results have not been reproduced in more recent works (Güvener *et al.*, 2006). Others studies show McpB as a predicted cytoplasmic protein with a PAS domain, related to aerotaxis (Hong *et al.*, 2004, 2005), but this finding has not been confirmed.

Despite of being studied for decades, the main role of Cluster II in *P. aeruginosa PAO1*, still needs to be elucidated. In this work we performed several bioassays according to previous work (Vílchez *et al*., 2016) using different organism models, to evaluate the impact of this gene in virulence pathways Our results suggest that Cluster II and the McpB chemoreceptor are both involved in virulence and pathogenesis in *P. aeruginosa* PAO1.

3. Material and Methods

3.1 Organisms, plasmids and oligonucleotides used in this study

The bacterial strains used in this study are listed in Table 11 and 12. All strains were grown on Luria-Bertani (LB) medium, at 30^{o}C (37^{o}C in the case of the pathogenic strains) unless otherwise noted.

Table 11. Strains and plasmids used in this study.

Strains	Use	Reference or source
Escherichia coli MC4100	*F- araDI39 Δ(argF-lac) U169 rpsL150 relAl flb5301 deoCl ptsF24 rbsR*. Used as microbial model in sensitivity and microbial metabolism assays.	Bachmann, 1972; Silhavy *et al.*, 1984
Escherichia coli OP50	Used to feed *Caenorhabditis elegans.*	Brenner, 1974
Burkholderia cepacia CC-Al74	Risk Group 2 bacteria and proposed as PGPR. Used as a positive control in virulence assays	(Eberl and Tümmler, 2004; Vial *et al.*, 2011
Pseudomonas putida KT2440	Risk Group 1 bacteria and PGPR. Used as negative control in virulence assays	Planchamp *et al.*, 2015
Vibrio fischeri ATCC 49387	Used as bioluminescent strain in MicroTox assays	Onorati and Mecozzi, 2004; Perry *et al.*, 2005
Pseudomonas aeruginosa PAO1	Used to compare the effect of mutant McpB strain.	This study
Pseudomonas aeruginosa ΔMcpB	Used to check the effect of McpB gene in virulence	This study
Caenorhabditis elegans Bristol strain N2	Used in virulence assays. Provided by the Laboratory of Nematology, National Museum of Nature Sciences-CSIC (Madrid, Spain)	Navas *et al.*, 2007
Adalia bipunctata	Used in virulence assays. Provided by ControlBio Co. (Almería, Spain)	Ref. CBi K04884
Chrysoperla carnea	Used in virulence assays. Provided by ControlBio Co. (Almería, Spain)	Ref. CBi 124 K04280
Eisenia foetida	Used in virulence assays. Provided by Lombriventa (Gerona, Spain).	Dorn *et al*., 1998

Escherichia coli β2163	*F' RP4-2-Tc::Mu ΔdapA*::(erm-pir), $Km^R Em^R$. Used for conjugation to get McpB mutant.	Demarre *et al.*, 2005
Escherichia coli DH5α	*supE44 lacU169 (Ø80lacZΔ M15) hsdR17* (rk-mk-) *recA1 endA1 gyrA96 thi-1 relA1*. Used for cloning to get McpB mutant.	Woodcock *et al.*, 1989
Plasmids	**Use**	**Reference**
pUC18NotI	Ap^R; identical to pUC18 but with two NotI sites flanking pUC18 polylinker. Used for mutagenesis.	Herrero *et al.*, 1990
pKNG101	Sm^R; *oriR6K mob sacBR.* Used for mutagenesis.	Kaniga *et al.*, 1991
pMAMV249	Ap^R; 1.4-Kb PCR product containing a 1116 bp in-frame deletion of *mcpB* inserted into the *Eco*RI/*Hind*III sites of pUC18Not. Used for mutagenesis.	This study
pMAMV250	Sm^R; 1.5-kb *Not*I fragment of pMAMV249 was cloned at the same site in pKNG101. Used for mutagenesis.	This study

[a]Ap, ampicillin; Sm, streptomycin; Em, *e*rythromycin

Table 12. Oligonucleotides used in this study.

Name	Sequence (5´-3´)	Description	Reference
mcpB up-*Eco*RI-F	TAAT**GAATTC**CACTGAGGCAAGCCAGGCG	Forward primer to clone upstream flanking region of *mcpB* for in-frame deletion	This study
mcpB up-*BamH*I-R	TAAT**GGATCC**ACCTGCTGGAGTTCGGC	Reverse primer to clone upstream flanking region of *mcpB* for in-frame deletion	This study
mcpB dw-*BamH*I-F	TAAT**GGATCC**GAATGCCCGCCAGGCCAACTC	Forward primer to clone downstream flanking region of *mcpB* for in-frame deletion	This study
mcpB dw-*Hind*III-R	TAAT**AAGCTT**TCAGAACTCTTCCCAGCCGTCT	Reverse primer to clone downstream flanking region of *mcpB* for in-frame deletion	This study

3.2 Construction of in-frame deletion McpB mutant

A mutant defective in *mcpB* (PA0176) was constructed by homologous recombination using a derivative plasmid of the suicide vector pKNG101. The plasmid for the construction of the in-frame deletion mutant was generated by amplifying the up- and downstream flanking regions of *mcpB*. The resulting PCR products were digested with

*EcoR*I and *BamH*I for upstream McpB region and with *BamH*I and *Hind*III for the downstream McpB region (Table 12). These products were ligated in a three-way ligation into pUC18Not, obtaining a plasmid called pMAMV249. This plasmid carries a 1.4-Kb PCR product containing a 1116 bp in-frame deletion of *mcpB* inserted into the *EcoR*I-*Hind*III sites. Then, the 1.5 Kb resulting fragment inserted between *Not*I sites, were mobilized from pMAMV249 to the marker exchange vector pKNG101 linearized with *Not*I restriction enzymes. The resulting plasmid was named pMAMV250, and the sequence cloned into, was confirmed by DNA sequencing and it carried an in-frame deletion of *mcpB* gene for the replacement of wild type gene in the chromosome. For the construction of the mutant, biparental conjugations were performed as described below. Briefly, in a biparental mating as described in Matilla *et al*. (2012), 100 µl of saturated cultures of *E. coli* β2163 containing pMAMV250 Sm^R, and *P. aeruginosa* PAO1 were mixed. Then, cells were washed with LB medium to eliminate the antibiotic, and collected by centrifugation. After that, they were resuspended in 30 µl of fresh LB, and spotted on an LB agar plate supplemented with 300 µM 2,6-diaminopimelic acid (DAPA), that allows the growth of DAPA auxotrofic mutant *E. coli* β2163. After overnight incubation at 37^{o}C, cells were scraped off the plate and resuspended in 1 ml of LB. Serial dilutions were plated on LB agar medium containing 400 µg ml^{-1} streptomycin for the selection of the cells containing the plasmid. DAPA was not added to the LB agar medium plates to avoid *E. coli* donor growth. To select derivatives that had undergone a second cross-over event during marker exchange mutagenesis, sucrose was added to a final concentration of 10 % (w/v). Final mutants lacking *mcpB* gene, were confirmed by PCR and sequencing. The resultant mutant was called *P. aeruginosa* ΔMcpB.

3.3 Virulence assays

Virulence and ecotoxicity assays were performed as is described in Vílchez *et al*. (2016), as summarized below.

3.3.1 *Escherichia coli* MC4100 Sensitivity and Microbial Metabolism Assays by *Vibrio fischeri* ATCC 49387

Sensitivity assays were performed in *Escherichia coli* MC4100 (Peters *et al.*, 2003; Small *et al.*, 1994) with some modifications. An aliquot of 0.5 ml of filtered and sterilized supernatants from stationary-phase cultures of *P. aeruginosa* PAO1 wild type strain or its mutant *P. aeruginosa* ΔMcpB, were mixed with 0.5 mL of *E. coli* MC4100 culture, containing about 10^8 to 10^9 cells collected from a mid-log phase suspension. These cells were collected and resuspended in M9 sterile buffer to perform this study. The mixtures were incubated for 1.5 h at room temperature, and then serial dilutions were prepared from each mixture. As a negative control, samples containing 0.5 ml of *E. coli* suspensions were mixed with 0.5 ml of TSB. The mixtures were spread on TSA plates to estimate $CFU \cdot ml^{-1}$.

Light emission by *Vibrio fischeri* ATCC 49387 is finely tuned to the microorganism's metabolism. To detect secondary metabolites with negative effects on cell metabolism independently of their lethal potential, Microtox® was used and EC50 values were calculated using Microtox software®. Experiments were done according to the manufacturer's recommendations using 1 ml of the sterile supernatants.

3.3.2 Pathogenicity Bioassay Based on *Caenorhabditis elegans*

Bacterial killing of *C. elegans* was assayed as previously described Darby *et al* (Darby *et al.*, 1999) , with some modifications (Navas *et al.*, 2007; Ruiz-Díez *et al.*, 2003). Briefly, potato dextrose agar plates were spread with *P. aeruginosa* ΔMcpB strain and *P. aeruginosa* wild type strain. Plates were incubated at 30°C for 24 h and then, each plate was seeded with 5 adult hermaphrodite individuals. Plates were incubated at 24°C, and nematodes were examined at 10x objectives and adults, eggs, young individuals (L2-L3) and dead individuals were counted every 24 h over 7 days. *E. coli* OP50 was used as a control to estimate the natural death rate of the nematode, and *Burkholderia cepacia* CC-Al74 was used as pathogenic strain control.

3.3.3 Ecotoxicity Tests in Green Lacewings (*Chrysoperla carnea*) and Ladybirds (*Adalia bipunctata*)

These bioassays were carried out according to Medina *et al.* and Álvarez-Alfageme *et al.* (Alvarez-Alfageme *et al.*, 2011; Medina *et al.*, 2004) with slight modifications. Five L1 larval stage insects *Adalia bipunctata* and *Chrysoperla carnea* were grown in 15x15x25 cm individuals terrariums and were fed with frozen Mediterranean fruit fly (*Ceratitis capitata*) eggs (Dept. of Parasitology, University of Granada, Spain) combined with the bacterial strain of interest (approx. 1,000 eggs every 3 days).

To preserve bacterial strains, each culture was resuspended in trehalose as a lyoprotectant (trehalose 10 % (w/v)) and lyophilized for 24 h (Manzanera *et al.*, 2004). The feed was prepared by mixing approximately 1,000 eggs with 0.1 g of each lyophile containing the test strain at 10^8 to 10^9 CFU·g^{-1}. Insects were fed three times per week. Body length, weight and mortality under each condition were recorded at 0, 7 and 15 days. Eggs combined with an equivalent amount of sterile trehalose were used as a negative control. All experiments were conducted in a climate chamber at 25 ± 1^{o}C, 60 ± 5 % relative humidity, and a 16 h photoperiod.

3.3.4 Ecotoxicity Tests in Earthworms (*Eisenia foetida*)

Earthworm reproduction tests (*E. foetida*) were carried out according to OECD Test Guideline Test No. 222 (Neuhauser and Callahan, 1990) with some modifications. Five earthworms of the same generation (at least 1 month old and about 5.5-6 cm long) were placed in containers filled with 0.5 l of a mixture of vegetal substrate and sphagnum peat (3:1 w/w). Each container was kept at pH 6.7-7.4, 70 % relative humidity and 20-25^{o}C for 30-60 days in the dark. The earthworms were fed with 5 g per week of moistened chickpea flour mixed with lyophilized bacteria cultures containing 10^8 to 10^9 CFU/g, prepared as described above. As a control for earthworm death rate, NaCl 2 % (w/w) was added to the substrate mixture as a dry powder. Moistened chickpea flour without bacteria was used to feed earthworms used as a negative control. At each sampling time (15, 30 and 60 days), length and weight of the

initial individuals, clitellum formation, the number of ootheca and the number of juveniles were recorded.

3.3.5 *Daphnia magna* Toxicity Bioassay: DaphToxKit®

The toxicity of bacterial extracts to *D. magna* (Cladocera) was assayed with DaphToxKit F® (Microbiotests, BE) (Hernando *et al.*, 2003) accordingly to ISO 6341 and OECD Guideline No. 211 (OECD, 1998). Twenty newly hatched animals (24 h old form ephippia) were transferred to a multiwell microplate system (10 ml/well; 5 animals/well) for each tested concentration of bacterial extract. Dilutions of bacterial extracts were done using test "freshwater" in the absence of cosolvents or vehicles. Assays were done in the dark at 20°C for 24 and 48 h, when immobility was checked. An individual was considered immobile when it did not swim (even if moving the antennae) during a 15 s observation period.

3.4 Bacterial Effects in Pepper (*Capsicum annuum*) plants

Virulence assays in pepper plants was tested according to Vílchez *et al*. with some modifications (Vílchez *et al*., 2016). Pots containing pepper plants about 10 cm height were inoculated with 4 ml of bacterial inoculum (10^8-10^9 CFU/ml) in 0.5 X M9 sterile saline solution directly over the ground. The plants were irrigated twice a week with 4 ml per plant. After 21 days after inoculation, height, fresh weight, fully turgid weight and dry weight were recorded. As a negative control 0.5 X M9 buffer without the bacterial inoculum was used.

3.5 Statistical Analyses

For statistical testing, T-Student test was used with a significance level of $p<0.05$. All analyses were done with SPSS software.

4. Results

4.1 *P. aeruginosa PAO1* ΔMcpB strain shows attenuated virulence in *Caenorhabditis elegans*

C. elegans is a model organism to study bacterial virulence mechanisms (Kurz and Ewbank, 2003; Tan *et al.*, 1999). For instance, virulence factors responsible in the killing of *C. elegans* are relevant for virulence in mammalian hosts (Ewbank, 2002; Mahajan-Miklos *et al.*, 2000).

In this study, we generated a *P. aeruginosa* PAO1 McpB mutant to test the virulence role of McpB chemoreceptor in *C. elegans*. We evaluated the effect of wild-type and *P. aeruginosa* PAO1 ΔMcpB bacterial strains on the number of eggs laid, number of juveniles, number of adults and death rate (Navas *et al*., 2007). *B. cepacia* and *E. coli* OP50 were used as pathogenic and non-pathogenic strains respectively.

When *C. elegans* was fed with *P. aeruginosa* wild type strain, the number of dead worms were significantly higher (p value < 0.05) practically in the whole assay (from 24 to 72 h), compared to the number of dead worms found when *C. elegans* was fed with *P. aeruginosa* ΔMcpB strain. However, at the last time point of the assay (96 h) the differences were not significant (Figure 60D). We observed an average of 2 ± 0.5, 6 ± 0.5, 7 ± 1 and 7 ± 1 dead worms at 24, 48, 72 and 96 h respectively in plates containing *P. aeruginosa* PAO1 wild type strain, compared to *P. aeruginosa* ΔMcpB strain that showed 0, 0, 2 ± 0.5 and 5 ± 2 dead worms at the same time points respectively. In the case of eggs production and number of adults recorded (Figure 60A and 60C), we observed a significant decreased (p value < 0.05) in wild type *P. aeruginosa* strain compared to the ΔMcpB strain at each time point recorded.

Regarding to the number of eggs deposited by *C. elegans* when fed with the wild type strain, they showed an important reduction of 10-fold at the beginning of the assay, to 60-fold reduction at the end of the process compared to values observed when they were fed with the ΔMcpB strain .

Adult individuals decreased as well, from an around 2-fold reduction at the beginning, to a 30-fold reduction observed at 96 h in wild type strain compared to *P. aeruginosa* ΔMcpB strain.

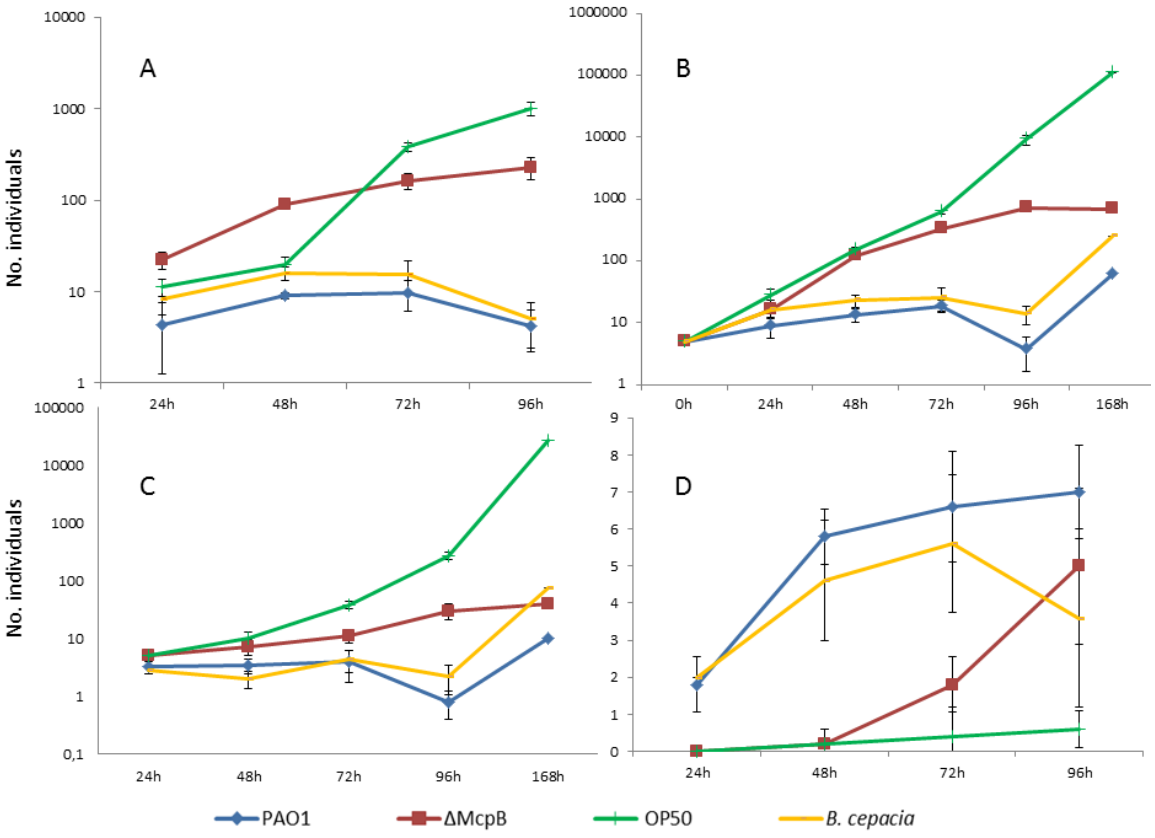

Figure 60. Development of *C. elegans* fed with different strains. *P. aeruginosa* PAO1 wild type (blue line), ΔMcpB strain of *P. aeruginosa* PAO1 (red line), *E.coli* OP50 (green line) and *B. cepacea* (yellow line). In A, B, C and D panels are shown eggs production, young individuals (L2-L3), adults and dead individuals in each condition respectively. X axis shows time (hours) and Y axis shows number of individuals. The values shown are the mean and standard deviation of three measurements.

When the number of juveniles individuals was studied (Figure 60B), we observed a significant decreased in plates containing *P. aeruginosa* wild type *strain* compared to those containing *P. aeruginosa* ΔMcpB strain (p value < 0.05) after 48 h and through the end of this assay. At time point 24 h, wild type strain showed a reduction of 2-fold in juveniles and these differences got higher at the end of the assay, observing a reduction by about 100-fold when *C. elegans* was fed with the wild type strain at 96 h

compared to when it was fed with *P. aeruginosa* ΔMcpB strain. The effect observed in *P. aeruginosa* PAO1 wild type strain, was similar to the effect caused by *B. cepacia* CC-A174, the standard pathogenic control strain used for these assays. At the end of the assay, we collected the total of adult and juvenile individuals, and we observed an increase in the survival of both of them, between 4- and 10-fold, respectively, when they were fed with *P. aeruginosa* ΔMcpB strain. At this point, the differences between both strains, wild type and mutant, got lower. With these results, we can conclude that *P. aeruginosa* ΔMcpB strain is less virulent than wild type *P. aeruginosa* strain for the *C. elegans* model.

4.2 *P. aeruginosa* ΔMcpB shows less pathogenicity in *Chrysoperla carnea* and *Adalia bipunctata* than wild type strain

P. aeruginosa has been described to be pathogenic to different types of insects (Andersen *et al.*, 2010). We performed virulence assays in the model insects green lacewings (*Chrysoperla carnea*) and ladybirds (*Adalia bipunctata*) because they are considered good biological control agents and these insects have been used in other virulence studies to validate the virulence impact by different strains of the Proteobacteria group (v d Schulenburg *et al*., 2002). In addition, these insects, in combination with other assays (human tissue culture, mammalian tissues) have been used as comparative models to validate the effect of these pathogens in humans (Fernández *et al*., 2015). We measured the variations in weight and length, and the numbers of dead insects of 5 biological replicates. Both types of insects were fed with a solid diet supplemented with different microorganism cultures including *P. aeruginosa* ΔMcpB strain, *P. aeruginosa* wild type strain or *P. putida* KT2440 and *B. cepacea;* the latter two strains were used as respective control strains for safe and pathogenic bacteria. These microorganisms were freeze-dried with trehalose as a xeroprotectant and we ensured that bacteria were distributed evenly in the diet. An additional control was introduced by feeding the insects with an equivalent amount of trehalose in absence of any bacterial strain. When *C. carnea* and *A. bipunctata* were fed with lyophilized *P. putida* KT2440, weight and length were similar as when fed with

trehalose in absence of bacteria used as a negative control. When they were fed with *P. aeruginosa* wild type strain, insects were smaller and lighter; nevertheless we observed that in those insects fed with the ΔMcpB strain (specially at 14 days after initial point), both, *A. bipunctata* and *C. carnea* showed increases in weight and length (Figures 61-64).

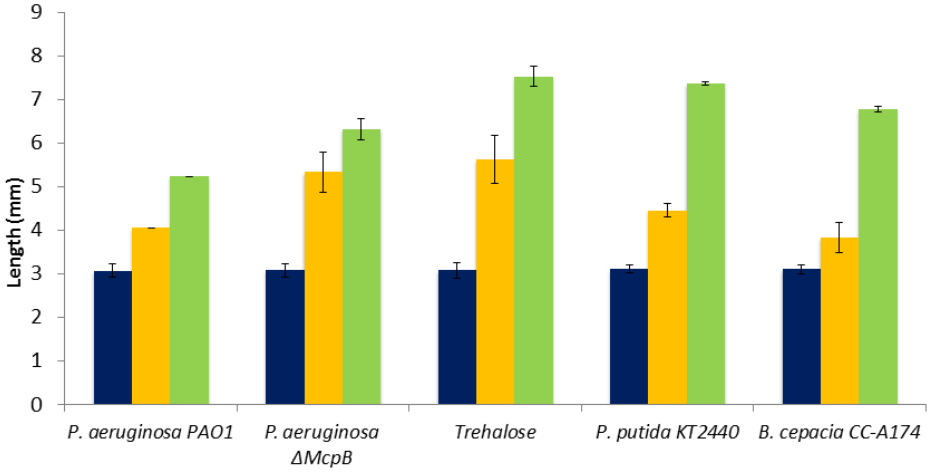

Figure 61. Length measures of *A. bipunctata* individuals fed with different bacterial strains. Shown are means and standard deviations of 5 biological replicates. Measures were done at time 0 (blue bars), 7 days (yellow bars) and 14 days (green bars) after feeding.

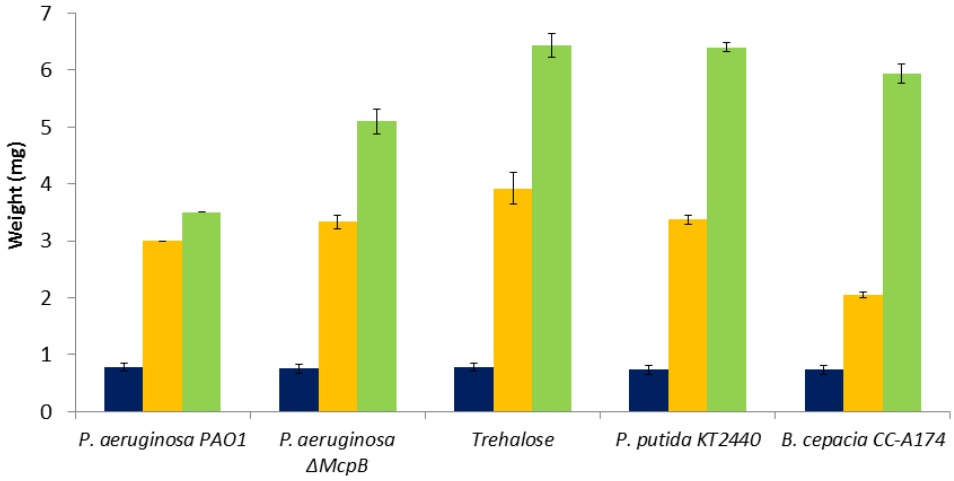

Figure 62. Weight measures of *A. bipunctata* individuals fed with different bacterial strains. Shown are means and standard deviations of 5 biological replicates. Measures were done at time 0 (blue bars), 7 days (yellow bars) and 14 days (green bars) after feeding.

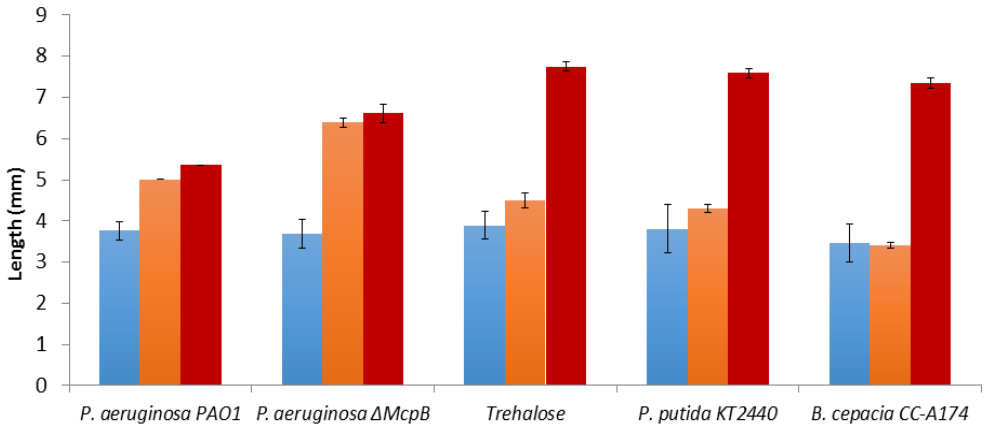

Figure 63. Length measures in *C. carnea* individuals fed with different bacterial strains. Shown are means and standard deviations of 5 biological replicates. Measures were done at time 0 (blue bars), 7 days (orange bars) and 14 days (red bars) after feeding.

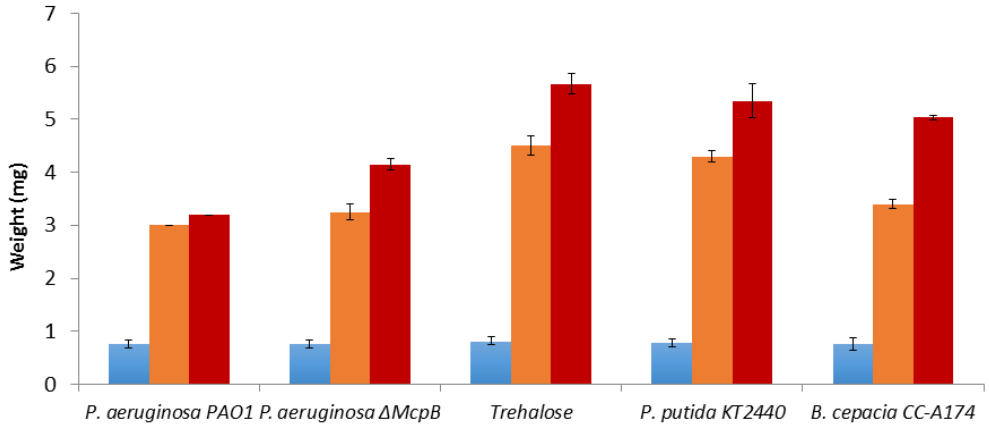

Figure 64. Weight measures in *C. carnea* individuals fed with different bacterial strains. Shown are means and standard deviations of 5 biological replicates. Measures were done at time 0 (blue bars), 7 days (orange bars) and 14 days (red bars) after feeding.

The number of dead individuals was also studied, and it was observed that in the case of insects fed with the wild type strain, 80 % of individuals were killed with respect 20 % and 40 % of death shown in the case of insects fed with *P. aeruginosa* ΔMcpB strain

in *A. bipunctata* and *C. carnea* respectively (Figure 65). It should nevertheless be noted that mortality rates between 10 % and 20 % are within the normal range for this type of assay.

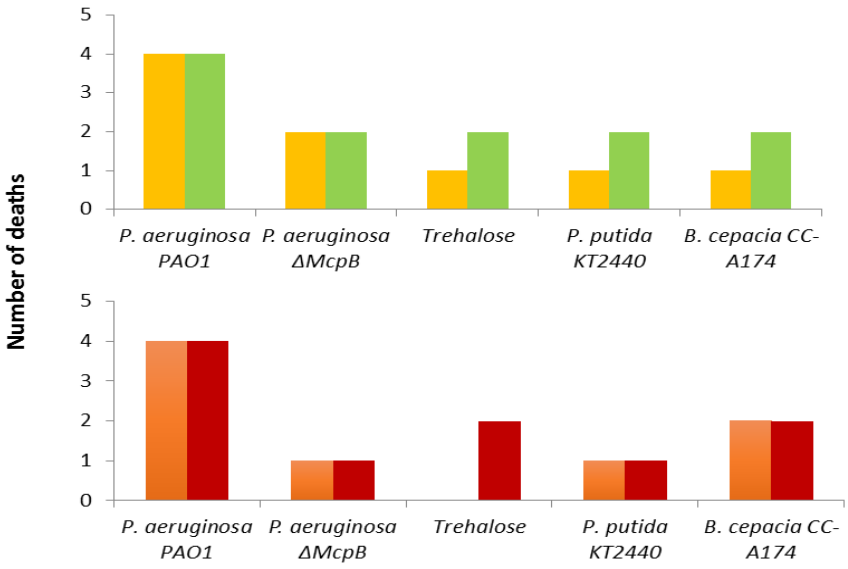

Figure 65. Dead individuals. Panel A shows dead individuals in *A. bipunctata* at 7 days (yellow bars) and 14 days (green bars). Panel B shows dead individuals in *C. carnea* at 7 days (orange bars) and 14 days (red bars).

4.3 Ecotoxicity testing on earthworms (*Eisenia foetida*) shows atenuated virulence for the P. *aeruginosa* ΔMcpB strain

Earthworms (*Eisenia foetida*) are highly resistant organisms and there are only few descriptions of bacterial strains able to infect them due to their highly developed immune system (Schulenburg et al., 2007). However, the presence of some bacterial pathogens on the ground can affect their developmental and reproductive capacity (Neuhauser and Callahan, 1990).

To study *E. foetida* development, we measured weight and length gain as well as their reproductive efficiency (number of juveniles and oothecas) after adding different bacterial strains to their diet, to analyze possible adverse effects on earthworm community. A sample containing moistened chickpea flour with 10 % trehalose (w/v) lyophilized and in absence of any bacterial strain was used as negative control, while

the composition to test the different strains, was the same but adding approximately 10^9 CFU of each strain as described in Material and Methods section.

P. putida KT2440 freeze-dried in 10 % trehalose and mixed with moistened chickpea flour as previously described was used as non-pathogenic control strain. As a control of the detrimental effect on the development of *E. foetida*, moistened chickpea flour with NaCl 2 % (w/v) in absence of bacteria was included. Also, the same composition supplemented with 10^9 CFU of *B. cepacea* as a pathogenic strain was tested.

Bioassays with the wild-type strain *P. aeruginosa* PAO1 and *P. aeruginosa* ΔMcpB strain, showed similar values for length increase during the whole study (Figure 66), however, weight gain was significantly higher for *E. foetida* fed with *P. aeruginosa* ΔMcpB than those fed with the wild type strain (*p* value < 0.05) from 15 to 60 days (Figure 67).

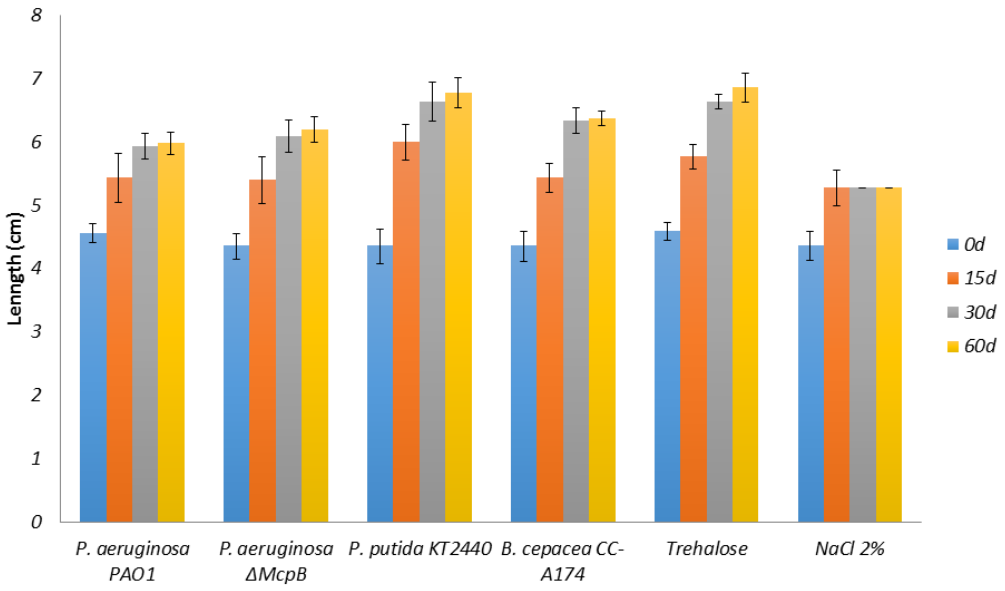

Figure 66. Length of *E. foetida* over 60 days fed with different bacterial strains, trehalose and NaCl. Bars correspond to the average length of 5 individuals at 0, 15, 30 and 60 days and standard deviation is shown in form of Error bars.

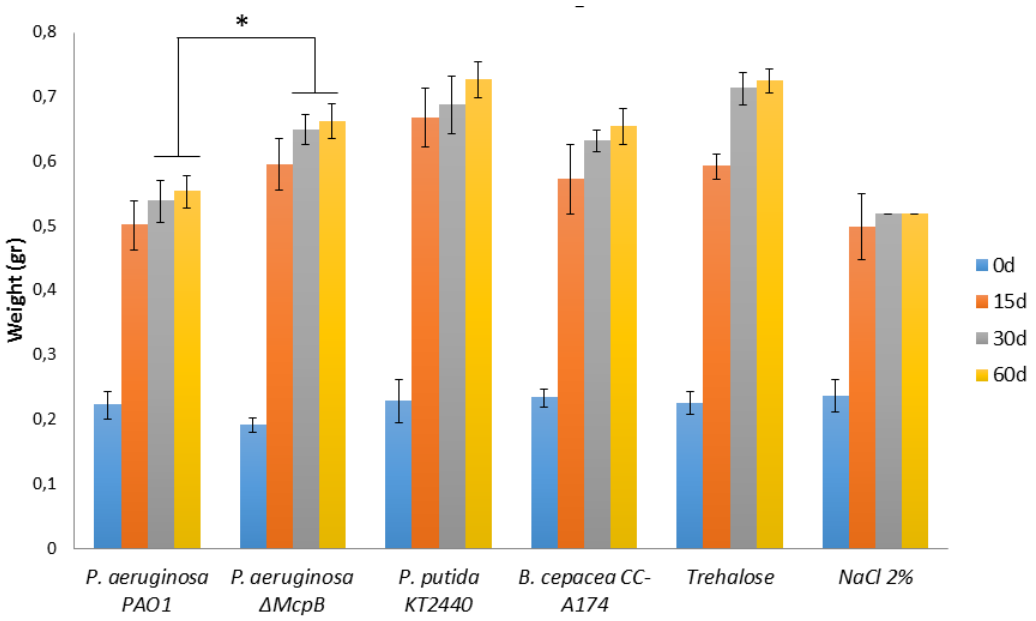

Figure 67. Weight of *E. foetida* over 60 days fed with different bacterial strains, trehalose and NaCl. Bars correspond to the average length of 5 individuals at 0, 15, 30 and 60 days and standard deviation is shown in form of Error bars. Asterisk shows significant differences between wild type strain and ΔMcpB strain based in T-Student test.

Regarding the reproductive efficiency of *E. foetida* in the presence of different strains, the number of eggs deposited (ootheca) in presence of *P. aeruginosa* ΔMcpB, was 1.2 times higher than the number of eggs deposited in presence of the wild type *P. aeruginosa* PAO1 (Figure 68A). With regard to the number of young individuals observed on these conditions, we observed a 2.5 times increase when *E. foetida* was fed with *P. areuginosa* ΔMcpB compared to wild type strain (Figure 68B). At 60 days of study, we observed 1 dead worm when fed with wild type strain, and no casualties were detected when fed with *P. aeruginosa* ΔMcpB (Figure 68C).

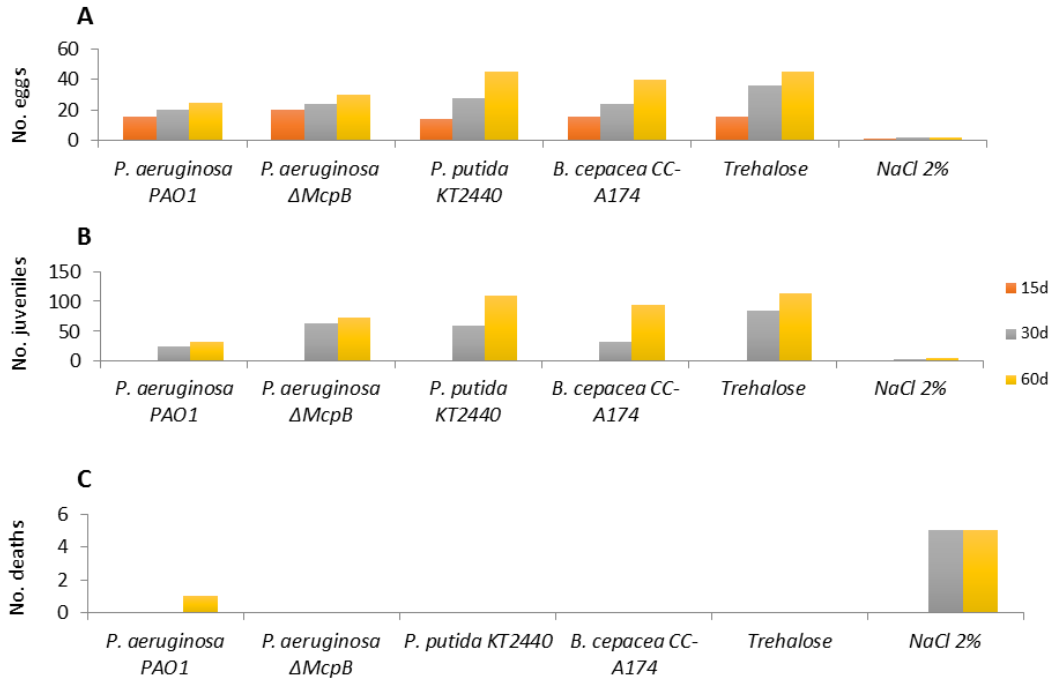

Figure 68. Reproductive efficiency and deaths of *E. foetida* for 60 days fed with different bacterial strains, trehalose and NaCl. Eggs formation (panel A), young individuals (panel B) and dead individuals (panel C) were studied over 5 individuals at 15, 30 and 60 days.

4.4 Wild type *P. aeruginosa* and *P. aeruginosa* ΔMcpB supernatants present similar effect on microbial communities and aquatic crustacean.

Secondary metabolites produced and released to the environment can alter the composition of microbial communities and their metabolic interactions. In the case of *P. aeruginosa* as an animal pathogen, these secondary metabolites could affect the gut microbiome of some animals producing several types of diseases (Rogers *et al*., 2015).

We compared the effect of those secondary metabolites produced and released to the media by *P. aeruginosa* PAO1 and *P. aeruginosa* ΔMcpB. In saturated cultures collected by centrifugation, the potential effect of these supernatants on microbial communities were tested on *E. coli* MC4100 cells to study the microbial viability upon exposition of cells with the supernatants. *V. fischeri* was used to study the effect of both strains on the microbial metabolism by alterations in its bioluminescence. *B. cepacea CC-A174* and *P. putida* KT2440 were used respectively as controls for pathogenic and non-pathogenic strains.

About 48 % reduction in survival of *E. coli* MC4100 was found when bacteria were exposed to the supernatant of both cultures (*P. aeruginosa* PAO1 wild type strain, and *P. aeruginosa* ΔMcpB strain) (Figure 69). These values were similar to those obtained in *B. cepacea* control.

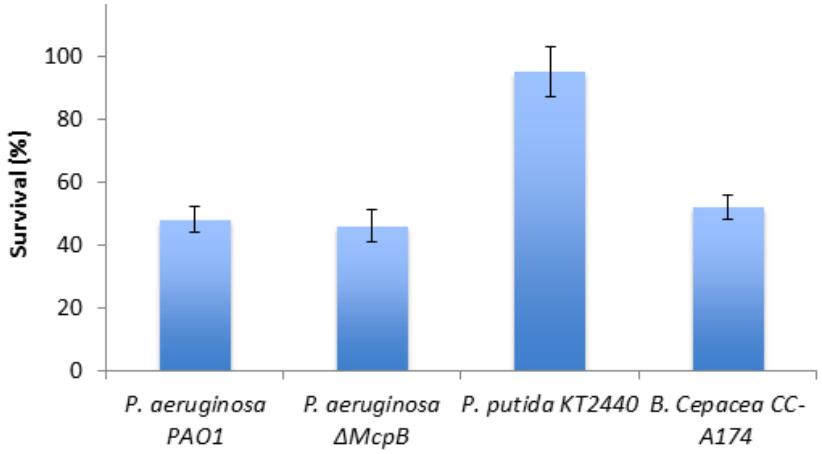

Figure 69. Survival of *E. coli* MC4100 after 1.5 h of exposure to bacterial culture supernatants. It is shown the survival (%) after the contact time. As a positive control for toxicity, supernatant from culture of *B. cepacia* CC-A174 was used while supernatant from culture of *P. putida* KT2440 was used as negative control of pathogenicity.

The Microtox® test was used to determine whether *V. fischeri* is affected in its metabolism by the presence of these two *P. aeruginosa* strains by changes in light emission (Onorati and Mecozzi, 2004). The results were recorded as half maximal effective concentration (EC_{50}), defined as the concentration of supernatant that caused a 50 % decrease in the light emitted by *V. fischeri*. Exposure to supernatants of the wild type *P. aeruginosa* PAO1, resulted in a very low EC_{50} (12.8 % ± 2.5), with was statisticaly not different to the value obtained with *P. aeruginosa* ΔMcpB (15.4 % ± 3), indicating that both strains present a marked effect on bacterial metabolism (Figure 70), which is comparable to that caused by the pathogenic control strain *B. cepacea* CC-A174 which showed an EC_{50} value of 19 % ± 0.8. The non-pathogenic strain *P. putida* KT2440, showed an EC_{50} value close to 100 %.

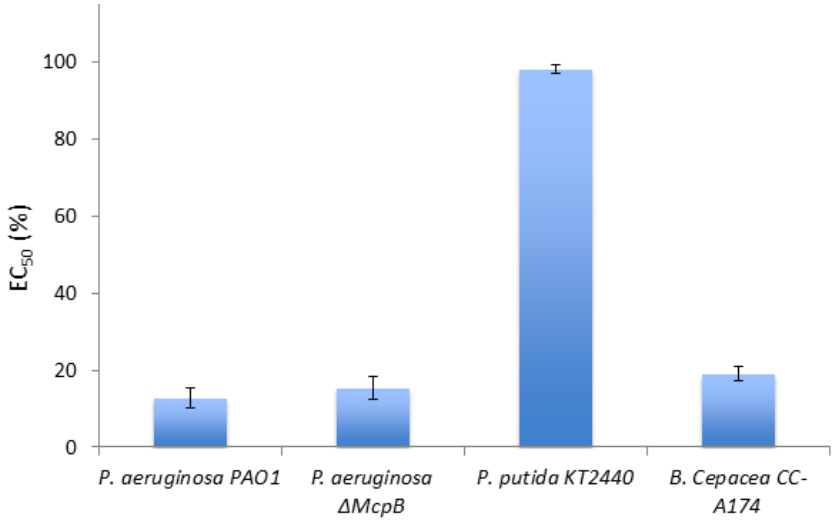

Figure 70. EC_{50} of *Vibrio fischeri* obtained in test Microtox® for different supernatants used in the test. It is shown the required percentage of each sample to achieve an effect of 50 % of reduction in bioluminescence emitted by *V. fischeri* . As a positive control for toxicity, we used *B. cepacia* CC-A174 while *P. putida* KT2440 was used as negative control of pathogenicity. The strains with values close to 100 % are considered as safe strains.

Since *P. aeruginosa* PAO1 is an ubiquitous environmental bacterium that colonizes a wide range of habitats (Blanc *et al*., 2007) and we therefore assessed the potential effect of these strains on aquatic communities using *D. magna* bioassays. These tests allowed us to assess the impacts of introduced substances, including microorganisms, on aquatic ecosystems. Bioassays with DaphToxKit® revealed EC_{50} values 14.46 % ± 4 or 18.61 % ± 3.93 for the wild-type and *P. aeruginosa* ΔMcpB strain respectively. These values do not showed significant differences between both strains, indicating that both of them affect *D. magna* mobility and survival in a similar way. EC_{50} values obtained using "fresh water" as negative control and *B. cepacea* CC-A174 supernatant, as pathogenic strain, were 100 % ± 3.08 and 12.5 % ± 1.54 respectively.

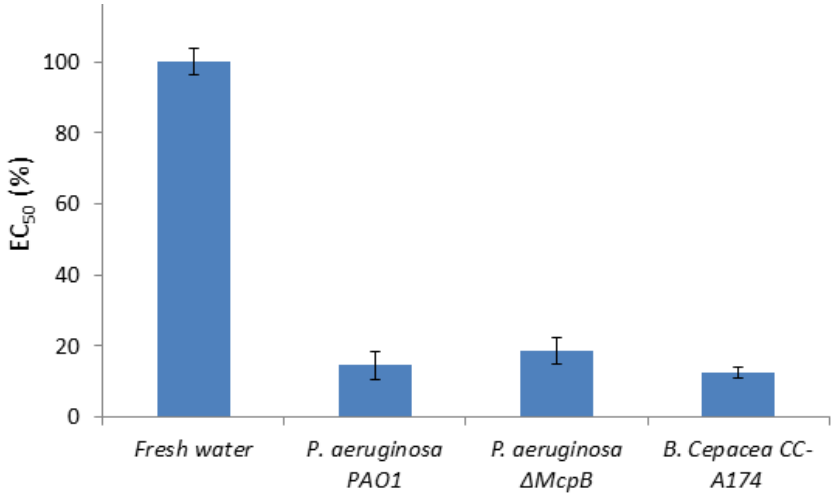

Figure 71. EC_{50} of *Daphnia magna* obtained in test Daphtoxkit® for different supernatants used in the test. It is shown the required percentage of each sample to achieve an effect of 50 % of reduction in *D. magna* mobility. As a positive control for toxicity, we used *B. cepacia* CC-A174 while *"fresh water"* was used as negative control of pathogenicity.

4.5 Wild type *P. aeruginosa* and ΔMcpB strain do not affect pepper plants

There are some studies that describe virulence pathways in *P. aeruginosa* that are required for the infection of human and plant hosts (Aragon *et al*., 2015). For this reason, virulence assays using pepper plants were performed according to the method described by Vílchez *et al.* (2016) with some modifications as described in Material and Methods section.

After 21 days post inoculation, pepper plants showed no statistical differences between plants inoculated with *P. aeruginosa* PAO1 compared to those inoculated with *P. aeruginosa* ΔMcpB. No differences were observed when height, fresh weight, fully turgid weight and dry weight were recorded. The values obtained were similar to those obtained in control plants inoculated with 0.5 X M9 medium suggesting this strain is not able to infect this type of plants.

4.6 Environmental and Human Safety Index (EHSI) calculation

To quantify differences in pathogenicity of *P. aeruginosa* PAO1 and its McpB mutant, we used the model described by Vílchez *et al*. (2016) that permits to determine whether a candidate strain is safe for human health and for the environment. They describe a rating scale called "Environmental and Human Safety Index" (EHSI), with values from 0 to 100. The higher this value, the higher is the probability that the corresponding bacterial strain is a safe one.

Based on this model, we calculated the index value in each strain, to compare the global effect in virulence. The results obtained in this study are shown in Table 13.

Table 13. Environmental and Human Safety Index (EHSI) calculation.

EHSI		
Bioassay	***P. aeruginosa* PAO1**	***P. aeruginosa* ΔMcpB**
E. coli MC4100	5	5
Microtox (*V. fischeri*)	1.25	1.25
C. elegans	0	6
C. carnea	3.18	6.75
A. bipunctata	4.125	6.75
E. foetida	8.75	10.625
D. magna	1.875	1.875
C. annuum	4	4
Final score	**28.18**	**42.25**

Values were obtained using standard values proposed by Vílchez *et al*. (2016).

The ESHI calculation shows both strains are to be considered as pathogenic strains, because both of them have less than 50 points (Vílchez *et al*., 2016), however, *P. aeruginosa* ΔMcpB presented a higher score than *P. aeruginosa* PAO1, indicative of an attenuation in virulence due to the deletion of the *mcpB* gene.

5. Discussion

There are several studies which relate this chemoreceptor with chemotaxis (Ferrández *et al*., 2002), or aerotaxis (Hong *et al*., 2004), but the reproducibility of these results is questioned (Güvener *et al*., 2006). Due to the contradictory results published in the past, the functional role of the chemosensory pathway encoded by gene Cluster II has not been established un-ambiguously. In this work, we show the relation between Cluster II and virulence in *Pseudomonas aeruginosa* PAO1, using a panel of virulence assays in different animal and microorganism models recently described by Vílchez *et al.* (2016) as Environmental and Human Safety Index (EHSI).

Our hypothesis that McpB is involved in virulence is reinforced by some observations and other previous studies: the presence of the Cluster II appears to be specific to some pathogenic bacteria, as exemplified by *Pseudomonas syringae* pv. *tomato* (plant pathogen bacteria (Xin and He, 2013)), *Shewanella oneidensis* MR-1 (opportunistic human pathogens (Khashe and Janda, 1998)), *Vibrio cholera* and *Pseudomonas aeruginosa* (animal pathogens), that were found to harbor homologues of this Cluster. This notion is supported by the fact that the non-pathogenic *Pseudomonas putida* and *Pseudomonas fluorescens* do not present Cluster II chemotaxis genes, although they have a complete sets of gene homologous to Cluster I, III, IV, and V chemosensory genes with high identity in their amino acid sequences (on the order of 70 to 80 % identity) to the orthologous *P. aeruginosa* Cluster I genes (Ferrández *et al.*, 2002). This observation, could suggest a relationship between the Cluster II encoded signal transduction pathway and the virulence processes.

On the other hand, previous studies show the relationship between inorganic phosphate (Pi) limitation and virulence in several microorganisms (Aoyama *et al.*, 1991; von Krüger *et al.*, 2006; Rüberg *et al.*, 1999; Winans, 1990). It has been described that Pi limitation in *Pseudomonas* alters the production of quorum-sensing signals (Jensen *et al*., 2006) and, consequently, it is related to biofilm formation, virulence process, and type III secretion system expression in *P. aeruginosa* (Haddad *et al*., 2009). Pi limitation can be considered as a signal that induces virulence and Bains *et al.*

(2012) show that under conditions of Pi starvation in *P. aeruginosa,* virulence routes are activated and the expression of genes of Cluster II is induced.

The hypothesis of a relationship between Cluster II and virulence is also reinforced by Garvis and coworkers. They showed that a mutant in CheB2 methylesterase in addition to be affected in motility, reduced considerably virulence in *C. elegans* and in murine models, recovering the virulence phenotype by complementation of this mutant in CheB2. Moreover, the mutant in methyltransferase CheB1 belonging to Cluster I, showed no effect on virulence in animal models previously tested, however, a significant involvement in motility was observed in this mutant, suggesting that the role of Cluster II pathway is related to the chemotactic response during infection due to a unknown signal molecule (Garvis *et al.*, 2009) while Cluster I plays the dominant role in *P. aeruginosa* chemotaxis and flagellar mobility. In addition, Burrowes and collaborators reported that RsmA exerted control over *cheB2* with a 10-fold reduction in expression of *cheB2* in an *rsmA* mutant (Burrowes *et al.*, 2006). It has been reported that RsmA works in conjunction with small non-coding RNA to regulate the expression of multiple virulence genes, including the quorum sensing *lasI* and *rhlI* genes (Pessi *et al.*, 2001). Therefore they propose that Cluster II signaling pathway could be regulated by RsmA, suggesting this fact again, Cluster II could be related to virulence.

These observations are in consonance with our results that show *C. elegans* mortality is highly reduced in plates containing *P. aeruginosa* ΔMcpB strain as compared with plates containing *the* wild type strain, suggesting that the function of the Che2 pathway is linked to virulence. Studies in other animal models like *A. bipunctata* and *C. carnea* show a drastic decrease in weight and more dead individuals in assays performed with *P. aeruginosa* PAO1 wild type strain, compared with its mutant in the *mcpB* gene.

Similar results were observed in the earthworm *E. foetida* that gained a significant higher weight when fed with *P. aeruginosa* ΔMcpB compared to worms fed with the wild type strain. Regarding to the reproductive success, we could observe 1.2 times more eggs and 2.5 times more young individuals in the case of worms fed with *P. aeruginosa* ΔMcpB, compared to those fed with wild type strain. In these bioassays

that imply a direct contact with the organism, we could observe an attenuated virulence effect in *P. aeruginosa* ΔMcpB. However, those assays to evaluate the environmental effect on microorganisms and aquatic organisms through the production of secondary metabolites exported to extracellular media by *P. aeruginosa* and its mutant *P. aeruginosa* ΔMcpB, we observed no significant differences between both strains. This implies that virulence pathway affection in the mutant, would not be related to general virulence systems described in *P. aeruginosa*, through secondary metabolites production constitutively (Strateva and Mitov, 2011), but it is necessary the direct contact between the strain and the host cells. However, it could be possible, that the mutation in *mcpB* gene, could have affected some others virulence pathways through a potential modulation of second messenger levels, which in turn may affect the expression of genes involved in virulence and pathogenesis. However, further studies are necessary to elucidate these mechanisms. Regarding de ESHI calculation, we can observe that both strains are considered as pathogenic strains, because both of them have less than 50 points in the classification proposed by Vilchez *et al*. (2016), however, *P. aeruginosa* ΔMcpB presents a phenotype less virulent than the wild type strain. Since this strain has other virulence factors (Strateva and Mitov, 2011), *mcpB* gen could be involved in other alternative virulence pathway.

In this work, we show the relationship between Cluster II and virulence in *Pseudomonas aeruginosa*. Our data reveal that McpB and Cluster II in *Pseudomonas aeruginosa* PAO1 are related to virulence and pathogenesis through different kinds of analyses performed but we do not know the complete mechanism involved in the phenotype observed in McpB mutant. To consolidate this hypothesis, and to identify the corresponding underlying mechanism(s), further experimentation is necessary like RNA-Seq or microarray assays.

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DISCUSIÓN GENERAL

Como se ha mencionado anteriormente, los sistemas de transducción de señales comprenden un conjunto de mecanismos que permiten a los microorganismos, adaptarse al medio en el que se encuentran, desarrollando las respuestas fisiológicas adecuadas.

La mayoría de sistemas de transducción de señales, pueden englobarse en tres grupos: sistemas de un componente (OCS), compuestos por una única proteína, sistemas de dos componentes (TCS), que entrañan una mayor complejidad y que parece ser que proceden evolutivamente de los anteriores (Ulrich *et al*., 2005) y sistemas de señalización química (Stock *et al*., 2000; Ulrich *et al*., 2005; Wuichet and Zhulin, 2010) en los que intervienen varias proteínas.

En relación al grupo de sistemas de dos componentes, en esta tesis doctoral, se ha llevado a cabo la caracterización del Sistema TmoS/TmoT presente en *P. mendocina* KR1, relacionándolo con sistemas de dos componentes previamente estudiados en otras cepas del género *Pseudomonas,* para determinar si las características de este sistema son específicas de la especie, o si por el contrario, presenta características comunes del género *Pseudomonas.*

En cuanto a los sistemas de señalización química en *Pseudomonas*, los análisis de genomas muestran una elevada complejidad en estos sistemas en comparación con el grupo de las enterobacterias, observando la existencia de varias copias de las proteínas núcleo involucradas en la cascada de señalización, entre otras características. En este trabajo se han intentado caracterizar estos complejos sistemas a través de distintas cepas del género *Pseudomonas.*

Con respecto al sistema de dos componentes TmoS/TmoT, la interpretación conjunta de los datos obtenidos junto con los datos previamente descritos para el sistema TodS/TodT nos llevaron a la identificación de algunas características que podrían ser comunes a la familia de proteínas. Con respecto a la topología, pudimos observar que la mayoría de las sensor Kinasas descritas, presentan una o varias regiones transmembrana (Krell *et al*., 2010; Mascher *et al*., 2006), mientras que TodS al igual que TmoS son proteínas solubles. Además, atendiendo a la presencia de dominios en

ambas proteínas, se ha observado que tanto TmoS como TodS, presentan 7 dominios: un primer dominio PAS que es capaz de unir tolueno con una elevada afinidad (Lacal *et al*., 2006), dos módulos transmisores AutoKinasa (AK1 y AK2) compuestos por un dominio catalítico y un dominio Dhp, de dimerización y fosfotransferencia, ambos separados por un dominio receptor de regulador de respuesta (RRR) y un dominio PAS 2, que podría estar implicado en la detección de oxígeno (Lau *et al*., 1997) (Figura 5). Este tipo de composición en cuanto a dominios, se clasifica como TRTR según Williams y Whitworth (2010) ya que el grupo fosforilo va pasando secuencialmente del primer dominio **t**ransmisor al dominio receptor del **r**egulador de respuesta, pasando seguidamente al segundo dominio **t**ransmisor, y por último al segundo dominio receptor del **r**egulador de respuesta. En este caso, los dominios transmisores, presentan actividad catalítica de hidrólisis de ATP, por lo que presentan capacidad de autofosforilarse, a diferencia de lo que ocurre con los TCS de tipo TRPR, donde el grupo fosforilo pasa del módulo **t**ransmisor al dominio receptor del **r**egulador de respuesta, para pasar seguidamente al módulo de fosfotransferencia (**p**hospotransfer), que no presenta actividad catalítica de hidrólisis de ATP. Este dominio de fosfotransferencia, cede el grupo fosforilo al dominio receptor del **r**egulador de respuesta del segundo componente del sistema. En este caso, el grupo fosforilo es el mismo desde el principio del proceso hasta el final, ya que sólo el primer módulo transmisor presenta dominio catalítico, catalizando la ruptura de la molécula de ATP (Williams and Whitworth, 2010) (Figura 20). El análisis de secuencias de las proteínas de ambos sistemas, muestra un elevado grado de homología en estos dominios, manteniéndose muy conservados los aminoácidos implicados en esta cascada de fosfotransferencia. Estos datos sugieren que este mecanismo de acción, podría ser una característica común en ambos sistemas.

Otra característica común, consiste en que ambos sistemas de dos componentes, TodS/TodT, y TmoS/TmoT, están implicados en la regulación de las vías de degradación de hidrocarburos aromáticos. La concentración de estos compuestos, puede ser distinta en el periplasma y en el citosol, ya que existen mecanismos de expulsión de estos contaminantes a partir de bombas de extrusión (Ramos *et al*., 2009). Sin

embargo, al ser TodS y TmoS proteínas citosólicas, ambas pueden detectar los sustratos disponibles que han sido capaces de atravesar la membrana plasmática, siendo por tanto las respuestas resultantes más precisas. Estos compuestos aromáticos, son bastante tóxicos para las células, y es por este motivo, por lo que estos sistemas han evolucionado para detectar concentraciones muy bajas de estos contaminantes para activar las respuestas necesarias para metabolizar estos contaminantes. En este contexto, se ha observado que TmoS y TodS, son capaces de unir muchos compuestos aromáticos con afinidades en el rango nanomolar, sin embargo, la concentración necesaria a la que se alcanza la estimulación máxima de autofosforilación, es bastante mayor (Busch *et al*., 2007). Ésto puede deberse a que el reconocimiento inicial del ligando no desencadena los mecanismos moleculares posteriores en la cascada de señalización de una manera inmediata. El mecanismo de degradación de tolueno ha coevolucionado con el mecanismo de resistencia al tolueno. Rojas y colaboradores describieron la bomba de eflujo TtgGHI como principal determinante para la resistencia tolueno (Rojas *et al.*, 2001). Esta bomba está bajo el control del represor TtgV, que en presencia de tolueno desreprime la expresión de la misma (Guazzaroni *et al*., 2004). Sin embargo, TtgV presenta una afinidad de unión a tolueno relativamente débil (118 μM) (Guazzaroni *et al*., 2005), en comparación con la concentración a la que se produce la activación transcripcional (10 μM) (Busch *et al*., 2007). Ésto nos hace suponer, que el primer paso de actuación frente a este contaminante, consiste en la degradación del tolueno mediante la ruta TOD, de manera que cuando la concentración de este contaminante es muy baja, el microorganismo es capaz de usar este contaminante como fuente de carbono mediante esta vía. Sin embargo, cuando las concentraciones de contaminante aumentan considerablemente, entraría en acción el sistema de expulsión de tolueno a través de la bomba de eflujo TtgGHI. Otra característica común de esta familia, viene determinada por las afinidades descritas para el tolueno, siendo para TmoS de 0,15 μM y para TodS, de 0,69 μM. Estas afinidades en las constantes de disociación son las más altas conocidas en TCS hasta el momento. Sugerimos que la naturaleza tóxica de estos efectores es una de las razones para esta alta afinidad observada.

Con respecto al estudio de la expresión génica de los promotores P_{todX} y P_{tmoX}, pudimos observar que en presencia de los 54 efectores ensayados, se mostró que el mismo conjunto de 22 compuestos, a los que denominamos agonistas, indujo la transcripción en ambos casos, siendo el tolueno el inductor más potente (Figura 29), mientras que otros 30 compuestos no indujeron la transcripción en ninguno de los dos promotores. La característica estructural común de estos 22 compuestos inductores fue la presencia de un solo anillo aromático. En estudios previos, se demostró que la fenilalanina situada en posición 79 en la HK, es esencial para la unión a su efector, ya que la mutación en este aminoácido, impide la unión a dicho efector (Busch *et al*., 2007). Este residuo está muy conservado, entre todos los miembros esta familia de TCS (Figura 21). Los datos presentados sugieren por tanto, que la existencia de agonistas y antagonistas no es una característica específica del TCS TodS/TodT, sino que se trata de una característica común de toda la familia. Tras analizar cuáles de los compuestos ensayados actuaban como agonistas, se seleccionaron varios de ellos para realizar ensayos de autofosforilación con TmoS. La exposición al tolueno provocó el aumento más pronunciado en la autofosforilación de la proteína, seguido del benceno y clorobenceno (Figura 28). Con este ensayo, podemos confirmar que las moléculas efectoras que activan la autofosforilación de TmoS, se pueden clasificar en agonistas y antagonistas dependiendo de si son capaces de activar o no, la transcripción de los genes de la ruta T4MO difiriendo los agonistas, en su capacidad de aumentar la expresión de genes de la ruta T4MO. Ésto podría ser debido a diferentes modos de unión de los agonistas en el bolsillo de unión de TmoS, que tiene capacidad de unir diferentes tipos de compuestos, como se ha confirmado en estudios posteriores (Silva-Jiménez *et al*., 2015).

En este contexto, se ha observado que existen bacterias capaces de expresar genes implicados en vías de degradación de contaminantes, y sin embargo no son capaces de degradarlos de forma eficaz (Cases and de Lorenzo, 2005). Debido a que los contaminantes ambientales están constituidos por una mezcla compleja, formada tanto por agonistas como por antagonistas, la ineficacia en el proceso de degradación observada en estos microorganismos podría deberse en parte, a la acción combinada

de los agonistas y antagonistas. Los antagonistas podrían actuar como inhibidores competitivos con los agonistas bloqueando la transcripción de los genes implicados en la degradación. Para poder diseñar una estrategia válida de biorremediación de mezclas de agonistas y antagonistas, como por ejemplo el petróleo, podrían llevarse a cabo ensayos de mutagénesis al azar para intentar generar un mutante en la HK que no sea reprimido por agonistas. Por otro lado, la obtención de un modelo de la estructura tridimensional de la HK unida a un agonista y a un antagonista, podría ser de gran utilidad para identificar los aminoácidos implicados en la unión de estos compuestos. De esta forma, si los aminoácidos implicados en la unión al antagonista, difieren de los implicados en la unión al agonista, se podría llevar a cabo una estrategia de mutagénesis dirigida, de manera que los antagonistas no pudieran interaccionar con el centro activo, evitando por tanto la inhibición competitiva que causan estas moléculas.

Se han identificado sistemas homólogos a estos TCS en otras bacterias, como por ejemplo el TCS StyS/StyR, implicado igualmente en la degradación de compuestos aromáticos en varias cepas de *Pseudomonas* (Milani *et al*., 2005; Velasco *et al*., 1998), por lo que este tipo de TCS, parece estar relacionado con la degradación de este tipo de contaminantes.

En este primer capítulo por tanto, hemos podido definir una serie de características en este tipo de sistema de dos componentes, que parecen ser comunes dentro del género *Pseudomonas,* características que demuestran la enorme complejidad que entrañan estos sistemas de transducción de señal en este género.

Con respecto a los sistemas de señalización química, a lo largo de los capítulos 2, 3 y 4 de este trabajo, se han desarrollado los estudios necesarios para intentar responder a algunas preguntas relacionadas con estos complejos sistemas de señalización química, y que se irán discutiendo a continuación.

A diferencia de lo que ocurre en las enterobacterias, las especies del género *Pseudomonas* presentan un elevado número de parálogos de una misma proteína dentro de los sistemas de señalización química. Por ejemplo, *P. aeruginosa* presenta

26 quimiorreceptores y se han descrito múltiples copias de genes que codifican las proteínas de señalización que se agrupan en cinco Clusters (Ferrández *et al*., 2002; Hong *et al*., 2004; Stover *et al*., 2000) (Figura 11). Cada uno de estos Clusters, está relacionado con una vía distinta de señalización.

En *P. putida* KT2440, se ha observado la existencia de tres parálogos de la metiltransferasa CheR sugiriendo este hecho, la existencia de tres vías diferentes de señalización química tal y como se ha descrito en el patógeno *P. aeruginosa*.
En esta tesis doctoral se realizó un estudio comparativo de los tres parálogos de CheR de *P. putida* KT2440, para tratar de definir la función de cada uno de ellos, y saber si intervienen en rutas de señalización independientes, o si por el contrario, son capaces de interactuar en varias vías de señalización.

Se ha observado que el operón de genes que contiene el parálogo *cheR1* en *P. putida*, es homólogo al Cluster III en *P. aeruginosa* (Hickman *et al*., 2005). CheR1 es pues, un homólogo de la metiltransferasa WspC de la vía WSP.

En este trabajo, hemos demostrado que la mutación del homólogo WspC en *P. putida*, (la metiltransferasa CheR1), reduce la formación de biopelículas, mientras que los mutantes en los otros dos parálogos, mostraron un fenotipo de formación de biopelículas comparable al de la cepa silvestre, lo que indica la existencia de una vía WSP funcional en *P. putida* y que CheR1 es el único parálogo de metiltransferas que actúa en esta vía (Figura 41).

Con respecto a CheR2, hemos demostrado que sólo este parálogo es capaz de metilar dos receptores de quimiotaxis, mientras que en los dos parálogos restantes, no se observó capacidad de metilación de dichos receptores (Figura 36). Estos resultados concuerdan con los resultados obtenidos en el mutante en el gen *cheR2*, que presenta un fenotipo defectuoso en motilidad por quimiotaxis general, mientras que los mutantes en los genes *cheR1* y *cheR3* mostraron motilidad quimiotáctica comparable con la observada en la cepa silvestre (Figuras 39 y 40). Estos datos sugieren que existe una elevada especificidad entre las metiltransferasas CheR y los quimiorreceptores, de manera que no todos los parálogos son capaces de metilar cualquier quimiorreceptor.

Con respecto al parálogo CheR3, pensamos que quizás podía formar parte de una vía homóloga a la vía CHP, codificada por el Cluster IV en *P. aeruginosa* (Darzins, 1994; Kearns *et al*., 2001). Para evaluar si este parálogo podía estar relacionado potencialmente con este tipo de la motilidad a través de pilus tipo IV, se realizaron varios ensayos de motilidad tipo *twitching* tanto con la cepa silvestre, como con el correspondiente mutante en el gen *cheR3*. A pesar de numerosos intentos en diferentes condiciones experimentales, no se observaron diferencias significativas que nos permitieran relacionar este parálogo con este tipo de motilidad. Para establecer su posible función a través de secuencia, se hizo un alineamiento y un análisis de agrupamiento de secuencias de los 4 parálogos de CheR de *P. aeruginosa* y los 3 parálogos de CheR de *P. putida* (Figura 42). Como se esperaba, el parálogo WspC de *P. aeruginosa* está estrechamente relacionado con CheR1 de *P. putida*, ambos implicados en formación de biopelículas como demuestran estudios previos realizados en *P. aeruginosa* (Hickman *et al*., 2005) y los resultados de esta tesis. También pudimos observar, que CheR2 de *P. putida*, comparte gran similitud y presenta una relación importante con el parálogo CheR1 de *P. aeruginosa,* ambos relacionados con quimiotaxis, tal y como se ha demostrado en estudios previos realizados con un mutante en *cheR1* de *P. aeruginosa* (Ferrández *et al*., 2002; Kato *et al*., 1999; Masduki *et al*., 1995), y como hemos podido observar en esta tesis. Así, los datos demuestran que las similitudes en la función de estas metiltransferasas CheR pertenecientes a especies bacterianas distintas, se reflejan en las similitudes que presentan a nivel de secuencia. Estas observaciones, pueden resultar de utilidad para la anotación de homólogos de metiltransferasas en otras cepas y relacionarlos con una determinada función. En el caso de CheR3 de *P. putida*, el análisis de secuencia realizado (Figura 42B) mostró que este parálogo no presenta equivalentes, por lo que no pudimos asignarle una posible función por comparación con otros homólogos previamente descritos. Lo mismo ocurrió con los homólogos de CheR2 y PilK de *P. aeruginosa*. El alineamiento de estas 7 secuencias (Figura 42A) muestra que CheR1 de *P. aeruginosa* y CheR2 de *P. putida*, ambos implicados en la quimiotaxis, tienen un inserto que comparte una secuencia consenso “E[R/K][S/T]N”. Este inserto se encuentra en una

hélice cerca del sitio de unión al pentapéptido (Figura 32A) descrito por Wu y colaboradores (1996) en *E. coli* y por lo tanto podría ser parte de la interfaz de interacción con quimiorreceptores.

La metiltransferasa CheR de *S. typhimurium* se caracteriza por la inhibición por producto, ya que el producto SAH se une con más afinidad a CheR que el sustrato SAM (Simms and Subbaramaiah, 1991). En estos estudios, quisimos comprobar si esta inhibición por producto final, era exclusiva de enterobacterias, o si por el contrario, era una característica común de las metiltransferasas. Para ello, se realizaron estudios de interacción mediante microcalorimetría isoterma de titulación (ITC), determinando que los tres parálogos de CheR en *P. putida*, reconocían SAH con mayor afinidad que SAM (Figura 34, Tabla 5). Se obtuvieron resultados similares al analizar los cuatro parálogos de CheR de *P. aeruginosa*, (Figura 45, Tabla 8). La relación entre las afinidades de metiltransferasas por SAM y SAH indican la magnitud de dicha inhibición en cada caso. En el caso de *P. aeruginosa* no se observaron diferencias significativas en el grado de inhibición para los parálogos CheR1, CheR3 y WspC, sin embargo, las diferencias fueron particularmente pronunciadas en el caso de CheR2, que unía SAH con una afinidad aproximadamente 360 veces superior a la afinidad observada para SAM. Ésto indica una elevada inhibición por producto final en el caso de este parálogo, sugiriendo que un aumento en la concentración celular de SAH, podría inhibir selectivamente a la metiltransferasa CheR2, mientas que el resto de parálogos podrían seguir activos, desempeñando su función celular. Estos datos indican que la inhibición por producto final, es una característica común de este tipo de proteínas.

Como se ha mencionado anteriormente, únicamente el parálogo CheR2 es capaz de metilar dos receptores de quimiotaxis. Estos quimiorreceptores son el McpS de *P. putida* KT2440 y el McpT de *P. putida* DOT-T1E (Molina *et al*., 2011). Este último receptor es responsable del fenotipo de hiperquimiotaxis hacia hidrocarburos tóxicos aromáticos tales como tolueno (Lacal *et al*., 2011). Se ha demostrado que existe una relación directa entre la degradación de los hidrocarburos aromáticos y la quimiotaxis hacia estos compuestos ya que la quimiotaxis aumenta la biodisponibilidad del

sustrato de biodegradación incrementando por tanto, la eficiencia de su metabolización (Lacal *et al*., 2013). En estudios previos, se demostró que la transferencia del gen *mcpT* a otras especies confiere este fenotipo de hiperquimiotaxis hacia compuestos aromáticos, lo que sugiere el establecimiento de complejos de señalización funcionales entre McpT y proteínas del huésped (Lacal *et al*., 2011). Ésto, se ha demostrado en este trabajo, mediante la observación de una metilación eficiente de McpT por una metiltransferasa CheR de otra cepa (Figura 36). Esta observación ofrece la posibilidad de realizar "ingeniería genética quimiotáctica" de cepas relacionadas por la transferencia de genes que codifican para determinados quimiorreceptores con interés biotecnológico. Ésto abre una nueva ventana en la investigación de estrategias en el campo de la biorremediación de zonas contaminadas.

Hasta el momento, hemos podido averiguar que los distintos parálogos de metiltransferasas en *Pseudomonas* interactúan con determinados quimiorreceptores activando distintas vías de señalización, pero en este punto, surge la necesidad de responder a la siguiente pregunta: ¿cuáles son los mecanismos moleculares relacionados con esta elevada especificidad de reconocimiento? En este trabajo intentamos resolver esta pregunta y darle un sentido biológico a los resultados obtenidos, así como buscar futuras aplicaciones biotecnológicas relacionadas con los resultados obtenidos.

En general, las interacciones proteína-proteína están mediadas por una única interfaz. Sin embargo, en el caso de CheR y CheB de algunas especies de enterobacterias, parece existir una excepción, ya que ambas proteínas se unen a dos sitios distintos dentro del quimiorreceptor. Por un lado, se unen a los sitios propios de metilación, y por otro lado al pentapéptido C-terminal presente en algunos quimiorreceptores (Lai and Hazelbauer, 2005; Lai *et al*., 2006). Ensayos en enterobacterias, demostraron que al eliminar el pentapéptido de los receptores de alta abundancia, se mantiene una actividad de metilación residual (Le Moual *et al*., 1997; Wu *et al*., 1996). Estos resultados contrastan con los obtenidos en este trabajo, donde se ha demostrado que

en el modelo de estudio *P. aeruginosa* PAO1, al eliminar el pentapéptido presente en el quimiorreceptor McpB, se pierde cualquier tipo de unión y de actividad de metilación por parte de la metiltransferasa CheR2 (Figuras 48A, 48B y 59). Estos datos sugieren que la existencia de este pentapéptido es esencial en *Pseudomonas* mientras que en enterobacterias, facilita la unión al sito de metilación pero no es esencial.

Los genes que codifican estas dos proteínas en *P. aeruginosa* (McpB y CheR2) se encuentran situados dentro del genoma uno junto al otro y forman parte del Cluster II, que codifica las proteínas implicadas en quimiotaxis de la vía Che2. Esta colocación adyacente de ambos genes (Figura 44), nos hace pensar que existe una relevancia fisiológica en esta interacción, de manera que se produce una interacción específica de un receptor concreto con una metiltransferasa particular, y no con cualquier parálogo (Figura 46). Esta hipótesis se ve reforzada por las observaciones de Güvener y colaboradores (2006), que demostraron que las proteínas pertenecientes al Cluster II no están colocalizadas con las proteínas pertenecientes al Cluster I, que constituye la vía principal descrita para la quimiotaxis en *P. aeruginosa*, sino que se localizan en polos celulares distintos (Güvener *et al*., 2006). Las proteínas de la vía de Che2 forman sus propios complejos de señalización. También observaron que de los 26 quimiorreceptores presentes en *P. aeruginosa*, McpB es el único que participa en la formación de complejos de señalización en la vía Che2, sugiriendo estas observaciones que la formación de los complejos de señalización de esta vía es altamente específico. Nuestros resultados, concuerdan totalmente con las conclusiones extraídas por Güvener y colaboradores, ya que demostramos que únicamente la metiltransferasa CheR2 interacciona con el quimiorreceptor McpB, mediante un mecanismo de unión a través de pentapéptido presente en el extremo C-terminal del receptor. Esto sugiere una alta especificidad entre estas dos proteínas pertenecientes al Cluster II (Figura 48).

Se han descrito muchas especies bacterianas que contienen múltiples quimiorreceptores con y sin pentapéptidos en sus extremos C-terminales, y que además, contienen múltiples copias de CheR (Perez and Stock, 2007). Es muy probable que este mecanismo de direccionamiento específico descrito aquí para CheR2 y McpB

también pueda ser aplicable a otras interacciones CheR-quimiorreceptor en otras especies.

Dentro del Cluster II, también se localiza el gen *mcpA* que codifica para otro quimiorreceptor que presenta igualmente una extensión en su extremo C-terminal (Figuras 43 y 44). Sin embargo, en este trabajo, concluimos que el pentapéptido de McpA, no es reconocido por ninguno de los parálogos CheR, ya que en la titulación de todos los parálogos de CheR presentes en *P. aeruginosa* con el pentapéptido del extremo C-terminal de este receptor, no se observó ninguna interacción. La relevancia del pentapéptido presente en el quimiorreceptor McpA, aún no ha sido identificada.

Todos estos datos nos indican que en *P. aeruginosa*, McpB parece ser el único receptor que participa en la vía Che2 (Güvener *et al*., 2006), y CheR2 es la única metiltransferasa capaz de unirse al receptor McpB (Figuras 46 y 48A) y de producir una metilación eficaz de dicho quimiorreceptor (Figura 58).

Estas observaciones, nos llevan a plantearnos el motivo de esta elevada especificidad. ¿Existen motivos estructurales o a nivel de secuencia en los parálogos de CheR que produzcan el reconocimiento de este pentapéptido sólo en CheR2?

Al estudiar la estructura de un cristal de un complejo proteico formado por CheR de *S. typhimurium* unido a SAH y al pentapéptido del receptor Tar, se puede observar que la base de la especificidad de interacción entre los quimiorreceptores y CheR, radica en un motivo de unión al receptor específico incorporado en el dominio de la metiltransferasa CheR. Esta metiltransferasa, presenta un subdominio β dentro del dominio de 7 hebras C-terminal, que es donde se localiza el sitio de unión al pentapéptido (Djordjevic and Stock, 1998; Martin and McMillan, 2002).

El hecho de que este subdominio contiene el sitio de unión al pentapéptido, podría hacer pensar que el reconocimiento del pentapéptido podría estar relacionado con la inserción de este subdominio permitiendo esta interacción. Sin embargo, los cuatro parálogos de CheR de *P. aeruginosa* contienen este subdominio β (Figura 52), mientras que sólo CheR2, es capaz de unir pentapéptidos (Figura 46), por lo tanto, la mera

presencia del subdominio β en metiltransferasas, no es la responsable del reconocimiento específico del receptor. Gracias al alineamiento y al estudio de agrupamiento realizado (Figuras 51 y 52), se identificaron dos familias diferentes de metiltransferasas CheR que se distinguen en función de su capacidad de unirse a receptores que contienen pentapéptidos.

Como resultado de este estudio, se observó que existe una inserción de tres aminoácidos en el bucle que une las hebras 2 y 3 del subdominio β, que sólo está presente en las metiltransferasas que son capaces de unir pentapéptidos (Figura 52). El uso de mutagénesis dirigida para eliminar esta inserción de tres aminoácidos de la metiltransferasa CheR2, demostró que este tripéptido es esencial para la unión al pentapéptido del extremo C-terminal del receptor McpB (Figura 54 y 55).

La inspección de la estructura CheR-pentapéptido en *S. typhimurium*, ilustra que estos tres aminoácidos forman pocas interacciones con el pentapéptido unido (Djordjevic and Stock, 1998). Sin embargo, nuestra hipótesis consiste en que la presencia de este tripéptido puede ser esencial para orientar las hebras 2 y 3 del subdominio β para que pueda interactuar correctamente con el pentapéptido. La presencia de esta inserción se podría utilizar como herramienta de predicción basándose en las secuencias de metiltransferasas. De esta manera, en organismos con varias copias de metiltransferasas se podría predecir cuales de ellas, podrían interaccionar con pentapéptidos presentes en quimiorreceptores, en función de la presencia o ausencia de la inserción de este tripéptido dentro del subdominio β.

En el caso de *P. aeruginosa*, CheR2 se une exclusivamente al pentapéptido C-terminal del receptor McpB, con una afinidad aproximadamente 20 veces mayor que la afinidad con la que se une CheR de *E. coli* al pentapéptido NWETF, presente en receptores de alta abundancia (Yi and Weis, 2002). Aunque este último péptido no está presente en los receptores de *P. aeruginosa*, CheR2 también lo reconoció con alta afinidad tal y como se muestra en la figura 47.

Un análisis bioinformático de pentapéptidos de diferentes especies ha permitido establecer la siguiente secuencia consenso: [D/N]-[W/F]-[E/Q]-[T/E]-[F] (Perez and

Stock, 2007). Shiomi y colaboradores (2000) observaron que el triptófano (W), y la fenilalanina (F) de este pentapéptido eran esenciales para la unión de la metiltransferasa CheR en enterobacterias, mientras que mutaciones en los tres aminoácidos restantes, no mostraban efectos importantes en la interacción (Shiomi *et al*., 2000). Estos datos muestran, que las posiciones más relevantes dentro del pentapéptido son los residuos de triptófano (W) en posición 2, y de fenilalanina (F), en posición 5. Ambos residuos están presentes en idénticas posiciones en el pentapéptido GWEEF de *P. aeruginosa*, y en el pentapéptido NWEEF de *E. coli*, pudiendo ser éste, el motivo por el cual la metiltransferasa CheR2 de *P. aeruginosa* es capaz de unirse al pentapéptido presente en *E. coli* (Figura 47). Para demostrar esta hipótesis, podría realizarse una mutagénesis dirigida para cambiar estos residuos, por otros distintos, y estudiar la interacción de este pentapéptido con la metiltransferasa CheR2.

Por otro lado, la afinidad con la que se une CheR2 de *P. aeruginosa* al pentapéptido de *E. coli*, es aproximadamente 4 veces mayor que la afinidad reportada para la interacción de CheR de la enterobacteria con su propio pentapéptido (Yi and Weis, 2002). Ésto puede estar indicando, que la interacción CheR2-pentapéptido, al ser un requisito estricto para cualquier actividad de metilación, a diferencia de lo que ocurre en enterobacterias, debe estar sometido a niveles de regulación y control altamente estrictos, lo que nos da una idea de la relevancia fisiológica de esta vía.

Estos datos sugieren que cada parálogo de CheR es capaz de interaccionar específicamente con un número determinado de quimiorreceptores. Para asegurar que cada parálogo interacciona con los componentes adecuados y que se produzca la activación de las vías correctas, deben existir mecanismos moleculares para garantizar la especificidad de las interacciones entre estas proteínas. Aquí, hemos identificado uno de estos mecanismos.

Los quimiorreceptores que contienen pentapéptidos, se encuentran en muchas especies diferentes, lo que sugiere que este mecanismo está muy extendido entre los microorganismos. Pero, ¿cuál es el sentido de este modo de interacción quimiorreceptor-pentapéptido en bacterias con sólo una copia de CheR/CheB, y con

receptores con y sin pentapéptidos? En *E. coli*, existe una única copia de CheR que actúa sobre los quimiorreceptores con y sin pentapéptido, produciéndose en todos los casos la activación de una misma vía que es la quimiotaxis (Webre *et al*., 2003). Para explicar este fenómeno se concluyó que la razón fisiológica para la unión de CheR con el pentapéptido presente en quimiorreceptores de alta abundancia, es aumentar la concentración local de CheR, lo que permitiría la metilación de los receptores vecinos (Levin *et al*., 2002; Muppirala *et al*., 2009; Wu *et al*., 1996; Yi and Weis, 2002). Los quimiorreceptores de alta abundancia, presentan un brazo flexible o *linker* de aproximadamente 25-30 aminoácidos que termina con el pentapéptido, lo que permitiría cierta movilidad de la metiltransferasa unida, para la metilación de receptores vecinos (Li and Hazelbauer, 2006).

Otra hipótesis para explicar este sistema en enterobacterias es la existencia de un sistema rudimentario de quimiotaxis en *E. coli*, debido a la pérdida de genes que no le han sido necesarios durante la evolución (Borziak *et al*., 2013). Basándonos en esta hipótesis, la existencia de quimiorreceptores con y sin pentapéptido, podría indicarnos que *E. coli* podría haber presentado un sistema de señalización química ancestral más complejo, con varias copias de metiltransferasas capaces de reconocer quimiorreceptores en función de la presencia de pentapéptidos en el extremo C-terminal de sus quimiorreceptores. Durante la evolución, podría haberse promovido la pérdida de algunos genes no necesarios. Esta hipótesis podría explicar la existencia de quimiorreceptores con y sin pentapéptidos, y una sola copia de CheR y CheB en enterobacterias.

Tras la obtención de estos resultados, nos planteamos el análisis del genoma de *P. aeruginosa* para determinar cuántos parálogos de metilesterasa CheB existen en esta cepa. Los análisis muestran la existencia de 4 parálogos de CheB en *P. aeruginosa*, y según estudios previos realizados en enterobacterias (Lai and Hazelbauer, 2005; Lai *et al*., 2006), podríamos suponer que alguno de estos parálogos, podrían interaccionar con los pentapéptidos de algunos de los quimorreceptores de esta cepa. Esta cuestión queda planteada para futuros ensayos.

Con estos resultados, queda de manifiesto la importancia de la vía Che2 de señalización por lo que el siguiente objetivo de esta tesis, fue la elucidación del papel fisiológico de esta vía. Existen varios estudios previos en este campo, que muestran resultados controvertidos. Aunque actualmente el papel fisiológico de la ruta Che2 todavía no está claro (Güvener *et al*., 2006), en principio, se pensó que la vía Che2 de *P. aeruginosa*, estaba relacionada únicamente con quimiotaxis (Ferrández *et al*., 2002), sin embargo, estos resultados no han sido reproducibles en estudios más recientes (Güvener *et al*., 2006). Otros estudios muestran McpB como una proteína citoplasmática relacionada con aerotaxis (Hong *et al*., 2004, 2005), pero este hallazgo no ha sido confirmado en estudios posteriores.

Los científicos han estado estudiando durante décadas, el papel principal de este Cluster en *P. aeruginosa*, de manera que las últimas evidencias parecen relacionarlo con un papel quimiotáctico llevado a cabo durante el periodo de infección en diferentes modelos animales (Garvis *et al.*, 2009). En el trabajo de Garvis y colaboradores (2009), observaron que el Cluster II, además de estar implicado en quimiotaxis, estaba implicado en virulencia, ya que un mutante en la metilesterasa CheB2, además de verse afectado en la motilidad, reducía considerablemente la virulencia en *C. elegans* y en modelos murinos, recuperándose el fenotipo de virulencia cuando se complementaba dicho mutante (Garvis *et al.*, 2009).

En el análisis de secuencias realizado en esta tesis, usando los 4 parálogos de CheR de *P. aeruginosa* y los 3 parálogos de CheR descritos para *P. putida*, se observó que el parálogo CheR2 (que es el único parálogo capaz de metilar el quimiorreceptor McpB de *P. aeruginosa*), no presentó ninguna homología con ningún otro parálogo (Figura 42B). Ésto indica que este parálogo, que interacciona con componentes del Cluster II puede estar interviniendo en vías de señalización distintas, como pueden ser, vías relacionadas con virulencia, hipótesis que se confirmó, en esta tesis doctoral.

La hipótesis de que McpB está implicado en virulencia, se ve reforzada por algunas observaciones y otros estudios anteriores: la presencia del Cluster II parece ser una característica común en bacterias patógenas, como por ejemplo *P. syringae* pv. *tomato*

(patógeno de plantas (Xin and He, 2013)), *Shewanella oneidensis* MR-1 (patógeno oportunista de humanos (Khashe and Janda, 1998)), *Vibrio cholera* y *P. aeruginosa* (patógenos animales), donde se encontraron genes homólogos pertenecientes a este Cluster (Ferrández *et al*., 2002). Sin embargo, cepas no patógenas pertenecientes a las especies de *P. putida* y *P. fluorescens*, no presentan este Cluster II pero sí presentan un set completo de los genes homólogos a los codificados por los Clusters I, III, IV y V cuyas proteínas comparten un elevado grado de identidad (70-80%) con las proteínas codificadas por el Cluster I en *P. aeruginosa* (Ferrández *et al*., 2002). Esta observación, podría sugerir una relación entre la ruta de señalización química perteneciente al Cluster II y algunos procesos de virulencia.

Por otro lado, estudios previos, muestran la relación entre la limitación de fosfato en determinados microorganismos y la virulencia (Aoyama *et al*., 1991) . El cuerpo animal se caracteriza por un bajo nivel de fosfato, de manera, que esta señal de limitación de fosfato, hace que el patógeno *P. aeruginosa* una vez se encuentra en el interior del animal, induzca distintas rutas relacionadas con la virulencia. En estas condiciones de limitación de fosfato, se observó a su vez una inducción de la expresión de los genes del Cluster II considerándose el fosfato por tanto, una señal clave para la regulación de la expresión de factores de virulencia (Bains *et al*., 2012).

Todas estas observaciones están en consonancia con nuestros resultados, que muestran que la mortalidad en *C. elegans* es más reducida en placas que contienen un mutante en *mcpB* que en placas que contienen *P. aeruginosa* silvestre (Figura 60), por lo que se corrobora que el Cluster II podría tener algún papel relacionado con la virulencia. Estos resultados se ven reforzados por estudios en otros modelos animales como *A. bipunctata* y *C. carnea*, donde se observó una disminución drástica en el peso de los individuos (Figuras 62 y 64) así como un mayor número de individuos muertos en los ensayos realizados con *P. aeruginosa* silvestre en comparación con los realizados usando el mutante en *mcpB* (Figura 65). Por otro lado, se observaron resultados similares cuando se realizaron ensayos de toxicidad en lombrices de tierra *E. foetida*, donde los individuos mostraron una significativa ganancia de peso en el caso de

lombrices alimentadas con un liófilo del mutante en *mcpB* en comparación con los individuos alimentados usando el liófilo de *P. aeruginosa* silvestre (Figura 67). Así mismo, se observó un mayor éxito reproducivo de estos individuos, (Figuras 68A y 68B) en el caso del mutante con respecto a la cepa silvestre. *E. foetida* es un organismo muy resistente, debido a que es especialmente tolerante a las infecciones causadas por microorganismos (Schulenburg et al., 2007), por lo que los resultados obtenidos en este bioensayo, indican que el grado de virulencia que podemos asociar a McpB es importante.

Sin embargo, este efecto de reducción en la virulencia, sólo se pudo observar en los experimentos que implican un contacto directo con el organismo. Para comprobar el efecto que tenía la mutación en el gen *mcpB* en la producción de metabolitos secundarios secretados al medio, se realizaron ensayos usando distintos tipos de organismos como *E. coli* MC4100, *V. fischeri* y *D. magna*. En estos casos, no se observaron diferencias significativas entre ambas cepas. Esto implica que las rutas de virulencia codificadas por el Cluster II, no están relacionadas con los sistemas de virulencia descritos en *P. aeruginosa*, como la producción de toxinas o enzimas extracelulares expresadas de manera constitutiva, sino que es necesario el contacto directo entre el patógeno y el huésped, para la activación de esta vía. Al analizar los valores obtenidos en el modelo *ESHI* propuesto por Vílchez y colaboradores (2016), pudimos observar que ambas cepas obtuvieron una puntuación por debajo de 50 puntos, por lo que según este índice, serían consideradas como patógenas. Ésto nos indica que además de este sistema, hay otros sistemas de virulencia independientes de McpB que siguen realizando su función en patogénesis (Strateva and Mitov, 2011). Sin embargo, la puntuación obtenida para el mutante en *mcpB*, fué más alta que la obtenida para la cepa silvestre, indicando, que la cepa silvestre presenta un fenotipo más virulento que el mutante, por lo que podríamos sugerir que la mutación del gen *mcpB* podría haber afectado algunas otras vías de virulencia a través de la modulación de segundos mensajeros y, en consecuencia, ocurra la inducción o represión de algunos genes implicados en virulencia y patogénesis aunque estos mecanismos aún no están claros.

Nuestros datos revelan que McpB y el Cluster II están relacionados con la virulencia y la patogénesis a través de los diferentes tipos de análisis realizados, pero no sabemos el mecanismo molecular correspondiente. Para identificar este mecanismo debemos realizar ensayos futuros como ensayos de RNA-Seq o microarrays para estudiar qué genes se activan o se reprimen en la cepa mutante en comparación con la silvestre para saber las vías que están involucrados en este caso.

Ésto nos abre las puertas para poder diseñar estudios encaminados al tratamiento de enfermedades producidas por *P. aeruginosa*, un patógeno que ocupa un lugar preponderante dentro de las infecciones nosocomiales, que constituyen un problema de salud de extraordinaria importancia en el mundo, afectando a la calidad y la eficiencia de los servicios médicos.

CONCLUSIONES/ CONCLUSIONS

De acuerdo con los resultados obtenidos en este trabajo así como con la bibliografía consultada, se derivaron las siguientes conclusiones en esta tesis doctoral:

1. TmoS es soluble en solución y en ausencia de detergentes, característica que comparte con la Histidin Kinasa (HK) TodS, por lo que ésta parece ser una característica común de esta familia de proteínas.

2. El perfil de ligandos de TmoS es muy similar al perfil descrito para TodS, clasificándose dichos ligandos como agonistas y antagonistas, activándose ambas kinasas en respuesta a compuestos que no son degradables por las vías correspondientes. Esta similitud en el funcionamiento de TmoS y TodS sugiere que este mecanismo puede ser una característica común de esta familia de TCS.

3. *P. putida* KT2440 presenta tres parálogos funcionales de la metiltransferasa CheR, con capacidad de unir SAM y SAH. En los tres parálogos se observó un efecto de inhibición por SAH lo que sugiere que este mecanismo es una característica general de la familia de metiltransferasas del tipo CheR.

4. Sólo el parálogo CheR2 de *P. putida* KT2440 es capaz de metilar los quimiorreceptores McpS y McpT, presentando el mutante en el gen *cheR2* un fenotipo defectuoso en quimiotaxis, concluyendo que este parálogo está relacionado con procesos quimiotácticos.

5. Un mutante en el gen *cheR1* de *P. putida* KT2440 presenta un fenotipo defectuoso en formación de biopelículas, lo que sugiere la existencia de una vía análoga a la WSP en esta cepa. Se concluye que CheR1 es el único parálogo implicado en esta vía.

6. El parálogo CheR3 en *Pseudomonas putida* KT2440 no se ha podido relacionar con ninguna vía de señalización, por lo que hasta el momento, su función es desconocida.

7. *P. aeruginosa* PAO1 presenta tres quimiorreceptores con extensiones en sus extremos C-terminales, y presenta 4 parálogos funcionales de CheR, todos ellos sujetos a una inhibición por SAH.

8. Únicamente el parálogo CheR2 de *P. aeruginosa* PAO1 es capaz de unir el pentapéptido C-terminal del receptor McpB, pero no los pentapéptidos de otros receptores. La presencia de este pentapéptido es necesaria para la metilación del receptor concluyendo que la relevancia de esta secuencia es la de garantizar la interacción específica entre CheR2 y McpB.

9. No existen efectos de unión cooperativa entre el sitio de unión al pentapéptido C-terminal, y el sitio de unión a SAM y SAH dentro de la metiltransferasa CheR2 de *P. aeruginosa* PAO1.

10. A través de análisis de secuencias y estudios de mutagénesis concluimos que existen dos familias de metiltransferasas del tipo CheR en base a su capacidad de unión a pentapéptidos C-terminales de quimiorreceptores. La presencia del tripéptido GPN en la metiltransferasa es determinante para la unión con el pentapéptido, pudiendo utilizar esta característica como herramienta predictiva de metiltransferasas capaces de unir pentapéptidos.

11. El quimiorreceptor McpB de *P. aeruginosa* PAO1 está implicado en procesos de virulencia a través de la ruta Che2, aunque el mecanismo molecular de esta vía es desconocido.

The results obtained, together with the literature review performed, lead us to draw the following conclusions:

1. TmoS is soluble in solution and in the absence of detergents, a feature that it shares with TodS, suggesting it to be a common feature of this protein family.

2. The ligand profile of TmoS is very similar to the profile described for the TodS, classifying them as agonists and antagonists. Both kinases are activated in response to compounds which are not degradable by the corresponding pathways. This similarity in the TmoS and TodS suggests that this mechanism can be a common feature of this family of TCS.

3. *P. putida* KT2440 has three functional paralogs of CheR methyltransferase that are able to bind SAM and SAH. For the three paralogs an inhibitory effect by SAH was observed suggesting that this mechanism is a general feature of the CheR type methyltransferases.

4. Only the CheR2 paralog of *P. putida* KT2440 can methylate McpT and McpS chemoreceptors, presenting a *cheR2* mutant, a defective phenotype in chemotaxis, concluding that the CheR2 paralog is related to chemotactic processes.

5. A mutant in the *cheR1* gene has a defective phenotype in biofilm formation, suggesting the existence of an analogous pathway to WSP in this strain. It is concluded that the CheR1 paralog is the only one involved in this pathway.

6. The CheR3 paralog in *P. putida* KT2440 has not been linked to any signaling pathway and so far its function remains unknown.

7. *P. aeruginosa* PAO1 has three chemoreceptors with extensions at their C-terminal ends and four functional paralogs of CheR methyltransferase presenting all of them inhibition by SAH.

8. Only the CheR2 paralog of *P. aeruginosa* PAO1 can effectively bind to C-terminal pentapeptide of McpB chemoreceptor, but no pentapeptides of other receptors. The presence of this pentapeptide is necessary for receptor methylation concluded that the significance of this sequence is to ensure the specific interaction between CheR2 and McpB.

9. There is no cooperative binding effect between the binding site of C-terminal pentapeptide and the binding site of SAM and SAH within the CheR2 methyltransferase in *P. aeruginosa* PAO1.

10. Through sequence analysis and mutagenesis studies we conclude that there are two families of type CheR methyltransferases based on their ability to bind to C-terminal pentapeptide of chemoreceptors. The presence of the tripeptide GPN in the methyltransferase is crucial for the pentapeptide binding, and this feature can be used as a predictive tool to identify methyltransferases able to bind pentapeptides.

11. The McpB chemoreceptor of *P. aeruginosa* PAO1 is involved in virulence processes through Che2 pathway, however the molecular mechanism of this pathway remains unknown.

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PRODUCCIÓN CIENTÍFICA

La producción científica desarrollada en esta etapa de investigación ha sido publicada, o está en vías de publicación en distintas revistas científicas, capítulos de libro, o ha sido presentada en distintas comunicaciones a congresos, como se detalla a continuación:

Artículos derivados de esta tesis doctoral:

1. Silva-Jiménez, H.*, **García-Fontana, C***., Hilal Cadirci, B., Ramos- González, M.B., Ramos, J.L., Krell, T. * (ambos autores han contribuido de la misma manera en este artículo). Título: Study of the TmoS/TmoT two-component system: Towards the functional characterisation of the family of TodS/TodT like systems.
   Microb. Biotechnol 2012: 5:489-500
   Factor de impacto: 3.081 (Q1)

2. **Garcia-Fontana C**, Reyes-Darias JA, Munoz-Martinez F, Alfonso C, Morel B, Ramos JL, Krell T. High specificity in CheR methyltransferase function: CheR2 of *Pseudomonas putida* is essential for chemotaxis whereas CheR1 is involved in biofilm formation.
   J Biol Chem 2013: 288(26):18987-99
   Factor de impacto: 4.6 (Q1)

3. **García-Fontana C**, Corral Lugo A, Krell T. Specificity of the CheR2 methyltransferase in *Pseudomonas aeruginosa* is directed by a C-terminal pentapeptide in the McpB chemoreceptor.
   Sci Signal 2014: 7(320):ra34
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4. McpB soluble chemoreceptor in *Pseudomonas aeruginosa* PAO1 has an essential role in virulence and pathogenesis. (Manuscrito en preparación).

Otros artículos:

5. Lacal J, **García-Fontana C**, Muñoz-Martínez F, Ramos JL, Krell T. Sensing of environmental signals: classification of chemoreceptors according to the size of their ligand binding regions.
   Environ Microbiol 2010: 12(11):2873-84
   Factor de impacto: 5.53 (Q1)

6. Jesús Lacal, **Cristina García-Fontana**, Carla Callejo-García, Juan-Luis Ramos and Tino Krell. Physiologically relevant divalent cations modulate citrate recognition by the McpS chemoreceptor
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7. Krell T, Lacal J, Muñoz-Martínez F, Reyes-Darias JA, Cadirci BH, **García-Fontana C**, Ramos JL. Diversity at its best: bacterial taxis.
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9. Krell T, Lacal J, Guazzaroni ME, Busch A, Silva-Jiménez H, Fillet S, Reyes-Darías JA, Muñoz-Martínez F, Rico-Jiménez M, **García-Fontana C**, Duque E, Segura A, Ramos JL. Responses of *Pseudomonas putida* to toxic aromatic carbon sources.
   J Biotechnol 2012: 160(1-2):25-32
   Factor de impacto: 3.18 (Q1)

10. Lacal J., Reyes-Darias J.A., **García-Fontana C**., Ramos J.L., Krell T. Tactic responses to pollutants and its potential to increase biodegradation efficiency.
    Appl. Microbiol 2013: 114(4):923-33
    Factor de impacto: 2.38 (Q2)

11. Miriam Rico-Jimenez, Francisco Muñoz-Martinez, **Cristina Garcia-Fontana**, Matilde Fernández, Bertrand Morel, Álvaro Ortega, Juan Luis Ramos and Tino Krell. Paralogous chemoreceptors mediate chemotaxis towards protein amino acids and the non-protein amino acid gamma-aminobutyrate (GABA).
    Molecular Microbiology 2013: 88(6):1230-43
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12. Silva-Jimenez, Hortencia; Ortega-Retuerta, Álvaro; **García-Fontana, Cristina**; Ramos, Juan Luis; Krell, Tino. Multiple agonistic and antagonistic signals modulate the activity of the hybrid sensor kinase TodS.
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13. Maximino Manzanera; **Cristina García-Fontana**; Juan Ignacio Vílchez; Jesús González-López. Genome Sequence of *Rhodococcus* sp. 4J2A2, a Desiccation-Tolerant Bacterium Involved in Biodegradation of Aromatic Hydrocarbons Genome Sequence of *Leucobacter* sp. 4J7B1, a Plant-Osmoprotectant Soil Microorganism.
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14. Maximino Manzanera Ruiz; Lucía Santa Cruz Calvo; Juan Ignacio Vilchez Morillas; **Cristina Garcia Fontana**; Gloria Andrea Silva Castro; Concepción Calvo Sainz; Jesús González López. Genome Sequence of *Arthrobacter siccitolerans* 4J27, a Xeroprotectant Producing Desiccation-Tolerant Microorganism.
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15. Maximino Manzanera Ruiz; **Cristina Garcia Fontana**; Juan Ignacio Vilchez Morillas; Jesús González López. Genome Sequence of *Microbacterium* sp. Strain 3J1, a Highly Desiccation-Tolerant Bacterium That Promotes Plant Growth
    Genome Annoucements 2015: 713-715

16. Maximino Manzanera Ruiz; Juan Jesús Narváez Reinaldo; **Cristina Garcia Fontana**; Juan Ignacio Vilchez Morillas; Jesús González López. Genome Sequence of *Arthrobacter koreensis* 5J12A, a Plant Growth-Promoting and Desiccation-Tolerant Strain.
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17. Maximino Manzanera Ruiz;Juan Ignacio Vilchez Morillas; **Cristina Garcia Fontana**; Concepción Calvo Sainz; Jesús González López. Genome Sequence of *Leucobacter* sp. 4J7B1, a Plant-Osmoprotectant Soil Microorganism
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Capítulos de libro:

1. Krell T, Lacal J, **García-Fontana C**, Silva-Jiménez H, Rico-Jiménez M, Lugo AC, Darias JA, Ramos JL. Characterization of molecular interactions using isothermal titration calorimetry.
   Methods Mol Bio 2014: 1149:193-203
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2. Reyes Darias J.A., **García-Fontana C**., Lugo A., Rico-Jiménez M., Krell, T. Qualitative and quantitative assays for flagellum-mediated chemotaxis
   Methods Mol Bio 2014: 1149:87-97
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Comunicaciones a congresos:

1. Identification of a chemoreceptor for the TCA cycle intermediates in P*seudomonas putida*
   3rd FEMS Congress of European Microbiologists
   Goteborg, Suecia (2009)
   Jesús Lacal Romero; Parales, Rebecca; **Cristina Garcia Fontana**; Juan Luis Ramos Martin; Tino Krell.
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2. Characterization of the TmoS/TmoT two component system of *Pseudomonas mendocina* KR1 involved in the regulation of toluene degradation
   Molecular Microbiology Meeting Würzburg
   Würzburg, Alemania (2011)
   Hortencia Silva Jimenez; **Cristina Garcia Fontana**; Bilge Hilal; Juan Luis Ramos Martin; Tino Krell.
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3. The diversity in molecular architecture and function of chemoreceptors that mediate chemotactic responses to environmental signals
   6th International Meeting on Biotechnology
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   Tino Krell; Jesús Lacal Romero; Jose Antonio Reyes Darias; **Cristina Garcia Fontana**; Rico-Jimenez, Miriam; Estela Pineda Molina; Jose Antonio Gavira Gallardo; Juan Manuel Garcia Ruiz; Juan Luis Ramos Martin.
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4. Isothermal Titration Calorimetry as an universal research tool in biochemistry
   Biacore and MicroCal User Meeting 2013
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   Tino Krell, **Cristina Garcia Fontana**; Jose Antonio Reyes Darias; Rico, Miriam; Alvaro Ortega Retuerta; Andres Corral Lugo; Saray Santamaria Hernando; María Isabel Ramos González; Juan Luis Ramos Martin.
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5. Bacterial sensor domain that recognizes its cognate signals in the monomeric state
   9th European Biophysics Congress
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6. Differential analysis of root proteome obtained in microbe-plant interaction as a tool for identification of proteins involved in resistance to drought events in pepper plants
   XXXVII Congreso Sociedad Española Bioquimica y Biología Molecular.
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   Juan Ignacio Vilchez Morillas; **Cristina Garcia Fontana**; Jesús González López; Maximino Manzanera Ruiz.

7. *Microbacterium* sp. 3J1 reduces the expression of enzymes involved in ethylene pathway in roots of pepper plants under drought conditions
   XXXVII Congreso Sociedad Española Bioquimica Y Biología Molecular.
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8. Servicio de Texto Orientado iBiotecSTO: multiplataforma interactiva para la docencia de Biotecnología
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10. Paralogous chemoreceptors mediate chemotaxis towards protein amino acids and the non-protein amino acid gamma-aminobutyrate (GABA)
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11. Sensor proteins with bimodular ligand binding domain: do multiple signals define the regulatory output?
    ESF-EMBO Symposium Bacterial Networks (Bacnet 15)
    Sant Feliu de Guixols, España (2015)
    Tino Krell, Estela Pineda Molina, Alvaro Ortega, Jose Antonio Reyes Darias, Bertrand Morel, Miriam Rico Jiménez, **Cristina García Fontana**, Andrés Corral Lugo and Jose Antonio Gavira.
    Presentación Oral Invitada

Cristina García Fontana

Tesis Doctoral

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