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Bases moleculares de la tolerancia a disolventes orgánicos: estudio de la regulación de la bomba de expulsión de disolventes TtgGHI en *Pseudomonas putida* DOT-T1E

TESIS DOCTORAL

María Eugenia Guazzaroni

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Bases moleculares de la tolerancia a disolventes orgánicos: estudio de la regulación de la bomba de expulsión de disolventes TtgGHI en *Pseudomonas putida* DOT-T1E

Memoria que presenta la licenciada en Biología, María Eugenia Guazzaroni, para aspirar al Título de Doctora

Fdo.: María Eugenia Guazzaroni

Vº Bº El director
Fdo: Juan Luis Ramos Martín
Doctor en Biología
Profesor de Investigación del C.S.I.C

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Portada: micrografía electrónica de barrido de *Pseudomonas putida* DOT-T1E cultivada en medio rico (LB) en fase exponencial y modelo estructural del dominio carboxilo-terminal de TtgV que incluye el posible bolsillo de unión a efectores.

A mamá y papá

A Naty y Viqui

A Rafa

Una Tesis Doctoral consiste en una contribución científica, original, al cuerpo de conocimientos universal de la Ciencia. Y, fundamentalmente, reside en el desarrollo y formación del doctorando.

Entre otras cosas, se aprende que…

A veces, las ideas más brillantes y ocurrentes terminan siendo refutadas por un argumento más elemental y una observación rigurosa se derrumba ante la sencillez de lo obvio…

Pero a pesar de eso, aprendemos a no desistir, a luchar contra la dificultad, a intentarlo otra vez…

Y al final…..lo conseguimos!

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RESUMEN

Los hidrocarburos aromáticos son compuestos tóxicos para cualquier organismo vivo, ya que muchos de ellos tienen propiedades carcinogénicas, mutagénicas y teratogénicas. La producción industrial de fármacos, plásticos, explosivos y compuestos agroquímicos entre otros, ha generado una dispersión pronunciada de estos contaminantes en el medio ambiente. Un factor limitante en la biodegradación de algunos de estos compuestos es su alta e inherente toxicidad. Entre los hidrocarburos aromáticos más tóxicos para los microorganismos se encuentran los disolventes orgánicos del tipo BTEX (benceno, tolueno, estireno, y xilenos) debido a que su acumulación en la membrana citoplasmática provoca daños irreparables en la estructura de la misma y alteran funciones vitales de ésta que conducen a la lisis celular.

Sin embargo, en la naturaleza existen microorganismos capaces de degradar los disolventes orgánicos que contienen uno o más anillos aromáticos, probablemente debido a que durante miles de años los microorganismos han estado expuestos a bajas concentraciones de estos compuestos. Entre las bacterias se han aislado cepas de la especie *Pseudomonas putida*, las cuales no sólo son capaces de degradar estos compuestos sino que además exhiben una amplia serie de mecanismos que las hacen tolerantes a los disolventes orgánicos. Entre estos últimos se incluye la modificación de la composición lipídica de la membrana celular que tiende a una mayor rigidificación y la expulsión del disolvente orgánico mediante un proceso dependiente de energía en el que intervienen bombas o transportadores de extrusión.

Pseudomonas putida DOT-T1E se aisló de aguas de la planta de tratamiento de aguas residuales de la cuidad de Granada por su capacidad de crecimiento con tolueno como única fuente de carbono y energía. Además, es capaz de tolerar concentraciones muy altas, de hasta el 90% (vol/vol) de este disolvente orgánico, lo que la ha convertido en un organismo modelo para el estudio de los mecanismos de tolerancia a estos tóxicos. Se ha demostrado que la expulsión activa de los disolventes implica varias bombas de extrusión, siendo éste un mecanismo clave en la tolerancia bacteriana a estos tóxicos. En *P. putida* DOT-T1E, se han identificado tres bombas de expulsión de disolventes (TtgABC, TtgDEF y TtgGHI) pertenecientes a la familia RND de transportadores bacterianos. La bomba TtgGHI tiene un nivel basal de expresión alto y se induce significativamente por disolventes orgánicos, lo cual la convierte en el elemento clave de la tolerancia intrínseca basal e inducible de *P. putida*

DOT-T1E a disolventes. Es por ello que es de sumo interés conocer los detalles de su mecanismo de regulación, objetivo central del trabajo de esta Tesis.

A continuación se exponen los principales logros alcanzados en este trabajo de Tesis Doctoral. Así, se estableció que el operón *ttgGHI* se transcribe a partir de un único promotor, el cual posee cajas -10 y -35 con una significativa similitud al consenso de las reconocidas por la ARN polimerasa con el factor sigma 70. Mediante ensayos de *footprint* se determinó que TtgV interacciona con la región promotora del operón *ttgGHI* y que cubre alrededor de 40 pb. Mediante experimentos de titulación microcalorimétrica y de ensayos de retardo en gel se determinó que la constante de afinidad de TtgV por su operador se encuentra en el rango nanomolar. También mediante el uso de estas técnicas y ultracentrifugación analítica se estableció que TtgV reconoce en su operador dos secuencias repetidas invertidas diferentes, uniéndose como tetrámero, siendo esta última la forma polimérica en la que se encuentra la proteína ya sea unida al ADN o en solución. La presencia de sus efectores en una solución de TtgV no llevó a la disociación del tetrámero, mientras que sí condujo al despegue del tetrámero del ADN en una solución del complejo TtgV-ADN. La visualización de imágenes del complejo TtgV-ADN mediante microscopía de fuerza atómica permitió establecer que TtgV induce una curvatura convexa de 57º en el ADN.

Se estableció el perfil de efectores de TtgV y se determinó la constante de unión de los mismos a la proteína reguladora. Los efectores son fundamentalmente compuestos aromáticos de uno o dos anillos y alcoholes alifáticos. Entre los compuestos que presentaron mayor afinidad por TtgV se encuentran 1-naftol, indol, 2,3-dihidroxinaftaleno, 4-nitrotolueno y benzonitrilo. La constante de afinidad de TtgV por sus efectores fue del orden micromolar, y se estableció que existe una tendencia clara entre las K_D determinadas *in vitro* mediante microcalorimetría y la eficiencia de inducción *in vivo* estudiada mediante ensayos de actividad β-galactosidasa utilizando una fusión del promotor *ttgGHI* a ´*lacZ*. Con ello se dedujo que la afinidad de TtgV por los efectores es el determinante principal de la eficiencia de los mismos. También se propuso un modelo tridimensional del posible dominio de unión de efectores de TtgV, y se generaron mutaciones puntuales a alanina de los aminoácidos que podrían contactar con los efectores. La afinidad de unión de las proteínas mutantes por sus efectores se vio alterada, siendo mayor en el caso de los efectores bicíclicos indol y 1-naftol y menor en el de los monocíclicos 4-nitrotolueno y benzonitrilo. Los resultados sugieren que los residuos F134 y H200 podrían jugar un papel fundamental en la unión de efectores. La eficacia en la

liberación del ADN cuando TtgV está unida al mismo en presencia de efectores es mayor para efectores bicíclicos que monocíclicos.

Por tanto, este trabajo de Tesis Doctoral ha permitido profundizar en la caracterización molecular del promotor P_{ttgGHI} y analizar el papel del regulador TtgV en la expresión génica de la bomba TtgGHI mediante un estudio pormenorizado a nivel bioquímico y molecular de sus propiedades de unión al operador y efectores.

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Apéndice 1: Abreviaturas

ADN, ácido desoxirribonucléico

ADNasa, desoxirribonucleasa

ADNc, ADN complementario

ARN, ácido ribonucléico

ATP, adenosina trifosfato

AUC, Ultracentrifugación Analítica

CRP, Proteína receptora de AMPc

ΔG, energía libre de Gibbs

ΔH, entalpía

ΔS, entropía

Da, Dalton o g/mol

DMSO, dimetil sulfóxido

DMS, dimetil sulfato

dNTPs, desoxinucleótidos trifosfato

DO660, turbidez medida a 660 nm

DTT, di-tiotreitol

EDTA, tetra acetato de etilendiamina

HTH, hélice-giro-hélice

IPTG, isopropil β-D-tio galactopiranósido

ITC, calorimetría de titulación isotérmica

K_A, constante de asociación al equilibrio

kb, kilobase(s)

K_D, constante de disociación al equilibrio

Km, kanamicina

LB, medio Luria Bertani

MDR, resistencia múltiple a fármacos

MIC, concentración inhibitoria mínima

ONPG, *o*-nitrofenilgalactopiranósido

pb, pares de bases

P*ttgG*, Promotor del operón *ttgGHI*

P*ttgV*, Promotor del operón *ttgV*

SDS, dodecilsulfato sódico

SDS-PAGE, electroforesis en gel de poliacrilamida en presencia de SDS

v/v, volumen/ volumen

wt, cepa silvestre

X-gal, 5-bromo 4-cloro 3-indolil β-D- galactopiranósido

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Introducción

1. EL GÉNERO *PSEUDOMONAS*

1.1. Clasificación científica y generalidades

El género *Pseudomonas* fue descrito por primera vez en 1894 por el Dr. W. Migula como: "Células con órganos polares para su movilidad. Algunas especies forman esporas, aunque en general es un evento anómalo". Esta definición fue inicialmente aceptada pero a lo largo de los años fueron surgiendo criterios nuevos que llevaron a que se incluyeran o excluyeran algunos de sus miembros. En 1986 Palleroni, sobre la base del análisis de hibridación ADN-ADN, propuso cinco grupos taxonómicos dentro del género. Posteriormente la secuenciación de los ARNr 16S reflejó la diversidad entre grupos y el denominado grupo I del ARNr se reconoció como el de las verdaderas *Pseudomonas* habiéndose reclasificado las cepas de otros grupos en nuevos géneros bacterianos. Dentro del grupo de las *Pseudomonas* "verdaderas" se incluyen las especies *P. aeruginosa, P. fluorescens, P. stutzeri* y *P. putida* entre otras (Palleroni y Moore, 2004). La actual afiliación del género *Pseudomonas* es como sigue:

Reino:	Bacteria
Filo:	Proteobacteria
Clase:	Gamma Proteobacteria
Orden:	Pseudomonadales
Familia:	Pseudomonadaceae
Género:	***Pseudomonas***

Las *Pseudomonas* son bacilos rectos o ligeramente curvados, Gram-negativos, aerobios estrictos aunque en algunos casos pueden utilizar nitrato como aceptor final de electrones. El catabolismo de los glúcidos se realiza por la ruta de Etner-Doudoroff y el ciclo de los ácidos tricarboxílicos. Algunos miembros del género son psicrófilos. Otros sintetizan sideróforos fluorescentes de color amarillo-verdoso con gran valor taxonómico. Es común la presencia de plásmidos. No forman esporas. Generalmente son móviles gracias a la presencia de flagelos polares. Algunas especies sintetizan una cápsula de exopolisacáridos que facilita la adhesión celular, la formación de biofilms y protege de la fagocitosis, de los anticuerpos o del complemento aumentando así su patogenicidad (Palleroni, 1984).

El género ***Pseudomonas*** contiene bacterias saprofitas del suelo y del agua, que intervienen en los ciclos del carbono y del nitrógeno. En general son inocuas para el hombre

aunque también existen cepas patógenas oportunistas de animales y plantas. Debido a su gran versatilidad metabólica utilizan distintas fuentes de carbono, siendo algunas cepas de gran utilidad en biodegradación de xenobióticos (Gibson *et al.*, 1984; Ramos *et al.*, 1994, Timmis *et al.*, 1988; Parales & Haddock, 2004). La mayoría de las cepas codifica un buen número de mono y dioxigenasas que son de interés en procesos industriales de fabricación de compuestos de valor añadido (Hüsken *et al.*, 2001 y 2002; Rojas *et al.*, 2004; Neumann *et. al.*, 2005). Algunas cepas de *Pseudomonas* del suelo sintetizan productos tóxicos para otros microorganismos o quelan hierro con altísima afinidad siendo útiles en procesos de biocontrol (Shoda, 2000; Walsh *et al.*, 2001 and Kiely *et al.*, 2006).

1.2 Hábitat y versatilidad metabólica de *Pseudomonas*

Las bacterias del género *Pseudomonas* se encuentran ampliamente distribuídas en la naturaleza, particularmente en suelo, agua, alimentos y plantas enfermas. La ubicuidad de estas bacterias y la capacidad para crecer en medios de cultivo muy simples hicieron que se las consideraran protagonistas en el proceso de mineralización de materia orgánica en el medioambiente, un papel que fue claramente demostrado a principios del siglo pasado por den Dooren de Jong (1926).

Aunque hay más de 200 especies agrupadas en este género, sólo tres de ellas se conocen como patógenas para el hombre: *P. aeruginosa, P. mallei y P. pseudomallei.* Existen patógenos de plantas como *P. syringae*, y cepas que estimulan el crecimiento de plantas como *P. fluorescens* (Lugtenberg, 1999).

El uso de medios químicamente definidos con compuestos orgánicos simples ha revelado que una de las propiedades más notables de los miembros del género *Pseudomonas* es su versatilidad nutricional (den Dooren de Jong, 1926; Palleroni, 1986; Stainer *et al.*, 1966). Los compuestos orgánicos empleados como fuentes de carbono y energía por muchas especies de *Pseudomonas* incluyen hidrocarburos lineales y aromáticos, ácidos alifáticos, aminas, amidas, aminoácidos, alcoholes y compuestos aromáticos. Su potencial catabólico se ha visto corroborado por la extensa batería de rutas y enzimas catabólicos codificados por sus genomas (Jiménez *et al.*, 2002; Nelson *et al.*, 2002).

La reciente secuenciación del genoma completo de 6 miembros de este género: *P. aeruginosa* PAO1 (Stover *et al.*, 2000), *P. putida* KT2440 (Nelson *et al.*, 2002), *P. syringae* pv tomato DC3000 (Buell *et al.*, 2003), *P. syringae* pv. syringae B728a (Field *et al.*, 2005), *P. fluorescens* Pf-5 (Paulsen *et al.*, 2006) y *P. entomophila* (Vodovar *et al.*, 2006) ha abierto nuevos frentes para entender la singular capacidad adaptativa de este género oportunista,

con importantes repercusiones, tanto en el ámbito ecológico-agronómico como clínico. Destacan el gran tamaño de sus genomas (entre 6 y 7 x 10^6 pb), con unos 5500 genes por genoma, la mitad de los cuales no tienen función conocida, y su alta proporción de genes reguladores. En *P. putida* 450 fases de lectura abierta corresponden a genes cuya traducción rendiría reguladores transcripcionales, con 24 factores sigma, 19 de ellos del tipo ECF (*extracytoplasmic function*) y 55 sensores de sistemas de dos componentes (Nelson *et al.*, 2002; Martínez-Bueno *et al.*, 2002; Ventre *et al.*, 2004). También poseen un amplio repertorio de sistemas de transporte (más de 300) y porinas. Estas características reflejan la gran capacidad de integración de señales y de adaptación a los cambios medioambientales, así como la versatilidad metabólica de estas cepas, ambas necesarias para su singular ubicuidad medioambiental. Estas propiedades explícitamente descriptas para *P. putida* son extensibles a otras *Pseudomonas*.

2. HIDROCARBUROS AROMÁTICOS

2.1. Origen

Los hidrocarburos aromáticos policíclicos constituyen una familia de compuestos ampliamente distribuida en el medio ambiente. El interés en conocer sus niveles ambientales radica fundamentalmente en las propiedades carcinogénicas, mutagénicas y teratogénicas que poseen algunos de sus miembros (Internacional Agency for Research of Cancer (IARC), 1983) y es por lo que la Agencia Americana de la Protección del Medio Ambiente (EPA) y la Unión Europea los considera contaminantes de eliminación prioritaria.

En muchos suelos se han detectado hidrocarburos aromáticos monocíclicos como benceno, tolueno, etilbenceno y xilenos (Dagley, 1981). Estos últimos se encuentran entre los 50 productos sintetizados industrialmente en mayor volumen (millones de toneladas por año). Son ampliamente usados como combustibles y disolventes de uso industrial. También constituyen la materia prima para la producción de compuestos farmacéuticos, agroquímicos, polímeros, explosivos y muchos otros. Por esta razón no es sorprendente detectarlos en el medio ambiente.

El origen de estos hidrocarburos en el medio ambiente es muy diverso, aunque básicamente podemos diferenciar tres fuentes distintas:

Origen pirolítico, procedente de la combustión incompleta de la materia orgánica, reciente o fósil, bien por causas naturales (incendios de bosques, erupciones volcánicas, etc.) o antropogénicas (utilización de combustibles fósiles, incineración de residuos, emisiones de

vehículos, procesos industriales de gasificación y licuefacción del carbón, fraccionamiento del petróleo, etc.). Predominan los compuestos no alquilados sobre sus alquilhomólogos.

Origen petrogénico, producido por vertidos accidentales o intencionados de derivados del petróleo. Se caracterizan por ser mezclas complejas formadas por compuestos con cadenas alquílicas de hasta 5 ó 6 átomos de carbono, que predominan sobre sus homólogos no sustituidos.

Origen diagenético, de la materia orgánica sedimentaria, la cual puede sufrir una serie de procesos geoquímicos naturales, como son la descarboxilación, aromatización o desfuncionalización, para convertirse en hidrocarburos aromáticos policíclicos de origen natural.

2.2. Biodegradación por microorganismos

Un factor restrictivo en la biodegradación de hidrocarburos aromáticos, ya sea en zonas contaminadas con altas concentraciones o a escala industrial en reactores, es su alta toxicidad. Entre los hidrocarburos aromáticos más nocivos para los microorganismos se encuentran los disolventes orgánicos conocidos como BTEX (benceno, tolueno, estireno y xilenos), ya que se acumulan en la membrana citoplasmática perturbando su estructura y funciones vitales: se pierden metabolitos esenciales, iones, proteínas y lípidos, se disipa el gradiente de protones y se pierde el potencial eléctrico, lo que conduce a la lisis celular (De Smet *et al.*, 1978; Sikkema *et al.*, 1995).

La capacidad de las bacterias para utilizar hidrocarburos aromáticos fue demostrada por primera vez en 1908 por Stormer, quien aisló una cepa de *Bacillus hexacarbovorum* con capacidad de utilizar tolueno y xileno como fuente de carbono. La biodegradación de los hidrocarburos aromáticos puede considerarse como parte de un proceso normal del ciclo del carbono y la amplia distribución de microorganismos capaces de degradarlos es probablemente debida a que durante miles de años los microorganismos han estado expuestos a bajas concentraciones de estos compuestos provenientes de procesos naturales, tales como la combustión de maderas o erupciones volcánicas (Gibson y Subramanian, 1984; Ramos y Rojo, 1990).

En los últimos años se ha avanzado mucho en la comprensión de los mecanismos que utilizan los microorganismos para superar la energía de activación necesaria para romper el anillo aromático y generar estructuras carbonadas lineales, que son las únicas asimilables por los seres vivos. Se han descrito numerosas rutas de degradación de hidrocarburos aromáticos por microorganismos pertenecientes a diferentes géneros de bacterias tanto

gram-negativas como gram-positivas, que podrían ser utilizadas en el tratamiento biológico para eliminar compuestos nocivos (Ramos *et al.*, 1994).

3. MECANISMOS DE TOLERANCIA A DISOLVENTES ORGÁNICOS

La toxicidad de un disolvente se correlaciona con el logaritmo del coeficiente de partición del mismo en una mezcla definida de octanol-agua (log P_{ow}) (Sikkema *et al.*, 1995). Una de las funciones principales de la membrana celular de un microorganismo es la de formar una barrera de permeabilidad que regula el paso de solutos entre la célula y el medio que la rodea (Nikaido, 1996[a] y 1999), y es de importancia vital para los procesos de transducción de energía de la célula (Sikkema *et al.*, 1992). Los disolventes orgánicos con un log P_{ow} entre 4,0 y 1,5 aproximadamente, son extremadamente tóxicos para los microorganismos porque se acumulan en la membrana citoplasmática y alteran su estructura hasta provocar lisis celular (de Smet *et al.*, 1978; Sikkema *et al.*, 1992). La toxicidad de un disolvente no depende solamente de sus propiedades, sino también de la tolerancia intrínseca de las especies y cepas bacterianas.

El fenómeno de tolerancia de las bacterias a disolventes orgánicos tiene gran utilidad en aplicaciones biotecnológicas. En las últimas dos décadas la tolerancia bacteriana a disolventes orgánicos ha sido muy estudiada (Ramos *et al.*, 2002), y se han propuesto varios mecanismos para explicarla. Dentro de ellos destacan las modificaciones de los lípidos de la membrana celular que llevan a una mayor rigidez de la misma, inhibiendo en parte la entrada de los disolventes, y la expulsión activa del disolvente orgánico a través de un proceso dependiente de energía mediado por transportadores.

3.1. Modificaciones a nivel de membrana

Los lípidos de las membranas, tanto externa como interna, cumplen entre sus múltiples funciones la de barrera a la entrada al interior celular de compuestos perjudiciales para la célula. Sin embargo, los disolventes orgánicos atraviesan rápidamente la envoltura celular de las bacterias Gram-negativas, acumulándose en la membrana citoplasmática, lo que provoca un aumento de la fluidez de ésta. Esto genera en la bacteria una respuesta a corto plazo que incluye una inmediata isomerización de *cis* a *trans* de los ácidos grasos insaturados (Keweloh *et al.*, 1990; Heipieper *et al.*, 1992; Weber *et al.*, 1994; Pinkart *et al.*, 1996; Junker y Ramos, 1999), y un cambio de los grupos de cabeza de los fosfolípidos de membrana (Weber y de Bont, 1996). La respuesta a largo plazo consiste en el aumento del contenido de ácidos

grasos saturados en detrimento de los ácidos grasos insaturados y un aumento de la velocidad de síntesis de fosfolípidos (Pinkart y White, 1997).

Otros agentes de estrés como alcoholes, acidez, presencia de sales o metales pesados y cambios de temperatura, también generan estas modificaciones en la membrana, por lo cual no es una respuesta específica a la presencia de disolventes orgánicos (Hamamoto *et al.*, 1994; Suutari y Laakso, 1994; Heipieper *et al.*, 1996; Keweloh y Heipieper, 1996).

3.2. Expulsión activa del disolvente por bombas

Numerosos estudios han demostrado que la expulsión activa de disolventes orgánicos por bombas es el recurso más efectivo y concluyente en la tolerancia bacteriana a estos compuestos. Los primeros trabajos que pusieron de manifiesto esto fueron los de Isken y de Bont (1996) y Ramos y colaboradores (1997), cuando aislaron las cepas tolerantes a disolventes orgánicos *P. putida* S12 y DOT-T1E, respectivamente. Se identificaron nuevas bombas implicadas en la expulsión de disolventes tras el aislamiento de mutantes sensibles a tolueno en las cepas *P. putida* S12 (Kieboom *et al.*, 1998a), *P. putida* KT2442 (Fukumori *et al.*; 1998), *P. putida* DOT-T1E (Ramos *et al.*, 1998) y *P. putida* GM73 (Kim *et al.*, 1998).

Se demostró por primera vez que disolventes orgánicos y antibióticos podrían ser expulsados por un mismo transportador en *Escherichia coli* K12, ya que la bomba de resistencia múltiple a antibióticos (MDR, *Multiple Drug Resistance*) AcrAB estaba implicada en la tolerancia intrínseca de esta bacteria a disolventes orgánicos (White *et al.*, 1997; Aono, 1998). También Li y colaboradores (1998) pusieron de manifiesto que las bombas de tipo MDR, MexAB-OprM, MexCD-OprJ y MexEF-OprN de *P. aeruginosa* (*mex* de *mulpiple eflux*) eran capaces de expulsar disolventes orgánicos además de antibióticos.

Todas las bombas de expulsión de disolventes orgánicos identificadas hasta ahora en bacterias Gram-negativas pertenecen a la familia de transportadores RND (*Resistance, Nodulation, cell Division*) (Saier *et al.*, 1994; Tseng *et al.*, 1999).

3.2.1. Bombas MDR

Los transportadores MDR (*Multiple Drug Resistance*) expulsan compuestos orgánicos de diferente estructura química, lo cual hace que la célula posea resistencia múltiple a diferentes compuestos. El primer transportador MDR descrito fue la glicoproteína-P presente en células de mamíferos (Juliano & Ling, 1976), dicha bomba es dependiente de ATP y permite a la célula ser resistente a un amplio espectro de compuestos, incluyendo drogas anti-cancer utilizadas en quimioterapia (Ambudkar *et al.*, 1999). En años posteriores, numerosos estudios demostraron que los sistemas MDR están ampliamente distribuidos en bacterias y que

pueden conferir resistencia a antibióticos, disolventes orgánicos, biocidas, metabolitos antimicrobianos de plantas, detergentes o metales pesados (Lewis, 1994; Nikaido, 1994 y 2001; Paulsen *et al*, 1996b; van Veen y Konings, 1997; Levy, 2002; Poole, 2001 y 2003; Ramos *et al.*, 2002). La aparición de resistencia cruzada es el producto del uso inmoderado de antibióticos en terapia clínica, antisépticos y detergentes en productos domésticos de uso habitual, herbicidas en producción agropecuaria o el vertido de residuos industriales tóxicos en ríos y suelos, lo que contribuye a crear una presión selectiva de cepas multiresistentes (Witte 2000 a y b; Levy, 2001; Russell, 2002 a y b).

Las bombas MDR bacterianas se clasifican en cinco familias (Figura 1): (i) la superfamilia MFS (*Major Facilitator Superfamily*) (Pao *et al.*, 1998, Saier *et al.*, 1999), cuyos miembros QacA en *S. aureus* y Bmr en *Bacillus subtilis* (Brown y Skurray, 2001; Neyfakh, 1992) son los mejor caracterizados; (ii) la familia SMR (*Small Multidrug Resistance*) (Paulsen *et al.*, 1996a), cuyos miembros constituyen los transportadores más pequeños existentes (~100 aminoácidos) como EmrE en *E. coli* y Smr en *S. aureus* (Schuldiner *et al.*, 2001; Grinius *et al.*, 1992); (iii) la familia MATE (*Multidrug And Toxic compound Extrusion*) (Brown *et al.*, 1999), representada por el transportador NorM de *Vibrio parahaemolyticus* (Morita *et al.*, 2000); (iv) la superfamilia RND (*Resistance, Nodulation, cell Division*) (Tseng *et al.*, 1999), cuyos miembros AcrAB en *E. coli* y MexAB en *P. aeruginosa* son los transportadores MDR mejor caracterizados genética, bioquímica y estructuralmente y (v) la superfamilia ABC (*ATP Binding Cassette*) (Saurin *et al.*, 1999; van Veen *et al.*, 2001).

Estas bombas funcionan como transportadores secundarios, acoplando la expulsión del sustrato a la entrada de un protón o catión (Na^+), con excepción de la familia ABC, que utiliza la hidrólisis del ATP como fuente de energía para el transporte (Figura 1). Algunos miembros de las familias ABC, MFS y RND requieren para su funcionamiento proteínas accesorias pertenecientes a las familias de proteínas de fusión de membrana (*Membrane Fusion Protein*, MFP; Dinh *et al.*, 1994), y de factores de membrana externa (*Outer Membrane Factor*, OMF; Paulsen *et al.*, 1997).

Los miembros de la familia RND son los más relevantes desde el punto de vista clínico en bacterias Gram-negativas (Poole, 2001), ya que poseen la especificidad de sustrato más extensa. Por otro lado, son los únicos transportadores implicados en tolerancia a disolventes orgánicos (Ramos *et al.*, 2002). En *P. aeruginosa* la resistencia a múltiples antibióticos es el resultado de una sinergia entre la impermeabilidad de la membrana externa y la acción de hasta siete bombas de la familia RND codificados en su cromosoma: MexAB-OprM, MexCD-

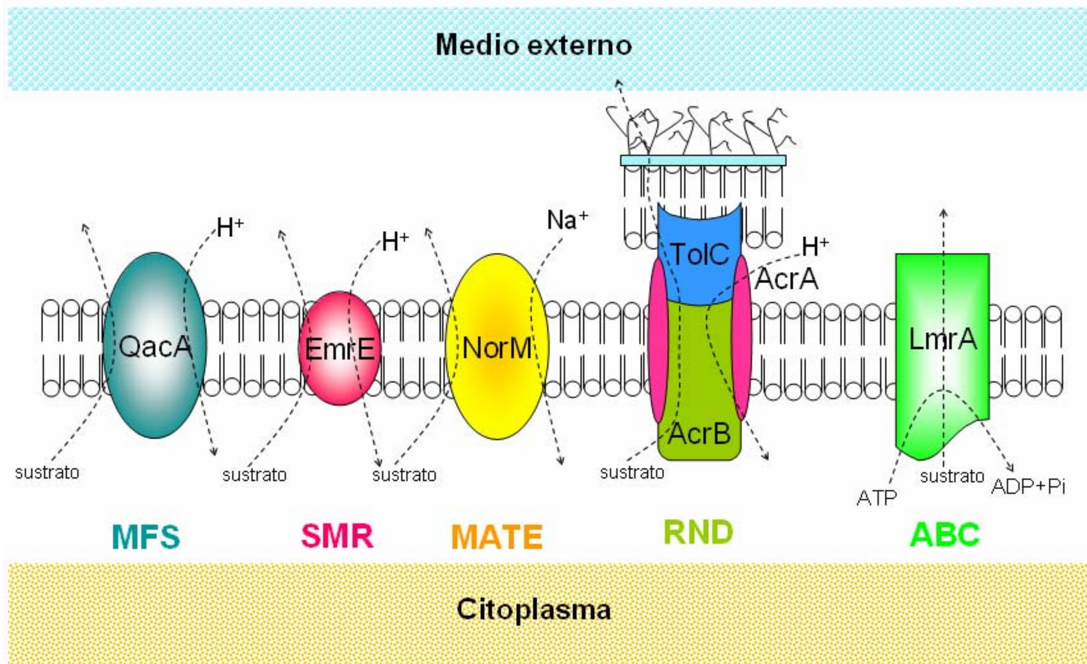

Figura 1. Miembros representativos de las cinco familias caracterizadas de transportadores MDR. QacA, de *S. aureus*; EmrR, de *E. coli*; NorM, de *V. parahaemolyticus*; AcrAB-TolC, de *E. coli* y LmrA de *Lactococcus lactis*. Los esquemas de los transportadores que se presentan son semejantes a los mostrados por Paulsen (2003).

OprJ, MexEF-OprN, MexXY-OprM y MexJK-OprM (ver Figura 4) (Germ *et al.*, 1999; Li *et al.*, 2000a; Poole, 2001; Poole & Srikumar, 2001). La bomba de extrusión MexGHI-OpmD proporciona resistencia a vanadio, y está relacionada con procesos dependientes de *quorum sensing* (Aendekerk *et al.*, 2002). MexVW-OprM (Li *et al.*, 2003) participa en el transporte de fluoroquinolonas y otros compuestos. Cabe destacar la presencia de un elemento extra (MexG) en el sistema MexGHI-OpmD, aunque éste no presenta homología con ninguna proteína descrita hasta el momento y no parece ser necesaria para el funcionamiento de la bomba (Aendekerk *et al.*, 2002). Estas bombas se caracterizan por tener un amplio perfil de sustrato (con la excepción de MexGHI-OpmD), siendo capaces de expulsar no sólo antibióticos de distinta estructura, sino también disolventes orgánicos, colorantes, detergentes, inhibidores metabólicos y energéticos, homoserín lactonas y posiblemente, factores de virulencia (Zhang *et al.*, 2001).

También se han identificado bombas de la familia RND en bacterias patógenas de humanos y oportunistas como *E. coli*, *Neisseria* spp., *Klebsiella pneumoniae, Haemophilus influenzae, Salmonella* spp, *Stenotrophomonas maltophilia*, *Serratia marcescens*, *Campylobacter jejuni*, *Vibrio cholerae* o *Burkholderia* spp.; en patógenas y simbiontes de plantas como *Agrobacterium tumefaciens, Erwinia amylovora*, *Bradyrhizobium japonicum* y

Rhizobium spp; y en bacterias del suelo inocuas como *P. putida* y *P. fluorescens* (Poole, 2001 y 2003).

3.2.1.1. El transportador RND

Las estructuras tridimensionales de los tres componentes de la bomba RND se han desvelado en los últimos años, éstos son: las proteínas de membrana externa TolC (Koronakis *et al.*, 2000) y VceC (Federici *et al.*, 2005), el transportador interno AcrB (Murakami *et al.*, 2002; Pos y Diederichs, 2002) y la proteína de fusión periplásmica MexA (Akama *et al.*, 2004; Higgins *et al.*, 2004). Mediante el ensamblaje de las estructuras de las tres unidades de la bomba tripartita se ha podido conocer más sobre el reconocimiento de los sustratos y el funcionamiento alostérico de la bomba (Eswaran *et al.*, 2004). Existen varias hipótesis sobre la vía de expulsión del sustrato al medio extracelular, pudiendo ser translocado desde el citoplasma, la membrana interna o el periplasma (Elkins y Nikaido, 2003) (Figura 2).

La combinación de las tres unidades permite la creación de un canal a través del periplasma de forma tal que el compuesto tóxico puede ser expulsado atravesando directamente las dos membranas: el transportador interno se ubica en la membrana citoplasmática y se acopla a una proteína de membrana externa (OMF), la cual se expande dentro del espacio periplásmico creando así un canal continuo entre ambas proteínas. Una proteína de fusión periplásmica (MFP) permite el acoplamiento físico y funcional entre el transportador interno y la proteína de membrana externa (Figura 2).

4. REGULACIÓN DE TRANSPORTADORES MDR EN BACTERIAS

Es necesario un control de la expresión de proteínas integrales de membrana que utilizan la fuerza protón motriz ya que su producción excesiva reduce la velocidad de crecimiento de la cepa. De hecho, la expresión constitutiva de los transportadores TetA(B) y TetA(C), que expulsan tetraciclina, es perjudicial para *E. coli*. En ensayos realizados en ausencia de tetraciclina (efector que al unirse al represor TetR permite la expresión del gen *tetA* y *tetR*) estos transportadores dejan a las células en una severa desventaja cuando compiten con cepas que no expresan los sistemas TetA (B) y TetA (C) constitutivamente (Lee & Edwin, 1985; Nguyen *et al*, 1989). Con excepción de los transportadores pertenecientes a la familia SMR, cuya expresión parece ser constitutiva, la expresión de la mayoría de las bombas MDR tiene algún tipo de regulación. La expresión de genes MDR está generalmente controlada a nivel transcripcional aunque existen algunos casos de regulación traduccional. Por ejemplo,

se ha propuesto que la modulación de la síntesis del determinante de resistencia a tetraciclina de Gram-positivas TetA(K) es a través de la atenuación traduccional (Speer *et al.*, 1992) mientras que la reiniciación traduccional regularía la síntesis de la proteína de resistencia a tetraciclina de *B. subtilis* TetA(L) (Stasinopoulos *et al.*, 1998).

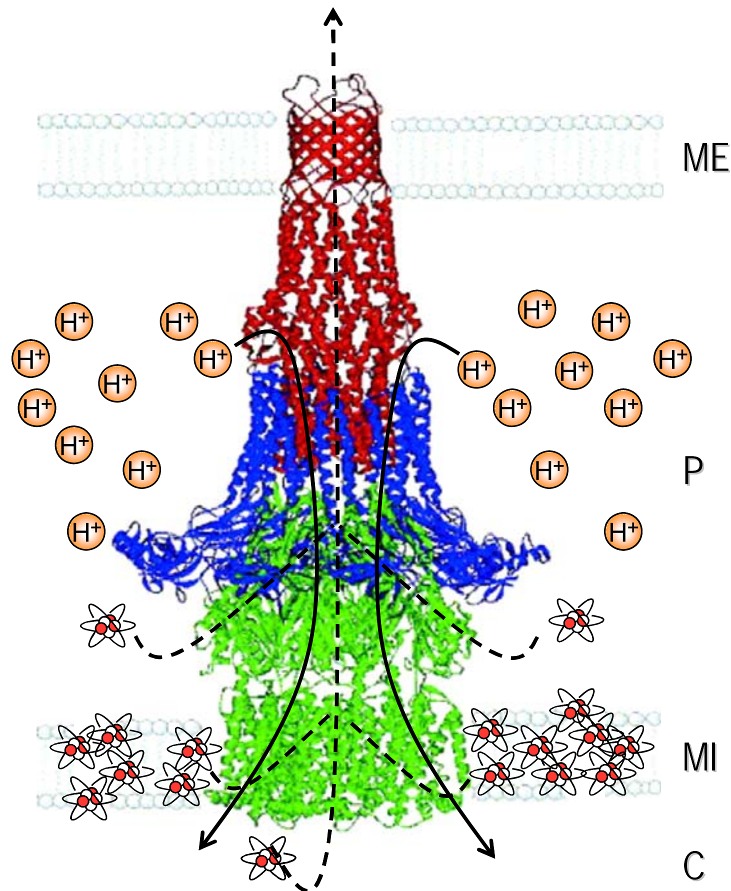

Figura 2. Modelo de la disposición de las tres unidades de una bomba RND. El esquema se basa en las estructuras tridimensionales del tranportador AcrB (Murakami *et al.*, 2002), la porina TolC de *E. coli* (Koronakis *et al.*, 2000) y la proteína de fusión MexA de *P. aeruginosa* (Akama *et al.*, 2004; Higgins *et al.*, 2004). La bomba la forman tres componentes estructurales: el transportador de membrana interna (verde), formado por un trímero que atraviesa la membrana interna con 12 fragmentos transmembrana por monómero y se expande en el periplasma, hasta contactar con el canal de membrana externa (OMF, en rojo). Éste, posiblemente trimérico, atraviesa el espacio periplásmico desde la membrana externa. La proteína de fusión de membrana (MFP, en azul) aparentemente formando un anillo de 9 monómeros estabilizaría los otros dos componentes. Los sustratos, representados como moléculas rojas y blancas, se acumulan principalmente en la membrana interna por sus características hidrofóbicas y polares (anfifílicas). Con flechas punteadas se indican las posibles vías de expulsión del sustrato. El gradiente de protones, que proviene de un sistema antiporte sustrato/H^+ es el generador de la energía necesaria para la translocación. El esquema que se presenta fue propuesto por Eswaran *et al.* (2004). C, citosol; MI, membrana interna; P, periplasma; ME, membrana externa.

La búsqueda y conocimiento de los mecanismos que regulan los transportadores MDR permiten entender las funciones celulares y fisiológicas de éstos y ofrecen una nueva diana

de acción para la lucha contra microorganismos multi-resistentes presentes en las infecciones nosocomiales. Además, la particularidad de que algunos reguladores puedan reconocer múltiples compuestos, ofrece soluciones a la dificultad de purificar y cristalizar proteínas de membrana con estás características, permitiendo así un estudio más factible del fenómeno de promiscuidad de reconocimiento de sustratos.

4.1. Regulación local

El gen regulador local de las bombas MDR normalmente se sitúa de manera adyacente al gen (o genes) del transportador MDR. Este regulador transcripcional, ya sea represor o activador, actúa de manera específica controlando directamente la expresión del transportador en cuestión. En la actualidad, entre los reguladores más ampliamente caracterizados se encuentran el represor TetR, que controla la expresión del gen *tetA* que codifica el transportador de tetraciclinas en *E. coli* (Altenbuchner *et al.*, 1983); el represor QacR de *S. aureus*, regulador local del gen *qacA*, que codifica el transportador implicado en la extrusión de aminas cuaternarias y otros cationes lipofílicos (Grkovic *et al.*, 1998), y el regulador BmrR, que estimula la expresión del gen *bmr* en *B. subtilis,* cuyo producto génico está implicado en la extrusión de cationes lipofílicos (Ahmed *et al.*, 1994); Los reguladores TetR, BmrR y QacR se han cristalizado, tanto en su forma libre como unida a sus efectores o a su ADN operador, lo que los hace proteínas modelos en el estudio de la regulación transcripcional (Hinrichs *et al.*, 1994; Hillen y Berens, 1994; Kisker *et al.*, 1995; Orth *et al.*, 1998 y 2000; Zheleznova *et al.*, 1997 y 1999; Heldwein y Brennan, 2001; Schumacher *et al.*, 2001 y 2002, Murray *et al.*, 2004). A continuación se hace una descripción más detallada de estos reguladores, que revelan la diversidad y diferentes grados de regulación que existen en el mecanismo de control de la expresión de las bombas MDR.

4.1.1. Los represores QacR de *S. aureus* y TetR de *E. coli*

El represor QacR junto con TetR son dos reguladores de la familia de represores TetR (Aramaki *et al.*, 1995b; Ramos *et al.*, 2005). Estos reguladores se encuentran entre los mejores caracterizados, con un mecanismo global de represión e inducción muy similar. Estos represores se unen a sus operadores que se localizan entre las regiones -10 y -35 del promotor que regulan. La inducción de los genes ocurre tras la unión del efector provocando un cambio conformacional que causa la disociación del represor del operador y permite el acceso de la ARN-polimerasa para transcribir. Sin embargo, existen diferencias importantes entre ambos reguladores, tanto en la unión al operador, como en la unión a los efectores: se necesitan dos dímeros de QacR, unidos de forma cooperativa y solapante a una secuencia

palindrómica de 28 pb para reprimir la transcripción de *qacA*, mientras que TetR se une como un único dímero a un operador de 18 pb (Figura 3C y 3D). Además, QacR no reprime totalmente, a diferencia de TetR, lo cual permite una cierta expresión basal del transportador QacA, y tampoco existe autorregulación negativa por parte de QacR (Grkovic *et al.*, 1998 y 2001). Otra diferencia radica en la capacidad de QacR para unirse a múltiples agentes antimicrobianos de distinta estructura con una estequiometría de unión de un efector por dímero. En el caso de TetR una molécula de su efector (tetraciclina-Mg^{2+}) se une a cada monómero de TetR. Los efectores de QacR incluyen cationes hidrofóbicos sintéticos (decualinio, bromuro de etidio, rodamina, entre otros) o naturales (berberina), todos sustratos del transportador QacA. Esta capacidad de reconocimiento múltiple es posible gracias a la presencia de un voluminoso bolsillo de unión a efectores, compuesto por dos subsitios distintos pero solapantes, en los que cuatro glutamatos y numerosos residuos aromáticos e hidrofóbicos permiten distintas combinaciones de interacción con las cargas positivas y los anillos aromáticos de los efectores. Una vez acomodado en el bolsillo, el efector es estabilizado por enlaces de hidrógeno en los que intervienen algunos residuos polares del bolsillo (Schumacher *et al.*, 2001; Murray *et al.*, 2004).

4.1.2. El activador BmrR de *B. subtilis*

La información dada por la estructura tridimensional de BmrR unido a distintos efectores fue la primera gran contribución al entendimiento de los principios de reconocimiento de múltiples ligandos a una misma proteína (Zheleznova *et al.*, 1999). BmrR es un activador homodimérico, miembro de la familia de reguladores MerR, capaz de reconocer, al igual que QacR, diferentes cationes hidrofóbicos con distintas estructuras. Cuando se une el efector a BmrR (dos por dímero), el complejo activador se une a una secuencia palindrómica imperfecta de 22 pb ubicada entre las regiones -10 y -35 del promotor de *bmr*. En ausencia del activador, estas regiones están separadas por 19 pb, lo que provoca que estén situadas en lados opuestos de la doble hélice de ADN, una configuración incompatible con la unión de la ARN polimerasa. La obtención de la estructura cristalina del complejo tripartito activador-efector-operador (Figura 3B), ha mostrado el mecanismo íntimo de activación de los reguladores de esta familia (Heldwein y Brennan, 2001): la unión del complejo BmrR-efector al operador genera una distorsión elemental en el ADN que, acompañada del desapareamiento de dos pares de bases (AT), lleva a una reducción de la distancia entre las cajas -10 y -35 de modo que ambos hexámeros quedan disponibles para la unión de la ARN polimerasa y la subsiguiente transcripción de los genes estructurales de la bomba MDR.

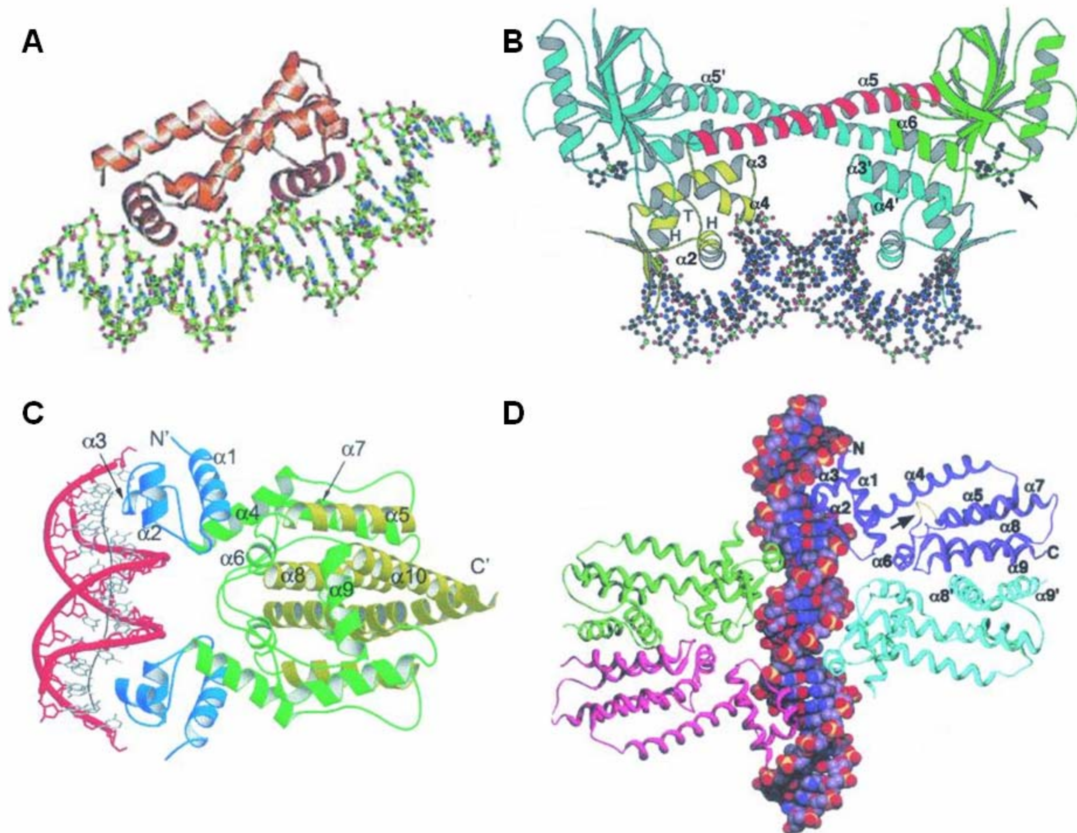

Figura 3. Estructuras de los reguladores MarA (A), BmrR (B), TetR (C) y QacR (D) unidos a su ADN-operador. A) MarA unida al promotor *marRAB*, se observa la curvatura del ADN que facilita el posicionamiento de las dos HTH del monómero (Kwon *et al.*, 2000). B) Estructura tripartita del complejo formado por el dímero BmrR, su operador y el ligando tetrafenilantimonio, el cual se señala con una flecha (Zheleznova *et al.*, 2001). C) Estructura del complejo ADN-TetR, con una línea gris se perfila la curvatura del operador inducida por dímero TetR (Orth *et al.*, 2000). D) Estructura del complejo ADN-QacR. Se observa la unión de dos dímeros de QacR al operador simétrico OR1. Con una flecha se indica el cambio que se genera en la hélice 5 tras la unión del ligando (Schumacher *et al.*, 2002). Se indican con números las α-hélices que se localizan en la estructuras de las proteínas. C y N muestran el extremo C-terminal y N-terminal respectivamente del polipéptido. HTH indica el motivo hélice-giro-hélice de reconocimiento del ADN.

Los reguladores TetR, QacR y BmrR son las proteínas reguladoras locales más estudiadas en la regulación de la expresión de los transportadores MDR. Aunque también se han identificado muchos otros reguladores específicos de bombas MDR que están siendo estudiados en la actualidad. Hasta la fecha, los reguladores que se han descrito en la modulación de sistemas MDR pertenecen a las familias de reguladores transcripcionales TetR, MarR, IclR, LysR y LacI/GalR.

Muchos represores han sido identificados por la aparición de fenotipos con alta resistencia, sobre todo en bacterias patógenas. Dichos fenotipos eran el resultado de

mutaciones espontáneas que inactivaban el gen represor, afectaban los promotores reconocidos por éste o las secuencias de transposición que los inactivaban, lo cual daba lugar a una sobreexpresión de los genes de las bombas reguladas por el represor, y un consecuente aumento de la resistencia. Entre los casos con fenotipos de alta resistencia se encuentran las bombas Acr y Emr de *E. coli* (Lomovskaya *et al.*, 1995; Jellen-Ritter y Kern, 2001; Wang *et al.*, 2001); los sistemas MexAB, MexCD, MexJK, y MexXY de *P. aeruginosa* (Okazaki y Hirai, 1992; Poole *et al.*, 1996 a y b; Ziha-Zarifi *et al.*, 1999; Adewoye *et al.*, 2002; Beinlich *et al.*, 2001; Chuanchuen *et al.*, 2002); el transportador MtrCDE de *N. gonorrhoeae* (Shafer *et al.*, 1995; Lucas *et al.*, 1997); la bomba SrpABC de *P. putida* S12 implicada en la resistencia disolventes orgánicos (Wery *et al.*, 2001) o SmeDEF de *S. maltophilia* (Alonso y Martínez, 2000 y 2001; Sánchez *et al.*, 2002a y 2004). La secuenciación de los genomas bacterianos ha permitido otra forma de identificación de estos represores, la cual es mediante el uso de mutagénesis dirigida de los genes adyacentes a los operones MDR. Es el caso, por ejemplo, de los represores de las bombas de expulsión de disolventes orgánicos TtgABC, TtgDEF y TtgGHI de *P. putida* DOT-T1E (Duque *et al.*, 2001; Ramos *et al.*, 2002; Rojas *et al.*, 2003).

4.1.3. Reguladores locales y su implicación en la regulación de otros sistemas

Existen algunos casos de regulación cruzada entre los reguladores locales de las bombas MDR. El represor MtrR de *N. gonorrhoeae* es el regulador local de los genes *mtrCDE*, sin embargo, activa también la transcripción del operón de genes de resistencia *farAB* indirectamente, ya que reprime la expresión del regulador negativo local de dicho operón, la proteína FarR (Lee *et al.*, 2003). Además, MtrR es el responsable de forma directa o indirecta del control de la expresión de 14 genes relacionados con el establecimiento y/o mantenencia de la infección en *N. gonorrhoeae* (Shafer *et al.*, 2001). En *P. aeruginosa* el regulador MexT no sólo activa la expresión del operón *mexEF-oprN,* sino que regula negativamente la expresión de la porina OprD (Köhler *et al.*, 1999). Posiblemente el gen *mexS* (de supresor) impediría la sobreexpresión de MexT y, en consecuencia, la de los genes de la bomba (Maseda *et al.*, 2000). En *B. subtilis* el activador BltR activa la expresión del gen de resistencia *blt* adyacente uniéndose a su región promotora (Ahmed *et al.*, 1995). BltR es homólogo al extensamente caracterizado BmrR de la misma bacteria, sin embargo, no se han logrado identificar sus efectores. Por otro lado, recientemente se ha confirmado que los sistemas de extrusión de disolventes orgánicos TtgGHI y TtgDEF de la bacteria *P. putida*

DOT-T1E son co-regulados por sus reguladores locales, TtgV y TtgT respectivamente (Segura *et al.*, 2003; Terán *et al.*, 2007).

4.1.4. Sistemas de dos componentes que regulan bombas MDR

Debido a la sensibilidad y especificidad de respuesta que presentan los sistemas de dos componentes para responder a una señal externa, estos sistemas también se han encontrado en la regulación de la expresión de los genes de los transportadores MDR. En *E. coli*, se han descrito dos sistemas: BaeS-BaeR y EvgS-EvgA. El regulador de respuesta BaeR activa la expresión del operón *mdtABC*, que confiere resistencia a sales biliares, novobiocina y deoxicolato (Baranova *et al.*, 2002; Nagabuko *et al.*, 2002). MdtB y MdtC son transportadores RND, los cuales comparten la proteína de fusión MdtA, mientras que MdtD es una bomba de tipo MFS, que no participa en la resistencia y se desconoce su papel. Las proteínas EvgS-EvgA también regulan dos operones de bombas pertenecientes a familias diferentes: *emrKY* (familia MFS) e *yhiUV* (familia RND), ambos transportadores utilizan la proteína de membrana externa TolC (Nishino y Yamagoshi, 2001 y 2002). El sistema EvgS-EvgA está involucrado en la inducción de hasta 37 genes en *E. coli*, la mayoría de los cuales participan en la resistencia a condiciones de acidez (Masuda y Church, 2002).

También se han caracterizado sistemas de dos componentes que regulan la expresión de transportadores MDR en otras bacterias, entre ellos se encuentran el sistema SmeS-SmeR de *S. maltophilia* que controla el operón *smeABC* (Li *et al.*, 2002); el sistema AdeS-AdeR de *Acinetobacter baumannii* que regula los genes *adeABC* (Marchand *et al.*, 2004) y el sistema ArlS-ArlR de *S. aureus* que controla la expresión del transportador NorA (Fournier *et al.*, 2000).

4.2. Regulación global

4.2.1. Control del operón *acrAB* de *E. coli* por activadores locales y globales

El operón *acrAB* y el gen *tolC* de *E. coli* se inducen por distintos tipos de estrés (osmótico, etanol o la entrada en fase estacionaria) de forma independiente del regulador local AcrR (Ma *et al.* 1995 y 1996). Dicho operón codifica el transportador AcrB y la proteína de fusión periplásmica AcrA. Por otro lado, el gen *tolC* codifica la proteína de membrana externa que se expande por el periplasma, constituyendo así la bomba tripartita funcional RND (Ma *et al.* 1995). Estudios en cepas que expresaban constitutivamente los reguladores globales MarA, SoxS y Rob (Ma *et al.*, 1995; Okusu *et al.*, 1996; Miller y Sulavik, 1996; Tanaka *et al.*, 1997; White *et al.*, 1997; Alekshun y Levy, 1999) mostraron que el operón *acrAB* y el gen *tolC* estaban controlados por estos reguladores globales y eran los responsables de la resistencia

a múltiples antibióticos (fenotipo *mar*, *multiple antibiotic resistance*), biocidas y resistencia a disolventes orgánicos de la enterobacteria. A su vez, el gen del activador global MarA está controlado localmente por el represor MarR que forma parte del operón *marRAB* (Cohen *et al.*, 1993) y modulado por el regulador accesorio Fis (Martin y Rosner, 1995). Otro mecanismo por el cual se interrumpen las actividades de MarR es por la unión de la proteína de unión a periplasma MppA a tripéptidos de mureína. El nivel bajo de tripéptidos de mureína en el periplasma podría ser un indicador de estrés, lo que genera la activación de un mecanismo de transducción de señal que terminaría en la fosforilación de MarR, lo cual lo dejaría inactivo (Li y Park, 1999). MarR es capaz de inducir la expresión de *marA* y, por tanto, de los genes *acrAB* y *tolC,* al unirse a diversos compuestos antimicrobianos (Alekshun y Levy, 1999). Fis es otro regulador global que modula la actividad transcripcional de promotores en función de las condiciones de crecimiento (Travers *et al.*, 2001). También se ha demostrado que AcrAB está regulada positivamente por SdiA, una proteína asociada a *quorum sensing* cuya expresión depende de la fase de crecimiento (Rand *et al.*, 2002), y que regula genes involucrados en la división celular (Rahmati *et al.*, 2002), sugiriendo un probable papel de esta bomba en la extrusión de señales de *quorum sensing.*

Por lo tanto, la modulación de la expresión de la bomba AcrAB-TolC de *E. coli*, parece ser el producto de una red compleja de señales y reguladores, en la cual la función primaria de AcrR es la de evitar una producción excesiva de la bomba, mientras que MarR y los reguladores globales MarA, Rob, SoxS, SdiA y Fis son los principales responsables de la inducción de los genes *acrAB* y *tolC.*

4.2.1.1. El activador global MarA

MarA, miembro de la familia AraC de activadores transcripcionales, activa su propia expresión y la de un gran número de genes del regulón *mar* por unión a secuencias de 20 pb (cajas "mar") que se localizan en la vecindad de los promotores de sus genes diana.

La activación transcripcional de MarA tiene varias características inusuales: se une al ADN como monómero, y los sitios de unión (cajas "mar") que reconoce en el ADN son asimétricos, sin ninguna secuencia repetida directa o indirecta propia de las secuencias reguladoras bacterianas (Alekshun *et al.*, 1997). La estructura tridimensional del complejo MarA-ADN reveló que la proteína posee dos HTH diferentes unidos por una α-hélice muy larga, otra disposición muy atípica en un regulador procariótico (Rhee *et al.*, 1998). En este activador, la presencia de dos HTH en una sola cadena polipeptídica explica el por qué de su funcionamiento como monómero. Cuando MarA se une a su operador el ADN es curvado

aproximadamente 35º, de esta forma, se facilitan los contactos simultáneos de dos surcos mayores sucesivos con las hélices de reconocimiento de sus dos HTH, ver Figura 3A (Rhee *et al.*, 1998).

Otro rasgo interesante de MarA es que su modo primario de activación involucra la formación de un complejo entre el activador y la ARN polimerasa, dicho complejo puede buscar más eficientemente en el cromosoma bacteriano promotores del regulón *mar* que la ARN polimerasa o MarA individualmente (Martin *et al.*, 2002). De esta forma, el complejo MarA-ARN polimerasa tiene la habilidad de identificar selectivamente cajas mar reales y distinguirlas de otras parecidas ubicadas en la proximidad de los promotores del regulón *mar* (Martin *et al.*, 2002).

4.2.1.2. El represor EmrR

El transportador EmrAB de *E. coli*, que confiere resistencia a múltiples agentes antimicrobianos a la bacteria, no está regulado por MarA ni por MarR. Este hecho es inesperado, ya que aunque EmrAB es un transportador de la familia MFS, también parece formar un complejo tripartito con la porina de membrana externa TolC (la cual está regulada por MarA y MarR, como ya se mencionó), de manera análoga a los transportadores RND (Lewis K., 2000). La inducción de la expresión del operón *emrRAB* está controlada por el represor EmrR, el cual es el producto del primer gen del operón.

EmrR es un miembro de la familia de represores MarA y se ha demostrado que es capaz de reprimir el promotor *marRAB* cuando se sobreexpresa (Sulavik *et al.*, 1995). Este represor actúa uniéndose a una secuencia repetida invertida imperfecta centrada alrededor de la región -10 del promotor *emrRAB*. Dicha interacción es interrumpida por la unión de ligandos al complejo EmrR-P_{emrRAB}, entre otros el 2,4-nitrofenol y el ácido nalidíxico, con una estequiometría de unión de un ligando por dímero de proteína reguladora (Brooun *et al.*, 1999). Recientemente se ha cristalizado una proteína homóloga de EmrR, la proteína StEmrR de la arqueabacteria termófila *Sulfolobus tokodaii* cepa 7 (Miyazono *et al.*, 2007). La estructura tridimensional puso de manifiesto que a pesar de la relativamente baja similitud estructural general entre StEmrR y proteínas miembros de la familia MarR, la estructura del dominio de unión al ADN está muy conservada (Miyazono *et al.*, 2007).

4.2.2. Regulación local y coordinada de las bombas *mex* de P. aeruginosa

En *P. aeruginosa* varios indicios sugieren que las distintas bombas de extrusión se encuentran reguladas a nivel transcripcional por reguladores locales y reguladores globales que controlan la expresión de sus numerosas bombas MDR (ver Figura 4). MexR, miembro

de la familia de proteínas MarR, actúa como represor de la transcripción de *mexAB-oprM* y autorregula su expresión mediante la represión del promotor de *mexR* (P_{mexR}). (Evans *et al.*, 2001; Sánchez *et al.*, 2002b). La inducción de la expresión de la bomba MexAB-OprM en fase estacionaria (Evans *et al.*, 2001; Sanchéz *et al.*, 2002b), así como su implicación en el transporte de moléculas señal de *quorum sensing* (Evans *et al.*, 1998; Pearson *et al.*, 1999), y más recientemente, en el transporte de moléculas asociadas a virulencia (Hirakata *et al.*, 2002), son elementos a favor de la hipótesis de control por reguladores globales y de un posible papel fisiológico de este transportador. El aislamiento de mutantes *nalC* que sobreexpresan los genes *mexAB*, pero que no están afectados ni en el correspondiente regulador MexR, ni en las regiones promotoras correspondientes, han permitido identificar que el producto del gen PA3721 es un regulador que actúa como represor. PA3721 pertenece a la familia TetR. Este regulador reprimiría la expresión de un operón de dos genes (PA3720-PA3719), cuyo producto (PA3719), una pequeña proteína de 53 aminoácidos, sería la responsable de este fenotipo *nalC* (Cao *et al.*, 2004).

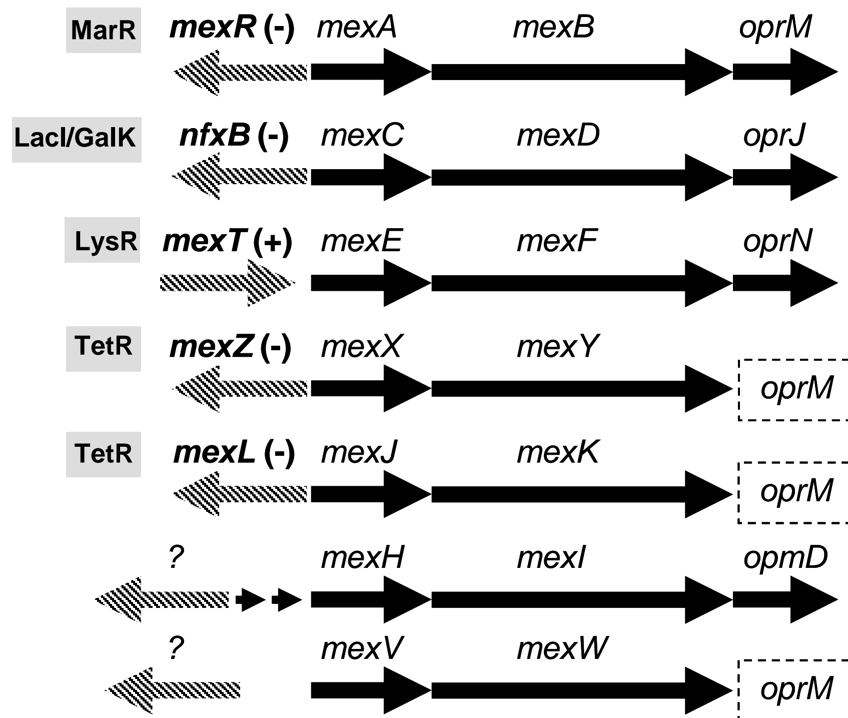

Figura 4. Organización génica de las bombas Mex y sus reguladores. Los genes estructurales de las bombas de extrusión se muestran en flechas negras, los genes reguladores en flechas rayadas perpendicularmente, la función de los mismos se indica con (-) si es represor y (+) si es activador. En cajas sombreadas se señala la familia a la que pertenece el regulador de cada bomba. El símbolo de interrogación indica que aún se desconoce el regulador local de dichos genes estructurales. En cajas

punteadas se muestra la proteína de membrana externa (OprM) que completa los sistemas tripartitos Mex XY, Mex JK y Mex VW. Adaptado y actualizado de Rojas A., Tesis Doctoral, 2004.

El gen *nfxB* se localiza adyacente a los genes *mexCD-oprJ* y codifica un represor de la familia LacI-GalR (Nguyen y Saier, 1995) que reprime la expresión de la bomba (Poole *et al.*, 1996a) y se autorregula negativamente (Shiba *et al.*, 1995). Por otro lado, MexT es un regulador positivo de *mexEF-oprN*, que pertenece a la familia LysR (Schell, 1993), y a su vez es también el represor responsable de la disminución de la expresión de la porina OprD en las cepas *nfxC* (Köhler *et al.*, 1999a; Ochs *et al.*, 1999).

Westbrock-Wadman *et al.* (1999) identificaron el gen *mexZ* aguas arriba de *mexXY*, el cual codifica un represor de la familia TetR, y controla la expresión de *mexXY* (Aires *et al.*, 1999). Las mutaciones en *mexZ* han demostrado ser suficientes para producir la hiperexpresión de los genes *mexXY* adyacentes, pero no para explicar el aumento de la resistencia a aminoglicósidos observado, quizá porque se están omitiendo otros componentes reguladores necesarios. Por ello, la resistencia a aminoglicósidos dependiente de la hiperexpresión de MexXY en los mutantes llamados "de tipo impermeable" es debida a mutaciones en otros genes diferentes de MexZ. Por otro lado, Llanes *et al.* (2004) encontraron tres cepas (llamados mutantes *agrW*) que hiperexpresaban MexXY sin mutaciones en su gen regulador *mexZ* y otras cuatro cepas que hiperproducían la bomba MexAB-OprM con los genes *mexR* y PA3721 (mutantes *nalD*) intactos, lo cual sugiere la existencia de reguladores adicionales.

A pesar de que cada bomba de extrusión dispone de un regulador local, algo muy interesante es que los sistemas de bombas Mex de *P. aeruginosa* están sujetos a una regulación coordinada, como lo demuestra el que la expresión de unas bombas disminuya en respuesta a un incremento de expresión de otras (Li *et al.*, 2000 b).

4.2.3. El regulador global Mta de *B. subtilis*

El cromosoma de *B. subtilis* codifica dos transportadores MDR de la familia MFS muy similares, Bmr y Blt. Estas proteínas tienen una identidad de secuencia de aminoácidos del 51% y confieren resistencia a un espectro idéntico de sustratos tóxicos cuando se las sobreexpresa (Ahmed *et al.*, 1995; Neyfakh *et al.*, 1991). Los transportadores Bmr y Blt están regulados por el producto del gen adyacente a los genes *bmr* y *blt*, que codifican los activadores transcripcionales BmrR (Ahmed *et al.*, 1994) y BltR (Ahmed *et al.*, 1995) respectivamente, ambos miembros de la familia de reguladores MerR. Sin embargo, los sustratos del transportador Blt no son ligandos del activador BltR, ni se conoce hasta la fecha cuáles lo son (Ahmed *et al.*, 1995).

Además de los activadores locales BmrR y BltR, los genes *bmr* y *blt* son modulados por el activador global Mta, que pertenece también a la familia MerR (Baranova *et al.*, 1999). El dominio N-terminal de Mta (MtaN) se une a los promotores *bmr* y *blt* en la misma posición que sus respectivos reguladores locales BmrR y BltR. Aparentemente, la supresión del extremo C-terminal de unión a efectores de Mta imita el efecto *in vivo* del inductor unido a la proteína. La estructura tridimensional de MtaN reveló que la proteína dimérica es estructuralmente muy similar a BmrR, sin embargo, cambios en la orientación de algunas de las hélices del dominio N-terminal sugieren que la forma de interacción de MtaN con el ADN es diferente al mecanismo descrito para BmrR (Godsey *et al.*, 2001).

4.3. Familia IclR de reguladores transcripcionales

La familia IclR de reguladores transcripcionales recibe su nombre del miembro más ampliamente caracterizado a nivel genético y bioquímico de la misma: la proteína IclR de *E. coli*, la cual controla el operón de utilización del acetato en *E. coli* (Maloy & Nunn, 1982; Sunnarborg *et al.*, 1990). Los reguladores de la familia IclR se encuentran ampliamente distribuidos en el reino procariota.

Los miembros de esta familia se definen por un perfil que se localiza en el dominio carboxilo terminal, entre los residuos 151 y 229 de IclR de *E. coli*, ya que este segmento es el que presenta mayor similitud en la secuencia primaria en el alineamiento múltiple de miembros de la familia (Krell *et al.*, 2006; Molina-Henares *et.al.*, 2006; ver también www.bacTregulators.com).

Las proteínas de esta familia tienen alrededor de 250 aminoácidos, poseen un dominio de unión al ADN del tipo hélice-giro-hélice (*helix-turn-helix, HTH)* en la región N-terminal y un dominio C-terminal involucrado en el reconocimiento de efectores. Estas proteínas controlan genes implicados en el ciclo del glioxilato en bacterias de la familia Enterobacteriaceae (Sunnarborg *et al.*, 1990; Song *et al.*, 2004), resistencia múltiple a compuestos tóxicos (Mosqueda & Ramos, 2000; Rojas *et al.*, 2003), degradación de compuestos aromáticos (Bertani *et al.*, 2001; DiMarco *et al.*, 1993; Gerischer *et al.*, 1998; Kok *et al.*, 1998; Guo & Houghton, 1999), inactivación de señales de *quorum sensing* (Zhang *et al.*, 2004), determinantes de patogenicidad de plantas (Nasser *et al.*, 1992; Liu *et al.*, 1999; Thomson *et al.*, 1999) y esporulación (Nomura *et al.*, 1998; van Wezel *et al.*, 2000).

Tabla 1. Características más relevantes de los miembros más estudiados de la familia IclR

Regulador	Función	Estado oligomérico	Operador	Efector	Referencia
IclR *E. coli*	Represor del operón *aceBAK* del desvío del glioxalato	Dímero (Negre *et al.*, 1992) o tetrámero (Donald *et al.*, 2001)	Palíndrome de 7 pb separado por 1 nucleótido espaciador (caja IclR)	glioxilato	Sunnarborg *et al.* (1990)
KdgR *Erwinia* sp.	Represor de genes del catabolismo de pectina y secreción de pectinasas	Dímero (Nasser *et al.*, 1992)	Secuencia de 17 pb que contiene dos medios sitios no idénticos (KdgR box). Puede unirse con baja afinidad a un medio sitio individual	2-keto-3-deoxigluconato	Nasser *et al.*, 1991, 1994
PcaU *Acinetobacter* sp. ADP1	Activador del metabolismo del protocatecuato	Dímero	Secuencia de 3 repeticiones de 10 pb, 2 formando un palíndrome y 1 directa separada por 10 pb	protocatecuato	Gerischer *et al.*, 1998 Guo & Houghton, 1999
PobR *Acinetobacter* sp. ADP1	Activador del metabolismo del p-hidroxibenzoato	No estudiado	Palíndrome de 8 pb separado por 1 nucleótido espaciador	p-hidroxibenzoato	DiMarco *et al.*, 1993 DiMarco & Ornston, 1994 Clubb *et al.*, 1994
PcaR *P. putida*	Activador de genes de degradación del protocatecuato	Dímero	Palíndrome de 7 pb separado por 1nucleótido espaciador.	β-ketoadipato (Weickert & Adhya, 1992; Harwood *et al.*, 1994)	Guo & Houghton, 1999 Romero-Steiner *et al.*, 1994
AllR (GclR) *E. coli*	Represor del metabolismo de la alantoína	Tetrámero	Palíndrome casi perfecto de 8 pb separado por 1 nucleótido espaciador	glioxilato	Donald *et al.*, 2001 Walker *et al.*, 2006
MhpR *E. coli*	Activador del catabolismo del 3-(3-hidroxipropil) ácido propiónico	No estudiado	Palíndrome imperfecto de 8 pb separado por 1 nucleótido espaciador. La unión de MhpR en el promotor P_a es esencial para la unión del activador CRP.	3-(3-hidroxipropil) ácido propiónico	Ferrández *et al.*, 1997 Torres *et al.*, 2003

No existe un consenso claro en la secuencia del ADN reconocida por los miembros de esta familia. Así, por ejemplo, el regulador MhpR reconoce una secuencia palindrómica de 15 pb (Ferrández *et al.*, 1997), mientras que el sitio reconocido por PcaU y PobR en sus respectivos promotores son tres secuencias repetidas perfectas de 10 pb que forman una secuencia repetida invertida y una directa separada de la anterior por 10 pb (Gerischer *et al.*,

1998; Guo & Houghton , 1999). En cuanto a la estequiometría de unión al ADN se ha visto que pueden unirse como dímeros o como dímeros de dímeros (Molina-Henares *et al.*, 2006). En la Tabla 1 se muestran las características más relevantes de los miembros más estudiados de la familia.

Los reguladores de la familia IclR actúan como represores, activadores y proteínas con ambas funciones. Los activadores se unen a la secuencia promotora de los genes que controlan en ausencia de efectores. El mecanismo de represión difiere entre los miembros de la familia. En la mayoría de ellos el sitio de unión de la ARN-polimerasa y el del represor solapan, de tal forma que impide la unión de la ARN-polimerasa. En otros casos, el sitio de unión del represor es distante con respecto al de la ARN-polimerasa, y el mecanismo de represión se lleva a cabo por una desestabilización del complejo de iniciación. En el caso de IclR de *E. coli*, Yamamotto & Ishihama (2003) propusieron que ambos tipos de mecanismos de represión coexisten en la regulación de los promotores *aceB* e *iclR*.

4.3.1. Estructuras tridimensionales de miembros de la familia IclR

Actualmente se encuentran disponibles 7 estructuras de la proteínas de la familia IclR. Estas son: la proteína completa TM0065 de *Thermotoga maritima* (Zhang *et al.*, 2002), la proteína completa del posible regulador del operón de degradación de catecol de *Rhodococcus* Sp. Rha1 (Chruszcz, 2006), los dominios de unión al efector de dos proteínas reguladoras del ciclo del glioxilato (registro PDB 1TF1 y 1TDJ); el dominio de unión al efector de YaiJ, el cual actúa como activador del metabolismo de la xilulosa de *E. coli* (registro PDB 1YSQ); el dominio de unión al efector del represor de la producción de exoenzimas KdgR de *Erwinia* sp. (registro PDB 1YSP) y el complejo del dominio de unión al efector con glioxilato del represor AllR de *E. coli* (Walker *et al.*, 2006). Actualmente no existe disponible ninguna estructura del complejo proteína reguladora con ADN.

5. BOMBAS DE EFLUJO EN LA CEPA TOLERANTE A DISOLVENTES ORGÁNICOS *PSEUDOMONAS PUTIDA* DOT-T1E

5.1. Bombas responsables de la tolerancia

En el año 1995, en el laboratorio de Degradación de Tóxicos Orgánicos de Granada, se aisló una cepa altamente tolerante a tolueno: *P. putida* DOT-T1E. Dicha cepa, que provenía de una muestra de una planta de tratamiento de aguas residuales de la cuidad de Granada, tenía la capacidad de crecer dentro de la varilla de tolueno que se utilizaba para proveer la atmósfera saturada del disolvente al medio de cultivo. Esto ponía en evidencia la gran

tolerancia de esta bacteria ya que era capaz de crecer en presencia de altas concentraciones (90% [v/v]) de tolueno, un compuesto letal para cualquier célula viva.

Como se mencionó anteriormente, la expulsión activa de disolventes por bombas es el recurso más efectivo en la tolerancia de bacterias a estos compuestos. El estudio de la tolerancia en *P. putida* DOT-T1E mostró la existencia de tres bombas RND implicadas en este proceso como elementos fundamentales para la supervivencia de la bacteria, aunque también existen modificaciones en la membrana que contribuyen a mejorar la tolerancia, estas son la isomerización *cis-trans* de ácidos grasos insaturados (Junker y Ramos, 1999) y cambios en los grupos de cabeza de fosfolípidos, jugando la cardiolipina un rol importante en la rigidez de la membrana y la resistencia a tolueno (Bernal *et al.*, 2006).

En el trabajo original Ramos *et al.* (1995) pusieron de manifiesto que la tolerancia a tolueno en la cepa *P. putida* DOT-T1E estaba influenciada por las condiciones de cultivo. La preexposición de células a bajas concentraciones del hidrocarburo aromático condujo a la supervivencia de casi el 100% de las células tras el choque de tolueno; en contraste, sólo sobrevivían el 0,1% de las células que no había sido preexpuestas a concentraciones subletales de tolueno. Cuando los ensayos se hicieron con un mutante obtenido por mutagénesis al azar por transposón, la cepa no sobrevivía al choque de tolueno, y sólo una pequeña fracción (aproximadamente 1 de cada 10^5 células) sobrevivía al choque si había sido preexpuesta a concentraciones subletales de tolueno. Dicho mutante estaba afectado en un gen que codifica la translocasa de un transportador de la familia RND (*ttgB*) el cual forma parte del operón de la bomba TtgABC (se la nombró de esta forma en relación a su descripción en inglés: "***t**oluene **t**olerance **g**enes*"). Esto llevó a pensar que esta bomba contribuía a la tolerancia innata de *P. putida* DOT-T1E a disolventes orgánicos (Ramos *et al.*, 1998).

Ramos y colaboradores (1998) postularon también la existencia de al menos otra bomba de eflujo implicada en el transporte de tolueno. Se identificó una segunda bomba inducible, TtgDEF, que transportaba tolueno en la cepa *P. putida* DOT-T1E, ligada a los genes *tod* de degradación de tolueno (Mosqueda y Ramos, 2000). El hecho de que los genes que codifican una ruta catabólica estén ligados a los genes implicados en el transporte del compuesto es un rasgo conservado entre otras cepas de *Pseudomonas* sp. que degradan tolueno a través de la ruta TOD (Huertas *et al.*, 2000).

En un mutante de *P. putida* DOT-T1E con las bombas TtgABC y TtgDEF inactivas, todavía una fracción de cultivo (10^{-4} células) sobrevivía si éste había sido preexpuesto a

concentraciones subletales de tolueno, aunque casi ninguna célula (<10^{-8}) sobrevivía sin inducción (Mosqueda y Ramos, 2000). Esto sugería la presencia de al menos otra bomba implicada en la tolerancia, lo cual fue demostrado por Rojas y colaboradores un año después, cuando presentaron la tercera bomba involucrada: TtgGHI. En la Tabla 2 se muestran las proteínas homólogas en otras bacterias a las tres proteínas que conforman el sistema TtgGHI.

Se pudo establecer que las tres bombas participan en la resistencia a disolventes orgánicos de forma aditiva y complementaria mediante el estudio de mutantes sencillos en cada uno de estos transportadores y combinaciones de mutantes dobles y un mutante triple deficiente en las tres bombas (Rojas *et al.*, 2001). La bomba TtgABC se expresa constitutivamente en las condiciones de laboratorio estudiadas, por lo cual formaría parte de la resistencia intrínseca basal a disolventes, mientras que la expresión basal mayor e importante inducción del transportador TtgGHI hacen que cumpla un papel clave en la resistencia intrínseca, tanto basal como inducible, de esta cepa a disolventes orgánicos. El rol de la bomba TtgDEF queda oculto por su expresión basal mínima y sus niveles modestos de inducción en comparación al transportador TtgGHI (Mosqueda y Ramos, 2000; Rojas *et al.*, 2001).

Tabla 2. Proteínas homólogas a los componentes de la bomba TtgGHI

Organismo	MFP - TtgG	Translocasa - TtgH	OMF- Ttg I	Referencia
P. putida S12	SrpA (98%)	SrpB (96%)	SprC (99%)	Kieboom *et al.*, 1998b
P. putida DOT-T1E	TtgD (72%)	TtgE (83%)	TtgF (59%)	Mosqueda y Ramos, 2000
P. aeruginosa	MexA (58%)	MexB (63%)	OprM (59%)	Stover *et al.*, 2000
P. putida DOT-T1E	TtgA (57%)	TtgB (62%)	TtgC (56%)	Ramos *et al.*, 1998
P. putida S12	ArpA (57%)	ArpB (62%)	ArpC (56%)	Kieboom *et al.*, 2001
E. coli	AcrA (50%)	AcrB (57%)	TolC (19%)	Blattner *et al.*, 1997

MFP, proteína de fusión periplásmica; OMF, proteína de membrana externa. Entre paréntesis se indica el porcentaje de identidad en la secuencia de aminoácidos para cada proteína respecto a cada componente: TtgG, TtgH y TtgI.

5.2. Identificación de los reguladores transcripcionales de las bombas Ttg

La mayoría de los genes reguladores que codifican proteínas involucradas en el control de la expresión de bombas de eflujo de la familia RND se localizan adyacentes a los genes estructurales de la bomba y, generalmente, el gen regulador se transcribe divergentemente desde el operón de la bomba. Divergentemente con respecto al sistema *ttgABC* se identificó el gen *ttgR* (Duque *et al.*, 2001), mientras que el gen *ttgT* se localizó adyacente y divergente con respecto al sistema *ttgDEF* (Segura *et al.*, 2003). El operón *ttgVW* constituyó el par de genes reguladores que a priori podrían regular el sistema *ttgGHI* (Rojas *et al.*, 2001) (ver Figura 5). La secuencia de la proteína TtgR mostraba similitud a la de otras proteínas de la familia de reguladores TetR como AcrR (Ma *et al.*, 1993) y MexZ (Aires *et al.*, 1999), también involucradas en regulación de bombas. Los genes TtgV y TtgT, miembros de la familia IclR de reguladores transcripcionales, reprimen la expresión de sus correspondientes operones *ttgGHI* (Rojas *et al.*, 2003) y *ttgDEF* (Segura *et al.*, 2003). Sin embargo, no se ha encontrado una función reguladora para TtgW (Rojas *et al.*, 2003), probablemente porque se trata de una proteína truncada por inserción de un codón terminador dentro de la secuencia del gen en la cepa silvestre (Rojas, 2003). Como ya se mencionó, existe regulación cruzada de los operones *ttgDEF* y *ttgGHI* por parte de sus reguladores locales TtgT y TtgV (Segura *et al.*, 2003; Terán et al., 2007). La alta identidad de secuencia entre estas proteínas (63%), así como la existente entre las regiones promotoras de los respectivos operones que controlan y el hecho de que se inducen por el mismo tipo de efectores son las posibles respuestas a la existencia de esta co-regulación.

Desde el punto de vista cuantitativo, el sistema TtgGHI es el más importante para la supervivencia de la bacteria en presencia de disolventes orgánicos. En un estudio de diferentes cepas de *P. putida* en el que se analizaba la resistencia a disolventes orgánicos de las mismas, se encontró que sólo las tres cepas que presentaban alta tolerancia a tolueno (*P. putida* DOT-T1E , S12 y MTB6) hibridaron contra una sonda *ttgH*. Este hallazgo sugería que las bombas de eflujo semejantes a TtgGHI son factores claves en el alto nivel de tolerancia a tolueno en varias cepas de *P. putida* (Segura *et al.*, 2003).

El patrón de expresión de los genes *ttgGHI* se determinó por análisis de extensión de cebador y ensayos de β-galactosidasa del promotor *ttgGHI* fusionado a *´lacZ*. La expresión del operón aumentó alrededor de cuatro veces en presencia de tolueno, un resultado que mostraba otra vez la naturaleza inducible del operón, el cual ya se había observado en los experimentos de choque de tolueno (Rojas *et al.*, 2001).

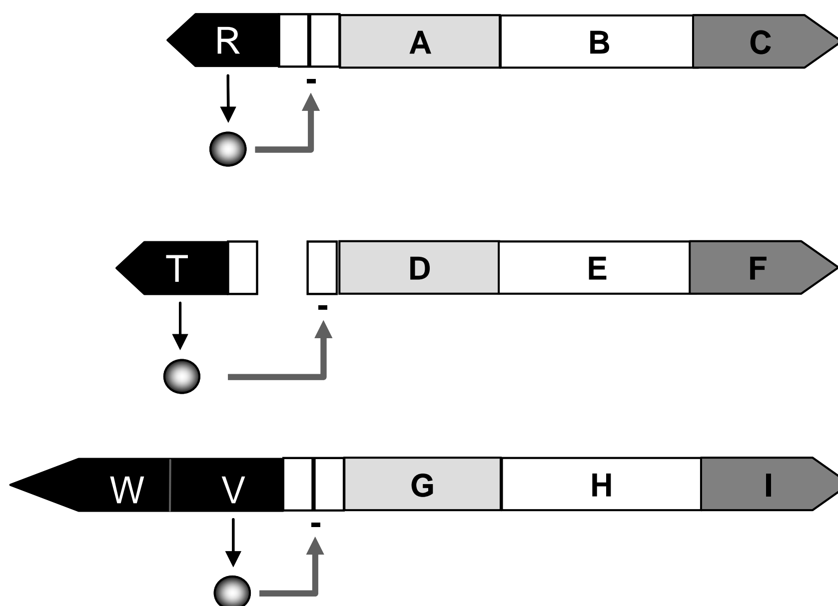

Figura 5. Organización de los operones *ttgABC*, *ttgDEF*, *ttgGHI* y de sus respectivos genes reguladores locales. En gris claro se indica el gen que codifica la proteína periplásmica, en blanco el transportador interno y en gris oscuro la proteína de membrana externa. Los genes reguladores se muestran en negro y la proteína represora se simboliza con un círculo y con una flecha se indica la unión a su operador, el cual se ubica dentro a la zona intergénica (rectángulos blancos pequeños). Adaptado de Ramos, *et al.*, 2002.

Tabla 3. Proteínas con las que TtgV comparte identidad en otras especies y cepas bacterianas. El porcentaje de identidad es con respecto a la secuencia de aminoácidos de TtgV.

Proteína	Organismo	Aminoácidos	Identidad	Sistema que regula	Referencia
SrpS	*P. putida* S12	259	96 %	Bomba de eflujo *srpABC*	Dennis y Zylstra, 1999
TtgT	*P. putida* DOT-T1E	260	63 %	Bomba de eflujo *ttgDEF*	Mosqueda y Ramos, 2000
SepR	*P. putida* F1	260	63 %	Bomba de eflujo	Phoenix *et al.*, 2003
IclR	*Azotobacter vinelandii* AvOP	267	52 %	Regulador trasncripcional	Copeland *et al.*, 2005a
KdgR	*Erwinia chrysanthemi*	291	47 %	Genes de degradación de pectina	Reverchon *et al.*, 1991
PcaR	*Mesorhizobium loti*	256	45 %	Genes degradación *p*-hidroxibenzoato	Kaneko *et al.*, 2000
IclR	*Marinobacter aquaelei* VT8	258	36 %	Regulador transcripcional	Copeland *et al.*, 2005b
PcaR	*P. putida*	291	29 %	Genes degradación *p*-hidroxibenzoato	Romero-Steiner *et al.*, 1994
IclR	*Escherichia coli* K12	287	28 %	Ciclo del glioxilato	Sunnarborg *et al.*, 1990
PcaU	*Acinetobacter calcoaceticus*	278	27 %	Genes de utilización de protocuatecuato	Gerischer *et al.*, 1998
AttJ	*Agrobacterium tumefaciens*	275	24 %	Recambio de moléculas de *quorum sensing*	Zhang *et al.*, 2002

Como se mencionó anteriormente, TtgV se transcribe divergentemente con respecto al operón de la bomba TtgGHI. En la Figura 6 se muestra la naturaleza solapante de los promotores para *ttgGHI* y *ttgV*. TtgV muestra un 97% de identidad con el regulador SrpS de la bomba SrpABC de *P. putida* S12 (Kieboom *et al.*, 1998b), la cual tiene una identidad del 96-99% con los elementos que conforman la bomba TtgGHI (ver Tabla 2). Ambas proteínas reguladoras muestran alrededor de 50-60% de identidad con un número de reguladores transcripcionales de la familia IclR. En la Tabla 3 se muestra el porcentaje de identidad de TtgV con respecto a algunas proteínas miembros de la familia. En el sistema SrpABC existe un segundo elemento regulador (SprR) que muestra una alta identidad a nivel de secuencia de nucleótidos (96%) con *ttgW* (el otro elemento regulador del operón *ttgW-ttgV*), aunque *srpR* codifica una proteína de 213 aminoácidos, mientras que *ttgW* codifica una proteína de sólo 134 aminoácidos. Estas dos proteínas muestran similitud con la familia de represores transcripcionales TetR, aunque como se mencionó anteriormente, TtgW parece ser un miembro truncado de la familia.

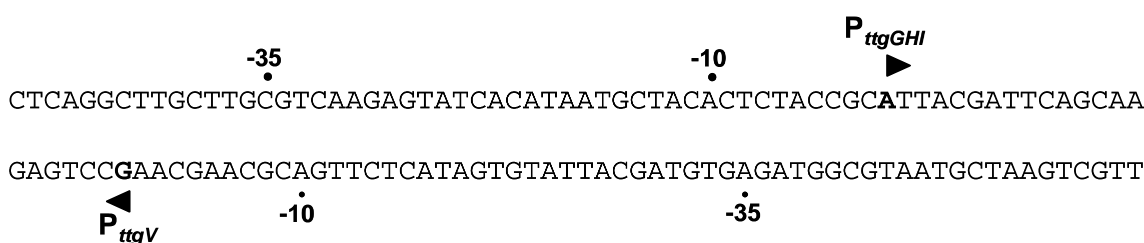

Figura 6. Detalle de la región intergénica *ttgV-ttgGHI* y de las regiones promotoras de cada operón. En triángulos se muestran los puntos de inicio de la transcripción y sus respectivas regiones -10 y -35 (Rojas *et al.*, 2001 y 2003).

En ensayos de choque de tolueno bajo condiciones sin inducción una cepa deficiente en *ttgV* mostró una mayor resistencia cuando se comparó con la cepa silvestre. Esto indicaba que TtgV se comportaba como un represor del operón *ttgGHI*. Además, el análisis de la expresión de los operones *ttgGHI* y *ttgV* en esta cepa mutante mostró que su expresión aumentaba alrededor de cuatro veces en ausencia de tolueno (Rojas *et al.*, 2003). Estos datos indican claramente que TtgV actúa como un represor que previene la expresión de los genes de la bomba TtgGHI y que regula su propia síntesis. En este estudio de Tesis Doctoral se aportan nuevos datos sobre la represión de los genes *ttgGHI* efectuada por TtgV y la desrepresión inducida por compuestos aromáticos.

Objetivos

OBJETIVOS

En la bacteria altamente tolerante a tolueno *P. putida* DOT-T1E existen tres bombas de eflujo de disolventes (TtgABC, TtgDEF y TtgGHI), las cuales participan de forma aditiva en la tolerancia a disolventes orgánicos. Dadas las características esenciales que otorga la bomba de eflujo TtgGHI a la tolerancia a disolventes orgánicos en esta cepa, el objetivo principal de este trabajo de Tesis fue el estudio de la regulación de la expresión de dicha bomba, centrado en la caracterización bioquímica, molecular y funcional de su regulador TtgV. Para ello, se plantearon los siguientes objetivos específicos:

1. Caracterización molecular del promotor P_{ttgG}.

2. Caracterización bioquímica y funcional de la proteína reguladora TtgV en su unión a su ADN operador.

3. Caracterización bioquímica de las interacciones de efectores con TtgV.

Resultados

CAPÍTULO 1.

Evidencia *in vivo* e *in vitro* de que TtgV es el regulador específico de la bomba de eflujo de disolventes TtgGHI de *Pseudomonas putida*

Autores: Rojas, A., Segura, A., Guazzaroni, M.E., Terán, W., Hurtado, A., Gallegos, M.T. y Ramos, J. L.

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La bomba de eflujo TtgGHI de *Pseudomonas putida* DOT-T1E cumple un papel clave en la tolerancia innata e inducible a disolventes orgánicos y antibióticos en esta bacteria. El operón *ttgGHI* se expresa a un nivel basal medio desde dos promotores solapantes en ausencia de disolventes, y su nivel aumenta varias veces en presencia de éstos, pero no de antibióticos. El operón *ttgVW* se transcribe divergentemente con respecto al operón *ttgGHI*. En un mutante carente del gen *ttgV*, aunque no en un mutante deficiente en el gen *ttgW*, la expresión de los operones *ttgGHI* y *ttgVW* aumentó cuatro veces. Esto sugiere que TtgV reprime la expresión del promotor *ttgG* y la suya propia. La proteína TtgW no parece cumplir un rol importante en la regulación de la expresión de estos promotores. Mediante experimentos de extensión del cebador se demostró que los promotores divergentes *ttgG* y *ttgV* solapan, y ensayos de retardo en la movilidad indicaron que TtgV se une a la región intergénica que contiene ambos promotores con alta afinidad. Ensayos de impronta *in vitro* con ADNasaI revelaron que TtgV cubre cuatro vueltas de hélice del ADN y que esta región incluye las cajas -10 y -35 de los promotores *ttgV* y *ttgG*.

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In Vivo and In Vitro Evidence that TtgV Is the Specific Regulator of the TtgGHI Multidrug and Solvent Efflux Pump of *Pseudomonas putida*

Antonia Rojas, Ana Segura, María Eugenia Guazzaroni, Wilson Terán, Ana Hurtado, María Trinidad Gallegos, and Juan L. Ramos*

Department of Plant Biochemistry and Molecular and Cellular Biology, Consejo Superior de Investigaciones Cientificas, Estación Experimental del Zaidín, E-18008 Granada, Spain

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The TtgGHI efflux pump of *Pseudomonas putida* DOT-T1E plays a key role in the innate and induced tolerance of this strain to aromatic hydrocarbons and antibiotics. The *ttgGHI* operon is expressed constitutively from two overlapping promoters in the absence of solvents and at a higher level in their presence, but not in response to antibiotics. Adjacent to the *ttgGHI* operon is the divergently transcribed *ttgVW* operon. In TtgV-deficient backgrounds, although not in a TtgW-deficient background, expression of the *ttgGHI* and *ttgVW* operons increased fourfold. This suggests that TtgV represses expression from the *ttgG* promoters and controls its own. TtgW plays no major role in the regulation of expression of these promoters. Primer extension revealed that the divergent *ttgG* and *ttgV* promoters overlap, and mobility shift assays indicated that TtgV binds to this region with high affinity. DNaseI footprint assays revealed that TtgV protected four DNA helical turns that include the −10 and −35 boxes of the *ttgV* and *ttgG* promoters.

In their natural habitats, living organisms are exposed to a wide range of natural and human-made toxic compounds, and survival in sites that have become hostile involves an extensive series of protective mechanisms. Multidrug efflux pumps are one of the most efficient tools that eukaryotic and prokaryotic cells use to extrude toxic chemicals and are very efficient in the removal of anticancer agents, antibiotics, dyes, superoxide-generating chemicals, organic solvents, and other toxic chemicals (31, 33, 34, 39). Efflux pumps of the resistance-nodulation-cell division (RND) family are very efficient in the removal of different drugs (33, 37, 45). These bacterial efflux pumps are made of three components (20, 28, 54): an efflux pump transporter located in the cytoplasmic membrane that recognizes substrates in the periplasm or in the outer leaflet of the cytoplasmic membrane (11, 28, 50), an outer membrane protein which forms a trimeric channel that penetrates into the periplasm (20) and contacts the efflux pump transporter, and a lipoprotein anchored to the inner membrane which expands into the periplasmic space and may serve as a bracket for the other two components (54).

Toluene, xylene, and styrene are among the most toxic chemicals that bacterial cells can be exposed to because they dissolve in the cytoplasmic membrane, disorganize it, and collapse the cell's membrane potential. This, together with the loss of lipids and proteins, leads to irreversible damage, resulting in the death of the cell (8, 47). However, a number of *Pseudomonas putida* strains are able to grow in the presence of high concentrations of toluene and other aromatic hydrocarbons (7, 14, 18, 41, 52). Tolerance to these chemicals in these strains is achieved mainly by a series of RND efflux pumps, called Ttg (toluene tolerance genes) in *P. putida* DOT-T1E (15–18, 21, 27, 29, 42, 43). In *P. putida* DOT-T1E, three different efflux pumps, TtgABC, TtgDEF, and TtgGHI, function additively to prevent the accumulation of toluene and other aromatic hydrocarbons in the cell membrane (43). Two of these efflux pumps, TtgABC and TtgGHI, also extrude antibiotics such as chloramphenicol, tetracycline, ampicillin (40, 43), and, in the case of TtgABC, probably also heavy metals (P. Godoy and J. L. Ramos, unpublished data).

Some of these efflux pumps have a relatively high basal level of expression and confer so-called intrinsic resistance, whereas others are inducible by the transported product and confer induced resistance. In *P. putida* DOT-T1E, the *ttgABC* operon is expressed at a certain basal level, which increases slightly in response to solvents and significantly in response to chloramphenicol (9; W Terán, A. Felipe, A. Segura, A. Rojas, J. L. Ramos, and M. T. Gallegos, submitted for publication), whereas the *ttgDEF* operon is induced by aromatic hydrocarbons such as toluene or styrene (27). The *ttgGHI* operon seems to be expressed from two overlapping promoters at a certain basal level in the absence of solvents, and its expression increases severalfold in the presence of aromatic hydrocarbons (43). The RND efflux genes are often regulated by the gene product of an adjacent and divergently transcribed regulatory gene (1, 5, 9, 22, 24, 29, 30, 31, 33, 43, 45). In fact, the *ttgR* gene is adjacent to the *ttgABC* operon and is transcribed divergently from *ttgA*. In a TtgR-deficient background, expression from the *ttgA* promoter increased about 10-fold, suggesting that TtgR represses expression from the *ttgA* promoter (9). In this mutant background, expression from the *ttgD* and *ttgG* promoters followed the same pattern of expression as in the wild type, suggesting that other regulators are involved in the control of expression of the other two efflux pumps.

In this study, we report the identification of two genes, *ttgV*

* Corresponding author. Mailing address: CSIC-EEZ, C/Prof. Albareda, 1, E-18008 Granada, Spain. Phone: 34 958 181602/08. Fax: 34 958 135740. E-mail: jlramos@eez.csic.es.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
P. putida DOT-T1E	Rif^r	41
P. putida DOT-T1E-PS52	Rif^r Km^r *ttgW*::ΩKm	This study
P. putida DOT-T1E-PS61	Rif^r Km^r *ttgV*::ΩKm	This study
P. putida DOT-T1E-PS62	Rif^r Km^r *ttgV*::*aphA3*	This study
E. coli DH5αF′	F′/*endA1 hsdR17*(r_K^- m_K^+) *glnV44 thi1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169* *deoR* [φ80*dlac*Δ(*lacZ*)M15]	4
E. coli B834 (DE3)	F$^-$ *omp* (r_B^- m_B^-) *gal dcm met* auxotroph	Promega
pANA82	pUC18Not plasmid bearing a 4.5-kb *Bam*HI-*Eco*RV insert with the *ttgVW* genes	This study
pANA83	*ttgW*::ΩKm, derived from pANA82	This study
pANA95	Promoter of *ttgV* cloned in pMP220	This study
pANA96	Promoters of *ttgG* cloned in pMP220	This study
pANA117	2.3-kb fragment cloned in pGEM-T, bearing *ttgV* and *ttgW* genes	This study
pANA118	*ttgV*::*aph*A3, derived from pANA117	This study
pANA119	*ttgV*::ΩKm, derived from pANA117	This study
pANA125	0.8-kb *Nde*I-*Bam*HI fragment bearing *ttgV* in pGEM-T	This study
pANA126	*ttgV*-His$_6$ tag cloned in pET28b(+)	This study
pET-28b(+)	Km^r, expression vector	Novagen
pGEM-T	Ap^r, cloning vector	Promega
pGG1	pUC18 bearing an 8-kb *Bam*HI fragment with *ttgGHI* and *ttgVW*	43
pHP45Ω-Km	Ap^r, carries the 2.25-kb ΩKm fragment	37
pMP220	Tc^r, promoterless *lacZ* vector	48
pSB*aph*A	pSB plasmid with a nonpolar Km^r cassette	25
pUC18Not	Ap^r, cloning vector	32

[a] Ap^r, Km^r, Nal^r, Rif^r, and Tc^r, resistance to ampicillin, kanamycin, nalidixic acid, rifampin, and tetracycline, respectively.

and *ttgW*, that form an operon and that are transcribed divergently from the *ttgGHI* operon. In the *ttgV*-deficient background, but not in the *ttgW*-deficient background, the *ttgGHI* and *ttgVW* operons are expressed at a higher level. This suggests that TtgV is a repressor of the expression from the *ttgG* and *ttgV* promoters. We overexpressed and purified TtgV and showed that this repressor binds to the *ttgV*-*ttgG* intergenic region protecting a region covering four DNA helical turns where the overlapping *ttgV* and *ttgG* promoters lie.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture medium. The bacterial strains, cloning vectors, and plasmids constructed in this study are shown in Table 1. Bacterial strains were grown in Luria-Bertani (LB) medium (4). *Escherichia coli* cultures were incubated at 37°C, whereas *P. putida* cultures were incubated at 30°C. Liquid cultures were shaken on an orbital platform operating at 200 strokes per min. When *P. putida* cultures were supplemented with toluene via the gas phase, we used culture flasks with a central vessel in which 0.15 ml of toluene was introduced to avoid direct contact with the liquid medium. The flasks were tightly closed with a Teflon screw cap. Under these culture conditions, the concentration of toluene in the liquid medium was about 2 mM.

E. coli DH5αF′ (4) was used as a recipient for recombinant plasmids, and *E. coli* B834(DE3) was chosen as a host for the production of His$_6$-tagged TtgV protein. When required, cultures were supplied with the following antibiotics (at the indicated concentrations unless otherwise stated): ampicillin (100 μg/ml), kanamycin (50 μg/ml), nalidixic acid (100 μg/ml), piperacillin (100 μg/ml), rifampin (20 μg/ml), streptomycin (300 μg/ml), and tetracycline (20 μg/ml).

DNA techniques. Preparation of chromosomal DNA, digestion with restriction enzymes, electrophoresis, and Southern blotting were done using standard methods (4). For hybridizations, we used a digoxigenin DNA labeling and detection kit (Roche Laboratories) in accordance with the manufacturer's instructions. A Qiagen kit was used for plasmid isolation. Plasmid DNA was sequenced on both strands with universal, reverse, or specifically designed primers by using an automatic DNA sequencer (ABI-PRISM 3100; Applied Biosystems). Electroporation of *P. putida* cells was done as described previously (43).

Preparation of RNA, primer extension analysis, and RT-PCR. *P. putida* DOT-T1E was grown overnight in LB medium. Cells were then diluted 100-fold in fresh medium, and aliquots were incubated in the absence or presence of 3 mM toluene, 1.5 mM styrene, or sublethal concentrations of antibiotics (chloramphenicol, 30 μg/ml; nalidixic acid, 30 μg/ml; tetracycline, 1 μg/ml; gentamicin, 0.25 μg/ml; and carbenicillin, 120 μg/ml) until the culture reached a turbidity of about 1.0 at 660 nm. Cells (30 ml for primer extension and 1.5 ml for reverse transcriptase [RT] PCR) were harvested by centrifugation (5,000 × *g* for 10 min) and processed for RNA isolation according to the method of Marqués et al. (23). Extracts were treated with RNase-free DNase I (50 U) in the presence of 3 U of an RNase inhibitor cocktail (Roche Laboratory). For primer extension analysis, we used as primers specific oligonucleotides complementary either to the *ttgV* mRNA or the *ttgG* mRNA. Primers were labeled at their 5′ ends with [γ-^{32}P] ATP and T4 polynucleotide kinase as described previously (4). About 10^5 cpm of the labeled primer was hybridized to 20 μg of total RNA, and extension was carried out using avian myeloblastosis virus RT as described previously (23). Electrophoresis of cDNA products was done in a urea-polyacrylamide sequencing gel to separate the reaction products. The relative intensity of the bands was quantitated using the Molecular Imager System GS 525 (Bio-Rad Laboratories) with the Multianalyst program.

RT-PCR was done with 1 μg of RNA in a final volume of 20 μl using the Titan OneTube RT-PCR system according to the manufacturer's instructions (Roche Laboratories). The annealing temperature used for RT-PCR was 60°C, and the cycling conditions were as follows: 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. Positive and negative controls were included in all assays. The primers used to test contiguity in the mRNA of the *ttgV* and *ttgW* genes are available from the authors upon request.

Construction of *ttgV* and *ttgW* knockout mutant strains. To facilitate site-directed mutagenesis, the 4.5-kb *Bam*HI-*Eco*RV fragment from pGG1 (43) harboring the *ttgV* and *ttgW* genes was ligated to pUC18Not digested with *Bam*HI-*Sma*I to produce pANA82. Plasmid pANA82 was in turn digested with *Bgl*II (which cuts once in the plasmid within the *ttgW* gene), made blunt with the four deoxynucleoside triphosphates and Klenow enzyme, and then ligated to a 2.25-kb *Bam*HI fragment (blunted as above) from pHP45Ω-Km (37) that contained the kanamycin resistance cassette to yield pANA83 (Ap^r Km^r). About 600 ng of the suicide pANA83 plasmid was electroporated into *P. putida* DOT-T1E cells, and transformants that integrated the pANA83 plasmid into the host chromosome via homologous recombination were selected on LB solid medium with kanamycin and piperacillin. Successful integration was confirmed by Southern blot analysis (data not shown). A random merodiploid clone was grown overnight in LB medium to allow for a second recombination event in which the wild-type gene was replaced by the mutant allele. For this selection, colonies were plated again on LB medium with kanamycin. Among these colonies, those that did not grow in the presence of piperacillin were selected as putative resolved clones. The mutant clones were checked again by Southern blotting, and one of the clones, which exhibited the correct replacement (data not shown), was kept for further studies and named *P. putida* strain DOT-T1E-PS52.

To construct a *ttgV* polar mutant, we first amplified by PCR the *ttgV* gene by

using appropriate primers and pGG1 as a source of the gene. The amplified DNA was ligated to pGEM-T to yield pANA117. This plasmid was digested with *Xcm*I (which cuts once in the plasmid within the *ttgV* gene), and the sticky ends were made blunt. The plasmid was then ligated to an *Eco*RI 2.25-kb blunt-end Ω-Km fragment from pHP45Ω-Km to yield plasmid pANA119. Plasmid pANA119 was subsequently electroporated into *P. putida* DOT-T1E cells. Transformants and resolved clones were selected as described above to generate *P. putida* DOT-T1E-PS52. The *ttgV* polar mutant was called *P. putida* strain DOT-T1E-PS61.

A nonpolar mutation in *ttgV* was constructed using the strategy described above, except that we inactivated the *ttgV* gene with the nonpolar *aphA*3 gene cassette (25) to produce pANA118. The mutation was then transferred into the host chromosome to produce *P. putida* strain DOT-T1E-PS62.

MIC assays. Concentration assays were done in LB medium using the microtiter broth dilution method (3). Microtiter plates were inoculated with 10^5 CFU/ml and incubated for 20 h at 30°C.

Survival in response to toluene shocks. Cells were grown overnight in 30 ml of LB medium with or without toluene in the gas phase. On the following day, cultures were diluted 1:100 and grown under the same conditions until the cultures reached a turbidity of about 0.8 at 660 nm. These cultures contained about 10^8 CFU/ml. The cultures were divided in two halves; to one we added 0.3% (vol/vol) toluene, and the other was kept as a control. The number of viable cells was determined before toluene was added and 10, 30, and 60 min later.

β-Galactosidase assays. We constructed fusions of the promoters of the *ttgGHI* and *ttgVW* operons to a promoterless *lacZ* gene in the low-copy-number pMP220 vector (48). The *ttgV-ttgG* intergenic region (196 bp) was amplified by PCR with primers incorporating restriction sites (an *Eco*RI site in the primer designed to meet the 5′ end and a *Pst*I site in the primer designed to meet the 3′ end) to create a fusion of the promoters of the *ttgGHI* operon to *′lacZ*. The same oligonucleotides, except with a *Pst*I site in the one meeting the 5′ end and an *Eco*RI site in the one meeting the 3′ end, were used to create a fusion of the *ttgVW* operon promoter to *′lacZ*. Upon amplification, DNA was digested with *Eco*RI and *Pst*I and ligated to *Eco*RI-*Pst*I-digested pMP220 to produce pANA95 (P_{ttgV}::*lacZ*) and pANA96 (P_{ttgG}::*lacZ*). Plasmids pANA95 and pANA96 were sequenced to make sure that no mutations were introduced in the corresponding promoter regions. These plasmids were electroporated into the wild-type *P. putida* DOT-T1E strain and the mutant strains DOT-T1E-PS52, DOT-T1E-PS61, and DOT-T1E-PS62. The corresponding transformants were grown overnight on LB medium with tetracycline, and then the cultures were diluted 100-fold in the same medium. After 1.5 h of incubation at 30°C with shaking, the cultures were supplemented or not with 3 mM toluene, and 4 h later β-galactosidase activity was assayed in permeabilized whole cells according to Miller's method (26). We also tested the response to other solvents by adding 3 mM benzene, *p*-xylene, ethylbenzene, or propylbenzene and 1.5 mM styrene. Assays were run in duplicate and were repeated for at least three independent experimental rounds.

Overexpression and purification of His-tagged TtgV. The *ttgV* gene was amplified by PCR from plasmid pGG1 (43) with primers incorporating *Nde*I and *Bam*HI restriction sites at their 5′ and 3′ ends, respectively, and cloned in pGEM-T to yield pANA125. The 0.8-kb *Nde*I-*Bam*HI fragment was subsequently subcloned in pET28b(+) to produce pANA126 so that the gene product, when expressed, would carry an N-terminal histidine tag. For His_6-tagged N-TtgV purification, pANA126 was transformed in *E. coli* B834(DE3). Cells were grown in 1-liter batches at 30°C in LB medium (4) with 50 μg of kanamycin/ml to an A_{660} between 0.5 and 0.7 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 3 h of induction at 20°C; resuspended in 25 mM sodium phosphate (pH 7.0), 0.5 M NaCl, 5% (vol/vol) glycerol, and protease inhibitor cocktail (Complete; Roche); and broken in a French press after treatment with 20 μg of lysozyme/ml. Following centrifugation at 20,000 × *g* for 30 min, the protein was found predominantly (more than 80%) in the soluble fraction. His_6-tagged TtgV was purified by nickel affinity chromatography using a Ni^+-Sepharose matrix (Amersham-Biosciences) as described by the supplier, and the bound protein was eluted with an imidazole gradient in the abovementioned buffer. Homogenous peak fractions were pooled and dialyzed against TGED (50 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol), to which 50% (vol/vol) glycerol and 50 mM NaCl were added, and stored at −70°C (long-term storage) or −20°C (short-term storage). Protein concentrations were determined using a Bio-Rad protein assay kit.

Electrophoresis mobility shift assay. A 210-bp DNA fragment containing the divergent *ttgG* and *ttgV* promoters was amplified by PCR from plasmid pGG1 and isolated from agarose gels. This fragment was end-labeled with ^{32}P as described above. Labeled DNA (about 1 nM; 1.5×10^4 cpm) was incubated with increasing amounts of TtgV-His_6 for 10 min at 30°C in 10 μl of TGED binding buffer containing 20 μg of poly(dI-dC)/ml and 200 μg of bovine serum albumin/ml. Reaction mixtures were then electrophoresed in a nondenaturing 4.5% (wt/vol) polyacrylamide gel in Tris-glycine buffer (0.2 M glycine–0.025 M Trizma base [pH 8.6]). Results were analyzed using a Molecular Imager GS525. Competition experiments were performed using increasing amounts of unlabeled probe, with unlabeled-to-labeled DNA ratios ranging from 1:1 to 1,000:1.

DNase I footprinting. For these assays, a 228-bp PCR fragment generated with the primers indicated below was used. For the footprint on the top strand of the *ttgGHI* operon, we amplified DNA using primers 5′-GTTCATATGTTTCCTC TGCG-3′ (end labeled with [γ-^{32}P]ATP as described above) and 5′-GTTTGG CTCCATCTCTCTGC-3′. For the footprint on the bottom strand, the same primers were used but the latter primer rather than the former primer was end labeled. About 10 nM concentrations (~10^4 cpm) of each labeled probe was incubated in 10-μl reaction mixtures with or without His_6-tagged TtgV (1 and 3 μM) in TGED supplemented with poly(dI-dC) (20 μg/ml) and bovine serum albumin (200 μg/ml). Reaction mixtures were incubated for 10 min at 30°C before being treated with 40 μl of DNase I (final concentration, 10^{-5} U/μl) diluted in 10 mM Tris-HCl (pH 8.0) supplemented with 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM dithiothreitol, and 100 mM KCl. After 4 min at 30°C, reactions were stopped with 2 μl of 0.5 mM EDTA, the sample was extracted with phenol, and then DNA was precipitated with 2 volumes of ethanol and resuspended in 6 μl of TE (10 mM Tris–0.1 mM EDTA [pH 8.0]) and 3 μl of loading dye. Equal amounts of DNA (5,000 to 6,000 cpm) were heated at 90°C for 3 min and electrophoresed through a 6.5% (wt/vol) denaturing polyacrylamide gel. Sequencing ladders were generated in each case with the corresponding labeled primer using a T7 DNA polymerase sequencing kit (USB-Amersham) and the pGG1 plasmid.

Computer analysis. Open reading frames (ORFs) in DNA sequences were predicted with various programs included in the DNA Strider 1.1 package. Sequences were compared with those in the BlastX programs (2), available from the National Institute for Biotechnology Information server. Protein sequences were aligned with the multiple-sequence alignment ClustalW program (49).

RESULTS AND DISCUSSION

Identification of an operon upstream from the *ttgGHI* genes that is divergently transcribed with respect to the efflux pump genes. DNA sequencing upstream from the *ttgGHI* genes by using plasmid pGG1 as a template (43) revealed two ORFs, of 780 and 405 bp (Fig. 1A), separated from each other by 5 bp. These genes were transcribed divergently with respect to the *ttgGHI* operon. Sequence homology search of the deduced 259- and 134-amino-acid proteins with sequences deposited in several databases showed that the first ORF showed an overall 50 to 60% similarity with a number of transcriptional regulators belonging to the IclR family (i.e., PsrA, IclR, GltR, PcaR, PcaU, PobR, SrpS, etc. [35, 36, 53]), whereas the second ORF showed similarity to members of the TetR family of repressors (i.e., TetR, TtgR, AcrR, MtrR, SrpR, and QacR [6, 9, 12, 13, 19, 22, 24, 46, 53]). We hypothesized that these ORFs encoded proteins involved in the transcriptional control of the *ttgGHI* efflux pump operon, and we called the first and second ORFs *ttgV* and *ttgW*, respectively.

To test whether *ttgV* and *ttgW* were part of the same transcriptional unit, we prepared total RNA from *P. putida* DOT-T1E growing on LB medium and RT-PCR assays were done with primers based on *ttgV* and *ttgW* on the one hand and *ttgV* and *ttgG* on the other. Amplification was obtained only when the *ttgV* and *ttgW* primers were used, with the size of the amplified fragment being that predicted from the DNA sequence (Fig. 1B). This result confirmed the presumed operon structure of the cluster. Using primers based on *ttgG*, *ttgH*, and *ttgI*, we also showed that *ttgGHI* was transcribed as a single transcriptional unit (Fig. 1B).

Transcriptional analysis of the *ttgVW* operon. The transcription initiation point of the *ttgVW* operon was mapped, as de-

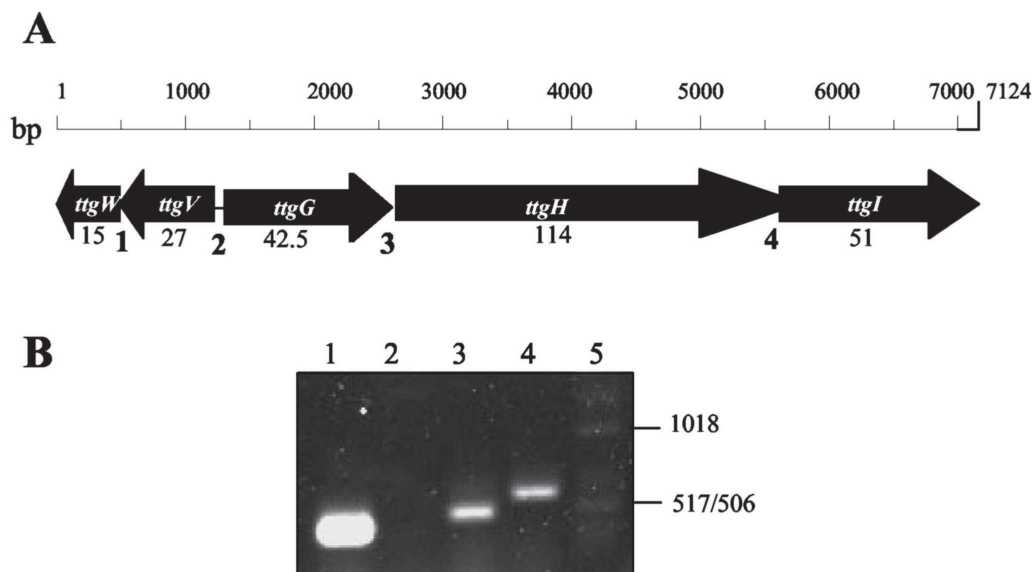

FIG. 1. Physical organization of the *ttgGHI-ttgVW* cluster and evidence that *ttgVW* is an operon. (A) The sequence of the *ttgGHI* and *ttgVW* operons can be accessed from GenBank (accession no. AF299253.2). The approximate sizes of the products of each ORF are given in kilodaltons below each gene. (B) The products resulting from each RT-PCR were separated on agarose gels, as described in Materials and Methods. Lane 1, *ttgV-ttgW* amplification (expected size, 398 bp); lane 2, *ttgV-ttgG* amplification (expected size, 437 bp); lane 3, *ttgG-ttgH* amplification (expected size, 503 bp); lane 4, *ttgH-ttgI* amplification (expected size, 606 bp); lane 5, molecular weight markers, whose sizes are provided on the right.

scribed in Materials and Methods, in cells growing in the absence or presence of toluene. Regardless of the growth conditions, the operon was transcribed from a single promoter (Fig. 2A). The 5′ end of the *ttgVW* mRNA starts at the G marked +1 in the sequence shown in Fig. 2A. The −10 and −35 regions of the *ttgV* promoter exhibit low similarity to promoters recognized by RNA polymerase with sigma-70 at the −10 region (5′-TGACGC-3′) and the −35 region (5′-TG TAGC-3′). The location of the start sites of the *ttgV* and *ttgG* genes indicates that the promoters of the divergently transcribed operons overlap (Fig. 2B). In fact, the −10 and −35 boxes of the *ttgVW* promoter totally overlap the −35 and −10 boxes, respectively, of the P_{G2} promoter (Fig. 2B).

The intensity of the cDNA bands determined densitometrically in Fig. 2A was used to estimate variations in the expression of *ttgV* in the absence or presence of toluene. We observed that in the presence of toluene the level of expression of the *ttgVW* operon was three- to fourfold higher than in the absence of the aromatic hydrocarbon. Our group has shown before that there was also a similar level of induction of the *ttgGHI* operon in the presence of toluene (43). Given that the TtgGHI efflux pump expels a large number of solvents and antibiotics, we decided to determine which of these compounds induced expression from the *ttgV* and *ttgG* promoters by analyzing the relative levels of mRNA expressed from these promoters in *P. putida* DOT-T1E cells growing in LB medium in the presence of solvents and of sublethal concentrations of antibiotics. We observed that both operons were induced about fourfold by toluene and styrene, but not in response to any of the antibiotics tested (i.e., carbenicillin, chloramphenicol, nalidixic acid, tetracycline, or gentamicin). The same results were obtained when expression was analyzed using fusions of the corresponding promoters to *′lacZ* in the low-copy-number promoter probe pMP220 (data not shown).

The set of results presented above indicates that the pattern of inducibility of the *ttgVW* operon is similar to that of the efflux pump *ttgGHI* operon, probably because the two promoters are regulated in the same way.

The *ttgV* gene product, but not that of the *ttgW* gene, is a repressor of the *ttgV* and *ttgG* promoters. We constructed three different mutant strains in the *ttgVW* operon as described in Materials and Methods: DOT-T1E-PS62 ($TtgV^-$ $TtgW^+$); DOT-T1E-PS61 ($TtgV^-$ $TtgW^-$), and DOT-T1E-PS52 ($TtgV^+$ $TtgW^-$). To determine the effect of these mutations on the expression of the *ttgGHI* and *ttgVW* operons, we used transcriptional fusions of the corresponding promoters to *′lac*Z. Plasmids pANA96 (P_{ttgG}::*lacZ*) and pANA95 (P_{ttgV}::*lacZ*) were transformed in the wild-type DOT-T1E strain and in the three mutant strains. β-Galactosidase activity was measured in the presence and absence of 3 mM toluene (Table 2), and we found that expression from the *ttgG* promoter was about four times higher in DOT-T1E-PS62 than in the wild-type strain in the absence of toluene (Table 2). This indicates that TtgV is a repressor that prevents the expression of the *ttgGHI* operon. Expression of the *ttgVW* promoter was about threefold higher in the *ttgV* mutant than in the wild-type strain in the absence of toluene, indicating that TtgV controls negatively its own expression.

Expression from the *ttgG* and *ttgV* promoters in the mutant background DOT-T1E-PS52 deficient in TtgW but proficient in TtgV was similar to that found in the wild-type strain (Table 2), which suggested that the TtgW protein, under our assay conditions, does not play a major role in the regulation of the expression from either of these two operons. The level of expression from the *ttgV* and *ttgG* promoters in the DOT-T1E-PS61 mutant deficient in TtgV and TtgW was similar to that obtained in mutant DOT-T1E-PS62 (*$ttgV^-$ $ttgW^+$*) (Table 2).

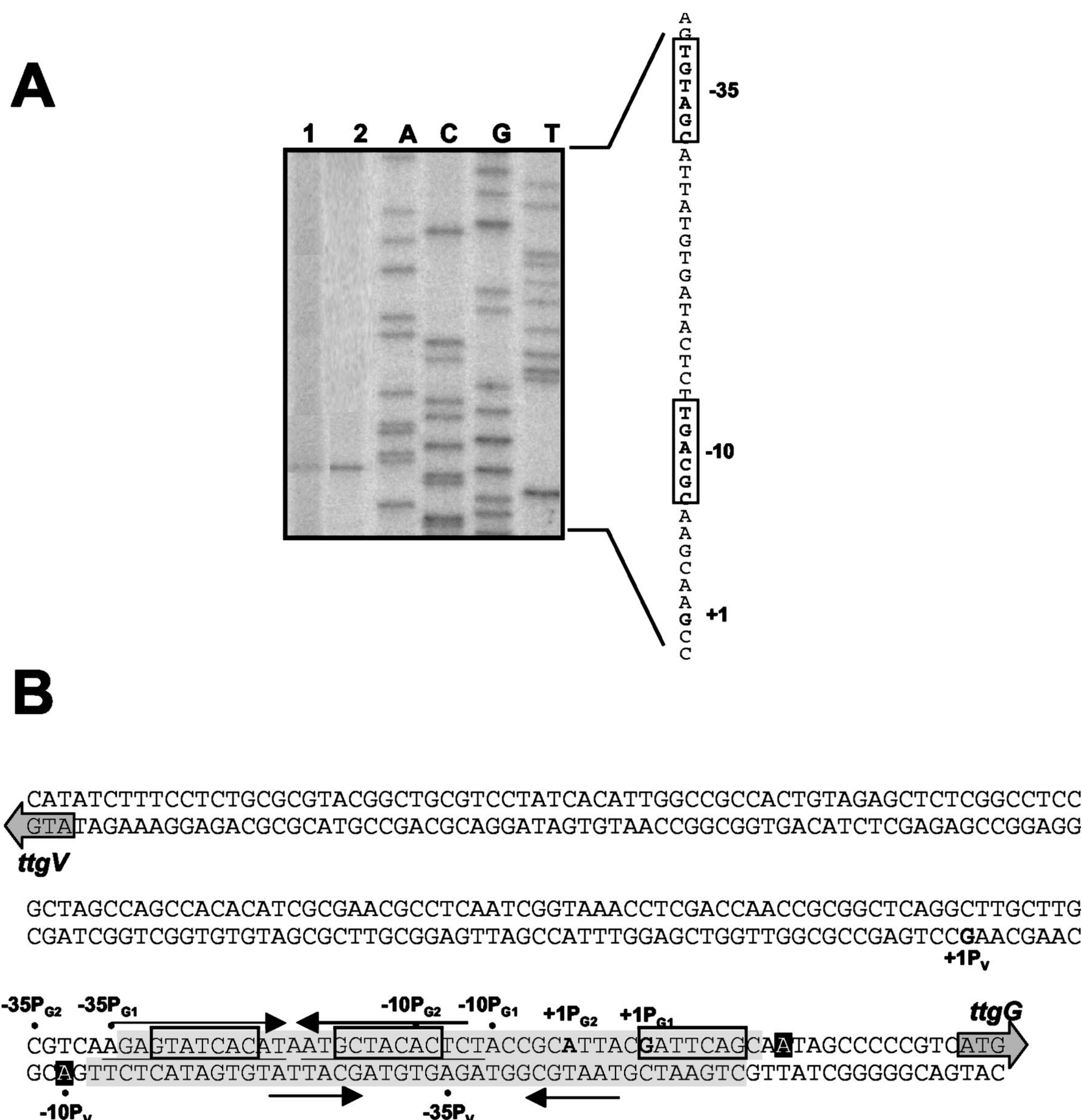

FIG. 2. Transcription initiation point of the *ttgV* gene in *P. putida* DOT-T1E growing in the absence or presence of toluene. (A) Transcription initiation mapping of the *ttgV* gene. RNA in lane 1 was isolated from cells grown in LB medium, whereas RNA in lane 2 was extracted from cells grown in LB medium with toluene supplied via the gas phase. The other lanes correspond to a sequencing ladder. The DNA sequence corresponding to the *ttgV* promoter region is shown on the right. The transcription initiation point is in boldface type and marked +1. The proposed −10 and −35 regions are boxed. (B) Overlap of the *ttgV* and *ttgG* promoters and binding region of TtgV protein as determined by in vitro DNaseI footprinting assays. The directions of transcription of *ttgVW* and *ttgGHI* are indicated by arrows followed by the notation *ttgV* or *ttgG*. The +1 of the single promoter for *ttgV* and the +1 of the tandem promoters for *ttgG* are in boldface type and designated $+1P_V$, $+1P_{G1}$, and $+1P_{G2}$, respectively. The −10 and −35 positions of each promoter are indicated. The region protected by TtgV is shaded, and hyperreactive sites are shown with white letters in black boxes. The potential inverted repeats are indicated by thin arrows, and potential direct repeats are boxed.

These results suggest that the TtgV protein is a repressor of its own synthesis as well as of the expression of the *ttgGHI* operon.

To further corroborate that TtgV is a repressor of expression from *ttgG* and *ttgV*, transcription from pANA95 and pANA96 was assayed in the heterologous *E. coli* DH5αF′ host with or without pANA126 that overproduced TtgV. We found that in the constructions in which TtgV was overproduced the level of expression for P_{ttgG} and P_{ttgV} was 10 to 20% of the level found in the strain without TtgV, which confirms that TtgV is a repressor of the expression of both operons.

The *ttgV* mutant is more resistant to toluene than is the wild-type strain. It has previously been reported that the TtgGHI efflux pump is involved in both intrinsic and induced resistance to organic solvents of *P. putida* DOT-T1E (43). Given that expression from P_{ttgG} increased in the mutant deficient in the TtgV protein, we analyzed solvent tolerance in the three mutant strains by determining the survival rate of the cells after a sudden 0.3% (vol/vol) toluene shock when the cultures were pregrown on LB liquid medium with or without toluene in the gas phase. Strain DOT-T1E-PS52 ($TtgV^+$ W^-)

TABLE 2. Transcription from the *ttgVW* and *ttgGHI* operon promoters determined as β-galactosidase activity using fusions of the operon promoters to *'lacZ*[a]

Fusion and strain	Genetic background	β-Galactosidase activity and growth conditions	
		Without toluene	With toluene
P_{ttgG}::*lacZ*			
Wild type	V^+ W^+	320 ± 20	1,400 ± 60
PS62	V^- W^+	1,100 ± 120	1,600 ± 250
PS61	V^- W^-	1,350 ± 120	1,500 ± 130
PS52	V^+ W^-	470 ± 50	1,230 ± 70
P_{ttgV}::*lacZ*			
Wild type	V^+ W^+	440 ± 80	1,600 ± 30
PS62	V^- W^+	1,100 ± 200	1,420 ± 200
PS61	V^- W^-	1,050 ± 150	1,720 ± 180
PS52	V^+ W^-	470 ± 30	1,310 ± 190

[a] The strains were transformed with pANA95 (P_{ttgG}::*lacZ*) or pANA96 (P_{ttgV}::*lacZ*), and cells were grown in LB medium in the absence or presence of 3 mM toluene. β-Galactosidase activity was determined 4 h later in duplicate samples. The data are rounded values of the averages of at least three independent assays.

survived the toluene shock to the same extent as wild-type DOT-T1E (Fig. 3A and C). The survival rate when the cells were not preinduced was around 1 cell per 10,000, whereas almost 1 in 10 cells survived the shock when the cultures were preinduced with toluene in the gas phase. These results correlate with the data presented above, i.e., since the expression of the efflux pump is not significantly increased in this mutant, the tolerance to toluene remains as in the wild type. However, in DOT-T1E-PS62 (*ttgV*$^-$ *ttgW*$^+$) and in the double mutant DOT-T1E-PS61 (*ttgV*$^-$ *ttgW*$^-$), we observed an increase in the survival rate of about 2 orders of magnitude, with the levels of survival being almost identical to the levels obtained when the cells were preinduced (compare Fig. 3A, B, and D). These results indicate that an increase in the expression of the TtgGHI efflux pump, such as that found in the mutant deficient in TtgV, increased the survival rate of cells that were suddenly exposed to toluene. Therefore, these results support the notion that TtgV is strongly involved in the solvent-dependent induction of the TtgGHI pump.

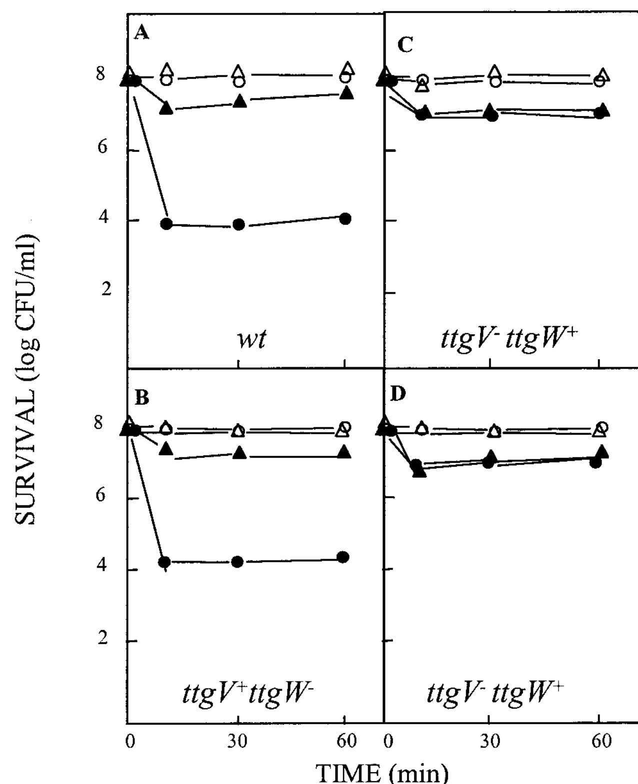

FIG. 3. Survival of *P. putida* DOT-T1E (A), DOT-T1E-PS62 (B), DOT-T1E-PS52 (C), and DOT-T1E-PS61 (D) after a sudden toluene shock. Cells were grown in 30 ml of LB medium (circles) or LB medium with toluene in the gas phase (triangles) until the culture reached a turbidity of about 0.8 at 660 nm. The culture was divided in two halves; to one we added 0.3% (vol/vol) toluene (closed symbols), while the other was kept as a control (open symbols). The number of viable cells was determined at the indicated times.

Rojas et al. (43) showed that the TtgGHI efflux pump contributed to resistance to ampicillin, chloramphenicol, and tetracycline only in a Δ*ttgABC* mutant background. This indicated a modest role for the TtgGHI pump in antibiotic efflux from the cell. We have now compared the sensitivities of the wild-type and the DOT-T1-PS52, -PS61, and -PS62 mutants to the three antibiotics cited above by determining the MICs that prevented growth. We found no difference in antibiotic resistance between each of the mutants and the wild-type strain (data not shown). These results suggest that the primary role of the TtgGHI efflux pump is to extrude solvents, whereas other antibiotic extrusion pumps operate efficiently in DOT-T1E (Terán et al., unpublished) so that the increased level of TtgGHI in the *ttgV* mutant backgrounds does not result in a significant increase in antibiotic resistance.

In vitro study of the interactions between the *ttgV* and *ttgG* promoter region and the TtgV protein. The results presented above suggest that the *ttgV* and *ttgG* promoters overlap and that the TtgV protein is a transcriptional repressor of the expression of both promoters. A gel mobility shift assay was done to study protein-DNA interactions within the *ttgV-ttgG* promoter region. A 210-bp DNA fragment containing the sequence between the first nucleotide before the start codon of *ttgV* and *ttgG* was synthesized by PCR and labeled with ^{32}P. When this fragment was incubated with increasing concentrations of TtgV, a single shifted band was found (Fig. 4). The retarded band was gradually lost in competition assays in which increasing concentrations of cold DNA probe were used, but not when unspecific competitor DNA [poly(dI-dC)] was used (data not shown). Based on the concentration of the protein that was used and on the degree of retard, we estimated that TtgV had a high affinity for its target, whose range is around 5×10^{-7} M.

Footprinting assays were carried out to define the region in which TtgV binds within the *ttgV-ttgG* intergenic region. A 228-bp DNA fragment, which contained the region between +134 and −94 of the *ttgV* promoter, was generated by PCR and labeled at each of its 5′ ends, incubated with or without His$_6$-TtgV, and then treated with DNase I as described in Materials and Methods. The digestion pattern for the two strands is shown in Fig. 5. The pattern revealed a 40-bp region where the abundance of certain fragments decreased as the TtgV concentration increased. The protected region covers the −10 and −35 regions of each of the *ttgG* promoters and the divergently oriented *ttgV* promoter (Fig. 1B). In fact, the pro-

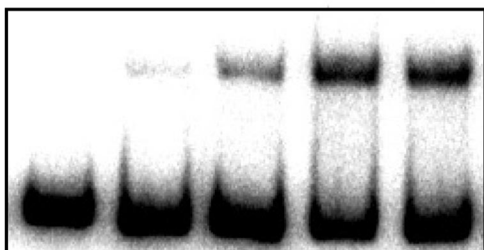

FIG. 4. Interaction of TtgV with the *ttgG-ttgV* intergenic region. The 210-bp (1 nM) fragment containing the *ttgG-ttgV* intergenic region was incubated without TtgV or with increasing concentrations of His_6-tagged TtgV (from 50 to 300 nM).

tected region extends from +7 to −34 with respect to the *ttgG1* promoter (+12 to −29 for the overlapping *ttgG2* promoter) and from −13 to −55 for the *ttgV* promoter (Fig. 1B). Given the overlapping organization of these promoters and the fact that TtgV represses the expression of both operons to the same extent, it is likely that the binding of TtgV to the intergenic region blocks the entry of RNA polymerase to transcribe these promoters (44).

We found two inverted repeats within the protected region: one located between −12 and −34 with respect to the *ttgG1* promoter (−7 to −29 for the *ttgG2* promoter and −12 to −36 with respect to *ttgV*) (Fig. 1B), and the other located between −2 and −24 in $P_{ttgG}1$ (+4 to −19 in $P_{ttgG}2$) and −24 to −46 in the *ttgV* promoter (Fig. 1B). If the first inverted repeat were the target for TtgV, then it is difficult to explain how TtgV protects the right-most region shown shaded in Fig. 2B. The position of the second inverted repeat is more central and could in principle explain why TtgV bound to this motif covers the adjacent region. It is also interesting to note the presence of three direct repeats in the protected region whose consensus sequence is 5′-GNA/TT/ACAC/G-3′ (Fig. 1B). Examples of transcriptional regulators that recognize direct repeats have been described in the AraC/XylS family of positive transcriptional regulators (10, 51). Although at present we cannot discern which nucleotides are specifically recognized by TtgV, it is worth noting that the QacR regulator interacts with its cognate promoters by recognizing direct repeats within inverted-repeat sequences (46). We cannot, therefore, exclude the possibility that TtgV binds to the left-most inverted repeat and that it recognizes a half site in the right-most protected region. Therefore, further in vitro assays using mutants in the potential target sequences and a wide series of footprint analyses are needed to define the precise motif recognized by TtgV.

In the *acrAB* operon of *E. coli*, the AcrR protein functions as the local specific regulator (22), but it has been shown that its level of expression is influenced by global regulators such as MarA, SoxS, and SdiA (22, 38). The genome of *P. putida* KT2440, a strain highly similar to DOT-T1E, was recently sequenced and annotated (30). Our BLASTN search revealed the presence of a SdiA homolog, but no MarA or SoxS ho-

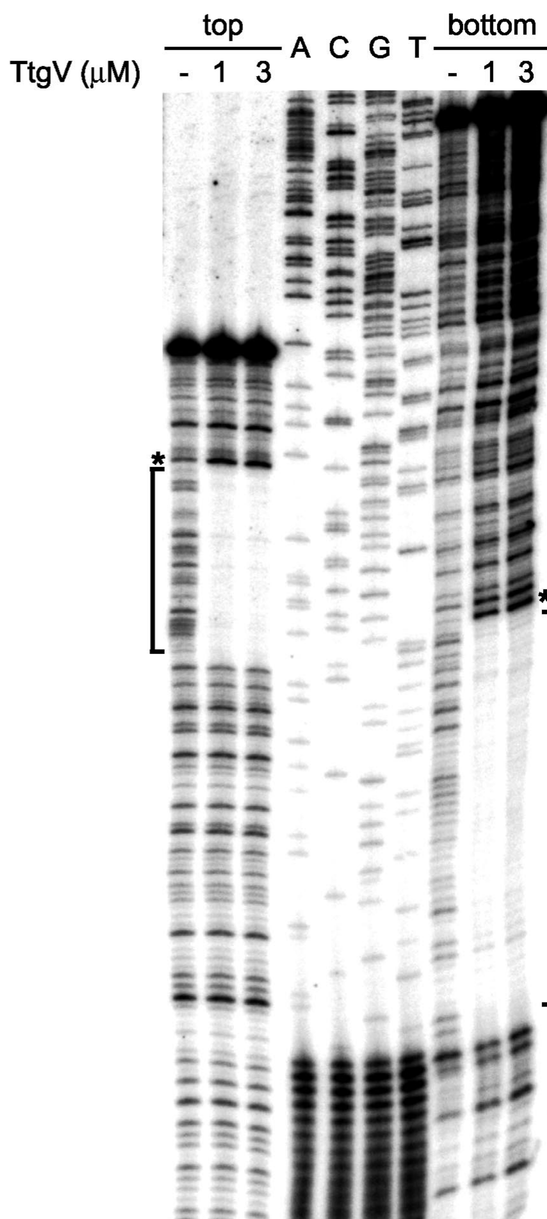

FIG. 5. Identification of the TtgV operator in the *ttgG-ttgV* intergenic region by DNase I footprinting. PCR fragments comprising the *ttgG-ttgV* intergenic region were labeled at one 5′ end and incubated without (−) or with a 1 or 3 μM concentration of purified TtgV before being subjected to DNaseI digestion and electrophoresis as described in Materials and Methods. The regions protected from DNase I digestion by TtgV are shown in brackets; asterisks indicate hyperactive sites. The sequence of the TtgV operator is marked in Fig. 2B.

mologs were found. Results from our laboratory seem to rule out the participation of SdiA in the control of *ttg* efflux pumps, because the overproduction of SdiA did not alter the expression pattern of a transcriptional fusion of each of the *ttg* promoters to *'lacZ* (E. Duque and J. L. Ramos, unpublished results). At present, we have no evidence that the *ttgGHI*/*ttgVW* system is integrated in any of the global regulatory circuits that operate in *Pseudomonas* sp., but future work with

the *ttgGHI*/*ttgVW* system should reveal more intimate details of its regulation.

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The first two coauthors contributed equally to this study.

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REFERENCES

1. **Adewoye, L., A. Sutherland, R. Srikumar, and K. Poole.** 2002. The MexR repressor of the *mexAB-oprM* multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. J. Bacteriol. **184:**4308–4312.
2. **Altschul, S. F., F. W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Amsterdam, D.** 1991. Susceptibility testing of antimicrobials in liquid media, p. 72–78. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams & Wilkins, Baltimore, Md.
4. **Ausubel, F. M., R. Brent, R. F. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Current protocols in molecular biology. Greene Publishing, New York, N.Y.
5. **Baranova, N., and H. Nikaido.** 2002. The BaeSR two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. J. Bacteriol. **184:**4168–4176.
6. **Baumeister, R., P. Flache, O. Melefors, A. von Gabain, and W. Hillen.** 1991. Lack of a 5′ non-coding region in Tn*1721* encoded *tetR* mRNA is associated with a low efficiency of translation and a short half-life in *Escherichia coli*. Nucleic Acids Res. **19:**4595–4600.
7. **Cruden, D. L., J. H. Wolfram, R. D. Rogers, and D. T. Gibson.** 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. **58:** 2723–2729.
8. **de Smet, M. J., J. Kingma, and B. Witholt.** 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. Biochim. Biophys. Acta **506:**64–80.
9. **Duque, E., A. Segura, G. Mosqueda, and J. L. Ramos.** 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. Mol. Microbiol. **39:**1100–1106.
10. **Egan, S. M.** 2002. Growing repertoire of AraC/XylS activators. J. Bacteriol. **184:**5529–5532.
11. **Elkins, C. A., and H. Nikaido.** 2002. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. J. Bacteriol. **184:**6490–6498.
12. **Hagman, K. E., and W. M. Shafer.** 1995. Transcriptional control of the *mtr* efflux system of *Neisseria gonorrhoeae.* J. Bacteriol. **177:**4162–4165.
13. **Hillen, W., and C. Berens.** 1994. Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. Annu. Rev. Microbiol. **48:**345–369.
14. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentrations of toluene. Nature **338:**264–265.
15. **Isken, S., and J. A. de Bont.** 1996. Active efflux of toluene in a solvent-resistant bacterium. J. Bacteriol. **178:**6056–6058.
16. **Kieboom, J., J. J. Dennis, J. A. M. de Bont, and G. J. Zylstra.** 1998. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J. Biol. Chem. **273:**85–91.
17. **Kieboom, J., J. J. Dennis, G. J. Zylstra, and J. A. M. de Bont.** 1998. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J. Bacteriol. **180:**6769–6772.
18. **Kim, K., S. Lee, K. Lee, and D. Lim.** 1998. Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J. Bacteriol. **180:**3692–3696.
19. **Kojic, M., C. Aguilar, and V. Venturi.** 2002. TetR family member PsrA directly binds the *Pseudomonas rpoS* and *psrA* promoters. J. Bacteriol. **184:** 2324–2330.
20. **Koronakis, V., A. Sharft, E. Koronakis, B. Luisi, and C. Hughes.** 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature **405:**914–919.
21. **Li, X., L. Zhang, and K. Poole.** 1998. Organic solvent-tolerant mutants of *Pseudomonas aeruginosa* display multiple antibiotic resistance. J. Bacteriol. **180:**2987–2991.
22. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of the *acrAB* gene *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
23. **Marqués, S., J. L. Ramos, and K. N. Timmis.** 1993. Analysis of the mRNA structure of the *Pseudomonas putida* TOL *meta* fission pathway operon around the transcription initiation point, the *xylTE* and the *xylFJ* region. Biochim. Biophys. Acta **1216:**227–237.
24. **Meier, I., L. V. Wray, and W. Hillen.** 1988. Differential regulation of the Tn*10*-encoded tetracycline resistance genes *tetA* and *tetR* by the tandem *tet* operators O1 and O2. EMBO J. **7:**567–572.
25. **Ménard, R., P. J. Sansonetti, and C. Parsot.** 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. **175:**5899–5906.
26. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Mosqueda, G., and J. L. Ramos.** 2000. A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1.E is linked to the *tod* genes for toluene metabolism. J. Bacteriol. **182:**937–943.
28. **Murakami, S., R. Nakashima, E. Yamashita, and A. Yamaguchi.** 2002. Crystal structure of bacterial multidrug efflux transporter AcrB. Nature **419:** 587–593.
29. **Nagakubo, S., K. Nishino, T. Hirata, and A. Yamaguchi.** 2002. The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. J. Bacteriol. **184:**4161–4167.
30. **Nelson, K. E., I. T. Paulsen, C. Weinel, R. J. Dodson, H. Hilbert, D. Fouts, S. R. Gill, M. Pop, M. Holmes, V. Martins Dos Santos, H. Khouri, I. Hance, P. C. Lee, E. Holtzapple, D. Scanlan, K. Tran, R. Deboy, A. Moazzez, L. Brinkac, M. Beanan, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Duesterhoft, B. Tümmler, and C. M. Fraser.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**799–808.
31. **Paulsen, I. T., J. Chen, K. E. Nelson, and M. H. Saier, Jr.** 2001. Comparative genomics of microbial drug efflux systems. J. Mol. Microbiol. **3:**145–150.
32. **Pérez-Martin, J., and V. de Lorenzo.** 1996. ATP binding to the sigma 54-dependent activator XylR triggers a protein multimerization cycle catalyzed by UAS DNA. Cell **86:**331–339.
33. **Poole, K.** 2001. Multidrug resistance in gram-negative bacteria. Curr. Opin. Microbiol. **4:**500–508.
34. **Poole, K., and R. Srikumar.** 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr. Top. Med. Chem. **1:**59–71.
35. **Poole, K., K. Tetro, Z. Zhao, S. Neshat, D. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
36. **Popp, R., T. Kohl, P. Patz, G. Trautwein, and U. Gerischer.** 2002. Differential DNA binding of transcriptional regulator PcaU from *Acinetobacter* sp. strain ADP1. J. Bacteriol. **184:**1988–1997.
37. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
38. **Rahmati, S., S. Yang, A. L. Davidson, and E. L. Zechiedrich.** 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. Mol. Microbiol. **43:**677–685.
39. **Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-González, A. Rojas, W. Terán, and A. Segura.** 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Annu. Rev. Microbiol. **56:**743–768.
40. **Ramos, J. L., E. Duque, P. Godoy, and A. Segura.** 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J. Bacteriol. **180:**3323–3329.
41. **Ramos, J. L., E. Duque, M. J. Huertas, and A. Haïdour.** 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J. Bacteriol. **177:**3911–3916.
42. **Ramos, J. L., E. Duque, J. J. Rodríguez-Herva, P. Godoy, A. Haïdour, F. Reyes, and A. Fernández-Barrero.** 1997. Mechanisms for solvent tolerance in bacteria. J. Biol. Chem. **272:**3887–3890.
43. **Rojas, A., E. Duque, G. Mosqueda, G. Golden, A. Hurtado, J. L. Ramos, and A. Segura.** 2001. Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. J. Bacteriol. **183:**3967–3973.
44. **Rojo, F.** 1999. Repression of transcription initiation in bacteria. J. Bacteriol. **181:**2987–2991.
45. **Saier, M. H., and I. T. Paulsen.** 2001. Phylogeny of multidrug transporters. Semin. Cell Dev. Biol. **12:**205–213.
46. **Schumacher, M. A., M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, and R. G. Brennan.** 2002. Structural basis for cooperative DNA binding by two dimers of the multidrug-binding protein QacR. EMBO J. **21:**1210–1218.
47. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59:**201–222.
48. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. Plant Mol. Biol. **9:**27–39.
49. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. Clustal W: improv-

ing the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
50. **Tikhonova, E. B., Q. Wang, and H. I. Zgurskaya.** 2002. Chimeric analysis of the multicomponent multidrug efflux transporters from gram-negative bacteria. J. Bacteriol. **184:**6499–6507.
51. **Tobes, R., and J. L. Ramos.** 2002. AraC-XylS database: a family of positive transcriptional regulators in bacteria. Nucleic Acids Res. **30:**318–321.
52. **Weber, F. J., S. Isken, and J. A. M. de Bont.** 1994. *Cis/trans* isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. Microbiology **140:**2013–2017.
53. **Wery, J., B. Hidayat, J. Kieboom, and J. A. M. de Bont.** 2001. An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. J. Biol. Chem. **276:**5700–5706.
54. **Zgurskaya, H., and H. Nikaido.** 1999. AcrA is a highly asymmetric protein capable of spanning the periplasm. J. Mol. Biol. **285:**409–420.

CAPÍTULO 2.

El regulador TtgV unido a un operador complejo reprime la transcripción del promotor de la bomba de eflujo de disolventes TtgGHI

Autores: Guazzaroni, M.E., Terán, W., Zhang, X., Gallegos, M.T. y J.L. Ramos.

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La bomba de eflujo TtgGHI de *Pseudomonas putida* extruye una variedad de disolventes orgánicos y antibióticos. En este trabajo hemos demostrado que el operón *ttgGHI* se transcribe tanto *in vivo* como *in vitro* desde un único promotor y no desde dos promotores solapantes como había sido propuesto inicialmente. La expresión de este promotor es controlada por el represor TtgV, cuyo operador comprende cuatro vueltas de hélice del ADN en el cual solapa la región -10 del promotor *ttgG*. También hemos puesto de manifiesto que TtgV se libera de su operador por la unión de efectores tales como alcoholes alifáticos. Un análisis mutacional del promotor *ttgGHI* reveló que la substitución de las posiciones -13, -12 y -8 dan lugar a promotores que son incapaces de dirigir la transcripción. Por otro lado, substituciones de las posiciones -9, -11 y de -6 a -3 llevaron a un aumento de la transcripción. La causa del aumento de la expresión fue una disminución de la afinidad de unión del represor TtgV por su operador o un aumento en la afinidad de la ARN polimerasa por los promotores mutantes.

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TtgV Bound to a Complex Operator Site Represses Transcription of the Promoter for the Multidrug and Solvent Extrusion TtgGHI Pump

María-Eugenia Guazzaroni,[1] Wilson Terán,[1] Xiaodong Zhang,[2] María-Trinidad Gallegos,[1] and Juan L. Ramos[1]*

Department of Biochemistry and Molecular and Cell Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, E-18008 Granada, Spain,[1] and Center for Structural Biology, Department of Biological Sciences, Imperial College, London, United Kingdom[2]

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The TtgGHI efflux pump of *Pseudomonas putida* extrudes a variety of antibiotics and solvents. We show that the *ttgGHI* operon is transcribed in vitro and in vivo from a single promoter and not from two overlapping promoters as previously proposed. The expression of this promoter is controlled by the TtgV repressor, whose operator expands through four helical turns that overlap the −10 region of the promoter. We also show that TtgV is released from its operator on binding of effectors such as aliphatic alcohols. Mutational analysis of the *ttgGHI* promoter revealed that substitutions at −13, −12, and −8 yielded promoters that were unable to drive transcription whereas certain mutations at −9, −11, and −6 to −3 increased expression in vivo. The cause of the increased expression was either a decrease in the affinity of the TtgV protein for its operator or an increase in the affinity of RNA polymerase for the mutant promoters.

Multidrug efflux pumps are widely distributed among prokaryotic and eukaryotic organisms (6, 16–18, 25, 29). In bacteria, resistance to a number of antibiotics, superoxide-generating agents, dyes, and organic solvents is mediated by different families of multidrug transporters. Toluene, styrene, and toxic solvents with log P_{ow} values between 1.5 and 3.5 (i.e., the logarithm of the partition coefficient of the target compound in a mixture of octanol and water) partition preferentially in the cell membrane, leading to the disorganization of the inner membrane, which causes cell death. In solvent-tolerant gram-negative bacteria, these organic solvents are removed by pumps which belong to the resistance-nodulation-division (RND) family, so that the solvents are kept below their toxicity threshold (19).

RND efflux pumps are made of three components: an inner membrane transporter (13, 30), an outer membrane channel (8), and a lipoprotein anchored in the inner membrane that extends in the periplasm (31) and that is probably involved in the correct assembly of the other two elements and is needed for optimal functioning of the efflux pump. These efflux pumps are energized by a proton motive force (15).

Pseudomonas putida DOT-T1E is able to thrive in liquid medium containing toluene (21). This strain exhibits an innate high resistance to solvents, which increases when bacteria are preexposed to sublethal concentrations of toluene (2, 20, 23). This is achieved through the cooperative efflux of the solvent by three RND pumps called TtgABC, TtgDEF, and TtgGHI (12, 23). The expression of *ttgABC* is constitutive, and its level does not vary significantly in the presence of toluene (2). The *ttgDEF* operon is not expressed at all in the absence of solvents, and its expression is higher in the presence of aromatic hydrocarbons (23). The *ttgGHI* operon is expressed at a basal level in the absence of solvents, and its expression increases about threefold in response to toluene. The TtgGHI efflux pump plays a pivotal role in the innate and induced tolerance to solvents in this strain (12, 23), and a *ttgH* knockout mutant is extremely sensitive to solvent shocks (22).

We suggested that *ttgGHI* expression occurred from two overlapping tandem promoters (23) and that it was controlled by the *ttgV* gene product (22). In the present study we show that expression from the *ttgGHI* operon takes place from a single promoter and that solvents such as 1-hexanol trigger the release of the repressor from the operator site, allowing transcription.

* Corresponding author. Mailing address: Consejo Superior de Investigaciones Científicas, Biochemistry and Molecular and Cell Biology of Plants, Calle Profesor Albareda, no. 1, E-18008 Granada, Spain. Phone: 34 958 181608. Fax: 34 958 135740. E-mail: jlramos@eez.csic.es.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture medium. The bacterial strains, cloning vectors, and plasmids constructed in this study are listed in Table 1. Bacterial strains were grown in Luria-Bertani (LB) medium at 30°C as described previously (22). Liquid cultures were shaken on an orbital platform operating at 200 rpm. *Escherichia coli* DH5α was used as the host strain to construct and maintain different plasmids (1). *P. putida* cultures were supplemented with solvents at 3 mM when indicated. When required, cultures were supplemented with the following antibiotics: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; rifampin, 20 μg/ml; and tetracycline, 20 μg/ml.

Nucleic acid techniques. DNA preparation, digestion with restriction enzymes, analysis by agarose gel electrophoresis, isolation of DNA fragments, ligations, and transformations were done by standard procedures (1). Plasmid DNA was sequenced on both strands with specifically designed primers, using an automatic DNA sequencer (ABI-PRISM 3100; Applied Biosystems). *P. putida* cells were electroporated done as described previously (23). RNA preparation and primer extension were done as described by Marqués et al. (11).

For footprint assays, we used the TtgV protein prepared as described by Rojas et al. (22). DNase I footprint assays were carried out as described previously for

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. putida DOT-T1E	Rif^r	21
E. coli DH5α	*recA1*	1
Plasmids		
pANA96	Tc^r, *ttgG* promoter cloned in pMP220	22
pET28b(+):ttgV	Ap^r, vector use to produce TtgV	22
pGG1	pUC18 bearing an 8-kb BamHI fragment with *ttgGHI* and *ttgVW*	23
pMP220	Tc^r, promoterless *lacZ* expression vector	26
pMEx[b]	Mutant *ttgG* promoter cloned in pMP220	This study
pET103-P $_{ttgG}$	Ap^r, promoter of *ttgG* cloned upstream of the T7 terminator in pET103	This study

[a] Ap^r, Km^r, Rif^r, and Tc^r stand for resistance to ampicillin, kanamycin, rifampin, and tetracycline, respectively.
[b] x indicates the plasmid number bearing different mutant promoters fused to *'lacZ*.

identification of the operator to which TtgV binds (22), whereas dimethyl sulfate (DMS) footprint analyses were carried out as described by Ausubel et al. (1).

Electrophoretic mobility shift assay (EMSA). A 210-bp DNA fragment containing the sequence between the nucleotides before the first start codons of *ttgV* and *ttgG* was amplified by PCR from plasmid pGG1 using the following set of primers: 5′-GGAATTCATCTTTCCTCTGCGCGTACG-3′ and 5′-AACTGCA GACGGGGGCTATTGCTGAATCG-3′. Cycling parameters were 4 min at 96°C followed by 30 cycles at 96°C for 1 min, 60°C for 30 s, and 72°C for 30s, and ending with 3 min at 72°C. These fragments were isolated from agarose gels and end labeled with ^{32}P as described previously (22, 27). About 1 nM labeled DNA ($\sim 1.5 \times 10^4$ cpm) was incubated with increasing amounts of purified TtgV for 10 min at 30°C in 10 μl of TGED binding buffer (10 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) containing 20 μg of poly(dI-dC) per ml and 200 μg of bovine serum albumin per ml. Reaction mixtures were electrophoresed in a nondenaturing 4.5% (wt/vol) polyacrylamide gel in Tris-glycine buffer (0.2 M glycine, 0.025 M Tris-HCl [pH 8.6]). The results were analyzed with Molecular Imager FX equipment (Bio-Rad, Madrid, Spain).

Construction of P_{ttgG} mutant promoters by PCR. The P_{ttgG} mutant promoters were generated by overlap extension PCR mutagenesis, as described previously (5). The internal oligonucleotide primers used for mutagenesis exhibited one mismatch with the wild-type sequence. After DNA amplification, the resulting DNA was digested with EcoRI and PstI and the 210-bp EcoRI-PstI P_{ttgG} mutant was inserted between the EcoRI and PstI sites of pMP220 to yield plasmid pMEx (the x indicates the plasmid number). All mutant P_{ttgG} promoters generated in this study were confirmed by DNA sequencing.

Single-round in vitro transcription assays with supercoiled plasmid DNA. Reactions (20-μl reaction mixtures) were performed with STA buffer (10 mM Tris-acetate [pH 8.0], 8 mM magnesium acetate, 3.5% [wt/vol] polyethylene glycol, 10 mM KCl, 1 mM dithiothreitol) containing 10 or 100 nM σ^{70}-holoenzyme (Epicentre), 20 U of RNAsin (Promega), and around 10 nM supercoiled plasmid DNA template. The reaction mixtures were incubated for 10 min at 30°C before the addition of the following elongation mixture: 0.1 mM each ATP, CTP, GTP, and UTP; 0.3 μCi of [α-^{32}P]UTP (20 μCi/μl); and 100 μg of heparin per ml. After a further 10-min incubation at 30°C, the reactions were stopped by chilling to 4°C and the products were precipitated with 0.25 volume of 10 M ammonium acetate and 2.5 volume of ethanol. The pellets were washed with 80% (vol/vol) ethanol. Dried pellets were resuspended in 8 μl of water plus 4 μl of formamide sequencing dye. Samples were electrophoresed in a 6.5% (wt/vol) polyacrylamide denaturing sequencing gel. The results were analyzed using Molecular Imager GS525 equipment with Quantity One software (Bio-Rad, Madrid, Spain).

RESULTS

In vitro evidence suggests that the *ttgGHI* promoter is expressed from a single promoter rather than two overlapping promoters. Rojas et al. (23) suggested that in *P. putida* DOT-T1E, the *ttgGHI* operon was transcribed from two promoters, P_{ttgG1} and P_{ttgG2}, since two potential transcription start points separated by 5 nucleotides were found in vivo regardless of the growth phase and growth in the absence or presence of toluene. To determine whether the two bands represented two promoters or whether the 5-nucleotide-shorter band was a degradation product of the larger one, we carried out independent in vitro transcription assays with two plasmids carrying the *ttgGHI* promoter region upstream of the early phage T7 terminator. We found that with RNA polymerase in excess, the *ttgGHI* promoter drove the synthesis of a single mRNA (Fig. 1A). The RNAs produced from these plasmids were 320 and 141 nucleotides. Based on the size, we determined that the transcription start point of the in vitro mRNA corresponded to the longer mRNA obtained in vivo. In further assays, we used the plasmid that yielded the 320-nucleotide mRNA. To confirm that the *ttgG* promoter was regulated by TtgV, we carried out in vitro transcription assays in the presence of 0.5, 1, and 5 μM TtgV, which was added before or after the RNA polymerase. When TtgV was added before the RNA polymerase, transcription from the *ttgGHI* promoter decreased to almost undetectable levels for the highest TtgV concentration tested (Fig. 1A). However, when TtgV was added after the RNA polymerase, inhibition of transcription was moderate and the amount of mRNA synthesized was 70 to 80% of that produced in the absence of TtgV. As a control we used a promoter (P_{sp}) of the plasmid that yielded a 488-nucleotide mRNA (Fig. 1A). As expected, the level of mRNA synthesized from the P_{sp} promoter did not change significantly in the presence of TtgV. These results suggested that RNA polymerase may recognize a single promoter upstream from the *ttgGHI* operon and that modulation of its expression is mediated by TtgV.

Site-directed mutagenesis of the region upstream from the *ttgGHI* promoter. The position of the −10 box in promoters is variable. To define with precision the promoter of the *ttgGHI* operon, we mutagenized the region of this promoter from positions −3 to −16 (Fig. 1B) by introducing as many single-point mutations as possible using oligonucleotide site-directed mutagenesis (Table 2) and analyzed the mutant promoters for expression in vivo by using fusions of the mutant promoters to *'lacZ*, and in vitro by performing in vitro transcription assays.

The transcriptional fusions to *'lacZ* revealed that point mutations at positions −13, −12, and −8 produced mutants with negligible activity in comparison to the wild-type promoter (Table 2). Mutations at −10 (A→C or G), −9 (C→G or T), and −7 (C→G or T) also produced mutant promoters with lower activity than the wild-type promoter (the activities

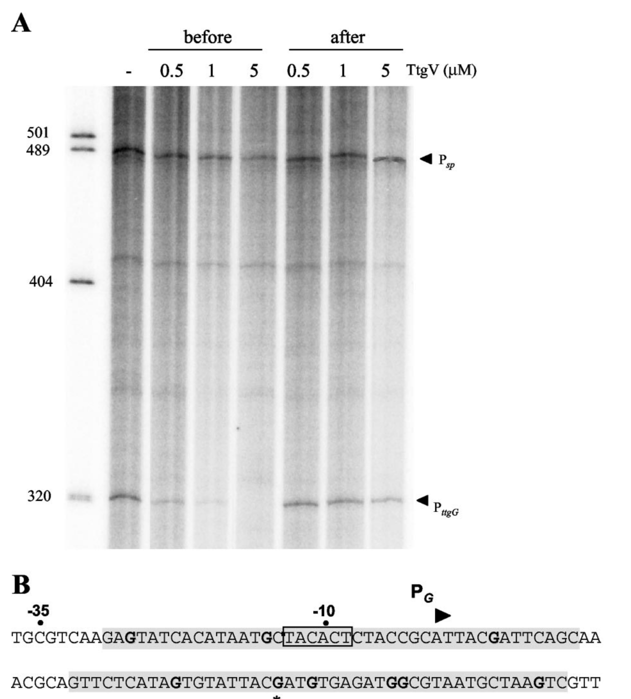

FIG. 1. In vitro transcription from P_{ttgG} in pET103-P_{ttgG}. (A) Single-round transcription assays were carried out as described in Materials and Methods. The assays were performed for 10 min at 30°C in the absence of TtgV (−) or in the presence of 0.5, 1, or 5 μM TtgV that was added before or after the addition of 100 nM RNA polymerase. The 320- and 488-nucleotide mRNAs synthesized from P_{ttgG} and P_{sp}, respectively, are indicated by arrowheads. (B) Binding region of TtgV protein as determined by in vitro DNase I and DMS footprinting assays. The region protected by TtgV deduced from DNase I digestion is shaded. The protected G's (DMS footprinting) are shown in bold. The asterisk identifies a hyperreactive position. The −10 and −35 positions of P_{ttgG} are indicated by dots. The +1 position and the direction of transcription are marked with a triangle.

TABLE 2. β-Galactosidase expression with fusions of the wild-type *ttgG* promoter or single-point *ttgG* mutant promoters to *'lacZ*[a]

Location and base changed	β-Galactosidase activity[b]	
	−Toluene	+Toluene
Wild type	290	700
T-16→A	280	725
T-13→A	5	10
T-13→G	10	10
T-13→C	2	2
A-12→C	10	8
A-12→G	7	10
A-12→T	10	15
C-11→A	570	610
C-11→T	2,755	3,360
A-10→C	130	235
A-10→G	70	150
C-9→A	1,690	2,700
C-9→G	68	180
C-9→T	175	390
T-8→A	10	30
T-8→G	10	22
T-8→C	3	4
C-7→G	15	30
C-7→T	180	465
T-6→A	15	40
T-6→C	455	1,100
T-6→G	460	1,080
A-5→C	265	710
C-4→A	1,410	1,560
C-4→G	1,580	1,825
C-3→G	1,570	1,820

[a] *P. putida* DOT-T1E bearing pANA96 (P_{ttgG}::*'lacZ*) or pMEx bearing a fusion of the indicated mutant P_{ttgG} promoter to *'lacZ* were grown on Luria-Bertani medium with tetracycline in the absence or presence of 3 mM toluene. After 4 h of incubation, the β-galactosidase activity (Miller units) in permeabilized cells was determined in triplicate (3).

[b] Values are the average of three to five independent duplicate assays. Standard deviations were below 10% of the given values.

ranged between 23 and 62% of that of the wild type). We also found that certain mutant promoters had higher activity than the wild type (between 1.9- and 9.3-fold that of the wild-type): C-11→T, C-9→A, T-6→C or G, C-4→A or G, and C-3→G (Table 2). Mutations at −14 and −16 had no significant effect on the level of expression of the promoter. Primer extension assays confirmed the results of the β-galactosidase assays; namely, when we found no β-galactosidase activity, we found no mRNA, and when we found lower or higher β-galactosidase activity than those in the wild type, the mRNA levels estimated by primer extension correlated with the results of the enzymatic assay (data not shown).

In vitro transcription assays with mutant promoters revealed that whereas mutant promoters altered at −13, −12, −8 and other mutants altered at −7 and −6 were not transcribed in vitro, promoters altered at any of the other positions were transcribed (data not shown).

Given that mutations at positions −13, −12, and −8 yielded no β-galactosidase activity (or very low levels of activity) and did not drive the expression of mRNA in vivo and in vitro, we considered that these positions delimited the −10 hexamer with the 5′-TACACT-3′ sequence. This sequence exhibits four of six identities to the −10 consensus of the promoters recognized by σ^{70} (5′-TATAAT-3′) (9).

Why do certain point mutations in the *ttgG* promoter result in an increase in expression? DNase I footprint assays showed that TtgV covered four DNA helical turns that overlap the *ttgGHI* promoter (22). The increase in expression of certain mutant promoters (i.e., C-3→G, C-4→G or A, C-9→A, and C-11→T) in the *ttgG* promoter may have resulted from either an improved −10 box for RNA polymerase or a decrease in the affinity of TtgV for its target sequences. We tested whether the mutant promoters that exhibited a higher activity than the wild type in vivo (i.e., C-4→A or G, C-3→G, C-9→A, and C-11→T) also exhibited increased expression in vitro with respect to the wild-type promoter. These assays were done with limiting amounts of RNA polymerase. The level of transcripts obtained in vitro from mutant promoters C-4→A or G and C-3→G was similar to that obtained from the wild-type promoters (see Fig. 3 for the C-4→A mutant), whereas with mutant promoters C-9→A and C-11→T, the level of expression was higher (Fig. 2). We suggest that the increased expression with C-9→A and C-11→T may have resulted from a promoter whose sequence, TATACT in C-11→T and TACAAT in C-9→A, was more similar to the consensus sequence since only one mismatch was found in each mutant promoter.

To explain the higher expression in vivo of the other mutant

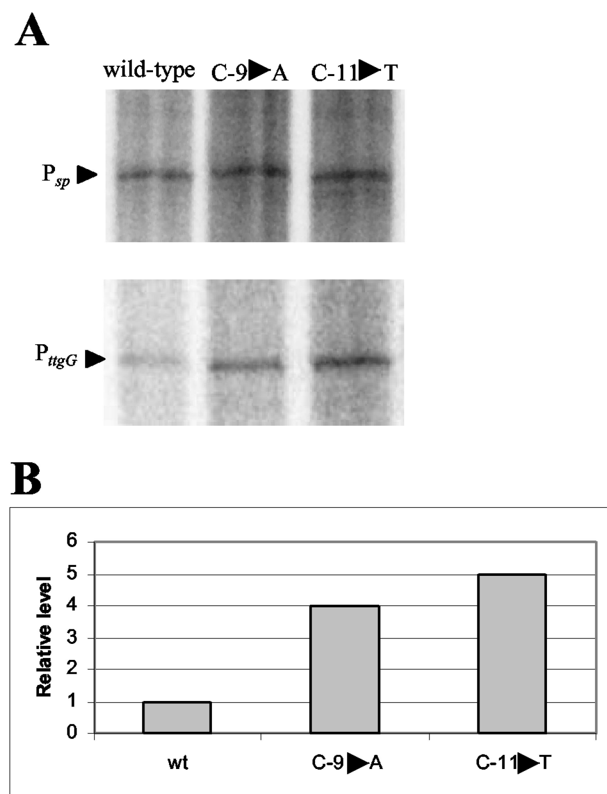

FIG. 2. Single-round transcription in vitro with limiting amounts of RNA polymerase. Conditions are as described in the legend to Fig. 1A, except that 10 nM RNA polymerase was used instead of 100 nM to transcribe the wild-type and the indicated mutant promoters. (A) Section of the gel corresponding to the control P_{sp} promoter, giving rise to a 488-nucleotide mRNA, and the wild-type P_{ttgG} or its single-point mutation derivative. (B) Relative levels of transcripts from P_{ttgG}. We assigned a relative value of 1 to the amount made from the wild type.

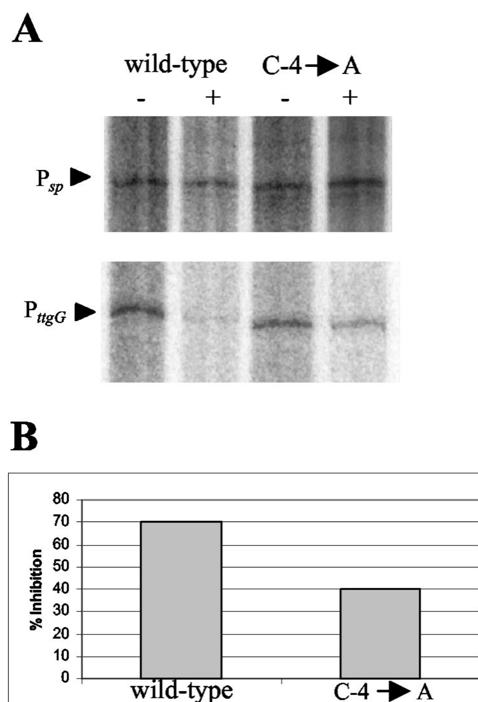

FIG. 3. In vitro transcription from P_{ttgG} and a single-point mutant (C-4→A) in the absence and presence of TtgV. (A) Single-round in vitro transcription assays were carried out as described in the legend to Fig. 1A. The templates contained the wild-type promoter or the mutant (C-4→A) *ttgG* promoter. The TtgV protein was either not present (−) or added at a concentration of 0.5 μM prior to the addition of RNA polymerase (+). (B) Relative levels of transcription inhibition for each of the promoters tested. The level of transcription was considered 100% for each promoter in the absence of TtgV. The degree of transcription inhibition was calculated from the equation (relative level in the presence of TtgV/relative level in the absence of TtgV) × 100.

promoters (C-4→A or G, C-3→G), we hypothesized that the affinity of TtgV for its operator might be reduced in the C-4→A or -G and C-3→G promoters and that therefore it competes less effectively with the RNA polymerase for its binding site. To test this hypothesis we performed single-round transcription assays with the wild-type and mutant promoters. While the addition of 0.5 μM TtgV prior to RNA polymerase led to 70% inhibition in the in vitro transcription assay with the wild-type promoter, inhibition with the mutant promoters was only around 40%. Figure 3 shows the results obtained for the mutant promoter C-4→A. Similar results were obtained for the C-3→G mutant (data not shown).

We also carried out EMSAs with the wild-type and mutant promoters. Figure 4 shows the results for the wild-type and mutant promoters C-4→A and C-9→A. We found that the C-4→A mutant promoter was less likely to be bound by TtgV. Similar results were obtained when the mutant promoters C-4→G or C-3→G were used (data not shown). In contrast, when EMSAs were done with the up mutant promoter C-9→A, the results were similar to those obtained with the wild-type promoter (Fig. 4), which supports the notion that increased expression in this mutant promoter could be the result of a higher affinity of the RNA polymerase for the mutant promoter.

1-Hexanol is an effector for TtgV and releases the repressor from its binding site in P_{ttgG}. Rojas et al. (22) showed that the TtgGHI efflux pump expels a large number of aromatic hydrocarbons (toluene, styrene, and ethylbenzene), aliphatic alcohols (1-octanol and 1-hexanol), and antibiotics such as ampicillin, carbenicillin, nalidixic acid, and tetracycline. We determined the in vivo effector profile for TtgV by measuring β-galactosidase activity using P_{ttgG}::′*lacZ* fusions in the presence of different compounds. Our assays revealed that antibiotics were not inducers of the expression of the *ttgGHI* operon, in accordance with the results of Rojas et al. (22). Aromatic compounds such as toluene, styrene, propylbenzene, *m*-xylene, and indole and aliphatic alcohols, i.e., 1-octanol and 1-hexanol, induced expression of the operon between 2.3- and 3-fold (data not shown). The in vivo assays did not reveal whether this was due to a direct or an indirect effect on the TtgV repressor. When we tested the effect of 1-hexanol on TtgV target binding in EMSA, we observed that hexanol had an in vitro effect on the binding of TtgV to the *ttgGHI-ttgV* operator site. Figure 5 shows the increase in dissociation of TtgV from the operator

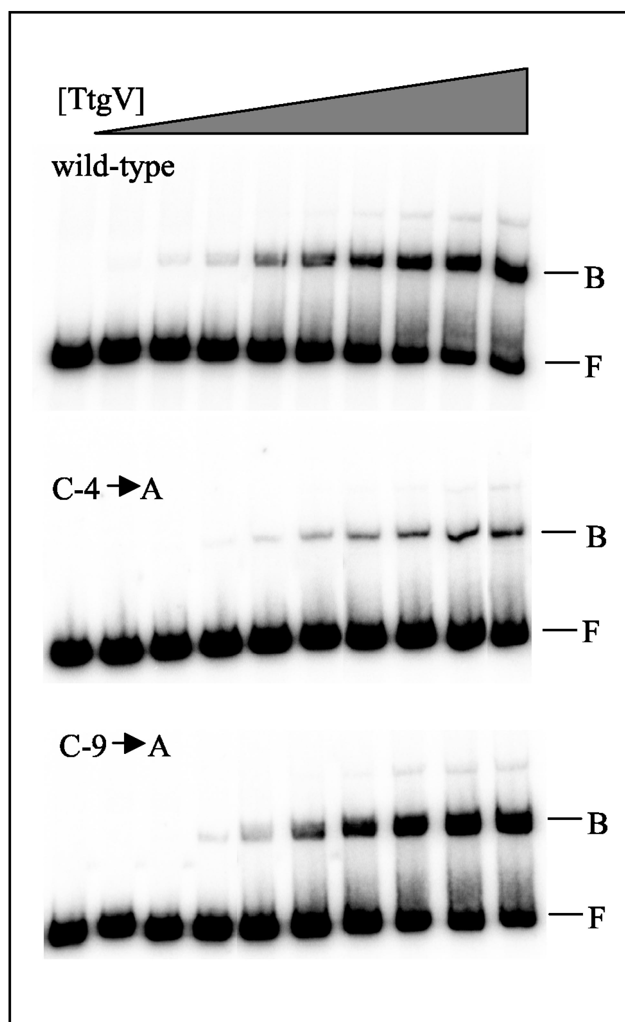

FIG. 4. EMSAs with the wild-type and C-4→A and C-9→A mutant promoters. The promoters were amplified by PCR as 210-bp fragments and labeled at the 5′ ends. About 1 nM DNA containing the *ttgG* promoter region was incubated with increasing concentrations of TtgV (from left to right, 0, 10, 50, 75, 100, 200, 300, 400, 500, and 700 nM) for 10 min and electrophoresed as indicated in Materials and Methods. B, bound DNA; F, free DNA.

containing the DNA fragment in EMSA when increasing concentrations of 1-hexanol were added to the binding region.

We also tested the effect of this compound in in vitro transcription assays using 20 to 200 μM 1-hexanol. In this range of concentrations, 1-hexanol did not interfere with RNA polymerase activity since the control P_{sp} promoter yielded the expected 488-nucleotide mRNA. We then performed in vitro transcription assays with TtgV added before RNA polymerase and with increasing concentrations of 1-hexanol. We found that the higher the concentration of 1-hexanol, the higher the level of transcription from the wild-type P_{ttgG} (Fig. 6).

DMS footprint assays. DNase I footprint assays revealed that TtgV protected the region in the P_{ttgG} promoter corresponding to positions +13 to −29 from digestion (22). To characterize the binding region in greater detail, DMS methylation protection assays were carried out. Figure 7 shows that

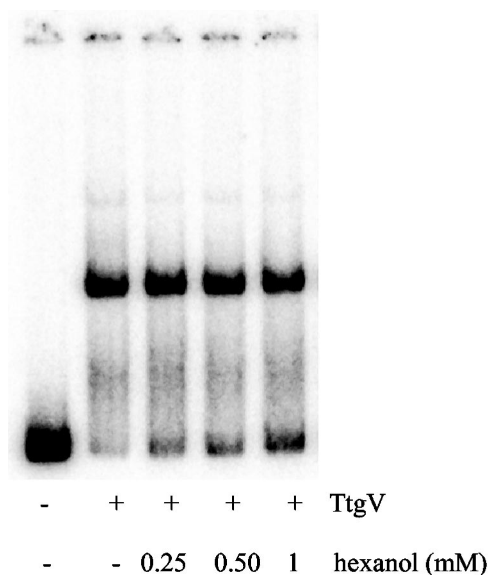

FIG. 5. EMSAs with the wild-type promoter in the presence of 1-hexanol. Conditions are as described in the legend to Fig. 4, except that DNA corresponded to the wild-type *ttgG* promoter and a fixed concentration of TtgV (500 nM) was used. Samples were incubated for 10 min with the indicated 1-hexanol concentration before electrophoretic separation.

G at position −14 in the bottom strand was hypermethylated in the presence of TtgV. This suggests that binding of TtgV to target DNA triggered local DNA conformational changes. We observed that G's at positions +6, −15, and −27 were protected in the top strand and G's at positions −23, −11, −4, −3, and +10 were protected in the bottom strand, suggesting that they might be contacted by TtgV on binding to its operator. All these positions are in accordance with our previous DNase I footprinting since they are located within the proposed binding site. Furthermore, the importance of G-4 and G-3 in TtgV operator recognition is consistent with our results showing that G-4→A and G-3→C are less prone to be recognized by TtgV.

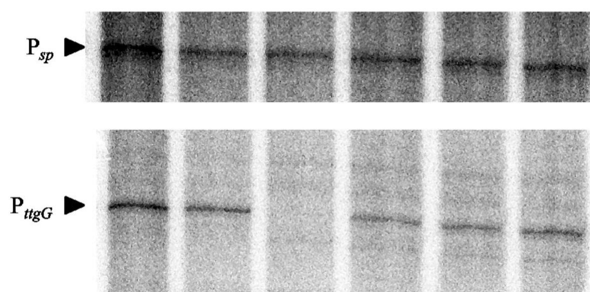

FIG. 6. TtgV repression of the transcription of P_{ttgG} is alleviated by 1-hexanol. Single-round in vitro transcription assays were performed as described in the legend to Fig. 1A in the absence and presence of 0.5 μM TtgV added before the addition of RNA polymerase. Increasing concentrations of 1-hexanol were added as indicated. Other conditions as are described in Materials and Methods.

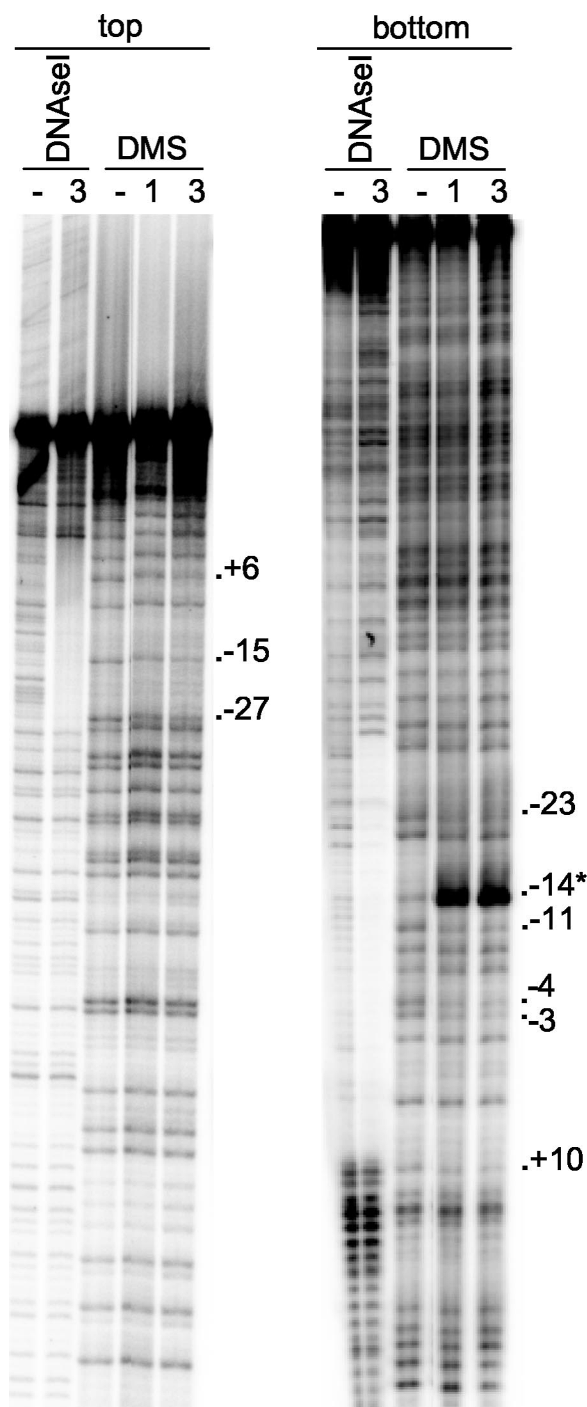

FIG. 7. In vitro methylation protection assay of the *ttgGHI* promoter region. PCR fragments comprising the *ttgG* promoter region were labeled at one 5′ end and incubated without (−) and with 1 or 3 μM purified TtgV before being subjected to treatment with DMS or DNase I digestion. The positions of the G's that are protected from methylation by the repressor are indicated; the asterisk indicates a hyperreactive position.

DISCUSSION

Our results establish that the *ttgGHI* operon is probably transcribed from a single promoter rather than from two tandem promoters, as proposed before (23). This assumption is supported by in vitro transcription analysis, which revealed a single transcription start point, and mutational analysis of the region upstream from the +1 position. Mutational studies support the notion that the most probable −10 hexamer of the promoter lies between positions −8 and −13 since a number of mutations in this stretch resulted in mutants that could not be transcribed either in vivo or in vitro. In contrast to these mutants, which lacked activity, we found that certain mutations at −9 and −11 increased promoter activity. This seems to be the result of a better −10 region that is recognized more efficiently by RNA polymerase.

DNase I footprint analysis revealed that TtgV covers four helical turns that cover the promoter region between positions +13 and −29 (22), which suggests that TtgV represses transcription from the *ttgG* promoter by competing with the RNA polymerase for promoter binding. To gain insight into the TtgV repression mechanism, we performed in vitro transcription assays. When TtgV was incubated with the *ttgG* promoter before the addition of the RNA polymerase, transcription from P_{ttgG} was repressed but that from a reference promoter, P_{sp}, was unaffected (Fig. 1A). The repression level of *ttgG* transcription correlated with the dose of TtgV added. However, TtgV did not repress *ttgG* transcription significantly when it was added after the formation of the RNA polymerase-*ttgG* promoter open complex (Fig. 1). This indicates that TtgV represses *ttgG* transcription by physically competing with the RNA polymerase for promoter binding, as in the case of the well-established classic model of repressor action (4, 7, 24, 28). DMS methylation protection assays revealed that in the presence of TtgV, G-14 in the bottom strand becomes hypermethylated. This probably indicates that TtgV binding to the operator region provokes a distortion immediately upstream from the −10 region, which may prevent proper recognition of the promoter by RNA polymerase. DMS footprinting, however, did not help us to discern whether TtgV was able to recognize a relatively highly conserved direct repeat [5′-(A/C)T(G/A)N (C/T)NCA-3′] that appeared in four consecutive helical turns (Fig. 1B) or whether TtgV recognized some of the imperfect inverted repeats in the protected region. Analysis of mutant promoters revealed that mutations C-4→A or G and C-3→G resulted in increased expression in vivo but not in vitro and correlated with a decrease in the affinity of TtgV for the target operator as determined in EMSAs, as well as the protection of the bases at these positions in DMS footprint assays.

The TtgV protein belongs to the IclR family of regulators (10, 14). Members of this family repress the transcription of specific cognate genes in the absence of the target chemical and detach from the operator in response to the presence of a specific signaling molecule. A similar mechanism seems to operate for TtgV: EMSAs revealed that in the presence of increasing concentrations of 1-hexanol, TtgV dissociated from its target operator. This was corroborated by in vitro transcription assays showing that when TtgV was present, there was no mRNA synthesis, but in the presence of 1-hexanol, mRNA levels increased. This suggests that in the presence of 1-hexanol, TtgV was released from its operator site and RNA polymerase was able to access and transcribe the *ttgG* promoter. These are the first experimental indications of a compound able to directly promote TtgV dissociation from its operator

site. Future work on the *ttgGHI-ttgV* system should reveal more intimate details of the molecular interactions between the regulator, its effectors, and its target DNA.

ACKNOWLEDGMENTS

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
2. **Duque, E., A. Segura, G. Mosqueda, and J. L. Ramos.** 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. Mol. Microbiol. **39:**1100–1106.
3. **Gallegos, M. T., S. Marqués, and J. L. Ramos.** 1996. Expression of the TOL plasmid *xylS* gene in *Pseudomonas putida* occurs from a σ^{70}-dependent promoter or from σ^{70}- and σ^{54}-dependent tandem promoters according to the aromatic compound used for growth. J. Bacteriol. **178:**2356–2361.
4. **Geanacopoulos, M., and S. Adhya.** 2002. Genetic analysis of GalR tetramerization in DNA looping during repressosome assembly. J. Biol. Chem. **277:** 33148–33152.
5. **González-Pérez, M. M., J. L. Ramos, M. T. Gallegos, and S. Marqués.** 1999. Critical nucleotides in the upstream region of the XylS-dependent TOL *meta*-cleavage pathway operon promoter as deduced from analysis of mutants. J. Biol. Chem. **274:**2286–2290.
6. **Gottesman, M. M., and I. Pastam.** 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. **62:**385–427.
7. **Heldwein, E. E., and R. G. Brennan.** 2001. Crystal structure of the transcription activator BmrR bound to DNA and a drug. Nature **409:**378–382.
8. **Koronakis, V., A. Sharff, E. Koronakis, B. Luisi, and C. Hughes.** 2001. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature **405:**914–919.
9. **Lisser, S., and H. Margalit.** 1993. Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids Res. **21:**1507–1516.
10. **Maloy, S. R., and W. D. Dunn.** 1982. Genetic regulation of the glyoxylate shunt in *Escherichia coli* K-12. J. Bacteriol. **149:**173–180.
11. **Marqués, S., J. L. Ramos, and K. N. Timmis.** 1993. Analysis of the mRNA structure of the *Pseudomonas putida* TOL *meta*-fission pathway operon. Biochim. Biophys. Acta **1216:**227–236.
12. **Mosqueda, G., and J. L. Ramos.** 2000. A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the *tod* genes for toluene metabolism. J. Bacteriol. **182:**937–943.
13. **Murakami, S., R. Nakashima, E. Yamashita, and A. Yamaguchi.** 2002. Crystal structure of bacterial multidrug efflux transporter AcrB. Nature **419:**587–593.
14. **Nasser, W., S. Reverchon, and J. Robert-Baudouy.** 1992. Purification and functional characterization of the KdgR protein, a major repressor of pectinolysis genes of *Erwinia chrysanthemi*. Mol. Microbiol. **6:**257–265.
15. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
16. **Nikaido, H.** 1998. Multiple antibiotic resistance and efflux. Curr. Opin. Microbiol. **1:**516–523.
17. **Poole, K.** 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob. Agents Chemother. **44:**2233–2241.
18. **Poole, K.** 2000. Efflux-mediated resistance to fluoroquinolones in gram-positive bacteria and the mycobacteria. Antimicrob. Agents Chemother. **44:**2595–2599.
19. **Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-González, A. Rojas, W. Terán, and A. Segura.** 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Annu. Rev. Microbiol. **56:**743–768.
20. **Ramos, J. L., E. Duque, P. Godoy, and A. Segura.** 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J. Bacteriol. **180:**3323–3329.
21. **Ramos, J. L., E. Duque, M.-J. Huertas, A. Haïdour.** 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J. Bacteriol. **177:**3911–3916.
22. **Rojas, A., A. Segura, M. E. Guazzaroni, W. Terán, A. Hurtado, M. T. Gallegos, and J. L. Ramos.** 2003. In vivo and in vitro evidence show that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. J. Bacteriol. **185:**4755–4763.
23. **Rojas, A., E. Duque, G. Mosqueda, G. Golden, A. Hurtado, J. L. Ramos, and A. Segura.** 2001. Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. J. Bacteriol. **183:**3967–3973.
24. **Rojo, F.** 1999. Repression of transcription initiation in bacteria. J. Bacteriol. **181:**2987–2991.
25. **Saier, J., and I. Paulsen.** 2001. Phylogeny of multidrug transporters. Cell Dev. Biol. **12:**205–213.
26. **Spaink, H. P., R. J. H. Okker, C. A., Wijffelman, E. Pees, and B. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. **9:**27–39.
27. **Sunnaborg, A., D. Klumpp, T. Chung, and D. C. LaPorte.** 1990. Regulation of the glyoxylate bypass operon: cloning and characterization of *iclR*. J. Bacteriol. **172:**2642–2649.
28. **Terán, W., A. Felipe, A. Segura, A. Rojas, J. L. Ramos, and M. T. Gallegos.** 2003. Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. Antimicrob. Agents Chemother. **47:**3067–3072.
29. **Yu, E. W., J. R. Aires, and H. Nikaido.** 2003. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J. Bacteriol. **185:**5657–5664.
30. **Yu, E. W., G. McDermott, H. I. Zgurskaya, H. Nikaido, and D. E. Koshland, Jr.** 2003. Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump. Science **300:**976–980.
31. **Zgurskaya, H. I., and H. Nikaido.** 1999. AcrA is a highly asymmetric monomeric protein capable of spanning the periplasm. J. Mol. Biol. **285:**409–420.

CAPÍTULO 3.

El regulador TtgV reconoce un amplio espectro de efectores estructuralmente diferentes estando tanto en solución como en complejo con el ADN

Autores: Guazzaroni M.E., Krell T., Felipe A., Ruiz R., Meng C., Zhang X., Gallegos M.T. y Ramos J.L.

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El regulador TtgV modula la expresión del operón *ttgGHI*, el cual codifica una bomba de eflujo que expulsa una amplia variedad de compuestos tales como hidrocarburos aromáticos de uno o dos anillos, alcoholes alifáticos y antibióticos de diferentes estructuras químicas. Mediante el empleo de una fusión del gen *'lacZ* al promotor *ttgG* hemos demostrado que los inductores más eficientes *in vivo* son 1-naftol, 2,3-dihidroxinaftaleno, 4-nitrotolueno, benzonitrilo e indol. A través de calorimetría de titulación isotérmica se determinaron los parámetros termodinámicos de la unión de TtgV a diferentes moléculas efectoras. Para la mayoría de los efectores, la interacción fue dirigida por la entalpía y contrabalanceada por cambios desfavorables de entropía. La afinidad de unión de TtgV por sus efectores fue de entre 2 y 890 μM. Se estableció que existe una tendencia entre las K_D determinadas *in vitro* mediante microcalorimetría y la eficiencia de inducción *in vivo* estudiada mediante ensayos de actividad β-galactosidasa utilizando una fusión del promotor *ttgGHI* a *lacZ*. Esto indica que la afinidad de TtgV por los efectores es el determinante principal de la eficiencia de los mismos en la inducción de la expresión génica. A través de ensayos de diálisis al equilibrio y calorimetría de titulación isotérmica se estableció que se une un dímero de TtgV por molécula de efector. No se obtuvo ninguna evidencia que indique unión simultánea de TtgV a múltiples efectores. La afinidad de unión de TtgV a un fragmento de ADN de 63 pb que contenía su operador fue alta y dirigida por la entropía (K_D = 2.4 ± 0.35 nM, ΔH = 5.5 ± 0.04 kcal/mol). Se demostró que el complejo TtgV-ADN se une a 1-naftol con una afinidad comparable al de la proteína TtgV libre, K_D = 4.8 ± 0.19 y K_D = 3.0 ± 0.15 μM, respectivamente. Se discute la relevancia fisiológica de este hallazgo.

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The Multidrug Efflux Regulator TtgV Recognizes a Wide Range of Structurally Different Effectors in Solution and Complexed with Target DNA

EVIDENCE FROM ISOTHERMAL TITRATION CALORIMETRY*

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María-Eugenia Guazzaroni‡, Tino Krell‡, Antonia Felipe‡, Raquel Ruiz‡, Cuixiang Meng§, Xiaodong Zhang§, María-Trinidad Gallegos‡, and Juan L. Ramos‡¶

From the ‡Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Profesor Albareda, 1, E-18008 Granada, Spain and the §Centre for Structural Biology, Department of Biological Sciences, Imperial College London, Flowers Building, South Kensington, London, SW7 2AZ, United Kingdom

TtgV modulates the expression of the *ttgGHI* operon, which encodes an efflux pump that extrudes a wide variety of chemicals including mono- and binuclear aromatic hydrocarbons, aliphatic alcohols, and antibiotics of dissimilar chemical structure. Using a *'lacZ* fusion to the *ttgG* promoter, we show that the most efficient *in vivo* inducers were 1-naphthol, 2,3-dihydroxynaphthalene, 4-nitrotoluene, benzonitrile, and indole. The thermodynamic parameters for the binding of different effector molecules to purified TtgV were determined by isothermal titration calorimetry. For the majority of effectors, the interaction was enthalpy-driven and counterbalance by unfavorable entropy changes. The TtgV-effector dissociation constants were found to vary between 2 and 890 μM. There was a relationship between TtgV affinity for the different effectors and their potential to induce gene expression *in vivo*, indicating that the effector binding constant is a major determinant for efficient efflux pump gene expression. Equilibrium dialysis and isothermal titration calorimetry studies indicated that a TtgV dimer binds one effector molecule. No evidence for the simultaneous binding of multiple effectors to TtgV was obtained. The binding of TtgV to a 63-bp DNA fragment containing its cognate operator was tight and entropy-driven (K_D = 2.4 ± 0.35 nM, ΔH = 5.5 ± 0.04 kcal/mol). The TtgV-DNA complex was shown to bind 1-napthol with an affinity comparable with the free soluble TtgV protein, K_D = 4.8 ± 0.19 and 3.0 ± 0.15 μM, respectively. The biological relevance of this finding is discussed.

Pseudomonas putida DOT-T1E is a paradigm of solvent-tolerant microorganisms because it can grow in the presence of high concentrations of extremely toxic and harmful compounds such as aromatic hydrocarbons (1, 2). These compounds preferentially partition in the cell membrane, disorganizing it and leading to cell death (3). Efflux pumps have been shown to play a critical role in the removal of toxic compounds such as antibiotics, biocides, dyes, detergents, fatty acids, and organic solvents from the cell membranes (2, 4–13). In *P. putida* DOT-T1E, the cooperative action of up to three efflux pumps, TtgABC, TtgDEF, and TtgGHI, is needed to achieve maximal tolerance against toluene, one of the most toxic aromatic hydrocarbons. TtgGHI appears to be the most important extrusion element since, in contrast to the other two efflux pumps, a knock-out mutant in which this efflux pump is not functional was not able to withstand a sudden 0.3% (v/v) toluene shock regardless of the growth conditions (14). TtgGHI, like other multidrug-resistant pumps, possesses a broad substrate specificity reflected in its capacity to extrude not only aromatic hydrocarbons such as toluene, xylenes, or styrene but also aliphatic alcohols such as octanol, nonanol, and decanol, as well as antibiotics of different chemical structure, *e.g.* ampicillin, tetracycline, and nalidixic acid (14, 15).

The expression of the *ttgGHI* operon is regulated by the TtgV protein (16). The *ttgV* gene is transcribed divergently from the *ttgGHI* operon, and the corresponding promoters, called P_{ttgV} and P_{ttgG}, overlap each other. The two start codons are separated by only 210 bp, 40 bp of which constitute the TtgV operator so that TtgV covers the −10 region of *ttgG* promoter and the −35 region of *ttgV* promoter (16, 17). Basal expression from the *ttgG* and *ttgV* promoters occurs, but expression has been shown to increase in response to the presence of some, but not all, of the pump substrates in the culture medium. Direct evidence of *in vitro* TtgV binding to drugs has only been obtained with 1-hexanol; Guazzaroni *et al.* (17) showed in EMSA[1] that this aliphatic alcohol released TtgV from its target operator. The present study was undertaken to determine the effector profile of TtgV, elucidate the TtgV-effector stoichiometry, and determine the thermodynamic parameters for the binding of the most potent effectors. Furthermore, the binding of 1-naphthol, one of the most potent effectors, by free TtgV and the protein complexed to its operator DNA has been compared using ITC.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Medium—The bacterial strains and plasmids used in this study are shown in Table I. Bacterial strains were grown in LB medium at 30 °C as described before (14) or in 2×YT for the production of the TtgV protein (18). Liquid cultures were shaken on an orbital platform operating at 200 rpm. When re-

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¶ To whom correspondence should be addressed. Tel.: 34-958181600 (ext. 204); Fax: 34-958135740; E-mail: jlramos@eez.csic.es.

[1] The abbreviations used are: EMSA, electrophoretic mobility shift assay; ITC, isothermal titration calorimetry; Km, kanamycin; LB, Luria-Bertani culture medium.

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TABLE I
Strains and plasmids used in this study

Ap^r, $K_m{}^r$, Rif^r, Tc^r, and Tol^r stand for resistance to ampicillin, kanamycin, rifampicin, tetracycline and toluene, respectively.

Strains and plasmids	Characteristics	Relevant source or references
P. putida		
DOT-T1E	Rif^r, Tol^r	2
E. coli		
B834 (DE3)	F^-, *ompI hsdS*$_B$ *(r− $_B$ m− $_B$) gal dem met*	Novagen
pANA96	Tc^r, *ttgG* promoter cloned in pMP220	16
pET29a(+)	$K_m{}^r$, protein expression vector	Novagen
pGG1	Ap^r, pUC18 bearing an 8-kb BamHI fragment with *ttgGHI* and *ttgVW*	14
pMP220	Tc^r, promoterless *lacZ* expression vector	37
pTE103-P$_{ttgG}$	Ap^r, promoter of *ttgG* cloned upstream of the T7 terminator in pTE103	17
pTGF2	Km^r, pET29a(+) derivative vector used to produce TtgV	This work

quired, the following antibiotics were added to the cultures: Km, 50 μg/ml; rifampicin, 20 μg/ml; and tetracycline, 20 μg/ml.

β-Galactosidase Assays—Plasmid pANA96 carries a transcriptional fusion of the P$_{ttgG}$ promoter region to the *'lacZ* gene in the low copy pMP220 promoter probe vector. *P. putida* DOT-T1E (pANA96) was grown overnight on LB medium with tetracycline. Cultures were diluted to an initial OD_{660} of 0.05 in the same medium supplemented or not with the chemicals under study at 1 mM. These compounds were dissolved in Me_2SO when needed (note that the latter did not interfere with the induction assays performed in this study). When cultures reached an OD_{660} of 0.9–1.0, β-galactosidase activity was determined in triplicate in permeabilized cells (19).

TtgV Expression and Purification—Plasmid pTGF2 was constructed by cloning a 784-bp NdeI-BamHI fragment bearing the *ttgV* open reading frame in the Km^r pET29a(+) plasmid (Novagen) digested with the same enzymes to allow the expression of the native TtgV protein. Plasmid pTGF2 was transformed in *Escherichia coli* B834 (DE3) cells. The cells were grown in two-liter conical flasks containing 500 ml of 2×YT culture medium with 50 μg/ml Km, incubated at 30 °C with shaking, and induced with 1 mM isopropyl β-D-thiogalactopyranoside when the turbidity of the culture was around 0.7. Then cultures were grown at 22 °C for 3 h, and cells were harvested by centrifugation (10 min at 4000 × *g*). The cell pellet was resuspended in 0.2 M sodium acetate, 50 mM NaCl, 0.1 mM EDTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and lysed by sonication. After centrifugation at 13,000 × *g* for 40 min, the supernatant was loaded onto an S-cation column (16/10, Amersham Biosciences) and eluted with a sodium chloride gradient. The fraction containing TtgV was then dialyzed against buffer containing 10 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 100 mM NaCl, 0.1 mM EDTA, and 2 mM dithiothreitol, concentrated to 2 ml and loaded onto a Superdex 200 16/20 column (Amersham Biosciences) for gel filtration. The proteins were then concentrated to 4 mg/ml as determined by the Bio-Rad protein assay kit.

Electrophoresis Mobility Shift Assay—A 228-bp DNA fragment containing the wild-type P$_{ttgG}$ promoter was amplified by PCR from pGG1 using appropriate primers, isolated from agarose gels, and end-labeled with ^{32}P as described before (18, 20). About 1 nM labeled DNA (∼1.5 × 10^4 cpm) was incubated with the indicated amounts of purified TtgV for 10 min at 30 °C in 10 μl of TAPS binding buffer (50 mM Tris-acetate, pH 8.0; 100 mM potassium acetate; 8 mM magnesium acetate; 27 mM ammonium acetate; 3.5% (w/v) polyethylene glycol, and 1 mM dithiothreitol) containing 20 μg/ml poly(dI-dC) and 200 μg/ml bovine serum albumin. Electrophoresis in nondenaturing polyacrylamide gels and analyses were as described before (17).

Single-round in Vitro Transcription Assays with Supercoiled Plasmid DNA—Reactions (20 μl) were performed in STA buffer (25 mM Tris-acetate, pH 8.0, 8 mM magnesium acetate, 3.5% (w/v) polyethylene glycol, 10 mM KCl) containing 100 nM σ^{70}-holoenzyme (Epicenter), 20 units of RNAsin (Promega), 0.1 mM GTP, and 10 nM supercoiled pTE103-P$_{ttgG}$ DNA template (10). The reactions were incubated for 20 min at 30 °C before the addition of the following elongation mixture: 0.1 mM each of ATP, CTP, and UTP; 0.3 μCi of [α-^{32}P]UTP (20 μCi/μl); and 100 μg/ml heparin. After incubation for a further 10 min at 30 °C, the reactions were stopped by chilling to 4 °C, and the product was precipitated with 0.25 volumes of 10 M ammonium acetate and 2.5 volumes of ethanol. The pellets were washed with 80% (v/v) ethanol. Dried pellets were resuspended in 8 μl of water and 4 μl of formamide sequencing dye. Samples were submitted to electrophoresis using a 6.5% (w/v) polyacrylamide denaturing sequencing gel. The results were analyzed using Personal FX equipment software (Bio-Rad).

Isothermal Titration Calorimetry—Measurements were performed on a VP-Microcalorimeter (MicroCal, Northampton, MA) at 30 °C. The protein was thoroughly dialyzed against 25 mM Tris acetate, pH 8.0, 8 mM magnesium acetate, 10 mM KCl, and 1 mM dithiothreitol. The protein concentration was determined using the Bradford assay. Stock solutions of 1-naphthol, 2,3-dihydroxynaphthalene, indole, and 4-nitrotoluene at a concentration of 500 mM were prepared in Me_2SO and subsequently diluted with dialysis buffer to a final concentration of 0.3 mM (1-naphthol and 2,3-dihydroxynaphthalene), 1.5 mM (indole), and 1 mM (4-nitrotoluene). The appropriate amount of Me_2SO (0.1%) was added to the protein sample in each assay. Solutions of benzonitrile (4 mM) and hexanol and toluene (5 mM) were directly prepared in dialysis buffer. All chemicals were manipulated in glass vessels, and effector samples were neither degassed nor filtered, to avoid evaporation or nonspecific binding. Each titration involved a single 2-μl injection and a series of 4-μl injections of effector molecules into the protein solution. For DNA binding studies, oligonucleotides corresponding to both strands of the TtgV operator were synthesized (5′-GGAATTCTCAAGAGT-ATCACATAATGCTACACTCTACCGCATTACGATTCAGCAACTGCA-GAA-3′ and its corresponding complementary oligonucleotide). Annealing was carried out by mixing equimolar amounts (at a concentration of 60 μM) of each complementary oligonucleotide in 0.5 mM Tris-HCl, pH 8.0, 0.5 mM $MgCl_2$. The mixture was incubated 95 °C for 5 min and then chilled on ice and dialyzed in the buffer used for ITC studies. The mean enthalpies measured from injection of the ligand in the buffer were subtracted from raw titration data before data analysis with ORIGIN software (MicroCal). Titration curves were fitted by a nonlinear least squares method to a function for the binding of a ligand to a macromolecule (21). From the curve thus fitted, the parameters ΔH (reaction enthalpy), K_A (binding constant, $K_A = 1/K_D$), and *n* (reaction stoichiometry) were determined. From the values of K_A and ΔH, the change in free energy (ΔG) and in entropy (ΔS) were calculated with the equation: $\Delta G = -RT \ln K_A = \Delta - T\Delta S$, where R is the universal molar gas constant and T is the absolute temperature.

Measurements of TtgV/Ligand Binding Ratio Using Equilibrium Dialysis Assays—Four samples of TtgV with different protein concentrations were dialyzed against protein buffer containing effectors using Slide-a-Lyzer (Pierce) equipment for 5 days at 4 °C with stirring to ensure equilibrium. The proteins inside the cassette were then denatured by incubating at 100 °C for 5 min to release the bound effectors into the buffer. The denatured proteins were then centrifuged for 2 min at 13,000 × *g*. Ultraviolet light absorption at the appropriate wavelength was then measured in the supernatants that contained the effectors, and the effector concentrations were determined using the corresponding ∈ extinction coefficients. The concentration of protein-bound effectors was obtained after correction for the effector concentration in the buffer. Protein concentrations were determined with the Bradford assay (Pierce). The binding ratio and dissociation constant were obtained with the following equation: binding ratio = [protein bound]/[effectors bound], K_D = [effectors free][protein free]/[effectors bound].

RESULTS

In Vivo Effector Profile of TtgV—As an initial approach to the identification of the effectors recognized by TtgV, we used a P$_{ttgG}$::*'lacZ* fusion (pANA96) to measure β-galactosidase activity in *P. putida* DOT-T1E cells grown in the absence or in the presence of 1 mM compounds (Fig. 1). The basal level of expression from the *ttgG* promoter was 438 ± 34 Miller units, and expression increased up to 5-fold in response to 1 mM tested

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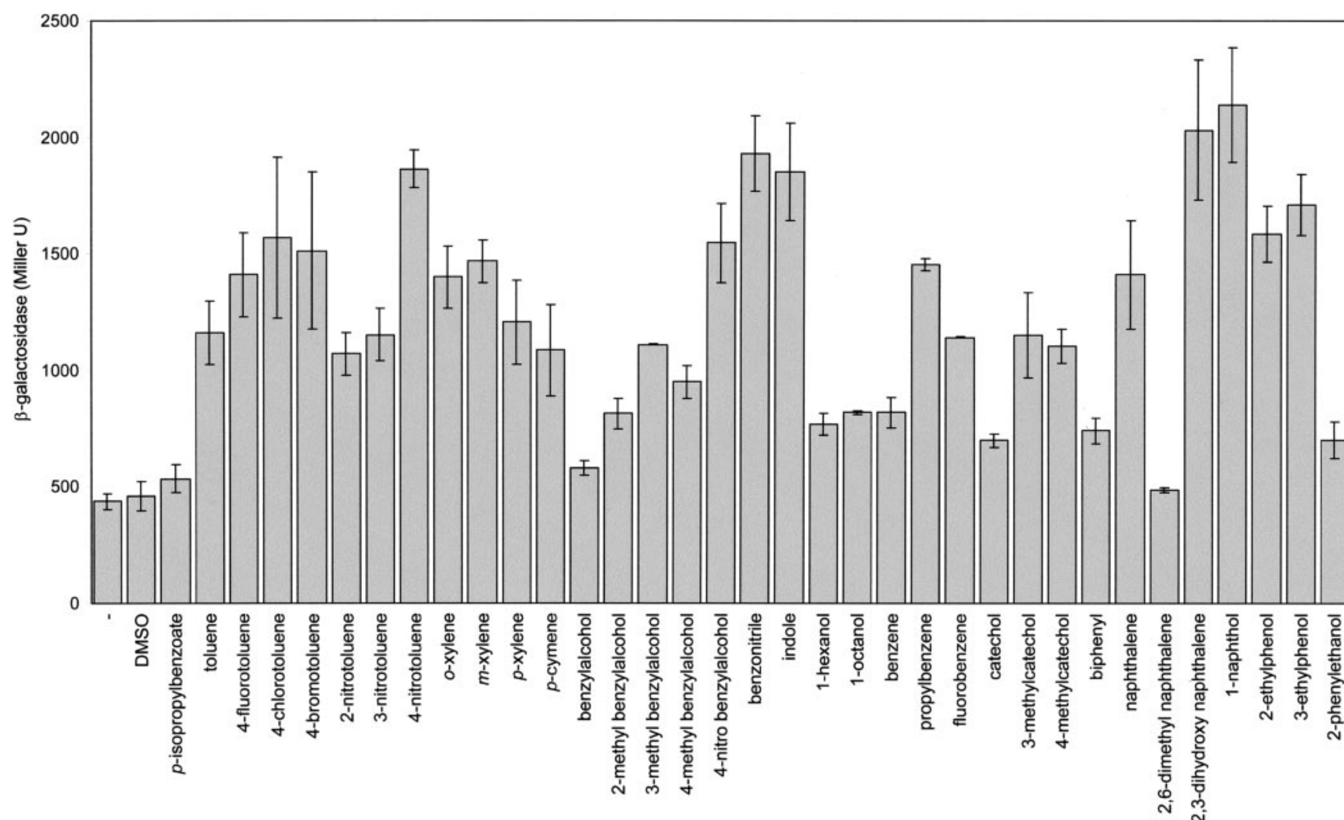

FIG. 1. **Expression from the *ttgGHI* promoter in the presence of different compounds.** β-galactosidase activity was determined from the *P. putida* DOT-T1E *ttgGHI* promoter (pANA96 plasmid) in cultures grown in the absence and in the presence of 1 mM indicated chemicals. Results are the mean and standard error of eight different experiments. *DMSO*, Me_2SO; *Miller* U, Miller units.

chemicals. The effectors that yielded the highest induction levels (4–5-fold increase) were two-ring aromatic compounds such as 1-naphthol, 2,3-dihydroxynaphthalene, and indole and one-ring aromatic compounds such as benzonitrile and 4-nitrotoluene (Fig. 1). Other compounds such as alkylphenols, halogenated aromatic rings, and aliphatic and aromatic alcohols also behaved as effectors and increased expression from the P_{ttgG} promoter by at least 2-fold.

In Vitro TtgV-Effector Interactions—Equilibrium dialysis experiments were carried out to shed light into the effector-TtgV stoichiometry. The results for the binding of 1-naphthol and 2,3-dihydroxynaphthalene to TtgV, as shown in Tables II and III, indicated that one effector molecule binds to the TtgV dimer. The apparent affinity for these molecules was in the low micromolar range. The thermodynamic parameters for the interaction of these two effectors as well as those of other effectors (biaromatic, monoaromatic compounds, and aliphatic alcohols) were subsequently determined by ITC at 30 °C. These effectors were chosen to cover the spectrum of chemically different compounds that behaved as inducers *in vivo*.

The titration of TtgV with different effectors (1-naphthol, 2,3-dihydroxynaphthalene, benzonitrile, indole, 4-nitrotoluene, toluene, and 1-hexanol) is characterized by exothermal heat changes, giving rise to hyperbolic binding curves. This was exemplified by the titration with 1-naphthol shown in Fig. 2 (*left-hand panel*). ITC data were analyzed using *n* fixed at 0.5 (one effector molecule/dimer) determined by equilibrium dialysis experiments (see above), and satisfactory curve fits were obtained. The corresponding thermodynamic parameters are shown in Table IV. For all tested effectors, with the exception of 2,3-dihydroxynaphthalene, the binding was driven by favorable enthalpy changes and counterbalanced by unfavorable entropy changes (Table IV). In contrast, the thermodynamic mode of the binding of 2,3-dihydroxynaphthalene was different from that of the other effectors. Binding gave rise to only very small exothermic heat changes and was thus driven by entropy changes. It should be noted that dissociation constants for the binding of naphthol and 2,3-dihydroxynaphthalene determined by ITC and equilibrium dialysis were very close (3.0 ± 0.15 and 2.2 ± 0.8 μM for 1-naphthol and 2.3 ± 0.42 and 1.2 ± 0.6 μM for 2,3-dihydroxynaphthalene, respectively). These two bi-aromatic compounds are clearly bound by TtgV with the highest affinity, which is also reflected in its superior *in vivo* efficiency (Fig. 1).

The dissociation constants for the different effectors span the micromolar range (Table IV). Although benzonitrile and indole were shown to be efficient effectors *in vivo*, the affinity of TtgV for these two chemicals was lower (in the range of 50–70 μM) than for 1-naphthol. Substantially lower affinities were determined for toluene (K_D = 118 μM) and 1-hexanol (K_D = 892 μM), which may account for the relatively modest activity of these effectors *in vivo*. The nitro substitution of toluene at position 4 resulted in a substantial increase in ΔH (−6.3 to −14.9 kcal/mol) and an 8-fold increase in affinity (Table IV), which is in agreement with 4-nitrotoluene being a more efficient effector than toluene *in vivo*. This is compatible with a potential direct recognition of the nitro group at position 4 by TtgV since a nitro group at position 2 or 3 resulted in a less efficient effector.

Different effectors have been shown to bind to different sites in the large binding pocket of the QacR protein (22–25). This raises the question whether the effector binding pocket of TtgV can accommodate different molecules at a time. We carried out a series of sequential ITC experiments that involved the initial saturation of TtgV with a first effector followed by the titration with a second effector. In a first series of experiments, TtgV (6.4 μM) was saturated by the addition of aliquots of 300 μM 1-naphthol. This complex was titrated with 300 μM 2,3-dihydroxynaphthalene. In a second series of experiments, TtgV (6.7

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TABLE II
Characterization of 1-naphthol binding to TtgV determined by equilibrium dialysis

TtgV at the different concentrations given below were dialyzed against buffers containing effectors for 5 days at 4 °C with stirring to ensure equilibrium. Other experimental conditions are given in "Experimental Procedures."

[1-naphthol-bound]	[1-naphthol-free]	[TtgV-bound]	[TtgV-free]	Binding ratio (effector/dimer TtgV)	K_d
μM	*μM*	*μM*	*μM*		*μM*
3.3	23.6	3.7	0.4	0.89	2.9
3.8	23.3	4.1	0.3	0.93	1.7
10.4	22.9	11.7	1.3	0.89	2.9
11.8	22.6	12.5	0.7	0.94	1.3
			average	0.91 ± 0.03	2.2 ± 0.8

TABLE III
Characterization of 2,3-dihydroxynaphthalene binding to TtgV determined by equilibrium dialysis

TtgV at the different concentrations given below were dialyzed against buffers containing effectors for 5 days at 4 °C with stirring to ensure equilibrium. Other experimental conditions are given in "Experimental Procedures."

[2,3-dihydroxynaphthalene-bound]	[2,3-dihydroxynaphthalene-free]	[TtgV-bound]	[TtgV-free]	Binding ratio (effector/dimer TtgV)	K_d
μM	*μM*	*μM*	*μM*		*μM*
15.9	19.1	16.2	0.3	0.98	0.8
16.7	18.9	17.4	0.8	0.96	2.1
19.8	19.7	21.9	2.2	0.90	1.0
20.4	19.2	21.5	1.1	0.95	0.7
			average	0.95 ± 0.03	1.2 ± 0.6

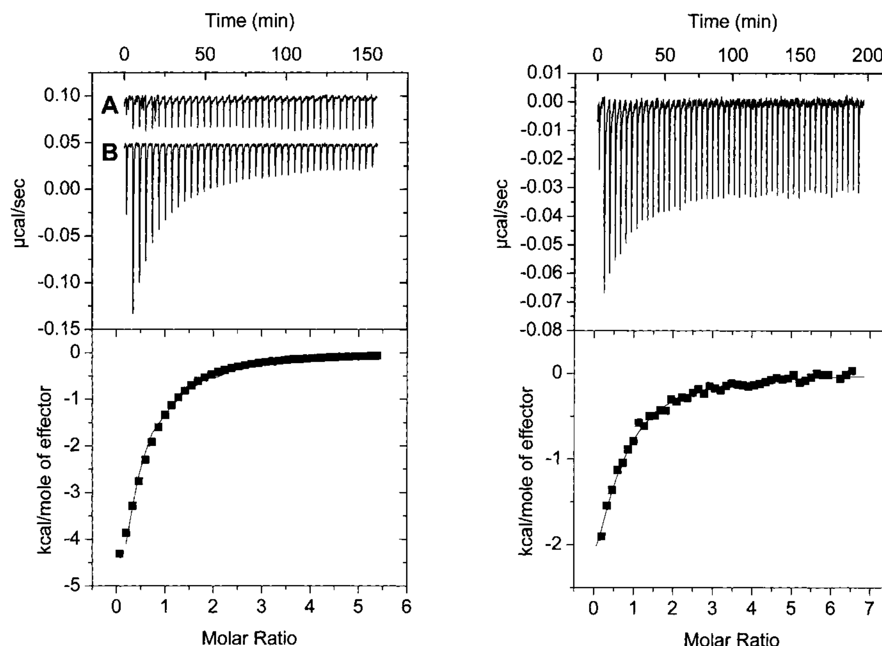

FIG. 2. **Isothermal titration calorimetry data for the binding of 1-naphthol to TtgV (*left-hand panel*) and to TtgV saturated with a 63-bp DNA fragment containing the operator (*right-hand panel*).** *Left panel*, heat changes (*upper panel*) and integrated peak areas (*lower panel*) for the injection of a single 2-μl and a series of 4-μl aliquots of 300 μM 1-naphthol in a solution of 6.4 μM TtgV (*B*) and buffer (*A*). *Right panel*, heat changes (*upper panel*) and integrated peak areas (*lower panel*) for the injection of a single 2-μl and a series of 4-μl aliquots of 300 μM 1-naphthol in a solution of 6.4 μM TtgV saturated with a DNA fragment containing the operator. Data were fitted with ORIGIN using an *n* value of 0.5 (one effector molecule/dimer). Derived thermodynamic parameters are given in Table IV.

TABLE IV
Thermodynamic parameters derived from the calorimetric titration of TtgV with effector molecules

TtgV solutions at 6–8 μM in 25 mM Tris-acetate, 8 mM magnesium acetate, 10 mM KCl, 1 mM dithiothreitol, pH 8.0, were titrated with 0.3–5 mM solutions of effectors. Experiments were carried out at 30 °C. Further assay conditions are given under "Experimental Procedures."

Effector	Ligand	K_D	K_A	ΔH	$T\Delta S$	ΔG
		μM	M^{-1}	*kcal/mol*	*kcal/mol*	*kcal/mol*
1-Naphthol	TtgV	3.0 ± 0.15	(3.4 ± 0.17) × 10^5	−8.6 ± 0.16	−1.1 ± 0.15	−7.5 ± 0.03
1-Naphthol	TtgV-DNA complex	4.8 ± 0.19	(2.1 ± 0.08) × 10^5	−5.2 ± 0.08	2.1 ± 0.09	−7.3 ± 0.02
2,3-Dihydroxynaphthalene	TtgV	2.3 ± 0.42	(4.4 ± 0.80) × 10^5	−0.8 ± 0.06	6.9 ± 0.10	−7.7 ± 0.10
4-Nitrotoluene	TtgV	16.9 ± 0.43	(5.9 ± 0.15) × 10^4	−14.9 ± 0.18	−8.5 ± 0.18	−6.4 ± 0.01
Benzonitrile	TtgV	51.3 ± 1.26	(1.9 ± 0.48) × 10^4	−12.5 ± 0.14	−6.7 ± 0.15	−5.8 ± 0.02
Indole	TtgV	67.1 ± 1.58	(1.5 ± 0.04) × 10^4	−13.0 ± 0.17	−7.3 ± 0.18	−5.7 ± 0.01
Toluene	TtgV	118.0 ± 2.64	(8.5 ± 0.19) × 10^3	−6.3 ± 0.07	−0.9 ± 0.09	−5.4 ± 0.01
1-Hexanol	TtgV	892.8 ± 135	(1.1 ± 0.17) × 10^3	−14.9 ± 0.15	−10.8 ± 1.56	−4.1 ± 0.09

μM) was saturated with 2,3-dihydroxynaphthalene and subsequently titrated with 1 mM benzonitrile. In both cases, heat changes were very small and corresponded to the competition of two effectors to a single site and not to the simultaneous binding of both effectors to the protein with a physiological relevant affinity (data not shown). We were thus unable to provide evidence for the simultaneous binding of multiple effectors to TtgV.

ITC Binding Studies of 1-nNaphthol to the TtgV-DNA Complex—As stated above, TtgV exerts its biological function by an up-regulation of gene expression as a result of the effector-

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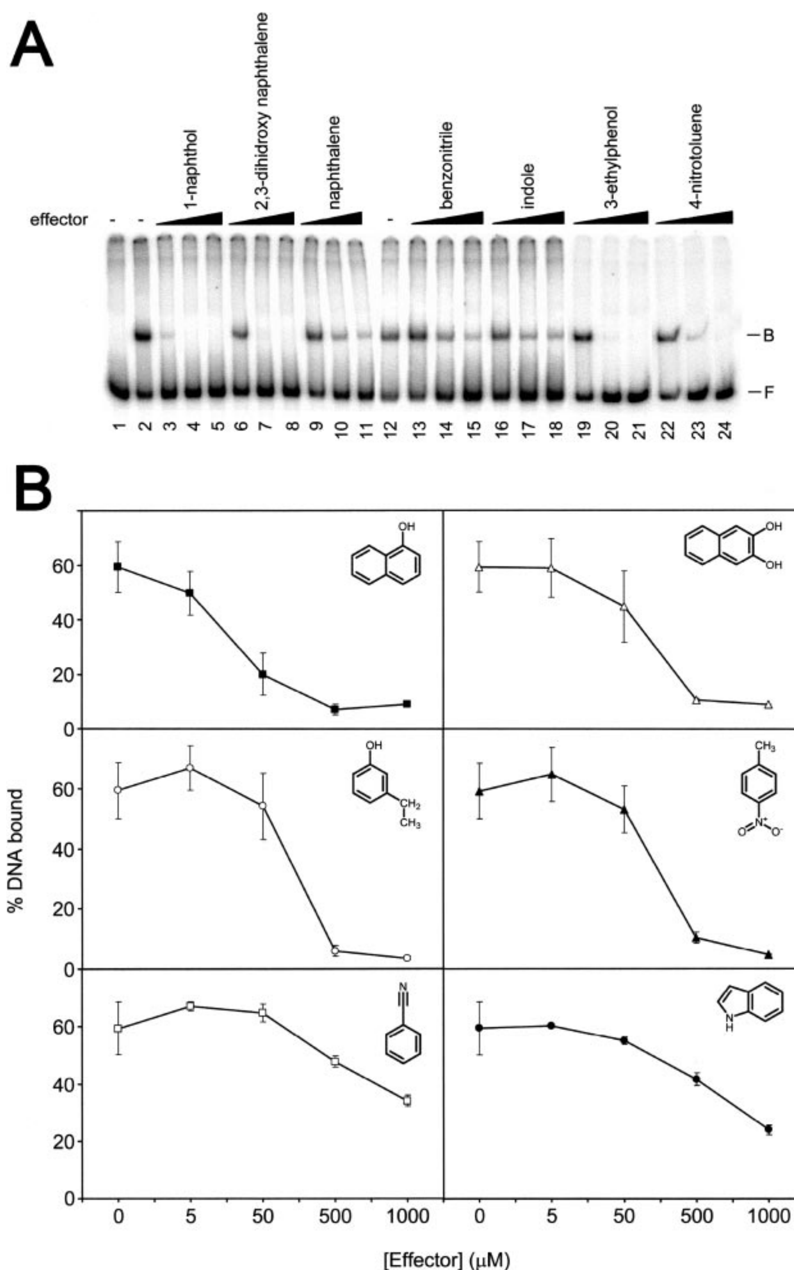

FIG. 3. **Effect of the concentration of different chemicals on TtgV binding to its operator site.** Binding reactions were carried out as described under "Experimental Procedures" with 1 nM DNA and 50 nM TtgV. *A*, electrophoretic mobility shift assays were carried out in the absence (*lanes 2* and *12*) and in the presence of 0.05, 0.5 and 1 mM indicated chemicals (*lanes 3–24*). *Lane 1* shows the migration of the target DNA fragment. *B*, bound DNA; *F*, free DNA. *B*, the percentage of DNA-TtgV complex remaining after incubation with different amounts of the indicated effectors as estimated from electrograms. The results shown are the mean and standard errors of four different assays.

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mediated dissociation of the regulatory protein from its operator. ITC experiments were carried out to study the interaction of 1-naphthol with the TtgV-DNA complex. Synthetic 63-bp oligonucleotides corresponding to both strands of the operator sequence protected by DNaseI footprint experiments were synthesized and annealed (see "Experimental Procedures") (17). In a first series of ITC assays, 8.1 μM TtgV was titrated with 10-μl aliquots of 32 μM DNA at 30 °C in STA buffer. Binding was entropy-driven (ΔH = 5.5 ± 0.04 kcal/mol, $T\Delta S$ = 17.5 ± 0.09 kcal/mol) and very tight (K_D = 2.4 ± 0.35 nM). Experiments were designed so that the protein concentration after saturation with DNA corresponded exactly to the protein concentration used for the titration of unliganded protein with 1-naphthol. After saturation, the TtgV-DNA complex was titrated with 1-naphthol in a similar fashion as the titration of the unliganded protein (Fig. 2, *right-hand panel*). The resulting heat changes corresponded to the binding of the effector to the protein and to the dissociation of the protein from DNA. Peaks were narrow, indicating that operator/TtgV dissociation occurred immediately upon effector binding by TtgV. After titration, the sample was subjected to EMSA, which demonstrated that protein has dissociated quantitatively from its target operator DNA (data not shown). In a control assay, free DNA at the same concentration as in the titration of the DNA-protein complex was titrated with 1-naphthol. Resulting peaks were small and uniform, indicative of that dilution. ITC data for the titration of the DNA-TtgV complex with 1-naphthol were analyzed assuming that one effector molecule was bound per dimer, and derived thermodynamic parameters are given in Table IV. 1-Naphthol binds with a K_D of 4.8 ± 0.19 μM to the TtgV/operator complex, which is comparable with the affinity of this effector for the TtgV protein free in solution (3.0 ± 0.15, Table IV). Most interestingly, binding was less exothermic as compared with the binding to the TtgV protein in solution, which was unexpected.

Effectors That Increased ttgGHI Expression Released TtgV from Its Target Operator and Allowed Transcription from P_{ttgG}—We previously showed that TtgV was released from its operator upon binding of 1-hexanol (17). The above *in vivo* and *in vitro* results suggested that the different effectors should

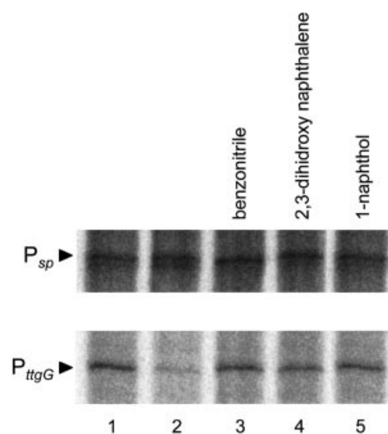

FIG. 4. **TtgV repression of the transcription of P_{ttgG} is alleviated by different ligands.** Single-round transcription assays were carried out as described under "Experimental Procedures." The assays were performed at 30 °C for 20 min in the absence of TtgV (*lane 1*) or in the presence of 0.5 μM TtgV added prior to 100 nM RNA polymerase (*lanes 2–5*). Different effectors were added at 100 μM: benzonitrile, 2,3-dihydroxynaphthalene, and 1-naphthol. The mRNAs synthesized from P_{ttgG} and P_{sp} (a plasmid promoter used as a control) are indicated by *arrowheads*.

release TtgV from its target site. To test this hypothesis, we used EMSA assays with TtgV at 50 nM, a concentration that shifted about 50% of the DNA (Fig. 3), and the most efficient inducers of P_{ttgG} *in vivo* were tested at different concentrations. EMSA studies revealed that there was a direct correlation between the concentration of the tested compound and the amount of TtgV that was released from its target DNA (see Fig. 3 for 1-naphthol, 2,3-dihydroxynaphthalene, benzonitrile, naphthalene, indole, 3-ethylphenol, and 4-nitrotoluene). In fact, with 0.5 mM 1-naphthol, 2,3-dihydroxynaphthalene, 3-ethylphenol, and 4-nitrotoluene, 90% of TtgV was released from its operator DNA (Fig. 3*B*). Indole and benzonitrile seemed to be similarly recognized by TtgV (around 70–80% of the DNA was freed from TtgV at a concentration of 1 mM compound (Fig. 3*B*)). These results are, in general, in agreement with the *in vivo* measurements of β-galactosidase activity since the compounds that yielded the highest induction level of the P_{ttgG} promoter *in vivo* also released more TtgV from its operator site in the *in vitro* experiments. Furthermore, compounds such as *p*-isopropylbenzoate, tetracycline, and chloramphenicol, which did not induce *in vivo* (not shown), failed to release TtgV from its target operator *in vitro*.

From a mechanistic point of view, TtgV was suggested to prevent RNA polymerase from accessing the promoter region (17). This suggestion was based on the observation that transcription inhibition was more effective when TtgV was added before the formation of the open complex by RNA polymerase. It was further reasoned that upon ligand binding, TtgV was released from its operator site and *ttgGHI* transcription occurred. To test whether the chemicals capable of releasing TtgV from its operator allowed expression from the P_{ttgG} promoter, we performed *in vitro* transcription assays in which TtgV was added before RNA polymerase in the absence and in the presence of some of the most efficient effectors (Fig. 4). As expected, expression from the *ttgG* promoter was inhibited (*lane 2*), whereas the presence of 100 μM benzonitrile, 2,3-dihydroxynaphthalene, or 1-naphthol enabled transcription from P_{ttgG} at a level similar to that observed in the absence of TtgV. These results support a mechanism by which TtgV represses *ttgG* expression by blocking the RNA polymerase binding site. Nevertheless, in the presence of ligand molecules of different structures and substituents, TtgV dissociates from its operator site, allowing RNA polymerase to start transcription from P_{ttgG}.

DISCUSSION

EMSA, equilibrium dialysis, and ITC experiments (Figs. 2 and 3 and Tables II–IV) suggested that TtgV is able to bind a large number of structurally different compounds and that this interaction leads to the induction of *ttgGHI* expression. This is the first case in which a regulator belonging to the IclR family is shown to interact directly with different chemicals. Our findings contrast with the relatively narrow effector specificity found for other regulators of the IclR family that recognize a single aromatic compound. For example, PobR and PcaU of *Acinetobacter calcoaceticus* interact only with 4-hydroxybenzoate and 3,4-dihydroxybenzoate, respectively (26–28). However, the ability of TtgV to recognize various effectors of dissimilar structure seems to be a particular feature of the regulators that control the expression of multidrug efflux pumps. This is consistent with a mechanism based on the direct recognition of structurally dissimilar compounds rather than the involvement of a secondary messenger, which has also been shown for a limited number of other multidrug pump regulators such as QacR (29) and BmrR (30, 31).

Our data indicate that one effector molecule binds to a TtgV dimer. This stoichiometry has also been seen for the QacR regulator (25) but is different from TetR, where two molecules of tetracycline bind to the protein dimer (32, 33). QacR accommodates structurally diverse ligands in different parts of the large binding pocket (25). However, signal transduction for all ligands is mediated by an identical induction mechanism, and QacR-drug complexes undergo the same transition (25, 33). Our results support that TtgV, a member of the IclR family of repressors, may function similarly to QacR since there is a relation between the *in vitro* affinity of TtgV for its effectors and their *in vivo* efficiency. However, details of TtgV-effector interactions await the resolution of the three-dimensional structure of TtgV bound to these chemicals.

The affinity of TtgV for its effectors spans the micromolar range, and the binding constants are similar to those of other repressor proteins such as TrpR (34) or QacR (35), the latter having a K_D around 1 μM for rhodamine 6G (25). Although there is no strict correlation ($R^2 = 0.76$) for the linear fit of the plot of $\ln K_D$ against β-galactosidase activity, a clear relation was observed between the affinity of TtgV for different effectors and their potential to induce gene expression (measured by β-galactosidase assays). Effectors with the highest affinity, such as 1-naphthol and 2,3-dihydroxynaphthalene, were shown to be more efficient *in vivo*. Effectors with slightly lower *in vivo* induction activities, such as indole, 4-nitrotoluene, and benzonitrile, were also found to bind less strongly to the protein *in vitro*, whereas effectors characterized by moderate *in vivo* activity (toluene and hexanol) showed significantly reduced affinity. The lack of a strict correlation between *in vitro*-determined K_D values and β-galactosidase levels induced *in vivo* is mainly due to differential cell extrusion of the tested compounds, which is mediated by the TtgGHI pump and other multidrug extrusion elements rather than to the existence of secondary layers of regulators influenced by these chemicals.[2] Furthermore, it should be taken into account that the positive correlation between the affinity of TtgV for an effector molecule and the release of the protein from the promoter is not always found in transcriptional regulators. This is exemplified by FadR, for which the effector palmitoyl-CoA is around 50-fold more efficient than myristoyl-CoA in inhibiting FadR from

[2] M. E. Guazzaroni, M. T. Gallegos, and J. L. Ramos, unpublished results.

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DNA binding. However, ITC assays showed that palmitoyl-CoA binds around one-sixth as strongly to FadR as does myristoyl-CoA (36). The binding affinity of the effector to TtgV can thus be considered the major determinant of gene expression.

In general, the binding modes of structurally similar ligands to proteins with narrow substrate specificity are comparable. This was not the case for binding of TtgV to 1-naphthol and 2,3-dihydroxynaphthalene (Table IV), which were enthalpy- and entropy-driven, respectively. It remains to be explored whether the different binding modes observed here for structurally similar effectors is a general feature of multidrug recognition. TtgV is a dimer in solution and binds to its corresponding target, DNA covering four potential direct repeats within the *ttgG* promoter (17). In the absence of effectors, TtgV binds tightly to its target promoter (K_D 2.4 ± 0.35 nM).

The effector 1-naphthol was shown to bind with high affinity to the protein-DNA complex ($K_D = 4.8$ μM), an affinity similar to that determined for the binding to free TtgV ($K_D = 3.0$ μM). Furthermore, TtgV bound to 1-naphthol (or other effectors) does not interact with its target DNA promoter. This set of observations is of physiological importance and can be critical for survival since the K_D of 1-naphthol for the TtgV-DNA complex is lower than the toxicity threshold of this compound (around 1 mM). This implies that 1-naphthol triggers a TtgV response at a concentration at which its toxicity is low. The up-regulation of the expression of the efflux pump and consequently the extrusion of toxic substances probably lead to a dynamic equilibrium between uptake and expulsion, which is characterized by an intracellular concentration of the toxic compound below its toxicity threshold. This model could be a general feature for the regulation of bacterial multidrug transporters.

The finding that the binding of the TtgV-DNA complex to 1-naphthol was less exothermal than the binding to free protein was unexpected (Fig. 2, *right-hand panel*; Table IV). As stated above, the heat signal from the titration of the TtgV-DNA complex with 1-naphthol should be considered as the sum of heats originating from effector binding and protein dissociation from DNA. We have demonstrated that the binding of the effector to TtgV is characterized by a favorable enthalpy change of −8.6 ± 0.16 kcal/mol, whereas the binding of protein to DNA is endothermic ($\Delta H = 5.5 \pm 0.04$ kcal/mol). This implies that protein dissociation from DNA should give rise to an exothermic signal. During the binding of 1-naphthol to the TtgV-DNA complex, the exothermic heat of TtgV-operator dissociation was expected to combine with the exothermic heat generated by the effector binding, giving rise to a stronger exothermic signal than in the binding of TtgV to the effector. However, this was not the case, and this heat change for the binding of 1-naphthol to the TtgV-DNA complex was less exothermic than that the one observed in the binding to TtgV (Fig. 2, Table IV). The above hypothetical combination effect of heat is only valid for fully reversible systems, in which the nature of the binding reactions corresponds to the inverse of the dissociation reactions. In general, very little is known about the mechanisms of effector-induced protein dissociation. Our data indicate either that the system studied here is not entirely reversible or that the molecular mechanisms of protein-DNA association and dissociation differ. Further studies are necessary to elucidate the conformational changes caused by effector binding to free and TtgV-bound DNA.

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REFERENCES

1. Ramos, J. L., Duque, E., Gallegos, M. T., Godoy, P., Ramos-González, M. I., Rojas, A., Terán, W., and Segura, A. (2002) *Annu. Rev. Microbiol.* **56,** 743–768
2. Ramos, J. L., Duque, E., Huertas, M. J., and Haïdour, A. (1995) *J. Bacteriol.* **177,** 3911–3916
3. Sikkema, J., de Bont, J. A. M., and Poolman, B. (1995) *Microbiol. Rev.* **59,** 201–222
4. Chatterjee, A., Chaudhuri, S., Saha, G., Gupta, S., and Chowdhury, R. (2004) *J. Bacteriol.* **186,** 6809–6814
5. Lee, E. H., Rouquette-Loughlin, C., Folster, J. P., and Shafer, W. M. (2003) *J. Bacteriol.* **185,** 7145–7152
6. Lee, E. W., Huda, M. N., Kuroda, T., Mizushima, T., and Tsuchiya, T. (2003) *Antimicrob. Agents Chem.* **47,** 3733–3738
7. Middlemiss, J. K., and Poole, K. (2004) *J. Bacteriol.* **186,** 1258–1269
8. Nikaido, H. (1996) *J. Bacteriol.* **178,** 5853–5859
9. Ramos, J. L., Duque, E., Godoy, P., and Segura, A. (1998) *J. Bacteriol.* **180,** 3323–3329
10. Schnappinger, D., Schubert, P., Berens, C., Pfleiderer, K., and Hillen, W. (1999) *J. Biol. Chem.* **274,** 6405–6410
11. van Dyk, T., Templeton, L. J., Cantera, K. A., Sharpe, P. L., and Sariaslani, F. S. (2004) *J. Bacteriol.* **186,** 7196–7204
12. Yoshida, K. I., Ohki, Y. H., Murata, M., Kinehara, M., Matsouka, H., Satomura, T., Ohki, R., Kumano, M., Yamane, K., and Fujita, Y. (2004) *J. Bacteriol.* **186,** 5640–5648
13. Yu, E. W., Aires, J. R., and Nikaido, H. (2003) *J. Bacteriol.* **185,** 5657–5664
14. Rojas, A., Duque, E., Mosqueda, G., Golden, G., Hurtado, A., Ramos, J. L., and Segura, A. (2001) *J. Bacteriol.* **183,** 3967–3973
15. Rojas, A., Duque, E., Schmid, A., Hurtado, A., Ramos, J. L., and Segura, A. (2004) *Appl. Environ. Microbiol.* **70,** 3637–3643
16. Rojas, A., Segura, A., Guazzaroni, M. E., Terán, W., Hurtado, A., Gallegos, M. T., and Ramos, J. L. (2003) *J. Bacteriol.* **185,** 4755–4763
17. Guazzaroni, M. E., Terán, W., Zhang, X., Gallegos, M. T., and Ramos, J. L. (2004) *J. Bacteriol.* **186,** 2921–2927
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1991) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
19. Gallegos, M. T., Marqués, S., and Ramos, J. L. (1996) *J. Bacteriol.* **178,** 2356–2361
20. Terán, W., Felipe, A., Segura, A., Rojas, A., Ramos, J. L., and Gallegos, M. T. (2003) *Antimicrob. Agents Chemother.* **47,** 3067–3072
21. Wiseman, T., Williston, S. Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem.* **179,** 131–137
22. Grkovic, S., Brown, M. H., Schumacher, M. A., Brennan, R. G., and Skurray, R. A. (2001) *J. Bacteriol.* **183,** 7102–7109
23. Murray, D. S., Schumacher, M. A., and Brennan, R. G. (2004) *J. Biol. Chem.* **279,** 14365–14371
24. Schumacher, M. A., Miller, M. C., and Brennan, R. G. (2004) *EMBO J.* **23,** 2923–2930
25. Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A., and Brennan, R. G. (2001) *Science* **294,** 2158–2163
26. Kok, R. G., D'Argenio, D. A., and Ornston, L. N. (1998) *J. Bacteriol.* **180,** 5058–5069
27. Gerischer, U., Segura, A., and Ornston, L. N. (1998) *J. Bacteriol.* **180,** 1512–1524
28. Trautwein, G., and Gerischer, U. (2001) *J. Bacteriol.* **183,** 873–881
29. Grkovic, S., Brown, M. H., Roberts, N. J., Paulsen, I. T., and Skurray, R. A. (1998) *J. Biol. Chem.* **273,** 18665–18673
30. Heldwein, E. E., and Brennan, R. G. (2001) *Nature* **409,** 378–382
31. Vazquez-Laslop, N., Markham, P. N., and Neyfakh, A. A. (1999) *Biochemistry* **38,** 16925–16931
32. Berens, C., and Hillen, W. (2003) *Eur. J. Biochem.* **270,** 3109–3121
33. Hinrichs, W., Kisker, C., Düvel, M., Müller, A., Tovar, K., Hillen, W., and Saenger, W. (1994) *Science* **264,** 418–420
34. Kavanoor, M., and Eftink, M. R. (1997) *Biophys. Chem.* **66,** 43–55
35. Grkovic, S., Hardie, K. M., Brown, M. H., and Skurray, R. A. (2003) *Biochemistry* **42,** 15226–15236
36. DiRusso, C. C., Tsvetnitsky, V., Højrup, P., and Knudsen, J. (1998) *J. Biol. Chem.* **273,** 33652–33659
37. Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. (1987) *Plant Mol. Biol.* **9,** 27–39

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CAPÍTULO 4.

Unión diferencial de TtgV a efectores monoaromáticos y biaromáticos. Papel en la desrepresión diferencial de su operador

Autores: Guazzaroni, M.E., Gallegos, M.T., Ramos J.L. y Krell, T.

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Los miembros de la familia IclR de reguladores transcripcionales muestran un dominio de unión a efectores muy conservado e interaccionan con un número limitado de efectores. En contraste con la mayoría de los miembros de la familia IclR, TtgV, el represor transcripcional de la bomba de eflujo TtgGHI, exhibe propiedades de reconocimiento de múltiples ligandos. En este trabajo se generó un modelo tridimensional del dominio de unión a efectores de TtgV basado en cuatro estructuras tridimensionales de otros miembros de la familia y se realizó una serie de mutaciones puntuales en el posible bolsillo de unión de efectores. Mediante el empleo de calorimetría de titulación isotérmica se determinaron los parámetros de unión de TtgV y sus mutantes puntuales a los efectores más eficientes *in vivo*. Todas las proteínas mutantes se unieron a los compuestos aromáticos de dos anillos con mayor afinidad que la proteína nativa, por otro lado, las proteínas mutantes se unieron con menor afinidad que TtgV nativa a los hidrocarburos aromáticos de un único anillo. Esta tendencia se vio aún más pronunciada en los mutantes F134A y H200A. La proteína TtgVF134A se unió a 4-nitrotolueno con una afinidad 13 veces más baja que TtgV (17,4 ± 0,6 μM). Sin embargo, esta proteína mutante se unió a 1-naftol con una afinidad de 5,7 μM, la cual es 7 veces mayor que la de TtgV (40 μM). El mutante TtgVV223A se unió al ADN con una afinidad similar a la de TtgV, pero permaneció unido a éste aún en presencia de efectores, lo que podría sugerir que el residuo V223 podría formar parte de la vía de reconocimiento de la señal dentro de TtgV. El análisis termodinámico de la unión de efectores a TtgV y sus mutantes en complejo con el ADN reveló que la unión de compuestos biaromáticos resultó en un despegue más eficiente del represor que los compuestos monoaromáticos. Se discute la relevancia fisiológica de estos datos.

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Different Modes of Binding of Mono- and Biaromatic Effectors to the Transcriptional Regulator TTGV

*ROLE IN DIFFERENTIAL DEREPRESSION FROM ITS COGNATE OPERATOR**

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María-Eugenia Guazzaroni, María-Trinidad Gallegos, Juan L. Ramos[1], and Tino Krell

From the Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, C/Professor Albareda, 1, E-18008 Granada, Spain

Members of the IclR family of regulators exhibit a highly conserved effector recognition domain and interact with a limited number of effectors. In contrast with most IclR family members, TtgV, the transcriptional repressor of the TtgGHI efflux pump, exhibits multidrug recognition properties. A three-dimensional model of the effector domain of TtgV was generated based on the available three-dimensional structure of several IclR members, and a series of point mutants was created. Using isothermal titration calorimetry, we determined the binding parameters of the most efficient effectors for TtgV and its mutant variants. All mutants bound biaromatic compounds with higher affinity than the wild-type protein, whereas monoaromatic compounds were bound with lower affinity. This tendency was particularly pronounced for mutants F134A and H200A. TtgVF134A bound 4-nitrotoluene with an affinity 13-fold lower than that of TtgV (17.4 ± 0.6 μM). This mutant bound 1-naphthol with an affinity of 5.7 μM, which is seven times as great as that of TtgV (40 μM). The TtgVV223A mutant bound to DNA with the same affinity as the wild-type TtgV protein, but it remained bound to the target operator in the presence of effectors, suggesting that Val-223 could be part of an intra-TtgV signal recognition pathway. Thermodynamic analyses of the binding of effectors to TtgV and to its mutants in complex with their target DNA revealed that the binding of biaromatic compounds resulted in a more efficient release of the repressor protein than the binding of monoaromatics. The physiological significance of these findings is discussed.

The DOT-T1E strain of *Pseudomonas putida* has the extraordinary capacity to withstand, and even grow in, the presence of high concentrations of organic solvents such as an aqueous solution saturated with toluene, a highly toxic compound (1). The main mechanism underlying this resistance lies in the action of three RND (resistance-nodulation-cell Division) efflux pumps, termed TtgABC, TtgDEF, and TtgGHI (pump encoded by toluene tolerance genes *ttgGHI*) (2), which extrude organic solvents and other toxic compounds from the cells. These three efflux pumps show a high degree of similarity to the AcrAB-TolC multidrug efflux pump, which is the best characterized member of this family (3–7, 29). Expression of the *P. putida* efflux pumps is controlled by transcriptional repressors; TtgR controls the expression of the *ttgABC* operon (8, 9), whereas TtgV (10–12) is the main regulator controlling the expression of the *ttgDEF* and *ttgGHI* operons.

TtgV, a member of the IclR family of regulators (13, 14), exhibits multidrug binding properties (12) in contrast to other members of this family, which are generally characterized by their high specificity for effector molecules (15–17). TtgV is a repressor that operates according to effector-mediated derepression. In the absence of effector, the protein is bound to the promotor DNA region repressing transcription. Effector binding to the TtgV-DNA complex is thought to produce an intramolecular signal that is transmitted to the DNA-binding domain, giving rise to protein dissociation from the operator. The RNA polymerase then accesses the promoter and transcribes the corresponding genes (11). The most efficient effectors *in vivo* are two-ring aromatic compounds such as 1-naphthol (1NL)[2] and indole (IND) and one-ring compounds such as 4-nitrotoluene (4NT) and benzonitrile (BN) (12).

In the framework of structural genomic studies, two research groups reported the three-dimensional structure of two members of the IclR family members, that of the IclR-TM protein isolated from *Thermotoga maritima* (PDB: 1MKM (18)) and that of a regulator of unknown function purified from *Rhodococcus* sp. RHA1 (PDB: 2G7U). Both proteins consist of two well separated domains. The N-terminal DNA-binding domain is linked by a long helix to the conserved effector-binding domain. Furthermore, the coordinates of several individual effector-binding domains of IclR family members have been released on the PDB data base. However, none of the structures available forms a complex with a physiologically relevant ligand.

A certain body of information is available on the interaction of multidrug-binding transcriptional regulators with effectors

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[1] To whom correspondence should be addressed: Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Calle Profesor Albareda, número 1, E-18008 Granada, Spain. Tel.: +34 958 181600, Ext. 326; Fax: +34 958 135740; E-mail: jlramos@eez.csic.es.

[2] The abbreviations used are: 1NL, 1-naphthol; BN, benzonitrile; EMSA, electrophoretic mobility shift assay; IND, indole; ITC, isothermal titration calorimetry; MIC, minimal inhibitory concentration; 4NT, 4-nitrotoluene; PDB, Protein Data Bank.

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(19, 20). In several cases, affinities do not correlate with the potential of the effectors to release protein *in vitro* or with their capacity to modulate gene expression *in vivo* (21, 22). This raises questions concerning the efficiency of effector-induced intramolecular signal transmission, an issue that has rarely been addressed. From structural studies, it appears that the effector structure determines with which set of amino acids the effector interacts in the binding pocket (23), which in turn determines the efficiency of signal transduction (21). It is unknown whether this efficiency can also be modulated by mutations in the regulator binding pocket, which would be of relevance for understanding the evolution of multidrug-binding proteins. Based on the three-dimensional homology model of TtgV, we have identified the potential effector-binding pocket of TtgV. Six site-directed alanine replacement mutants of amino acids located in this pocket were generated and characterized in this study. Isothermal titration calorimetry (ITC) was used to determine the thermodynamic parameters for the binding of four different effectors to the wild type and to all TtgV mutants. Further experiments were aimed at evaluating the impact of the mutations on DNA binding and at characterizing the efficiency of the effector in triggering the release of the bound repressor.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—TtgV mutants, in which amino acid residues at positions 118, 134, 140, 200, 204, and 223 were replaced by alanine and valine (positions 134 and 200), were generated by overlapping PCR mutagenesis (24) using plasmid pANA126 (10) as a source of the *ttgV* wild-type allele. For each mutant, three PCRs were carried out. The initial two PCRs involved amplifications using the upstream primer (5′-CGCTCCACCGTTCAGAGAAT-3′, corresponding to nucleotides 139–158 of *ttgV* coding sequence) and a mismatch primer covering the segment to be mutated as well as a PCR amplification using the downstream primer (5′-CTTGTCGACGGAGCTCGAAT-3′, nucleotides 791–810 of the *ttgV* coding sequence) and an oligonucleotide complementary to the mismatch primer. The following mismatch primers were used, and the mismatch codon is underlined: I118A, 5-AGACAAA<u>GCG</u>TACGTGCTT-3; F134A, 5′-GGTAGTG<u>GCG</u>CCGATTGGTA-3′; V140F, 5′-GTATTAAC<u>TTC</u>CCCGCGCA-3′; V140A, 5′-GTATTAAC<u>GCG</u>CCCGCCGCA-3′; H200A, 5′-TGGACGAG<u>GCG</u>ATTGATGGC-3′; V204A, 5′-ATTGATG<u>GCG</u>CGTGCTCATT-3′; V223A: 5′-CTCGCGATC<u>GCG</u>ATGCCGAG-3′. The resulting overlapping PCR products were annealed, supplemented with upstream and downstream primers, and submitted to the third PCR. For the I118A, F134A, and V160A mutations, the final PCR product was cut with BbvCI and BlnI, which produced a 223-bp fragment that was cloned into pANA126 linearized with the same enzymes. For the H200A, H200V, V204A, and V223A mutations, the final PCR product was digested with BlnI and PstI, and the resulting 200-bp fragment was equally cloned into pANA126.

Cell Culture and Protein Expression—*Escherichia coli* B834 (DE3) was transformed with pANA126 bearing the wild-type *ttgV* allele (10) and a series of pANA126 derivatives that encode the six different TtgV mutants. Cells were grown in 2-liter conical flasks with 500 ml of LB supplemented with 25 μg/ml kanamycin. Cultures were incubated at 30 °C with shaking and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside when the culture reached a turbidity at 660 nm (OD_{660}) of 0.7. Cultures were then transferred at 18 °C, and after growth for 3 h, cells were harvested by centrifugation (10 min at 4000 *g*) and stored at −80 °C.

Protein Purification—Cells from a 1-liter culture were suspended in 50 ml of buffer A (25 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole, 5% (v/v) glycerol, 0.1 mM dithiothreitol, pH 7.5) containing 10 units/ml Benzonase (Novagen, Madrid, Spain) and one tablet of Roche Applied Science Complete™ EDTA-free protease inhibitor mixture. Cells were broken by two passages through a French press at 1000 p.s.i., and the resulting suspension was centrifuged at 19,000 × *g* for 45 min. The supernatant was filtered and loaded onto a 5-ml Hi-Trap chelating column (GE Healthcare, St. Gibes, UK). His_6-TtgV and its mutant variants were eluted with a 45–500 mM gradient of imidazole in buffer A. Protein was dialyzed against 20 mM Tris-HCl, 8 mM magnesium acetate, 300 mM NaCl, pH 7.2. For storage at −80 °C, the samples were mixed with 10% (v/v) glycerol. The purity of the protein was between 90 and 95%, as judged from SDS-PAGE gels. Protein samples were aliquoted prior to freezing. All experiments were carried out with a single batch of each protein. Protein aliquots were thawed for immediate use, and excess protein was discarded.

ITC—Measurements were made with a VP-Microcalorimeter (MicroCal, Northampton, MA) at 25 °C. The protein was thoroughly dialyzed against 20 mM Tris-HCl, 8 mM magnesium acetate, 100 mM NaCl, 10% (v/v) glycerol, and 1 mM dithiothreitol, pH 7.2. The buffer used in our initial analysis (12) and in the present study differed in that the buffer used for the assays reported here included 10% (v/v) glycerol, which had a stabilizing effect on the protein, as well as a higher ionic strength and lower pH (7.2 rather than 8.0), which corresponds more closely to physiological conditions. Protein concentration was determined with the Bradford assay. Stock solutions of 1NL, BN, 4NT, and IND at a concentration of 500 mM were prepared in dimethyl sulfoxide, and the solutions were diluted with dialysis buffer to final concentrations of 0.5 to 1 mM. The corresponding amount of dimethyl sulfoxide was added to the protein sample. DNA duplex samples were prepared as described by Guazzaroni *et al.* (12). Each titration involved a single 1.6-μl injection and a series of 4.8-μl injections of effectors into a 34–40 μM protein solution. The mean enthalpies measured from injection of the ligands into the buffer were subtracted from raw titration data prior to data analysis with a model for the binding of a ligand to identical independent sites of a macromolecule (MicroCal). Data analysis with this model produced satisfactory statistical data.

Circular Dichroism—The CD spectra of each protein were recorded on a Jasco 715 spectropolarimeter (Great Dunmow, UK). Spectra in the far UV region (195–260 nm) were recorded in cylindrical quartz cells (0.02-cm path length) at a protein concentration of 0.6 mg/ml. All protein solutions were dialyzed against the buffer used for ITC.

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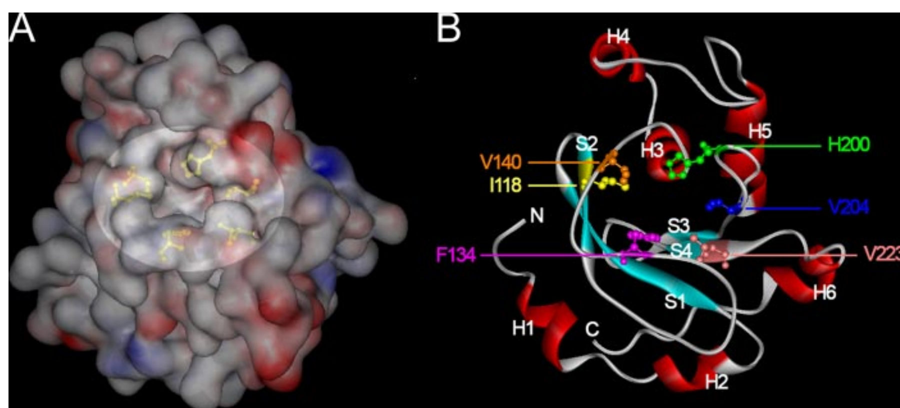

FIGURE 1. **The three-dimensional model of the effector-binding domain of TtgV.** *A*, a surface plot colored according to electrostatic potential. The assumed ligand-binding pocket is *highlighted*, and the amino acids replaced by alanine are shown in *ball-and-stick mode*. *B*, ribbon representation of the model, annotated to show secondary structure elements (*H* for helix, *S* for strand) and the mutated amino acids. The figures were prepared with WebLabViewer software (Accelrys, San Diego, CA).

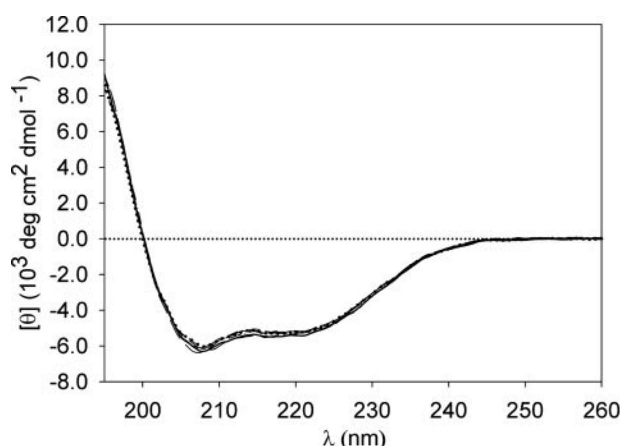

FIGURE 2. **Circular dichroism analysis of mutant and wild-type TtgV.** Superimposed far-UV circular dichroism spectra of TtgV (——), TtgVH200A (····), TtgVI118A (—–), TtgVF134A (–··–··), TtgVV140A (– – –), TtgVV204A (·–·–·–), and TtgVV223A (–||–||–).

EMSA—Experiments were carried out as described previously (12). One nM labeled DNA probe ($\sim 1.5 \times 10^4$ cpm) was incubated with increasing concentration of TtgV (10–1500 nM) for 10 min at 30 °C in 10 μl of 20 mM Tris-HCl, 8 mM magnesium acetate, 100 mM NaCl, 10% (v/v) glycerol, and 1 mM dithiothreitol, pH 7.2, containing 20 μg/ml poly(dI-dC) and 200 μg/ml bovine serum albumin.

Determination of MICs—P. putida DOT-T1E and isogenic mutants lacking one or several Ttg efflux pumps (2, 25) were grown overnight in LB medium with the appropriate antibiotics. Cultures were diluted 100-fold in LB supplemented with solvents at different concentrations and incubated for 20 h at 30 °C. The MIC value corresponds to the lowest concentration that reduced growth by more than 90%. Results are the average of at least five independent assays.

RESULTS

Homology Modeling of TtgV and Identification of Amino Acids in the Effector-binding Site—We have previously shown that the IclR family of regulators comprises more than 500 members and that a distinct signature profile can be derived from the alignment of the whole set of proteins. In contrast with other families of regulators, the region that best defines the IclR family is not the DNA-binding domain but the effector-binding region (13, 25). The predicted secondary structure of TtgV can be closely aligned to the secondary structure elements found in the three-dimensional structure of the full-length IclR-TM protein of *T. maritima* (18). The TtgV sequence corresponding to the effector-binding domain was subsequently subjected to homology modeling with Geno3D (26) software. The model was based on the four following templates: *T. maritima* IclR-TM (PDB: 1MKM (18)), the effector-binding domains of *E. coli* IclR (PDB: 1TD5), the glyoxylate regulatory protein (PDB: 1TF1), and KdgR (PDB: 1YSP). These templates share 21–29% sequence identity with TtgV. The model obtained was submitted to What_check (27) and was found to have an acceptable geometry. A Ramachandran plot showed that over 97% of the residues were in allowed regions. With the DALI algorithm, it was possible to superimpose the TtgV effector-binding domain model onto its templates with C_α root mean square deviation values between 1.8 and 2.4 Å. A surface representation of this model (Fig. 1*A*) shows a hydrophobic cavity with a volume of 1200 Å^3 as determined by PASS software (28). This model proposes that the ligand-binding pocket of TtgV is formed by a long loop connecting S2 with H3 and the β-sheet (Fig. 1*B*). A number of residues within this cavity were selected for mutagenesis according to two criteria: 1) their location and projection in the binding pocket of the model and 2) their conservation in TtgT, a transcriptional regulator sharing 56% sequence identity with TtgV, which has a very similar effector profile.[3]

Based on these criteria, 6 residues, Ile-118, Phe-134, Val-140, His-200, Val-204, and Val-233, were chosen (Fig. 1*B*), and alanine replacement mutants were generated in each position. In the model, 2 amino acids were located on β-strands: Ile-118 on S2 and Val-223 on S4. Residues Phe-134 and Val-140 were located on the loop connecting S2 with H3. The side chain of Phe-134 lies in the lower side of the effector-binding pocket and appears to play a central role in effector binding. Amino acids His-200 and Val-204 were mutated in the upper part of the pocket.

Mutations in the predicted effector-binding pocket seemed not to alter the secondary protein structure of the mutant proteins, as deduced from analysis of mutant and wild-type TtgV proteins by far UV circular dichroism spectroscopy. The spectra of all proteins could be closely superimposed (Fig. 2), indicating that the amino acid replacements did not significantly affect the protein secondary structure.

[3] W. Terán, A. Felipe, M.-E. Guazzaroni, T. Krell, R. Ruiz, J. L. Ramos, and M.-T. Gallegos, submitted for publication.

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Most Mutants Show Increased Affinities for Biaromatic Effectors but Bind Monoaromatic Compounds with Lower Affinity— We recently showed that TtgV effectors are primarily monoaromatic and biaromatic compounds (12). Two representatives of each class, which are efficient effectors *in vivo*, the monoaromatic compounds 4NT and BN and the biaromatic effectors 1NL and IND, were chosen to study their binding parameters to native and mutant proteins using ITC. With this technique, all thermodynamic binding parameters can be determined in a single experiment.

Fig. 3*A* shows the titration of TtgV and the F134A mutant with 4NT. The derived binding parameters are given in Table 1. Binding to the TtgV protein was enthalpy-driven ($\Delta H = -9.7 \pm 0.2$ kcal/mol) and characterized by a K_D of 17.4 ± 0.6 μM. When the same experiment was repeated with the F134A mutant, the heat changes were much smaller, and affinity decreased by a factor of almost 13-fold (Fig. 3*A* and Table 1). An even more dramatic decrease in affinity for 4NT (27-fold) was obtained for the H200A mutant. For both mutants, enthalpy changes were reduced to −3.0 and −3.5 kcal/mol, respectively (Table 1), which is consistent with fewer molecular interactions between the effector and these mutants. Affinity of the TtgVV140A, TtgVV204A, and TtgVV223A mutants was reduced by one-half to two-thirds of that of the native TtgV protein (Table 1). We also found that the single mutant TtgV I118A showed 2-fold higher affinity for 4NT than the wild-type protein.

The tendency in affinity observed for 4NT was completely opposite when the same experiments were repeated with the biaromatic effector 1NL; all mutants showed a higher affinity than the wild-type protein (Table 1). This is illustrated in Fig. 3*B* for the wild type and the F134A mutant, which bound 1NL

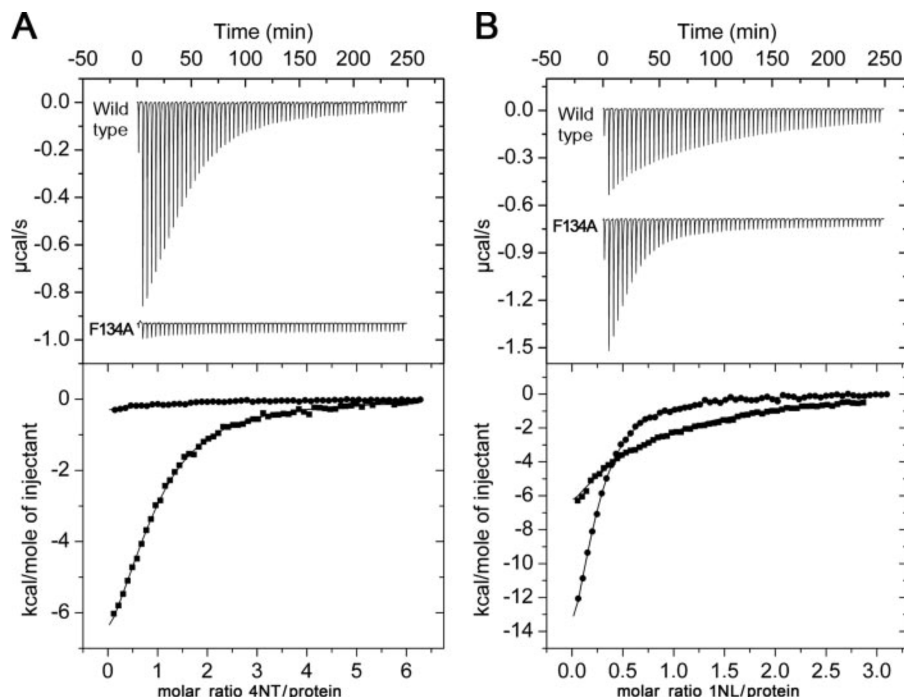

FIGURE 3. **Isothermal titration calorimetry study of the binding of 4NT and 1NL to the TtgV and TtgV F134A mutant.** *A*, *upper panel*, Raw ITC data, injection of 1.6- and 4.8-μl aliquots of 4NT (1 mM) into 37 μM TtgV and 35 μM F134A mutant (tetramer concentration). For clarity, the traces have been displaced arbitrarily along the *x* axis. *Lower panel*, integrated and corrected-for-dilution peak areas. *B*, microcalorimetric titration of 40 μM TtgV and 37 μM TtgVF134A mutant with 0.5 mM 1NL, using the same injection protocol as in *panel A*. The symbols are as follows: *squares*, wild-type TtgV protein; *circles*, TtgVF134A mutant protein. Thermodynamic parameters are shown in Table 1.

TABLE 1

Thermodynamic parameters derived from the microcalorimetric titration of TtgV with one- and two-ring aromatic effectors

Data were derived from ITC experiments; for further details, see "Experimental Procedures." One-ring aromatic compounds were 4-nitrotoluene (4NT) and benzonitrile (BN); two-ring aromatic compounds were 1-naphthol (1NL) and indole (IND). Experiments were carried out in 20 mM Tris-HCl, 8 mM magnesium acetate, 100 mM NaCl, 10% (v/v) glycerol, and 1 mM dithiothreitol, pH 7.2.

Protein	Effector	K_D	$K_D wt / K_D$	ΔH	Effector	K_D	$K_D wt / K_D$	ΔH
		μM		kcal/mol		μM		kcal/mol
WT[a]	4NT	17.4 ± 0.6	1.0	−9.7 ± 0.2	BN	50 ± 3	1.0	−5.7 ± 0.1
F134A	4NT	221 ± 8	0.08	−3.0 ± 0.1	BN	330 ± 10	0.15	−3.5 ± 0.1
F134V	4NT	No binding	BN	No binding				
H200A	4NT	470 ± 20	0.04	−3.5 ± 0.1	BN	No binding		
H200V	4NT	83 ± 10	0.2	−13 ± 2	BN	No binding		
I118A	4NT	8.9 ± 0.4	2.0	−8.6 ± 0.2	BN	46 ± 2	1.1	−14 ± 1
V140A	4NT	30 ± 1	0.6	−6.6 ± 0.2	BN	85 ± 3	0.6	−6.3 ± 0.5
V140F	4NT	24 ± 1	0.7	−5.9 ± 0.1	BN	77 ± 7	0.6	−4.8 ± 0.8
V204A	4NT	41 ± 2	0.4	−14.3 ± 0.1	BN	71 ± 5	0.7	−13 ± 4
V223A	4NT	59 ± 3	0.3	−6.4 ± 0.4	BN	58 ± 4	0.9	−10 ± 2
WT	1NL	40 ± 3	1.0	−21 ± 3	IND	78 ± 7	1.0	−6 ± 2
F134A	1NL	5.7 ± 0.2	7.0	−22.5 ± 0.6	IND	32 ± 2	2.4	−12 ± 1
F134V	1NL	8 ± 1	6.7	−47 ± 2	IND	44 ± 6	1.8	−11 ± 1
H200A	1NL	2.9 ± 0.4	13.8	−16 ± 3	IND	39 ± 3	2.0	−2.9 ± 0.6
H200V	1NL	5.1 ± 0.8	7.8	−26.5 ± 0.1	IND	49 ± 5	2.0	−13 ± 9
I118A	1NL	24 ± 2	1.7	−16 ± 1	IND	46 ± 2	1.7	−16 ± 2
V140A	1NL	6.5 ± 0.5	6.2	−22 ± 1	IND	21 ± 2	3.7	−14 ± 2
V140F	1NL	2.5 ± 0.1	16	−20.5 ± 0.5	IND	19 ± 2	4.1	−15.7 ± 0.6
V204A	1NL	23.8 ± 0.8	1.7	−23 ± 1	IND	37 ± 2	2.1	−10 ± 1
V223A	1NL	6.2 ± 0.4	6.5	−16.8 ± 0.6	IND	35 ± 2	2.2	−9 ± 1

[a] Wild-type TtgV protein.

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with K_D values of 40.1 ± 3 and 5.7 ± 0.2 μM, respectively. All six mutants exhibited affinities for 1NL that were between 1.7- and 13.8-fold higher. Most interestingly, the gain in affinity for 1NL was most pronounced for the mutants that had the lowest affinity for the monoaromatic 4NT, *i.e.* the F134A and H200A mutants.

To establish whether the differential binding behavior of these two effectors is a general phenomenon for mono- and biaromatic effectors, ITC experiments were conducted with two other representative effectors, namely BN (monoaromatic) and IND (biaromatic). In a manner exactly analogous to 4NT, all mutants except I118A had decreased affinity for the monoaromatic compound BN. This reduction in affinity was again particularly pronounced for F134A and H200A (Table 1). In analogy to 4NT, the enthalpic contribution to the binding of BN to F134A was again reduced, and no detectable affinity was observed for the H200A protein. As in the experiments with 1NL, all mutants were found to have increased affinities with respect to IND (Table 1).

We hypothesized that the increase in affinity for bicyclic compounds is founded on the increase in the volume of the binding pocket caused by the replacement of bulky amino acids. To further study this unexpected finding, we generated mutants in which Phe-134 and His-200 were replaced by valine. The microcalorimetric titrations also showed that both mutants bound monocyclic compounds with decreased affinity when compared with the wild-type protein (Table 1). Although F134A and H200V bound bicyclic compounds with higher affinity than the parental wild-type protein, affinity was lower than that of the corresponding alanine replacement mutants. Therefore, these results support the above hypothesis. We have also generated the V140F change. In this case, we found that the decrease in the size of the pocket resulted in small changes in affinity for the 4NT, BN, and IND compounds when compared with the V140A mutant. In contrast, 1NL bound with a higher affinity to V140F than to V140A. This indicates that apart from the volume increase, there are other factors, such as the establishment of additional van der Waals interactions between Val-140 and 1-NL, that can influence effector recognition (3, 5, 20, 21).

We also determined the affinity of the wild-type TtgV and its mutant variants when complexed with DNA for mono- and biaromatic effectors. We found, in agreement with previous findings (12), that affinity of TtgV and TtgV mutants for their effectors when bound to DNA was not altered in a significant manner. To illustrate this, we performed experiments involving the titration of free and DNA-bound TtgVV223A with 1NL (Fig. 4). 1NL bound to free and DNA-bound TtgVV223A with similar affinities, as evidenced by their respective dissociation constants of 6.2 ± 0.4 and 4.8 ± 0.1 μM.

Effect of Mutations on Affinity for Operator DNA—Subsequent experiments were aimed at elucidating whether the mutations in the effector-binding site altered the DNA binding characteristics of the mutants. EMSAs using 1 nM 210-bp fragment corresponding to the entire *ttgV-ttgGHI* intergenic region were carried out with increasing concentrations of each protein (10–1500 nM). From the fractions of bound and free DNA, the apparent dissociation constants were calculated, which are listed in Table 2. The wild-type TtgV protein had a K_{Dapp} of 150 ± 5 nM. The TtgVV223A mutant exhibited affinity similar to that of the wild type, whereas the remaining five proteins had lower affinities for their target DNA operator (their affinity

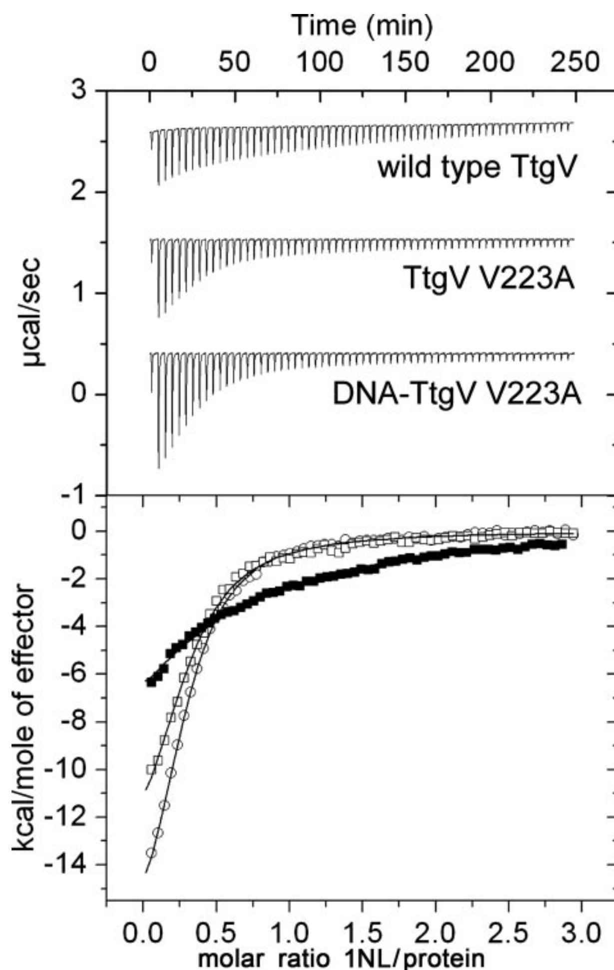

FIGURE 4. **1NL binding to the TtgV-DNA complex and quantification of the amount of protein released.** *Upper panel*, raw data for the microcalorimetric titration of TtgV, TtgVV223A, and TtgVV223A-DNA complex with 0.5 mM 1NL (initial injection of 1.6 μl followed by 4.8-μl aliquots). In all cases, the protein concentration was 40 μM, and 15 μM 63-bp operator DNA fragment was present in the latter sample. The sequence and preparation of the 63-bp DNA duplex were as described by Guazzaroni *et al.* (12). For clarity, the traces have been displaced arbitrarily along the *y* axis. *Lower panel*, integrated peak areas of the raw titration data and curve fit. The symbols are as follows: ■, wild-type TtgV; □, TtgVV223A; ○, DNA-TtgVV223A.

TABLE 2

Determination of apparent dissociation constants of wild-type and TtgV mutants for its operator

Data were obtained from the densitometric analyses of EMSA in the presence of protein concentrations ranging from 10 to 1500 nM. Results shown are the means of at least two independent assays done in duplicate. The DNA probe was 1 nM of a 210-bp fragment comprising the entire intergenic *ttgV-ttgG* region.

TtgV protein variants	K_D app	K_{Dappwt}/K_D app
	nM	
Wild type	150 ± 5	1
F134A[a]	550 ± 12	0.27
H200A[a]	640 ± 15	0.24
I118A[a]	680 ± 18	0.21
V140A[a]	800 ± 18	0.19
V204A[a]	800 ± 27	0.19
V223A[a]	160 ± 5	1

[a] The amino acid change in the mutant protein is indicated.

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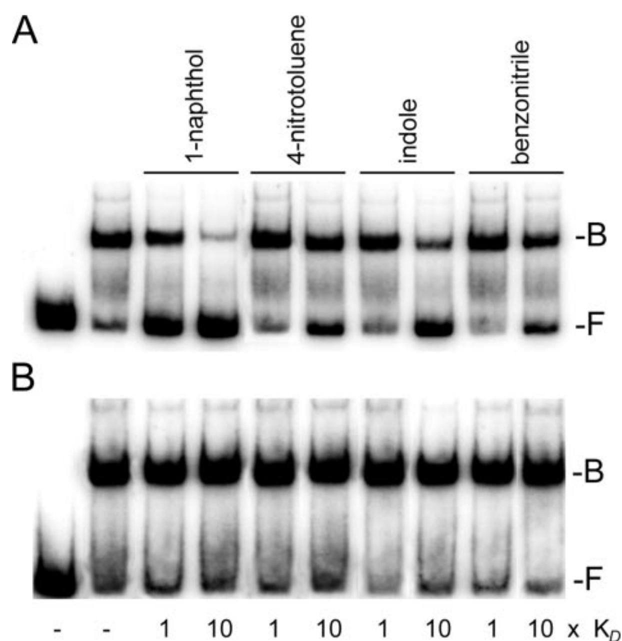

FIGURE 5. **EMSA of the *ttgV-ttgGHI* intergenic region in the presence of TtgV (*A*) and TtgVV223A (*B*) with different effectors.** One nM DNA fragment comprising the 210-bp intergenic *ttgV-ttgG* region was incubated without (*first lane*) and with 1 μM wild-type TtgV (*A*) or the mutant TtgVV223A (*B*) in the absence of effectors (−) or with the indicated effectors at concentrations corresponding to 1 or 10 times their K_D as determined by ITC (Table 1). *B*, bound DNA; *F*, free DNA.

decreased by factors of 3.7–5.4). An additional control experiment was done with mutant TtgVC205S. This mutation is vicinal to Val-204, but according to the homology model, it is unlikely to be involved in effector binding. Mutant TtgVC205S was found to have unaltered DNA binding properties when compared with the wild-type protein. This set of results suggested that mutations in the effector-binding pocket had a measurable effect on the DNA binding properties of this regulator, which is consistent with the existence of an intra-TtgV signal transmission chain connecting the two critical domains of this protein. This has been documented with other regulators such as TetR (30, 31), AraC (32, 33), MelR (34), XylS (35, 36), and XylR (37).

Differential Efficiency of Derepression of the Four Effectors—We then reasoned that if an intra-TtgV signal chain exists, it might be possible for different effector molecules to influence the efficiency of transmission, and subsequently, the functioning of TtgV. To test this hypothesis, we conducted EMSA with constant amounts of DNA and TtgV but with effector concentrations corresponding to multiples of the K_D. Under these conditions, occupancy of a protein-DNA complex with the effector is constant. Differences in effector-mediated protein release can thus be assumed to reflect differences in the efficiency of intra-TtgV signal transduction rather than differences in affinity.

Fig. 5*A* shows the EMSA results for TtgV in the presence of 1 and 10 times the K_D value determined for each effector. The densitometric analysis of this gel revealed that at a concentration of $1 \times K_D$ of 1NL, about 70% of the DNA was freed from the DNA/TtgV complex. However, in the corresponding experiments with 4NT, IND, and BN, the amount of freed DNA was low (between 1 and 3%). At effector concentrations corresponding to $10 \times K_D$, almost complete release of DNA was observed for the two biaromatic effectors 1NL (97%) and IND (92%), whereas the amount of freed DNA in the presence of the monoaromatic compounds 4NT and BN was only 33 and 30%, respectively. These data are consistent with the different efficiency in signal transduction by different effectors, biaromatic compounds being more effective than monoaromatic effectors. Similar assays were conducted with all mutants, and the results were similar to those reported for the wild-type protein (not shown) except for TtgVV223A. EMSAs revealed that this mutant protein was not readily released from its target operator in the presence of effectors, in contrast to the wild-type protein. The experiment illustrated in Fig. 5*B* shows that with concentrations of 1NL corresponding to 10 times its K_D, a large amount (>80% in a series of three independent assays) of the TtgVV223A mutant remained bound to DNA, which is not the case for the wild-type protein (Fig. 5*A*).

Analogous experiments with TtgVV223A were carried out using the effectors 4NT and BN. Both these effectors bind with weaker affinity to mutant proteins (Table 1), which implies that the concentrations used were superior to those added to the wild-type TtgV. However, in analogy to the experiments with 1NL, the amount of TtgVV223A released by 4NT and BN was almost negligible. These results are consistent with TtgVV223A being less efficient in effector-mediated protein release.

TABLE 3
MIC of TtgV effectors for the wild-type *P. putida* DOT-T1E strain and its isogenic mutant strains

The mutant strains had a knock-out in the genes encoding the indicated efflux pumps (2, 25). Data are the average and standard deviation of at least three independent assays.

Strain	Nonfunctional pump(s)	MIC			
		1NL	IND	4NT	BN
		mM			
DOT-T1E		7 ± 0.1	9 ± 0.4	10[a]	15 ± 1.0
DOT-T1E-PS28	TtgGHI	4 ± 0.1	5 ± 0.1	10[a]	15 ± 0.8
DOT-T1E-1	TtgDEF	4 ± 0.1	7 ± 0.2	10[a]	13 ± 0.7
DOT-T1E-PS34	TtgDEF, TtgGHI	2 ± 0.2	3 ± 0.2	8 ± 0.4	10 ± 0.3

[a] This concentration corresponds to the solubility limit of this compound in water.

Biaromatic TtgV Effectors Are Substrates for the TtgDEF and TtgGHI Efflux Pumps—To evaluate the physiological relevance of the above data, it appeared indispensable to elucidate whether the effectors tested are substrates of the efflux pumps TtgDEF and TtgGHI. The MICs for these four compounds were determined with the parental *P. putida* DOT-T1E strain as well as in three mutants lacking the TtgDEF, TtgGHI, or both RND pumps (Table 3). A clear increase in susceptibility to 1NL and IND was seen in the strains that lacked the TtgDEF or TtgGHI pumps, which is direct evidence that these two compounds are substrates of the pumps under study. However, susceptibility to 4NT and BN was only slightly affected. These data are consistent with primarily biaromatic compounds acting as substrates for the TtgDEF and TtgGHI pumps.

DISCUSSION

Localization of the Effector-binding Site—A homology model of the effector-binding domain of TtgV was generated, and 6

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amino acids located in an apparent binding pocket were mutated. All mutants had altered binding parameters for the four effectors used (Fig. 3 and Table 1). Recently, the three-dimensional structure of the IclR family member AllR in complex with its effector glyoxylate was reported by Walker *et al.* (38). The alignment of AllR and TtgV sequences (24% identity) reveal that 4 of the 6 amino acids mutated in this study (Val-140, His-200, Val-204, Val-223) were found to correspond to amino acids (Leu-149, His-211, Leu-215, Ser-234, respectively) that are involved in glyoxylate binding by AllR. Furthermore, the mutation of each of His-211, Leu-215, and Ser-234 amino acids rendered AllR fully insensitive to 1 mM glyoxylate, as evidenced by EMSA. Based on the data reported for AllR and those obtained in this study for TtgV, we suggest that the amino acids mutated in this study in TtgV may be part of the effector-binding pocket of this repressor. Five of the six amino acids present in the effector-binding site of TtgV were hydrophobic. This contrasts with the results for AllR since of the 10 amino acids involved in glyoxylate binding, only two were hydrophobic. A similarly hydrophobic composition was found for other multidrug-recognizing regulators such as QacR (23) and BmrR (19), which supports the notion that hydrophobic interaction is the main driving force in multidrug recognition.

Different Binding Modes for Monoaromatic and Biaromatic Effectors—The ITC studies of mutant proteins revealed altered affinity for effectors with respect to the wild-type protein. Indeed, TtgV variants bound monoaromatic compounds with a lower affinity than TtgV, whereas biaromatic compounds were recognized with a higher affinity (Table 1 and Fig. 3). In this respect, a clear parallelism exists with BmrR, a well studied transcriptional regulator with multidrug binding capacity (39). Based on the co-crystal structure of BmrR in complex with the effector tetraphenylphosphonium (40), Vázquez-Laslop *et al.* (19) prepared alanine mutants of amino acids involved in the binding of this effector and studied the interaction of the mutant proteins with six different effectors. With the exception of 3 key residues for which affinity decreased for all effectors, the remaining mutations caused an irregular pattern characterized by an increase in affinity for some effectors and a decrease for others. The authors concluded that each effector contacts with a different set of amino acids, giving rise to multiple effector binding modes. In this respect, TtgV appears to be different since a clear pattern emerged for the binding of mono- and bicyclic compounds. Our data are consistent with the existence of two different binding modes specific for either mono- or biaromatic compounds.

The central feature of the binding mode for monoaromatic compounds appears to be extensive contacts between the effector and Phe-134 and His-200 side chains. Besides the strong reduction in the binding constants of F134A and H200A for the monoaromatic compounds (Table 1), the respective enthalpy changes are much less favorable for both mutant proteins. This is exemplified by the enthalpy changes of 4NT binding to F134A and H200A, which, at −3.0 and −3.5 kcal/mol, were significantly below the value of the wild-type protein (−9.7 kcal/mol). A similar decrease in enthalpy is observed for BN binding to TtgVF134A (Table 1). Favorable enthalpy changes are typically attributed to the extent of direct contacts between the two ligands, and the decrease in enthalpy observed in alanine mutants has been used to estimate the contribution of single side chains to the binding energetics (41, 42). This observation is in consonance with previous reports that showed that phenylalanine side chains play essential roles in the recognition of monoaromatic compounds such as in toluene monooxygenase (43) or nitrotoluene dioxygenase (44).

The binding mode of bicyclic compounds is unlikely to involve close contacts between Phe-134 and His-200 and the effector. The replacement of both amino acids by alanine caused an increase in affinity, and no clear tendency was observed for the corresponding enthalpy changes (Table 1). Why then does the replacement of both of these residues, which are essential for the binding of the monoaromatic compounds, increase the affinity for the biaromatic ones? According to the model, the TtgV effector-binding site appears to be a closed, buried space with well defined lateral borders (Fig. 1*A*), forming a binding pocket that is 13 Å deep. Attempts to model 1NL in this pocket resulted in the conclusion that a degree of steric hindrance may exist that impedes the entrance or optimal accommodation of biaromatic compounds in the binding site. All mutations resulted in an increase in the volume of the binding pocket. This change, however, was particularly pronounced for F134A and H200A, causing increases from 1200 Å^3 (wild type) to 1610 and 1540 Å^3, respectively, as determined by PASS (28). This significant increase in volume is thought to relieve the steric constrains, enabling higher affinity binding due to the optimized accommodation of the effector. The volumes of the effector binding pocket of mutants F134V and H200V are in between the volumes of the corresponding alanine mutants and the wild-type protein. The fact that the affinities for bicyclic compounds for valine mutants also lie between the affinities observed for the alanine mutant and the wild-type protein supports the aforementioned hypothesis on the relief of steric constraints on volume increase. However, the analysis of mutant V140F demonstrates that the volume increase is not the sole determinant of the binding behavior of bicyclic compounds. 1-NL bound with significantly higher affinity to V140F than to V140A. This increase in affinity despite the decrease in the volume of the binding pocket is attributed to the establishment of additional van der Waals contacts between the hydrophobic side chain of Phe-140 and the effector. Van der Waals interactions were reported to play a key role in substrate of effector recognition in other multidrug-binding proteins (3, 5, 20, 21). In summary, data presented here reinforce the notion that multidrug-binding proteins have not evolved to recognize a given substrate with high affinity but to recognize instead a large range of structurally different compounds with physiologically relevant affinity.

Different Effectors Have Different Signal Transduction Efficiencies in Vitro, and Val-223 Might Play a Key Role—We were able to show, with EMSAs of wild-type TtgV in the presence of effector concentrations corresponding to either 1 or 10 × the K_D, that the bicyclic compounds analyzed were more efficient than the monocyclic compounds in inducing release of the protein (Fig. 5*A*). This is consistent with the idea that the signal generated by the binding of biaromatic 1NL and IND is transmitted more efficiently than the signal transmitted by the two

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monoaromatic compounds. Furthermore, our data also suggest that the binding mode for biaromatic compounds may be associated with greater efficiency in triggering the release of the repressor.

The V223A mutation did not alter the affinity of the protein for operator DNA (Table 2). The experiments with ITC showed that TtgVV223A in its free and DNA-bound form binds 1NL with similar affinity (Fig. 4). We also performed EMSA studies of TtgVV223A in the presence of 1NL concentrations corresponding to 1–10 times the K_D value (Fig. 5*B*). The data showed that TtgVV223A was less efficient in the release of the protein when compared with the wild-type protein (Fig. 5). We then hypothesized that the interdomain signaling cascade, in which the signal generated by the binding of an effector is transmitted to the DNA-binding domain, can be modulated by amino acid substitutions in the effector-binding site. This hypothesis is thus consistent with the notion that a single amino acid can have two roles that need not be necessarily related to each other, *i.e.* roles in effector binding and signal transmission. Mutation of such amino acids can have different effects with respect to both roles.

Indole as an Effector of Physiological Relevance—A central but often neglected question concerning multidrug-binding proteins is whether ligands are physiologically relevant. In this study, we have shown that the two-ring compounds 1NL and IND are substrates of the TtgDEF and TtgGHI efflux pumps (Table 3). Indole can be synthesized by *P. putida* and other microorganisms (45) and is excreted into the medium as a final metabolic product (46). Indole is toxic for other microorganisms, and incubation of *E. coli* with 5 mM IND led to a severe loss of viability (47). It was shown recently that IND induces the expression of multidrug exporter genes in *E. coli*, which confers acquired multidrug resistance (48). Some of the genes induced are highly homologous to pump genes controlled by TtgV, as exemplified by *E. coli* AcrD and AcrE, which share 53 and 44% sequence identity with TtgH and TtgG, respectively. Furthermore, it has been demonstrated that the *E. coli* AcrEF pump extrudes IND (49).

The toxicity of IND is particularly acute in dense microbial populations found in sewage, where concentrations in the millimolar range have been reported (50). In this context, IND can be considered an environmental pollutant (50, 51). *P. putida* DOT-T1E was isolated from sewage (1) in work aimed at isolating bacteria that exhibit tolerance to toluene. Subsequently, the TtgDEF and TtgGHI systems were shown to play a key role in toluene tolerance (2). It should be recalled that the mechanisms through which IND and toluene exert their toxicity are the same. They both dissolve in the membrane, leading to cell disorganization (47, 52). The parallels in the toxic mechanisms of these two aromatic compounds reflect their common chemical traits and might explain why TtgV, TtgGHI, and TtgDEF act on IND and toluene. The data we report here therefore suggest that one of the physiological reasons for the existence of the TtgV-TtgGHI/TtgDEF systems may be the extrusion of IND in dense bacterial populations, but whether the extrusion of other drugs (including toluene) occurs opportunistically is unknown.

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REFERENCES

1. Ramos, J. L., Duque, E., Huertas, M. J., and Haïdour, A. (1995) *J. Bacteriol.* **177,** 3911–3916
2. Rojas, A., Duque, E., Mosqueda, G., Golden, G., Hurtado, A., Ramos, J. L., and Segura, A. (2001) *J. Bacteriol.* **183,** 3967–3973
3. Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002) *Nature* **419,** 587–593
4. Zgurskaya, H. I., and Nikaido, H. (2000) *J. Bacteriol.* **182,** 4264–4267
5. Yu, E. W., McDermont, G., Zgurskaya, H. I., Nikaido, H., and Koshland, D. E., Jr. (2003) *Science* **300,** 976–980
6. Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hugues, C. (2000) *Nature* **405,** 914–919
7. Su, C.-C., Li, M., Gu, R., Takatsuka, Y., McDermont, G., Nikaido, H., and Yu, E. W. (2006) *J. Bacteriol.* **188,** 7290–7296
8. Duque, E., Segura, A., Mosqueda, G., and Ramos, J. L. (2001) *Mol. Microbiol.* **39,** 1100–1106
9. Terán, W., Krell, T., Ramos, J. L., and Gallegos, M. T. (2006) *J. Biol. Chem.* **281,** 7102–7109
10. Rojas, A., Segura, A., Guazzaroni, M. E., Terán, W., Hurtado, A., Gallegos, M.-T., and Ramos, J. L. (2003) *J. Bacteriol.* **185,** 4755–4763
11. Guazzaroni, M.-E., Terán, W., Zhang, X., Gallegos, M.-T., and Ramos, J. L. (2004) *J. Bacteriol.* **186,** 2921–2927
12. Guazzaroni, M.-E., Krell, T., Felipe, A., Ruiz, R., Meng, C.,Zhang, X., Gallegos, M.-T., and Ramos, J. L. (2005) *J. Biol. Chem.* **280,** 20887–20893
13. Molina-Henares, A.-J., Krell, T., Guazzaroni, M.-E., Segura, A., and Ramos, J. L. (2006) *FEMS Microbiol. Rev.* **30,** 157–186
14. Krell, T., Molina-Henares, A. J., and Ramos, J. L. (2006) *Protein Sci.*, **15,** 1207–1213
15. Kok, R. D., D'Argenio, D. A., and Ornston, L. N. (1998) *J. Bacteriol.* **180,** 5058–5069
16. Rintoul, M. R., Cusa, E., Baldoma, L., Badia, J., Reitzer, L., and Aguilar, J. (2002) *J. Mol. Biol.* **324,** 599–610
17. Arias-Barrau, E., Olivera, E. R., Luengo, J. M., Fernández, C., Galán, B., García, J. L., Díaz, E., and Miñambres, B. (2004) *J. Bacteriol.* **186,** 5062–5067
18. Zhang, R. G., Kim, Y., Skarina, T., Beasley, S., Laskowski, R., Arrowsmith, C., Edwards, A., Joachimiak, A., and Savchenko, A. (2002) *J. Biol. Chem.* **277,** 19183–19190
19. Vázquez-Laslop, N., Markham, P. N., and Neyfakh, A. A. (1999) *Biochemistry* **38,** 16925–16931
20. Schumacher, M. A., Miller, M. C., and Brennan, R. G. (2004) *EMBO J.* **23,** 2923–2930
21. Grkovic, S., Brown, M. H., Roberts, N. J., Paulsen, I. T., and Skurray, R. A. (1998) *J. Biol. Chem.* **273,** 18665–18673
22. DiRusso, C. C., Tsvetnitsky, V., Højrup, P., and Knudsen, J. (1998) *J. Biol. Chem.* **273,** 33652–33659
23. Schumacher, M. A., Millar, M. C., Grkovic, S., Brown, M., Skurray, R. A., and Brennan, R. G. (2001) *Science* **294,** 2158–2163
24. Ho, S. N., Hunt, H. D., Horton, M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
25. Mosqueda, G., and Ramos, J. L. (2000) *J. Bacteriol.* **182,** 937–943
26. Combet, C., Jambon, M., Deléage, G., and Geourjon, C. (2002) *Bioinformatics* **18,** 213–214
27. Hooft, R. W. W., Vriend, G., Sander, C., and Abola, E. E. (1996) *Nature* **381,** 272–272
28. Brady, Jr., G. P., and Stouten, P. F. W. (2000) *J. Comput. Aided Mol. Des.* **14,** 383–401
29. Yu, E. W., Aires, J. R., McDermont, G., and Nikaido, H. (2005) *J. Bacteriol.* **187,** 6804–6815

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30. Orth, P., Schnappinger, D., Hillen, W., Saenger, W., and Hinrichs, W. (2000) *Nat. Struct. Biol.* **7,** 215–219
31. Scholz, O., Kostner, M., Reich, M., Gastiger, S., and Hillen, W. (2003) *J. Mol. Biol.* **329,** 217–227
32. Soisson, S. M., McDouglas-Shackleton, B., Schleif, R., and Wolberger, C. (1997) *Science* **276,** 421–425
33. Reed, W. L., and Schleif, R. F. (1999) *J. Mol. Biol.* **294,** 417–425
34. Kahramanoglou, C., Webster, C. L., Samir-el-Robn, M., Belyaeva, T. A., and Busby, S. J. W. (2006) *J. Bacteriol.* **188,** 3199–3207
35. Ramos, J. L., Stolz, A., Reineke, W., and Timmis, K. N. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8467–8471
36. Zhou, L., Timmis, K. N., and Ramos, J. L. (1990) *J. Bacteriol.* **172,** 3707–3710
37. Salto, R., Delgado, A., Michán, C., Marqués, S., and Ramos, J. L. (1998) *J. Bacteriol.* **180,** 600–604
38. Walker, J. R., Altamentova, S., Ezersky, A., Lorca, G., Skarina, T., Kudritska, M., Ball, L. J., Bochkarev, A., and Savchenko, A. (2006) *J. Mol. Biol.* **358,** 810–828
39. Ahmed, M., Borsch, C. M., Taylor, S. S., Vázquez-Laslop, N., and Neyfakh, A. A. (1994) *J. Biol. Chem.* **269,** 28 506–28 513
40. Zheleznova, E., Markham, P. N., Neyfakh, A. A., and Brennan, R. G. (1999) *Cell* **96,** 353–362
41. Desrosiers, D. C., and Peng, Z. Y. (2005) *J. Mol. Biol.* **354,** 375–384
42. Fasolini, M., Wu, X., Flocco, M., Jean-Yves Trosset, J.-Y., Oppermann, U., and Knapp, S. (2003) *J. Biol. Chem.* **278,** 21092–21098
43. Sazinsky, M. H., Bard, J., Di Donato, A., and Lippard, S. J. (2004) *J. Biol. Chem.* **279,** 30600–30610
44. Friemann, R., Ivkovic-Jensen, M. M., Lessner, D. J., Yu, C., Gibson, D. T., Parales, R. E., Eklund, H., and Ramaswamy, S. (2005) *J. Mol. Biol.* **348,** 1139–1151
45. Snell, E. E. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **42,** 287–333
46. Claus, G., and Kutzner, H. J. (1983) *Syst. Appl. Microbiol.* **4,** 169–180
47. Garbe, T. R., Kobayashi, M., and Yukawa, H. (2000) *Arch. Microbiol.* **173,** 78–82
48. Hirakawa, H., Inazumi, Y., Masaka, T., Hirata, T., and Yamaguchi, A. (2005) *Mol. Microbiol.* **55,** 1113–1126
49. Kawamura-Sato, K., Shibayama, K., Horii, T., Iimuma, Y., Arakawa, Y., and Ohta, M. (1999) *FEMS Microbiol. Lett.* **179,** 345–352
50. Madsen, E. L., Francis, A. J., and Bollag, J.-M. (1988) *Appl. Environ. Microbiol.* **54,** 74–78
51. Kamath, A. V., and Vaidyanathan, C. S. (1990) *Appl. Environ. Microbiol.* **56,** 275–280
52. Ramos, J. L., Duque, E., Gallegos, M.-T., Godoy, P., Ramos-González, M. I., Rojas, A., Terán, W., and Segura, A. (2002) *Annu. Rev. Microbiol.* **56,** 743–768

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CAPÍTULO 5.

El represor transcripcional TtgV reconoce como tetrámero un operador complejo e induce una curvatura convexa del operador

Autores: Guazzaroni M.E., Krell T., Gutiérrez del Arroyo P., Vélez M., Jiménez M., Rivas G. y Ramos J.L.

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El represor TtgV forma parte de la amplia pero escasamente estudiada familia IclR de reguladores transcripcionales. Aunque los miembros de esta familia generalmente muestran una especificidad alta de efectores, TtgV tiene la propiedad de reconocer múltiples efectores. La proteína TtgV regula la expresión del operón *ttgGHI* que codifica la bomba principal de eflujo de disolventes de la bacteria extremófila *Pseudomonas putida* DOT-T1E. En este estudio hemos utilizado un enfoque multidisciplinar para estudiar el estado oligomérico funcional de TtgV durante los mecanismos de represión y desrepresión de la expresión, como también en el estudio de las bases moleculares de la interacción TtgV-ADN. Los estudios de ultracentrifugación analítica mostraron que TtgV es un tetrámero en solución y que su estado oligomérico no cambia en presencia de efectores. También hemos puesto de manifiesto que la unión de efectores al complejo TtgV-ADN lleva a la disociación de la proteína como tetrámero. Experimentos previos de ensayos de impronta *in vitro* con ADNasaI y DMS mostraron que TtgV protege una región de 42 pb. A través de análisis de ultracentrifugación analítica, ensayos de retardo en gel y calorimetría de titulación isotérmica hemos demostrado que TtgV puede interaccionar con secuencias invertidas diferentes intercaladas dentro de un operador de 34 pb. La estequiometría de unión es un tetrámero de TtgV por molécula de ADN y la afinidad de unión es de 200 nM. Los análisis de dicroísmo circular revelaron que TtgV se une provocando un cambio conformacional en el ADN y mediante microscopía de fuerza atómica se estableció que TtgV induce una curvatura convexa de 57º en su ADN operador. En este trabajo se propone que el mecanismo de represión de TtgV se basa en el impedimento estérico de la unión de la ARN polimerasa reforzado por la curvatura que provoca la proteína represora en la región del promotor *ttgG*.

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The Transcriptional Repressor TtgV Recognizes a Complex Operator as a Tetramer and Induces Convex DNA Bending

María-Eugenia Guazzaroni[1]†, Tino Krell[1]†
Paloma Gutiérrez del Arroyo[2,3], Marisela Vélez[3], Mercedes Jiménez[4]
Germán Rivas[4] and Juan L. Ramos[1]*

[1]*Consejo Superior de Investigaciones Científicas Estación Experimental del Zaidín, Department of Environmental Protection C/Prof. Albareda, 1 E-18008 Granada, Spain*

[2]*Centro Nacional de Biotecnología (CNB) CSIC, Campus de la Universidad Autónoma de Madrid, E-28049 Madrid, Spain*

[3]*Instituto Universitario de Ciencias de los Materiales Nicolás Cabrera, Facultad de Ciencias, Universidad Autónoma de Madrid E-28049 Madrid, Spain*

[4]*Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas E-28040 Madrid, Spain*

**Corresponding author*

The TtgV repressor belongs to the large but infrequently investigated IclR family of transcriptional regulators. Although members of this family usually exhibit high effector specificity, TtgV possesses multidrug binding properties. The TtgV protein regulates the expression of the *ttgGHI* operon encoding the main solvent extrusion pump of the extremophile *Pseudomonas putida* DOT-T1E strain. Here we used a multidisciplinary approach to study the functional oligomeric state of TtgV during repression and derepression events, as well as the molecular basis of TtgV–DNA operator interactions. Analytical ultracentrifugation studies (AUC) show that TtgV is a tetramer in solution and that this oligomeric state does not change in the presence of effectors. We also show that the binding of effectors leads to the dissociation of TtgV as a tetramer from the DNA-TtgV complex. Previous dimethyl sulfate and DNase I footprints revealed that TtgV protected a 42 bp region. Based on AUC, electrophorectic mobility shift assays and isothermal titration calorimetry analyses we show that TtgV recognition specificity is restricted within this operator to a 34-nucleotide stretch and that TtgV may interact with intercalated inverted repeats that share no significant DNA sequence similarities within this short 34-nucleotide segment. Binding stoichiometry is one TtgV tetramer per operator, and affinity for its target DNA is around 200 nM. Circular dichroism analysis reveals that TtgV binding causes DNA distortion and atomic force microscopy imaging of TtgV-DNA operator complexes shows that TtgV induces a 57° convex bend in its operator DNA. We propose that the mechanism of TtgV repression is based on the steric occlusion of the RNA polymerase binding site reinforced by DNA-bending of the *ttgV-ttgG* promoter region.

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Keywords: TtgV; DNA binding; DNA bending; AFM; IclR family

Introduction

The IclR family of transcriptional regulators includes almost 500 bacterial and archaeal proteins that can act as positive transcriptional regulators or as repressors.[1] Members of this family are involved in the regulation of a variety of metabolic processes such as the glyoxylate shunt in Enterobacteriaceae, multidrug resistance, degradation of aromatic compounds, inactivation of quorum sensing signals and sporulation.[1]

Members of the IclR family typically have an N-terminal winged helix-turn-helix (wHTH) DNA binding domain[2] followed by the effector-binding domain,[1,2] which is the trait that best defines the members of this family. At present there is no clear consensus on the architecture of DNA binding sites for IclR regulators, and there is a general lack of

† M-E.G. and T.K. contributed equally to this work.

Abbreviations used: AFM, atomic force microscopy; AUC, analytical ultracentrifugation; DMS, dimethylsulfate; IR, inverted repeats; ITC, isothermal titration calorimetry; EMSA, electrophoretic mobility shift assays; wHTH, winged helix-turn-helix.

E-mail address of the corresponding author: jlramos@eez.csic.es

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information concerning functional interactions between the DNA binding domain of these regulators and their cognate operators. Nonetheless, a number of experimentally identified IclR family member operator regions contain palindromic or pseudo-palindromic sequences.[3–9]

The little information available on the oligomeric state of IclR family members in solution and when interacting with DNA is contradictory. Zhang *et al*.[10] proposed that the *Thermotoga maritima* TM-IclR is a dimer in solution, but that it binds as a dimer of dimers to its cognate operator. However, no experimental support for this hypothesis is available. Other members of the family, such as Pir, PacR, and PobR,[8,9,11,12] were shown to be dimers in solution, and no information is available concerning their oligomeric state when in complex with their operator DNA. Nègre *et al.*[13] suggested that the IclR protein of *Escherichia coli* was a dimer in solution, based on equilibrium sedimentation analysis with a glycerol gradient. However, mass spectroscopy studies indicated that IclR was predominantly a tetramer in solution.[14]

The TtgV protein of *Pseudomonas putida* controls the expression of the *ttgGHI* operon, which encodes the TtgGHI efflux pump. This pump is chiefly responsible, from a quantitative point of view, for the extrusion of toluene and other solvents from the cell membrane.[15,16] The TtgGHI pump confers the strain the ability to thrive in a culture medium saturated with extremely toxic chemicals.[17,18] TtgV operates according to a de-repression mechanism, so that in the absence of effectors it is bound to its operator and represses transcription. Upon the binding of effectors it dissociates from the target DNA, allowing transcription.[19] TtgV exhibits multi-drug effector specificity and recognizes a large number of drugs, including mono- and bi-aromatic compounds and aliphatic alcohols, among other compounds.[19,20]

The *ttgV* gene is transcribed divergently from the *ttgGHI* operon, and the corresponding promoters, termed P_{ttgV} and P_{ttgG}, overlap each other. DNase I and dimethylsulfate (DMS) footprint assays established that TtgV protects a 42-bp region that covers the −10/–35 regions of the *ttgG* promoter and the −10 region of the divergently oriented *ttgV* promoter.[16,19] However, DNA sequence analysis did not reveal the presence of a clear single motif as the potential target of TtgV, since no obvious inverted or direct repeats were identified. Binding of effectors to TtgV causes its dissociation from the operator, and RNA polymerase subsequently enhances transcription from the *ttgV* and *ttgG* promoters.[20]

This study was undertaken to gain insights on the oligomeric functional state of TtgV in solution and when bound to its operator sequence, and to provide new insights on the molecular basis of TtgV–operator interactions. To this end we used a multidisciplinary approach involving analytical ultracentrifugation (AUC), atomic force microscopy (AFM), circular dichroism (CD), site-directed mutagenesis, and isothermal titration calorimetry (ITC). The results that we obtained showed that TtgV is a tetramer in its free form as well as in complex with effectors and with its target DNA. Binding of TtgV to its operator creates a convex 57° bend. The TtgV tetramer seems to recognize a set of intercalated inverted repeats. The binding of effectors to the TtgV-operator complex leads to dissociation of TtgV tetramers from their cognate promoter.

Results

Oligomeric state of TtgV in the absence and in the presence of effector molecules

The oligomeric state of a regulator can influence its interactions with its target operator. Until now the only available information on TtgV:DNA stoichiometry was derived from electrophoretic mobility shift assays (EMSA), which indicates a TtgV/DNA molar ratio of 4:1, but whether this was the result of the binding of four monomers, two dimers or one tetramer remained unknown (M-E.G. and J.L. R., unpublished). To determine the oligomeric form of TtgV we purified it to homogeneity as described in Materials and Methods, and subjected the protein to sedimentation velocity experiments at different protein concentrations. Data were analysed in terms of distribution of sedimentation coefficients, which made it possible to evaluate protein homogeneity and self-association. Figure 1(a) shows sedimentation velocity data for a 10 μM TtgV solution. The major species (>95%) sedimented with a standard *s*-value of 4.9(±0.2) S.

To determine the size of the protein, sedimentation equilibrium assays were carried out as described in Materials and Methods (Figure 1(b)). The sedimentation equilibrium gradient of TtgV fitted best with a single species with a molecular weight of 112,000±3000. This is compatible with TtgV being a tetramer in solution, taking into account that the sequence-derived molecular mass of the TtgV monomer is 28,232 Da. The protein concentration (in the 1–50 μM range) had no significant impact on the sedimentation velocity and equilibrium of the protein, which indicates that TtgV forms stable tetramers. The combined information on protein size and hydrodynamic behaviour[21] led to the conclusion that TtgV, under the experimental conditions that we used, acts essentially as a globular protein (frictional ratio $f/f_0=1.3$).

Our previous results showed that TtgV in solution and in complex with DNA binds effectors such as 1-naphthol or 4-nitrotoluene,[19,20] and ITC analysis revealed that the affinity of TtgV for these compounds was around 40(±3) μM for 1-naphthol and 17.4(±0.6) μM for 4-nitrotoluene.[20] To determine whether the binding of effectors altered the oligomeric state of TtgV, we repeated the analytical ultracentrifugation sedimentation velocity studies with 10 μM TtgV in the presence of 200 μM 1-naphthol (a concentration that corresponds to five

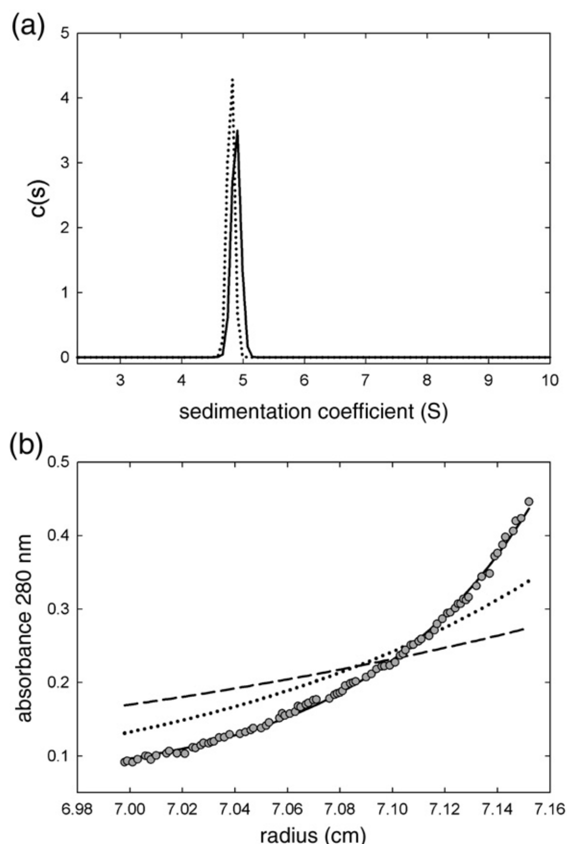

Figure 1. Analytical ultracentrifugation of TtgV. (a) Distribution of sedimentation coefficients $c(s)$ of 10 μM TtgV at 40,000 rpm monitored by interference optics in the absence (continuous line) and the presence (dotted line) of a 20 molar excess of naphthol. (b) Gradients at sedimentation equilibrium of 10 μM TtgV at 12,000 rpm (grey circles) monitored at 280 nm. The lines represent the theoretical gradient expected for the monomer (broken), dimer (dotted) and tetramer TtgV (continuous), respectively.

times the K_D of TtgV for this bi-aromatic compound[22]). Figure 1(a) shows that in the presence of this effector a single predominant TtgV oligomeric species was present, characterized by a sedimentation coefficient of 4.8(±0.2) S, which was similar to that found in the absence of the effector. This led us to suggest that TtgV in its complex with 1-naphthol is also tetrameric. Similar results were obtained for 4-nitrotoluene (data not shown). These results are in agreement with far-UV CD spectroscopy measurements of TtgV in the absence and in the presence of 0.2 mM 1-naphthol. The spectra of TtgV both with and without 1-naphthol were closely superimposable, indicating that effector binding did not significantly affect the secondary structure of the protein (data not shown).

Interaction of TtgV with synthetic operators studied by ITC

As mentioned above, DNase I and DMS footprint assays revealed that TtgV protected a 42-bp region in the *ttgV/ttgG* intergenic region.[16,19] Sequence analysis of the operator region did not reveal a clear binding motif for recognition by TtgV. Rojas *et al*.[16] had suggested previously that a series of four imperfect direct repeats might represent the binding sites of four TtgV monomers. This proposal does not seem to be supported by the above results, which demonstrate that TtgV is a tetramer. In addition, Zhang *et al*.[10] showed that two monomers in the IclR-TM protein are related to each other by 2-fold symmetry, which is consistent with binding to inverted rather than direct repeats. We therefore proceeded to re-analyse the protected region to look for potential binding motifs. We identified two different intercalated inverted repeats (IR), which we termed IR1, an almost perfect palindrome made up of 14 nucleotides separated by 11 nucleotides (Figure 2(a), black arrows) and IR2, a palindrome of 12 nucleotides separated by 11 nucleotides (Figure 2(a), grey arrows).

To elucidate the significance of each IR in the TtgV–operator binding, different-size DNA oligomers were synthesized (Figure 2(a)) and the binding characteristics of TtgV were determined by ITC (Figure 2). We first synthesized a 63-bp double-strand oligomer that included the entire centred 42-bp region flanked by ~10-bp stretches on either side (Figure 2(a)). The thermodynamic parameters of titration of the 63-bp oligonucleotide with TtgV derived from the raw data in Figure 3 are given in Table 1. Binding was entropy-driven and characterized by a dissociation constant (K_D) of 215(±40) nM and a stoichiometry of one TtgV tetramer per DNA oligomer. When this assay was done with the 38-bp double-stranded DNA with the two IR motifs defined above, we found a very similar titration curve to that determined with the larger 63-bp DNA fragment (Figure 3). The derived data revealed a K_D of TtgV for the 38-mer oligonucleotide of 200(±20) nM, and a TtgV/DNA operator stoichiometry of one tetrameter. These results support that the fragment containing the two IRs (38 nucleotides) that forms part of the 42-bp DNase I-protected region is sufficient for specific TtgV recognition.

To shed further light on the binding process we synthesized the 29-bp and the 27-bp double-strand oligomers shown in Figure 2(a). In the 29-mer double-strand oligomer, the right-hand submotif of IR2 was not present, whereas in the 27-bp double-strand oligomer the left submotif of IR1 was missing (Figure 2(a)). When the right IR2 half-site was eliminated (29-bp oligomer), TtgV still bound to this oligomer; however its affinity decreased around 1.5-fold compared to the affinity for the 38-bp double strand (Table 1). This suggests that TtgV is still able to recognize an operator bearing the entire IR1 and the proximal IR2 half-site, although binding affinity was slightly affected. To test whether the decrease in binding affinity was due to the loss of the right IR2 half-site or merely to the fact that ten nucleotides were missing, we synthesized a 38-bp oligomer in which the 29-bp of the operator sequence were kept, and we added nine extra random nucleotides (38 bp** in Figure 2(a)) on the

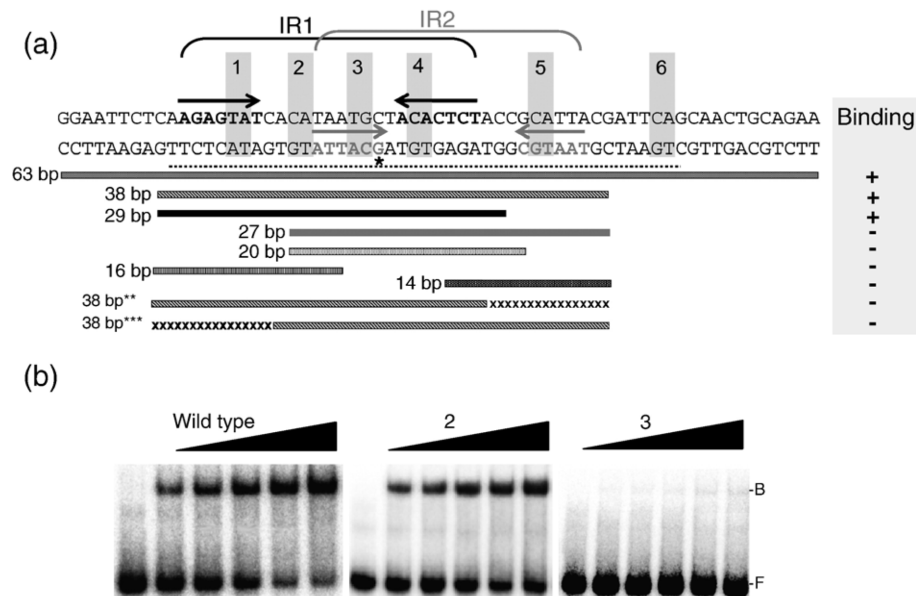

Figure 2. Oligonucleotides used in ITC and EMSA experiments. (a) Part of the intergenic sequence between *ttgV* and *ttgG*. The region protected from DNase I digestion is shown on the bottom strand and represented by a dotted line.[16] The asterisk marks the hypermethylated G deduced from DMS footprint assays.[19] Black arrows represent palindromic sequences of IR1. Grey arrows are used to represent palindromic sequences of IR2. Brackets point to IR1 and IR2 element sequences. Bars below the DNA sequence indicate the length and sequences of oligonucleotides used in ITC experiments. Shaded boxes 1, 2, 3, 4, 5 and 6 mark the base-pairs changed in 63-bp oligomers used in EMSA and ITC analysis. The sequence of the 38 bp** oligoprimer was 5′-CAAGAGTATCACATAATGCTACACTCTACATTGCAGAA-3′. The underlined sequence corresponds to the variation with respect to the wild-type sequence. The corresponding complementary oligonucleotide was used to create the double-strand target. The 38 bp*** sequence was 5′-TGTCGTCCGTTCATAATGCTACACTCTACCGCATTACG-3′. The underlined sequence corresponds to the variation with respect to the wild-type sequence. The corresponding complementary oligonucleotide was used to create the double-strand target. (b) Electrophoretic mobility shift assays of the wild-type and mutated 63-bp oligomer (box 2 and 3) of the TtgV-binding site in the presence of TtgV. 1 nM of each DNA 63-bp oligomer was incubated without (first lane) and with 125, 250, 375, 500, 750 nM of TtgV (remaining lanes). B, Bound DNA; F, free DNA.

3′ end. The corresponding ITC assay revealed that TtgV did not bind at all, which suggests that the decrease in affinity is mainly due to the absence of the IR2 right half-site. When the experiment was done with the 27-bp double strand oligomer lacking the left IR1 half-site and maintaining the entire IR2 element (27-bp oligomer), no binding was observed. When the assay was done with a 38-bp*** oligomer which has nine random nucleotides in its 5′ end and with the 27-bp of the TtgV operator, no binding was found either.

Isothermal titration calorimetry binding assays with DNA oligomers that contain a half-site of IR1 and IR2 (20 bp) or only a half-site of IR1 (16 bp) or IR2 (14 bp) (see Figure 2(a)) were also carried out. The results revealed that TtgV does not bind to half-sites of IR1 or IR2. The above series of results point towards IR1 being critical for TtgV recognition, and towards a measurable effect of IR2 on binding affinity. Taking into account the entire set of data, we believe that the 34-nucleotide region containing IR1 and IR2 corresponds to the functional operator recognized by TtgV. However, the data were not unequivocal and further complementary assays were done (see below). The aim underlying these assays was, primarily, to confirm that a TtgV tetramer bound to all oligomer variants, and secondly, to determine more precisely the role of the two IRs in the binding of TtgV.

Functional TtgV protomer binding to 38-bp and 29-bp DNA-oligonucleotides

Isothermal titration calorimetry experiments suggested that one tetramer of TtgV binds to 63-bp, 38-bp or 29-bp DNA duplex samples. To further shed light on the binding stoichiometry of TtgV with its target DNA operator, we carried out sedimentation velocity analysis of a mixture containing a 2.5-fold molar excess of TtgV tetramer (10 μM TtgV and 1 μM 38 or 29-bp DNA duplex). We found a biphasic behaviour that gave rise to two separate peaks in the distribution of the sedimentation coefficients (Figure 4(a)). Sedimentation was monitored at 260 nm, a wavelength at which the extinction coefficient of DNA is predominant, showing that the main sedimenting species are the free and complexed DNA forms. The slower sedimentation species was characterized in both cases by standard *s*-values of 2.8(±0.2) S and 3.1(±0.15) S, which correspond to free DNA. The TtgV/DNA complexes sedimented faster with standard *s*-values of 5.8(±0.2) S (38-bp fragment) and 5.4(±0.2) S (29-bp fragment).

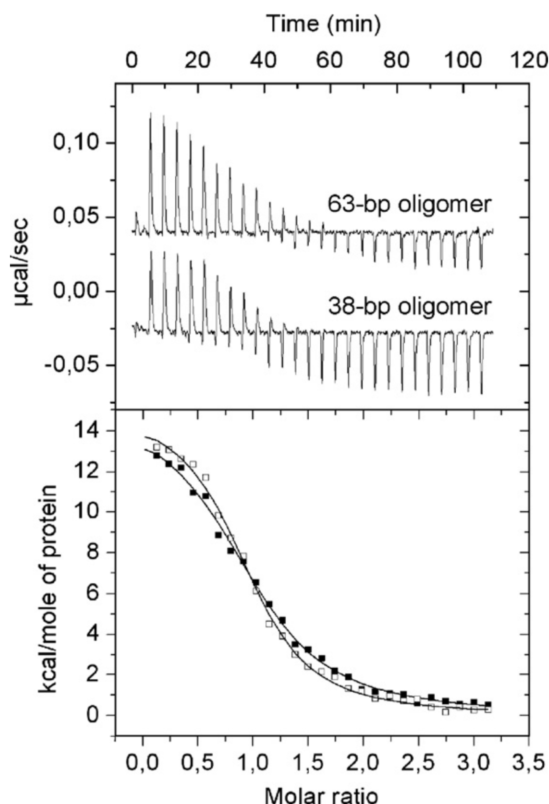

Figure 3. ITC study of the binding of TtgV to 63-bp and 38-bp DNA oligomers. Upper panel: Raw data, injection of 1.6-µl and 11.22-µl aliquots of TtgV (27.5 µM) into 2 µM of the respective DNA solutions. For clarity, data have been moved arbitrarily on the *Y* axis. The exothermic signal observed in the second part of the titration represents heats of dilution. Lower panel: Integrated and corrected-for-dilution peak areas. Symbols: filled squares, 63-bp oligomer; open squares, 38-bp oligomer. Thermodynamic parameters are shown in Table 1.

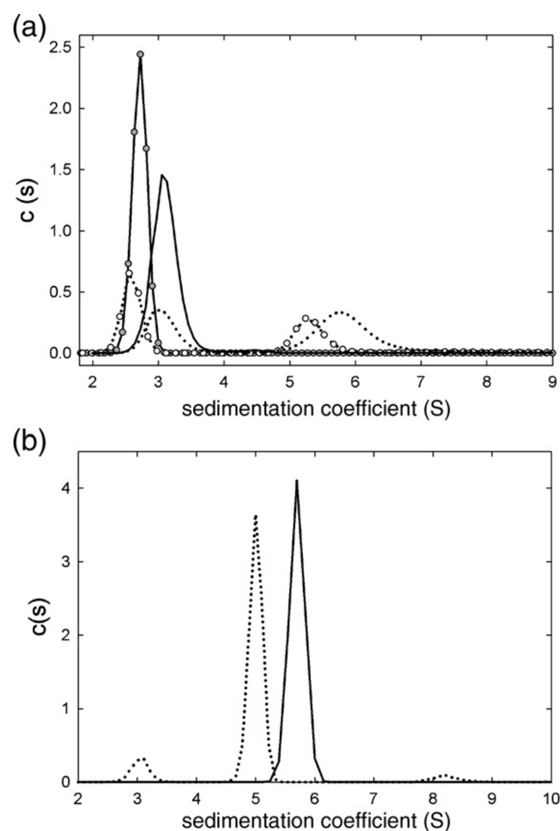

Figure 4. Sedimentation velocity analysis of TtgV-DNA complex formation. (a) Distribution of sedimentation coefficients $c(s)$ of 1 µM DNA alone (continuous lines) or in the presence of 10 µM TtgV (dotted lines) at 40,000 rpm, monitored at 260 nm. The data shown with open circles correspond to 29-bp DNA, and data without symbols correspond to 38-bp DNA. (b) Distribution of sedimentation coefficients $c(s)$ of mixtures of TtgV-DNA at 40,000 rpm in the absence (continuous line) and in the presence of an excess of naphthol (dotted line), monitored by interference optics.

Zhang *et al*.[10] hypothesized that the IclR protein of *T. maritima* forms tetramers when bound to DNA, and that effector binding to this complex leads to the dissociation of the protein into dimers, causing its dissociation from DNA. To test this hypothesis we used sedimentation velocity to analyse the behaviour of the TtgV-DNA complex in the presence and in the absence of 1-naphthol. These studies were performed on the TtgV-DNA complex (2.5 µM TtgV and 1 µM 38-bp oligomer) in the absence and presence of 400 µM 1-naphthol, which corresponds to ten times its K_D for TtgV. Previous EMSA experiments demonstrated that under these conditions quantitative protein release

Table 1. Thermodynamic parameters derived from the calorimetric titration of TtgV with different-size oligomers

DNA fragment (bp)	N[a]	K_D (nM)	K_A (M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
63	1.02±0.02	215±40	(4.6±0.9)×10^6	14.1±0.6	23.2±0.6	−9.1±0.1
38	0.97±0.01	200±20	(5.0±0.4)×10^6	15.2±0.3	24.3±0.3	−9.1±0.1
29	0.87±0.01	294±20	(3.4±0.3)×10^6	16.5±0.4	25.4±0.4	−8.9±0.1
27	No binding					
20	No binding					
16	No binding					
14	No binding					

Assay conditions are given in Materials and Methods. The sequence of the DNA fragments is given in Figure 2. Data are the average of two to three independent determinations with the indicated assay error.
[a] Tetramer concentration was used for data analysis.

from DNA occurs.[22] In this case the sedimentation profiles were monitored by interference optics. In the absence of effector, the main species sedimented with an *s*-value compatible with the DNA-protein complex (Figure 4(b)). In the presence of 1-naphthol, the sedimentation coefficient distribution was shifted toward higher *s*-values (5.0 S, compatible with the behaviour of TtgV reported above). In addition, a sedimentation species appeared with a velocity of 2.8(±0.1) S, which corresponded to the expected sedimentation of free DNA. Our data thus demonstrate that when TtgV is dissociated from DNA, the dissociated repressor exists in its tetrameric state.

Binding of TtgV to variants of the 63-bp oligomer

Isothermal titration calorimetry assays shortened the target sequence of TtgV to a 34-bp segment, but left unresolved a number of questions, such as the potential role of the right-half submotif of IR2, as well as the potential contribution of the right IR1 half-side to binding. To shed light on these issues we decided to carry out further assays with variants of the operator region bearing point changes. For this series of assays, synthetic 63-bp oligonucleotides were designed to exhibit two-base-pair changes with respect to the wild-type sequence (the changes are highlighted with a grey bar in Figure 2(a) and labelled 1 through 6). On the left–half-site of IR1 (box 1), AG replaced the original TA; in the short inter-sequence between the left half-site of IR1 and IR2 (box 2), GT replaced CA; in the left half-site of IR2 (box 3), AT replaced TG; in the right half-site of IR1 (box 4), GT replaced CA; and in the right half-site of IR2 (box 5) AT replaced the original CA. Two base-pairs were also changed to GT in the rightmost protected CA (box 6).

Electrophoretic mobility shift assays using wild-type and 63-bp variant duplex DNAs were carried out with increasing concentrations of TtgV. From the fractions of bound and free DNA we calculated that the apparent affinity of TtgV for these oligomers was around 0.75 μM. Changes within the IRs sequences (boxes 1, 3 and 4) had a marked negative effect on TtgV binding, namely, affinity for the mutant variants notably decreased (see Figure 2(b) for the wild-type and variant in box 3). Mutations in boxes 2 and 5 had only a moderate effect (see Figure 2(b) for the wild-type and variant with changes in box 2). No effect was observed with the mutant in box 6 (not shown).

To more precisely determine the nature of the interactions between TtgV and the variant DNA operators, we carried out ITC titration assays. Figure 5 shows the results for titration of the wild-type sequence and variants in boxes 1 and 2. The data derived from ITC titration are shown in Table 2, together with the data derived from the binding of TtgV to variant operators with changes in boxes 3, 4, 5, and 6. Isothermal titration calorimetry data confirmed that changes in boxes 1, 3 and 4 prevented the binding of TtgV, whereas mutations in boxes 2 and 5 resulted in a noticeable decrease in affinity by a factor of 2 to 2.5. This set of results supports the *sine qua non* requirement of both half-sites of IR1 and the left-hand site of IR2 for TtgV binding.

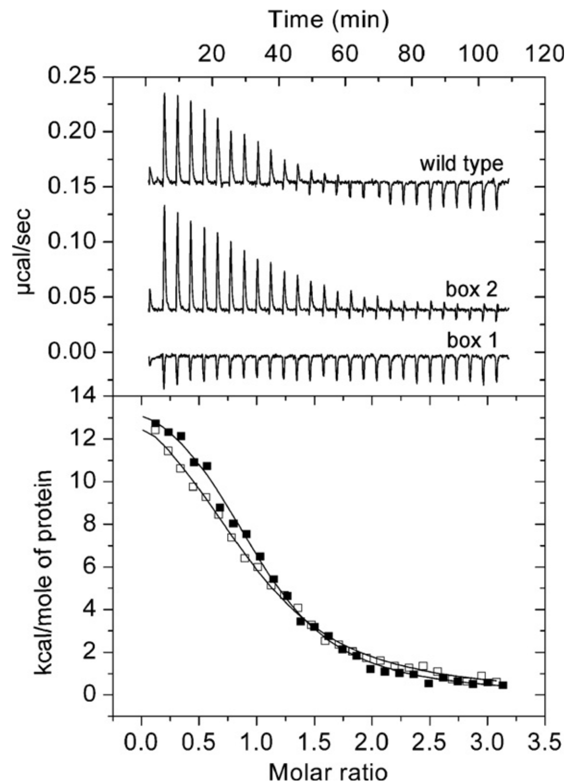

Figure 5. ITC of the binding of TtgV to 63-bp oligomer variants. Conditions were as in the legend to Figure 3 except that wild-type or variant 63-mer DNA samples were placed in sample cells. For clarity, data have been moved arbitrarily on the *Y* axis. The exothermic signal observed in the second part of the titration represents heats of dilution. In the lower panel, filled squares indicate the wild-type sequence, and open squares indicate box 2 variants.

DNA bending by TtgV protein

In previous DMS footprinting assays we showed that the recognition of target DNA by TtgV could lead to DNA distortion, because we observed hypermethylation of guanine −14, which is almost centred between the two IRs (Figure 2(a)).[19] We hypothesized that binding of the repressor to its target operator deforms the DNA molecule.

To test this hypothesis we used CD spectroscopy to analyse potential TtgV-mediated changes in the secondary structure of a 38-bp duplex DNA containing the TtgV binding site. Protein contributions to the CD signal in the near-UV range were minimal, and above 240 nm ellipticity was dominated by contributions from the purine and pyrimidine bases of the DNA duplex. The CD spectrum of 38-bp DNA oligomer was characteristic of *B*-form DNA (Figure 6). Under stoichiometric binding conditions with a

Table 2. Thermodynamic parameters derived from the calorimetric titration of TtgV with different 63-bp DNA oligomers

DNA oligomers	K_D (nM)	K_A (M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
Wild-type	215±40	$(4.6\pm0.9)\times10^6$	14.1±0.6	23.2±0.6	–9.1±0.1
Box 1	No binding				
Box 2	420±50	$(2.4\pm0.3)\times10^6$	13.4±0.5	22.1±0.5	–8.7±0.1
Box 3	No binding				
Box 4	No binding				
Box 5	500±40	$(2.0\pm0.2)\times10^6$	14.2±0.3	22.8±0.3	–8.6±0.1
Box 6	260±40	$(3.8\pm0.1)\times10^6$	16.1±0.4	24.6±0.5	–8.5±0.1
Box 4+5	No binding				

Assay conditions are given in Materials and Methods. Data are the average and standard deviation of two to three independent assays. The sequence of the DNA fragments is given in Figure 2.

molar ratio of TtgV tetramer:DNA of 1:1 yielding 100% saturation of 38-bp DNA oligomer, TtgV significantly decreased the magnitude of the positive CD band at 275 nm (Figure 6). Changes in the ellipticity band at 275 nm have been correlated with changes in the DNA winding angle,[23–25] and are consistent with a decrease in the number of basepairs per turn of the DNA, or an increase in the helical twist of the DNA duplex.

To analyse this phenomenon in further detail we used AFM, a well-established technique for the characterization of conformational changes in DNA.[26–30] We amplified by PCR a 716-bp DNA fragment bearing the TtgV operator located towards the centre of the duplex oligomer. TtgV and the 716-bp DNA fragment were incubated at a molar ratio of TtgV tetramer:DNA of 1:1 under conditions similar to those used for EMSAs. Atomic force microscopy images showed that TtgV bound specifically to DNA at the expected position within the operator, producing a convex bend in the DNA helix (Figure 7(a)). The position of TtgV bound to DNA was determined by measuring the length of the DNA from the centre of the protein in about 100 protein-DNA complexes, as the distance between the centre of the protein and the nearest end of the DNA molecule. In most of the DNA molecules, TtgV was not centred but located at roughly 43% from the fragment end, as expected from a previous characterization of the operator.[19]

We also analysed the DNA contour length of the 716-bp DNA fragment by tracing the DNA molecules from one end to the other in the absence and in the presence of TtgV. There were no significant differences in DNA contour length upon TtgV binding (data not shown), meaning that TtgV does not compact DNA upon binding, nor does DNA wrap around the TtgV protein.

The DNA bend angle is defined as the angle by which a DNA segment departs from linearity. To investigate whether binding of TtgV to the operator induced bending of the template, we subjected 111 images of TtgV-DNA complexes to statistical analysis. Measurements of the DNA bending angle (θ) yielded a distribution with the mean bend angle of 57(±3)° (Figure 7(a) and (b)). To determine whether the bend was an inherent characteristic of this specific DNA molecule, we examined 75 DNA molecules not incubated with TtgV. The bend angle distribution was determined at the position where the TtgV operator was located. Uncomplexed DNA molecules showed Gaussian distribution centred at 0°, indicating no intrinsic curvature of the DNA fragments at this location (Figure 7(b)). This confirms that the bending observed in the complexed DNA molecules was dependent on binding of the TtgV repressor.

Discussion

TtgV forms stable tetramers in solution in the presence and in the absence of effectors

Members of the IclR family were found to act either as transcriptional activators or repressors, or to have a dual function.[1,2] The oligomerization form has been studied for only a few members of the Ic1R

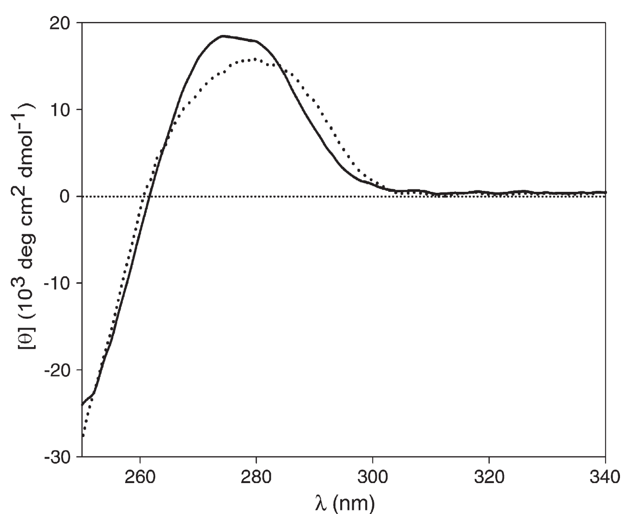

Figure 6. Circular dichroism analysis of operator DNA in the presence and in the absence of TtgV. Near-UV CD spectra of 3.7 μM 38-bp oligomer containing the TtgV operator in the absence of protein (continuous line) or in complex with 4 μM TtgV (dotted line). Data were corrected for protein and solvent contributions to the signal. TtgV-DNA complex formation was verified by electrophoretic mobility shift assay (data not shown).

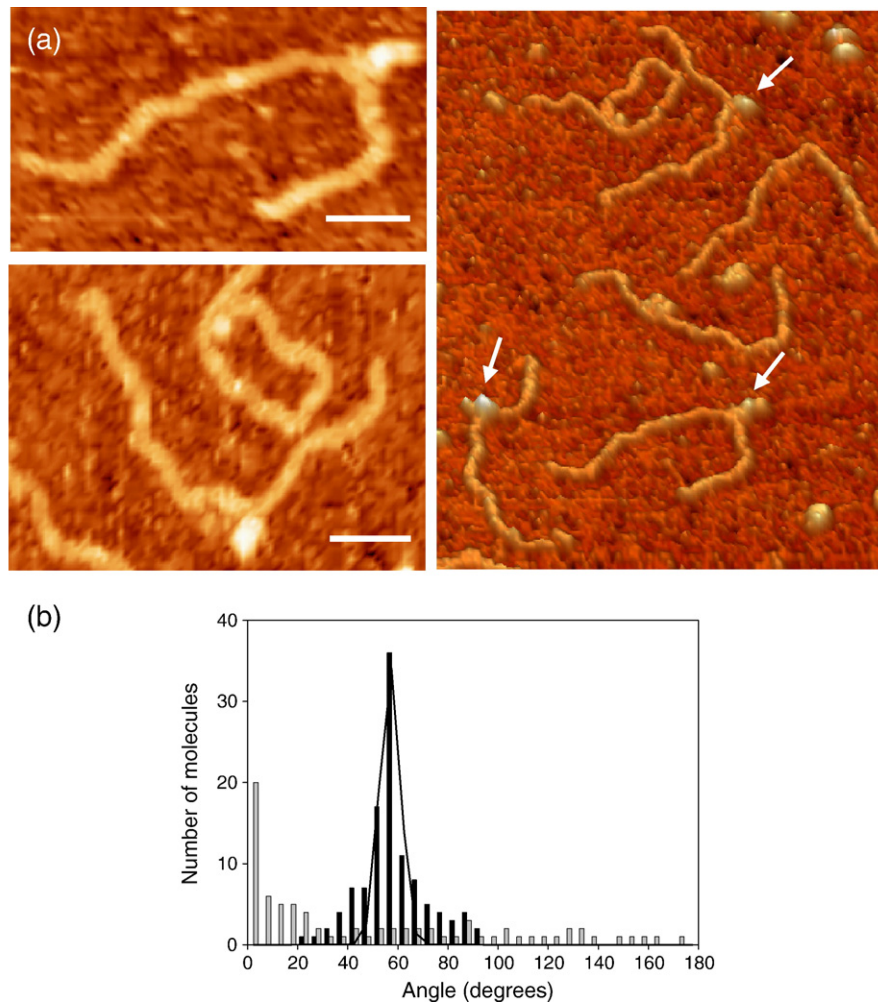

Figure 7. AFM study of the TtgV-DNA complex. (a) Small and large-scale AFM images of representative TtgV-DNA complexes. A 716-bp DNA fragment was used that contains the TtgV recognition sequence 43% from the DNA fragment end. The arrows indicate TtgV bound to DNA (scale bars represent 40 nm). (b) Histograms represent the DNA bend angle distributions of protein-free DNA molecules (light grey bars) and TtgV-DNA complexes (black bars) of representative DNA molecules (75 and 111, respectively). The black line represents the Gaussian fitting of the distribution as defined in equation (1) of Schulz *et al*.[55] The mean angle value of TtgV-bound DNA molecules is 57° with a standard deviation of 3°.

family of regulators. The activator of the protocatechuate pathway in *Acinetobacter* sp. ADP1, the PcaU protein,[3] the PcaR protein in *Pseudomonas*,[9] and the Pir protein of *Erwinia*[31] involved in the stimulation of pectinase synthesis, were found to be homodimers in solution. In contrast, the AllR repressor of *E. coli* (also called GclR), which controls allantoin metabolism, was found to exist predominantly as a tetramer in solution, although it forms an equilibrium with the monomeric and dimeric forms.[32] The addition of the effector glyoxylate caused a dramatic increase in the percentage of tetrameric states of AllR, which was interpreted as evidence that the binding of glyoxylate to AllR stabilizes and favours the tetrameric state.[32] Controversial results have been reported for the founding member of the family, the *E. coli* IclR protein, which has been described as a dimer by some[13] and as a tetramer by other investigators.[14]

To establish the oligomeric state of TtgV in solution, we carried out AUC experiments. This technique provides excellent sensitivity and resolution, and makes it easy to distinguish between the different sedimenting forms.[33] The sedimentation coefficient (*s*) of TtgV in solution matches the value for the tetrameric form of the TtgV protein (Figure 1(a) and (b)). The sedimentation coefficient of TtgV in the presence of two different effectors was similar to that obtained for TtgV in their absence (Figure 1(b)), which was interpreted as evidence that TtgV forms stable tetramers with or without effectors.

TtgV binds to a complex operator formed by two inverted repeats as a tetramer

Several attempts have been made before to identify the specific TtgV target within the 42-bp DNase I protected operator recognized by TtgV.[16,19]

Here we used a series of complementary techniques (AUC, ITC and EMSA), and taken together, the findings lead us to propose that TtgV recognizes a set of 34 bp in which two different intercalated IRs are decisive for operator recognition by TtgV. Furthermore, ITC and AUC techniques showed that the minimal TtgV operator corresponded to 29 bp, and that binding to this or other extended operators occurred with a binding stoichiometry of 1:1 between the TtgV tetramer and the DNA operator molecule. There was no significant difference in binding parameters between 63-bp and 38-bp oligomers (Table 1, Figure 3), indicating that the oligomer bearing both IRs (38-bp) was sufficient for complete TtgV DNA recognition. However, removal of the left half-site of IR1 or alteration of the right half-site of IR2 prevented binding of TtgV to the target operator. Alteration of the left half-site of IR2 or removal of the right half-site of IR1 resulted in low albeit measurable binding of TtgV to its operator. Taken together, the data are consistent with a first event in which TtgV recognizes the IR1 sequence and a second event in which it interacts with the IR2 sequence, needed to maintain and stabilize the TtgV/DNA complex. If these interactions are sequential, initial IR1 binding may distort the DNA and thus allow the subsequent recognition of IR2 and stabilization of the complex.

Interactions between regulators with distinct motifs within an operator have not often been described, but recognition of intercalated IRs has been described for CbnR, a LysR-type transcriptional regulator that activates the genes involved in chlorocatechol degradation.[34] Crystallographic and biochemical data showed that CbnR recognizes two different IRs, called ABS (activation binding site) and RBS (recognition binding site) in an operator of approximately 50 to 60 bp.[34,35] These authors proposed that the CbnR tetramer might be considered as a pair of dimers, and that one of the dimers might recognize the RBS motif whereas the other dimer might bind to the ABS motif, anchoring the whole tetramer in the target DNA.[35] Whether this is also the case for TtgV awaits resolution of TtgV/operator complexes.

Others regulators such as LacI and FruR also recognize asymmetric operators.[36,37] Moreover, FruR, the fructose repressor of *E. coli*, is a tetramer in solution and interacts asymmetrically with the two half-sites of its operator.[37]

In the presence of effectors TtgV dissociates from the DNA-TtgV complex as a tetramer

The mechanism of action of several repressor proteins has been shown to be effector-mediated derepression. In most cases the repressor protein binds to the promoter in the absence of effectors. Effector binding leads to dissociation of the protein from the operator DNA, facilitating the entry of RNA polymerase and the subsequent increase in gene expression. This mechanism was shown to operate for TtgV in a series of *in vitro* transcription assays,[19,20] and seems to operate as well for other IclR protein members such as AllR[32] and KdgR.[38]

For some repressors it has been shown that binding of the effector triggers a conformational change in the DNA-binding domain, which changes the oligomeric state of the regulator protein.[39–41] In this context, Zhang *et al*.[10] proposed that the TM-IclR protein binds to its target DNA as a tetramer, and that effector binding induces tetramer dissociation in dimers. To study whether effector binding induces alterations in the oligomeric state in TtgV, we carried out AUC experiments with TtgV complexed to a 38-bp oligomer in the presence of 1-naphthol and 4-nitrotoluene, two of the best *in vivo* inducers of the P_{ttgG} promoter. The addition of these effectors to the sedimenting mixture caused dissociation of TtgV from the complex, but in the same oligomeric form (tetramer) in which it was bound to DNA.

TtgV induces a 57° convex bend in DNA

Previous DMS footprint assays revealed that in the presence of TtgV, G −14 in Figure 2(a) becomes hypermethylated. This suggested that binding of TtgV to its operator target might cause a distortion immediately upstream from the −10 region, which could prevent proper recognition of the promoter by RNA polymerase.[19] Our analysis of AFM images shows that TtgV binding produces an unusual 57° convex bending, with the DNA bent away from the bound protein (Figure 7(a)). Several repressor proteins have been shown to bend the DNA at their target regions[42–44]; however, the most common type of bending observed in protein-DNA complexes is concave, in which the DNA helix is smoothly curved around the bound protein.[45] In fact, the analysis of the 31 Protein Data Bank three-dimensional structures available for DNA-repressor complexes revealed in 55% of the cases that repressor binding produced no significant bending, that 32% of the repressors caused concave bending, and that only 13% of the repressors induced convex bending.

Three of the proteins that induce convex bending belong to the LacI-GalR family of transcriptional regulators, and induce global bend angles of DNA of ~50° (PurR),[46] ~40° (LacI)[47] and 35° (CcpA).[48] LacI-GalR family members use their leucine levers to reconfigure their operator so that major groove bases can be specifically read. Helix-turn-helix motifs of CcpA and LacI display significant plasticity in their ability to bind half-sites with altered sequences.[36,49] As discussed above, this is similar to the binding recognition mechanism of TtgV, which recognizes two different IRs.

This is the first time that convex bending has been shown for a protein of the IclR family. Repressors of this family have been shown to impede RNA polymerase binding through steric hindrance, as is usual for others repressor proteins. Bending induced by TtgV might increase the degree of repression, since DNA distortion can hamper the ability of the RNA polymerase to enter the promoter. These two

complementary repression mechanisms may constitute a way to fine-tune the expression of membrane proteins such as those involved in efflux pumps.

Materials and Methods

Bacterial strains and culture media

E. coli BL21 (DE3) carrying plasmid pANA126 (a pET29b [+] derivative vector that encodes a His_6-tagged TtgV protein) was used for protein production. Cells were grown in 2-l conical flasks with 500 ml of LB medium supplemented with 25 μg/ml kanamycin (Km). Flasks were incubated at 30 °C and shaken on an orbital platform at 200 rpm.

Isothermal titration calorimetry (ITC)

Protein expression and purification were carried out as described.[16] Protein concentration was determined with the Bradford assay. Measurements were made with a VP-Microcalorimeter (MicroCal, Northampton, MA, USA) at 25 °C. The protein and DNA oligonucleotides were thoroughly dialysed against ITC buffer (20 mM Tris-HCl (pH 7.2), 8 mM magnesium acetate, 300 mM NaCl, 10% (v/v) glycerol, 1 mM DTT). Oligonucleotides corresponding to both strands of the whole or partial TtgV operator were synthesized by Sigma, and complementary oligonucleotides were annealed by mixing 50 μM of each oligonucleotide in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 185 mM NaCl. The mixture was incubated 95 °C for 5 min and then chilled on ice and dialysed in the buffer used for ITC studies.

Each ITC titration involved a single 1.6-μl injection and a series of 11.22-μl injections of protein (27.5 μM) into a 2 μM DNA solution. The mean enthalpies measured from injection of the ligand in the buffer were subtracted from raw titration data before data analysis with ORIGIN software (MicroCal). Titration curves were fitted by a non-linear least squares method (ORIGIN) to a function for the binding of a ligand to a macromolecule.[50]

Circular dichroism

Complementary synthetic oligonucleotides were annealed as described above to generate a 38-bp duplex DNA containing the TtgV-binding site. CD spectroscopy was performed on a Jasco 715 spectropolarimeter using a quartz cuvette with 1-cm path length. Protein and DNA solutions were dialysed against the buffer used for ITC. DNA was diluted to a final concentration of 3.7 μM in ITC buffer and titrated with concentrated TtgV to a final concentration of 4 μM. Ellipticity measurements were collected at 25 °C from 340 nm to 200 nm at intervals of 1 nm. All data were corrected for additional ellipticity contributions from the protein. Experiments were performed in duplicate.

Electrophoretic mobility shift assays (EMSA)

Five pmol of 5′ ^{32}P-end-labelled oligonucleotides was incubated with a slight molar excess of the complementary unlabelled strands in 20 μl of 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$. Samples were heated to 95 °C for 3 min and then transferred to ice for 5 min to allow heteroduplex formationLabelled DNA probe. (1 nM) (~1.5×10^4 cpm) was incubated with increasing amounts of TtgV for 10 min at 30 °C in 10 μl of 20 mM Tris-HCl (pH 7.2), 8 mM magnesium acetate, 300 mM NaCl, 10% (v/v) glycerol, 1 mM DTT containing 20 μg/ml poly(dI-dC) and 200 μg/ml bovine serum albumin. Samples were subsequently electrophoresed with non-denaturing 4.5% (w/v) polyacrylamide gels in 0.2 M glycine, 25 mM Tris (pH 8.6). Images were analysed with Molecular Imager FX equipment (Bio-Rad).

Atomic force microscopy (AFM)

DNA-protein complexes were prepared by incubating TtgV (88 nM) together with 17 nM of the DNA fragment. A 716-bp DNA fragment containing the TtgV binding site was obtained by PCR (from −368 to +347 nucleotides with respect to the transcription start point of the *ttgGHI* promoter). TtgV operator is not at the centre in this fragment, but is located at a distance of 43% from the nearest end. The mixture was incubated in binding buffer (25 mM Tris-HCl (pH 7.5), 8 mM magnesium acetate, 10 mM KCl, 3.3% (w/v) PEG, 1 mM DTT) at room temperature for 10 min, and then deposited onto freshly cleaved mica (Agar Scientific Limited) in the presence of 15 mM $MgCl_2$ and incubated for 1 min at a final DNA concentration of 3 nM.

Images were acquired in solution with a Nanotec Electrónica Atomic Force Microscope operating in jumping mode, using silicon nitride cantilevers (0.76-0.05 N/m, Olympus OMCL-RC800PSA). Images were processed with WSxM software (Nanotec Electrónica, Tres Cantos, Spain). Angles were measured with ImageJ software (NIH, Bethesda, Maryland, USA).

Analytical ultracentrifugation (AUC)

Analytical ultracentrifugation analysis of TtgV, DNA and TtgV-DNA mixtures was performed at several protein concentrations (in the range 1–50 μM) and two DNA concentrations (0.5 and 1 μM). Effector concentrations corresponded to ten times their K_D for TtgV (0.4 mM for 1-naphthol and 0.17 mM for 4-nitrotoluene), which were determined before by ITC.[22] All samples were processed in 20 mM Tris-HCl (pH 7.2), 8 mM magnesium acetate, 50 mM KCl, 1 mM TCEP.

Sedimentation velocity runs were carried out at 40,000 rpm and 20 °C in an XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) with an UV-VIS optics detection system, using an An60Ti rotor and 12-mm double-sector centrepieces. Absorbance scans were run at either 260 nm or 275 nm depending on the composition of the samples, as stated in the text. Sedimentation coefficient distributions were calculated by least-squares boundary modelling of sedimentation velocity data using the $c(s)$ method,[33] as implemented in the SEDFIT program. These s-values were corrected to standard conditions (water, 20 °C, and infinite dilution[51]) using the SEDNTERP program[52] to obtain the corresponding standard s-values ($s_{20,w}$).

Sedimentation equilibrium studies were conducted to determine the state of association of TtgV. The sedimentation equilibrium runs were carried out at multiple speeds (10,000, 12,000 and 15,000 rpm) and wavelengths (250, 280 and 290 nm) with short column (80–100 μl), using the same experimental conditions and instruments as for the sedimentation velocity experiments. After the equilibrium

scans a high-speed centrifugation run (40,000 rpm) was done to estimate the corresponding baseline offsets. Weight-average buoyant molecular weight of TtgV was determined by fitting data to the single species model using either the MATLAB program (kindly provided by Dr Allen Minton, NIH), based on the conservation of signal algorithm,[53] or the HeteroAnalysis program (retrieved from the FTP site of the Analytical Ultracentrifugation Facility of the University of Connecticut, Storrs, CT)‡. Both analyses gave similar results. Molecular weight of the protein was determined from the experimental buoyant masses using 0.734 as the partial specific volume of TtgV (calculated from the amino acid composition using the SEDNTERP program[52]). The HeteroAnalysis program was also used to determine the association scheme (in terms of stoichiometry and affinity) for TtgV-DNA complex formation. A second global sedimentation analysis (SEDPHAT[54]) gave essentially the same results.

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References

1. Molina-Henares, A. J., Krell, T., Guazzaroni, M. E., Segura, A. & Ramos, J. L. (2006). Members of the IclR family of transcriptional activators and repressors exhibit conserved features outside the DNA binding domain. *FEMS Microbiol. Rev.* **30**, 157–186.
2. Krell, T., Molina-Henares, A. J. & Ramos, J. L. (2006). The IclR family of transcriptional activators and repressors can be defined by a single profile. *Protein Sci.* **15**, 1207–1213.
3. Gerischer, U., Segura, A. & Ornston, L. N. (1998). PcaU, a transcriptional activator of genes for protocatechuate utilization in *Acinetobacter*. *J. Bacteriol.* **180**, 1512–1524.
4. Nasser, W., Reverchon, S., Condemine, G. & Robert-Baudouy, J. (1994). Specific interactions of *Erwinia chrysanthemi* KdgR repressor with different operators of genes involved in pectinolysis. *J. Mol. Biol.* **236**, 427–440.
5. Ferrández, A., García, J. L. & Díaz, E. (1997). Genetic characterization and expression in heterologous hosts of the 3-3-(hydroxyphenyl)-propionate catabolic pathway of *Escherichia coli* K-12. *J. Bacteriol.* **170**, 2573–2581.
6. Di Marco, A. A. & Ornston, L. N. (1994). Regulation of *p*-hydroxybenzoate hydroxylase synthesis by PobR bound to an operator in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **176**, 4277–4284.
7. Pan, B., Unnikrishnan, I. & La Porte, D. C. (1996). The binding site of the IclR repressor protein overlaps the promoter of aceBAK. *J. Bacteriol.* **178**, 3982–3984.
8. Popp, R., Kohl, T., Patz, P., Trautwein, G. & Gerischer, U. (2002). Differential DNA. binding of transcriptional regulator PcaU from *Acinetobacter* sp. strain ADP1. *J. Bacteriol.* **184**, 1988–1997.
9. Guo, Z. & Houghton, J. E. (1999). PcaR-mediated activation and repression of pca genes from *Pseudomonas putida* are propagated by its binding to both the −35 and the −10 promoter elements. *Mol. Microbiol.* **32**, 253–263.
10. Zhang, R. G., Kim, Y., Skarina, T., Beasley, S., Laskowski, R. & Arrowsmith, C. (2002). Crystal structure of *Thermotoga maritima* 0065, a member of the IclR transcriptional factor family. *J. Biol. Chem.* **277**, 19183–19190.
11. Thomson, N. R., Nasser, W., McGowan, S., Sebaihia, M. & Salmond, G. P. (1999). *Erwinia carotovora* has two KdgR-like proteins belonging to the IclR family of transcriptional regulator: identification and characterization of the RexZ activator and the KdgR repressor of pathogenesis. *Microbiology*, **145**, 1531–1545.
12. Krell, T., Terán, W., López-Mayorga, O., Rivas, G., Jiménez, M., Molina-Henares, A., Gallegos, M.T. & Ramos, J.L. (2007). Optimisation of the palindromic order of TtgR operator enhances binding cooperativity. *J. Mol.Biol*. In the press.
13. Negré, D., Cortay, J. C., Galinier, A., Sauve, P. & Cozzone, A. J. (1992). Specific interactions between the IclR repressor of the acetate operon of *Escherichia coli* and its operator. *J. Mol. Biol.* **228**, 23–29.
14. Donald, L. J., Hosfield, D. J., Cuvelier, S. L., Ens, W., Standing, K. G. & Duckworth, H. W. (2001). Mass spectrometric study of the *Escherichia coli* repressor proteins, Ic1R and Gc1R, and their complexes with DNA. *Protein Sci.* **10**, 1370–1380.
15. Rojas, A., Duque, E., Mosqueda, G., Golden, G., Hurtado, A., Ramos, J. L. & Segura, A. (2001). Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. *J. Bacteriol.* **183**, 3967–3973.
16. Rojas, A., Segura, A., Guazzaroni, M. E., Terán, W., Hurtado, A., Gallegos, M. T. & Ramos, J. L. (2003). *In vivo* and *in vitro* evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. *J. Bacteriol.* **185**, 4755–4763.
17. Ramos, J. L., Duque, E., Huertas, M. J. & Haïdour, A. (1995). Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol.* **177**, 3911–3916.
18. Ramos, J. L., Duque, E., Godoy, P. & Segura, A. (1998). Efflux pumps involved in toluene tolerance in *P. putida* DOT-T1E. *J. Bacteriol.* **180**, 3323–3329.
19. Guazzaroni, M. E., Terán, W., Zhang, X., Gallegos, M. T. & Ramos, J. L. (2004). TtgV bound to a complex operator site represses transcription of the single promoter for the multidrug and solvent extrusion TtgGHI pump. *J. Bacteriol.* **186**, 2921–2927.
20. Guazzaroni, M. E., Krell, T., Felipe, A., Ruíz, R., Meng, C., Zhang, X. *et al.* (2005). The multidrug efflux regulator TtgV recognizes a wide range of structurally different effectors in solution and complexed with

‡ ftp://spin6.mcb.uconn.edu

target DNA. Evidence from isothermal titration calorimetry. *J. Biol. Chem.* **280**, 20887–20893.

21. Waxman, E., Laws, W. R., Laue, T. M., Nemerson, Y. & Ross, J. (1993). Human factor VIIa and its complex with soluble tissue factor: evaluation of asymmetry and conformational dynamics by ultracentrifugation and fluorescence anisotropy decay methods. *Biochemistry*, **32**, 3005–3012.
22. Guazzaroni, M.E., Gallegos, M.T., Ramos, J.L. & Krell, T. (2007) Different modes of binding of mono- and biaromatic effectors to the transcriptional regulator TtgV. Role in differential derepression from its cognate operator. *J. Biol. Chem*. In the press.
23. Chan, A., Kilkuskie, R. & Hanlon, S. (1979). Correlations between the duplex winding angle and the circular dichroism spectrum of calf thymus DNA. *Biochemistry*, **18**, 84–91.
24. Woody, R. W. (1995). Circular dichroism. *Methods Enzymol.* **246**, 34–71.
25. Johnson, W. C., Jr (1985). Circular dichroism and its empirical application to biopolymers. *Methods Biochem. Anal.* **31**, 61–163.
26. Guthold, M., Bezanilla, M., Erie, D. A., Jenkins, B., Hansma, H. G. & Bustamante, C. (1994). Following the assembly of RNA polymerase–DNA complexes in aqueous solutions with the scanning force microscope. *Proc. Natl Acad. Sci. USA*, **91**, 12927–12931.
27. Bustamante, C. & Rivetti, C. (1996). Visualizing protein–nucleic acid interactions on a large scale with the scanning force microscope. *Annu. Biophys. Biomol. Struct.* **25**, 395–429.
28. Hansma, H. G. (2001). Surface biology of DNA by atomic force microscopy. *Annu. Rev. Phys. Chem.* **52**, 71–92.
29. Erie, D. A., Yang, G., Schultz, H. C. & Bustamante, C. (1994). DNA bending by Cro protein in specific and nonspecific complexes: implications for protein site recognition and specificity. *Science*, **266**, 1562–1566.
30. Seong, G. H., Kobatake, E., Miura, K., Nakazawa, A. & Aizawa, M. (2002). Direct atomic force microscopy visualisation of integration host factor-induced DNA bending stucture of the promoter regulatory region on the *Pseudomonas* TOL plasmid. *Biochem. Biophys. Res. Commun.* **291**, 361–366.
31. Nomura, K., Nasser, W., Kawagishi, H. & Tsuyumu, S. (1998). The pir gene of *Erwinia chrysanthemi* EC16 regulates hyperinduction of pectate lyase virulence genes in response to plant signals. *Proc. Natl Acad. Sci. USA*, **95**, 14034–14039.
32. Walker, J. R., Altamentova, S., Ezersky, A., Lorca, G., Skarina, T., Kudritska, M. *et al.* (2006). Structural and biochemical study of effector molecule recognition by the *E. coli* glyoxylate and allantoin utilization regulatory protein AllR. *J. Mol. Biol.* **358**, 810–828.
33. Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys. J.* **78**, 1606–1619.
34. Muraoka, M., Okumura, R., Uragami, Y., Nokata, T., Ogawa, N., Miyashita, K. & Senda, T. (2003). Purification and crystallization of a LysR-type transcriptional regulator CBNR from *Ralstonia eutropha* NH9. *Protein Pept. Letters*, **10**, 325–329.
35. Ogawa, N., McFall, S., Klem, T. J., Miyashita, K. & Chakrabarty, A. M. (1999). Transcriptional activation of the chlorocatechol degradative genes of *Ralstonia eutropha* NH9. *J. Bacteriol.* **181**, 6697–6705.
36. Kalodimos, C. G., Bonvin, A. M., Salinas, R. K., Wechselberger, R., Boelens, R. & Kaptein, R. (2002). Plasticity in protein-DNA recognition: Lac repressor interacts with its natural operator 01 through alternative conformations of its DNA-binding domain. *EMBO J.* **21**, 2866–2876.
37. Penin, F., Geourjon, C., Montserret, R., Bockmann, A., Lesage, A., Yang, Y. S. *et al.* (1997). Three-dimensional structure of the DNA-binding domain of the fructose repressor from *Escherichia coli* by ^{1}H and ^{15}N NMR. *J. Mol. Biol.* **270**, 496–510.
38. Nasser, W., Reverchon, C., Condemine, G. & Robert-Baudouy, J. (1994). Specific interactions of *Erwinia chrysanthemi* KdgR repressor with different operators of genes involved in pectinolysis. *J. Mol. Biol.* **236**, 427–440.
39. Chen, S. & Calvo, J. M. (2002). Leucine-induced dissociation of *Escherichia coli* Lrp hexadecamers to octamers. *J. Mol. Biol.* **318**, 1031–1042.
40. Schleif, R. (2003). AraC protein: a love-hate relationship. *Bioessays*, **25**, 274–282.
41. Vilar, J. M. & Saiz, L. (2005). DNA looping in gene regulation: from the assembly of macromolecular complexes to the control of transcriptional noise. *Curr. Opin. Genet. Dev.* **15**, 136–144.
42. Zwieb, C., Kim, J. & Adhya, S. (1989). DNA bending by negative regulatory proteins: Gal and Lac repressors. *Genes Dev.* **3**, 606–611.
43. Travers, A. A. (1989). DNA conformation and protein binding. *Annu. Rev. Biochem.* **58**, 427–452.
44. García, R. A., Bustamante, C. J. & Reich, N. O. (1996). Sequence-specific recognition by cytosine C5 and adenine N6 DNA methyltransferases requires different deformations of DNA. *Proc. Natl Acad. Sci. USA*, **93**, 7618–7622.
45. Dickerson, R. E. & Chiu, T. K. (1997). Helix bending as a factor in protein/DNA recognition. *Biopolymers*, **44**, 361–403.
46. Schumacher, M. A., Choi, K. Y., Zalkin, H. & Brennan, R. G. (1994). Crystal structure of LacI member, PurR, bound to DNA: minor groove binding by alpha helices. *Science*, **266**, 763–770.
47. Lewis, M., Chang, G., Horton, N. C., Kercher, M. A., Pace, H. C., Schumacher, M. A. *et al.* (1996). Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. *Science*, **271**, 1247–1254.
48. Schumacher, M. A., Allen, G. S., Diel, M., Seidel, G., Hillen, W. & Brennan, R. G. (2004). Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. *Cell*, **118**, 731–741.
49. Miwa, Y., Nakata, A., Ogiwara, A., Yamamoto, M. & Fujita, Y. (2000). Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. *Nucl. Acids Res.* **28**, 1206–1210.
50. Wiseman, T., Williston, S., Brandts, J. F. & Lin, L. N. (1989). Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **179**, 131–137.
51. van Holde, K. E. (1986). Sedimentation In Physical Biochemistry. 2nd edit. pp. 110–136, Prentice-Hall, Englewood Cliffs, NJ.
52. Laue, T. M., Shah, B. D., Ridgeway, T. M. & Pelletier, S. L. (1992). Interpretation of analytical sedimentation data for proteins. In Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J. & Horton, J. C., eds), pp. 90–125, Royal Society of Chemistry, Cambridge, UK.
53. Minton, A. P. (1994). Conservation of signal: a new algorithm for the elimination of the reference concentration as an independently variable parameter in

the analysis of sedimentation equilibrium. In Modern Analytical Ultracentrifugation (Schuster, T. M. & Laue, T. M., eds), pp. 81–93, Birkhauser, Boston, MA.
54. Cole, J. A. (2004). Analysis of heterogeneous interactions. *Methods Enzymol.* **384**, 212–232.
55. Schulz, A., Mucke, N., Langowski, J. & Rippe, K. (1998). Scanning force microscopy of *Escherichia coli* RNA polymerase sigma 54 holoenzyme complexes with DNA in buffer and in air. *J. Mol. Biol.* **283**, 821–836.

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Discusión

Discusión

La biotecnología pretende el aprovechamiento y la explotación de productos y procesos biológicos. Sus múltiples aplicaciones en campos de medicina, agricultura, química industrial, o protección medioambiental, entre otros, son en la actualidad - y lo seguirán siendo en el futuro - uno de los mayores productores de progreso, así como la fuente más importante de soluciones técnicas a diferentes problemas en éstas áreas y la mejora de la calidad de vida.

Desde un punto de vista medioambiental los microorganismos tolerantes a disolventes orgánicos son una herramienta muy importante en la lucha contra la contaminación ambiental, sobre todo si poseen rutas catabólicas para la degradación y/o mineralización de contaminantes del tipo de los hidrocarburos aromáticos. En zonas contaminadas con este tipo de compuestos, frecuentemente no crece ninguna bacteria degradadora debido a la alta toxicidad de los mismos. Sin embargo, la capacidad de tolerancia a estos compuestos y la plasticidad genética de la cepa *Pseudomonas putida* DOT-T1E la convierten en un organismo potencialmente exitoso para su utilización en tareas de recuperación de sitios contaminados, así como en un biocatalizador de interés para la producción de compuestos industriales. La capacidad de esta bacteria de transformar *m*-xileno en 3-metilcatecol, un precursor de la síntesis de compuestos farmacéuticos y aditivos sintéticos, ha sido ya demostrada por Rojas *et. al.* (2004). Asimismo Ramos-González *et al.* (2003) han demostrado la viabilidad de la cepa en la síntesis de 4-hidroxibenzoato a partir de tolueno.

Por lo cual, el conocimiento de los mecanismos moleculares de la tolerancia a disolventes orgánicos nos permitirá avanzar en el discernimiento y mejor utilización de cepas con capacidades biodegradadoras. En este sentido, esta Tesis Doctoral aporta un estudio profundo sobre la regulación de uno de los mecanismos esenciales para la tolerancia a disolventes orgánicos de la cepa tolerante *P. putida* DOT-T1E. A continuación se discuten los hallazgos más relevantes de este trabajo de Tesis.

Pseudomonas putida DOT-T1E es una cepa altamente tolerante a disolventes orgánicos que se aisló de la planta de tratamiento de aguas residuales de la cuidad de Granada (Ramos *et al.*, 1995). La tolerancia en esta cepa se debe a su capacidad para reajustar la composición lipídica de la membrana celular para lograr una mayor rigidez (Junker y Ramos, 1999; Ramos *et al.*, 2002; Bernal *et al.*, 2006), y en mayor medida a la actuación de unos sistemas de bombas de eflujo que son capaces de expulsar los disolventes orgánicos desde el citoplasma

o el espacio periplásmico hasta el exterior, mediante un proceso acoplado a la fuerza protón-motriz (Ramos *et al.*, 2002; Godoy *et al.*, 2001).

En *P. putida* DOT-T1E, se han identificado tres bombas de expulsión de disolventes (TtgABC, TtgDEF y TtgGHI) pertenecientes a la familia RND de transportadores bacterianos (Ramos *et al.*, 1998; Mosqueda y Ramos, 2000; Rojas *et al.*, 2003). La bomba TtgABC participa en la expulsión de antibióticos, disolventes orgánicos y moléculas señal de plantas con actividad antimicrobiana. Tiene un nivel basal de expresión medio y se induce por todos estos compuestos (Ramos *et al.*, 1998; Terán *et al.*, 2006). La bomba TtgDEF transporta disolventes orgánicos y no parece estar implicada en la expulsión de antibióticos, su expresión sólo se detecta por la inducción por disolventes (Mosqueda y Ramos, 2000). La bomba TtgGHI participa fundamentalmente en la expulsión de disolventes orgánicos y en menor medida en la expulsión de los antibióticos ampicilina, cloramfenicol, tetraciclina y ácido nalidíxico. Tiene un nivel basal de expresión alto y se induce significativamente por disolventes orgánicos, lo cual la convierte en el elemento más importante desde el punto de vista cuantitativo en cuanto a expulsión de disolventes orgánicos (Rojas *et al.*, 2003).

Al inicio de esta Tesis Doctoral se disentía si la bomba TtgGHI, factor clave de la tolerancia intrínseca basal e inducible de *P. putida* DOT-T1E a disolventes, se expresaba a partir de uno o dos promotores (Rojas *et al.*, 2001). Nuestros resultados establecieron que el operón *ttgGHI* se transcribe probablemente desde un único promotor en lugar de dos promotores solapantes, como había sido propuesto por Rojas *et al.* (2001). Esta propuesta se fundamenta en los resultados obtenidos en el análisis de transcripción *in vitro*, los cuales revelaron un único punto de inicio de la transcripción, y en el análisis mutacional de la región corriente arriba de la posición +1 de la transcripción (Guazzaroni *et al.*, 2004). El estudio de la transcripción tanto *in vivo* como *in vitro* demostraron que el promotor *ttgGHI* se puede transcribir con la ARN polimerasa sigma 70. Asimismo, en un trabajo reciente Duque *et al.* (2007) han puesto de manifiesto que *ttgGHI* forma parte del regulón de RpoT, un factor sigma alternativo. En *E. coli* se ha establecido que algunos promotores se transcriben tanto con σ^{70} como con σ^{S}, regulador central en la respuesta a estrés (Typas *et. al.*, 2007; Altuvia *et. al.*, 1994). Sin embargo, no se ha demostrado que σ^{S} esté implicado en la regulación de la respuesta a estrés por compuestos tóxicos. En el sistema TtgABC de *P. putida* DOT-T1E sólo pudo establecerse la implicación del factor σ^{70} en la transcripción de dicho operón (W. Terán, Tesis Doctoral. 2005); en *P. aeruginosa* y *E. coli* se estableció que la transcripción de los

genes *mex* y *acrAB* la realiza la ARN-polimerasa con σ^{70} pero no parecen formar parte del regulón RpoS (Rand *et al.*, 2002; Schuster *et al.*, 2004; Murakami *et al.*, 2005).

Un aspecto en el que se profundizó en este trabajo de Tesis fue en la caracterización funcional y molecular de la unión de TtgV a su operador en el ADN. A través del empleo de microcalorimetría y de ensayos de retardo en gel se determinó la constante de afinidad (K_D) de TtgV por su operador, la cual fue de 200 nM (Guazzaroni *et al.*, 2007). Esto indica que la fuerza de unión es alta, aunque permite un cierto nivel de expresión basal que mantiene las proteínas de la bomba siempre presentes en la membrana. Otros miembros de la misma familia de TtgV presentaron constantes de afinidad también en el orden nanomolar: el regulador PcaU del catabolismo del protocatecuato de *Acinetobacter* sp. ADP1 tiene una afinidad por la región intergénica *pcaU- pcaI* de 0.16 nM (Popp *et al.*, 2002), mientras que la proteína KdgR de *Erwinia chrysanthemi* se une *in vitro* a diversos operadores que participan en el metabolismo de la pectina con valores de K_D que varían entre 0.1 y 10 nM (Nasser *et al.*, 1994).

Mediante ensayos de improntas *in vitro* se determinó la zona protegida por TtgV, éstos revelaron que TtgV protege 4 vueltas de hélice de ADN que cubren la región promotora entre las posiciones +13 y -29 del promotor *ttgGHI*. Esto sugería que la proteína TtgV reprimía la transcripción del promotor *ttgGHI* por competencia con la ARN polimerasa por el sitio de unión al promotor. A través de ensayos de transcripción *in vitro* se profundizó más sobre este mecanismo: cuando se incubó TtgV con el plásmido que portaba el promotor P_{ttgG} previo a la adición de la ARN polimerasa, la transcripción desde P_{ttgG} se reprimió. Sin embargo, TtgV no reprimió significativamente la transcripción desde P_{ttgG} cuando el represor se agregó tras la formación del complejo abierto entre la ARN polimerasa y el promotor P_{ttgG}. Esto indicaba que TtgV reprimía la transcripción del promotor *ttgGHI* por competencia física con la ARN polimerasa por el sitio de unión al promotor (Guazzaroni *et al.*, 2004). Este mecanismo es el más ampliamente utilizado por diversos represores y ha sido estudiado en detalle en diversos trabajos (Rojo, 2001; Geanacopoulos y Adhya, 2002; Terán *et al.*, 2003; Yamamoto e Ishihama, 2003). Una característica muy interesante de este represor es que al unirse a su operador provoca un ángulo convexo de 57º. Esta particularidad probablemente aumente su eficiencia en el mecanismo de represión (Guazzaroni *et al*, 2007).

En la familia IclR no existe un consenso claro del tipo de organización física de las secuencias de ADN reconocidas por los miembros de esta familia. Por ejemplo la secuencia reconocida por el regulador MhpR está formada por un palíndrome de 15 pb (Ferrández *et al.*,

1997), mientras que el sitio reconocido por PcaU y PobR en sus respectivos promotores son tres secuencias repetidas perfectas de 10 pb que forman una secuencia repetida invertida y una directa separada de la anterior por 10 pb (Gerischer *et al.*, 1998; Guo & Houghton, 1999). En cuanto a la estequiometría de unión al ADN se ha visto que pueden unirse como dímeros, como dímeros de dímeros o como tetrámeros (Molina-Henares *et al.*, 2006, Walker *et al.*, 2006). Mediante el uso de microcalorimetría de titulación isotérmica, estudios de mutagénesis y ultracentrifugación analítica pudimos establecer que TtgV reconoce en su operador dos secuencias repetidas invertidas diferentes, uniéndose como tetrámero, siendo esta última la forma polimérica en la que se encuentra la proteína ya sea unida al ADN o en solución. La presencia de efectores en una solución TtgV-ADN provocó el despegue de la proteína del operador en forma tetramérica.

En cuanto al mecanismo de regulación de TtgV cabe señalar que los perfiles de expresión de los promotores *ttgGHI* y *ttgV* (como se mencionó en el apartado Introducción, TtgV también reprime su propia expresión) son similares, es decir, ambos se inducen en presencia de los mismos efectores. Esto puede probablemente explicarse por el hecho de que ambos promotores solapan (Figura 5 en el apartado Introducción) y de que el operador de TtgV cubre la caja -10 de la región promotora de P_{ttgG} y la caja -35 de P_{ttgV}. Este tipo de organización hace que exista continuamente una autorregulación negativa del sistema, lo cual permite alcanzar la expresión máxima de los genes regulados en tiempos muy cortos, ajustándose a cada condición fisiológica, con lo que se ahorra un costo metabólico innecesario (Sauvangeau, 1974; McAdams y Arkin, 1997; Rosenfeld *et al.*, 2002). Este mismo tipo de organización génica también se observó en el sistema TtgABC-TtgR de *P. putida* DOT-T1E y en AcrAB-AcrR de *E. coli*, aunque no en TtgDEF-TtgT, ya que TtgT se expresa independientemente del operón TtgDEF y no regula su propia síntesis (Ma et al., 1996; Terán *et al.*, 2003 y 2007). Esta característica de autorregular negativamente su propia síntesis también se ha encontrado en otros reguladores de la familia IclR como IclR de *E. coli* (Gui *et al.*, 1996) y PobR y PcaU de *Acinetobacter sp*. ADP1 (DiMarco *et al.*, 1993; DiMarco & Ornston, 1994; Gerischer *et al.*, 1998). Recientemente se ha demostrado por primera vez que la expresión de un miembro de esta familia, el activador de los genes de formación del flagelo de *Burkholderia glumae* QsmR (de *quorum sensing master regulador*), se regula en un proceso dependiente de *quorum sensing* (Kim *et al.*, 2007).

Otra propiedad relevante de la proteína TtgV puesta de manifiesto en esta Tesis Doctoral es su amplio perfil de efectores estructuralmente diferentes, característica que no había sido

encontrada en otros miembros de la familia IclR. El perfil de efectores incluye compuestos aromáticos de uno o dos anillos y alcoholes alifáticos. Entre los compuestos que presentaron mayor inducción de la expresión del operón TtgGHI se encuentran 1-naftol, indol, 2,3-dihidroxinaftaleno, 4-nitrotolueno y benzonitrilo. Se ha demostrado que TtgV es capaz de unirse a estos efectores con un amplio rango de afinidades (K_D), que van desde 2 μM en el caso del 2,3-dihidroxinaftaleno hasta 890 μM para el 1-hexanol (Guazzaroni *et al.*, 2005). Sin embargo, todas las K_D se encuentran por debajo de la concentración tóxica para la bacteria, que está en el orden milimolar, de modo tal que estos compuestos son capaces de inducir la transcripción de los genes estructurales que codifican las bombas de extrusión que los expulsarán del interior celular antes de que la célula sufra daños irreparables. También pudo establecerse que existe una tendencia clara entre las K_D observadas *in vitro* por microcalorimetría y la eficiencia de inducción *in vivo* estudiada mediante el ensayo de actividad β-galactosidasa (Figura D1). Con ello se dedujo que la afinidad de TtgV por sus efectores es el determinante principal de la eficiencia de los mismos (Guazzaroni *et al.*, 2005).

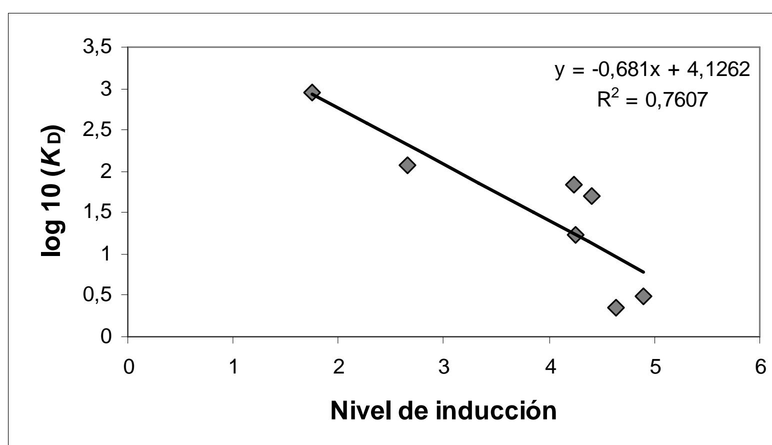

Figura D1: Relación entre la K_D de los efectores y la eficacia de inducción de la expresión génica de P_{ttgG} *in vivo* (ensayos de β-galactosidasa).

En los últimos 7 años se han obtenido las estructuras cristalinas de otros reguladores de sistemas MDR acomplejados con sus efectores que permitieron comenzar a conocer el fenómeno de promiscuidad en el reconocimiento de efectores de estas proteínas. Entre ellos se encuentran el activador BmrR (Zheleznova *et al.*, 1999) y el represor QacR (Schumacher *et al.*, 2001).Todos ellos presentan una característica común: los ligandos penetran dentro de

un bolsillo profundo en la proteína, donde establecen una serie de interacciones de van der Waals con los residuos hidrofóbicos, estos residuos apolares y aromáticos serían los responsables de recibir el correspondiente núcleo hidrofóbico de los efectores, formado a su vez por uno o varios anillos aromáticos. En el caso de QacR y BmrR la unión está aún más fortalecida por interacciones electrostáticas entre ligandos cargados positivamente y residuos de la proteína cargados negativamente. El ambiente hidrofóbico del sitio de unión hace esta interacción electrostática especialmente poderosa, sin que se generen grandes movimientos de las moléculas de agua (Zheleznova *et al.*, 1999; Schumacher *et al.*, 2001).

Para caracterizar funcionalmente el bolsillo de unión de efectores de TtgV se obtuvo un modelo tridimensional de dicho dominio en base a la estructura de cuatro proteínas de la familia cristalizadas, y se realizaron mutaciones puntuales a alanina (residuos I118, F134, V140, H200, V204 y V223) y a valina (residuos F134, H200 y V140) de los aminoácidos que probablemente contactaban con los efectores, todos ellos de naturaleza hidrofóbica, lo cual concuerda con el tipo de efectores reconocidos por la proteína, a diferencia de otros reguladores que reconocen efectores cargados como QacR y BmrR. También el modelo reveló que el bolsillo se encontraba sepultado en la proteína, y el volumen predicho del mismo fue de 1200 $Å^3$, similar al volumen de 1100 $Å^3$ de QacR (Schumacher *et al.*, 2001). El tamaño del bolsillo de unión es lo suficientemente grande como para permitir que ligandos diferentes adquieran distintas orientaciones y establezcan diferentes interacciones con distintos conjuntos de residuos que forman la pared del bolsillo. Schumacher *et al.* (2001) pusieron de manifiesto en su estudio cristalográfico como dos ligandos diferentes, rodamina 6G y etidio, ocupaban sitios diferentes que casi no solapaban dentro del bolsillo de QacR. Mediante experimentos de microcalorimetría se pudo establecer que TtgV no unía simultáneamente dos ligandos diferentes, es decir que si diferentes efectores contactan distintos subsitios éstos son mutuamente excluyentes por no poder recibir al mismo tiempo dos efectores. Lo mismo se observó en la proteína reguladora TtgR, en la cual se propone la existencia de dos subsitios diferentes en donde uno de ellos aceptaría un núcleo hidrofóbico pequeño (un solo anillo bencénico) y otro que recibiría un núcleo hidrofóbico mayor (naftaleno) (W. Terán, Tesis Doctoral, 2005).

La afinidad de unión de las proteínas con mutaciones puntuales a alanina y valina por sus efectores se vio alterada con respecto a la proteína silvestre, siendo mayor en el caso de los efectores bicíclicos indol y 1-naftol y menor en el de los monocíclicos 4-nitrotolueno y benzonitrilo. Esta diferencia en el cambio de afinidad dependiente del efector también

sustenta la hipótesis de que ligandos diferentes contactan distintos conjuntos de residuos de la pared del bolsillo.

En un trabajo reciente se describe por primera vez la existencia de regulación cruzada entre dos reguladores locales de dos bombas de extrusión con el mismo perfil de sustrato: TtgV y TtgT, los cuales se transcriben divergentemente de los correspondientes genes estructurales *ttgGHI* y *ttgDEF* (Terán *et al.*, 2008). El hecho de que ambos promotores mostraran una alta identidad en su secuencia y de que se indujeran por el mismo tipo de efectores llevó a la búsqueda de una posible regulación en *trans* por parte de los reguladores específicos. Los estudios *in vitro* pusieron de manifiesto que TtgV y TtgT reconocen el mismo operador en ambos promotores. La afinidad de unión al operador del promotor P_{ttgD} fue similar para ambos reguladores, mientras que TtgV se unió con mayor afinidad que TtgT al operador del promotor P_{ttgG}. Los estudios *in vivo* establecieron que TtgV jugaba el principal rol en el control de ambos promotores, mientras que TtgT parecía cumplir un papel más modesto. Esto puede explicarse probablemente por la diferencia en la expresión de ambos reguladores, ya que TtgV tiene un nivel basal de expresión medio y se induce de igual forma que el operón *ttgGHI*, mientras que TtgT tiene un nivel basal de expresión bajo y no es inducible (Terán *et al.*, 2007). El perfil de reconocimiento de efectores de ambas proteínas reguladoras es amplio y tienen un perfil similar, aunque no idéntico. Esto sugería que ambas proteínas reconocen juntas un rango de compuestos tóxicos más amplio, de modo tal que le conferiría a la bacteria una ventaja evolutiva al estar protegida contra un conjunto más extenso de compuestos tóxicos.

Sin embargo, cabe señalar que existen otros transportadores MDR en otras bacterias que también parecen ser redundantes. En el caso de *B. subtilis*, los transportadores Bmr y Blt muestran un 51% de identidad de secuencia y transportan un conjunto idéntico de cationes lipofílicos con similar efectividad (Ahmed *et al.*, 1995). Su expresión está controlada por reguladores transcripcionales de la familia MerR: BmrR y BltR respectivamente. Sin embargo, los patrones de expresión de ambos son muy diferentes: mientras BmrR activa la expresión de la bomba Bmr en respuesta a múltiples sustratos de este transportador, ninguno de estos compuestos son capaces de inducir la bomba Blt (Ahmed *et al.*, 1994), sugiriendo que la función fisiológica de Blt podría no estar relacionada con la quimioprotección, sino más bien con el transporte de otro sustrato específico que aún no ha sido encontrado (Woolridge *et al.*, 1997). Sin embargo, el hecho de que ambos transportadores estén modulados por el regulador global Mta es contrario a esta hipótesis (Baranova *et al.*, 1999).

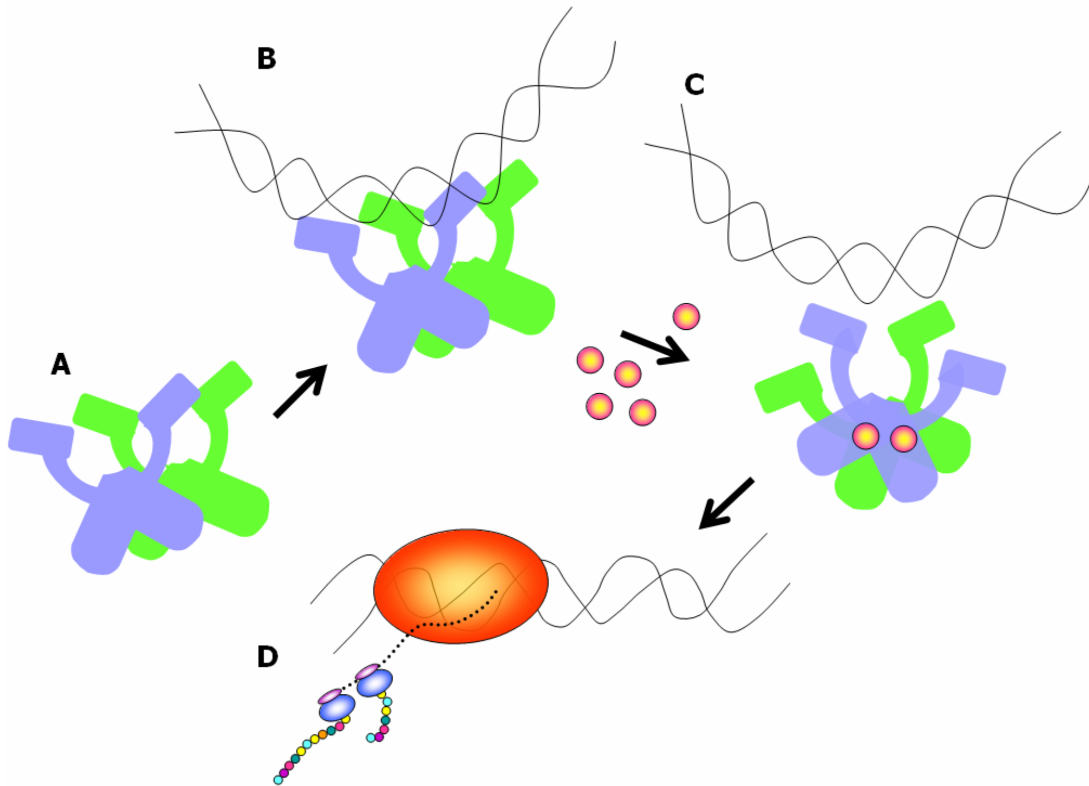

Figura D2. Representación esquemática del mecanismo funcional del represor TtgV. A, TtgV se encuentra como tetrámero en el interior celular. B, la proteína TtgV se une a su secuencia operadora en el ADN en ausencia de sus efectores curvando el ADN. C, presencia de efectores en el interior celular, dos moléculas de los mismos se unen al tetrámero y producen un cambio en su conformación que conlleva a su liberación del ADN. D, el promotor de los genes estructurales de la bomba TtgGHI queda libre para el acceso de la ARN polimerasa, comienza la transcripción del ARN mensajero policistrónico y la síntesis simultánea de las proteínas de la bomba TtgGHI.

Como ya se señaló en la Introducción de esta Tesis Doctoral, en *P. putida* DOT-T1E tres bombas de extrusión de disolventes funcionan de forma aditiva y sus tres operones se expresan de forma diferente. Estas diferencias en la expresión génica se correlacionan con la contribución de cada sistema a la "resistencia intrínsica" (*ttgABC* y *ttgGHI* se expresan siempre a un cierto nivel basal) y la "resistencia inducida" (*ttgDEF* y *ttgGHI* se inducen por disolventes). Esto nos hizo plantear la pregunta de si existe una regulación global coordinada. En nuestro laboratorio se observó que al igual que en las bombas Mex de *P. aeruginosa*, en *P. putida* DOT-T1E existe una expresión regulada de las bombas, es decir, cuando una no está presente el resto se expresa a un nivel mayor para compensar su falta (Li *et al.*, 2000; W. Terán, comunicación personal).

La cuestión aún sin resolver es conocer si esta compensación es parte de un circuito superior de regulación. En *E. coli*, se ha descrito que reguladores globales de estrés como MarA, Rob

y Sox y reguladores de respuesta a densidad celular y señales del *quorum sensing* (SdiA) inducen la bomba AcrAB-tolC (Ma *et al.*, 1995 y 1996; Rahmati *et al.*, 2002). Sin embargo, no se encontró homólogos de ninguno de estos reguladores globales en el genoma de *P. putida* KT2440. Por otro lado, estudios de genómica funcional con esta cepa en condiciones de presencia y ausencia de tolueno y *o*-xileno permitieron detectar variaciones significativas en la expresión de reguladores transcripcionales con función desconocida (Domínguez-Cuevas *et al.*, 2006), lo cual no descarta la posibilidad de que alguno de ellos esté implicado en un control global de la respuesta a disolventes.

En esta Tesis Doctoral se estudió en detalle los mecanismos de regulación de uno de los factores esenciales de tolerancia a disolventes orgánicos de la cepa tolerante *P. putida* DOT-T1E, haciendo hincapié en la caracterización molecular y funcional de su regulador local TtgV. En la Figura D2 se representa en un esquema las características más revelantes del mecanismo de acción del represor TtgV aportadas en este trabajo. Los aspectos que aún quedarían por conocer con más profundidad son, como se mencionó en el párrafo anterior, la existencia de un mecanismo global de regulación; los residuos de la proteína implicados en el reconocimiento del operador y los contactos que existen entre el efector y los residuos de la proteína reguladora, lo cual ayudará a la comprensión del mecanismo de transducción de la señal entre el dominio de unión al efector y el de unión al ADN, que conduce a la desrepresión del sistema. Asimismo, es sumamente interesante discernir entre el modo de acción molecular del regulador TtgT con respecto a TtgV y, si existe otro fin fisiológico para la existencia de estos transportadores. Para conocer algunos de estos aspectos, se está procediendo a la cristalización de la proteína TtgV en complejo con sus moléculas efectoras y su operador, y se están realizando mutaciones puntuales en la hélice de reconocimiento del dominio de unión al ADN de TtgV con el fin de establecer con exactitud los aminoácidos implicados en este reconocimiento.

Conclusiones

Las conclusiones de esta Tesis Doctoral se dividen en las secciones I, II y III en correspondencia con los objetivos de este trabajo.

I. Caracterización molecular del promotor P_{ttgG}.

1. El operón *ttgGHI* se transcribe desde un único punto de inicio de la transcripción, la correspondiente región se denominó promotor P_{ttgG}. El promotor P_{ttgG} es divergente con respecto al promotor P_{ttgV} y ambos promotores se inducen en presencia de disolventes orgánicos.

2. Ciertas mutaciones que llevan a una mejora del consenso del hexámero localizado en la región -10, que es reconocido por la ARN polimerasa con el factor σ^{70} incrementan la afinidad de la ARN polimerasa en el reconocimiento del promotor.

II. Caracterización bioquímica y funcional de la proteína reguladora TtgV en su unión al operador en el promotor P_{ttgG}.

1. La proteína TtgV es un tetrámero estable en solución, con un peso molecular aproximado de 110 KDa, y protege cuatro vueltas de hélice del ADN en la región intergénica *ttgGHI-ttgV* entre las posiciones +13 a -29 de P_{ttgG}, por lo cual solapa con las regiones -35 de P_{ttgV} y -10 de P_{ttgG}.

2. La afinidad de unión de TtgV por su operador es de 200 nM, con una estequiometría de unión de un tetrámero por operador. El operador tiene un tamaño de 36 pb y está formado por dos secuencias palindrómicas diferentes, sin nucléotidos espaciadores. Cuando a la solución se añaden efectores se libera el tetrámero del complejo.

3. El mecanismo de represión de TtgV se basa en la competencia física por el sitio reconocido por la ARN polimerasa. La curvatura convexa de 57º que produce TtgV en el ADN probablemente aumenta la eficacia de la represión. La presencia de efectores causa la separación de TtgV de su operador y permite el acceso a la ARN polimerasa, con la consecuente transcripción de los genes estructurales de la bomba.

III. Caracterización bioquímica y funcional de TtgV en su unión a efectores.

1. El perfil de efectores de TtgV está formado por compuestos aromáticos de uno o dos anillos y alcoholes alifáticos. La constante de afinidad de TtgV por sus efectores es del orden micromolar. La unión de TtgV a su operador no altera la afinidad de unión por los efectores. La estequiometría de unión de TtgV a efectores es de dos moléculas de efector por tetrámero.

2. El bolsillo de unión de efectores de TtgV está probablemente formado por residuos hidrofóbicos. La mutación de alguno de estos residuos a alanina altera la afinidad de unión por los efectores, siendo mayor para los compuestos bicíclicos y menor para los monocíclicos.

Bibliografía

Aendekerk, S., B. Ghysels, P. Cornelis, and C. Baysse. 2002. Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. *Microbiology* 148: 2371-2381.

Adewoye, L., A. Sutherland, R. Srikumar, and K. Poole. 2002. The mexR repressor of the mexAB-oprM multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. *J Bacteriol* 184:4308-4312.

Ahmed, M., C. M. Borsch, S. S. Taylor, N. Vazques-Laslop, and A. A. Neyfakh. 1994. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J. Biol. Chem.* 269: 28506-28513.

Ahmed, M., L. Lyass, P. N. Markham, S. S. Taylor, N. Vazquez-Laslop, and A. A. Neyfakh. 1995. Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. *J Bacteriol* 177: 3904-3910.

Aires, J. R., T. Kohler, H. Nikaido, and P. Plesiat. 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 43: 2624-8.

Akama, H., T. Matsuura, S. Kashiwagi, H. Yoneyama, S. Narita, T. Tsukihara, A. Nakagawa, and T. Nakae. 2004. Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. *J Biol Chem* 279: 25939-25942.

Alekshun, M. N., and S. B. Levy. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. *Antimicrob. Agents Chemother*. 41: 2067–2075.

Alekshun, M. N. and S. B. Levy. 1999. The mar regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends Microbiol* 7:410-413.

Altuvia S., Almiron M., Huisman G., Kolter R. and Storz G. 1994. The dps promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. *Mol Microbiol*. 13: 265-272.

Alonso, A. and J. L. Martinez. 2000. Cloning and characterization of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 44: 3079-3086.

Alonso, A. and J. L. Martinez. 2001. Expression of multidrug efflux pump SmeDEF by clinical isolates of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 45:1879-1881.

Altenbuchner, J., K. Schmid, and R. Schmitt. 1983. Tn1721-encoded tetracycline resistance: mapping of structural and regulatory genes mediating resistance. *J Bacteriol* 153:116-123.

Aono, R. 1998. Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes. *Extremophiles* 2: 239-248.

Aono, R., M. Kobayashi, H. Nakajima, and H. Kobayashi. 1995. A close correlation between improvement of organic solvent tolerance levels and alteration of resistance toward low levels of multiple antibiotics in *Escherichia coli*. *Biosci Biotechnol Biochem* 59: 213-218.

Aono R, K. Aibe., A. I Inoue and K. Horikoshi. **1991**. Preparation of organic solvent-tolerant mutants from *Escherichia Coli* K12. *Agric. Biole. Chem*. 55, 1935-1938.

Aramaki, H., N. Yagi, and M. Suzuki. 1995b. Residues important for the function of a multihelical DNA binding domain in the new transcription factor family of Cam and Tet repressors . *Protein Eng* 8:1259-66.

Baranova, N. N., A. Danchin, and A. A. Neyfakh. 1999. Mta, a global MerR-type regulator of the *Bacillus subtilis* multidrug efflux transporters. *Mol. Microbiol*. 31:1549–1559.

Baranova, N. and H. Nikaido. 2002. The baeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. *J Bacteriol* 184: 4168-4176.

Beinlich, K. L., R. Chuanchuen, and H. P. Schweizer. 2001. Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary clinical isolates of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 198:129-314.

Bertani, I., Kojic, M., and Venturi, V. 2001. Regulation of the *p*-hydroxybenzoic acid hydroxylase gene (*pobA*) in plant-growth-promoting *Pseudomonas putida* WCS358. *Microbiology* 147, 1611-1620.

Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science*. 277: 1453-1474.

Brooun, A., J. J. Tomashek, and K. Lewis. 1999. Purification and ligand binding of EmrR, a regulator of a multidrug transporter. *J. Bacteriol.* 181:5131–5133.

Brown, M. H., I. T. Paulsen, and R. A. Skurray. 1999. The multidrug efflux protein NorM is a prototype of a new family of transporters. *Mol Microbiol* 31: 394-395.

Brown, M. H. and R. A. Skurray. 2001. Staphylococcal multidrug efflux protein QacA. *J Mol Microbiol* Biotechnol 3:163-170.

Buell, C. R., V. Joardar, M. Lindeberg, J. Selengut, I. T. Paulsen, M. L. Gwinn, R. J. Dodson, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, S. Daugherty, L. Brinkac, M. J. Beanan, D. H. Haft, W. C. Nelson, T. Davidsen, N. Zafar, L. Zhou, J. Liu, Q. Yuan, H. Khouri, N. Fedorova, B. Tran, D. Russell, K. Berry, T. Utterback, S. E. Van Aken, T. V. Feldblyum, M. D´Ascenzo, W. L. Deng, A. R. Ramos, J. R. Alfano, S. Cartinhour, A. K. Chatterjee, T. P. Delaney, S. G. Lazarowitz, G. B. Martin, D. J. Schneider, X. Tang, C. L. Bender, O. White, C. M. Fraser, and A. Collmer. 2003. The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. Proc Natl Acad Sci U S A 100:10181-10186.

Chuanchuen, R., C. T. Narasaki, and H. P. Schweizer. 2002. The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of triclosan. *J Bacteriol* 184: 5036-5044.

Clubb, R.T., Omichinski, J.G., Savilahti, H., Mizuuchi, K., Gronenborg, A.M., and Clore, G. 1994. A novel class of winged helix-turn-helix protein: The DNA-binding domain of Mu transposase. *Structure* 2, 1041-1048.

Cohen, S. P., S. B. Levy, J. Foulds, and J. L. Rosner. 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the mar operon and a mar-independent pathway. *J Bacteriol* 175: 7856-7862.

Copeland A., Lucas S., Lapidus A., Barry K. and Detter C. 2005a. Sequencing of the draft genome assembly of *Azotobacter vinelandii* AvOP. Enviado (JUN-2005) to the EMBL/GenBank/DDBJ databases.

Copeland A., Lucas S., Lapidus A., Barry K. and Detter C. 2005b. Sequencing of the draft genome assembly of *Marinobacter aquaeolei* VT8. Enviado (JUN-2005) to the EMBL/GenBank/DDBJ databases.

Cronan, J. E. Jr. 1968. Phospholipid alterations during growth of *Escherichia coli*. *J Bacteriol* 95: 2054-2061.

Dagley, S. 1981. New perspectives in aromatic catabolism. In Lessinger, T., Cook, A. M., R. Hütter, and Nüesch, J. (eds). Microbial degradation of xenobiotics and recalcitrant compounds (pp 181-186). Academic Press. New York.

den Dooren Jong, L. E. 1926. Bigdrage tot de kennis van het mineralisatieproces. *Nijgh & van Ditmar*, Rotterdam.

Dennis, J. J., and G. J. Zylstra. 1999. Characterization of the regulators of the Srp solvent resistance efflux pump.

de Smet, M. J., J. Kingma, and B. Witholt. 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim Biophys Acta* 506:64-80.

De Vos, P., A. V. Landschoot, P. Segers, R. Tytgat, M. Gillis, M. Bauwens, R. Rossau, M. Goor, B. Pot, K. Kerster, P. Lizzaraga, and J. De Ley. 1989. Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxy-ribonucleic acid: ribosomal ribonucleic acid hybridizations. *Int. J. Syst. Bacteriol.* 39: 35-49.

DiMarco, A.A., Averhoff, B., and Ornston, L.N. 1993. Identification of the transcriptional activator *pobR* and characterization of its role in the expression of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in *Acinetobacter calcoaceticus*. *J. Bacteriol.* 175, 4499-4506.

DiMarco, A.A., and Ornston, L.N. 1994. Regulation of *p*-hydroxybenzoate hydroxylase synthesis by PobR bound to an operator in *Acinetobacter calcoaceticus*. *J. Bacteriol.* 176, 4277-4284.

Dinh, T., I. T. Paulsen, and M. H. Saier Jr. 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. *J Bacteriol* 176: 3825-3831.

Dominguez-Cuevas P., Gonzalez-Pastor J.E., Marques S., Ramos J.L. and de Lorenzo V. 2006. Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. *J Biol Chem.* 281: 11981-11991.

Donald, L.J., Hosfield, D.J., Cuvelier, S.L., Ens, W., Standig, K.G., and Duckworth, H.W. 2001. Mass spectrometric study of the *Escherichia coli* repressor protein, IclR and GalR, and their complexes with DNA. *Protein Sci.* 10, 1370-1380.

Duque E., Rodríguez-Herva J.J., de la Torre J., Domínguez-Cuevas P., Muñoz-Rojas J. and J. L. Ramos. 2007. The RpoT regulon of *Pseudomonas putida* DOT-T1E and its role in stress endurance against solvents *J. Bacteriol* 189: 207-219.

Duque, E., A. Segura, G. Mosqueda, and J. L. Ramos. 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. *Mol Microbiol* 39:1100-1106.

Elkins, C. A. and H. Nikaido. 2003. 3D structure of AcrB: the archetypal multidrug efflux transporter of *Escherichia coli* likely captures substrates from periplasm. *Drug Resist Updat* 6:9 - 13.

Eswaran, J., E. Koronakis, M. K. Higgins, C. Hughes, and V. Koronakis. 2004. Three´s company: component structures bring a closer view of tripartite drug efflux pumps. *Curr Opin Struct Biol* 14: 741-747.

Evans, K., L. Adewoye, and K. Poole. 2001. MexR repressor of the mexAB-oprM multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the mexAmexR intergenic region. *J Bacteriol* 183: 807-812.

Evans, K., L. Passador, R. Srikumar, E. Tsang, J. Nezezon, and K. Poole. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 180:5443-7.

Federici, L., D. Du, F. Walas, H. Matsumura, J. Fernandez-Recio, K. S. McKeegan, M. I. Borges-Walmsley, B. F. Luisi, and A. R. Walmsley. 2005. The crystal structure of the outer membrane protein VceC from the bacterial pathogen *Vibrio cholerae* at 1.8 A resolution. *J Biol Chem* 280:15307-15314.

Feil H., Feil W.S., Chain P., Larimer F., DiBartolo G., Copeland A., Lykidis A., Trong S., Nolan M., Goltsman E., Thiel J., Malfatti S., Loper J.E., Lapidus A., Detter J.C., Land M., Richardson P.M., Kyrpides N.C., Ivanova N., Lindow S.E. 2005. Comparison of the complete genome sequences of *Pseudomonas syringae* pv. syringae B728a and pv. tomato DC3000. *Proc Natl Acad Sci USA.* 102 (31): 11064-11069.

Ferrández, A., García, J.L., and Díaz, E. 1997. Genetic characterization and expression in heterologous hosts of the 3-3-(hydroxyphenyl)-propionate catabolic pathway of *Escherichia coli* K-12. *J. Bacteriol*. 170, 2573-2581.

Fournier, B., R. Aras, and D. C. Hooper. 2000. Expression of the multidrug resistance transporter NorA from *Staphylococcus aureus* is modified by a two-component regulatory system. *J Bacteriol* 182: 664-671.

Fukumori, F., H. Hirayama, H. Takami, A. Inoue, and K. Horikoshi. 1998. Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant: involvement of an efflux system in solvent resistance. *Extremophiles* 2: 395-400.

Geanacopoulos M. and Adhya S. 2002. Genetic analysis of GalR tetramerization in DNA looping during repressosome assembly. *J Biol Chem*. 277: 33148-33152.

Germ. M., E. Yoshihara, H. Yoneyama, and T. Nakae. 1999. Interplay between the efflux pump and the outer membrane permeability barrier in fluorescent dye accumulation in *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Commun*. 261: 452-455.

Gerischer, U., Segura, A., and Ornston, L.N. 1998. PcaU, a transcriptional activator of genes for protocatechuate utilization in *Acinetobacter*. J. Bacteriol. 180, 1512-1524.

Gibson, D. T. and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons. pp. 361-369 In D. T. Gibson [ed.], Microbial degradation of organic compounds. Marcel Dekker, New York.

Godsey, M. H., N. N. Baranova, A. A. Neyfakh, and R. G. Brennan. 2001. Crystal structure of MtaN, a global multidrug transporter gene activator. *J. Biol. Chem*. 276:47178–47184.

Grinius, L., G. Dreguniene, E. B. Goldberg, C. H. Liao, and S. J. Projan. 1992. A staphylococcal multidrug resistance gene product is a member of a new protein family. Plasmid 27:119-129.

Grkovic, S., M. H. Brown, N. J. Roberts, I. T. Paulsen, and R. A. Skurray. 1998. QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. *J Biol Chem* 273:18665-18673.

Grkovic, S., M. H. Brown, M. A. Schumacher, R. G. Brennan, and R. A. Skurray. 2001. The staphylococcal QacR multidrug regulator binds a correctly spaced operator as a pair of dimers. *J Bacteriol* 183:7102-7109.

Guazzaroni, M. E., Gallegos, M-T, Ramos J. L. and Krell, T. 2007. Different modes of binding of mono- and biaromatic effectors to the transcriptional regulator TtgV. Role in differential derepression from its cognate operator. *J. Biol. Chem*., 282: 16308-16316.

Guazzaroni M.E., Krell T., Felipe A., Ruiz R., Meng C., Zhang X., Gallegos M.T. and Ramos J.L. 2005. The multidrug efflux regulator TtgV recognizes a wide range of structurally different effectors in solution and complexed with target DNA. Evidence from isothermal titration calorimetry. *J. Biol. Chem.*, 280: 20887-20893.

Guazzaroni M.E., Krell T., Gutiérrez del Arroyo P., Vélez M., Jiménez M., Rivas G. and Ramos J.L. 2007. The transcripcional represor TtgV recognizes a complex operador as a tetramer and induces convex DNA bending. *J. Mol. Biol.*, 369: 927-939.

Guazzaroni, M.E., Terán, W., Zhang, X., Gallegos, M.T. and J.L. Ramos. 2004. TtgV bound to a complex operator site represses transcription of the single promoter for the multidrug and solvent extrusion TtgGHI pump. *J. Bacteriol.*, 186: 2921-2927.

Gui, L., Sunnarborg, A., Pan, B., and LaPorte, D.C. 1996. Autoregulation of *iclR*, the gene encoding the repressor of the glyoxylate bypass operon. *J. Bacteriol.* 178: 321-324.

Guo, Z., and. Houghton, J.E. 1999. PcaR-mediated activation and repression of *pca* genes from *Pseudomonas putida* are propagated by its binding to both the –35 and the –10 promoter elements. Mol. Microbiol. 32, 253-263.

Hamamoto T., N. T. T. K. a. K. H. 1994. Effect of temperature and growth phase on fatty acid composition of the psychrolophic *Vibrio* sp. strain no. 5710. FEMS Microbiol. Lett. 119, 77-81.

Harwood, C.S., Nichols, N.N., Kim, M.K., Ditty, J.L., and Parales, R.E. 1994. Identification of the *pcaRKF* gene cluster from *Pseudomonas putida*: involvement in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. *J. Bacteriol.* 176, 6479-6488.

Heipieper, H. J., R. Diefenbach, and H. Keweloh. 1992. Conversion of cis unsaturated fatty acids to trans, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl Environ Microbiol* 58:1847-1852.

Heipieper, H.-J., G. Meulenbeld Q. van Oirschot and J. A. M. de Bont. 1996. Effect of environmental factors on the *cis/trans* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. Appl. Environ. Microbiol **62**, 2773-2777.

Heldwein, E. E. and R. G. Brennan. 2001. Crystal structure of the transcription activator BmrR bound to DNA and a drug. Nature 409: 378-382.

Higgins, M. K., E. Bokma, E. Koronakis, C. Hughes, and V. Koronakis. 2004. Structure of the periplasmic component of a bacterial drug efflux pump. Proc Natl Acad Sci U S A 101: 9994-9999.

Hillen, W. and C. Berens. 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu Rev Microbiol 48:345-369.

Hinrichs, W., C. Kisker, M. Duvel, A. Muller, K. Tovar, W. Hillen, and W. Saenger. 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. Science 264:418-20.

Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. J Exp Med 196:109-118.

Hirakawa, H., Y. Inazumi, T. Masaki, T. Hirata, and A. Yamaguchi. 2005. Indole induces the expression of multidrug exporter genes in *Escherichia coli*. *Mol Microbiol* 55:1113-1126.

Huertas, M. J., E. Duque, L. Molina, R. Rossello-Mora, G. Mosqueda, P. Godoy, B. Christensen, S. Molin and J.L. Ramos. 2000. Tolerance to sudden organic solvent shocks by soil bacteria and characterization of *Pseudomonas putida* strains isolated from toluene polluted sites. *Env. Sci. Technol.* 34: 3395-3400.

Hüsken, L. E., R. Beeftink, J. A. M. de Bont, and J. Wery. 2001. High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. *Appl.Microbiol. Biotechnol.* 55: 571-577.

Hüsken, L. E., M. Oomes, K. Schoren, J. Tramper, J. A. M. de Bont, and R. Beeftink. 2002. Membrane-facilitated bioproduction of 3-methylcatechol in an octanol/water two-phase system. J. Biotechnol. 96: 281-289.

Isken, S. and J. A. de Bont. 1996. Active efflux of toluene in a solvent-resistant bacterium. *J Bacteriol* 178:6056-6058.

Jellen-Ritter, A. S. and W. V. Kern. 2001. Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants selected with a fluoroquinolone. *Antimicrob Agents Chemother* 45:1467-1472.

Jimenez, J. I., B. Minambres, J. L. Garcia, and E. Diaz. 2002. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. Environ Microbiol 4: 824-841.

Junker, F. and J. L. Ramos. 1999. Involvement of the cis/trans isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. *J Bacteriol* 181:5693-5700.

Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata. 2000. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. 9: 189-197.

Keweloh, H. and H. J. Heipieper. 1996. Trans unsaturated fatty acids in bacteria. Lipids 31:129- 137.

Keweloh, H., G. Weyrauch, and H. J. Rehm. 1990. Phenol-induced membrane changes in free and immobilized *Escherichia coli*. Appl Microbiol Biotechnol 33:66-71.

Kieboom, J. and J. de Bont. 2001. Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. *Microbiology* 147:43-51.

Kieboom, J., J. J. Dennis, J. A. de Bont, and G. J. Zylstra. 1998a. Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. *J Biol Chem* 273:85-91.

Kieboom, J., J. J. Dennis, G. J. Zylstra, and J. A. de Bont. 1998b. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *J Bacteriol* 180:6769-6772.

Kiely PD, Haynes JM, Higgins CH, Franks A, Mark GL, Morrissey JP, O'gara F. 2006. Exploiting New Systems-Based Strategies to Elucidate Plant-Bacterial Interactions in the Rhizosphere. *Microb. Ecol*. 51: 257-266.

Kim J., Kang Y., Choi O., Jeong Y., Jeong J.E., Lim J.Y., Kim M., Moon J.S., Suga H., Hwang I. 2007. Regulation of polar flagellum genes is mediated by quorum sensing and FlhDC in *Burkholderia glumae*. *Mol Microbiol*. 2007 64:165-179.

Kim, K., S. Lee, K. Lee, and D. Lim. 1998. Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. *J Bacteriol* 180:3692- 3696.

Kisker, C., W. Hinrichs, K. Tovar, W. Hillen, and W. Saenger. 1995. The complex formed between Tet repressor and tetracycline-Mg2+ reveals mechanism of antibiotic resistance. J Mol Biol 247:260-280.

Köhler, T., S. F. Epp, L. K. Curty, and J. C. Péchère. 1999a. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J. Bacteriol.* 181: 6300-6305.

Köhler, T., M. Michea-Hamzehpour, S. F. Epp, and J. C. Péchère. 1999b. Carbapenem activities against: respective contributions of OprD and efflux systems. *Antimicrob Agents Chemother* 43:424-427.

Kok, R. G., D'Argenio, D. A., and Ornston, L. N. 1998. Mutation analysis of PobR and PcaU, closely related transcriptional activators in *Acinetobacter*. J. Bacteriol. 180, 5058-5069.

Koronakis, V., J. Li, E. Koronakis, and K. Stauffer. 1997. Structure of TolC, the outer membrane component of the bacterial type I efflux system, derived from two-dimensional crystals. *Mol Microbiol* 23:617-626.

Krell T., Molina-Henares A.J., Ramos J.L. 2006. The IclR family of transcriptional activators and repressors can be defined by a single profile. *Protein Sci*. 15: 1207-1213.

Kwon, H. J., M. H. Bennik, B. Demple, and T. Ellenberger. 2000. Crystal structure of the *Escherichia coli* Rob transcription factor in complex with DNA. *Nat. Struct. Biol.* 7:424–430.

Lee, E. H., C. Rouquette-Loughlin, J. P. Folster, and W. M. Shafer. 2003. FarR regulates the farAB-encoded efflux pump of *Neisseria gonorrhoeae* via an MtrR regulatory mechanism. *J Bacteriol* 185: 7145-7152.

Lee, S. W. and G. Edlin. 1985. Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. Gene 39:173-180.

Levy, S. B. 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. *J Appl Microbiol* 92 Suppl 1:65-71.

Levy, S. B. 2001. Antibacterial household products: cause for concern. *Emerg Infect Dis* 7:512-515.

Lewis, K. 1994. Multidrug resistance pumps in bacteria: variations on a theme. *Trends Biochem Sci* 19:119-123.

Lewis, K. 2000. Translocases: a bacterial tunnel for drugs and proteins. *Curr. Biol*. 10: 678–681.

Li, X. Z., N. Barré, and K. Poole. 2000b. Influence of the MexA-MexB-OprM multidrug efflux system on expression of the MexC-MexD-OprJ and MexE-MexF-OprN multidrug efflux system in *Pseudomonas aeruginosa. J. Antimicrob. Chemother*. 46: 885-893.

Li, Y., T. Mima, Y. Komuri, Y. Morita, T. Kuroda, T. Mizushima, and T. Tsuchiya. 2003. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa. J. Antimicrob. Chemother.* 52 (4): 572-575.

Li, H., and J. T. Park. 1999. The periplasmic murein peptide-binding protein MppA is a negative regulator of multiple antibiotic resistance in *Escherichia coli. J. Bacteriol.* 181:4842–4847.

Li, X. Z., L. Zhang, and K. Poole. 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. *J Bacteriol* 180:2987-2991.

Li, X. Z., L. Zhang, and K. Poole. 2002. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia. Antimicrob Agents Chemother* 46:333-343.

Li, X. Z., L. Zhang, and K. Poole. 2000a. Interplay between MexAB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 45: 433-436.

Liu, Y., Jiang, G., Cui, Y., Mukherjee, A., Ma, W.L., and Chatterjee, A.K. 1999. *kdgR$_{Ecc}$* negatively regulates genes for pectinases, cellulase, protease, Harpin$_{Ecc}$, and a global RNA regulator in *Erwinia carotovora* subsp. *carotovora. J. Bacteriol.* 181, 2411-2421.

Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plesiat P. 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. *Antimicrob Agents Chemother*. 48: 1797-802.

Lomovskaya, O., K. Lewis, and A. Matin. 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. *J Bacteriol* 177: 2328-2334.

Lucas, C. E., J. T. Balthazar, K. E. Hagman, and W. M. Shafer. 1997. The MtrR repressor binds the DNA sequence between the mtrR and mtrC genes of *Neisseria gonorrhoeae*. *J Bacteriol* 179:4123-4128.

Lugtenberg, B. J. and L. C. Dekkers. 1999. What makes Pseudomonas bacteria rhizosphere competent? *Environ Microbiol* 1:9-13.

Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst. 1996. The local repressor AcrR plays a modulating role in the regulation of acrAB genes of *Escherichia coli* by global stress signals. *Mol Microbiol* 19:101-12.

Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst. 1995. Genes acrA and acrB encode a stress-induced efflux system of *Escherichia coli. Mol Microbiol* 16:45-55.

McAdams, H. H. and A. Arkin. 1997. Stochastic mechanisms in gene expression. *Proc Natl Acad Sci USA* 94: 814-819.

Maloy, S.R., and Nunn, W.D. 1982. Genetic regulation of the glyoxylate shunt in *Escherichia coli* K-12. J. Bacteriol. 149,173-180.

Marchand, I., L. Damier-Piolle, P. Courvalin, and T. Lambert. 2004. Expression of the RNDtype efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two component system. *Antimicrob Agents Chemother* 48:3298-3304.

Martin, R. G., W. K. Gillette, N. I. Martin, and J. L. Rosner. 2002. Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in *Escherichia coli. Mol. Microbiol.* 43:355–370.

Martin, R. G. and J. L. Rosner. 1995. Binding of purified multiple antiobiotic resistance repressor (MarR) to mar operator sequences. *Proc. Nat. Acad. Sci.* USA 92: 5456-5460.

Martinez-Bueno, M. A., R. Tobes, M. Rey, and J. L. Ramos. 2002. Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of *Pseudomonas putida* KT2440 and their counterparts in *Pseudomonas aeruginosa* PA01. Environ Microbiol 4:842-55.

Maseda, H., K. Saito, A. Nakajima, and T. Nakae. 2000. Variation of the mexT gene, a regulator of the MexEF-oprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa. FEMS Microbiol Lett* 192:107-112.

Masuda, N. and G. M. Church. 2002. Escherichia coli gene expression responsive to levels of the response regulator EvgA. *J Bacteriol* 184:6225-6234.

Matsuo, Y., S. Eda, N. Gotoh, E. Yoshihara, and T. Nakae. 2004. MexZ-mediated regulation of mexXY multidrug efflux pump expression in *Pseudomonas aeruginosa* by binding on the mexZ-mexX intergenic DNA. *FEMS Microbiol Lett* 238:23-28.

Migula, W. 1894. Ueber ein neues System der Bakterien. Arb. Bakteriol. Inst. Karlsruhe 1: 235-238.

Miller, P. F. and M. C. Sulavik. 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli. Mol Microbiol* 21:441-448.

Molina-Henares J., Krell T., Guazzaroni M. E., Segura A. and Ramos J. L. 2006. Members of the IclR family of transcriptional activators and repressors exhibit conserved features outside the DNA binding domain. FEMS Microbiol. Rev., 30: 157-186.

Morita, Y., A. Kataoka, S. Shiota, T. Mizushima, and T. Tsuchiya. 2000. NorM of *vibrio parahaemolyticus* is an Na(+)-driven multidrug efflux pump. *J Bacteriol* 182:6694-6697.

Mosqueda, G. and J. L. Ramos. 2000. A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the tod genes for toluene metabolism. *J Bacteriol* 182: 937-943.

Murakami, S., R. Nakashima, E. Yamashita, and A. Yamaguchi. 2002. Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* 419: 587-593.

Murakami K., Ono T., Viducic D., Kayama S., Mori M., Hirota K., Nemoto K. Miyake Y. 2005. Role for rpoS gene of *Pseudomonas aeruginosa* in antibiotic tolerance. *FEMS Microbiol Lett.* 242:161-167.

Murray, D. S., M. A. Schumacher, and R. G. Brennan. 2004. Crystal structures of QacR diamidine complexes reveal additional multidrug-binding modes and a novel mechanism of drug charge neutralization. *J Biol Chem* 279:14365-14371.

Nagakubo, S., K. Nishino, T. Hirata, and A. Yamaguchi. 2002. The Putative Response Regulator BaeR Stimulates Multidrug Resistance of *Escherichia coli* via a Novel Multidrug Exporter System, MdtABC. *J Bacteriol* 184:4161-4167.

Nasser, W., Reverchon, C., Condemine, G., and Robert-Baudouy, J. 1994. Specific interactions of *Erwinia chrysanthemi* KdgR repressor with different operators of genes involved in pectinolysis. *J. Mol. Biol.* 236, 427-440.

Nasser, W., Reverchon, S., and Robert-Baudouy, J. 1992. Purification and functional characterisation of the KdgR protein, a major repressor of pectinolysis genes of *Erwinia chrysanthemi. Mol. Microbiol.* 6, 257-265.

Nègre, D., Cortay, J.C., Galinier, A., Sauve, P., and Cozzone, A.J. 1992. Specific interactions between the IclR repressor of the acetate operon of *Escherichia coli* and its operator. *J. Mol. Biol.* 228, 23-29.

Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. A. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummler, and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* 4:799-808.

Neumann G, Kabelitz N, Zehnsdorf A, Miltner A, Lippold H, Meyer D, Schmid A, Heipieper HJ. 2005. Prediction of the adaptability of *Pseudomonas putida* DOT-T1E to a second phase of a solvent for economically sound two-phase biotransformations. *Appl Environ Microbiol.* 71: 6606-6612.

Neyfakh, A. A. 1992. The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the *Staphylococcus* NorA protein. *Antimicrob Agents Chemother* 36:484- 495.

Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen. 1991. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. *Proc. Natl. Acad. Sci.* USA 88:4781–4785.

Nguyen, T. N., Q. G. Phan, L. P. Duong, K. P. Bertrand, and R. E. Lenski. 1989. Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Mol Biol Evol* 6:213-225.

Nguyen, C. C., and M. H. Saier, Jr. 1995. Phylogenetic, structural and functional analyses of the LacI-GalR family of bacterial transcription factors. *FEBS Lett.* 377: 98-102.

Nikaido, H. 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin Cell Dev Biol* 12:215-223.

Nikaido, H., 1999. Microdermatology: cell surface in the interaction of microbes with the external world. *J. Bacteriol.* 181: 4-8.

Nikaido, H. 1996. En F. C. Neidhardt, R. Curtis, J. L. Ingraham, E. C. C. Lin, B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, y H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D. C. 29-47.

Nikaido, H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264: 382-388.

Nishino, K. and A. Yamaguchi. 2002. EvgA of the two-component signal transduction system modulates production of the yhiUV multidrug transporter in *Escherichia coli*. *J Bacteriol* 184: 2319-2323.

Nishino, K. and A. Yamaguchi. 2001. Overexpression of the response regulator evgA of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. *J Bacteriol* 183:1455-1458.

Nomura, K., Nasser, W., Kawagishi, H., and Tsuyumu, S. 1998. The *pir* gene of *Erwinia chrysanthemi* EC16 regulates hyperinduction of pectate lyase virulence genes in response to plant signals. *Proc. Natl. Acad. Sci. USA* 95,14034-14039.

Ochs, M. M., M. P. McCusker, M. Bains, and R. E. Hancock. 1999. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. *Antimicrob. Agents Chemother.* 43: 1085-1090.

Okazaki, T. and K. Hirai. 1992. Cloning and nucleotide sequence of the *Pseudomonas aeruginosa* nfxB gene, conferring resistance to new quinolones. *FEMS Microbiol Lett* 76:197-202.

Okusu, H., D. Ma, and H. Nikaido. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* 178: 306-308.

Olliver, A., M. Valle, E. Chaslus-Dancla, and A. Cloeckaert. 2005. Overexpression of the multidrug efflux operon acrEF by insertional activation with IS1 or IS10 elements in *Salmonella enterica* serovar typhimurium DT204 acrB mutants selected with fluoroquinolones. *Antimicrob Agents Chemother* 49:289-301.

Olliver, A., M. Valle, E. Chaslus-Dancla, and A. Cloeckaert. 2004. Role of an acrR mutation in multidrug resistance of in vitro-selected fluoroquinolone-resistant mutants of *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* 238:267-272.

Orth, P., F. Cordes, D. Schnappinger, W. Hillen, W. Saenger, and W. Hinrichs. 1998. Conformational changes of the Tet repressor induced by tetracycline trapping. J Mol Biol 279:439-447.

Orth, P., D. Schnappinger, W. Hillen, W. Saenger, and W. Hinrichs. 2000. Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nat Struct Biol* 7: 215-219.

Palleroni, N. J. 1984. Genus I. *Pseudomonas* Migula 1894. In R. G. Krieg and J. G. Holt (eds), *The Bergey´s Manual of Systematic Bacteriology*, 141-199. The Willians & Wilkins Co., Baltimore.

Palleroni, N. J. 1986. Taxonomy of *Pseudomonas*. In "The Bacteria". Vol. X. Sokatch, J. R. (ed.). Academic Press. London. 3-25.

Palleroni, N. J., and E. Moore. 2004. Taxonomy of *Pseudomonas*. Chapter 1 in *The Pseudomonads*. vol. I.

Pao, S. S., I. T. Paulsen, and M. H. Saier Jr. 1998. Major facilitator superfamily. Microbiol Mol Biol Rev 62:1-34.

Parales RE, Haddock JD. 2004. Biocatalytic degradation of pollutants. Curr Opin Biotechnol. 15 (4): 374-379.

Paulsen, I. T., M. H. Brown, and R. A. Skurray. 1996b. Proton-dependent multidrug efflux systems. *Microbiol Rev* 60:575-608.

Paulsen, I. T. and M. H. Saier Jr. 1997. A novel family of ubiquitous heavy metal ion transport proteins. *J Membr Biol* 156:99-103.

Paulsen I.T., Press C.M., Ravel J., Kobayashi D.Y., Myers G.S., Mavrodi D.V., Deboy R.T., Seshadri R., Ren Q., Madupu R., Dodson R.J., Durkin A.S., Brinkac L.M., Daugherty S.C., Sullivan S.A., Rosovitz M.J., Gwinn M.L., Zhou L., Schneider D.J., Cartinhour S.W., Nelson W.C., Weidman J., Watkins K., Tran K., Khouri H., Pierson E.A., Pierson L.S., Thomashow L.S., Loper J.E. 2006. Corrigendum: Complete genome sequence of the plant comensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol.* 23: 873-878.

Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier Jr, R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius. 1996a. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. *Mol Microbiol* 19:1167-1175.

Pearson, J. P., C. Van Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* 181:1203-1210.

Pinkart, H. C. and D. C. White. 1997. Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. *J Bacteriol* 179:4219-4226.

Pinkart, H. C. J., W. Wolfram, R. Rogers and D. C. White. 1996. Cell envelope changes in solvent-tolerant and solvent sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. *Appl. Environ. Microbiol.* 62: 1129-1132.

Phoenix, P., A. Keane, A. Patel, H. Bergeron, S. Ghoshal, and P. C. K. Lau. 2003. Characterization of a new solvent-responsive locus in *Pseudomonas putida* F1 and its functionalization as a versatile biosensor. *Environ. Microbiol.* 12:1309-27.

Poole, K. 2003. Efflux pumps, pp. 635-674 In J. L. Ramos [ed.], Pseudomonas. Kluwer Academic/Plenum Publishers, New York.

Poole, K. 2001. Multidrug resistance in Gram-negative bacteria. *Curr Opin Microbiol* 4:500-508.

Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J. Yamagishi, X. Z. Li, and T. Nishino. 1996a. Overexpression of the mexC-mexD-oprJ efflux operon in nfxB type multidrug-resistant strains of *Pseudomonas aeruginosa*. *Mol Microbiol* 21:713-724.

Poole, K., and R. Srikumar. 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr. Top. Med. Chem.* 1: 59-71.

Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco. 1996b. Expression of the multidrug resistance operon mexA-mexB-oprM in *Pseudomonas aeruginosa*: mexR encodes a regulator of operon expression. *Antimicrob Agents Chemother* 40: 2021-2028.

Pos, K. M. and K. Diederichs. 2002. Purification, crystallization and preliminary diffraction studies of AcrB, an inner-membrane multi-drug efflux protein. Acta Crystallogr D Biol Crystallogr 58:1865-1867.

Popp, R., Kohl, T., Patz, P., Trautwein, G., Gerischer, U. & Differential, D. N. A. 2002. Binding of transcriptional regulator PcaU from *Acinetobacter* sp. strain ADP1. *J. Bacteriol.* 184, 1988–1997.

Rahmati, S., S. Yang, A. L. Davidson, and E. L. Zechiedrich. 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. *Mol Microbiol* 43: 677-685.

Ramos, J. L., E. Diaz, D. Dowling, V. de Lorenzo, S. Molin, F. O´Gara, C. Ramos, and K. N. Timmis. 1994. The behavior of bacteria designed for biodegradation. Biotechnology (N Y).12:1349-1356.

Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-Gonzalez, A. Rojas, W. Terán, and A. Segura. 2002. Mechanisms of Solvent Tolerance in Gram-Negative Bacteria. *Annu Rev Microbiol.* 56: 743-768

Ramos, J. L., E. Duque, P. Godoy, and A. Segura. 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT- T1E. *J Bacteriol* 180:3323-3329.

Ramos, J. L., E. Duque, M. J. Huertas, and A. Haidour. 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J Bacteriol* 177:3911-3916.

Ramos, J. L., E. Duque, J. J. Rodriguez-Herva, P. Godoy, A. Haidour, F. Reyes, and A. Fernandez-Barrero. 1997. Mechanisms for solvent tolerance in bacteria. *J Biol Chem* 272: 3887-3890.

Ramos, J. L., M. Martinez-Bueno, A. J. Molina-Henares, W. Terán, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes. 2005. The TetR Family of Transcriptional Repressors. *Microbiol Mol Biol Rev* 69: 326-356.

Ramos, J. L., y F. Rojo. 1990. Biodegradación e ingeniería genética. *Investigación y Ciencia*. 164: 72-79.

Ramos-Gonzalez M.I., Ben-Bassat A., Campos M.J., Ramos J.L. 2003. Genetic engineering of a highly solvent-tolerant Pseudomonas putida strain for biotransformation of toluene to p-hydroxybenzoate. *Appl Environ Microbiol.* 69: 5120-5127.

Rand, J. D., S. G. Danby, D. L. Greenway, and R. R. England. 2002. Increased expression of the multidrug efflux genes acrAB occurs during slow growth of *Escherichia coli*. *FEMS Microbiol Lett* 207:91-95.

Rauha, J. P., S. Remes, M. Heinonen, A. Hopia, M. Kahkonen, T. Kujala, K. Pihlaja, H. Vuorela, and P. Vuorela. 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int J Food Microbiol* 56: 3-12.

Rhee, S., R. G. Martin, J. L. Rosner, and D. R. Davies. 1998. A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator. *Proc. Natl. Acad. Sci.* USA 95:10413–10418.

Reverchon, S., W. Nasser, and J. Robert-Baudouy. 1991. Characterization of *kdgR*, a gene of *Erwinia chrysanthemi* that regulates pectin degradation. *Mol. Microbiol.* 5: 2203-2216.

Rojas, A., E. Duque, A. Schmid, A. Hurtado, J. L. Ramos, and A. Segura. 2004. Biotransformation in double-phase systems: physiological responses of *Pseudomonas putida* DOT-T1E to a double phase made of aliphatic alcohols and biosynthesis of substituted catechols. Appl Environ Microbiol 70:3637-3643.

Rojas, A., E. Duque, G. Mosqueda, G. Golden, A. Hurtado, J. L. Ramos, and A. Segura. 2001. Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. *J Bacteriol* 183: 3967-3973.

Rojas, A., A. Segura, M. E. Guazzaroni, W. Terán, A. Hurtado, M. T. Gallegos, and J. L. Ramos. 2003. In vivo and in vitro evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. *J Bacteriol* 185:4755-4763.

Rojo, F. 2001. Mechanisms of transcriptional repression. *Curr Opin Microbiol* 4:145-151.

Romero-Steiner, S., R. E. Parales, C. S. Harwood, and J. E. Houghton. 1994. Characterization of the *pcaR* regulatory gene from *Pseudomonas putida*, which is required for the complete degradation of *p*-hydroxybenzoate. J. Bacteriol. 176: 5771-5779.

Rosenfeld, N., M. B. Elowitz, and U. Alon. 2002. Negative autoregulation speeds the response times of transcription networks. *J Mol Biol* 323: 785-793.

Russell, A. D. 2002a. Antibiotic and biocide resistance in bacteria: comments and conclusions. Symp Ser Soc Appl Microbiol. 171-173.

Russell, A. D. 2002b. Mechanisms of antimicrobial action of antiseptics and disinfectants: an increasingly important area of investigation. *J Antimicrob Chemother* 49: 597-599.

Saier, M. H. Jr, J. T. Beatty, A. Goffeau, K. T. Harley, W. H. Heijne, S. C. Huang, D. L. Jack, P. S. Jahn, K. Lew, J. Liu, S. S. Pao, I. T. Paulsen, T. T. Tseng, and P. S. Virk. 1999. The major facilitator superfamily. *J Mol Microbiol* Biotechnol 1: 257-279.

Saier, M. H. Jr, R. Tam, A. Reizer, and J. Reizer. 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol Microbiol* 11:841-847.

Sanchez, P., A. Alonso, and J. L. Martinez. 2002a. Cloning and characterization of SmeT, a repressor of the *Stenotrophomonas maltophilia* multidrug efflux pump SmeDEF. *Antimicrob Agents Chemother* 46: 3386-3393.

Sanchez, P., A. Alonso, and J. L. Martinez. 2004. Regulatory regions of smeDEF in *Stenotrophomonas maltophilia* strains expressing different amounts of the multidrug efflux pump SmeDEF. *Antimicrob Agents Chemother* 48: 2274-2276.

Sanchez, P., F. Rojo, and J. L. Martinez. 2002b. Transcriptional regulation of mexR, the repressor of *Pseudomonas aeruginosa* mexAB-oprM multidrug efflux pump. *FEMS Microbiol Lett* 207: 63-68.

Savageau, M. A. 1974. Comparison of classical and autogenous systems of regulation in inducible operons. *Nature* 252: 546-549.

Saurin, W., M. Hofnung, and E. Dassa. 1999. Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. *J Mol Evol* 48: 22-41.

Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. *Ann. Rev. Microbiol.* 47: 597-620.

Schneiders, T., S. G. Amyes, and S. B. Levy. 2003. Role of AcrR and ramA in fluoroquinolone resistance in clinical *Klebsiella pneumoniae* isolates from Singapore. *Antimicrob Agents Chemother* 47: 2831-2837.

Schuldiner, S., D. Granot, S. Steiner, S. Ninio, D. Rotem, M. Soskin, and H. Yerushalmi. 2001. Precious things come in little packages. *J Mol Microbiol* Biotechnol 3:155-162.

Schumacher, M. A. and R. G. Brennan. 2002. Structural mechanisms of multidrug recognition and regulation by bacterial multidrug transcription factors. *Mol Microbiol* 45: 885-893.

Schumacher, M. A., M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, and R. G. Brennan. 2002. Structural basis for cooperative DNA binding by two dimers of the multidrug-binding protein QacR. *EMBO J* 21:1210-1218.

Schumacher, M. A., M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, and R. G. Brennan. 2001. Structural mechanisms of QacR induction and multidrug recognition. *Science* 294: 2158-2163.

Schuster, M., A. C. Hawkins, C. S. Harwood, and E. P. Greenberg. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol.* 51:973-985.

Segura, A., A. Rojas, A. Hurtado, M. J. Huertas, and J. L. Ramos. 2003. Comparative genomic analysis of solvent extrusion pumps in *Pseudomonas* strains exhibiting different degrees of solvent tolerance. *Extremophiles* 7:371-376.

Segura, A. H., Heipieper., W. Terán, M. E. Guazzaroni, A. Rojas, E. Duque, M. T. Gallegos and J. L. Ramos. 2003. Enzymatic activation of the *cis-trans* isomerase and transcriptional regulation of efflux pumps in solvent tolerance in *Pseudomonas putida*, pp. 479-508 In Ramos J.L. [ed.], *Pseudomonas*. Kluwer Academic/Plenum Publishers, London, United Kingdom.

Shafer, W. M., W. L. Veal, E. H. Lee, L. Zarantonelli, J. T. Balthazar, and C. Rouquette. 2001. Genetic organization and regulation of antimicrobial efflux systems possessed by *Neisseria gonorrhoeae* and *Neisseria meningitidis. J. Mol. Microbiol. Biotechnol.* 3:219–224.

Shiba, T., K. Ishiguro, H. Takemoto, H. Koibuchi, and K. Sugimoto. 1995. Purification and characterization of *Pseudomonas aeruginosa* NfxB protein, the negative regulator of the *nfxB* gene. *J. Bacteriol.* 177: 5872-5877.

Shoda, M . 2000. Bacterial control of plant diseases. *J Biosci Bioeng*. 89: 515-521.

Sikkema, J., J. A. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* 59: 201-222.

Sikkema, J., B. Poolman, W. N. Konings, and J. A. M. de Bont. 1992. Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. *J. Bacteriol*. 174: 2986-2992.

Song, Y., Tong, Z., Wang, J., Guo, Z., Han, Y., Zhang, J., Pei, D., Zhou, D., Qin, H., Pang, X., Han, Y., Zhai, J., Li, M., Cui, B., Qi, Z., Jin, L., Dai, R., Chen, F., Yang, R. 2004. Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. *DNA Res*. 11:179-197.

Speer, B. S., N. B. Shoemaker, and A. A. Salyers. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clin. Microbiol. Rev*. 5:387–399.

Stainer, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol*. 43:159-271.

Stasinopoulos, S. J., G. A. Farr, and D. H. Bechhofer. 1998. *Bacillus subtilis tetA*(L) gene expression: evidence for regulation by translational reinitiation. *Mol. Microbiol*. 30:923–932.

Sulavik, M. C., L. F. Gambino, and P. F. Miller. 1995. The MarR repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*: prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. *Mol. Med*. 1:436–446.

Sunnarborg, A., Klumpp, D., Chung, T., and LaPorte, D.C. 1990. Regulation of the glyoxylate bypass operon: Cloning and characterization of IclR. *J. Bacteriol*. 172, 2642-2649.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406:959-964.

Suutari, M. and S. Laakso. 1994. Microbial fatty acids and thermal adaptation. Crit Rev Microbiol 20:285-328.

Tanaka, T., T. Horii, K. Shibayama, K. Sato, S. Ohsuka, Y. Arakawa, K. Yamaki, K. Takagi, and M. Ohta. 1997. RobA-induced multiple antibiotic resistance largely depends on the activation of the AcrAB efflux. *Microbiol Immunol* 41:697-702.

Terán, W. 2005. Regulación de la expresión del transportador TtgABC de *Pseudomonas putida* DOT-T1E. Caracterización bioquímica de su regulador TtgR. Tesis Doctoral. Universidad de Granada.

Terán, W., Felipe, A., Guazzaroni, M.-E., Krell, T., Ruiz, R., Ramos, J. L., and Gallegos, M.-T. 2008. Crossregulation of TtgDEF and TtgGHI solvent efflux pumps of *Pseudomonas putida* DOT-T1E. *Mol. Microbiol*. En prensa.

Terán W., Felipe A., Segura A., Rojas A., Ramos J.L. and Gallegos M.T. 2003. Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. Antimicrob. Agents Chemother. *47*, 3067-3072.

Terán W., Krell T., Ramos J.L., Gallegos M.T. 2006. Effector-repressor interactions, binding of a single effector molecule to the operator-bound TtgR homodimer mediates derepression. *J Biol Chem*. 17: 7102-7109.

Timmis, K. N., F. Rojo, and J. L. Ramos. 1988. Prospects for laboratory engineering of bacteria to degrade pollutants. Basic Life Sci 45:61-79.

Thomson, N.R., Nasser, W., McGowan, S., Sebaihia, M., and Salmond, G.P. 1999. *Erwinia carotovora* has two KdgR-like proteins belonging to the IclR family of transcriptional regulator: Identification and characterization of the RexZ activator and the KdgR repressor of pathogenesis. *Microbiology* 145, 1531-1545.

Torres, B., Porras, G., García, J.L., and Díaz, E. 2003. Regulation of the *mhp* cluster responsible for 3-(3-hydroxyphenyl)propionic acid degradation in *Escherichia coli*. *J. Biol. Chem*. 278, 27575-27585.

Travers, A., R. Schneider, and G. Muskhelishvili. 2001. DNA supercoiling and transcription in *Escherichia coli*: The FIS connection. Biochimie 83:213-217.

Tseng, T. T., K. S. Gratwick, J. Kollman, D. Park, D. H. Nies, A. Goffeau, and M. H. Saier Jr. 1999. The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. *J Mol Microbiol* Biotechnol 1:107-125.

Typas A., Becker G., Hengge R. 2007. The molecular basis of selective promoter activation by the sigma subunit of RNA polymerase. *Mol Microbiol*. 63: 1296-1306.

van Veen, H. W., C. F. Higgins, and W. N. Konings. 2001. Molecular basis of multidrug transport by ATP-binding cassette transporters: a proposed two-cylinder engine model. *J Mol Microbiol* Biotechnol 3:185-192.

van Veen, H. W. and W. N. Konings. 1997. Drug efflux proteins in multidrug resistant bacteria. *Biol Chem* 378:769-77.

van Wezel, G.P., van der Meulen, J., Kawamoto, S., Luiten, R.G., Koerten, H.K., and Kraal, B. 2000. *ssgA* Is essential for sporulation of *Streptomyces coelicolor* A3(2) and affects hyphal development by stimulating septum formation. *J. Bacteriol*. 182: 5653-5662.

Vodovar N., D. Vallenet, S. Cruveiller, Z. Rouy, V. Barbe, C. Acosta, L. Cattolico, C. Jubin, A. Lajus, B. Segurens, B. Vacherie, P. Wincker, J. Weissenbach, B. Lemaitre, C. Médigue and F. Boccard. 2006. Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. *Nature Biotechnology*. 24: 673-679.

Walker J.R., Altamentova S., Ezersky A., Lorca G., Skarina T., Kudritska M., Ball L.J., Bochkarev A. and Savchenko A. 2006. Structural and Biochemical Study of Effector Molecule Recognition by the *E.coli* Glyoxylate and Allantoin Utilization Regulatory Protein AllR. *J. Mol. Biol*. 358: 810-828.

Walsh, U.F., Morrissey and J.P., O'Gara, F. 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr Opin Biotechnol* 12: 289–295

Wang, H., J. L. Dzink-Fox, M. Chen, and S. B. Levy. 2001. Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of acrR mutations. *Antimicrob Agents Chemother* 45:1515-1521.

Weber, F. J. and J. A. de Bont. 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim Biophys Acta* 1286: 225-245.

Weber, F. J., S. Isken, and J. A. de Bont. 1994. Cis/trans isomerization of fatty acids as a defence mechanism of *Pseudomonas putida* strains to toxic concentrations of toluene. *Microbiology* 140: 2013-2017.

Weickert, M.J., and Adhya, S. 1992. A family of bacterial regulators homologous to Gal and Lac repressors. *J. Biol. Chem*. 267: 15869-15874.

Wery, J., B. Hidayat, J. Kieboom, and J. A. de Bont. 2001. An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. *J Biol Chem* 276: 5700-5706.

Westbrock-Wadman, S., D. R. Sherman, M. J. Hickey, S. N. Coulter, Y. Q. Zhu, P. Warrener, L. Y. Nguyen, R. M. Shawar, K. R. Folger, C. K. Stover. 1999. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrob. Agents. Chemother.* 43: 2975-2983.

White, D. G., J. D. Goldman, B. Demple, and S. B. Levy. 1997. Role of the acrAB locus in organic solvent tolerance mediated by expression of marA, soxS, or robA in *Escherichia coli*. *J Bacteriol* 179: 6122-6126.

Witte, W. 2000a. Ecological impact of antibiotic use in animals on different complex microflora: environment. *Int J Antimicrob Agents* 14: 321-325.

Witte, W. 2000b. Selective pressure by antibiotic use in livestock. *Int J Antimicrob Agents* 16 Suppl 1:S19-24.

Woese, C. R., W. G. Weisurg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt. 1985. The phylogeny of purple bacteria: the gamma subdivision. *Syst. Appl. Microbiol.* 6: 25-33.

Woolridge D.P., Vazquez-Laslop N., Markham P.N., Chevalier M.S., Gerner E.W., Neyfakh A.A. 1997. Efflux of the natural polyamine spermidine facilitated by the Bacillus subtilis multidrug transporter Blt. *J Biol Chem*. 272: 8864-8866.

Xiong, A., A. Gottman, C. Park, M. Baetens, S. Pandza, and A. Matin. 2000. The EmrR protein represses the *Escherichia coli* emrRAB multidrug resistance operon by directly binding to its promoter region. *Antimicrob Agents Chemother* 44: 2905-2907.

Yamamoto, K., and Ishihama, A. 2003. Two different modes of transcription repression of the *Escherichia coli* acetate operon by IclR. *Mol. Microbiol.* 47, 183-194.

Zhang, R.G., Kim, Y., Skarina, T., Beasley, S., Laskowski, R., Arrowsmith, C. 2002. Crystal structure of *Thermotoga maritima* 0065, a member of the IclR transcriptional factor family. *J. Biol. Chem.* 277, 19183-19190.

Zhang, L., Li X. Z, and K. Poole. 2001. Fluoroquinolone susceptibilities of efflux-mediated multidrug-resistant *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*. *J. Antimicrob. Chemother.*48: 549-552.

Zhang, H.-B., Wang, C., and Zhang, L.-H. 2004. The quormone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp. *Mol. Microbiol.* 52, 1389-1401.

Zheleznova, E. E., P. N. Markham, A. A. Neyfakh, and R. G. Brennan. 1997. Preliminary structural studies on the multi-ligand-binding domain of the transcription activator, BmrR, from Bacillus subtilis. *Protein Sci* 6: 2465-2468.

Zheleznova, E. E., P. N. Markham, A. A. Neyfakh, and R. G. Brennan. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. *Cell* 96: 353-362.

Zheng H, Skarina T, Chruszcz M, Cymborowski M, Grabowski M, Onopriyenko O, Savchenko A, Edwards A, Minor W. 2006. A Structure Of Putative Catechol Degradative Operon Regulator From *Rhodococcus Sp.* Rha1. Midwest Center, Structural Genomics (Mcsg).

Ziha-Zarifi, I., C. Llanes, T. Kohler, J. C. Pechere, and P. Plesiat. 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. *Antimicrob Agents Chemother* 43: 287-291.