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TRANSPORTADORES INVOLUCRADOS EN LA TOLERANCIA A DISOLVENTES ORGÁNICOS EN *Pseudomonas putida* DOT-T1E

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TRANSPORTADORES INVOLUCRADOS EN LA TOLERANCIA A DISOLVENTES ORGÁNICOS EN *Pseudomonas putida* DOT-T1E

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A la memoria de mi Padre

A mi Madre

A mis Hermanas

"… Y así después de esperar tanto, un día como cualquier otro decidí triunfar…
decidí no esperar a las oportunidades sino yo mismo buscarlas,
decidí ver cada problema como la oportunidad de encontrar una solución,
decidí ver cada desierto como la oportunidad de encontrar un oasis,
decidí ver cada noche como un misterio a resolver,
decidí ver cada día como una nueva oportunidad de ser feliz.

Aquel día descubrí que mi único rival no eran más que mis propias debilidades,
y que en éstas, está la única y mejor forma de superarnos.
Aquel día dejé de temer a perder y empecé a temer a no ganar,
descubrí que no era yo el mejor y que quizás nunca lo fui.
Me dejó de importar quién ganara o perdiera;
ahora me importa simplemente saberme mejor que ayer.

Aprendí que lo difícil no es llegar a la cima,
sino jamás dejar de subir.
Aprendí que el mejor triunfo que puedo tener,
es tener el derecho de llamar a alguien "Amigo".

Descubrí que el amor es más que un simple estado de enamoramiento,
"el amor es una filosofía de vida".
Aquel día dejé de ser un reflejo de mis escasos triunfos pasados
y empecé a ser mi propia tenue luz de este presente;
aprendí que de nada sirve ser luz
si no vas a iluminar el camino de los demás.

Aquel día decidí cambiar tantas cosas…
Aquel día aprendí que los sueños son solamente para hacerse realidad.
Desde aquel día ya no duermo para descansar…
ahora simplemente duermo para soñar."

Walt Disney

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ABREVIATURAS

(En esta sección sólo se comentan las abreviaturas de mayor uso en esta Tesis Doctoral, las demás están explicadas a lo largo del texto)

ABC	familia de casetes de unión a ATP (del inglés, *ATP-Binding Cassette*)
ATP	trifosfato de adenosina (del inglés, *Adenosin tri-phosphate*)
bcr	gen de resistencia a Biciclomicina (del inglés, *Bicyclomicin resistance*)
CFU	Unidades formadoras de colonias
CIM	Concentración inhibitoria mínima
COV's	Compuestos Orgánicos Volátiles
Cti	*Cis – trans* Isomerasa
***cyo*B**	gen de la Citocromo Ubiquinol Oxidasa
Log Pow	Logaritmo del coeficiente de partición en una mezcla de octanol-agua
LPS	Lipopolisacáridos
MATE	Familia de extrusión de múltiples antibióticos y compuestos tóxicos (del inglés, *multidrug and toxic compound extrusion*)
MDR	Transportadores de resistencia a múltiples drogas (del inglés, *multidrug resistance*)
MFP	Proteína de fusión de membrana (del inglés, *Membrane Fusion Protein*)
MFS	Superfamilia de facilitadores mayores (del inglés, *Major Facilitator Superfamily*)
OMP	Proteína de membrana externa (del inglés, *Outer Membrane Protein*)
RND	Familia de resistencia-nodulación-división celular (del inglés, *Resistance-Nodulation-cell Division*)
SBP	Proteína de unión al sustrato (del inglés, *Substrate binding protein*)
SMR	Familia de proteínas de bajo peso molecular que confieren resistencia a múltiples compuestos (del inglés, *Small Multidrug Resistance*)
STM	Segmentos Transmembranas
Ttg	Gen de tolerancia a tolueno (del inglés, *Toluene tolerance gene*)

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ÍNTRODUCCIÓN

I.) Importancia de estudiar microorganismos degradadores y/o resistentes a disolventes orgánicos

Desde la revolución industrial, la producción y el uso de productos químicos se ha incrementado enormemente, y como consecuencia de ello, se han producido todo tipo de residuos tóxicos, muchos de los cuales han sido liberados al medio ambiente. Algunos de estos productos se utilizan como insecticidas y pesticidas para el control de plagas y maleza mientras que otros, como los disolventes orgánicos y combustibles, alcanzan la biosfera como resultado de escapes accidentales durante su producción o almacenamiento (Ramos *et al.*, 2002; Ramos *et al*., 1994).

A medida que se ha ido disponiendo de mayores conocimientos sobre el efecto tóxico o carcinogénico de muchos de estos productos químicos, su liberación al medio ambiente se ha restringido por la legislación. No obstante, un buen número de contaminantes ya han alcanzado la biosfera y otros lo harán accidentalmente y por tanto sigue existiendo una necesidad real de disponer de medios que permitan su eliminación.

El uso de tratamientos biológicos para la eliminación de productos tóxicos es una opción prometedora. Para la utilización de microorganismos en la eliminación de contaminantes es necesario disponer de una gran diversidad de bacterias con propiedades degradativas que puedan ser utilizadas para la mineralización de diferentes compuestos. Además, en algunos casos la toxicidad de algunos productos químicos puede limitar la aplicación de microorganismos en la remoción de éstos (Ramos *et al*., 2002; Segura *et al*., 1999).

Los Compuestos Orgánicos Volátiles (COVs) son principalmente hidrocarburos derivados del petróleo, que exhiben en su mayoría una elevada inflamabilidad, toxicidad, carácter mutagénico y cancerígeno, lo cual los convierte en residuos peligrosos (Directivas 91/689/CE y 96/61/CE). Los vertidos, emisiones y escapes accidentales de COVs suponen un serio perjuicio para el ecosistema natural. La generación de residuos, efluentes líquidos y emisiones gaseosas procedentes de actividades industriales en sectores como el petroquímico, refino de petróleo, pintura, procesado de gomas y plásticos, textil, madera, acabado de materiales, semi-conductores, entre otros, se encuentran entre los principales focos emisores de COVs (Cárdenas *et al*. 2003; Yeom y Daugulis, 2001). Así la cantidad de COVs liberada a la atmósfera en España en 2005 fue de 1.300.000 Kg./año. Aunque muchas industrias ya han adaptado sus emisiones a la normativa vigente (Directiva 1999/13/EC, Real Decreto 117/2003) que persigue la prevención y reducción de los efectos directos e indirectos de sus emisiones, para poder cumplir con la normativa las empresas tienen que modernizar sus instalaciones adaptándolas a las nuevas tecnologías que permitan la

eliminación de estos contaminantes de forma más eficaz y a precios asequibles. Una alternativa al uso de métodos físico-químicos se basa en el uso de microorganismos con capacidad degradativa para este tipo de contaminantes. Estos procesos de biodegradación, aparte de requerir menores costes de inversión y operación, son medioambientalmente más amigables puesto que se basan en la mineralización del contaminante por la comunidad de microorganismos degradadores presentes en el biorreactor (bacterias, hongos, levaduras, etc.) (Arriaga *et a*l. 2005; Koutinas *et al*. 2005, Ortiz *et al*., 2006). No obstante, muchos de los COVs (por ejemplo el tolueno) tienen una elevada toxicidad lo que hace que los biofiltros desarrollados para la degradación de estos compuestos tengan limitaciones, sobre todo en cuanto a la estabilidad de proceso (Villaverde *et al*. 1997; Acuña *et al*. 1999; 2002; Song y Kinney, 2000; 2005). Por consiguiente, el desarrollo de nuevos procesos y estrategias de operación que eliminen o reduzcan estas limitaciones es crucial para aumentar la capacidad de carga y mejorar la estabilidad del proceso (Song y Kinney; 2005). Por tanto, la toxicidad de los disolventes orgánicos es uno de los factores limitantes tanto en la prevención de la contaminación como cuando se pretende llevar a cabo la biorremediación de zonas altamente contaminadas, y la utilización eficaz de microorganismos tolerantes a disolventes orgánicos una posible solución a estos problemas.

Los microorganismos tolerantes a altas concentraciones de disolvente orgánico también son interesantes desde el punto de vista comercial. La síntesis biológica de compuestos de alto valor añadido muchas veces no es rentable dado que en el proceso se utilizan sustratos altamente tóxicos, que en contacto con el biocatalizador lo desestabilizan haciendo que el rendimiento del proceso sea muy bajo. En otras ocasiones es el producto final el que es tóxico, de forma que por encima de una determinada concentración el proceso se ralentiza ó incluso el biorreactor se colapsa. Una forma de aumentar el rendimiento en este proceso es utilizar un reactor de doble fase consistente en una fase acuosa y otra orgánica en la que se disuelve preferentemente el sustrato/producto tóxico evitando así un mayor contacto con el biocatalizador que está en la fase acuosa (Daugulis *et al*., 1988; Rojas *et al*., 2004), consiguiendo así mayores rendimientos.

Por todos estos motivos a finales de los años 80, principios de los 90, se comenzó la búsqueda de bacterias capaces de crecer en presencia de una segunda fase de disolvente orgánico (el más utilizado como compuesto modelo es el tolueno). Como resultado de estos esfuerzos, se lograron aislar una serie de bacterias tolerantes al tolueno (Inoue y Horikoshi, 1989; Weber *et al*, 1994; Ramos *et al*., 1995; Kim *et al*., 1998). La toxicidad de los disolventes orgánicos, en general, se correlaciona con su log P_{ow}, que es el valor del

logaritmo del coeficiente de partición del disolvente en una mezcla definida de octanol y agua (log P_{ow}); dicho valor se usa comúnmente como medida de la lipofilicidad de un disolvente (Rekker y Kort, 1979). Los disolventes orgánicos con un valor de log P_{ow} entre 1 y 4 son extremadamente tóxicos para los microorganismos (Rekker y Kort, 1979), por ejemplo, benceno (log P_{ow} 2,0), estireno (log P_{ow} 3,6), xileno (log P_{ow} 3,2) y tolueno (log P_{ow} 2,5), porque se acumulan en la membrana de las bacterias modificando la estructura de la misma. Sin embargo, el efecto tóxico de los disolventes no sólo depende de la toxicidad inherente del compuesto sino también de la tolerancia intrínseca de las especies bacterianas. Por ejemplo, se han identificado algunas cepas de *E. coli* como tolerantes a ciclohexano (logP_{ow} 3,2) (Aono *et al*., 1991) pero no a tolueno (logP_{ow} 2,4). En este contexto, no es de extrañar que todas las cepas Gram negativas identificadas hasta el momento como altamente resistentes a disolventes orgánicos pertenezcan al género *Pseudomonas*. Una de estas cepas es *Pseudomonas putida* DOT-T1E, la cepa que se ha utilizado en este estudio de tesis doctoral, que es capaz de crecer hasta en el 90% (v/v) de tolueno (Ramos *et al*., 1995). Esta cepa se aisló de la planta de tratamiento de aguas residuales del puente de los Vados en Granada (Ramos *et al*., 1995) y no sólo es tolerante a tolueno, sino que también puede utilizarlo como fuente de carbono y energía a través de la ruta de la tolueno dioxigenasa (ruta TOD) (Gibson *et al*., 1970; Mosqueda *et al*., 1999).

II.) Biología de *Pseudomonas*

El género *Pseudomonas* fue descrito por primera vez por Migula (1894) en un simple párrafo de dos líneas cuya traducción sería: "Células con órganos polares para su movilidad. Algunas especies forman esporas, aunque en general es un evento raro (por ejemplo, *Pseudomonas violacea*)". Esta definición tan poco específica fue generalmente aceptada y algunas bacterias, que habían sido asignadas a otros géneros, fueron rebautizadas en años posteriores como pertenecientes al género *Pseudomonas.*

A principios del siglo XX los microbiólogos, en particular la escuela de Delft, establecieron que las cepas de *Pseudomonas* eran muy comunes en hábitats naturales, particularmente en suelo, agua, alimentos y plantas enfermas. La ubicuidad de estos microorganismos y la capacidad para crecer en medios de cultivo muy simples hicieron que se considerasen a las bacterias de este género como protagonistas en el proceso de mineralización de materia orgánica en la naturaleza, un papel que fue claramente demostrado por den Dooren de Jong (1926).

En 1986 Palleroni, en base a análisis de hibridación ADN-ADN, propuso cinco grupos taxonómicos (ARN-I a ARN-V) dentro del género *Pseudomonas* (Palleroni *et al*., 1973; Palleroni, 1986). Posteriormente la secuenciación de los ARNr 16S reflejó la diversidad entre grupos y el grupo denominado ARN-I dentro la subclase-γ de *Proteobacteria* (De Vos *et al*., 1989; Woese *et al*., 1985), que fue reorganizada como la clase "G*ammaproteobacteria*" se considera hoy en día como verdaderas *Pseudomonas* (Krieg y Garrity, 2001).

La actual definición de *Pseudomonas* que aparece en la edición del Manual de Bacteriología Sistemática de Bergey define este género como: "células en forma de bastón, curvadas o derechas, pero no helicoidales, de entre 0,5-1,0 μm de diámetro por 1,5-5,0 μm de longitud. La mayoría de las especies no acumula gránulos de poli-β-hidroxibutirato, pero se pueden formar poli-hidroxialcanoatos cuya longitud es mayor de cuatro carbonos cuando las células se cultivan en alcanos o gluconato. No se conocen estados de células viables pero no cultivables. Son gram-negativas. Son generalmente móviles y presentan uno o varios flagelos polares; aunque se han descrito flagelos laterales más cortos. Son aeróbicas, con un tipo de metabolismo respiratorio estricto con oxígeno como el aceptor terminal de electrones; en algunos casos el nitrato puede ser usado como un aceptor de electrones alternativo, lo que permite en estos casos el crecimiento anaerobio. No producen xantomonadinas. La mayoría, si no todas las especies, no crecen bajo condiciones ácidas (pH ≤ 4,5). La mayoría de las especies no requiere factores de crecimiento orgánicos. Pueden ser oxidasa positiva o negativa, y son catalasa positiva y quimiorganotróficas. Las cepas de las especies de este género incluyen en su composición los ácidos grasos hidroxilados 3-OH 10:0 y 12:0, y 2-OH 12:0, y ubiquinona Q-9. Están ampliamente distribuidas en la naturaleza. Algunas especies son patógenas de humanos, animales o plantas. El porcentaje de contenido en G+C del ADN está entre 58-69%".

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). El espectro nutricional de cada especie es característico y, en general, la variabilidad entre las cepas de una misma especie es menor. En contraste con muchos otros grupos bacterianos, muchas *Pseudomonas* no se pueden distinguir mediante la utilización de azúcares. De hecho, las cepas de la especie *P. palleronii* no utilizan estos compuestos como fuentes de carbono, y algunas cepas de *P. putida* sólo pueden utilizar los azúcares glucosa y

sacarosa. 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. La capacidad de utilizar los compuestos aromáticos es particularmente interesante, dadas las distintas rutas por las cuales *Pseudomonas* es capaz de metabolizarlos, y su posible utilización en procesos de descontaminación y biocatálisis.

La gran versatilidad del género no sólo se deriva de la gran cantidad y variedad de fuentes de carbono que pueden utilizar sino también de la capacidad de explotar diferentes nichos ecológicos. El género *Pseudomonas* incluye cepas patógenas oportunistas de animales y el hombre, como *P. aeruginosa*, patógenos de plantas como *P. syringae*, y cepas que estimulan el crecimiento de plantas actuando como fungicidas, como *P. fluorescens* y cepas de suelo como *P. putida* (Lugtenberg, 1999; Palleroni y Moore, 2004). En los últimos cinco años se ha secuenciado el genoma de las cepas *P. aeruginosa* PAO1 (Stover *et al*., 2000), *P. putida* KT2440 (Nelson *et al.*, 2002), *Pseudomonas enthomophila* (Vodovar *et al*., 2006), *P. syringae* pv. tomatoe DC3000 (http://pseudomonas-syringae.org/) y *P. fluorescens* Pf-5 (http://pseudomonas-fluorescens.org/). Estas bacterias presentan en promedio 5500 genes en sus genomas, la mitad de ellos sin función conocida. Vodovar *et al.* han identificado un grupo de casi 2100 genes que constituyen el núcleo del genoma del género *Pseudomonas*. Los datos de secuenciación sugieren que el número de genes "exclusivos" de *P. putida* KT2440 es mayor que el de genes que tiene en común con otras *Pseudomonas.* Estas regiones no compartidas se asocian a la versatilidad y "fitness" de *P. putida.*

III.) Mecanismos de tolerancia a disolventes orgánicos

La principal función de la membrana celular de los microorganismos es la de formar una barrera permeable, regulando el paso de solutos entre la célula y el medio externo. Las propiedades de la membrana citoplasmática como barrera son de especial importancia para la transducción de energía de la célula y para el mantenimiento de funciones vitales (Ramos *et al*., 2002; Segura *et al*., 1999, Sikkema *et al*., 1995). Los disolventes orgánicos se acumulan preferentemente en las bicapas lipídicas de las membranas, desorganizándolas y provocando la interrupción de las funciones vitales (pérdida de iones, metabolitos, lípidos y proteínas; la disipación del gradiente de pH y del potencial eléctrico). Todo esto conlleva a

la lisis y posterior muerte celular (Sikkema *et al*., 1995; Ramos *et a*l., 2002; Segura *et al*., 1999).

Aunque el metabolismo de los agentes químicos disminuya las concentraciones efectivas del mismo a las que está sometido el microorganismo, no parece que este sea un mecanismo de tolerancia en aquellas bacterias expuestas a altas concentraciones del disolvente orgánico. Por un lado, algunos microorganismos tolerantes a un disolvente orgánico en particular no pueden metabolizarlo, por ejemplo, *P. putida* S12 es tolerante a concentraciones supersaturantes de tolueno pero no utiliza este hidrocarburo como fuente de carbono y por otro lado, un mutante de *Pseudomonas putida* DOT-T1E en el que se ha inactivado la tolueno dioxigenasa, una enzima clave en la ruta de degradación de tolueno en esta cepa, no es más sensible al disolvente que la cepa silvestre (Mosqueda *et al.*, 1999).

En la tolerancia a disolventes orgánicos, se han descrito una serie de mecanismos involucrados en la respuesta a esos agentes tóxicos: rigidificación de la membrana celular, alteración de la composición de los fosfolípidos; alteración de la superficie celular que hace que las células sean menos permeables; expulsión de los compuestos tóxicos en un proceso dependiente de energía; y formación de vesículas que remueven los disolventes desde la superficie celular entre otros. (Ramos *et al*., 2002; Kadurugamuwa y Beveridge, 1995). A continuación se explican en detalle algunos de estos mecanismos.

III.a) Alteración en la composición de fosfolípidos de la membrana debido a la exposición a disolventes orgánicos

Cuando las células bacterianas se exponen a disolventes orgánicos, éstos entran en contacto con la bicapa lipídica y penetran en ella, provocando un aumento en la fluidez de la misma. Inmediatamente en la bacteria se desencadenan una serie de respuestas para disminuir los efectos perjudiciales de este cambio. La fluidez de la membrana es reajustada, alterando la composición de la bicapa lipídica por medio de mecanismos compensatorios que se asemejan a algunos de los observados en respuesta a cambios físicos y químicos impuestos por el medio ambiente (Sikkema *et al*., 1992; Ramos *et al*., 2001)

III.a.i) -Cambios en la composición de ácidos grasos

Existen distintos mecanismos que alteran la composición de ácidos grasos esterificados y por consiguiente la fluidez de la bicapa lipídica bacteriana: la isomerización *cis*/*trans* de

ácidos grasos como una respuesta a muy corto plazo; el cambio en la proporción de ácidos grasos saturados e insaturados, como una respuesta a más largo plazo y el cambio en la proporción de ácidos grasos de cadena corta y cadena larga (Ramos *et al*., 2002).

III.a.i.1. Isomerización *cis* / *trans* de los ácidos grasos insaturados

Los ácidos grasos insaturados son sintetizados mayoritariamente en la configuración *cis* y por tanto estos son los isómeros mayoritarios en las membranas celulares bacterianas. Sin embargo, se han descrito en algunas bacterias Gram negativas (del género *Pseudomonas* y *Vibrio*) la presencia de isómeros *trans* como resultado de una modificación postsintética catalizada por la enzima *cis*-*trans*-isomerasa (Cti).

La conformación estérica de ácidos grasos insaturados *trans* y de los ácidos grasos saturados es muy similar, ambos poseen una conformación extendida lo que permite un empaquetamiento más compacto de las cadenas acilo de los fosfolípidos en la membrana. Por el contrario, la configuración *cis* del doble enlace provoca un ángulo fijo de 30º en la cadena acílica lo que impide un empaquetamiento compacto con los ácidos grasos, provocando una mayor fluidez en las membranas donde están presentes (Figura 1). Precisamente por esta conformación diferente de los ácidos grasos insaturados, la isomerización *cis* / *trans* juega un papel importante en la adaptación de las bacterias frente a cambios de temperatura o presencia de disolventes orgánicos en el medio. Estos estreses ambientales provocan un aumento de la fluidez de membrana, que es compensado mediante la síntesis de isómeros *trans* que contrarrestan el efecto fluidificante del estrés.

Varias cepas de *P. putida* son capaces de cambiar rápidamente su proporción *cis/trans* en la membrana en respuesta a la exposición a disolventes orgánicos, compuestos aromáticos o temperatura; este cambio se observa tan sólo cinco minutos después de la adición del disolvente orgánico en *P. putida* DOT-T1E (Ramos *et al*., 1995). Ésta se considera una respuesta rápida, que no necesita de energía ya que no depende de la síntesis *de novo* de ácidos grasos. De esta manera, las células ganan tiempo para un ajuste metabólico y poner en marcha las respuestas a largo plazo. (Junker y Ramos, 1999; Bernal *et al.,* 2007a). En *P. putida* DOT-T1E esta modificación postsintética ha sido bien caracterizada (Junker y Ramos, 1999). Diversos estudios señalan que el gen *cti* se expresa constitutivamente (Heipieper *et al*., 2003; Bernal *et al*., 2007a) y sin embargo la actividad enzimática se induce en presencia del estrés. La hipótesis más plausible hasta el momento es que tan sólo cuando la membrana tiene una fluidez elevada la enzima Cti puede alcanzar el

doble enlace (sustrato de la reacción) que de otra manera permanece embebido en el interior de la membrana (Heipieper *et al*., 2003).

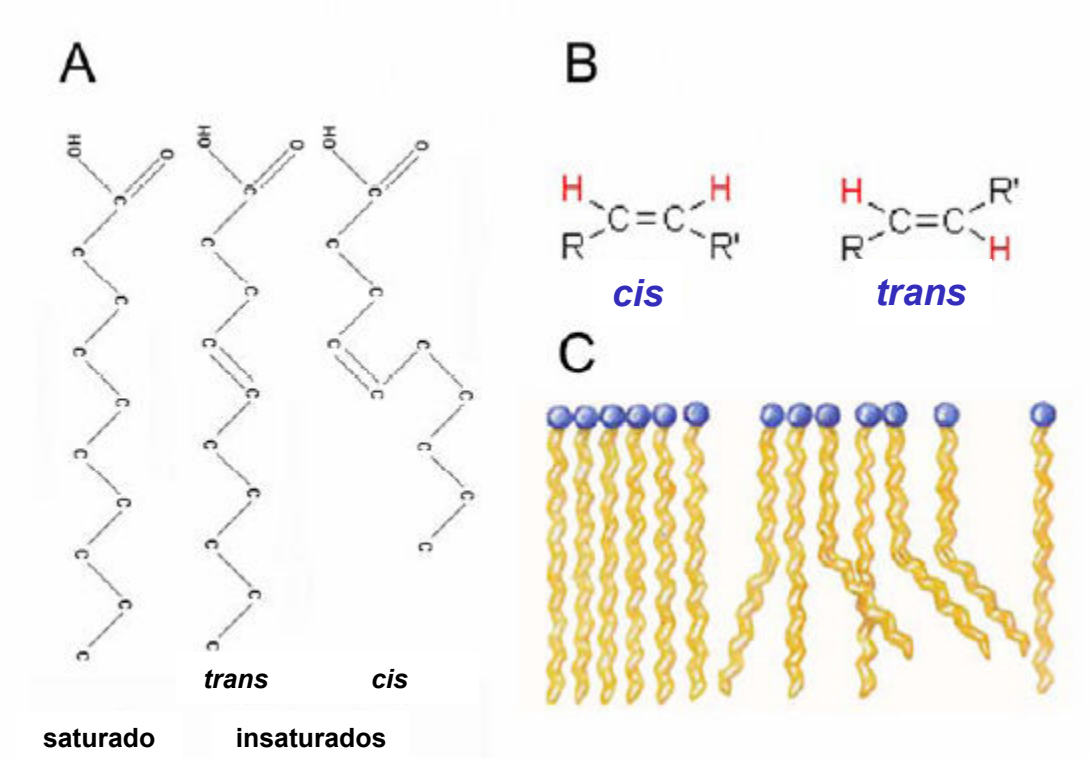

Figura 1. **Estructura de los ácidos grasos insaturados. A:** Estructura y conformación en el espacio de un ácido graso saturado y sus correspondientes isómeros *cis* y *trans*; **B:** conformación espacial de los átomos que forman los dobles enlaces *cis* y *trans*; **C:** Representación de una monocapa lipídica con fosfolípidos saturados e insaturados *cis* y *trans* (Bernal, 2007).

III.a.i.2 . Cambio en la proporción de ácidos grasos saturados e insaturados

En cepas de *E. coli* se ha observado un incremento en fosfolípidos con ácidos grasos saturados, en respuesta a cambios de temperatura y exposición a disolventes, en presencia de alcoholes de cadena larga y compuestos aromáticos (Aono *et al*., 1991).

En la cepa *P. putida* Idaho se observó que tras 15 minutos de exposición a *o*-xileno tenía lugar un aumento en el contenido de ácidos grasos, alcanzando niveles máximos a las 2 horas de exposición al disolvente (Pinkart y White 1997). Esta estrategia que permite regular la fluidez de la membrana depende de la síntesis de *novo* de ácidos grasos, un proceso que lleva cierto tiempo por lo que es considerado un mecanismo de adaptación a largo plazo (Ramos *et al*., 2002). Sin embargo, otras cepas de *Pseudomonas* no alteran su proporción de

ácidos grasos saturados/insaturados en respuesta a disolventes orgánicos como sucede en la cepa *Pseudomonas putida* DOT-T1E, que no varía esta proporción tras su exposición a distintos disolventes orgánicos (Ramos *et al*., 1997).

III.a.i.3 Cambios en la proporción de ácidos grasos de cadena larga y cadena corta.

La razón de ácidos grasos de cadena corta/cadena larga también se puede alterar para regular la fluidez de la membrana (Keweloh y Heipieper, 1996; Heipieper y de Bont, 1994; Heipieper *et al*., 1996; Holtwick *et al*., 1997; Pinkart y White, 1997; Pinkart *et al*., 1996; Ramos *et al*., 1997; Weber y de Bont, 1996). Este es uno de los mecanismos a largo plazo que ponen en marcha *Pseudomonas* spp., *E. coli* y otros microorganismos cuando son expuestos a concentraciones subletales de disolventes orgánicos, dando como resultado un incremento en la cantidad total de fosfolípidos y un aumento en la proporción de lípidos saturados de cadena larga. Todos estos cambios son concomitantes con las alteraciones producidas en los grupos de cabeza de los fosfolípidos. En suma estos cambios modifican la fluidez de la membrana celular con el fin de volverla más rígida (Ramos *et al*, 2002; Segura *et al*., 1999).

III.a.ii Cambio en los grupos de cabeza de los fosfolípidos

Los principales tipos de fosfolípidos en las membranas de *P. putida* son fosfatidiletanolamina (PE), fosfatidilglicerol (PG), y difosfatidilglicerol o cardiolipina (CL). Diferentes cepas de *Pseudomonas* sp. parecen haber desarrollado diferentes estrategias para responder a disolventes orgánicos mediante el cambio en la composición de los grupos de cabeza de los fosfolípidos. Se han analizado los cambios en los fosfolípidos de tres cepas: *P. putida* S12, *P. putida* DOT-T1E, y *P. putida* Idaho. En general, en todas se observó un aumento de los niveles de CL (Weber, 1994; Ramos *et al*., 1997) sin embargo se han visto diferencias significativas entre ellas. Un análisis detallado del recambio de los grupos de cabeza de los fosfolípidos en ausencia y presencia de *o*-xileno en la cepa *P. putida* Idaho reveló que mientras en ausencia del disolvente la incorporación de ^{32}P era mayor en PG, seguida de PE y CL, en presencia de 200 ppm de *o*-xileno, la incorporación de ^{32}P se producía mayoritariamente en PE, seguida muy de cerca por PG. Experimentos similares hechos con *P. putida* DOT-T1E sin embargo indicaron que la mayor parte del ^{32}P se incorporaba en CL cuando en el medio había tolueno (Ramos *et al*., 1997). Pinkart y White

(1997) también demostraron que en la cepa Idaho la isomerización *cis/trans* de ácidos grasos insaturados ocurría principalmente en los ácidos esterificados en PE como grupo de cabeza, sin embargo, en la cepa DOT-T1E no parece que haya una distribución preferente de ácidos grasos esterificados en los grupos de cabeza (Bernal *et al*., resultados no publicados). Aunque el incremento en PE para contrarrestar los efectos de los disolventes no es muy común entre las bacterias (Weber y de Bont, 1996), el PE tiene una temperatura de fusión más alta que PG, por tanto, un aumento en PE podría estabilizar la membrana celular. El aumento en los niveles de CL es algo más común; la CL tiene una temperatura de transición (10ºC) mayor que el PE, lo cual en teoría podría disminuir la fluidez de la membrana y estabilizarla en presencia del tóxico orgánico (Weber y de Bont, 1996). Sin embargo, mutantes de *P. putida* DOT-T1E en el gen *cls* (cardiolipina sintasa) que tienen menor proporción de este grupo de cabeza tienen membranas más rígidas. Este mutante es más sensible al tolueno y a otras drogas que son transportados por bombas de la familia RND (ver más abajo), lo que indica que la falta de CL provoca la desorganización de algunas proteínas de membrana y por tanto la sensibilidad al tolueno estaría provocada más por este efecto sobre las bombas que sobre la fluidez de la membrana (Bernal *et al*., 2007b).

III.a.iii.- Cambios en la velocidad de síntesis de los ácidos grasos

El análisis de la biosíntesis de fosfolípidos en la cepa tolerante *P. putida* Idaho mostró que el nivel basal de biosíntesis de fosfolípidos era mayor en esta cepa que en cepas sensibles a disolventes de la misma especie y que la velocidad de biosíntesis aumentaba en respuesta a la exposición de concentraciones subletales de disolventes orgánicos (Pinkart y White, 1997). También se ha constatado que mutantes sensibles a tolueno de *P. putida* DOT-T1E eran incapaces de incorporar $^{13}CH_3$-$^{13}COOH$ en ácidos grasos en presencia de cantidades subletales de tolueno (Segura *et al*., 2004). Esto sugiere que el tolueno afecta fuertemente a la síntesis *de novo* de ácidos grasos. El recambio limitado en el metabolismo de ácidos grasos en esta cepa mutante contribuye a la pérdida de la integridad de la membrana en presencia de disolventes, y el consecuente aumento de la sensibilidad a estos compuestos.

III.a. iv- Alteración de lipopolisacáridos

Los lipopolisacáridos (LPS) de las bacterias gram-negativas son uno de los principales componentes de la membrana externa y son considerados como una barrera de defensa. En varias cepas de *P. putida* la adición de altas concentraciones de cationes divalentes (Mg^{2+} y Ca^{2+}) al medio de cultivo con disolventes orgánicos resultó en un incremento en la supervivencia (Ramos *et al*., 1995; Weber y de Bont, 1996). La hipótesis desarrollada a partir de estos datos fue que los cationes divalentes se unían electrostáticamente a las moléculas polianiónicas adyacentes de LPS y así reducían la repulsión de cargas. Los cationes permitirían un empaquetamiento más denso de las moléculas aniónicas de las membranas. Sin embargo, un mutante en el gen *wbpL* de *P. putida* DOT-T1E, que codifica la enzima que inicia la síntesis de la cadena lateral del antígeno-O de los LPS, es tan tolerante como la cepa silvestre a disolventes orgánicos (tolueno, octanol, *p*-xileno, propilbenceno, y heptano) lo que sugiere que los LPS juegan un papel menor en la respuesta a disolventes (Junker *et al*., 2001)

III.b - Bombas de expulsión de disolventes

Probablemente las bombas de expulsión de disolventes orgánicos sean el principal responsable de la tolerancia, al menos en aquellas cepas que son altamente tolerantes a disolventes orgánicos. Casi todas las bombas identificadas hasta el momento como involucradas en los procesos de tolerancia pertenecen a la familia de las RND (Resistencia, Nodulación, División celular).

Los organismos vivos se comunican con el medio ambiente que les rodea en parte a través de sistemas de transporte sustrato-específicos. Estos sistemas consisten en proteínas integrales de membrana que se expanden múltiples veces por la membrana citoplasmática. Estas proteínas que forman canales específicos para cada sustrato y que están acopladas a una fuente de energía y/o a proteínas situadas en la membrana externa, pueden conferirle a la célula la capacidad para bombear un soluto y reconocerlo con una alta afinidad (Saier *et al*., 1998). Las proteínas que conforman estos transportadores han sido estudiadas extensamente desde el punto de vista estructural, funcional y filogenético, y como consecuencia de ello se han descrito familias de proteínas transportadoras, algunas de las cuales se ha visto que son ubicuas, mientras que otras están restringidas sólo a uno de los principales reinos de seres vivos (Saier *et al*., 1998). Actualmente hay descritas en bacterias cinco familias de transportadores; MFS (*Major Facilitator Superfamily*), ABC (*ATP*-

Binding-Cassette) y MATE (*multidrug and toxic compound extrusion*) son mayoritarias y ancestrales, mientras que las otras dos familias SMR (*Small Multidrug Resistance*) y RND (*Resistance-Nodulation-cell Division*) son minoritarias y con un origen evolutivo más reciente (Saier *et al*., 1998). Por lo general, el número de sistemas de transporte está en proporción al tamaño del genoma del microorganismo en cuestión, excepto en bacterias adaptadas a sobrevivir en medio ambientes pobres en materia orgánica y ricos en minerales donde llegan a tener hasta dos veces menos transportadores en relación al tamaño del genoma (Saier *et al*., 1998).

Muchos de estos transportadores tienen un papel importante en lo que se denomina resistencia a multidrogras (MDR, *multidrug resistance*). Estos sistemas catalizan la expulsión activa de compuestos biogénicos y xenobióticos no relacionados estructural o funcionalmente, desde el citoplasma o la membrana interna, hacia el medio externo (Krulwich *et al*., 2005; Piddock, 2006; Borges-Walmsley y Walmsley, 2001). Las evidencias experimentales indican que las características físico-químicas de los compuestos (carga, hidrofobicidad, o anfipatía), las interacciones de van der Waals que establecen éstos con los residuos de los sitios activos, y la flexibilidad de estos sitios para acomodar distintas moléculas, son los factores determinantes de la amplia especificidad de sustrato de estos sistemas de extrusión (Neyfakh, 2001; Murakami *et al*., 2002; Yu *et al*., 2003). Aunque se puede pensar que estos transportadores de antimicrobianos surgieron recientemente en respuesta a la quimioterapia antimicrobiana y a compuestos de síntesis humana, el hecho de que haya un número similar de bombas de extrusión que confieren multirresistencia en microorganismos patógenos y no patógenos y el hecho de que muchos estén codificados en el cromosoma, sugiere que juegan un rol fisiológico importante en la expulsión de sustancias tóxicas que normalmente tienen lugar en la naturaleza (tales como toxinas naturales, productos finales del metabolismo endógeno, y antibióticos) y que también expulsan nuevas drogas de fabricación reciente por ser éstas de estructura similar a los respectivos sustratos naturales (Saier *et al*., 1998).

Junto con las proteínas transportadoras, se han identificado dos familias de proteínas accesorias que funcionan conjuntamente con algunos de los transportadores. Estas dos familias han sido designadas de acuerdo a su localización celular en la envoltura de las bacterias Gram-negativas y su presunta función, y se conocen con el nombre de familia OMP (*Outer Membrane Protein*) y familia MFP (*Membrane Fusion Protein*). Se cree que el conjunto de estas tres proteínas (transportador, OMP y MFP) permiten el transporte a través de ambas membranas de la envoltura de las bacterias Gram-negativas en un simple paso

acoplado a una fuente energética. La construcción de este complejo tripartito sugiere que los compuestos tóxicos son expulsados directamente al medio externo y no al periplasma (Nikaido y Takatsuka, 2008). Esta es una gran ventaja para las células bacterianas, ya que los compuestos una vez expulsados al medio externo deben atravesar la membrana externa para volver a entrar a la célula (Figura 2).

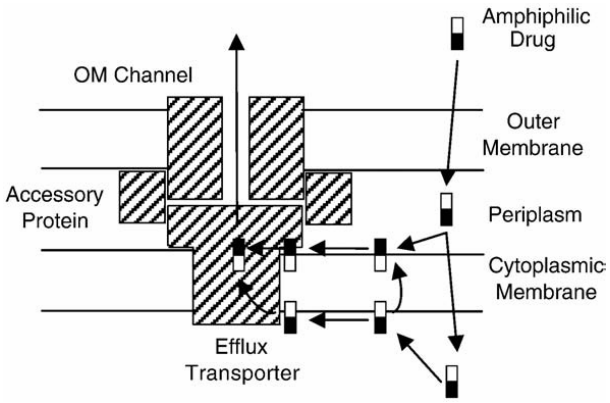

Figura 2. Esquema del complejo tripartito. Los compuestos anfifílicos (rectángulos mitad vacíos mitad llenos que representan las partes hidrofóbicas e hidrofílicas respectivamente, de la molécula) son hipotéticamente capturados tanto desde el periplasma (o de la interfaz periplasma- membrana plasmática) como desde el citosol (o de la interfaz citosol-membrana plasmática). Para el último proceso hay dos posibles caminos: en ambos el sustrato primero atraviesa la membrana hasta la superficie externa de la membrana citoplásmica y luego sigue el patrón de captura normal o es capturado directamente desde el citosol. (Nikaido, 1996)

III-b.i La familia RDN ("RND", *Resistance-Nodulation-cell Division*)

Esta familia de transportadores es ubicua, encontrándose en bacterias, arqueas y eucariotas. El transporte está, en este caso, acoplado a la fuerza protón-motriz. Los transportadores son complejos (1000 aminoácidos) y poseen una topología inusual y muy característica de esta familia. La proteína contiene 12 segmentos transmembrana (STM) y 2 dominios extracitoplásmicos entre las hélices 1 y 2, y las hélices 7 y 8 (Figura 3). Los análisis de la secuencia primaria de estos transportadores sugieren que probablemente hayan surgido como resultado de una duplicación intragénica en tandem. Los análisis filogenéticos de los miembros de esta familia realizados en los últimos años revelan la existencia de tres subfamilias cuyos miembros se agrupan según su función (Saier *et al*., 1998). Una de las subfamilias posee miembros que son específicos para iones divalentes de metales pesados,

otra subfamilia probablemente sea específica para el transporte de lipo-oligosacáridos, y otra subfamilia esta conformada por proteínas que expulsan múltiples drogas. Miembros de estas tres subfamilias se han descrito solamente en bacterias gram-negativas. (Marger y Saier, 1993; Saier *et al*., 1998; Borges-Walmsley y Walmsley, 2001).

La mayoría de los transportadores identificados como relevantes en la tolerancia a disolventes orgánicos pertenecen a esta familia. En *P. putida* DOT-T1E, tres bombas de esta familia, denominadas Ttg (Toluene tolerance genes) participan en la extrusión de disolventes orgánicos (TtgABC, TtgDEF y TtgGHI) (Ramos *et al*., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001); en *P. putida* S12 y *P. putida* GM73 también se han identificado bombas de esta familia como los principales determinantes de tolerancia a

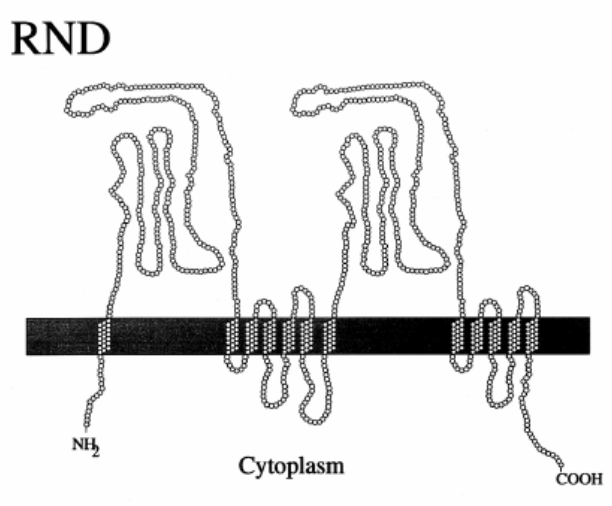

Figura 3. **Modelo estructural representativo de los miembros de la familia "RND".** La figura muestra los segmentos transmembrana y los dos lazos periplásmicos (Saier *et al*., 1998)

tolueno (Kieboom *et al*., 1998a, Kim *et al*, 1998). En *P. aeruginosa* y *E. coli* se ha relacionado este tipo de bombas con la tolerancia a disolventes (Aono *et al*., 1998; Fralick, 1996; Fukimori *et al*., 1998; Kieboom *et al*., 1998b; Kim *et al*., 1998; Li *et al*., 1998; Ma *et al*., 1993; Mosqueda y Ramos, 2000; Ramos *et al*., 1998; Rojas *et al*., 2001; Zgurskaya y Nikaido, 1999). En general, estos transportadores juegan un rol importante en la resistencia a una gran variedad de compuestos tóxicos en bacterias gram-negativas siendo también capaces de transportar una gran variedad de sustratos que no siempre guardan una gran similitud a nivel de su estructura, por ejemplo, antibióticos, compuestos flavonoides y

mutágenos como el bromuro de etidio (Piddock, 2006; Ramos et al., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001, Saier *et al*., 1998).

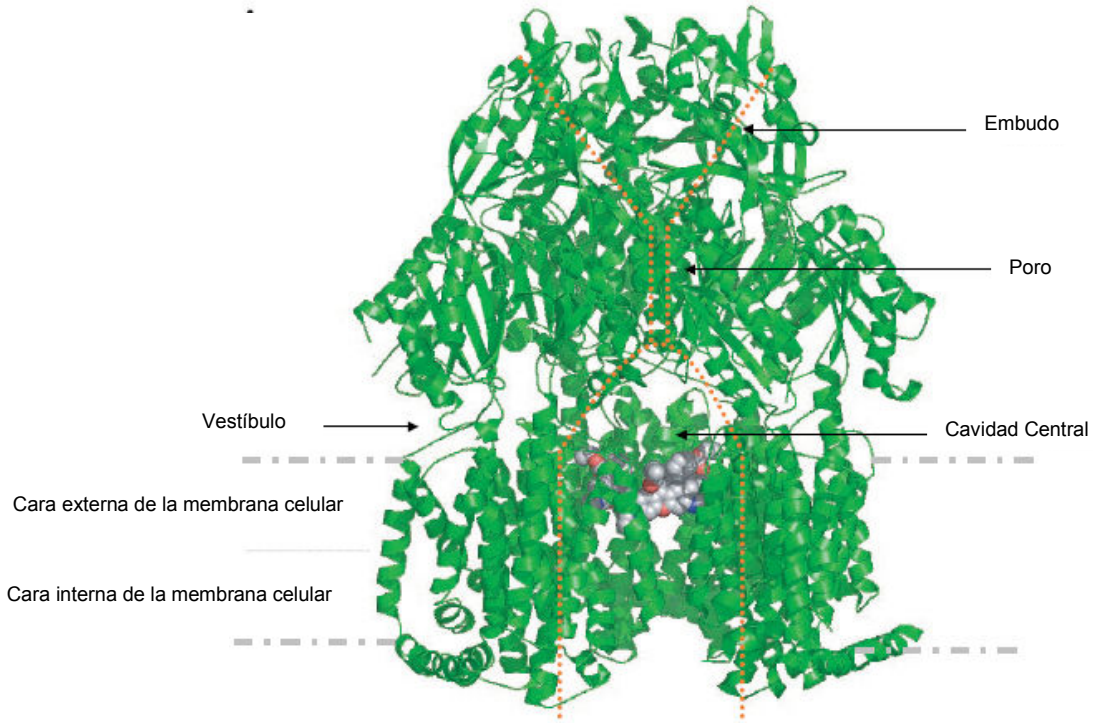

Figura 4. **Estructura del transportador trimérico AcrB**. La figura muestra el dominio transmembrana (caras interna y externa), el dominio periplásmico, la cavidad central, el vestíbulo, el poro y el embudo. Las drogas se unen aproximadamente a nivel de la superficie externa de la bicapa lipídica. (Yu *et al*., 2003; Yu *et al*., 2005).

Una de los transportadores de esta familia más estudiado es AcrB de *E. coli,* que actuando conjuntamente con AcrA (MFP) y TolC (OMP) es capaz de transportar colorantes catiónicos tales como acriflavina, cristal violeta, bromuro de etidio y rodamina 6G; antiobióticos como penicilinas, cefalosporinas, fluoroquinolonas, macrólidos, cloramfenicol, tetraciclinas, novobiocina, acido fusídico, oxazolidinonas, y rifampicina; detergentes como Tritón X-100, SDS (dodecilsulfato de sodio) y sales biliares, y también disolventes orgánicos (Nikaido, 1996). La estructura cristalina de este transportador y de sus proteínas asociadas, TolC y AcrA, han permitido conocer algunos detalles de su funcionamiento. AcrB se organiza como un trímero en una estructura en forma de medusa, con una pieza de "cabeza" de unos 100 Å que se localiza en el periplasma y una región anclada en la

membrana interna de unos 50 Å (Figura 4). Tres de los dominios transmembrana de cada protómero se organizan en una estructura a modo de anillo con un hueco central que cruza la membrana y se extiende hasta la parte de debajo de la pieza de cabeza. El transportador posee unas pequeñas aperturas (vestíbulos) entre las subunidades al final del dominio periplásmico cerca de la superficie externa de la membrana citoplasmática.

Estas aperturas conducen a la gran cavidad central dentro del dominio transmembrana (Figura 4). Se cree que los compuestos tóxicos difunden a través del vestíbulo hasta llegar a la cavidad central donde son capturados (Nikaido y Takatsuka, 2008). TolC también se organiza de forma trimérica adoptando una conformación cilíndrica que se abre hacia el extremo anclado en la membrana externa pero que se constriñe hacia el extremo periplasmático. Presenta dos dominios principales: una parte que se expande por la membrana externa con una estructura de barril β y una región α-helicoidal como un túnel que se expande por el periplasma. La proteína TolC tiene una longitud de 100 Å, lo cual se acerca a la longitud mínima que puede tener el espacio periplásmico (130 Å). El extremo periplásmico de AcrB tiene las dimensiones precisas para encajar con el final del túnel TolC (Borges-Walmsley *et al*., 2003; Nikaido y Takatsuka, 2008; Borges-Walmsley y Walmsley, 2001) (Figura 5). El componente AcrA tiene una estructura muy elongada en la que parece que el extremo N-terminal (que lleva unido un lípido) está insertado en la membrana. Medidas hidrodinámicas indicaron que AcrA (MFP) tiene una longitud aproximada de 210 Å, por lo que es posible que las MFP's sean necesarias para acercar las membranas entre sí (Borges-Wamsley y Walmsley, 2001, Borges-Walmsley *et al*., 2003). La capacidad de las MFP's de formar trímeros estables, sugieren que éstas juegan un papel importante en la formación de un canal conector entre la translocasa (AcrB) y TolC permitiendo el paso de los sustratos hacia el medio externo. La unión sería estable solo durante el transporte del sustrato según está descrito (Fernandez-Recio *et al*., 2004, Borges-Walmsley *et al*., 2003; Borges-Walmsley y Walmsley, 2001).

Aunque la ruta de extrusión no está clara en la estructura cristalina, se cree que los compuestos son capturados a través de los vestíbulos localizados justo en la superficie externa de la membrana citoplasmática, llegan al techo de la cavidad central, entran en el poro central y lo atraviesan hasta llegar al final del dominio periplásmico (Nikaido y Takatsuka, 2008). El extremo periplásmico interactúa transitoriamente con la proteína de fusión de membrana, abriéndose el canal para permitir el paso del sustrato. (Zgurskaya y Nikaido, 2000; Fernandez-Recio *et al*., 2004). Por tanto parece que los sustratos se reclutan en el periplasma más que en el citoplasma. Esto se ha demostrado para aquellos compuestos

que no son capaces de atravesar la membrana citoplasmática, como los compuestos dianiónicos (carbenicilina, ceftriaxona) y los aminoglicósidos, pero que sin embargo son sustrato de estas bombas (Nikaido, 1996). Si bien parece quedar claro que la captura de sustratos desde el citoplasma también tiene lugar, no queda tan claro si éste es el modo de captura que predomina.

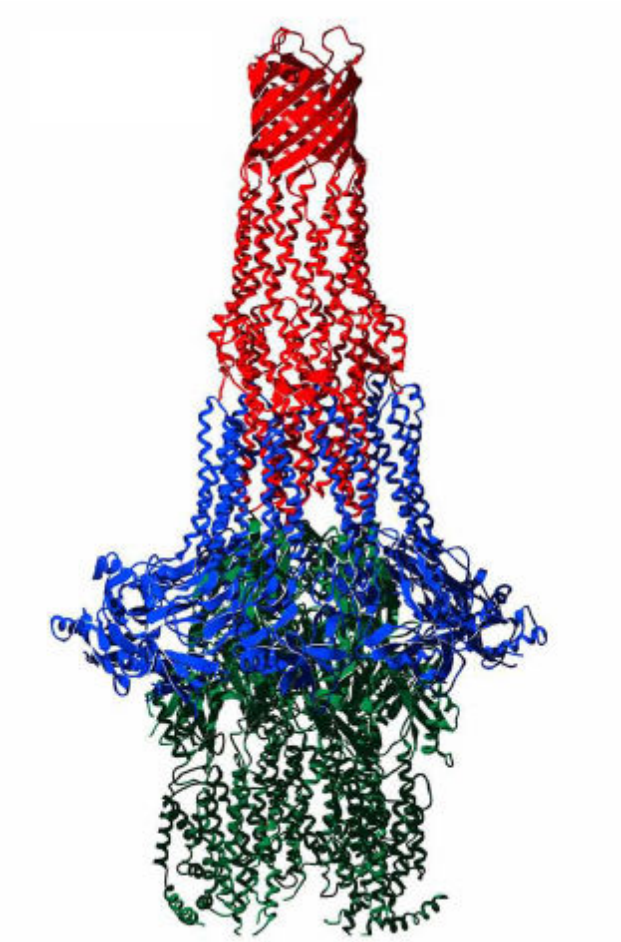

Figura 5. Estructura cristalina de TolC, AcrA y AcrB. Organización hipotética de la estructura tripartita TolC (rojo)- AcrA (azul)- AcrB (verde) basado en la estructura cristalina obtenida por difracción de rayos X (Nikaido y Takatsuka, 2008).

También se ha visto que las bombas RND actúan en sinergismo con otros transportadores. Un ejemplo de esto sucede en *P. aeruginosa*; TetA una bomba de la familia MFS que expulsa tetraciclina, confiere un nivel de resistencia relativamente bajo (CIM = 32 μg/mL) en ausencia del principal transportador RND, MexA-MexB-OprM; en ausencia de

TetA la CIM conferida por el transportador RND es de 4 μg/mL. Sin embargo, la CIM aumenta fuertemente (CIM = 512 μg/mL) cuando ambos transportadores se expresan a niveles normales y constitutivos. De acuerdo con lo expuesto, parece que estos dos transportadores actúan de modo sinérgico. La explicación más plausible de este resultado es que la tetraciclina es bombeada hacia el periplasma por TetA desde donde es capturada por el complejo tripartito (MexAB-OprM) y expulsada hacia el exterior de la célula (Lee *et al*, 2000). Esto a su vez enfatiza la importancia del mecanismo de captura desde el periplasma de las bombas RND (Nikaido y Takatsuka, 2008).

En algún momento se ha sugerido que estos transportadores trabajan sinergísticamente con la membrana externa. Sin embargo, la inactivación del transportador más importante de una bacteria (por ejemplo, AcrB en *E. coli* ó TtgGHI para disolventes en *P. putida* DOT-T1E) hace que ésta se vuelva susceptible al compuesto toxico en cuestión, a pesar de tener la membrana externa intacta (Nikaido y Takatsuka, 2008; Ramos *et al*., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001).

Cada uno de los componentes es imprescindible para la extrusión del compuesto tóxico, ya que la falta de alguno de ellos hace que el transportador no funcione (Nikaido y Takatsuka, 2008). Algunos de estos transportadores están codificados junto con sus correspondientes OMP y MFP (MexAB-OprM, MexEF-OprN, TtgABC, TtgDEF, TtgGHI entre otros (Poole *et al*., 1993; Koehler *et al*., 1997; Ramos *et al*., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001) mientras que en otros casos tan sólo se cotranscriben la translocasa con su correspondiente MFS, utilizando una OMP que puede ser compartida entre varios transportadores (por ejemplo AcrAB que utiliza TolC, MexXY que se une a OprM; (Srikumar *et al*., 1997; Aires *et al*, 1999; Fralick, 1996; Mine *et al*., 1999). No está claro cómo se ensamblan estos complejos y porqué en unos casos las tres proteínas se cotranscriben y en otros casos no. A diferencia de las proteínas de fusión de membrana (MFP), las proteínas de membrana externa (OMP) de los sistemas de extrusión descritos en bacterias Gram-negativas pueden intercambiarse entre diferentes sistemas de transporte y entre bombas de la misma familia (Yoneyama *et al*., 1998, Srikumar *et al*., 1997, Zgurskaya y Nikaido, 2000). TolC de *E. coli* (esencial para el funcionamiento del transportador AcrAB en esta cepa) también colabora en la actividad transportadora de las bombas MexD y MexY de *P. aeruginosa* cuando éstas son expresadas en células de *E. coli* en ausencia de su correspondiente proteína de membrana externa nativa (Srikumar *et al*., 1998). Otro ejemplo

lo constituye la proteína OprM de *P. aeruginosa* la cual se acopla al menos a dos transportadores de la familia RND, MexB y MexY (Zgurskaya y Nikaido, 2000). Además, TolC tiene la capacidad de interactuar con diferentes clases de antiportes de membrana interna pertenecientes a la misma familia o de familia diferente (RND, ABC y MFS) (Figura 6) (Piddock, 2006; Fernandez-Recio *et al*., 2004).

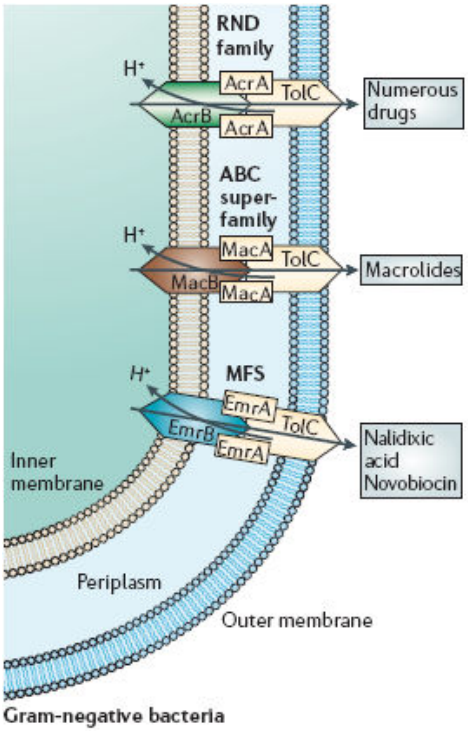

Figura 6. **Diagrama de transportadores de resistencia pertenecientes a las familias ABC, RND y MFS**, todos ellos formando un complejo tripartito con TolC en bacterias gram-negativas (Piddock, 2006).

III. b. ii. La Superfamilia "ABC" (*ATP-Binding-Cassette*)

Estos sistemas de transporte pertenecen al grupo de los transportadores activos primarios según la clasificación de Saier (2000), en el que el transporte está acoplado a la presencia de una fuente primaria de energía, en este caso, a la hidrólisis de ATP. En este tipo de sistemas, la proteína transportadora puede ser o no transitoriamente fosforilada, pero el sustrato que se transporta nunca es fosforilado durante su translocación (Saier, 2000). Esta familia de transportadores es ubicua, estando presente tanto en bacterias como en organismos superiores. Esta superfamilia está compuesta por más de 30 familias; cada familia transporta

una enorme variedad de sustratos, cada uno de los cuales es específico de un transportador. Estos sustratos incluyen moléculas pequeñas que pueden ser transportadas al interior o exterior de la célula dependiendo del transportador pero también transportan macromoléculas tales como proteínas y carbohidratos complejos que son sintetizados en el citoplasma y secretados a la envoltura celular o al medio externo; igualmente transportan nutrientes al citoplasma bacteriano y participan además en numerosos procesos como en la transducción de señales, en la resistencia a drogas y a antibióticos, en los mecanismos de patogénesis y esporulación, etc. (Higgins, 1992; Wandersman, 1998).

Los transportadores de la familia ABC se caracterizan por presentar una secuencia conservada, de aproximadamente 215 aminoácidos, que se estructura en un dominio soluble con actividad ATPasa (Young y Holland, 1999) y que se denomina dominio de unión a ATP o dominio ABC (*ATP-Binding-Cassette*). Es importante destacar la diferencia entre proteínas ABC y transportadores ABC. El término proteína ABC se refiere a proteínas que contienen un dominio de unión a ATP y/o de hidrólisis del ATP y que participan en muchos procesos fisiológicos no necesariamente (aunque sí normalmente) relacionados con el transporte. Por otro lado, un transportador ABC se forma cuando dicha actividad ATPasa se asocia con un domino hidrofóbico de membrana (Young y Holland, 1999).

Los transportadores ABC se componen normalmente de cuatro dominios: dos dominios hidrofóbicos integrales de membrana interna que consisten en 6 STM con estructura de α-hélice, y dos dominios hidrofílicos que se localizan en la cara citoplásmica de la membrana y catalizan la hidrólisis del ATP (Higgins, 1992) (Figura 7). En sistemas eucariotas los módulos se encuentran en una única cadena polipeptídica, mientras que en bacterias generalmente se conforman en subunidades diferentes (aunque no siempre). Dichos dominios del transportador ABC pueden estar formados por cuatro polipéptidos diferentes o pueden ser sintetizados como dos proteínas independientes que presentan un dominio hidrofóbico que se ensambla en la membrana y un dominio hidrofílico con actividad ATPasa (Wandersman, 1998). Esto es lo que sucede en la mayoría de los transportadores ABC de bacterias (dominios transmembrana codificados por uno o dos genes independientes al gen que codifica la proteína ABC) (Young y Holland, 1999).

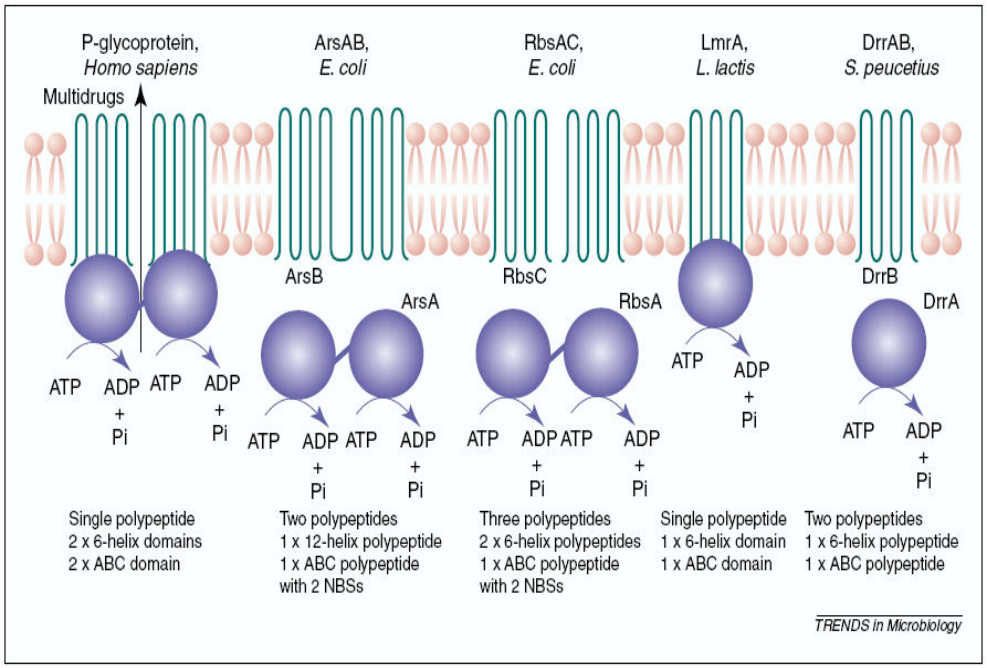

Figura 7. **Transportadores de la familia ABC.** Representación diagramática de la topología de la membrana de transportadores de tipo ABC (Borges-Walmsley y Walmsley, 2001).

Los dominios con actividad ATPasa son dominios conservados que presentan los denominados sitios Walker que parecen estar implicados en la unión a nucleósidos (Saraste *et al*., 1990; Walker *et al*., 1982). El sitio Walker A se caracteriza por presentar la secuencia GXXGXGKS/T (X representa cualquier aminoácido), y el sitio Walker B presenta la secuencia hhhhD, donde h suele ser un aminoácido hidrofóbico. Este sitio Walker B está precedido por un motivo muy conservado (LSGGQQ/R/KQR) que es único para los miembros de la familia de transportadores tipo ABC (Ames *et al*., 1990; Hyde *et al*., 1990; Schneider y Hunker, 1998). Entre esta secuencia y el sitio Walker A se localiza un dominio con estructura de α-hélice que parece ser el responsable de la interacción de las ATPasas con los componentes de la membrana (Ames *et al*., 1990; Higgins, 1992; Hyde *et al*., 1990; Mimura *et al*., 1991), interacción que es necesaria para que se lleve a cabo el cambio conformacional de estas subunidades de membrana que permite la translocación del compuesto como respuesta a la hidrólisis del ATP (Schneider y Hunker, 1998).

Los sistemas de transporte que transportan nutrientes al interior celular (azúcares, aminoácidos, péptidos, iones inorgánicos y vitaminas) son la subclase mejor caracterizada (Boos y Lucht, 1996). Estos dependen de una proteína de unión periplásmica que actúa como "buscador" de sustratos y a veces de quimiorreceptor. Se ha determinado que para que

se produzca la hidrólisis del ATP es necesaria la interacción de la proteína periplásmica con los dominios de las subunidades de membrana del transportador ABC presentes en la superficie de la monocapa externa de la membrana (Ames *et al*., 1990; Davidson *et al*., 1992). En estos sistemas de transporte, el sustrato y el ATP se encuentran situados en sitios distintos del transportador (en la cara periplásmica y citoplásmica del mismo, respectivamente) y es la proteína de unión periplásmica la que reconoce el compuesto que se va a transportar, lo que implica que la proteína ABC no participaría en el reconocimiento directo de dicha sustancia (Schneider y Hunker, 1998).

Sin embargo, en el caso de los sistemas exportadores tipo ABC, el sustrato y el ATP están presentes en la cara citoplásmica del transportador y en este caso existen evidencias de que las ATPasas implicadas en los sistemas de secreción de polipéptidos podrían participar en el reconocimiento de sus sustratos (Schneider y Hunker, 1998). Los exportadores tipo ABC de las bacterias gram-negativas están asociados con una proteína de la membrana externa y con la denominada proteína de fusión que presenta un dominio N-terminal de anclaje a la membrana interna y un dominio periplásmico (Young y Holland, 1999; Wandersman, 1998). Estos sistemas están implicados en la secreción de un gran número de compuestos hidrofílicos, desde moléculas pequeñas (como drogas o antibióticos), hasta polímeros complejos de carbohidratos (como el LPS, polisacáridos capsulares, etc.).

Los sistemas de transporte tipo ABC son sensibles a una gran variedad de compuestos que inhiben su actividad ATPasa. Uno de estos compuestos es el vanadato, y se ha determinado que dicho compuesto inhibe la actividad enzimática del transportador atrapando el ADP en el sitio catalítico del enzima e impidiendo su liberación de la proteína (Urbatsch *et al*., 1995). Se ha sugerido que en los sistemas de transporte tipo ABC la hidrólisis de ATP se llevaría a cabo de manera cooperativa entre todos sus componentes (Davidson *et al*., 1996).

Kim et al. (1998) identificaron en *P. putida* GM73 un transportador ABC que confería resistencia a tolueno y lo denominaron Ttg2ABCD, sin embargo en dicho trabajo no caracterizaron en detalle el sistema de transporte.

II.b.iii. La Superfamilia "MFS" (*Major Facilitator Superfamily*)

La superfamilia MFS está integrada por transportadores secundarios formados por un solo polipéptido de aproximadamente 400 aminoácidos que solo son capaces de transportar pequeños solutos en respuesta a gradientes quimiosmóticos de iones. Esta superfamilia, al

igual que la superfamilia ABC, es ancestral y está integrada por 17 familias cada una con una especificidad de sustrato diferente. Un estudio basado en la homología de las proteínas comprendidas dentro de esta superfamilia ha permitido subdividirla en 5 familias en donde podemos encontrar uniportes, antiportes y simportes, todos ellos específicos para azúcares, ácidos orgánicos y otras drogas. La primera familia comprende proteínas de resistencia a drogas, las cuales catalizan la extrusión activa de antimicrobianos como quinolonas, tetraciclinas, metilenomicina A, antisépticos y múltiples drogas en células bacterianas y aminotriazol en levaduras. Dentro de esta familia se distinguen dos tipos porque una posee 12 segmentos transmembrana (STM) y la otra familia posee 14 STM (Figura 8). El análisis de las secuencias sugiere que las proteínas con 14 STM surgieron de la inserción de un segmento dentro del gen que codifica para la proteína de 12 STM dando lugar a la aparición de los segmentos 7 y 8; esto sucedió durante los estadios tempranos en la evolución de estos dos transportadores, antes de la duplicación de los genes que codifican sus numerosos miembros (Jin *et al*., 2001; Saier *et al*., 1998; Borges-Walmsley y Walmsley, 2001).

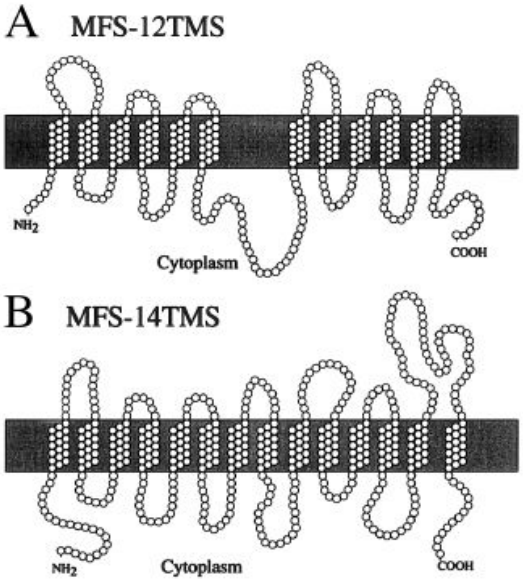

Figura 8. **Modelo estructural de las bombas de extrusión de la Superfamilia de los Facilitadores Mayores**, con A) 12 STM y B) 14 STM (Saier *et al*., 1998).

La segunda gran familia de transportadores incluye uniportes que transportan azucares, azúcar-protón simportes, y un transportador de quinato que está presente tanto en células eucarióticas como procarióticas. Las proteínas de la tercera familia son transportadores

específicos para los intermediarios del ciclo de Krebs (citrato y α-cetoglutarato) y probablemente funcionen como protón-simportes. La cuarta familia incluye un grupo de antiportes específicos para fosfo-ésteres orgánicos y sorprendentemente un regulador transcripcional de *E. coli* (UhpC) (Jin *et al.,* 2001; Marger y Saier, 1993; Schwöppe *et al*., 2003). La quinta familia consiste en protón-simportes para algunos oligosacáridos (rafinosa, sacarosa, lactosa) en *E. coli*. Las familias tercera, cuarta y quinta están presentes solo en bacterias.

En la literatura hay una vasta evidencia acerca de la topología de estas proteínas, que consiste en 6 STM (alfa-hélices) seguidos de un lazo citoplasmático que a su vez le siguen 6 STM adicionales (alfa-hélices), este patrón de 6 + 6 (12 segmentos) ha sido publicado para uniportes de glucosa en mamíferos, para una permeasa de lactosa de *E. coli* (LacY) y para los antiportes de tetraciclina en bacterias. (Saier *et al*., 1998; Borges-Walmsley y Walmsley, 2001) Para los transportadores con 14 STM el lazo citoplasmático suele ser de menor tamaño (Borges-Walmsley y Walmsley, 2001). Ninguno de estos transportadores ha sido implicado en la tolerancia a disolventes orgánicos hasta el momento.

III.b.iv. La familia "MATE" (*multidrug and toxic compound extrusion*).

Esta familia de transportadores fue descrita primeramente como familia de transportadores exclusiva de bacterias pero actualmente se sabe que están presentes tanto en procariotas como en eucariotas, y constituyen una de las familias de transportadores más conservadas en la naturaleza. Los transportadores bacterianos constan de unos 450 aminoácidos y forman 12 STM. Las proteínas de las levaduras tienen aproximadamente unos 700 aminoácidos, y probablemente ellos también formen un transportador con 12 STM. Dos de estos transportadores bacterianos descritos, utilizan un mecanismo de transporte inusual: antiporte Na^+/soluto (Moriyama *et al*., 2008).

Las proteínas NorM de *Neisseria gonorrhoeae* y YdhE de *Escherichia coli* pertenecen a esta familia. NorM de *Neisseria gonorrhoeae* es una proteína de membrana interna que consta de 459 aminoácidos, y reconoce como sustratos a compuestos catiónicos tóxicos como bromuro de etidio, acriflavina, y ciprofloxacina (Su *et al*., 2008; Long *et al*., 2008). YdhE de *E coli* tiene un 57% de identidad y un 88% de similitud con respecto a NorM de *V. parahaemolyticu*s, el cual transporta norfloxacina y ciproflofaxina además de bromuro de etidio, pero a pesar de la gran similitud entre ambas proteínas, YdhE no transporta bromuro

de etidio. Tanto NorM de *V. parahaemolyticu*s como YdhE de *E coli* constan de 456 aminoácidos (Morita *et al*., 1998).

III.b.v La familia "SMR" (*Small Multidrug Resistance*)

Algunos de los miembros de esta familia catalizan la multirresistencia a drogas. Estos transportadores homo-oligoméricos son pequeños, estando constituidos por cadenas polipeptídicas de 100 a 110 aminoácidos que se expanden por la membrana celular atravesándola 4 veces como α-hélices (Figura 9). Se cree que su estado nativo dentro de la membrana es como homotrímero. Debido a su naturaleza hidrófoba son solubles en disolventes orgánicos. Es una familia pequeña, bien conservada, que solo esta presente en bacterias (Saier *et al*., 1998)

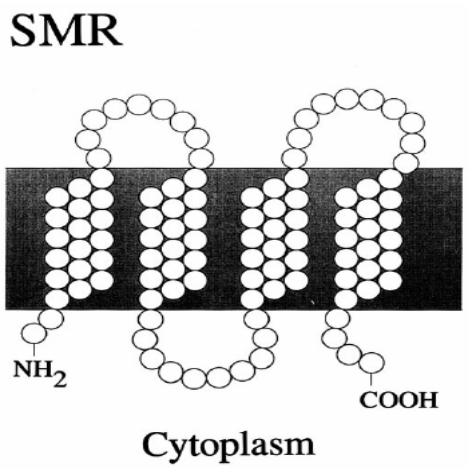

Figura 9. Modelo estructural de los transportadores de la familia "SMR" (Saier et al., 1998).

Esta familia está integrada por dos subfamilias, una de ellas tiene miembros que confieren multirresistencia a drogas y cataliza la extrusión de éstas vía antiporte droga/H^+ al igual que los miembros de la superfamilia MFS. Por el contrario, los miembros de la otra subfamilia parecen no tener ninguna de estas características, además se desconoce la naturaleza de sus sustratos naturales (Saier *et al*., 1998; Borges-Walmsley y Walmsley, 2001).

III.d. Otros factores implicados en la resistencia a tolueno

En bacterias gram-negativas se han descrito otros mecanismos que contribuyen a la tolerancia a disolventes. Algunos investigadores sugieren que la formación de vesículas en *Pseudomonas* sp. IH-20000, una cepa tolerante a tolueno, es una respuesta específica de esta estirpe cuando se añade tolueno al medio de cultivo con el fin de remover dicho disolvente (Kobayashi *et al*., 2000). Las vesículas de *Pseudomonas* sp. IH-20000, formadas por fosfolípidos, LPS y una pequeña cantidad de proteínas, contenían 0,17-0,63 mol tolueno/ mol lípidos. También se ha descrito la formación de vesículas en cultivos de *P. putida* DOT-T1E cuando crece en presencia de altas concentraciones de tolueno, sin embargo, no ha evidencias de que en *P. putida* DOT-T1E la función de estas vesículas sea la de expulsar tolueno (Ramos *et al*., 1995).

En nuestro grupo se ha demostrado que mutaciones producidas en genes que codifican los componentes que exportan las proteínas flagelares (i.e *flh*B) de *P. putida* DOT-T1E, tornaban a esta cepa en inmóvil y sensible a tolueno. También en la cepa *P. putida* S12, una mutación en el gen *flg*K, la hacía sensible a tolueno (Segura *et al*., 2001; Kieboom *et al*., 2001). Estos datos sugieren que algunas proteínas flagelares, aquellas implicadas en el sistema de transporte de los distintos componentes del flagelo, juegan un rol indirecto en la tolerancia a tolueno, probablemente permitiendo la exportación a través de la membrana de alguna proteína que tenga un papel importante en tolerancia.

Para mantener la homeostasis celular frente a una variedad de estreses ambientales, las bacterias usan factores sigma alternativos para redirigir la RNA polimerasa y expresar selectivamente algunos genes. En *P. putida* se han identificado 24 factores sigma, 20 de los cuales corresponden a la subfamilia de los factores sigma extracitoplasmáticos (ECF) (Ramos *et al*., 2001; Duque *et al*., 2007). En nuestro grupo se descubrió que alrededor del 1% del genoma de *P. putida* DOT-T1E está bajo la influencia directa o indirecta del factor sigma RpoT y que el mismo está involucrado en la tolerancia a tolueno, hecho que quedó demostrado al generar un mutante (DOT-T1E-Δ*rpo*T) sensible a tolueno. Esto se debe a que RpoT regula la expresión de TtgGHI, uno de los transportadores clave en la expulsión de tolueno (Duque *et al*., 2007; Rojas *et al*., 2001).

Aunque los factores anteriormente descritos son fundamentales en la respuesta al choque a altas concentraciones de tolueno, las células bacterianas expuestas a la presencia constante de un disolvente orgánico requieren otras adaptaciones que les permiten crecer en estas condiciones. Mediante el análisis de proteínas (Segura *et al*., 2005) que se expresan diferencialmente en cultivos de *P. putida* DOT-T1E creciendo en presencia y ausencia de

tolueno, se han identificado otros factores en respuesta a estas condiciones, Una de las principales aportaciones de este trabajo es que la presencia de tolueno, aun en concentraciones mucho más bajas del límite de resistencia de la cepa y pese a que no se muestra ningún efecto a nivel de crecimiento cuando se comparaba con el crecimiento en glucosa, es percibida por las células como un estrés y por tanto se inducen una serie de proteínas relacionadas con el mismo (CspA, GroES entre otras). También que las células necesitaban más energía en presencia de tolueno y por tanto aceleraban el metabolismo de la glucosa. Resultados similares se obtuvieron en la cepa *P. putida* S12 cultivada en quimiostatos con y sin tolueno (Volkers *et al*., 2006).

A su vez, ensayos de microarray realizados en nuestro grupo (Domínguez-Cuevas *et al*., 2006) y llevados a cabo en *P. putida* KT2440 (una cepa sensible), para analizar la respuesta global de esta cepa en presencia de compuestos aromáticos, revelaron que en esta estirpe se inducen, en presencia de tolueno, toda una batería de genes en respuesta a estrés.

En todos estos experimentos se identificaron como inducidos por tolueno una serie de transportadores de diversas familias, cuyo papel en la tolerancia a disolventes orgánicos no ha sido estudiado hasta el momento.

IV. Regulación de la tolerancia a disolventes orgánicos

La complejidad de la respuesta al tolueno queda de manifiesto no sólo en los experimentos de microarrays y proteómica que se han llevado a cabo en distintos laboratorios, sino por la gran cantidad de mecanismos de defensa diferentes que participan en la tolerancia a disolventes orgánicos.

El principal mecanismo de defensa frente a concentraciones supersaturantes de tolueno en *P. putida* DOT-T1E son las bombas de extrusión; hasta el momento sólo mutantes en algunas de estas bombas son totalmente incapaces de sobrevivir al choque con 0,3% (v/v) de tolueno. Otros mutantes en, por ejemplo, componentes de membrana son capaces de sobrevivir al choque, si bien el porcentaje de células que sobreviven es menor (Bernal *et al*., 2007 a; Pini *et al*., 2009)

La tolerancia a tolueno en *P. putida* DOT-T1E es un proceso inducible; aproximadamente 1 de cada 10.000 células sobrevive al choque con 0,3% (v/v) cuando las células se han cultivado en LB, sin embargo, si el cultivo se ha preinducido con bajas concentraciones de tolueno (tolueno en la fase gaseosa) casi el 90% de las células es capaz de sobrevivir al choque (Figura 10-A). Como se ha dicho anteriormente, en *P. putida* DOT-

T1E hay identificadas tres bombas implicadas en la tolerancia a tolueno (TtgABC, TtgDEF y TtgGHI) (Ramos *et al*., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001). TtgABC se expresa constitutivamente a niveles bajos y expulsa tanto disolventes orgánicos como antibióticos, pero solo es inducida por antibióticos y algunos flavonoides (Duque *et al*., 2001; Terán *et al*., 2003; Terán, 2006). Mutantes en esta cepa no son capaces de sobrevivir al choque con tolueno a no ser que hayan sido previamente preinducidos (Figura 10-B; Ramos *et al*., 1998). Además, esta bomba parece estar ampliamente distribuida en cepas de *P. putida* (Segura *et al*., 2003). TtgR es el regulador transcripcional de esta bomba y pertenece a la familia de TetR (Duque *et al*., 2000).

TtgDEF no se expresa bajo condiciones normales de laboratorio pero es inducida por disolventes. Así, en un mutante en esta cepa tan sólo se observa una disminución de su capacidad de sobrevivir al choque con tolueno cuando el cultivo ha sido previamente inducido con disolvente orgánico (Figura 10-C). Esta bomba no está involucrada en resistencia a antibióticos, solo expulsa disolventes (Mosqueda y Ramos, 2000) y está presente en todas las cepas que presentan la ruta *tod* para la degradación de tolueno (Segura *et al*., 2003). TtgGHI tiene un nivel de expresión basal relativamente alto y es inducida por disolventes orgánicos (Rojas *et al*., 2001). Es quizá la bomba que confiere mayor tolerancia a disolventes orgánicos lo que se manifiesta en que mutantes en esta cepa son incapaces de sobrevivir al choque de tolueno independientemente de las condiciones en las que se haya cultivado la cepa (Figura 10-D). En ensayos llevados a cabo en diferentes cepas de *P .putida* sólo se identificaron genes similares a los del operón *ttgGHI* en las cepas altamente tolerantes a disolventes orgánicos, como por ejemplo, *P. putida* MTB6 (Huertas *et al*., 2000), y *P. putida* S12 (Isken y de Bont, 1998). Esta bomba participa también en la extrusión de disolventes orgánicos y antibióticos, aunque su función en resistencia a antibióticos está enmascarada por la presencia de la bomba TtgABC (Rojas *et al*., 2001). Los reguladores TtgT (Terán *et al*., 2007) y TtgV (Rojas *et al*., 2003) son represores de las bombas TtgDEF y TtgGHI respectivamente. TtgT y TtgV pertenecen a la familia de reguladores transcripcionales IclR y muestran una regulación cruzada hacia las bombas TtgDEF y TtgGHI.

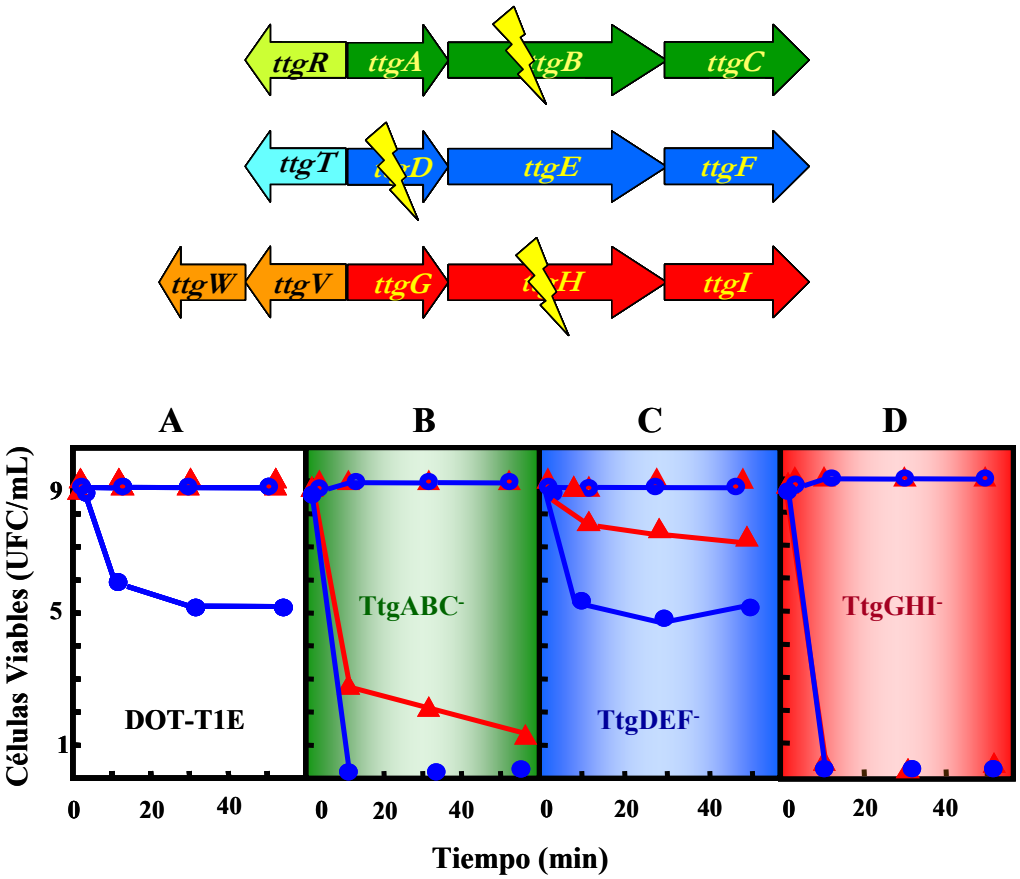

Figura 10. **Grado de supervivencia de las diferentes cepas de *Pseudomonas* putida DOT-T1E ante choques de tolueno. A)** cepa DOT-T1E salvaje, **B)** DOT-T1E TtgABC$^-$, **C)** DOT-T1E TtgDEF$^-$, **D)** DOT-T1E TtgGHI$^-$. Las células se cultivaron en medio LB en ausencia (círculos) o en presencia (triángulos) de tolueno en fase gaseosa. Cuando los cultivos alcanzaban una turbidez de 0.8-1, se dividieron en dos alícuotas, y a una de ellas se le añadió 0,3% (vol/vol) de tolueno (símbolos cerrados). La segunda alícuota se usaba como control (símbolos abiertos). El número de células viables se determinó después de la adición del disolvente a los tiempos indicados (Ramos *et al*., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001). En la parte superior de la figura se representa la organización en operones de las bombas de extrusión de *P. putida* DOT-T1E y los genes que fueron interrumpidos en cada uno de los mutantes construidos (Ramos *et al*., 1998; Mosqueda y Ramos, 2000; Rojas *et al*., 2001).

El hecho de que en una misma célula existan tres bombas de expulsión para el mismo compuesto con una regulación diferente sugiere que las células poseen un ajuste muy fino a la hora de responder frente al estrés por tolueno. La expresión diferencial de las bombas se complementa con los mecanismos a corto y largo plazo de las membranas y con la expresión de otros genes (respuesta general a estrés, aceleración del metabolismo, y otros) que hacen que finalmente las células sean capaces de adaptarse y sobrevivir en un ambiente hostil. Cómo la célula integra todos estos procesos es una de las preguntas que quedan por resolver.

OBJETIVOS

El objetivo general de esta Tesis Doctoral es el de profundizar en los mecanismos de tolerancia a disolventes orgánicos en la cepa *Pseudomonas putida* DOT-T1E, haciendo especial énfasis en mecanismos de transporte. Para ello se establecieron cuatro objetivos específicos:

1.- Identificación y caracterización de nuevos factores de tolerancia a disolventes orgánicos: participación del sistema *cyoB* en la tolerancia.

2.- Construcción y análisis del comportamiento de diferentes bombas híbridas (con distintas combinaciones de los tres elementos que las conforman) en cepas de *P. putida*.

3.- Profundización del análisis de los genes *ttgGHI:* Análisis y caracterización de un mutante espontáneo sensible al tolueno derivado de *Pseudomonas putida* DOT-T1E (*P. putida* DOT-T1E-100).

4.- Implicación de otros sistemas de transporte cuya expresión se ve afectada por la presencia de tolueno en *P. putida* KT2440, la cepa sensible a tolueno, y en la tolerancia a disolventes orgánicos en la cepa resistente a tolueno, *P. putida* DOT-T1E.

RESULTADOS

Plasmolysis induced by toluene in a *cyoB*mutant of *Pseudomonas putida*

Estrella Duque, Vanina García, Jesús de la Torre, Patricia Godoy, Patricia Bernal and Juan-Luis Ramos

El operón *cyo*ABCDE de *Pseudomonas putida* DOT-T1E codifica para una citocromo oxidasa terminal. Un fragmento de 500 pb del gen "*cyo*B" se clonó en el vector pCHESIW Km y se utilizó para generar un mutante knock-out en el gen *cyo*B in vivo. El mutante no se vio limitado en la generación de fuerza protón motriz, aunque cuando se cultivó en medio mínimo con glucosa o citrato, el mutante en CyoB exhibió un ligero incremento en el tiempo de duplicación con respecto a la cepa salvaje. Este efecto fue aun más pronunciado cuando se suplementó el medio con tolueno en fase gaseosa. En consonancia con el efecto negativo del tolueno en el crecimiento, se vio que el mutante CyoB era hipersensible a los choques de 0,3% (v/v) de tolueno, en contraste con la cepa silvestre. Este efecto se vio particularmente exacerbado en células que alcanzaban la fase estacionaria. El incremento en la sensibilidad a los disolventes en el mutante CyoB no parecía estar relacionado con la incapacidad de las células de reforzar el empaquetamiento de la membrana o de inducir las bombas de extrusión en respuesta a los disolventes, sino que la plasmólisis inducida por disolventes puede ser desencadenada por pliegues de la membrana citoplasmática en los polos de las células mutantes, y una invaginación de la membrana externa que eventualmente pueden conducir a la muerte celular.

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Plasmolysis induced by toluene in a *cyoB* mutant of *Pseudomonas putida*

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Summary

The *cyoABCDE* gene cluster of *Pseudomonas putida* DOT-T1E encodes a terminal cytochrome oxidase. A 500-bp '*cyoB*' DNA fragment was cloned in pCHESI-ΩKm and used to generate a cyoB knock-out mutant *in vivo*. The mutant strain was not limited in the generation of proton-motif force, although when grown on minimal medium with glucose or citrate, the CyoB mutant exhibited a slight increase in duplication time with respect to the wild-type strain. This effect was even more pronounced when toluene was supplied in the gas phase. In consonance with the negative effect of toluene on the growth was the finding that the CyoB mutant was hypersensitive to sudden 0.3% (v/v) toluene shocks, in contrast with the wild-type strain. This effect was particularly exacerbated in cells that reached the stationary phase. The increased sensitivity to solvents of the CyoB mutant did not appear to be related to the inability of the cells to strengthen the membrane package or to induce the efflux pumps in response to the solvent, but rather to solvent-induced plasmolysis that may be triggered by wrinkles in the cytoplasmic membrane at the poles of the mutant cells, and invagination of the outer membranes, which eventually lead to cell death.

Introduction

Aerobic bacteria generate energy through respiratory processes in which electrons flow through a series of electron carriers until they reach terminal oxidases that reduce oxygen to water. In *Pseudomonas* sp. two main terminal cytochrome oxidases are known, namely the cytochrome ubiquinol *o* oxidase (Cyo) and the cyanide insensitive oxidase (Cio) (Cunningham and Williams, 1995; Cooper *et al*., 2003). The *cyoABCDE* gene cluster encodes the cytochrome *o* oxidase complex (CyoA,B,C and D proteins), and the heme *o* synthase (CyoE) (Hirayama *et al*., 1998; Dinamarca *et al*., 2002). This cytochrome receives electrons from the ubiquinone pools (Nakamura *et al*., 1997). The CyoABCD terminal oxidase seems to play a major role in cells growing in conditions of high oxygen tension (Sweet and Peterson, 1978; Cotter *et al*., 1990; 1997), and is expressed preferentially in cells in the exponential phase although expression in the stationary phase also takes place but at a lower level (Dinamarca *et al*., 2003). In contrast, at least in *Escherichi coli* the CydAB terminal oxidase (CioAB in *P. aeruginosa*) is preferentially expressed in cells growing under low oxygen tension and in the stationary phase (Taylor and Zhulin, 1999). Nonetheless, in *Escherichia coli* each of these terminal oxidases replaces the other one in isogenic mutants deficient in their synthesis.

Mutants in the *cyo* genes in different strains of *Pseudomonas putida* have been isolated and the effects on gene regulation and solvent tolerance have been analysed. Mutants in the *cyoABCDE* cluster in *P. putida* KT2440 (Dinamarca *et al*., 2002; 2003) and *P. putida* strain H (Petruschka *et al*., 2001) escape from catabolite control, in the case of the alkane degradation pathway in the KT2440 strain, and the phenol degradation pathway, in the H strain. It was hypothesized that the catabolic repression system could monitor the physiological status of the cells by sensing the redox state of the elements of the electron transport chain, and that this could be used as a signalling system. However, the authors could not ascertain whether the Cyo terminal oxidase plays a direct or an indirect role in signal transmission. A mutant in the cytochrome ubiquinol oxidase system was also described in the solvent-tolerant *P. putida* IH2000 strain. This mutation lead to increased solvent sensitivity in comparison with wild-type strain (Hirayama *et al*., 1998). The reason for this increased tolerance was believed to be changes in the membrane hydrophobicity, although the intimate reasons remain unknown (Kobayashi *et al*., 1999).

Pseudomonas putida DOT-T1E is a solvent-tolerant strain (Ramos *et al*., 1995) that exhibits physical and biochemical barriers to decrease solvent toxicity (Ramos *et al*., 1997; 2002). In response to toluene in the culture medium, the cell membrane is packaged by transforming

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cis unsaturated fatty acids into *trans* isomers (Junker and Ramos, 1999), and by the increased synthesis of phospholipids (Segura *et al*., 2004). In addition to the physical barrier, this bacterium, and other solvent-resistance microorganisms, uses a series of efflux pumps, known in DOT-T1E as TtgABC, TtgDEF and TtgGHI, that remove toluene from the cell membranes (Isken and de Bont, 1996; Fukumori *et al*., 1998; Ramos *et al*., 1998; Mosqueda and Ramos, 2000; Rojas *et al*., 2001). The energy required by these efflux pumps for solvent extrusion is linked to the generation of the electron proton-motive force.

We decided to explore the organization of the *cyoABCDE* cluster in the solvent-tolerant *P. putida* DOT-T1E strain, and to generate a mutant in *cyoB* to analyse its behaviour under different growth conditions with regard to solvent sensitivity. Our results showed that the mutant strain grew in a wide variety of culture conditions, but that although the cells exhibited normal levels of efflux pump components, and were able to isomerize unsaturated fatty acids, they were hypersensitive to solvents. This does not seem to be result of the limited generation of proton-motive force, but rather to solvent-induced plasmolysis that may be triggered by wrinkles in the cytoplasmic membrane at the poles of the mutant cells, and invagination of the membranes as observed with scanning electron microscopy. These defects may account for the cells' extreme sensitivity to organic solvents.

Results

Transcriptional organization of the cyo *cluster of* Pseudomonas putida *DOT-T1E*

The *cyoB* gene cluster had been previously identified in several *P. putida* strains (Kobayashi *et al*., 1999; Petruschka *et al*., 2001; Dinamarca *et al*., 2002). Based on the sequence of the *cyo* cluster of *P. putida* KT2440, we designed suitable primers for the amplification of the chromosomal region of *P. putida* DOT-T1E, which was sequenced. Analysis revealed the presence of five open reading frames (ORF) corresponding to the *cyoABCDE* cluster (Fig. 1A, the sequence deposited at GenBank under accession number AY466718). Overall identity with the *P. putida* KT2440 *cyoABCDE* cluster was 99.1%, and when the translated products were compared it was close to 100%. Sequence analysis revealed short extragenic DNA sequences between contiguous genes (3–11 nucleotides) or overlapping ORFs (*cyoC* and *cyoD*), suggesting that the genes could form an operon. To determine whether all the genes were part of the same transcript, RT-PCR, with primers based on the 3′ and 5′ ends of contiguous genes was carried out with mRNA isolated from cells in the exponential and the stationary growth phase (Fig. 1B). Under both conditions, cDNA fragments of the expected sizes were found with all five genes. These results suggest that there is a single transcriptional unit made up of *cyoABCDE* in *P. putida* DOT-T1E.

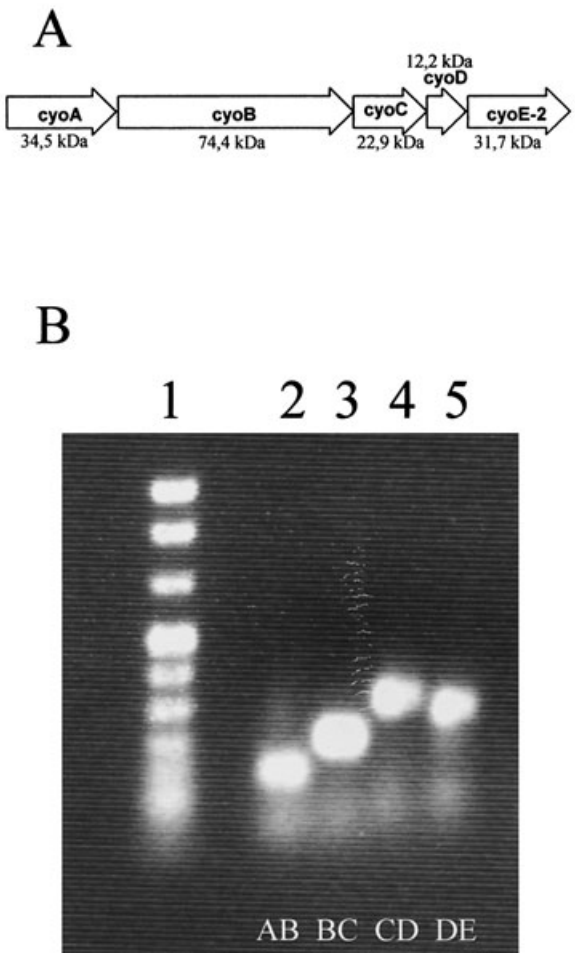

Fig. 1. Physical organization of the *cyoABCDE* cluster and evidence that the genes form an operon.
A. The sequence of the *cyoABCDE* operon can be accessed from GenBank (accession no. AY466718). The approximate sizes of the products of each ORF are given in kilodaltons below each gene.
B. The products resulting from each RT-PCR were separated on agarose gels. Lane 1, molecular weight markers. Lane 2, *cyoAB* amplification (expected size, 150 bp). Lane 3, *cyoBC* amplification (expected size, 210 bp). Lane 4, *cyoCD* amplification (expected size, 300 bp). Lane 5, *cyoDE* amplification (expected size, 290 bp). Control assays without RT yielded no cDNA.

Construction and characterization of a CyoB mutant

To generate a knock-out mutant in the *cyoB* gene we transferred the pEDX-1 plasmid into DOT-T1E as described in *Experimental procedures*, and selected Km^R clones. These appeared at a rate of about 10^{-7} per recipient cell, and 10 clones were examined by PCR with the appropriate primers for insertion of the Km^R cassette within the *cyoB* gene. We expected a 1.3 kb amplification band

in clones with a knock-out gene. Two of the clones yielded the expected band. Subsequently, we sequenced the mutant clones based on sequencing primers located at the end of the Km^R cassette. This allowed us to identify the position where the gene was disrupted by the Km^R cassette, which occurred between positions 2132 and 2133 in the sequence deposited at GeneBank. To further confirm the nature of the mutation we prepared total DNA from one of the clones, digested it with *Ava*l and hybridized it against the Km^R genes coding for the *cyoB* gene (not shown). This mutant was called *P. putida* DOT-T1E-CyoB.

We determined the growth rate of the wild type and the CyoB mutant in LB medium and minimal medium with glucose and citrate in the absence and in the presence of toluene ($logP_{ow}$ 2.5) supplied in the gas phase. In LB no significant difference was found in the absence of toluene (tg ≅ 46–49 min) or in the presence of toluene (tg ≅ 54–58 min) in the gas phase. In the presence of toluene, both the wild type and the mutant exhibited a 3 h lag before growth started. The wild-type strain grew at a similar rate on M9 minimal medium with glucose (tg ≅ 65–68 min) or citrate (tg ≅ 70–73 min) as the carbon source in the absence and in the presence of toluene. In contrast, growth of the mutant was slower than that of the wild type. In M9 minimal medium with glucose the growth rate was 83 min and 107 min in the absence and in the presence of toluene, respectively, whereas in M9 minimal medium but with citrate the doubling time was 88 and 119 min with and without toluene supplied in the gas phase respectively. Again a prolonged lag of about 5 h was found before the mutant or the wild type started to grow (not shown).

These results prompted us to test the effect of several solvents (supplied at 3 mM) *n*-decane ($logP_{wo}$ 5.6), *n*-heptane ($logP_{ow}$ 4.5), *n*-ethylbenzene ($logP_{wo}$ 3.1), *n*-propylbenzene ($logP_{ow}$3.6), *m*-xylene ($logP_{ow}$3.2), styrene ($logP_{ow}$3.0) and 1-octanol ($logP_{ow}$2.9) on the growth of the strain in M9 minimal medium with glucose. We found that octanol, styrene and toluene inhibited or fully prevented the growth of the CyoB mutant. In M9 minimal medium, ethyl- and propylbenzene delayed growth and *n*-heptane and *n*-decane did not affect growth as compared to a culture without solvent. The effect of solvent on growth of the CyoB mutant correlated with the log P_{ow} of the chemicals.

We then decided to study survival of the wild-type strain and the DOT-T1E-CyoB mutant in the log growth phase and in the stationary growth phase after a sudden toluene shock. Both types of cells were grown in LB or in M9 minimal medium with citrate in the absence and in the presence of toluene supplied at sublethal concentrations in the gas phase. The behaviour of the wild type grown in LB in the exponential phase was similar to our previous observation: one out of 10^4 non-induced cells survived the solvent shock, whereas >50% cells survived if preinduced with toluene (Fig. 2 and Ramos *et al*., 1998). We then analysed the survival of DOT-T1E in the stationary phase. Only one out of 10^6 non-induced cells in the stationary phase survived the solvent shock, whereas about one out of 10^3 cells survived the solvent shock when preinduced with toluene. This indicated that cells in the stationary growth phase were intrinsically more sensitive to toluene than cells in the exponential growth phase. The mutant DOT-T1E-CyoB cells were more sensitive to a sudden solvent shock than the wild type regardless of the growth conditions (Fig. 2). Particularly, in the stationary phase the number of viable cells after the solvent shock was negligible for non-induced cells and very low (one out of 10^4–10^5) for induced cells (Fig. 2).

We also analysed the behaviour of the wild type and mutant CyoB when grown on M9 minimal medium with citrate both in the absence and in the presence of toluene in response to a sudden toluene shock. We found that regardless of the growth conditions, the wild-type strain was more resistant than the mutant, particularly the CyoB mutant cells in the stationary phase were hypersensitive to the sudden toluene shock because no cells were recovered in non-induced cells, and fewer than one in 10^7 were recovered when induced cells were used (not shown). This contrasted with the survival rate of the wild-type strain which was in the order of 10^{-5} and 10^{-3} for non-induced and induced cells respectively.

Analysis of well-defined defence mechanisms against solvents in P. putida *DOT-T1E and its isogenic DOT-T1E-CyoB mutant*

Several possible mechanisms of organic solvent tolerance in bacteria have been reported (Diefenbach *et al*., 1992; Heipieper *et al*., 1992; Sikkema *et al*., 1994; Weber and de Bont, 1996; Ramos *et al*., 1997; White *et al*., 1997). The two main contributors are an increase in membrane packaging, achieved through *cis* to *trans* isomerization of unsaturated fatty acids, and a number of solvent efflux pumps.

As mentioned above, the growth curves of *Pseudomonas putida* DOT-T1E and the *P. putida* CyoB mutant in LB medium were similar when grown in the absence and in the presence of sublethal concentrations of toluene. We determined the composition of the fatty acids in the exponential and stationary phase in the wild-type and mutant cells under those growth conditions. The results revealed that in both the wild-type and the CyoB mutant cells, the pattern of fatty acid composition was similar, with an increase in the level of *trans* isomers in the presence of toluene which was particularly evident in the stationary phase (Table 1). It also should be noted that because of this high level of C16 : 1 *trans* in the presence of toluene, the level of C17 : cyclo was much lower in these cells

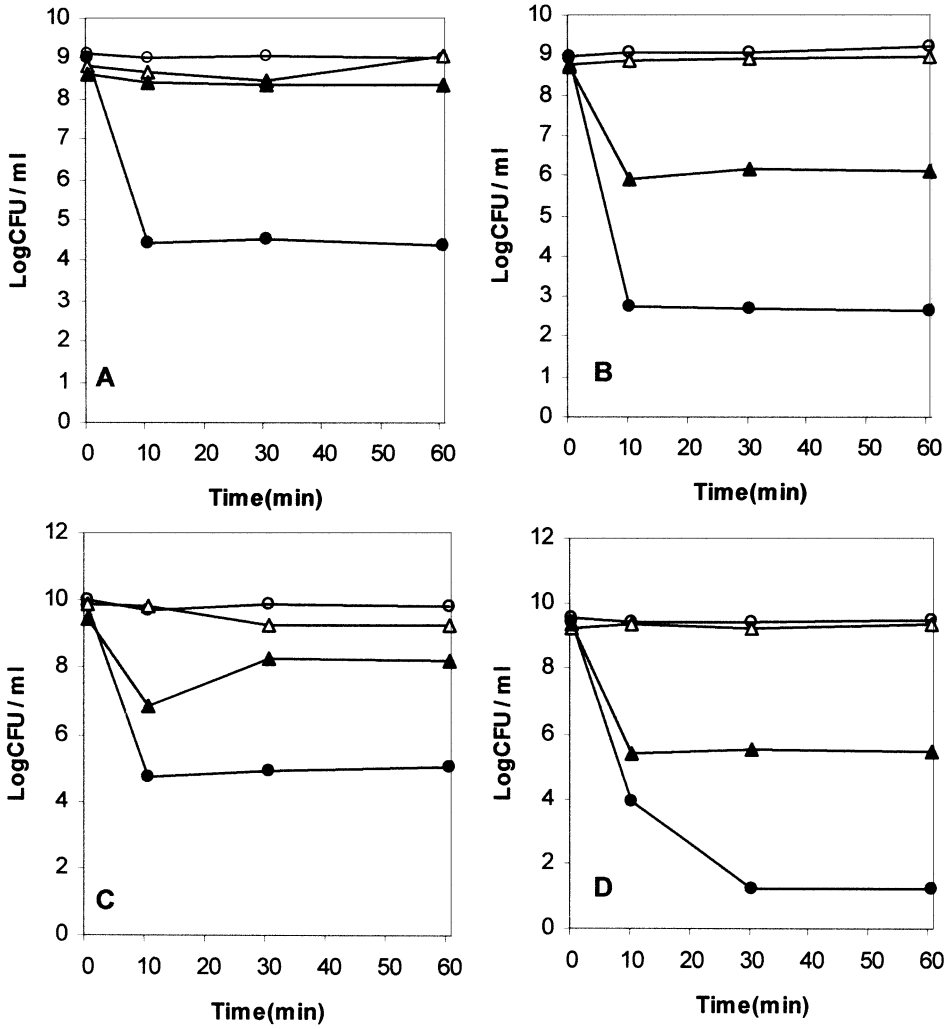

Fig. 2. Survival of *P. putida* DOT-T1E and the CyoB mutant. Cells were grown in 30 ml of LB medium (circle) or LB medium with toluene in the gas phase (triangles) until the cultures reached a turbidity of 0.8 at 660 nm (log phase) or a turbidity of about 3.0 at 660 nm (stationary phase). The cultures were divided in two halves; to one of them 0.3% (v/v) toluene (closed symbols) was added, whereas the other was kept as a control (open symbols). The number of viable cells was determined at the indicated times.
A and B. Wild-type in the log and stationary phase respectively.
C and D. *cyoB* mutant in the log and stationary phase respectively.

compared with cells grown in the absence of toluene (Table 1). We also determined the composition of the phospholipid head groups in the wild-type and the mutant strain in the log- and stationary growth phase in cells grown in the absence and in the presence of toluene. The composition was similar in the wild-type and the mutant strain regardless of the growth conditions. The main phospholipid head group was phosphatidylethanolamine (70%–76.6% of total), with similar levels of phosphatidylglycerol (12.7%–14%) and cardiolipin (10.3%–15.2%). Therefore the increased solvent sensitivity of DOT-T1E seems not to be related to defects in the pattern of phospholipids.

Rojas *et al*. (2001) demonstrated that up to three efflux pumps were involved in solvent tolerance. To analyse the level of expression of these pumps in the wild-type and in the CyoB mutant strain, we transformed wild-type and CyoB mutant cells with plasmids pED14, pMTT and pANA96, which carry a fusion of the *ttgA*, *ttgD* and *ttgG* promoter regions to a promoterless '*lacZ* gene (results are shown in Table 2). The pattern of expression of all three efflux pumps in the wild type and the mutant was similar. In the log phase, *ttgA*, *ttgD* and *ttgG* were expressed at a higher level than in the stationary phase, which suggest that the expression of the efflux pumps may not be altered in the CyoB mutant. It appears that the lower survival of the cells in the stationary phase with respect to cells in the log phase could be due the diminished ability of the cells in the stationary phase to remove solvents.

Table 1. Phospholipid composition of P. putida DOT-T1E and its isogenic CyoB mutant cells growing in the absence and in the presence of toluene in the exponential and the stationary phase.

	Exponential phase				Stationary phase			
Strain Media	T1E LB	CyoB LB	T1E LB + toluene	CyoB LB + toluene	T1E LB	CyoB LB	T1E LB + toluene	CyoB LB + toluene
C14 : 0	0.3	0.4	0.5	0.5	0.6	0.4	0	0
C15 : 0	0	0.2	0	0	0.5	0.4	0	0
C16 : 1 *cis*	35	36.0	26.3	31.4	12.3	18.5	6.4	5.3
C16 : 1*trans*	1.8	2.6	8.9	8.3	2.2	2.1	24.7	22.4
C16 : 0	34.1	35.9	38.6	42.6	52.0	47.8	40.9	45.9
C17 : cyclo	1.6	3.3	0.2	0.4	18.0	11.5	4.1	3.7
C17 : 0	0	0.2	0	0	0.4	0.4	0	0
C18 : *cis* vacc	26.4	20.7	24.6	16.5	13.0	18.1	8.8	12.8
C18 : *trans* vacc	0	0	0	0	0.4	0	14.	9.0
C18 : 0	0.8	0.7	0.8	0.5	0.6	0.7	0.8	0.9
C19 : ciclo	0	0	0	0	0	0	0	0
cis/trans	34.1	22.2	5.68	5.74	9.65	17.06	0.39	0.57
sat/unsat	0.55	0.63	0.66	0.77	1.94	1.29	0.77	0.95

Analysis of the proton-motive force in P. putida *DOT-T1E and its isogenic Cyo mutant*

The similar growth rate in the exponential phase of the wild type and the CyoB mutant suggested that, as occurs in *E. coli*, the Cyd terminal oxidase successfully replaces the Cyo terminal oxidase. However, the increased solvent sensitivity of CyoB may be linked to limited generation of proton-motive force. We used the method described by Llamas *et al*. (2003) to determine the ability to generate proton-motive force in cells of the wild-type and mutant CyoB strain grown on LB with or without toluene in the gas phase, when cells reached the mid-log phase or the early stationary phase. The results revealed that the magnitude of proton-motive force was similar in both strains, suggesting that proton-motive force seems not to be the limiting factor (not shown).

To rule out proton-motive force as the limiting factor, we tested the sensitivity of DOT-T1E CyoB to antibiotics which are removed through RND pumps, i.e. Tc, Ap, Cb, Pip, Gm, Cm and Nal. The MICs (μg ml^{-1}) of wild-type and CyoB mutant were identical [(ampicillin > 1000; carbenicillin > 400; Pip, 100; Tc, 20; Gm, 20; Cm, 324 and Nal, 50)], which supports no limitation in the generation of proton-motive force to the extrusion of drugs.

Table 2. Expression of the efflux pumps in cells growing in the absence and in the presence of toluene in the exponential and the stationary phase.

	Toluene	PttgA	PttgD	PttgG
Exponential				
Wild-type	–	120	20	310
Wild-type	+	140	45	1530
CyoB-mutant	–	120	20	305
CyoB-mutant	+	170	40	1930
Stationary				
Wild-type	–	80	1	130
Wild-type	+	50	5	420
CyoB-mutant	–	50	1	120
CyoB-mutant	+	40	5	480

The strains were transformed with pED14 (P_{ttgA}::*lacZ*), pMTT(P_{ttgD}::*lacZ*) or pANA96 (P_{ttgG}::*lacZ*) and cells were grown in the absence (–) or in the presence (+) of toluene until they reached the mid-exponential phase (i.e. OD_{660} 0.8–1) or the stationary phase (i.e. OD_{660} 3–3.5). β-galactosidase activity was determined in duplicate in two independent assays. The data are rounded values of the averages with standard deviations in the range of 5% to 17% of the given values.

Scanning electron microscopy revealed defects at the poles of the cyoB *mutant*

The above findings suggested that the basic solvent defence mechanisms such as efflux pumps, energy generation and fatty acid modifications were operational in the CyoB mutant. Consequently, the increased sensitivity of the CyoB mutant cannot be ascribed to defects in specific defence mechanisms. What, then, makes this strain more sensitive to solvents than the parental one? We previously reported that an OprL-null mutant that exhibited altered cell morphology was hypersensitive to toluene (Ramos *et al*., 1997). To test the integrity of the outer membrane we transformed the wild type and CyoB mutant with plasmid pJB3Km. We found that no β-lactamase was released to the outer medium, suggesting that outer membrane integrity is not affected (not shown). On the other hand, De Smet *et al*., 1978) showed that solvents lead to alterations in the cytoplasmic membrane. This led us to examine the wild type and CyoB mutant inner membrane in cells in the exponential and stationary phases under different growth conditions.

Cells were prepared for transmission electron microscopy as described in *Experimental procedures*. We found that in the absence of solvent, the wild-type and mutant

cells both in the log and stationary phase presented normal long rod morphology with a well-defined inner membrane and a highly electron-dense cytoplasm (Fig. 3). In the presence of toluene the wild-type cells in the log and stationary phase showed its typical morphology (Fig. 4); however, *cyoB* mutant cells, although they conserved the rod morphology, showed a number of vesicles on the cell surface and retraction of the inner membrane (Fig. 4B and C). In fact the cytoplasmatic membrane detached from the cell wall at the poles, retracted, and formed plasmolysis cavities starting at the poles, as described by Mulder and Woldring (1993) in *Escherichia coli* under hypertonic treatment. Further details were observed when cells were analysed under scanning electron microscopy. In contrast with the well-defined morphology of the wild-type cells, the mutant CyoB cells exhibited non-polar and polar invaginations (Fig. 5).

We wondered whether the defects observed in the CyoB mutant were specific to this strain or occurred in any other toluene-sensitive mutant. To investigate further we used scanning electron microscopy to examine *P. putida* DOT-T1E-18, a mutant that lacks the TtgABC efflux pump and exhibits increased solvent sensitivity (Ramos *et al*., 1998). In this strain we observed no invaginations in cells growing under different conditions. It would therefore seem that the mutation in the CyoB cluster leads to a series of morphological alterations that make cells hypersensitive to toluene.

Discussion

CyoB is reported to be one of the subunits of cytochrome *o* terminal oxidase, and is required for the assembly of the metal centres in the Cyo system (Chepuri *et al*., 1990;

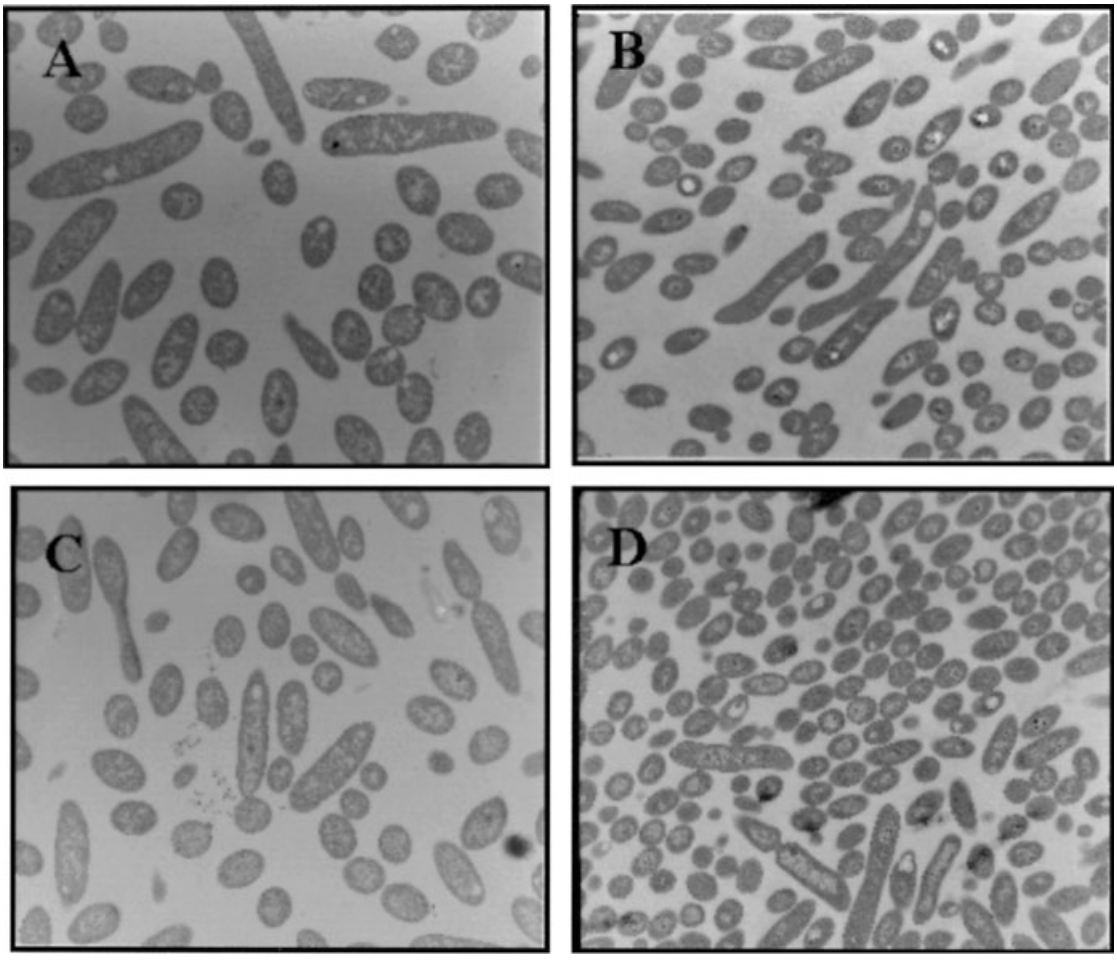

Fig. 3. Ultrastructure of *P. putida* DOT-T1E and the CyoB mutant. Cells were grown on LB medium and prepared for electron microscopy as described before (Rodríguez-Herva *et al*., 1996).
A. Wild-type strain in the exponential phase.
B. Wild-type strain in the stationary phase.
C. CyoB mutant in the exponential phase.
D. CyoB mutant in the stationary phase. A–D, magnification, 28 000×.

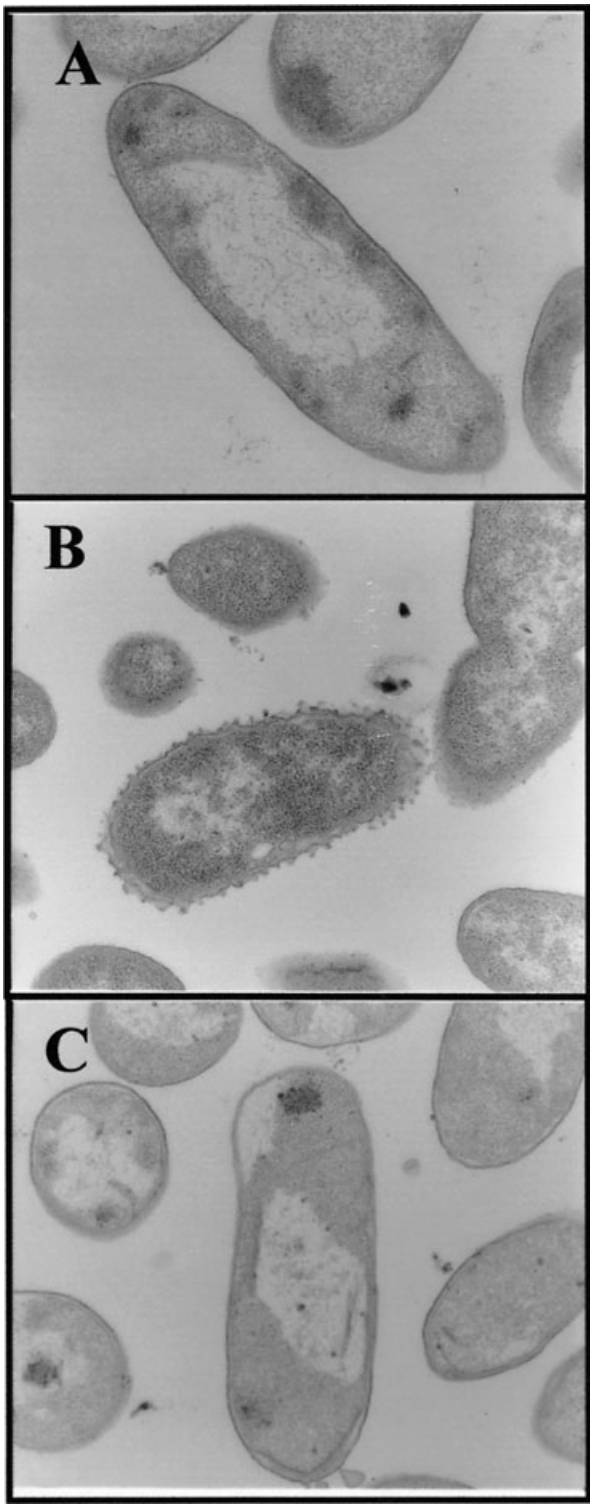

Fig. 4. Ultrastructure of *P. putida* DOT-T1E and CyoB mutant growing on LB medium with toluene supplied via the gas phase.
A. Wild-type cells in the stationary phase.
B and C. CyoB mutant cells in the log (B) and stationary phase (C).
Magnification, 40 000×.

Fukaya *et al*., 1993; Puustinen *et al*., 1989). The cytochrome *o* branch of the respiratory chain is expressed at a low level in cells in the stationary phase, and at a high level in cells growing exponentially (Anraku and Gennis, 1987; Armitage, 1997; Rice and Hempfling, 1978; Sweet and Peterson, 1978; Dinamarca *et al*., 2002). In contrast, the cytochrome *d* (*cio*) terminal oxidase is expressed mainly in the stationary growth phase in Gram-negative bacteria such as *E. coli* and *P. putida* (Bradford, 1976; Nakamura *et al*., 1997; Sweet and Peterson, 1978). Given that solvent extrusion requires energy, we were tempted to consider that the link between the lack of a component of the electron transport chain and the increased solvent sensitivity of the CyoB mutant resided in the mutant's inability to generate enough proton-motive force. However, our data rule out this possibility because the magnitude of the proton-motive force was similar, and the growth rate of the Cyo mutant was equivalent to that of the wild-

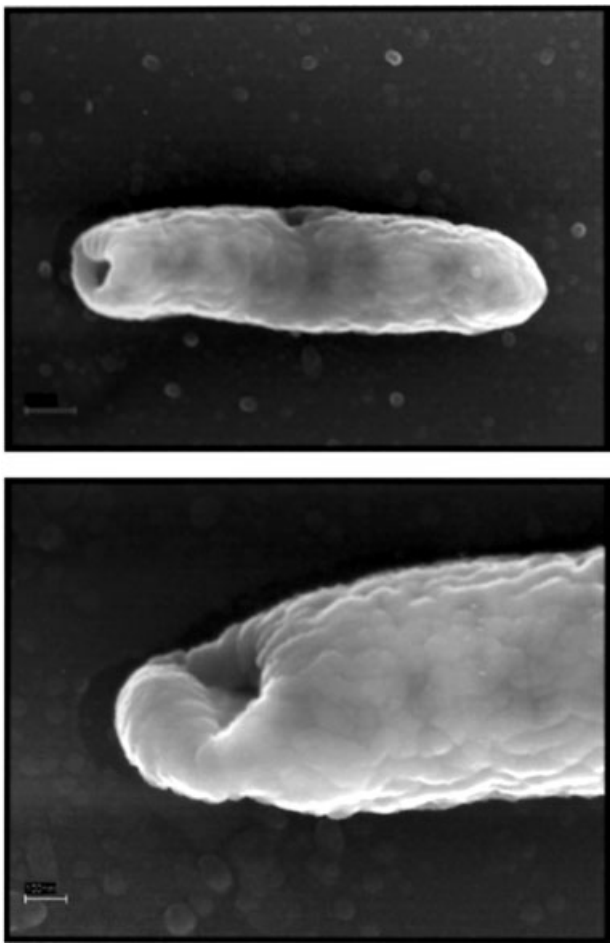

Fig. 5. SEM of *P. putida* CyoB mutant. Cells were grown on LB medium with toluene, harvested in the exponential phase of growth and treated for SEM as described in *Experimental procedures*.
A. Top, magnification, 40 000×.
B. Bottom, magnification, 100 000×.

type strain in different growth media, and also because the RND efflux pumps that extrude antibiotics were functional in the Cyo mutant, as revealed by a similar pattern of antibiotic resistance in the wild-type and the Cyo mutant. It was reported in *E. coli* that Cyd system was used instead of Cyo in *cyo*-deficient mutants. This seems also to be the case in *P. putida*, because the results presented here show that inactivation of cytochrome *o* ubiquinol oxidase (Cyo) had no significant effect on the growth rate in the logarithmic phase under the growth conditions we tested.

Dinamarca *et al*. (2002) showed that the *cyo* genes of *P. putida* were expressed preferentially in the log phase. Based on this finding we expected solvent sensitivity in CyoB mutants to be greater in the log phase that in the stationary phase. However, our results with the wild type revealed that as far as tolerance to highly toxic solvents was concerned, non-induced and induced cells of *P. putida* DOT-T1E were more sensitive to a solvent shock when cells reached the stationary phase (see Fig. 2). This situation was exacerbated in the CyoB mutant: no cells were found to survive a sudden toluene shock in the stationary phase. We found that the expression of the efflux pumps decreased in the stationary phase in the wild-type and Cyo mutant, a finding which correlates with the increased solvent sensitivity. Whether the reduction in *ttg* expression in the stationary phase is the result of a type of global regulation (Duque *et al*., 2001; Rojas *et al*., 2001) or whether it is mostly related to the physiological status of the cells remains unknown. In this regard, it should be noted that Dinamarca *et al*. (2002) and Petruschka *et al*. (2001) also found that Cyo mutants escape from catabolite repression, and speculated that the redox state of the respiratory chains could influence the expression of the Crc catabolite repression protein (O'Toole *et al*., 2000; Dinamarca *et al*., 2002). It may well happen that *P. putida* DOT-T1E cells sense the redox state of the membrane, which is transduced in a signal that influences the control of the expression of TtgABC, TtgDEF and TtgGHI in the stationary phase.

The lipid bilayer of the outer membrane is closely attached to the peptidoglycan through lipoproteins present in the inner leaflet, and non-covalent interactions with other outer membrane proteins (Mulder and Woldringh, 1993; Schwarz and Koch, 1995; Korber *et al*., 1996). The poles, however, are 'free' of peptidoglycan and can present a point of cell weakness. It is also known that ingrowth of the outer membrane can be dissociated from ingrowth of the division septum by mutations, and that this affects the attachment of the murein layer to the outer membrane, which in turn favours plasmolysis (Rothfield and Justice, 1997; Fung *et al*., 1978). Our EM studies have revealed that solvent exposure in the CyoB mutant induces plasmolysis from one of the poles. The lack of the CyoB mutant itself is unlikely to directly affect solvent sensitivity but the lack of CyoB could result an inappropriate signal transmission that negatively influences cell architecture in the poles. In this context we should note that Segura *et al*. (2001; 2004) demonstrated that solvent tolerance is compromised in certain flagellarless mutants in *P. putida* DOT-T1E, and that this strain has a single flagellum located in one of the poles of the cell.

Experimental procedures

Bacterial strains, plasmids and culture media

Pseudomonas putida DOT-T1E (Rif^R, Tol^R, Tol$^+$) (Ramos *et al*., 1995) and DOT-T1E-CYOB (this study) were grown at 30°C in LB medium or in M9 minimal medium supplemented with glucose (0.5% wt/vol) or citrate (10 mM), as the carbon source. *Escherichia coli* DH5α (F′/*hsdR17*, *recA1*, *gyrA*) cells were grown on LB medium at 37°C, and used for cloning experiments.

Plasmids pUC18 and pUC19 (Ap^R) were used for cloning (Sambrook *et al*., 1989). Plasmid pRK600 (Cm^R, *mob*$^+$, *tra*$^+$, ColE1 replicon) was used as a helper for the mobilization of *tra*$^-$, *mob*$^+$ plasmids (Herrero *et al*., 1990). Derivatives of the suicide plasmid pCHESIΩKm (Km^R, *mob*$^+$, ColE1 replicon) were used to generate *in vivo* knock-outs of target genes (Llamas *et al*., 2003).

The antibiotics used were ampicillin (Ap), 100 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; piperacillin (Pip), 100 μg ml^{-1}; rifampicin (Rif), 20 μg ml^{-1}; streptomycin (Sm), 50–100 μg ml^{-1}, and tetracycline (Tc), 15 μg ml^{-1}.

Solvent shock assay

Cells were grown in 30 ml LB medium or M9 minimal medium with 10 mM citrate with or without toluene in the gas phase overnight. On the following day the cultures were diluted 1 : 50 in the same medium and grown under the same conditions until the culture reached the mid-exponential growth phase (turbidity about 0.8 at 660 nm) or the early stationary phase (turbidity about 2.0 at 660 nm). The cultures were divided in two halves; to one we added 0.3% (v/v) toluene, and the other was kept as a control. The number of viable cells in the log and stationary growth phase was determined before toluene was added, and 10, 30 and 60 min later.

DNA techniques

Plasmid DNA was isolated by the alkaline lysis method with the QIAprep Spin Plasmid Minipreps Kit. Total DNA was isolated with a modification of the method of Kado and Liu as described by Ramos-González *et al*. (1991), except that the 30 min incubation step at 55°C was omitted. DNA digestions with restriction enzymes, ligations, and transformations were performed by standard procedures (Sambrook *et al*., 1989).

Polymerase chain reactions (PCRs)

The standard PCR mixture (25 μl) contained 10 ng of DNA, 200 μM each deoxynucleoside triphosphate, 50 pmol of each primer, 2 μl of dimethyl sulphoxide, and 0.25 U of *Taq* polymerase. The PCR conditions were as follows: 4 min at 95°C, and then 35 cycles of 60°C for 45 s, 72°C for 30–180 s, and 94°C for 4 s, followed by a final 5 min step at 72°C.

Generation of a cyoB *mutant by insertional inactivation*

To generate a ∇-*cyoB*:Km *P. putida* mutant strain, a 500 bp DNA fragment of the *cyoB* gene was amplified by PCR from the *P. putida* DOT-T1E chromosome using the primers: 5′-GGAATTCTGCACTCGCTCTGCGACTGTTGA-3′ and 5′-CGAATTCATGCCACCGATGGAGAAGGTGAT-3′, which contain *Eco*RI sites at their 5′-terminal ends. The amplified product was digested with *Eco*RI and cloned at the *Eco*RI site of plasmid pCHESIΩKm (Llamas *et al*., 2003), yielding plasmid pEDX-1. This plasmid was designed for marker exchange mutagenesis because it replicates in *E. coli* but not in *P. putida,* and carries a Km^R cassette to select for insertion of the plasmid in the host chromosome. Plasmid pEDX-1 was transferred to *P. putida* DOT-T1E in triparental matings with pRK600 as the donor of transfer functions. Transconjugants incorporating the Km^R cassette were selected on M9 minimal medium with citrate as the sole carbon source, and Km and Ap. These cells were expected to contain pEDX-1 integrated into the chromosome and to generate a mutant by insertional inactivation at the *cyoB* gene. The absence of the wild-type allele and the presence of the *cyoB*::Km allele were confirmed by PCR and Southern blot. One of the clones was retained for further studies and was called DOT-T1E-CyoB.

Southern hybridization and DNA labelling

DNA fragments were separated in agarose gels and transferred onto nylon membranes by capillary blotting as previously described (Sambrook *et al*., 1989). Specific probes for hybridization were recovered from agarose gels with an agarose gel DNA extraction kit (Boehringer Mannheim). All probes were labelled with digoxigenin by Klenow random primer extension according to the recommended procedure (Ausubel *et al*., 1991). Blotted filters were prehybridized, hybridized, washed and immunologically developed according to the supplier's instructions. High-stringency conditions [50% (v/v) formamide at 42°C] were used.

Assay for β-galactosidase

An overnight culture of the appropriate strain was diluted to a final turbidity (A_{600}) of ~0.04 in fresh LB medium and cultures were grown at 30°C. When turbidity reached 0.08 the inducer was added, and 4 h later aliquots were taken and β-galactosidase activity measured as described by Miller (1972). At least three independent assays were performed in each case.

Analysis of phospholipids

Phospholipids were extracted by the method of Bligh and Dyer (1959). To measure fatty acids, phospholipids were *trans* esterified by dissolving 3–10 mg of phospholipid extract in 350 μl of hexane. Then 40 μl of 2 M KOH dissolved in methanol was added and mixed vigorously. After the addition of 2 ml of hexane, the upper (hexane) phase was recovered and, within 1 h, methyl ester derivatives of fatty acids were identified by mass spectrometry after gas chromatographic separation as described previously (Ramos *et al*., 1997).

To measure the phospholipid headgroups, the chloroform layer was dried by evaporation under a stream of nitrogen. The lipid residue was dissolved in chloroform and the different phospholipids were separated on silica-gel 60 G thin layer chromatography plates, using a mixture of chloroform/methanol/acetic acid glacial (65 : 25 : 8) as solvent.

The spots corresponding to each phospholipid were visualized by exposing the plates to iodine vapours and scraped. The phospholipids were then quantified by Bartlett's method (1959).

Determination of $\Delta\psi$

Cells were converted to spheroplasts by treatment with lysozyme and ethylenediaminetetraacetic acid (EDTA). The cells were grown to an OD_{660} of ~0.5–0.7 in M9 minimal medium supplemented with glucose or citrate in the absence and in the presence of toluene supplied via the gas phase. Cultures (50–100 ml) were centrifuged (10.000 *g*, 5 min, at 4°C), and the cells were resuspended in 6.3 ml of 0.75 M sucrose and 10 mM Tris-HCl (pH 7.8), and then treated with 0.1 mg ml–1 of lysozyme for 2–5 min on ice. Then 13.2 ml of 1.5 mM EDTA (pH 7.5) solution was added. The cell suspension was incubated for 15 min at 30°C and observed under a phase-contrast microscope. More than 95% of the cells appeared as spheroplasts. The generation of Dy in spheroplasts of the wild-type strain and the CyoB mutant was monitored using the cationic dye 3,3'-diethylthiadicarbocyanine iodide [$DiSC_2(3)$], which translocates into the lipid bilayer of hyperpolarized membranes, resulting in quenching of its fluorescence. The reaction mixture (total volume 1 ml) contained buffer A [125 mM Hepes, 0.9% (w/v) NaCl, 1 mM KCl, 1 mM MgCl2 and 0.4% (wt/vol) glucose], spheroplasts (about 10^6) and 0.08 μM $DiSC_2(3)$. The fluorescence emission of $DiSC_2(3)$ was measured at 570 nm with excitation at 555 nm using an SLM Aminco SPF-500C fluorimeter. To dissipate the membrane potential, valinomycin (2 mM final concentration) was added to the reaction mixture. The membrane potential was determined as the difference between the fluorescence quenching of $DiSC_2(3)$ when it accumulated in the cytoplasmic membrane, and the fluorescence obtained when it was released upon the addition of valinomycin.

Microscopy studies

Pseudomonas putida cells grown in LB medium were harvested in the logarithmic or stationary growth phase and subjected to microscopic analysis. For transmission electron microscopy (TEM), samples were prepared and observed as previously described (Rodríguez-Herva *et al*., 1996). For

scanning electron microscopy (SEM), cells were fixed with glutaraldehyde vapors for 24 h in a humid chamber at 4°C. Then the cells were rinsed with distilled water, dehydrated with a graded series of ethanol solutions, suspended in amyl acetate, critical-point dried, and coated with gold. Samples were examined in Zeiss DSM950 scanning electron microscope.

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Functional replacement of the outer membrane protein TtgC in the TtgABC efflux pump in antibiotic resistance in *Pseudomonas putida* DOT-T1E

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La clave en la resistencia de la cepa altamente tolerante a disolventes orgánicos *Pseudomonas putida* DOT-T1E es la presencia de tres bombas de extrusión (TtgABC, TtgDEF, TtgGHI) que pertenecen a la familia RND la cual exporta compuestos tóxicos tales como antibióticos y disolventes orgánicos. Los transportadores de la familia RND normalmente son complejos tripartitos compuestos por un sistema de extrusión que está localizado en la membrana interna junto con una proteína de membrana externa (OMP) y una proteína de fusión de membrana (MFP) como proteínas auxiliares.

Para estudiar el intercambio de componentes entre ellas, construimos bombas de extrusión híbridas. Observamos que a nivel de resistencia un intercambio funcional tuvo lugar cuando TtgC (OMP) fue sustituida por componentes de otras bombas. Aunque en nuestros estudios el reemplazo de TtgA (MFP) fue funcional en cuanto a la expulsión de antibióticos, para confirmar este resultado son necesarios muchos análisis.

Manuscrito en preparación

Functional replacement of the outer membrane protein TtgC in the TtgABC efflux pump in antibiotic resistance in *Pseudomonas putida* DOT-T1E

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SUMMARY

In *Pseudomonas putida* DOT-T1E three efflux pumps (TtgABC, TtgDEF, TtgGHI) that belong to the RND family have been described as able to extrude toxic compounds such as antibiotics and organics solvents. Transporters of the RND family usually consist of an efflux system that is made up by one component that is located at inner membrane, and is often associated with an auxiliary outer membrane protein (OMP) and a membrane fusion protein (MFP). In order to study the exchange between components among these three efflux pumps, we have constructed hybrid efflux pumps. The results show that functional replacement took place when the TtgC (OMP) was substituted by other pump components. Although we found some indications of functional replacement of the TtgA (MFP) component, further analysis will be necessary to confirm that result.

INTRODUCTION

Pseudomonas putida DOT-T1E is an antibiotic- and solvent-tolerant strain isolated from a wastewater treatment plant in Granada (Ramos *et al*., 1995). The common basis of the resilience of this strain is its ability extrude toxic compound by means of efflux pumps (Ramos *et al*., 2002; Daniels and Ramos 2009). Two efflux pumps of the RND (resistance, nodulation, cell division) family, named TtgABC (Ramos *et al*., 1998; Mosqueda and Ramos, 2000) and TtgGHI (Rojas *et al*., 2001), have been shown to be involved in solvent and antibiotic resistance, while a third efflux pump, named TtgDEF, has been implicated in the efflux of solvents but not antibiotics (Rojas *et al*., 2006). The TtgGHI efflux pump is the main determinant of solvent-tolerance in *Pseudomonas putida* DOT-T1E, while its participation in antibiotic efflux is masked by the presence of the TtgABC efflux pump. The RND family of efflux pumps have been extensively studied in recent years due to their involvement in the multidrug resistance observed in clinically interesting strains such as *Pseudomonas aeruginosa* (Poole, 2004). Transporters of the RND family usually consist of a efflux system that is made up by one component that is located at inner membrane, and is often associated with an auxiliary outer membrane protein (OMP) and a membrane fusion protein (MFP).

The most studied RND efflux pump is AcrAB-TolC of *E. coli* (Osuku *et al.*, 1996). The genes that make up this pump form an operon (*acrAB*), while the OMP (*tolC*) is monocistronic. It is also known that TolC can act as the outer membrane element for other transport systems, such as the haemolysis transporter HylD (Dinh et al., 1994). In *Pseudomonas aeruginosa* some

efflux transporters are transcribed together with the corresponding OMP and MFP, e.g., *mexAB-oprM* or *mexCD-oprJ* (Li *et al.*, 1995; Poole *et al.* 1996), while others are only transcribed with the MFP, e.g., *mexXY* (Aires *et al.*, 1999). In *P. putida* DOT-T1E each of the three components of the TtgABC, TtgDEF and TtgGHI efflux pumps are transcribed together. The fact that multiple efflux pumps are present in the same cell has lead to the question of the specificity of the assembly of the different components of the efflux pumps. Previous experiments suggested that in some cases the OMP of one efflux pump can functionally replace the OMPs of other efflux pumps, as has been shown for OprM, which can replace OprJ and OprN in the MexCD-OprJ and MexEF-OprN systems (Srikumar *et al*., 1997; Gotoh, et al., 1998; Maseda *et al.,* 2000; Elkins and Nikaido, 2003). However, functional replacement between different MFPs was not possible (Yoneyama *et al*., 1998; Mokhonov *et al*., 2004).

To study if the different components of the three efflux pumps involved in antibiotic and solvent tolerance in *P. putida* DOT-T1E can be functionally exchanged, we constructed hybrid efflux pumps and studied their phenotypes. Our results show that, similar to what has been observed in other systems, functional replacement is possible between the different OMPs of the TtgABC, TtgDEF and TtgGHI efflux pumps. Surprisingly, we also observed signs of functional replacement between the MFPs proteins of these pumps, although further studies will be necessary to confirm the results.

RESULTS

Replacement of TtgC of the TtgABC efflux pump by other OMPs leads to functional efflux pumps.

The strain that we used to test the functionality of potential hybrid efflux pumps was *P. putida* DOT-T1E-34S. DOT-T1E-34S is a derivative of the DOT-T1E-PS34 triple mutant (*ttgB:phoA::km; ttgD::KilAB; ttgH:ΩSm*) (Rojas et al., 2001) that was selected because it has lost the pGRT-1 plasmid that bears the *ttgGHI* genes (Rodríguez-Hervá, 2007). As such, within this strain, the TtgABC, TtgDEF efflux pumps are inactivated and the TtgGHI system is absent. To facilitate the handling of the different efflux pump sequences, we introduced restriction sites at the 5' and at the 3' ends of each of the genes of each operon so that they could be recombined in a vector. Our cloning strategy is shown in Figure 1. To construct hybrid pumps each of the genes of the three operons were cloned and sequenced and those clones without errors were chosen to construct the hybrid efflux pumps. As a proof of principle, plasmid pVAN-1 bearing a reconstructed TtgABC pump (pBBR1MCS5:*ttgABC*) was introduced into *P. putida* DOT-T1E-34S to test its resistance towards different antibiotics. As shown in Table 1, the expression of *ttgABC* from the plasmid promoter was sufficient to increase resistance towards several antibiotics. A control experiment in which *P. putida* DOT-T1E-34S was transformed with an empty vector did not exhibit increased antibiotic resistance. This series of results indicated that the reconstructed efflux pump was functional in *Pseudomonas* and that the introduction of the restriction sites at the end of the genes did not prevent the expression of a functional efflux pump.

The *ttgC* gene was then replaced by *ttgF* or *ttgI* in plasmids pVAN-2 and pVAN-3. Transformation of these plasmids into *P. putida* DOT-T1E-34S lead to increased resistance toward different antibiotics (Table 1). Interestingly, the TtgABI efflux pump was more efficient at extruding piperacillin than the wild-type efflux pump, based on the level of resistance observed.

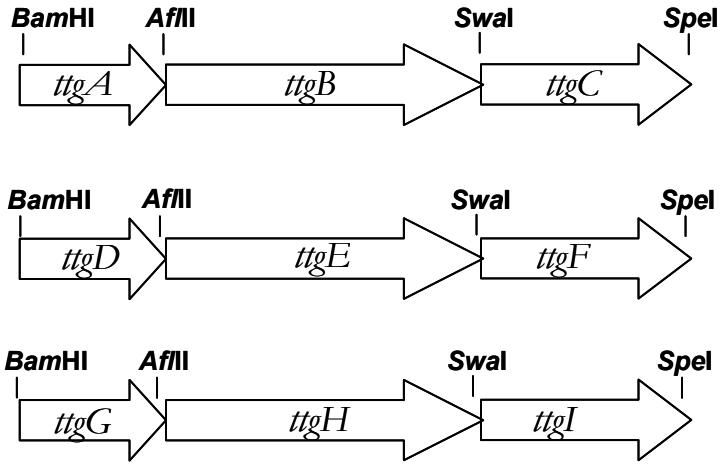
Replacement of TtgA in the TtgABC efflux pump by other MFPs

Replacement of the MFP, TtgA, by the homologous MFPs (TtgD or TtgG) to yield TtgDBC and TtgGBC resulted in pumps that also showed increased antibiotic resistance when compared with the recipient strain (Table 1). RT-PCR experiments showed that *P. putida* DOT-T1E-34S synthesized *ttgA* mRNA (not shown) so that in these two strains (*P. putida* DOT-T1E-34S (pVAN-3) and *P. putida* DOT-T1E-34S (pVAN-4)) *ttgA* may be expressed along with the introduced *ttgD* or *ttgG* component, and

TABLE 1. Antibiotic resistance of the strains containing the different hybrid efflux pumps.

Strain	pump	Ap	Tc	Cb	Pip
P. putida DOT-T1E		2000	15	2000	35
P. putida DOT-T1E-34S		125	0.23	7.8	1.1
P. putida DOT-T1E-34S (pVAN-1)	*ttgABC*	1000	0.94	250	4.4
P. putida DOT-T1E-34S (pVAN-2)	*ttgAB**F***	125	0.06	62.5	4.4
P. putida DOT-T1E-34S (pVAN-3)	*ttgAB**I***	500	0.23	500	35
P. putida DOT-T1E-34S (pVAN-4)	*ttg**D**BC*	500	0.94	500	4.4
P. putida DOT-T1E-34S (pVAN-5)	*ttg**G**BC*	500	0.47	500	8.8
P. putida DOT-T1E-34S (pVAN-6)	*ttg**D**B**F***	250	0.47	250	8.8
P. putida DOT-T1E-34S (pVAN-7)	*ttg**D**B**I***	500	0.94	250	8.8
P. putida DOT-T1E-34S (pVAN-8)	*ttg**G**B**F***	250	0.47	125	17.6
P. putida DOT-T1E-34S (pVAN-9)	*ttg**GBI***	250	0.23	125	4.4
P. putida DOT-T1E-34S (pVAN-10)	*ttgAEC*	62.5	0.47	7.8	0.56
P. putida DOT-T1E-34S (pVAN-11)	*ttgAEF*	62.5	0.23	7.8	0.56
P. putida DOT-T1E-34S (pVAN-12)	*ttgAEI*	62.5	0.12	7.8	0.56
P. putida DOT-T1E-34S (pVAN-13)	*ttgDEC*	62.5	0.47	7.8	1.1
P. putida DOT-T1E-34S (pVAN-14)	*ttgDEF*	125	0.47	7.8	0.56
P. putida DOT-T1E-34S (pVAN-15)	*ttgDEI*	62.5	0.23	7.8	0.56

therefore the possibility of the formation of the native TtgABC efflux pump can not be excluded. However, when both the OMP (TtgC) and the MFP (TtgA) were replaced by other pump components not only all of the combinations were functional regarding antibiotic extrusion but their antibiotic resistance was higher than that of the corresponding efflux pumps with the TtgA component alone. Differences were observed between the extrusion ability of these chimeras, as the TtgDBF and TtgGBF pumps were more resistant toward tetracycline than the TtgABF efflux pump. The strain containing TtgDBF also showed a 4-fold increased resistance toward carbenicillin in comparison to the TtgABF containing strain. Similarly, TtgDBI conferred higher tetracycline resistance than TtgABI. These results suggest that TtgA can be functionally exchanged by TtgD and TtgG.



ttgA:	Forward: pE2	CCCGCGCTTGTAGAAGGCCG
	Reverse: AflII-2	GAACTTCGACAT**CTTAAG**GTTTACTCCGCTT
ttgB:	Forward AflII-1	AAGCGGAGTAAAC**CTTAAG**ATGTCGAAGTTC
	Reverse: SwaI-2	GACTTGGTCA**TTTAAA**TCATTGCCCAGCCTC
ttgC:	Forward SwaI-1	GAGGCTGGGCAATGA**TTTAAA**TGACCAAGTC
	Reverse: Spe-C	**ACTAGT**GATCAGCCTTTCGGTGTTTCC
ttgD:	Forward: Bam-D	CCA**GGATCC**GACGAACGGTGAG
	Reverse: AflII-D	GAAGCGAGACAT**CTTAAG**AATCAATAACTCC
ttgE:	Forward: AflII-E	GGAGTTATTGATT**CTTAAG**ATGTCTCGCTTC
	Reverse: Swa-E	GTGAGTCTTCATA**TTTAAA**TCATGCCAATTCTTC
ttgF:	Forward: Swa-F	GAATTGGCATGA**TTTAAA**TATGAAGACTCAC
	Reverse: Spe-F	CCTGTT**ACTAGT**TGCTCAGTTCTGAGCGG
ttgG:	Forward: Bam-G:	GCCCCC**GGATCC**GTCATGCGGGCAG
	Reverse: Afl-G	GTGCCCC**CTTAAG**TCAGTTAGCCGAG
ttgH:	Forward:Afl-H	CTCGGCTAACTGA**CTTAAG**GGGGCAC
	Reverse:SwaI-H	CATGGGGAGTGACA**TTTAAA**TTAAACTTCAT
ttgI:	Forward:Swa-I	GGTGACTCA**TTTAAA**TATGAAGTTTAAGTCACTCCCCATG
	Reverse:Spe-I	**ACTAGT**GGATCTTAGTTTTGACTCACGC

Figure 1. PCR amplification of the corresponding Ttg genes was carried out using oligonucleotides to which the corresponding restriction sites were added. Amplified DNA was digested and cloned in pGEM-T (Promega) or pCR2.1 (Invitrogen, Ca) (single components) or pBBR1MCS5 (hybrids efflux pumps) (Kovach et al., 1995). pGEM-T/pCR2.1 constructs were introduced into *E. coli* KAM32 (Huda et al., 2003) and those in pBBR1MCS5 were electroporated into *P. putida* DOT-T1E-34S.

Chimeric efflux pumps with TtgE as transporter do no show increased antibiotic resistance

Mosqueda and Ramos (2000) suggested that TtgDEF did not confer antibiotic resistance to *Pseudomonas putida*. The above results suggest that the extrusion channels provided by both TtgF and TtgD can lead to functional pumps in regards to antibiotic extrusion. Hence, we hypothesize that TtgE does not function as an antibiotic effluxer. In order to check if the substrate specificity of an efflux pump was only conferred by the inner membrane element, we exchanged the *ttgB* gene for *ttgE* and studied if the hybrid efflux pump was able to expulse pump out

TABLE 2. sequence homology among the Mex and Ttg systems. A) MFP's B) RND's, C) OMP's components.

A

	AcrA	**MexA**	**TtgA**	**TtgD**	**TtgG**
AcrA	100	56	53	49	50
MexA		100	67	56	58
TtgA			100	58	59
TtgD				100	72
TtgG					100

B

	AcrB	**MexB**	**TtgB**	**TtgE**	**TtgH**
AcrB	100	70	65	58	57
MexB		100	78	64	64
TtgB			100	63	63
TtgE				100	84
TtgH					100

C

	TolC	**OprM**	**TtgC**	**TtgF**	**TtgI**
TolC	100	21	20	20	18
OprM		100	68	60	58
TtgC			100	58	56
TtgF				100	59
TtgI					100

antibiotics. As shown in table 1, expression of wild type and hybrid efflux pumps containing the TtgE component from plasmid promoter did not increase the antibiotic resistance of the recipient strain. Tetracycline resistance was increased two-fold when TtgAEC, TtgDEF or TtgDEC was expressed, however, two-fold changes are not significant enough as to speculate about the participation of the OMPs and MFPs in substrate specificity.

DISCUSSION

Resolution of the 3D structure of TolC, AcrA, AcrB and MexA and Z (Koronakis *et al.*, 2000; Avila-Sakar *et al.*, 2001; Murakami *et al.*, 2002; Yu *et al.*, 2003; Akama *et al.*, 2004), and mutational analysis and cross-linking experiments have provided information about the contacts between the three different components of the efflux pumps, however, how they interact with each other is still unclear.

MFPs are highly asymmetrical proteins that are anchored in the inner membrane via a fatty acid moeity. Of the three domains identified in AcrA and MexA (Akama et al., 2004), the α-helical hairpin was implicated as being important to the functional contacts between MexA and OprM, between AcrA and TolC, and between HylD and TolC (Schlor, et al., 1997; Gerken and Misra, 2004; Nehme *et al.*, 2004; Touzé *et al.*,

2004; Stegmeier *et al*., 2006; Lobedanz *et al.*, 2007). Only two (D^{73} and R^{104}) out of the eight AcrA residues reported to be involved in TolC recognition are conserved between AcrA, MexA, TtgA, TtgD and TtgG, while two other residues (A^{75}, Q^{112}) were conserved between AcrA, TtgA and MexA. The MexA residues involved in OprM recognition (Nehme *et al*., 2004; Nehme and Poole, 2007), A^{108} and V^{129} (located at hairpin domain), and P^{68} are well conserved between MexA and the three Ttg efflux pumps. Conservation of these residues among the different MFPs of the Ttg systems supports our observations that MFPs (i.e. TtgA) may interact with different OMPs (TtgF and TtgI).

The OMP structure shows that this protein folds to generate a hollow exit duct with an α-helical domain projecting across the periplasm and a β-barrel domain that is anchored to the outer membrane (Koronakis *et. al*, 2000). TolC residues involved in AcrA recognition are located in the lower α-barrel of the OMP (Lobedanz *et al.*, 2007). Although none of the TolC residues that have been shown to be involved in AcrA recognition (S^{124}, Q^{139}, S^{142} and S^{363}) are conserved among the Ttg proteins, OprM residues (T^{198} and F^{439}), which suppress the effect of the V129M mutation in MexA, are conserved in all Ttg OMPs. Conservation of the residues that establish contacts between MFPs and OMPs support the notion that, at least within these four systems (MexAB-OprM, TtgABC, TtgDEF and TtgGHI), the subunits may form contacts with different corresponding components to form hybrid complexes. This hypothesis is corroborated by our results, which show the resilient activity of hybrid efflux pumps such us TtgDBF, TtgDBI, TtgGBF or TtgGBI in which the OMP and the MFP have been exchanged.

In order for efflux pumps to be functionally active, MFPs and the OMPs must also establish contacts with their corresponding transporters. Tamura *et al*. (2005) identified contacts between residues Q^{255}, D^{256} and G^{257} of AcrB with Q^{142}, R^{143}, G^{147} and A^{150} of TolC. Of these residues, D^{256} and G^{257} are conserved between the five translocases analyzed in this study while only G^{147} is present in all the OMPs. MexB residues important for contact with OprM (V^{203}, G^{581}) were reported to not be conserved among different efflux pumps (Middlemiss and Poole, 2004); however, these two residues are present in all Ttg efflux proteins suggesting that it is a common feature within MexB and Ttg proteins. These commonalities further support the notion of functional interchangeability between OMPs that have been observed in Ttg (this work) and Mex efflux systems (Srikumar et al., 1997; Gotoh et al., 1998; Maseda *et al.* 2000; Elkins and Nikaido, 2003) .The conservation of three (G^{220}, A^{618} and R^{716}) of the four MexB residues involved in MexA binding within the three Ttg components (the fourth aminoacid, T^{578}, is conserved in TtgB and TtgH but not in TtgE) suggests again the possibility of functional replacement between the MFPs of members of the Ttg efflux pumps as observed in this study. Studies done by Nehme et al., (2004) and Nehme and Poole (2007) identified MexA mutations that suppressed the effect of the T578I or G220S mutations in MexB. All the MexA residues (V^{66}, A^{227}, V^{259}, A^{263}, V^{278}, and L^{282}) that restore the phenotype of the G220S mutants are conserved among the five efflux pumps, while residues that restore the phenotype of the T578I mutant are not well conserved. All these data indicated that despite the low sequence homology among the Mex and Ttg systems (Table 2) the residues that are involved in contacts between OMPs, MFPs and translocases in the MexAB-OprM efflux pump are relatively well conserved among the TtgABC, TtgDEF and TtgGHI pumps, and provide a strong case for the interchangeability of these components.

Whether hybrids efflux pumps can operate *in vivo* or not is still an open question and the answer will partially depend on the effects of expression of the three efflux pumps in the cell at the same time. The expression pattern of the three Ttg pumps is influenced by different effectors and mediated by distinct regulators under laboratory conditions (Duque et al., 2001, Terán *et al.*, 2003; Mosqueda and Ramos, 2000; Rojas et al., 2001 and Guazzaroni *et al*., 2005), however expression of the efflux pumps in the environment has yet been studied in detail. Future work, involving *In vitro* binding assays using the different components of the three efflux pumps and

inmunolocalization of the components within the membrane will help further our understanding of the principles governing the association between different components of the RND efflux pumps.

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The *ttgGHI* solvent efflux pump operon of *Pseudomonas putida* DOT-T1E is located on a large self-transmissible plasmid

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Pseudomonas putida DOT-T1E es una cepa tolerante a disolventes capaz de crecer en presencia de mas del 1% (v/v) de tolueno en el medio de cultivo. Se ha encontrado que un grupo de bombas que expulsan múltiples compuestos juegan un rol principal en la tolerancia de esta bacteria a disolventes orgánicos (Rojas *et al*., J Bacteriol 183: 3967–3973). Durante el estudio de los mecanismos subyacentes de tolerancia a disolventes de DOT-T1E, aislamos un mutante espontáneo sensible a disolventes derivado de esta cepa el cual había perdido los genes que codificaban para la bomba TtgGHI, el elemento de extrusión más importante en términos cuantitativos. Comparaciones de genoma por análisis de microarray entre la cepa mutante y su estirpe parental revelaron que además del grupo de genes ttgVW-ttgGHI, otro conjunto de genes muy similares a los encontrados en los elementos transponibles, Tn4653A y ISPpu12, del plasmido TOL (pWW0) de *P. putida* mt-2, estaban también ausentes. Análisis más detallados demostraron que la cepa DOT-T1E albergaba un plasmido de gran tamaño (llamado pGRT1) que se perdía en el mutante sensible a disolventes. Análisis de mapeo revelaron que los genes *ttgVW-ttgGHI* y el transposon similiar a Tn4653A formaban parte del esqueleto del plasmido pGRT1. El plásmido pGRT1 es muy estable y la frecuencia con la que se pierde es de 10^{-8} por célula por generación bajo una variedad de condiciones de crecimiento que incluyen estreses nutricional y físico. El plásmido pGRT1 es autotransferible, y su adquisición por las cepas sensibles a tolueno *P. putida* KT2440 y *Pseudomonas aeruginosa* PAO1 incrementa la tolerancia a tolueno del receptor a niveles similares a los exhibidos por *P. putida* DOT-T1E. En este trabajo se discute la importancia y el beneficio potencial del plasmido para el desarrollo de bacterias con la tolerancia a disolventes incrementada y su impacto potencial para la biorremediación y las biotransformaciones llevadas a cabo por células enteras.

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The *ttgGHI* solvent efflux pump operon of *Pseudomonas putida* DOT-T1E is located on a large self-transmissible plasmid

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Summary

Pseudomonas putida **DOT-T1E is a solvent-tolerant strain able to grow in the presence of > 1% (v/v) toluene in the culture medium. A set of multidrug efflux pumps have been found to play a major role in the tolerance of this bacterium to organic solvents (Rojas *et al.*, *J Bacteriol* 183: 3967–3973). In the course of studies of the mechanisms underlying solvent tolerance in DOT-T1E, we isolated a spontaneous solvent-sensitive mutant derivative which had lost the genes encoding the TtgGHI efflux pump, the most important extrusion element in quantitative terms. Genomic comparisons between the mutant and its parental strain by microarray analysis revealed that in addition to the *ttgVW-ttgGHI* gene cluster, another group of genes, highly similar to those found in the Tn*4653A* and IS*Ppu12* transposable elements of the TOL plasmid pWW0 from *P. putida* mt-2, were also absent from this strain. Further analysis demonstrated that strain DOT-T1E harboured a large plasmid (named pGRT1) that was lost from the solvent-sensitive mutant. Mapping analysis revealed that the *ttgVW-ttgGHI* genes and the Tn*4653A*-like transposon are borne by the pGRT1 plasmid. Plasmid pGRT1 is highly stable and its frequency of loss is below 10^{-8} per cell per generation under a variety of growth conditions, including nutritional and physical stresses. The pGRT1 plasmid is self-transmissible, and its acquisition by the toluene-sensitive *P. putida* KT2440 and *Pseudomonas aeruginosa* PAO1 increased the recipient's tolerance to toluene up to levels similar to those exhibited by *P. putida* DOT-T1E. We discuss the importance and potential benefits of this plasmid for the development of bacteria with enhanced solvent tolerance, and its potential impact for bioremediation and whole-cell biotransformations.**

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Introduction

Bacteria are exposed to a wide range of natural and man-made toxic compounds in their natural habitats, and survival in the environment requires protective mechanisms (Ramos *et al.*, 2001; Corcoran *et al.*, 2005; Bjornsdottir *et al.*, 2006; Velázquez *et al.*, 2006). Toluene is an organic solvent of natural origin that has been widely used in industry during recent decades, and is considered a ubiquitous contaminant by a number of environmental protection agencies. Therefore, microbes in the environment have been exposed to this membrane-disturbing agent for years, and it is not surprising that bacteria have evolved mechanisms to counteract solvent toxicity.

Pseudomonas putida DOT-T1E is a highly toluene-tolerant strain isolated from a wastewater treatment plant in Granada. This strain can grow in liquid medium with > 1% (v/v) toluene (Ramos *et al.*, 1995; Mosqueda *et al.*, 1999). The molecular mechanisms leading to solvent tolerance in this strain have been extensively studied in our laboratory (Ramos *et al.*, 2002; Segura *et al.*, 2003; 2004). Tolerance to organic solvents in *P. putida* DOT-T1E is achieved by a series of enzymatic tools that include the modification of fatty acids and phospholipid head groups in the membrane to compact it (Ramos *et al.*, 1997; Junker and Ramos, 1999; Bernal *et al.*, 2007), synthesis of chaperones that help proteins to fold properly in the presence of solvents (Segura *et al.*, 2005; Domínguez-Cuevas *et al.*, 2006; Volkers *et al.*, 2006), and the extrusion of solvents by means of three efflux pumps named TtgABC (Ramos *et al.*, 1998), TtgDEF (Mosqueda and Ramos, 2000) and TtgGHI (Rojas *et al.*, 2001). *Pseudomonas putida* DOT-T1E exhibits innate tolerance to solvents that allows 1 out of 10^4 cells to survive a sudden toluene shock [i.e. the addition of 0.3–1% (v/v) toluene]; however, nearly 100% of the cells survive sudden solvent shocks if pre-induced with sublethal concentrations of toluene (Ramos *et al.*, 1995). Although all of the mechanisms contribute to some extent to the

Table 1. Bacterial strains used in this study.

Pseudomonas putida strains	Relevant characteristics	References
KT2440 (pWW0)	Tol^S; ATCC 33015	Williams and Murray (1974)
KT2440	mt-2 pWW0 cured, Tol^S	Nakazawa (2002)
KT2442	KT2440 Rif^R	Franklin *et al*. (1981)
KT2440crc	KT2440*crc*::*aacC1*, Gm^R	Aranda-Olmedo *et al*. (2005)
DOT-T1E	Tol^R, Rif^R	Ramos *et al*. (1998)
DOT-T1E PS62	DOT-T1E *ttgV*::*aphA3*, Km^R	Rojas *et al*. (2003)
DOT-T1E PS51	DOT-T1E *ttgW*::ΩKm, Km^R	Rojas *et al*. (2003)
MTB5	Tol^S	Huertas *et al*. (2000)
MTB6	Tol^R	Huertas *et al*. (2000)
DOT-T1E-100	Spontaneous Tol^S derivative of DOT-T1E, Rif^R	This study
VAN1 through to VAN4	Spontaneous Tol^S derivative of DOT-T1E-PS51, Rif^R, Km^S	This study
VAN5 and VAN6	Spontaneous Tol^S derivative of DOT-T1E-PS51, Rif^R, Km^S (carbon starvation assay)	This study
VAN7 and VAN8	Spontaneous Tol^S derivative of DOT-T1E-PS51, Rif^R, Km^S (growth under sublethal tetracycline concentrations)	This study
DOT-T1E-109	DOT-T1E *fadD*::Tn*5phoA*, Tol^S, Rif^R, Km^R	Segura *et al*. (2004)
DOT-T1E-16	DOT-T1E *ttgX*::Km, Tol^S, Rif^R, Km^R	E. Duque and J.L. Ramos (unpublished)
Pseudomonas aeruginosa PAO1	Prototroph	Strain provided as a gift

Gm^R, Km^R, Rif^R, resistance to gentamicin, kanamycin and rifampicin respectively. Km^S stands for sensitivity to kanamycin (50 μg ml^{-1}). Tol^R and Tol^S stand for tolerance and sensitivity, respectively, to ≥ 0.3% (v/v) toluene in liquid medium.

solvent-tolerant phenotype of this strain, it has been shown that the main determinants of solvent tolerance are the efflux pumps.

Two of the efflux pump operons, *ttgDEF* and *ttgGHI*, are induced by sublethal toluene concentrations (Mosqueda *et al*., 1999; Rojas *et al*., 2003), whereas the level of expression of *ttgABC* does not change in response to solvents (Duque *et al*., 2001; Terán *et al*., 2006). The TtgGHI efflux pump plays a pivotal role in innate and induced tolerance to toluene, as shown by the finding that a mutant in this efflux pump was not able to survive the addition of 0.3% (v/v) toluene even if bacteria were pre-cultured with sublethal toluene concentrations (Rojas *et al*., 2001). Furthermore, in a survey of different *P. putida* strains, the presence of the *ttgGHI*-like genes was detected only in highly solvent-tolerant strains, i.e. *P. putida* MTB6 (Huertas *et al*., 2000) and *P. putida* S12 (Isken and de Bont, 1996), whereas the TtgDEF efflux pump was present only in those strains carrying the *tod* pathway for toluene degradation (Phoenix *et al*., 2003; Segura *et al*., 2003). TtgABC seems to be widespread among the *P. putida* strains tested, and its main role is the extrusion of antimicrobial agents (Rojas *et al*., 2001; Terán *et al*., 2006).

In the course of our research, we found that a *P. putida* DOT-T1E derivative clone spontaneously turned solvent sensitive (Table 1). We first determined whether the solvent-sensitive clone had all the three efflux pumps, and discovered that the clone had lost the *ttgGHI* genes, a phenomenon that was subsequently linked to the loss of a plasmid of about 121 kb, dubbed pGRT1, in which the *ttgGHI* operon is located. Plasmid stability is high, and under different growth and stress conditions its rate of loss is below 10^{-8} per cell and per generation. The plasmid is self-transmissible and constitutes a valuable tool for biotechnological processes.

Results

Genomic characterization of a P. putida *DOT-T1E derivative that is toluene sensitive*

Pseudomonas putida strain DOT-T1E-100 is a serendipitous toluene-sensitive derivative of *P. putida* DOT-T1E isolated from a culture in M9 minimal medium plus toluene in the gas phase that was stored at 4°C for several weeks. This mutant, in contrast to its parental strain, was unable to grow in Luria–Bertani (LB) medium supplemented with 0.3% (v/v) toluene. As efflux pumps are key for solvent tolerance, we decided to check whether the genes that encode these pumps were still present in the DOT-T1E-100 strain, using Southern hybridization analysis of total DNA of the mutant strain and the *ttgABC*, *ttgDEF* and *ttgGHI* genes as probes. We found that whereas the *ttgABC* and *ttgDEF* genes gave positive hybridization signals, the *ttgGHI* operon was no longer present in the mutant strain (not shown).

To further confirm this observation and to define more precisely the extent of the DNA region hypothetically deleted in DOT-T1E-100, we took advantage of the availability of a genome-wide microarray from the closely related strain *P. putida* KT2440. Total DNA preparations from *P. putida* DOT-T1E-100 and its parental strain DOT-T1E were fluorescently labelled with Cy3 and Cy5 dyes and analysed by comparative genomic hybridization as described in *Experimental procedures*. The results, sum-

Table 2. *Pseudomonas putida* DOT-T1E ORFs (present on the DNA chip) which are absent from the DOT-T1E-100 strain.

Gene[a]	Protein length (aa)	Closest relationship (protein/microorganism)[b]	Identity (%)	Accession No.
orf45	188	OsmC-like protein/*Burkholderia cenocepacia*	49.2 (88/179)	YP_624744
orf46	140	TniA transposase/*Xanthomonas* sp. W17	100 (126/126)	AAA98333
orf51	98	Probable transcriptional regulator, LysR family, HTH domain/*Polaromonas naphthalenivornas*	64.0 (57/89)	ZP_01021453
tnpR	220	TniC, serine recombinase family, resolvase-invertase (R751 plasmid)/*Enterobacter aerogenes*	71.7 (134/187)	NP_044261
orf55	145	Arsenical resistance operon repressor/*Magnetospirillum magnetotacticum* MS-1	84.1 (90/107)	ZP_00051419
orf155	107	EbrB multidrug resistance protein (pLM80 plasmid)/*Listeria monocytogenes*	50.7 (38/75)	ZP_00230415
orf156	110	Putative membrane transport protein EmrE/*Marinobacter aquaeoli* VT8	45.9 (28/61)	ZP_00818462
tnpA	404	Transposase TnpA (naphthalene catabolic plasmid pND6-1)/*Pseudomonas* sp. ND-6	99.7 (403/404)	NP_943156
lspA	170	Lipoprotein signal peptidase A8/*Marinobacter aquaeoli* VT8	100 (170/170)	ZP_00820188
orf163	298	CzcD, putative transporter Co/Zn/Cd, efflux system component/*Pseudomonas* sp. ND-6	100 (298/298)	NP_943154
orf164	135	Transcriptional regulator, MerR family/*Alcanivorax borkumensis* SK2	96.3 (130/135)	YP_693087
merB′	181	MerB Organomercurial lyase (pPB plasmid)/*Pseudomonas stutzeri*	100 (180/180)	AAC38230
merR	144	Organomercurial resistance regulatory protein (pPB plasmid)/*Pseudomonas stutzeri*	100 (144/144)	AAC38229
tnpR	186	Resolvase, serine recombinase family, resolvase-invertase subfamily, Tn*1721*/*Escherichia coli*	100 (186/186)	CAA43640
tnpA	988	TnpA transposase, Tn*501*/*Pseudomonas aeruginosa*	97.3 (961/988)	P06695
ttgG	391	Periplasmic efflux pump component/*Pseudomonas putida* DOT-T1E	NA	AAK69563
ttgH	1049	Inner membrane efflux pump component/*P. putida* DOT-T1E	NA	AAK69564
ttgI	470	Outer membrane efflux pump component/*P. putida* DOT-T1E	NA	AAK69565
ttgV	259	HTH-type transcriptional regulator/*P. putida* DOT-T1E	NA	Q93PU6
ttgW	134	Putative HTH-type transcriptional regulator/*P. putida* DOT-T1E	NA	Q93PU7

a. Homologies from the upper part of the table were calculated based on the amino acid sequences obtained from the published sequence of the TOL plasmid pWW0 (Greated *et al*., 2002).
b. Only the first BLASTP hit is reported.
HTH, helix–turn–helix; NA, not applicable.

marized in Table 2, showed that in addition to the *ttgGHI* genes, the adjacent *ttgV* and *ttgW* genes were also absent. Surprisingly, a group of spots corresponding to several open reading frames (ORFs) from the *P. putida* KT2440 TOL plasmid pWW0 gave a positive hybridization signal with the DNA obtained from *P. putida* DOT-T1E but not with that prepared from the DOT-T1E-100 strain. To further confirm the presence of TOL-like genes in DOT-T1E, we carried out comparative genomic hybridization assays between the parental DOT-T1E strain and *P. putida* KT2440 (pWW0). From these analyses it was clear that these genes were indeed present in DOT-T1E. Interestingly, besides the number of *P. putida* KT2440 chromosomal genes that were not present in the DOT-T1E strain, the majority of the pWW0 oligonucleotide targets deposited on the microarray did not hybridize with the DOT-T1E DNA, except for those mentioned above and that corresponding to the *xylT* gene (see Greated *et al*., 2002).

The TOL-like genes missing from DOT-T1E-100 are clustered within a 14.7 kb region in the TOL plasmid and form part of two encompassed transposons, Tn*4653A* (the probable ancestral of Tn*4653*) and IS*Ppu12* (included in the former transposon) (Fig. 1A). In pWW0, the Tn*4653* transposon contains, in addition to the IS*Ppu12* element, another 56 kb transposon (named Tn*4651*) inserted into *orf*55 (Greated *et al*., 2002) (see Fig. 1A). DNA sequencing analysis of the TOL-like genes in DOT-T1E revealed they were also clustered within a transposon, although some changes in gene order were found, and the four left-most ORFs of the TOL plasmid were absent (compare Fig. 1A and B).

It is well known that transposition can involve significant chromosomal rearrangements, including deletions, inversions and replicon fusions (Craig, 1996). Transposable elements can also facilitate occasional rearrangements by serving as substrates for other types of recombination reactions (i.e. by homologous recombination between elements at different genomic sites). Consequently, one could consider three possible explanations for the *ttgWV-ttgGHI* deletion event in the DOT-T1E-100 strain: (i) the *ttgWV-ttgGHI* genes were lost from the DOT-T1E chromosome as a consequence of Tn*4653A* re-transposition, (ii) the genes are located between two direct repeats of Tn*4653A* (or IS*Ppu12*) whose RecA-mediated homologous recombination resulted in the deletion of the inter-

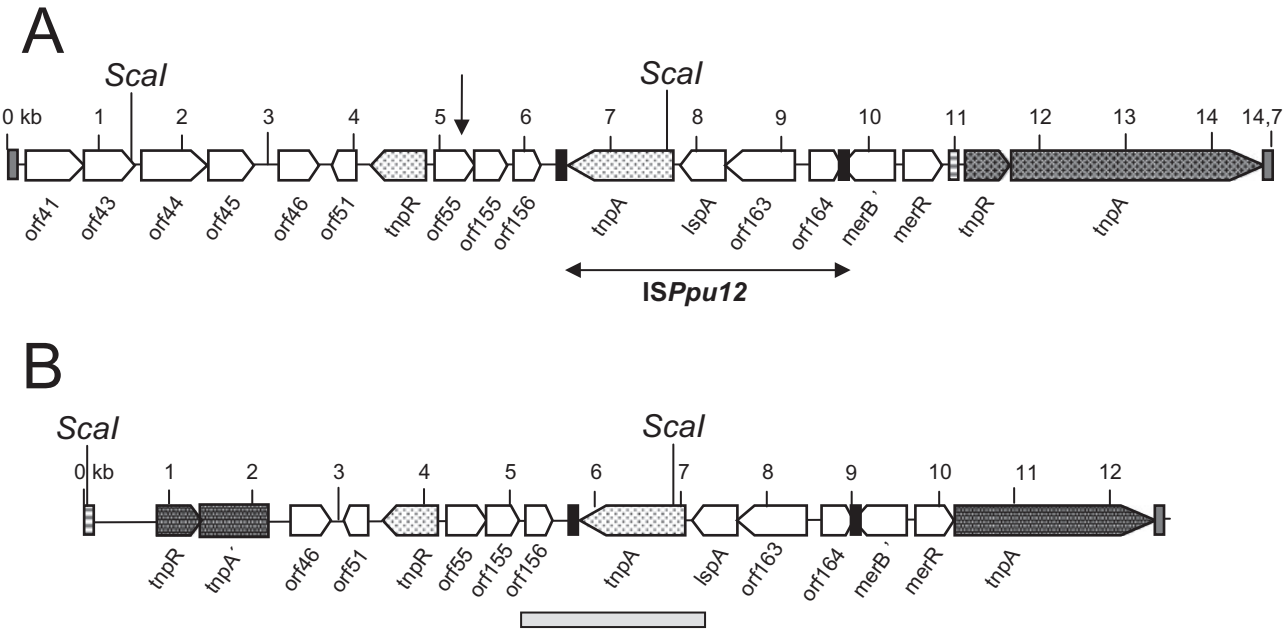

Fig. 1. Physical organization of the Tn*4653* region of pWW0 (A) and pGRT1 (B). Arrows represent the different genes, their relative sizes and the directions of transcription. Gene nomenclature and organization are based on the previously annotated sequence of the TOL plasmid pWW0 from *P. putida* KT2440 (Greated *et al*., 2002). Stippled arrows correspond to genes putatively involved in transposition functions. Among these, the Tn*4653*A transposase and resolvase genes are shaded in grey. The 38 bp inverted repeats (IRs) of Tn*4653*A are shown in grey boxes, and the 24 bp IRs of the IS*Ppu12* transposable element are shown in black boxes. The vertical arrow pointing to *orf55* in (A) indicates the point at which the Tn*4651* transposon is inserted in pWW0. The shaded box indicates the *res* region upstream of the *tnpR* resolvase gene of Tn*4653*, which is defective in pWW0 but not in pGRT1. The pGRT1 DNA sequence has been deposited in GenBank under Accession No. EF094543.

vening DNA region, or (iii) the *ttgWV-ttgGHI* genes are located on an as-yet-unidentified plasmid (which shares the presence of Tn*4653A* and IS*Ppu12* with pWW0) that is lost from DOT-T1E-100 under certain environmental conditions. The first hypothesis can be ruled out, as Tn*4653*A belongs to the Tn*3* family (class II) of transposable elements, which transpose via a replicative mechanism. These elements can yield chromosomal deletions by means of replicative intramolecular transposition, but this always leads to the appearance of a second transposon copy (Berg and Berg, 1996), which was not the case for DOT-T1E-100 according to the microarray results. Regarding the second hypothesis, homologous recombination between direct DNA repeats would leave a transposon copy in the chromosome, which, again, did not fit the deduced DOT-T1E-100 genotype. We therefore decided to test the last hypothesis: that the *ttgWV-ttgGHI* cluster is located in a large plasmid which has not previously been identified in DOT-T1E.

Pseudomonas putida *DOT-T1E carries a low-copy large plasmid*

The presence of extrachromosomal genetic elements in *P. putida* DOT-T1E has never been convincingly observed in DNA preparations of this strain (E. Duque, pers. comm.). To detect the possible presence of a high-molecular-weight plasmid in DOT-T1E, we adapted the plasmid isolation procedure developed by Zhou and colleagues (1990) for the purification of large plasmids (see *Experimental procedures*). Using this protocol, we were able to detect and purify a high-molecular-weight plasmid (named pGRT1) from the parental *P. putida* DOT-T1E, but not from strain DOT-T1E-100 (Fig. 2A). On the basis of the electrophoretic mobilities of the ScaI, EcoRV and XhoI fragments of pGRT1 (data not shown), the size of the plasmid was estimated to be about 121 $\pm$ 5 kb (not shown).

To further confirm the plasmid coding of the TtgGHI efflux pump and the Tn*4653* transposon, we performed Southern blot analysis of pGRT1 plasmid preparations digested with ScaI or XhoI, using the *P. putida* DOT-T1E *ttgH* gene (Fig. 3A) or the amplified *lspA-orf156* DNA region (Fig. 3B) as probes. Both probes gave positive hybridization signals, demonstrating that the genes mentioned above were indeed carried on the pGRT1 plasmid. No positive hybridization was observed when total DNA from strain DOT-T1E-100 was used as the target (data not shown).

Segura and colleagues (2003) carried out a comparative genomic analysis of solvent extrusion pumps in

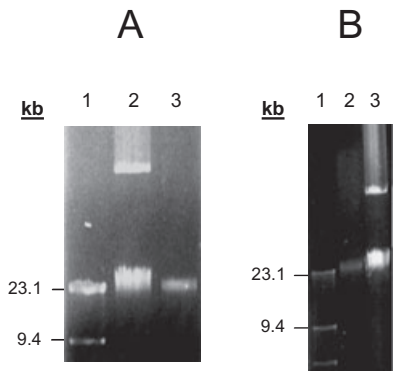

Fig. 2. A. High-molecular-weight plasmid preparations from strains DOT-T1E and DOT-T1E-100. A large plasmid (designated pGRT1) was detected in *P. putida* DOT-T1E (lane 2), which was not present in the DOT-T1E-100 strain (lane 3).
B. Plasmid DNA preparation from *P. putida* strains MTB5 (lane 2) and MTB6 (lane 3).
Plasmid isolation and purification were carried out as described in *Experimental procedures*. DNA electrophoresis was performed on 0.6% (w/v) agarose gels. Lane 1 corresponds to the molecular marker λ-HindIII.

P. putida, and found a strong correlation between the ability of strains to survive a 0.3% (v/v) sudden toluene shock and the presence of a TtgGHI-like efflux pump. Based on this finding, we investigated whether a correlation also existed between the presence of the *ttgGHI* genes and the presence of a large plasmid in different strains. To this aim we analysed strain MTB5 (toluene sensitive, *ttgH*$^{-}$) and strain MTB6 (highly toluene tolerant, *ttgH*$^{+}$), two *P. putida* strains isolated from agricultural soils in Granada, southern Spain (Huertas *et al*., 2000). Interestingly, we were able to identify a high-molecular-weight plasmid in strain MTB6, whereas no plasmid could be isolated from strain MTB5 (Fig. 2B). Plasmid preparations from *P. putida* strain MTB6 were also subjected to restriction analysis with EcoRV and ScaI, and compared with pGRT1 plasmid preparations from strain DOT-T1E digested with the same enzymes. Surprisingly, the restriction patterns obtained with these enzymes (Fig. 3C) suggest that both plasmids are highly similar.

pGRT1 stability in P. putida *DOT-T1E*

The serendipitous finding of the DOT-T1E-100 strain led us to wonder how stable plasmid pGRT1 was. To investigate this we decided to use *P. putida* DOT-T1E PS51, which carries a kanamycin-resistant (Km^{R}) marker inserted in the plasmid pseudogene *ttgW* (Table 1). This strain was grown for more than 100 generations in LB medium without kanamycin. Then bacteria were spread onto LB plates and kanamycin-sensitive (Km^{S}) clones searched for among 40 000 colonies. We found four Km^{S} derivative and estimated that the rate of plasmid loss was below 10^{-8} per cell and per generation. These clones were called *P. putida* DOT-T1E-VAN1 through to DOT-T1E-VAN4 (characterized below).

As *P. putida* DOT-T1E-100 was isolated from a M9 minimal medium plate supplemented with toluene in the gas phase as the only carbon source, and which had been stored in the refrigerator for several weeks, we thought that cells in these plates would be subjected to starvation conditions. To test whether carbon starvation increased the rate of loss of the solvent-tolerant phenotype, we cultured *P. putida* DOT-T1E PS51 on M9 minimal medium with low amounts of glucose. After a prolonged period under stationary phase conditions, cells were spread on LB plates. About 40 000 individual colonies were replica-plated on LB with and without kanamycin, and we found two Km^{S} clones. This suggested that carbon starvation might not influence the solvent-resistance character. These clones were called VAN5 and VAN6.

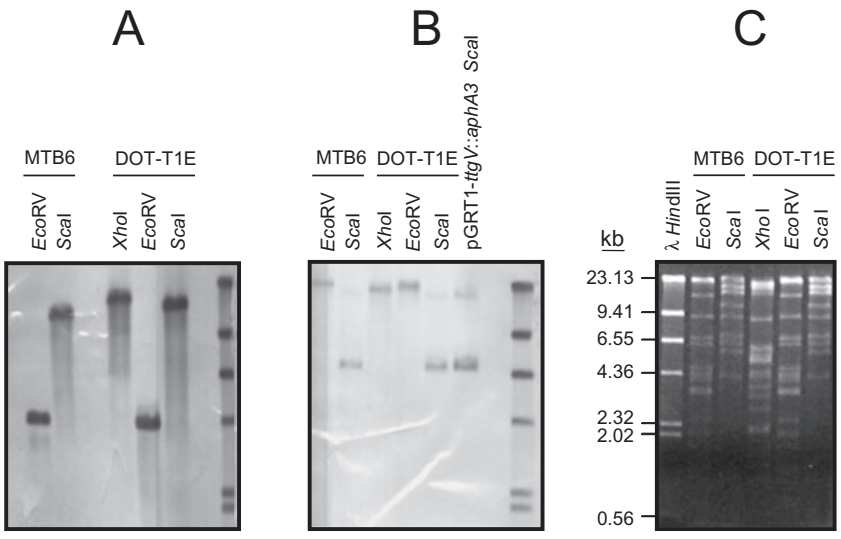

Fig. 3. Southern blot analysis of the pGRT1 plasmid. Plasmid DNA samples were digested with different restriction enzymes.
A and B. The probes used were a digoxigenin-labelled 0.9 kb PCR fragment corresponding to the *P. putida* DOT1E *ttgH* gene (A), or a 2.3 kb PCR fragment covering the *lspA-orf156* DNA region (B).
C. Enzymatic digestion of the large plasmids isolated from *P. putida* DOT-T1E and MTB6, with the indicated restriction enzymes. DNA electrophoresis was performed on 0.6% (w/v) agarose gels.

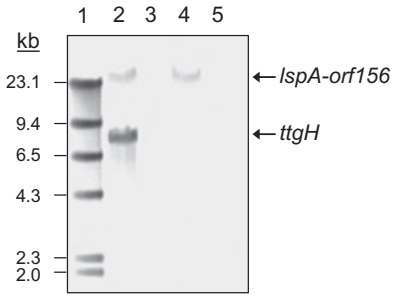

Fig. 4. Southern hybridization of some solvent-sensitive strains identified in this study. Total DNA from *P. putida* DOT-T1E (lane 2), *P. putida* DOT-T1E-100 (lane 3), *P. putida* DOT-T1E PS51-VAN5 (Km^R) (lane 4), *P. putida* DOT-T1E PS51-VAN1 (Km^R) (lane 5) was cut with BamHI and hybridized with the *ttgH* probe, and later the same filter was hybridized with the *lspA-orf156* probe. Molecular marker II (Roche) was included in the Southern blot (lane 1).

We also cultured *P. putida* DOT-T1E PS51 under different stress conditions induced by sublethal concentrations of chemicals, i.e. in the presence of membrane-disturbing agents, drugs and NaCl. No Km^S clones were found in a search of 40 000 clones derived from cells grown under suboptimal conditions in the presence of sodium dodecyl sulfate (SDS) or NaCl. After growth under sublethal tetracycline concentrations (2 μg ml^{-1}) two Km^S clones were found among the 20 000 clones we tested. These clones were called VAN7 and VAN8.

In our laboratory we had previously isolated two solvent-sensitive mini-Tn*5* mutants that exhibited the transposon inserted in a *fadD* allele and in the *ttgX* genes (Duque *et al*., 2001; Segura *et al*., 2004). Reconstruction of these two mutations by site-directed mutagenesis using *in vitro* interrupted alleles yielded the expected mutants; however, these mutants behaved like the parental strain regarding solvent tolerance, and we therefore proposed that neither the TtgX nor the FadD proteins are required for solvent tolerance (Segura *et al*., 2004; E. Duque and J.L. Ramos, unpubl. results). The original clones, called DOT-T1-109 and DOT-T1E-16, were reanalysed here regarding the presence of the *ttgGHI* genes.

To determine whether the solvent-sensitive character correlated with the absence of the plasmid in VAN1 through to VAN8, DOT-T1-E-109 and DOT-T1E-16, we first prepared plasmid DNA as above using DOT-T1E as a control. We found that VAN5 bore a plasmid that was smaller than pGRT1, whereas the rest of the clones bore no plasmids. To further confirm the loss of the plasmids and the genes we identified on pGRT1, we carried out Southern blot analysis with total DNA of the strains and used the *ttgGHI* and *lpsA-orf156* genes as probes. For all strains except for VAN5 we found no hybridization with the probes, which points toward the loss of the plasmid (see Fig. 4 for a number of clones).

With plasmid DNA of the VAN5 strain we found no hybridization with the *ttgVW-ttgGHI* genes, but positive hybridization bands against the *lspA-orf156* genes were found. This raised the possibility that plasmid pGRT1 had undergone deletion in the VAN5 strain. Subsequent digestion of pGRT1-VAN5 with ScaI revealed the loss of five fragments that added up to about 50 kb (Fig. 5A). It should be noted that the 6.8 kb ScaI fragment within the Tn*4653A*-like element was not present in the mutant plasmid (Figs 5B and 1B).

pGRT1 is a self-transmissible plasmid that replicates in different Pseudomonas *strains*

As part of the characterization of the pGRT1 plasmid, we tested whether it was able to self-transfer to other *P. putida* strains. We set up biparental matings between donor strain *P. putida* DOT-T1E PS62, which carries a Km^R marker in the *ttgV* gene (*ttgV*::*aphA3*), and *P. putida* KT2440*crc*, a gentamicin-resistant derivative strain of KT2440, as the receptor strain. Insertion of the Km^R marker in the regulatory gene *ttgV* increases solvent tolerance so pre-induction with toluene is not necessary to achieve the maximum level of survival after toluene shock (Rojas *et al*., 2003). Mating was allowed to proceed overnight at 30°C before plating suitable dilutions onto M9 minimal medium supplemented with 5 mM benzoate plus kanamycin and gentamicin. Kanamycin- and gentamicin-resistant transconjugants appeared at a frequency of about 1.5×10^{-5} per recipient. One clone was randomly chosen for further analysis. Colony PCR analysis was

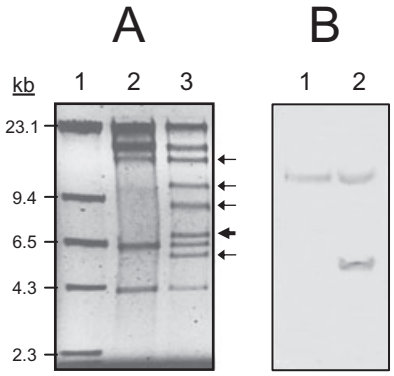

Fig. 5. Analysis of the plasmid present in strain DOT-T1E-PS51-VAN5.
A. Restriction analysis (ScaI) of the wild-type pGRT1 plasmid (lane 3) and the pGRT1-VAN5 plasmid in mutant strain *P. putida* DOT-T1E-51-VAN5 (lane 2). Lane 1 is the molecular marker λ-HindIII.
B. Southern blot of the wild-type (pGRT1) (lane 2) and the mutant plasmid (lane 1) hybridized with the *lspA-orf156* probe.
Arrows indicate the bands that are not present in the mutant plasmid. The bold arrow indicates the band corresponding to the 6.8 kb ScaI fragment of the Tn*4653* transposon.

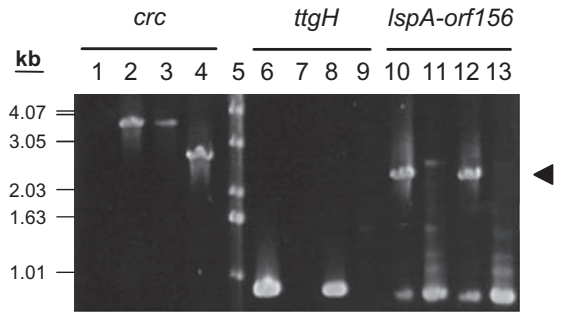

Fig. 6. Colony PCR analysis of the strains used in the mating experiment between *P. putida* DOT-T1E PS62 and *P. putida* KT2440*crc*. *P. putida* DOT-T1E PS62 was used as the donor strain (lanes 1, 6 and 10), and KT2440*crc* was used as the receptor strain (lanes 2, 7 and 11). A randomly chosen transconjugant clone of *P. putida* KT2440*crc* harbouring the pGRT1-*ttgV*::*aphA3* plasmid was used for the analysis (lanes 3, 8 and 12); *P. putida* KT2440 was included as a PCR control (lanes 4, 9 and 13). Specific primers were used to amplify the following genes *crc*, *crc*::*aacC1*, *ttgH* and the *lspA-orf156* region. The arrowhead on the right indicates the band corresponding to the *lspA-orf156* amplicon.

performed to verify the identity of the transconjugant colonies. Amplification with primers specific for the *P. putida* KT2440*crc* gene confirmed that, as expected, the chosen transconjugants derived from the recipient KT2440*crc* strain, as they exhibited the 3.8 kb fragment corresponding to the *crc*::*aacC1* allele (Fig. 6). The presence of a large plasmid in the transconjugant was confirmed by DNA electrophoresis of plasmid preparations obtained from this clone (not shown). Positive PCR amplification of the DOT-T1E *ttgH* gene (0.89 kb band in Fig. 6) in the transconjugant clone confirmed the transfer of the *ttgW-ttgV*::*aphA3-ttgGHI* genes from *P. putida* DOT-T1E PS62 to *P. putida* KT2440*crc*. Furthermore, the successful amplification of a 2.3 kb DNA fragment (Fig. 6) covering the *lspA-orf156* DNA region from Tn*4653*a- and IS*Ppu12*-like transposable elements (see Fig. 1B) proved that both transposons were also mobilized into the transconjugant strain.

To test the frequency of transfer of the pGRT1 plasmid between the same *P. putida* strain, we carried out a mating assay using *P. putida* KT2440*crc* (pGRT1-*ttgV*::*aphA3*) as the donor strain and *P. putida* KT2442, a rifampicin-resistant derivative of KT2440, as the recipient. The transconjugants, selected on M9 minimal medium supplemented with 5 mM benzoate plus kanamycin and rifampicin, appeared at a frequency of about 5×10^{-1} per recipient, indicating that pGRT1 is a self-transmissible plasmid which transfers at a very high frequency similar to that of pWW0. We also found pGRT1 can be transferred to other *Pseudomonas* species like *Pseudomonas aeruginosa* and *P. stutzeri*, but not to α-(*Sinorhizobium*, *Sphingomonas*), β-(*Burkholderia cepacia*) or γ-proteobacteria (*Escherichia coli* ET8000).

Pseudomonas putida *KT2440 and* P. aeruginosa *PAO1 cells carrying the pGRT1 plasmid are highly tolerant to toluene*

In *P. putida* KT2440 only the genes encoding the TtgABC pump (98.8% identical to those from DOT-T1E) are present and this strain is unable to survive in the presence of elevated toluene concentrations above 0.3% (v/v). *Pseudomonas aeruginosa* PAO1 exhibits a large number of efflux pumps (Poole, 2004), but does not grow in the presence of high concentrations of toluene. To test whether the presence of the pGRT1-*ttgV*::*aphA3* plasmid in KT2440*crc* and PAO1 had any influence on solvent tolerance, we analysed the toluene tolerance of these strains by determining the survival rate to a sudden 0.3% (v/v) toluene shock in cells that were not pre-exposed to the solvent. We also transferred the pGRT1-*ttgV*::*aphA3* plasmid to DOT-T1E-100 to test whether the reacquisition of the plasmid restored solvent tolerance. Figure 7 shows the survival rate of *P. putida* KT2440*crc* and KT2440*crc* (pGRT1-*ttgV*::*aphA3*) to a sudden toluene shock. Survival of KT2440*crc* was below our detection limit ($< 10^2$ cells ml^{-1}) (Fig. 7B), but surprisingly, strain KT2440*crc* harbouring the pGRT1-*ttgV*::*aphA3* plasmid exhibited a level of survival practically identical to that obtained for DOT-T1E PS62 (Fig. 7A and C), which denotes the predominant role of this efflux pump in the mechanisms of solvent tolerance. Similar results were obtained when the assays were performed with *P. putida* DOT-T1E-100 (pGRT1-*ttgV*::*aphA3*), *P. putida* KT2442 (pGRT1-

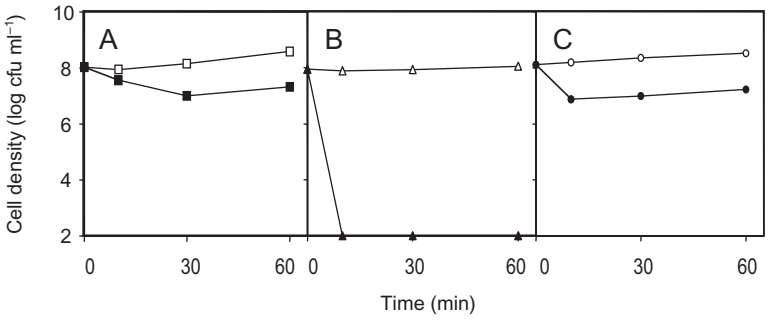

Fig. 7. Survival of *P. putida* DOT-T1E PS62 (A), KT2440*crc* (B) and KT2440*crc* (pGRT1-*ttgV*::*aphA3*) (C) after a toluene shock. Cells were grown in LB medium until they reached an OD_{660} of about 0.8. The cultures were then split into two halves; 0.3% (v/v) toluene was added to one half (closed symbols) and the other was used as a control (open symbols). The number of viable cells was determined at the indicated times.

ttgV::*aphA3*) and *P. aeruginosa* PAO1 (pGRT1-*ttgV*::*aphA3*) (not shown). Furthermore, the solvent sensitive *P. putida* KT2440, *P. putida* DOT-T1E-100 and *P. aeruginosa* PAO1 strains bearing the pGRT1 plasmid derivative were able to grow to high cell densities [$> 10^8$ colony-forming units ml^{-1}] in LB culture medium with 3% (v/v) toluene.

Discussion

It has been previously shown that in *P. putida* DOT-T1E, three efflux pumps are involved in solvent tolerance (TtgABC, TtgDEF and TtgGHI), although the TtgGHI efflux pump played a major role from a quantitative point of view in the extrusion of toluene and a wide range of toxic aromatic hydrocarbons (Rojas *et al*., 2003; Segura *et al*., 2003). The *ttgABC* genes are widely disseminated in genomes of different *P. putida* strains, and their chromosomal location was identified when the genome of KT2440 was sequenced. The *ttgDEF* genes are also chromosomally encoded, and Mosqueda and Ramos (2000) showed that they are located downstream from the *tod* genes encoding toluene degradation. This observation was further confirmed in other strains bearing the *tod* genes (Phoenix *et al*., 2003; Segura *et al*., 2003). One explanation proposed for this chromosomal organization is the co-evolution of the *tod* genes for toluene degradation and the *ttgDEF* genes for toluene extrusion. The initial difficulties in visualizing plasmids in *P. putida* DOT-T1E led us to assume that the *ttgGHI* genes were also present in the host chromosome. We have now obtained solid evidence to support that the *ttgGHI* operon and the adjacent set of regulatory *ttgVW* genes are both borne by the pGRT1 plasmid. Indeed, transfer of the pGRT1-*ttgV*::*aphA3* plasmid to the solvent-sensitive *P. putida* KT2440 strain [as well as transfer of the pGRT1 derivative mutated in the *ttgW* pseudogene (results not shown)] conferred solvent tolerance to this strain, and reciprocally, the spontaneous loss of the plasmid from DOT-T1E led to the loss of the solvent-tolerant phenotype. Solvent-tolerant strains *P. putida* DOT-T1E and *P. putida* MTB6 carry apparently similar plasmids, whereas solvent-sensitive soil strains *P. putida* KT2440 and *P. putida* MTB5 do not carry the pGRT1 plasmid.

It is known that large plasmids can impose a metabolic burden on the strains that carry them, and that this can be a source of instability (Lucas *et al*., 2005; Gammon *et al*., 2006). We have shown here that pGRT1 is stable under non-selective conditions, and that the rate of plasmid loss is below 10^{-8} per cell and per cell generation, even when cells are cultured under suboptimal laboratory conditions. This is of interest because the property of solvent tolerance is a phenotype that can be exploited in the bioremediation of heavily polluted sites (Ramos *et al*., 1995) or in industrial processes in which solvent-tolerant bacteria are used as whole-cell biocatalysts in double-phase systems for the production of toxic compounds that partition in the organic phase of a solvent/water mixture (Godoy *et al*., 2004; Rojas *et al*., 2004; Wery and de Bont, 2004; Meyer *et al*., 2005; Neumann *et al*., 2005; Nijkamp *et al*., 2005). Therefore, the presence of the *ttgGHI* genes in a self-transmissible plasmid opens a window for applications of this plasmid in biotechnological processes for which biochemically relevant properties are present in other strains. Nonetheless, this potential seems to be restricted to a limited number of strains – mainly those belonging to the genus *Pseudomonas* – which suggests that pGRT1 is a narrow-host-range plasmid. If the solvent character is to be extended to other microbes, the approach should be the one used by Jan de Bont's group (Kieboom *et al*., 1998; Wery and de Bont, 2004), and which insists on the cloning of the *ttgGHI* genes into a cassette to transfer the pump genes to the recipient strain. Although this approach is valid, it should be noted that we found gene instability associated with the *ttgH* gene cloned in low- and medium-copy-number expression vectors (V. García and A. Segura, unpublished). It thus seems that the presence of genes in a self-transmissible plasmid is advantageous for biotechnological exploitation, although restricted to hosts in which the plasmid replicates.

In our laboratory we have screened over 140 000 clones for solvent sensitivity, and found that the character was associated to loss of the pGRT1 plasmid in only nine cases. Nonetheless, plasmid loss is not the only way in which *P. putida* DOT-T1E can lose the solvent-tolerant phenotype as in the course of this study we identified a mutant that had lost approximately 50 kb of plasmid DNA that included the *ttgGHI* efflux pump genes. Therefore, two different mechanisms are involved in the loss of the main solvent-tolerance determinant in *P. putida* DOT-T1E, namely loss of the pGRT1 plasmid – which could be a consequence of uncoupling plasmid replication and cell daughter septation – and the deletion of a 50 kb DNA fragment of the plasmid. At present we do not know which mechanism leads to this large deletion, but we expect to provide clues on this phenomenon once pGRT1 is sequenced.

In biodegradation, loss of catabolic properties related to plasmid-encoded functions has been well described in the case of the TOL plasmid. TOL plasmid loss can be linked to a temperature increase as replication of the plasmid cannot occur at, for example, 41°C. Furthermore, genes can be lost from the TOL plasmid without plasmid loss due to the recombination of two 1400 bp *IS* sequences (Bailey *et al*., 1977). The mechanism through which pGRT1 is lost is unknown, as well as the reason for the deletion of a large fragment in the plasmid derivative found in strain VAN5.

In summary, our experiments showed that the *ttgGHI* genes are borne on a self-transmissible narrow-host-range pGRT1 plasmid. This plasmid is highly stable and its frequency of loss is very low, making it a potentially suitable biotechnological tool to enhance solvent tolerance in other microbes.

Experimental procedures

Bacterial strains, culture media and growth conditions

The *P. putida* strains used in this study are listed in Table 1. Bacteria were grown routinely at 30°C in liquid LB medium (Sambrook *et al*., 1989) or in M9 minimal medium supplemented with benzoate (5 mM) or glucose (10 mM) as the sole carbon source (Abril *et al*., 1989), on an orbital platform operating at 200 strokes per min. When required, antibiotics were added at the following final concentrations (μg ml^{-1}): gentamicin, 25; kanamycin, 50; and rifampicin, 20.

Standard DNA techniques

Standard molecular biology techniques were used for DNA manipulations (Sambrook *et al*., 1989). Southern blot analyses were performed as previously described (Ausubel *et al*., 2006). DNA probes were labelled by PCR with digoxigenin-11-dUTP (Roche, Cat. No. 11573179910) as recommended by the manufacturer. The sequences of the primer pairs used to generate the hybridization probes will be provided on request. In cases where two probes were used to hybridize the same Southern blot, hybridizations were performed sequentially; the filters were boiled in distilled water between hybridizations.

For total DNA isolation, 0.5 ml aliquots of overnight cultures were harvested by centrifugation and re-suspended in 400 μl of buffer A [25 mM Tris-HCl, pH 8.0; 25 mM EDTA; 20% (w/v) sucrose; 100 μg ml^{-1} RNase A]. Cells were lysed by adding 200 μl of a solution containing 2% (w/v) SDS and 0.3 N NaOH. After incubation for 10 min on ice, $MgCl_2$ was added to a final concentration of 10 mM, and the lysate was extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1) and ethanol precipitated with ammonium acetate 0.3 M, pH 4.8. After incubation for 20 min at –20°C, the precipitated DNA was collected by centrifugation (16 600 *g*, 15 min, 4°C), washed with 70% (v/v) ethanol, dried and dissolved in 50 μl of distilled water.

Mating procedure

Biparental matings between *Pseudomonas* strains were performed as described before (Ramos-González *et al*., 1991). For the conjugal transfer of pGRT1-*ttgV*::*aphA3* from *P. putida* KT2440*crc* (pGRT1-*ttgV*::*aphA3*) to *P. aeruginosa* PAO1, the receptor strain was grown on solid LB medium at 43°C for 2 days prior to mating, to transiently overcome the host restriction/modification system (Rolfe and Holloway, 1966). PAO1 (pGRT1-*ttgV*::*aphA3*) transconjugants were selected by growth on LB plates containing kanamycin (100 μg ml^{-1}), at 42°C.

Plasmid DNA isolation

Isolation of large plasmid DNA was performed according to the alkaline lysis procedure of Zhou and colleagues (1990) with several modifications. Briefly, 0.5 ml of an overnight bacterial culture was collected by centrifugation. Most of the supernatant was decanted and the pellet was thoroughly re-suspended in the remaining fluid and lysed for 5 min on ice with 300 μl of TENS [10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.1 N NaOH; 10% (w/v) SDS] and RNase A (100 μg ml^{-1}). Samples were neutralized with 3 M sodium acetate pH 5.2 (150 μl), and cell debris and chromosomal DNA were pelleted by centrifugation at 4°C (16 600 *g*, 10 min). Proteins were then removed from the resulting supernatant fraction by successive extractions with phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). Plasmid DNA was ethanol-precipitated (for 30 min at –20°C), concentrated by centrifugation, washed with 70% (v/v) ethanol and re-suspended in 15 μl of distilled water. Large plasmids were resolved by electrophoresis on 0.6% (w/v) agarose gels in a Tris-acetate buffer system (Sambrook *et al*., 1989).

Genomic DNA microarrays

The genome-wide DNA chip used in this work (printed by Progenika Biopharma; http://www.progenika.com) has been described in detail previously (Yuste *et al*., 2006). It consists of an array of 5539 oligonucleotides (50-mer) spotted in duplicate onto γ-amino silane-treated slides, and covalently linked with UV light and heat. The oligonucleotides represent 5350 of the 5421 predicted ORFs annotated in the *P. putida* KT2440 genome (Nelson *et al*., 2002; http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). In addition, 140 of the 148 putative ORFs predicted for the TOL plasmid pWW0 are also included, together with a suite of 49 commonly used reporter genes and antibiotic resistance markers. The chips also have homogeneity controls consisting of oligonucleotides for the *rpoD* and *rpoN* genes spotted at 20 different positions, as well as duplicate negative controls at 203 pre-defined positions.

To prepare fluorescently labelled DNA, 2 μg of total DNA was primed with 7.5 μg of pd(N)$_6$ random hexamers (Amersham, Cat. No. 27-2166-01). Probe synthesis was performed at 37°C for 3 h in a 30 μl reaction volume containing 0.5 mM (each) dATP, dCTP and dGTP; 0.25 mM (each) dTTP and amino allyl-dUTP (aa-dUTP; Sigma, Cat. No. A0410); 1× Klenow buffer and 50 U Klenow fragment (New England Biolabs, Madrid, Spain). The reaction was stopped by adding 3 μl of 0.5 M EDTA, and the DNA was purified by using a QIAquick PCR purification column (Qiagen, Germany) and evaporated in a Speed-Vac. The amino-modified cDNA was labelled with Cy3 or Cy5 fluorescent dyes (monoreactive NHS-esters; Amersham Biosciences, Cat No. PA23001 and PA25001 respectively) according to the manufacturer's instructions, and purified as described above. Labelling efficiency was assessed with a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). Equal amounts of Cy3- and Cy5-labelled cDNAs, one of them corresponding to the control and the other to the problem sample, were mixed, dried in a Speed-Vac and reconstituted in 35 μl of hybridization buffer [5× SSC, 25% (v/v) formamide, 0.5% (w/v) SDS, 5× Denhardt's solution, 5% (w/v) dextransulfate] pre-heated to 42°C.

Prior to the hybridization process, the microarray slides were blocked by immersion in 5× SSC, 0.1% (w/v) SDS, 1% (w/v) bovine serum albumin for 45 min at 42°C. The slides were washed by two successive immersions in distilled water at room temperature, followed by a final wash with isopropanol. The slides were spin-dried by centrifugation at 1500 *g* for 5 min, and used within the next hour.

The labelled probe was denatured at 98°C for 3 min, applied to the microarray slide and covered with a glass coverslip. The slide was then placed in a humidified hybridization chamber (AHC ArrayIt Hybridization Cassette; Telechem International) and incubated for 18–20 h in a water bath at 42°C, protected from light. After hybridization the microarrays were washed by gentle shaking in 2× SSC, 0.1% (w/v) SDS at 42°C for 5 min, followed by a 5 min wash at room temperature in 1× SSC, two 5 min washes in 0.2× SSC and a final 5 min wash in 0.1× SSC. The slides were spin-dried in a centrifuge at 1500 *g* for 5 min and scanned on a GenePix 4100A scanner (Axon Instruments). Images were acquired at 10 μm resolution, and the background-subtracted median spot intensities were determined with GenePix Pro 5.1 image analysis software (Axon Instruments). Spots with anomalies were removed manually with GenePix software before the data were processed. To filter out unreliable data, spots with signal intensity below twice the standard deviation of the background in the control channel, which reveals the genomic DNA printed on the slide, were discarded. The microarray data were processed according to the method by Kim and colleagues (2002) with the algorithm in the GACK software (http://falkow.stanford.edu/whatwedo/software/software.html). This algorithm uses the shape of the signal distribution to estimate the probability of any given gene being conserved (present) or divergent (absent) independently of any normalization, and thus provides a level of confidence for gene category assignment. This method has been shown to be more reliable to predict the presence or absence of genes than methods based on the empirical determination of cut-off values. At least two hydridizations (from independent DNA extractions) were performed and averaged across the GACK data sets.

Survival in response to toluene shocks

Cells were grown overnight in 30 ml of LB medium. On the following day cultures were diluted to reach a turbidity at 660 nm (OD_{660}) of 0.05, and grown under the same conditions until they reached an OD_{660} of about 0.8. Then the cultures were divided into two halves; 0.3% (v/v) toluene was added to one half, and the other was used as a control. The number of viable cells was determined (before toluene addition and after 10, 30 and 60 min of exposure to the solvent) by spreading suitable dilutions on LB plates. The assays were performed in duplicate and repeated at least three times.

Determination of the frequency of loss of solvent tolerance and the ttgH *gene*

To trace the stability of the pGRT1 plasmid we used *P. putida* strain DOT-T1E PS51 (Table 1). This strain carries a kanamycin resistance marker (ΩKm) inserted in the *ttgW* pseudogene (Rojas *et al*., 2003). This insertion does not influence solvent tolerance and allows plasmid stability to be easily determined. Cells were grown in batch at 30°C on LB medium for at least 100 generations, and then plated on large Petri dishes (20 × 20 cm) with solid LB to obtain around 10 000 independent colonies per plate. About 20 000 colonies were replica-plated on LB plus kanamycin using a colony-picking robot from the *Pseudomonas* Reference Culture Collection. Kanamycin-sensitive colonies were selected and DNA was extracted to confirm the lack of *ttgH* by PCR and Southern blot hybridization. The presence or absence of the plasmid was checked on agarose gels and by Southern blot hybridization using a Tn*4653A* internal probe generated with primers based on the *lspA* gene and *orf156* (see Fig. 1).

For carbon starvation assays, *P. putida* DOT-T1E PS51 cells were grown on liquid M9 minimal medium supplemented with 10 mM glucose for 5 days, and then cell suspensions were diluted and plated on LB medium. Around 20 000 colonies were replica-plated on LB with and without kanamycin. To test plasmid stability in response to membrane-disturbing agents, bacteria were grown for 100 generations in LB medium with sublethal concentrations of the stressing agents 0.1 mM SDS, 7 μg ml^{-1} tetracycline and 0.3 M NaCl. Cells were then spread on LB medium and Km^S clones were searched for among pools of about 20 000 colonies, as described above.

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New transporters involved in stress tolerance: from proteomic and transcriptomic data to functional analysis

Vanina García, Patricia Godoy, Ana Hurtado, Juan-Luis Ramos and Ana Segura

Pseudomonas putida DOT-T1E es una cepa muy tolerante a disolventes. Aunque el principal mecanismo que confiere tolerancia a disolventes en esta cepa es la bomba de extrusión TtgGHI, muchas otras proteínas están involucradas en la respuesta a tolueno. Análisis previos de transcriptómica y proteómica llevados a cabo en nuestro laboratorio con la cepa *P. putida* DOT-T1E y la cepa sensible a tolueno, *P. putida* KT2440, revelaron que una serie de transportadores fueron inducidos en presencia de tolueno. Para determinar el rol de esos transportadores preparamos mutantes de los correspondientes genes en *P. putida* DOT-T1E con el propósito de analizar sus fenotipos con respecto a la tolerancia a disolventes, resistencia a estrés y crecimiento en diferentes fuentes de carbono, nitrógeno y azufre. Con esos datos hemos asignado una función a tres de los cinco transportadores analizados. Uno de ellos es un transportador de sulfato/sulfito, mientras que los otros dos transportadores están involucrados en la resistencia múltiple a drogas y tolerancia a tolueno.

Manuscrito en preparación.

Manuscrito en preparación

New transporters involved in stress tolerance: from proteomic and transcriptomic data to functional analysis

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ABSTRACT

Pseudomonas. putida DOT-T1E is a highly solvent-tolerant strain. Although the main mechanism that confers the strain with solvent tolerance is the TtgGHI efflux pump, several other proteins are involved in the response to toluene. Previous proteomic and transcriptomic analysis carried out in our lab with *P. putida* DOT-T1E, and the solvent-sensitive strain, *P. putida* KT2440, revealed a number of transporters that were induced in the presence of toluene. To determine the role of these transporters we prepared mutants of the corresponding genes in *P. putida* DOT-T1E in order to analyse their phenotypes regarding solvent tolerance, stress endurance and growth in different carbon, nitrogen and sulphur sources. With these data we have assigned a function to three out of the five transporters under analysis. One of them is a sulphate/sulphite transporter, while other two transporters are involved in multidrug resistance and toluene tolerance.

INTRODUCTION

Pseudomonas putida DOT-T1E is a highly solvent-tolerant strain that was isolated from a waste water treatment plant in Granada (Ramos *et al*., 1995). Solvent tolerance in this strain is achieved by a combination of lipid modifications at the membrane level (Ramos *et al.*, 1997; Junker and Ramos, 1999; Ramos *et al*., 2002; Bernal *et al*., 2007) and the extrusion of the toxic compound via efflux pumps belonging to the RND (Resistance, Nodulation and cell Division) family. In this strain, three efflux pumps have been identified and found to be involved in solvent tolerance, namely TtgABC (Ramos *et al.*, 1998), TtgDEF (Mosqueda and Ramos, 2000) and TtgGHI (Rojas *et al*., 2001). TtgABC and TtgGHI have been shown to be able to extrude not only organic solvents such as toluene, xylenes or propylbenzene but also antibiotics and ethidium bromide (Terán *et al.*, 2005). The three efflux pumps identified in *P. putida* DOT-T1E are expressed under different conditions. The *ttgABC* operon is expressed under normal laboratory conditions and is induced by antibiotics such as tetracycline or chloramphenicol but is only slightly induced by organic solvents (Duque *et al*., 2001; Terán *et al*., 2003). The *ttgDEF* operon is not expressed under normal laboratory conditions but its expression is triggered by the presence of organic solvents (Mosqueda and Ramos, 2000), and

TABLE 1. Transporters induced in response to toluene (Segura *et al.*, 2002; Dominguez-Cuevas *et al.*, 2006). (*) Proteins induced in the presence of toluene in *P. putida* DOT-T1E; [a] Protein is only visible in the gel when toluene is present in the media.
n.s: not sequenced.

Description	Fold-change	Oligonucleotides used for amplification	Fragment size (bp)	GenBank number (*P. putida* DOT-T1E)
Sugar ABC transporter, periplasmic sugar-binding protein	5.75	---	---	n.s.
Major facilitator superfamily transporter	9.54	PP3349.1 ggaatcCAATACCCTGCAGTGAACCC PP3349.2 gcggatccGCCGTGCTCACCATCGGCTTG	913	---
ABC transporter, periplasmic binding protein*	[a]	PP1726.1 CAGCAAGTACGGCCTCAAGC PP1726.2 TGGGGTGCTTGGCGTACTTG	640	FJ853990
RND efflux transporter	2.41	PP1517.1 ggaattcGGCCTGCAGGACGAGAAAG PP1517.2 gcggatccGTGCTGATCAGCCAACTGCG	1390	FJ842959
Multidrug resistance transporter Bcr/CflA family	4.35	PP3588.1 ggaattcCGGTATTCGTGATCATCGCT PP3588.2 cgggatccACCATGAACAGCACGCCCA	610	FJ853990
Toluene tolerance ABC transporter	2.33	PP0960.5 GGACGCCTGATGAGCTGATG PP0960.7 TCGTCTTCACCGCCAACGC	1290	FJ842958
ABC transporter, periplasmic binding protein	2.38	PP0219.1 ggaattcCTGGTAGCCATACCGATAGG PP0219.2 cgggatccTCGGTGCAACACTCGCCGC	728	FJ853991

ttgGHI is expressed under normal laboratory conditions and is induced in the presence of organic solvents, but not antibiotics (Rojas *et al*., 2001). This differential expression suggests that solvent tolerance is a complex trait in which different processes are coordinated to achieve high level of solvent tolerance.

One of the most comprehensive methods to study such complex traits is the analysis of the reprogramming of global expression after exposure to a stressor by proteomic and/or transcriptomic analysis. Proteome analysis of *P. putida* cells grown in glucose and glucose plus toluene in the gas phase revealed that a number of proteins were specifically induced in response to toluene, mainly toluene degradation proteins, proteins related with stress responses, and proteins involved in energy generation (Segura *et al*., 2005; Volkers *et al*., 2006). Transcriptomic analysis in the solvent-sensitive strain *P. putida* KT2440 (Domínguez-Cuevas *et al*., 2006) revealed very similar responses, such that the presence of toluene induced the expression of genes related with stress endurance and toluene degradation. Therefore, proteomic and transcriptomic analysis provide an overall view of the functions involved in toluene response; however, these experimental approaches are limited in their ability to shed light about the specific role of the genes/proteins in the cell response.

In this study, we have focussed our interest in several transport proteins that were reported to be induced in response to toluene in *P. putida* DOT-T1E and *P. putida* KT2440. We have constructed the corresponding knock-out mutants in the solvent-tolerant *P. putida* DOT-T1E strain and we have studied the response of these mutants towards different stresses, including organic solvent shock. We have also monitored the growth of the strains on different carbon (C), nitrogen (N), and sulphur (S) sources. Our results show that two of these transporters are involved in toluene tolerance, a third one is related to sulphur metabolism, and no role could be assigned to other two transporters. Of the transporters that we show to be involved in toluene tolerance, one belongs to the Major Facilitator Superfamily (MFS) and exhibits homology to a putative transporter of the Bcr/CflA family that has not been previously described to be involved in toluene tolerance, while the second transporter (*ttg2ABCD*) belongs to the RND family of extrusion pumps, which are involved in multidrug resistance (MDR) in *P. putida* DOT-T1E.

TABLE 2. List of mutant strains constructed in this study.

Strain	Mutated gene (KT2440)	Transporter
P. putida DOT-T1E-PS127	PP_3588	Multidrug resistance (Bcr/CflA family)
P. putida DOT-T1E-PS128	PP_1517	RND efflux
P. putida DOT-T1E-PS129	PP_0219	ABC, inner membrane component (permease)
P. putida DOT-T1E-PS130	PP_0960	ABC, periplasmic substrate binding protein
P. putida DOT-T1E-PS168	PP_1726	ABC, periplasmic substrate binding protein

MATERIALS AND METHODS

Construction of mutants

For the construction of mutants, gene fragments of about 600 to 1400 bp (Table 1) were amplified with appropriate oligonucleotides (Table 1), based on the *P. putida* KT2440 genome sequence (Nelson *et al.*, 2002), while using *P. putida* DOT-T1E chromosomal DNA as a template. Amplified fragments were cloned into $pMBL_1$-T plasmid (Dominion MBL), except for the 610 bp fragment amplified from PP3588 that was cut with *Eco*RI and *Bam*HI and cloned into pUC18 (Norrander *et al*., 1983; GenBank: L08752) digested previously with the same restriction enzymes. All the plasmids containing the corresponding inserts were then cut with *Bam*HI, and the Ω::*Km* resistance cassette of plasmid pHP45Ω-Km (Prentki and Krisch, 1984), previously excised with *Bam*HI, was ligated into the *Bam*HI restriction site. The fragment amplified using oligonucleotides PP0958-5 and PP0958-7 contained two internal *Bam*HI restriction sites so that the DNA that was finally cloned into the plasmid containing the Ω::*Km* cassette was a 314 bp fragment.

About 600 ng of each plasmid was electroporated into *P. putida* DOT-T1E (Enderle and Farwell, 1998) and the resulting kanamycin resistance clones were selected and analyzed by Southern blot (not shown). Clones that contained the insertion in the appropriate location were kept for further analysis (Table 2).

Survival in response to organic solvent shocks. Cells were grown overnight in 30 ml of LB medium. 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 (≈ 10^8 CFU/ml). Then cultures were divided into two halves; 0.3% (v/v) of the organic solvent was added to one, and the other was kept as a control. The number of viable cells was determined before toluene was added and at 10, 30 and 60 minutes after the addition of toluene. Viable cells were counted before and 10 minutes after other organic solvent shocks (propylbenzene, *o*-, *p*- and *m*-xylene).

Phenotypic characterization

Strains were grown on M9 solid medium (Abril *et al*., 1989) supplemented with 0.1% (w/v) glucose. The following day cells were recovered with a loop and resuspended in M8 minimal medium (Sambrook *et al*., 1989). The wild-type and the mutant strains were inoculated in microwell plates in M9 minimal medium with different carbon (5 mM), nitrogen (5 mM) and sulphur (5 mM) sources, and growth at 30ºC with continuous shaking was monitored in a Bioscreen C (ThermoFisher Scientific) at 420-580 nm for 24 hours. Minimal medium M9 was prepared as in Abril *et al*. (1989) but when appropriate $MgSO_4$ or NH_4Cl were replaced by other sulphur or nitrogen sources. *Carbon sources*: D-glucose, D-fructose, D-glucuronic acid, glycerol, sodium acetate, trisodium citrate, fumaric acid, sodium succinate, lactic acid, sodium lactate, malic acid, sodium pyruvate, methyl pyruvate, propionic acid, sodium benzoate, sodium 4-hydroxybenzoate, quinic acid, aminobutyric acid, 5-amino-n-valeric acid, 2,4-dihydroxyphenylacetic acid, sodium decanoate, Tween 20, 2-phenyl ethanolamine, L-Leu, L-Lys, L-His, L-Gln, L-Glu, L-Phe, L-Arg, L-Asn, L-Ala, L-Pro, L-Tyr, L-Ile, L-Cys, L-Met, L-Val, glutaric acid, and xylose. *Nitrogen sources*: D- and L-Arg, D- and L-Lys, D- and L-Pro, D- and L-Val, D- and L-Ala, D- and L-Asn, D-Met, D-Leu, L-Asp, L-Cys, L-Phe, L-Glu, L-Gln, L-Gly, L-His, L-homoserine, L-Ile, L-*trans* hydroxyproline, L-Ser, L-Tyr, Ala-Glu, Ala-Gly, Ala-His, Ala-Leu, Ala-Phe, Gly-Gln, Gly-Gly, Gly-Leu, Gly-Ser, Gly-Val, Tyr-Ala, adenine, agmatine sulphate, hypoxanthine, phenylethanolamine, ethanolamine, putrescine, DL-ornithine, NH_4Cl and NH_4NO_3 and urea. *Sulphur sources:* L-Cys, D- and L-Met, L-cystine, cysteamine, D,L-ethionine, D,L-homocysteine, taurine, thiourea, 2-thiouracil, N-acetyl-cysteamine, 2-thiohidanthoin, sodium taurocholate, agmatine sulphate, Na_2SO_4, Na_2SO_3.

For stress experiments all strains were grown overnight in 1/5 LB medium plate, resuspended in M8 minimal media as before and inoculated in ½ LB liquid medium plus the corresponding stressor. Stressors used were: *Antibiotics*: ampicillin(Ap) 10 µg/ml; carbenicillin (Cb) 80 µg/ml; chloramphenicol (Cm) 15 µg/ml; cefotaxine (Ctx) 0.375 µg/ml; erythromycin (Ery) 15 µg/ml; gentamycin (Gm) 2 µg/ml; kanamycin (Km) 0.195 µg/ml; nalidixic acid (Nal) 0.012 mg/ml; neomycin (Neo) 1 µg/ml; norfloxacin (Nor) 0.05 µg/ml; novomycin (Nov) 100 µg/ml; piperacillin (Pip) 10 µg/ml; rifampicin (Rif) 30 µg/ml; streptomycin (Sm) 2 µg/ml; spectinomycin (Sp) 0.1 mg/ml; and tetracycline (Tc) 5 µg/ml. *Heavy metals and metaloids*: $AgNO_3$ (3 mM), $CdCl_2$ (1.56 mM), $CoCl_2$ (0.156 mM), $CuSO_4$ (1 mM), LiCl (0.25 mM), $MnSO_4$ (1 mM), $NiCl_2$ (1 mM), RbCl (6.2 µM), K_2TeO_3 (0.975 µg/ml); KH_2AsO_4 (0.9 mg/ml) and $ZnCl_2$ (0.5 mM). *Detergents and protein solubilising agents:* Cetyl trimethylammonium bromide (CTAB), 0.001%; N-lauroyl sarcosine (NLS), 0.078%; sodium dodecyl sulfate (SDS), 0.06%; deoxycholate (DOC), 1%; tritón X-100 (1%) and non-detergent sulphobetaines (NDSB-201), 1%.

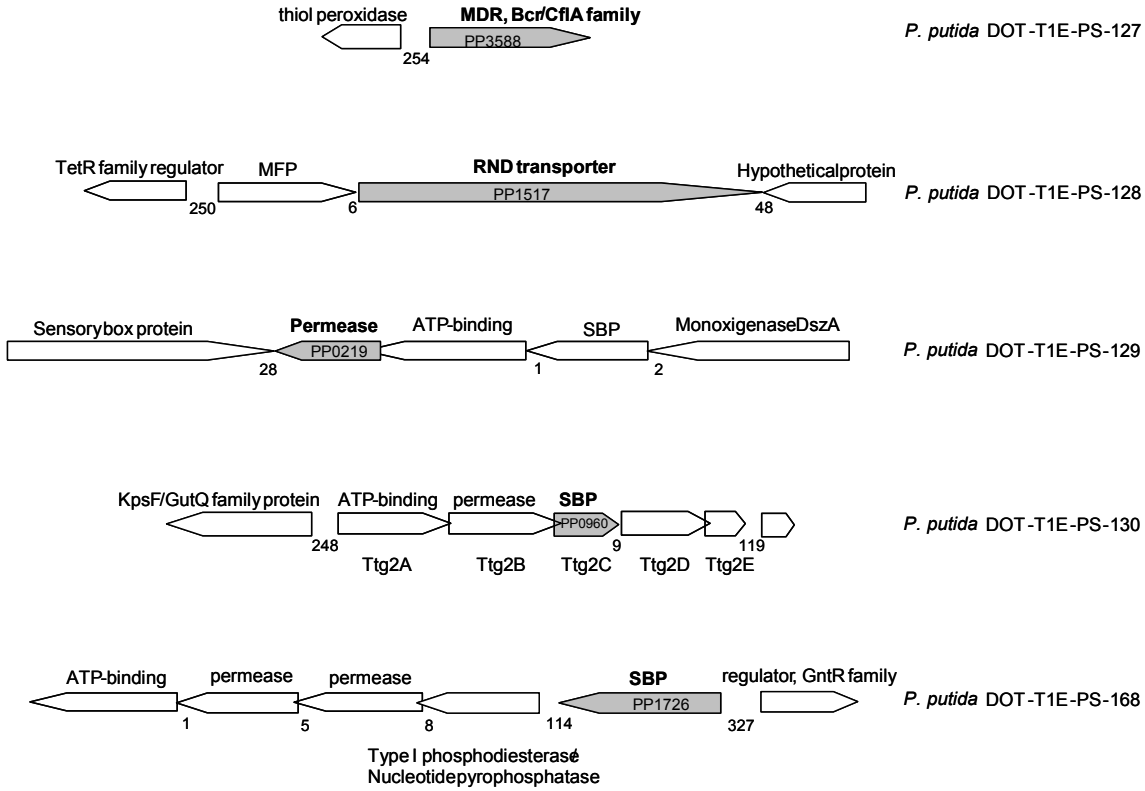

Figure 1. Schematic representation of the genes under study and the surrounding chromosomal area in *P. putida* DOT-T1E. Mutated genes are indicated as grey arrows and the corresponding gene number in the *P. putida* KT2440 genome is given inside the arrow. Numbers between arrows indicated the number of base pairs of the intergenic regions.; when not indicated the genes overlapped. We refer as permease to the inner membrane component of the ABC systems. SBP stands for periplasmic substrate binding protein. Ttg2ABCDE is the nomenclature of these genes in *P. putida* GM73.

Chelating agents: ethylenediaminetetraacetic acid (EDTA), 0.125 mM and 2,2'-bipyridyl (Bip) (0.5 mM). *Osmotic stress agents*: NaCl (0.5 M) *Oxidative stress:* $K_2Cr_2O_7$, 12.5 µg/ml; H_2O_2, 0.004%; NH_2OH, 30 µg/ml; methyl viologen (MV), 100 µM; Tert-butyl hydroperoxide (TBH), 0.0015%. *Others*: Ethidium bromide (EtBr) 0.1 mg/ml; KCN 0.325 mg/ml and KSCN (80 mM) and NaBr (0.25 M).

At least three independent experiments were performed for each condition. Growth curves were drawn using the media values of the three experiments (standard deviations were always less than 0.1 units of the O.D. value). Generation time (tg) was calculated using the formula tg = log2/r, with r being the slope of curve when bacteria were growing in exponential phase. When indicated, stress experiments were done in 100 ml flasks using 30 ml of LB liquid medium.

RESULTS

Identification of transporters in *P. putida* DOT-T1E and construction of the corresponding mutants

Seven *Pseudomonas* transporter systems were reported to be induced by toluene in previous proteomic and transcriptomic experiments (Segura *et al.*, 2005; Domínguez-Cuevas *et al.*, 2006, Table 1). PP1015, which encodes a sugar ABC transporter (del Castillo *et al.,* 2008), is likely induced in the proteomic analysis because of the higher energy demand of cultures growing in the presence toluene than those growing without toluene, therefore, it was discarded.

We were unable to amplify the predicted 913 bp fragment of the major facilitator

family transporter (PP3349) using *P. putida* DOT-T1E chromosomal DNA; furthermore hybridization of *P. putida* DOT-T1E chromosomal DNA with a probe synthesized by amplification from *P. putida* KT2440 DNA failed to reveal any hybridization band. These results suggested that a gene homologous to the PP3349 does not exist in *P. putida* DOT-T1E.

Five mutants were constructed that corresponded to knock-outs in *P. putida* genes homologous to PP3588, PP1517, PP0219, PP0960 and PP1726 (Table 2). The complete sequence of the chromosomic region surrounding these genes was determined. Gene organization of these chromosomal regions in strain *P. putida* DOT-T1E (Figure 1) was identical to that of the *P. putida* KT2440 and *P. putida* F1 strains. ORFs analysis revealed that the deduced protein sequences were around 98-99% identical to that of KT2440 and F1.

Solvent tolerance of the five transporters mutants and growth with toluene as the sole carbon source

Solvent tolerance in *P. putida* DOT-T1E is an inducible response (Ramos *et al.*, 1998); only 1 out of 10,000 cells survived the sudden addition of 0.3% (v/v) toluene. However, when cultures were grown in the presence of sublethal concentrations of toluene (i.e. toluene in the gas phase) almost 100% of the cells survived the toluene shock (Figure 2). This response was similar in mutants, *P. putida* DOT-T1E-PS128 (knocked out mutant in an RND transporter homologous to PP1517), *P. putida* DOT-T1E-PS129 (knocked out mutant in an ABC permease homologous to PP0219) , and *P. putida* DOT-T1E-PS168 (knocked out mutant in periplasmic substrate binding protein homologous to PP1726) (not shown). However, *P. putida* DOT-T1E-PS127 (knocked out mutant in

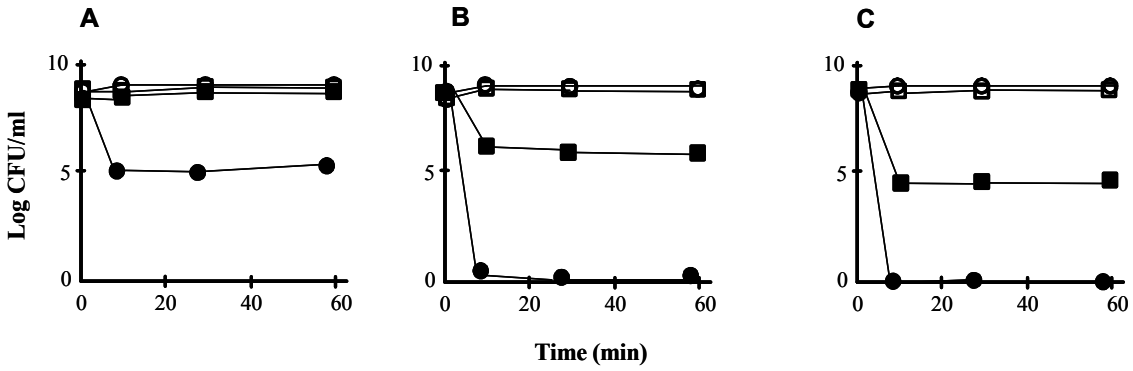

Figure 2. Survival of *P. putida* DOT-T1E (A), *P. putida* DOT-T1E-PS127 (B) and *P. putida* DOT-T1E-PS130 (C) after the addition of 0.3% (v/v) toluene. Circles: non-induced cultures, Squares: induced cultures. Closed symbols represent the survival after toluene addition, and open symbols represent controls without toluene.

MFS transporter homologous to PP3588) and *P. putida* DOT-T1E-PS130 (knocked out mutant in the substrate binding protein of the ABC transporter homologous to PP0960) mutants did not survive the sudden addition of 0.3% (v/v) toluene and their survival rate upon pre-induction with low toluene concentrations was lower (2 and 5 orders of magnitude, respectively) than in wild-type (Figure 2).

Solvent tolerance of the wild-type and mutant strains was also tested with other organic solvents, such as different xylene isomers and propylbenzene. No significant differences were observed between the wild-type and mutant strains (not shown), so sensitivity of *P. putida* DOT-T1E-PS127 and *P. putida* DOT-T1E-PS130 to organic solvents seemed to be restricted to toluene.

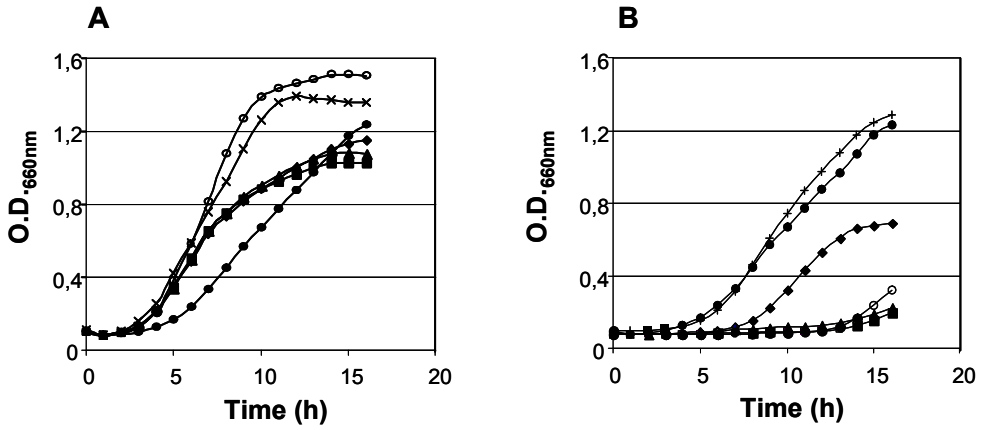

Figure 3. Growth of *P. putida* DOT-T1E (A) and *P. putida* DOT-T1E-PS129 (B) on different sulphur sources. Thiouracyl (black circles), L-cystine (crosses), L-methionine (diamonds), sodium sulphate (squares), sodium sulphite (triangles) and agmantine sulphate (white circles).

Resistance to other stresses

As many transporters have been reported to be involved in multidrug resistance (Krulwich *et al*., 2005; Piddock, 2006; Borges-Walmsley *et al*., 2003), we decided to subject the mutants to different stresses (heavy metals, chelating agents, mutagens, detergents, antibiotic and oxidative stress). We have previously determined the lethal concentration of each stressing agent for *P. putida* DOT-T1E (Godoy *et al.*, personal communication) and therefore performed the assays using sublethal concentrations of the stressors. The concentrations used for the growth curves were chosen such that a two-fold increase in concentration completely inhibited the growth of the wild-type strain.

Results clearly showed that *P. putida* DOT-T1E-PS130 was not only sensitive to toluene but also towards several oxidative stress agents ($Cr_2O_7^{2-}$ and TBH), ethidium bromide, CTAB, some heavy metals (Mn^{2+}, Ni^{+}), and antibiotics (Tc, Sm, Cm, Ery and Gm), suggesting that this is a multidrug resistance (MDR) transporter (Table 3). Mutant *P. putida* DOT-T1E-PS127 showed a longer lag phase than the wild-type when grown in the presence of Zn^{2+} or the chelating agent bypiridyl and the growth rate was lower in the presence of the detergent CTAB as well as the antibiotic Gm. *P. putida* DOT-T1E-PS128 was only affected by the presence of bypiridyl (lag = 10 h). *P. putida* DOT-T1E-PS129 was more susceptible than the wild-type to EtBr, DOC, Mn^{2+} and Ery (Table 3). *P. putida* DOT-T1E-PS168 was more sensitive toward MV (lag = 17 h *versus* lag = 11 h of the wild-type strain) and Gm (tg = 5.4 h).

Growth with different carbon, nitrogen and sulphur sources

To test if some of the transporters were required for the growth with certain carbon, nitrogen or sulphur sources, we decided to analyze the growth of the wild-type and mutant strains in minimal medium plus different carbon, nitrogen or sulphur sources. We did not find significant differences in the pattern of nitrogen source utilization among the wild-type and the mutant strains. However, under the conditions tested, *P. putida* DOT-T1E-PS129 grew very poorly, if at all, with any carbon or nitrogen source when the culture media was supplemented with magnesium sulphate. The growth of this mutant was also impaired when cultured with glucose as the sole carbon source and agmantine sulphate or sodium sulphite as the sulphur source. However, this mutant grew well in minimal media plus glucose when the sulphur source was L-cystine or 2-tiouracile (Figure 3). No differences were observed among the other mutants and the wild-type strain regarding the utilization of sulphur sources.

Table 3 : Generation time (tg) and duration of lag phase of the wild-type and mutant strains subjected to different stressors. Shaded numbers indicated the parameters that showed differences between the wild-type and mutant strains. Only differences in generation time and lag phase duration of 40% and higher are considered to be relevant and are shaded in the table. (*) These experiments have been carried out in 100 ml flasks

	DOT-T1E		DOT-T1E-PS127		DOT-T1E-PS129		DOT-T1E-PS130	
Stressor	**tg (h)**	**lag (h)**	**tg (h)**	**lag (h)**	**tg (h)**	**lag(h)**	**tg (h)**	**lag (h)**
$Cr_2O_7^{=}$	4.2	3	4.3	3	3.8	3	**8.3**	**5**
TBH	2.1	5	1.7	5	2.5	5	2.4	**11**
Bip	2.7	5	2.3	**11**	4.0	6	2.6	7
EtBr	1.9	5	2.3	5	**3.6**	5	**3**	6
CTAB	1.4	4	**3.3**	**13**	1.9	4	1.9	**7**
DOC	1.5	6	1.8	4	**2.8**	**7**	1.3	7
Mn^{2+}	2.3	11	2.0	10	**3.8**	12	**3.8**	12
Zn^{2+}	1.9	5	2.2	**8**	2.1	6	2.4	6
Ni^{+}	4.9	5	5.3	5	4.6	5	**12.4**	5
Sm*	0.8	2	0.8	2	0.9	2	**1.4**	2
Tc	2.5	6	2.4	6	3.0	6	**10.2**	**14**
Cm	2.2	4	1.9	4	2.1	4	**3.5**	5
Ery	4.9	6	3.3	5	**10.4**	6	**13.9**	6
Gm*	2.1	1	**3.1**	1	2.9	1	**16.8**	1

DISCUSSION

Cellular membranes are the first contact point between the cell and the environment. Transporters are important proteins for living organisms not only because permeability to nutrients and metabolites are basic for maintenance and growth but also because efflux of toxic compounds is crucial for survival in the presence of toxins such as antibiotics and organic solvents. In this study, five transporters belonging to three different superfamilies of bacterial transporters (Resistance Nodulation cell Division [RND] family, ATP-binding transporters [ABC], and major facilitator superfamily [MFS]), have been investigated. These genes were originally selected because they were induced in the presence of toluene within the solvent tolerant *P. putida* DOT-T1E strain or in the solvent sensitive *P. putida* KT2440 strain (Segura *et al.,* 2005; Domínguez-Cuevas *et al.*, 2006). However, preliminary analysis of transcriptomic data of *P. putida* DOT-T1E under conditions similar to those used by Domínguez-Cuevas *et al*. (2006), showed no significant transcriptional changes after 15 minutes of exposure to 1 mM of the organic solvent (Rodríguez-Hervá, personal communication). This was probably because

the stress condition was too mild to induce a response in such a highly solvent tolerant strain. Further analysis of the expression of these transporters in response to toluene and to other stresses are being carried out in our laboratory.

The construction and analysis of mutants in genes of interest is one possible approach that is used to assign functions to orphan genes. In this study, clear phenotypes for three transporters could be determined, while in order to assign a function to the other two transporters, more studies will be required.

Although RND transporters have been involved in organic solvent and antibiotic resistance in *P. putida* DOT-T1E, *P. putida* DOT-T1E-PS128 was as resistant as the wild-type towards toluene shocks, antibiotics and heavy metals. The lag phase of the mutant in the presence of bipyridil was double than that of the wild-type strain suggesting a role for this efflux pump in iron acquisition. In fact, when growing in the absence of Fe^{3+} this mutant released more fluorescent siderophores than the wild-type and the other transporter mutants (Figure 4); however more experimental data will have to be done to confirm this function. Efflux pumps of this family have been involved in siderophore transport in *E. coli* (Poole *et al.*, 1993).

Transporters belonging to the ATP Binding Cassette (ABC) superfamily can be either importers that mediate the uptake of nutrients or exporters involved in the secretion of various molecules such as toxins, peptides or hydrophobic drugs (Davidson *et al.*, 2008). ABC transporters contain two hydrophobic integral membrane domains and two hydrophilic domains and ABC-mediated transport is coupled with ATP hydrolysis. The three ABC transporters that have been studied here (mutated in *P. putida* DOT-T1E-PS129; *P. putida* DOT-T1E-PS130; and *P. putida* DOT-T1E-PS168) belong to the class 3 group of ABC transporters, whose functionality depends on the presence of a separate extracellular substrate-binding protein (SBP) that recognizes substrates with high affinity. Mutant *P. putida* DOT-T1E-PS168 grew with a longer lag phase in the presence of MV and showed a lower growth rate in the presence of Gm, however no significant differences were observed when growing in other oxidative-stress-related agents or antibiotics so that, as

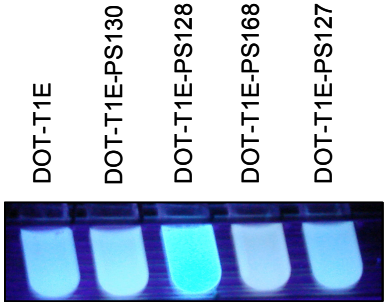

Figure 4. Siderophore released by wild-type and transporter mutants. Cells were grown overnight in BM2 iron-deficient media (Gilleland *et al*., 1974). Next day, tubes with fresh BM2 media were inoculated and fluorescence was observed after 16 hours with ultraviolet light. Tubes were washed with chromic mixture before used.

of yet, it is difficult to assign a specific role for this gene.

The ABC transporter gene that was knocked out in *P. putida* DOT-T1E-PS129 shows homology to putative methionine ABC transporters in other *Pseudomonas*. Methionine transport has been studied in *E. coli* (Gál *et al*., 2002; Merlin *et al.*, 2002) and in *Bacillus subtilis* (Hullo *et al.*, 2004). We found that *P. putida* DOT-T1E is not able to use L-Met as the sole carbon source, and that it is not able to use L- or D-Met as the sole nitrogen source, but that it can use L- or D-Met as a sulphur source. It is important to note that the utilization of L-Met as sulphur source by *P. putida* DOT-T1E-PS129 was not as efficient as in the wild-type (Figure 3), suggesting that this transporter may be involved in methionine uptake. This mutant exhibits even more limited growth when different sulphate or sulphite salts were used as the only sulphur source, indicating that this transporter plays an important role in sulphate/sulphite uptake. In the model organism *E. coli,* two different sulphate transporters have been identified (Kertesz, 2001); one of them belongs to the ABC superfamily and the other to the MFS superfamily. The *E. coli* ABC sulphate transporter is encoded by the *cysPTWA* genes plus one periplasmic sulphate binding protein (SBP) that is encoded by a separately located gene on the chromosome (Sirko *et al.*, 1990). These genes are also present in the *P. putida*

DOT-T1E chromosome. The presence of several transport systems for sulphate and other sulphur-related acquisition mechanisms with overlapping specificities have been described in different organisms (Kadner and Watson, 1974; Sirko *et al.*, 1995). We have found that culture conditions influence the growth of mutant *P. putida* DOT-T1E-PS129 when growing with sulphate as the only S source (not shown).

The last ABC transporter that we looked at, which was knocked out in the *P. putida* mutant DOT-T1E-PS130, was clearly shown to be involved in multidrug resistance. The mutant was not only sensitive to toluene but also to a wide range of antibiotics, several heavy metals, and oxidative stress agents (Table 3). The observation that *P. putida* DOT-T1E-PS130 is toluene sensitive is in agreement with previous results by Kim *et al.* (1998), which showed that mutations in this transporter of the solvent-tolerant strain *P. putida* GM73 lead to a solvent sensitive phenotype. However, to our knowledge, this is the first report to demonstrate that this transporter also confers multidrug resistance to the strain.

The gene that has been mutated in the *P. putida* strain DOT-T1E-PS127 encodes for a member of the MFS that has homology within the Bcr/CflA family of transporters that are involved in bicyclomycin resistance (Bentley *et al.*, 1993). This gene has been reported to confer moderate resistance also toward Tc, phosphomycin and Km when expressed from a plasmid in an *E. coli acrAB* deleted mutant (Nishino and Yamaguchi, 2001). It has also been shown to be involved in sulfathiazole resistance (Vedantam *et al.*, 1998) and in the efflux of L-Cys (Yamada *et al.*, 2006). We found that the DOT-T1E-PS127 mutant is toluene sensitive (Figure 2), that it was more sensitive than wild-type to CTAB and Gm, and it showed longer lag phases when growing in the presence of Bip and Zn^{2+}. No differences were found when Tc was added to the medium, possibly because of the presence of other efflux systems (i.e. TtgABC [Ramos *et al.*, 1998]) with overlapping specificity. In conclusion, while this transporter seemed to have a less broad transport capacity than that of Ttg2A, this is the first transporter belonging to the MFS that has been shown to be involved in solvent tolerance.

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DISCUSIÓN

La caracterización de los distintos mecanismos moleculares de la tolerancia a disolventes orgánicos en *Pseudomonas putida* DOT T1E ha sido uno de los temas de estudio en nuestro laboratorio durante los últimos 10 años. El transporte de tolueno vía bombas de extrusión es un factor fundamental en la tolerancia en esta cepa, siendo la bomba TtgGHI la principal implicada. Los transportadores que participan en mayor o menor medida en la tolerancia a disolventes que ya estaban descritos al inicio de esta tesis (TtgABC, TtgDEF y TtgGHI) pertenecen todos a la familia de transportadores secundarios RND (Resistencia, Nodulación, División celular).

Uno de los ejes conductores de este trabajo de Tesis Doctoral ha consistido en una profundización sobre diversos aspectos de estas bombas; por un lado caracterizando la localización genómica de la bomba TtgGHI y por otro explorando la posibilidad de intercambios funcionales entre los diferentes componentes de estas bombas.

Ensayos previos a esta Tesis Doctoral realizada en nuestro laboratorio, indicaron que los genes *ttgABC* estaban presentes en el cromosoma de todas las *Pseudomonas* analizadas y que la bomba *ttgDEF* aparecía siempre asociada a los genes cromosómicos *tod* de degradación de tolueno (Huertas *et al.*, 1998; Segura *et al.,* 2005). Sin embargo, la presencia de genes *ttgGHI* se restringía a cepas altamente tolerantes a tolueno, y su localización, cromosómica ó plasmídica, se desconocía. En este trabajo se han encontrado evidencias sólidas de la localización del operón *ttgGHI* en un plásmido de gran tamaño (≈ 121 ± 5 kb) al que denominamos pGRT1, y que no había sido descrito previamente. Este plásmido está presente al menos en dos cepas tolerantes a disolventes orgánicos (DOT-T1E y MTB6). Aunque es bien sabido que el mantenimiento de los plásmidos de gran tamaño pueden impone una carga energética grande en las cepas portadoras, y que esto es posiblemente una fuente de inestabilidad (Lucas *et al*., 2005; Gammon *et al*., 2006), en este trabajo se ha demostrado que la pérdida de pGRT1 es un evento raro, estando la tasa de pérdida del plásmido por debajo de 10^{-8} por célula y por generación, tanto en condiciones de cultivo no selectivas, como cuando las células se cultivaban bajo condiciones desfavorables como la presencia de concentraciones sub-letales de detergentes (SDS) y sales (NaCl). Además, aunque a lo largo de este estudio se identificó un mutante espontáneo (*P. putida* DOT-T1E-VAN5) que había perdido un fragmento del plásmido pGRT1 de aproximadamente 50 kb que incluía los genes *ttgGHI*, este evento es aún menos frecuente que el de la pérdida del plasmido pGRT1 completo. Estos resultados indican que la tolerancia a disolventes orgánicos es una

propiedad bastante estable y que la posible utilización de esta cepa en procesos biotecnológicos no va a estar limitada por la inestabilidad del plásmido. Al igual que la pérdida del plásmido provoca la pérdida de la tolerancia a tolueno en *P. putida* DOT-T1E, la transferencia del plásmido mediante conjugación desde *P. putida*-DOT-T1E a otras cepas de *Pseudomonas* sensibles a tolueno, tiene como consecuencia que éstas adquieran alta tolerancia a disolventes orgánicos. Este hecho es de gran importancia ya que abre una puerta a la mejora de cepas que, si bien poseen rutas catabólicas para la degradación y/o mineralización de algún contaminante del tipo de los hidrocarburos aromáticos, no son lo suficientemente tolerantes al mismo como para ser utilizadas en procesos de biorremediación de sitios contaminados con altas concentraciones del tóxico en cuestión. Aunque no se ha explorado en esta Tesis Doctoral, las implicaciones que pueda tener esta transferencia en la naturaleza es un aspecto importante que deberá ser estudiado en más detalle. Recientemente se ha llevado a cabo la secuenciación completa del plásmido pGRT1 y un análisis preliminar de los marcos de lectura abierta codificados en el plásmido revelan la existencia de un gran número de genes implicados en la transferencia y movilización del mismo.

Las bombas de la familia RND están constituidas por un componente de membrana interno (translocasa), la proteína de membrana externa (OMP) y una proteína de fusión de membrana (MFP). En el caso de *E. coli* y *P. aeuginosa* se había comprobado que las proteínas de membrana externa podían actuar con diferentes bombas de extrusión (Srikumar et al., 1997; Poole, 2004; Fralick, 1996; Buchanan, 2002; Mine *et al*., 1999; Yoneyama *et al*., 1998, Tikhonova *et al*., 2002). No obstante, no se tenían datos sobre si los componentes de las tres bombas Ttg de *P. putida* DOT-T1E podrían intercambiarse entre sí manteniendo su funcionalidad. Para estudiar esta posibilidad, analizamos el funcionamiento de la bomba TtgABC sustituyendo el componente MFP (TtgA) por los otros dos componentes de las otras dos bombas (TtgE ó TtgG) y el componente OMP (TtgC) por TtgF ó TtgI. La construcción de quimeras funcionales de ambos transportadores indica que algunos de los elementos de estos complejos tripartitos pueden ser intercambiables, fundamentalmente los componentes de membrana externa, hecho que concuerda con lo ya descrito para las bombas Mex de *P. aeruginosa* (Srikumar *et al*., 1997) que también pertenecen a la familia RND. Además los datos sugieren que también algunos de los componentes MFPs podrían intercambiarse. En cualquier caso, parece que estas quimeras no son tan eficaces como la bomba nativa. Estos resultados podrían interpretarse como que las bombas RND

podrían existir en las cepas silvestres como diferentes combinaciones de bombas quiméricas. Los distintos componentes de las bombas RND (IMP, OMP, y MFP) se encontrarían al azar en la membrana y se ensamblarían formando múltiples combinaciones. No obstante, el hecho de que los tres genes se co-transcriban en un mismo operón sugiere que ha habido una co-evolución de los mismos dando lugar a complejos tripartitos específicos. El hecho de que el patrón de expresión de estos genes también sea diferente para cada uno de los complejos (*ttgABC, ttgDEF y ttgGHI*) parece apoyar también la especificidad en el ensamblaje del complejo. Finalmente, el hecho de que estas bombas quiméricas sean menos eficaces que las nativas también indican que ó bien son menos estables ó la formación del complejo no permite un funcionamiento óptimo. Aunque no se han hecho estudios de afinidad entre componentes ó co-localización de componentes en la membrana, es muy probable que la combinación más afín y más estable sea la codificada por cada operón (TtgABC, TtgDEF y TtgGHI) y que las bombas quiméricas, si existen en las cepas silvestres sean en proporción minoritaria.

El segundo eje conductor de esta tesis se relaciona con la complejidad de la respuesta a tolueno. Aunque las bombas son el principal determinante de la tolerancia, distintos tipos de ensayos llevados a cabo en el laboratorio sugerían que en la respuesta a tolueno actúan otros factores cuya relevancia, en algunos casos, estaba todavía por determinar. La participación en la respuesta a tolueno de diferentes componentes de membrana (Junker y Ramos, 1999; Bernal *et. al*., 2007, Pini *et al*., 2009), de genes flagelares (Segura et al., 2001), la inducción de proteínas relacionadas con otros estreses (Segura *et al*., 2005; Domínguez-Cuevas *et al*., 2006), y de algunos transportadores sin función específica asignada (Segura et al., 2005; Domínguez-Cuevas *et al.*, 2006), entre otros, nos llevó a realizar un estudio detallado de algunos de estos genes. Para este fin se construyeron los correspondientes mutantes y se realizaron los ensayos pertinentes para la caracterización en relación a su papel en tolerancia a tolueno. En esta Tesis Doctoral, se ha determinado la implicación del complejo citocromo ubiquinol oxidasa (*cyoABCD*), y de dos transportadores (Ttg2ABCDE y Bcr) en la tolerancia a tolueno.

El gen *cyoB* se estudió inicialmente en relación a la tolerancia a tolueno porque forma parte de una de las dos oxidasas terminales que reducen oxígeno a agua y que representa el final del transporte de electrones en procesos respiratorios. No hay que

olvidar que la fuerza protón-motriz, de la que dependen los transportadores Ttg en *P. putida* DOT-T1E, es generada a través de procesos respiratorios. Los genes Ttg2ABC y el gen Bcr se seleccionaron inicialmente por su inducción en presencia de tolueno en la cepa sensible *P. putida* KT2440. Mutantes en estos dos genes son hipersensibles a tolueno, poniendo de manifiesto que no sólo transportadores de la familia RND, sino también de la familia ABC (Ttg2ABC) y MFS están implicados en la tolerancia a tolueno. Los otros tres transportadores inducidos en presencia de tolueno y estudiados durante esta Tesis Doctoral no participan, al menos de forma relevante, en la tolerancia a altas concentraciones de tolueno. A través de estos trabajos, se pone de manifiesto una vez más, que la construcción de mutantes en funciones que podrían estar implicadas en respuesta a estrés es una de las herramientas genéticas más útiles en el estudio de estos procesos. Hay que destacar, aunque para los objetivos de esta Tesis Doctoral no fue relevante, que los análisis funcionales llevados a cabo en los distintos transportadores han llevado a la asignación inequívoca de una función como transportador de sulfato/sulfito a uno de los transportadores de la familia ABC estudiados.

En conclusión, conjuntamente con algunos estudios previos, en los que se puso de manifiesto que un mutante en la bomba TtgGHI era hipersensible al choque de tolueno (Rojas *et al*., 2001), durante esta Tesis Doctoral se ha confirmado el papel esencial que la bomba TtgGHI, codificada en el plásmido pGRT1, tiene en la tolerancia a tolueno. También los datos obtenidos en esta Tesis Doctoral confirman la complejidad de la respuesta a tolueno. La participación de genes presentes tanto en cepas sensibles como en las tolerantes a tolueno en la respuesta a este disolvente orgánico tiene una fácil explicación cuando los correspondientes mutantes sólo presentan una reducción (aunque significativa) de la supervivencia del cultivo tras el choque con tolueno. Estos serían genes que participarían en la tolerancia como "ayuda" al mecanismo principal que sería la extrusión de tolueno por la bomba TtgGHI. Así por ejemplo, la isomerización *cis-trans* compacta las membranas celulares aumentando su rigidez y contrarrestando el efecto fluidificante del tolueno, aunque a concentraciones altas esta compactación de las membranas no sería suficientemente efectiva como para evitar la toxicidad (Junker y Ramos, 1999). Similar situación se presentaría con otros mecanismos como la síntesis de cardiolipina (Bernal *et al*., 2007), la participación de ácidos grasos ciclopropano (Pini *et al*., 2009) ó incluso el papel que jugaría *cyoB* en la tolerancia. Más difícil de explicar es el hecho de que proteínas presentes en los dos

tipos de cepas jueguen un papel esencial en la tolerancia. Mutantes en *ttgB* o los estudiados durante esta Tesis Doctoral (Ttg2ABCDE y Bcr) son incapaces de sobrevivir la adición repentina de 0,3% (v/v) de tolueno, pese a que la bomba TtgGHI siga funcionando y eliminando tolueno. Esto indica que aunque la bomba TtgGHI confiere el carácter de tolerancia a la cepa, ésta necesita de una batería adicional de mecanismos para poder sobrevivir a estos choques de tolueno. Algunos de estos mecanismos son también esenciales (TtgABC, Ttg2ABCDE y Bcr), mientras que otros (mecanismos relacionados con la modificación de membranas ó CyoB) permiten que las células respondan al choque con la mayor eficiencia posible.

Los resultados obtenidos en esta Tesis Doctoral por tanto son relevantes tanto desde el punto de vista biotecnológico, ya que se ha comprobado que el fenotipo de tolerancia es un carácter estable, y por tanto la cepa puede usarse en reactores aún sin presión selectiva, como desde el punto de vista de la biología básica ya que se han identificado nuevos factores implicados en la tolerancia, siendo la primera vez que se describe la participación de los genes *cyoB* y *bcr* en la tolerancia a tolueno. De hecho, la cepa se ha utilizado como base para la síntesis de productos tóxicos como son los catecoles (Rojas *et al*., 2004) y derivados nitroaromáticos (Meyer *et al*., 2005). Otro de los potenciales de esta cepa es el del desarrollo de biosensores para la detección de contaminantes, un área en la que se vislumbran avances al disponer la cepa de un sensor de aromáticos muy versátil como es el regulador TodS (Lacal *et al*., 2006).

CONCLUSIONES

1- La mutación en el gen *cyoB* no afecta a la producción de fuerza protón motriz en la cepa *P. putida* DOT-T1E, ni produce cambios significativos en el crecimiento de la misma independientemente de las condiciones de cultivo. La sensibilidad a tolueno en *P. putida* está influenciada por la fase de crecimiento del cultivo, así tanto células inducidas como sin inducir de *P. putida* DOT-T1E fueron más sensibles al choque de tolueno en fase estacionaria. Esta situación se exacerbó en el mutante CyoB.

2- Los resultados de Microscopia electrónica (ME) revelaron que la exposición a disolventes del mutante CyoB induce plasmólisis desde uno de los polos.

3- El intercambio funcional de proteínas de membrana externa (OMP) y de proteínas de fusión de membranas (MFP) es posible entre las bombas Ttg (RND) de *P. putida* DOT-T1E, debido a la conservación de los residuos de las zonas que están en contacto entre los elementos de estos complejos tripartitos.

4- Las bombas quiméricas construidas entre elementos de transportadores Ttg de *P. putida* DOT-T1E fueron menos efectivas que las respectivas bombas nativas.

5- Los operones *ttgVW-ttgGHI* están localizados en un plásmido de gran tamaño llamado pGRT1.

6- El plásmido pGRT1 de *P. putida* DOT-T1E es autotransferible a cepas del género *Pseudomonas*, confiriendo a las células receptoras la capacidad de tolerar altas concentraciones de tolueno.

7- La pérdida del plásmido pGRT1 es un evento raro que ocurre con una frecuencia de 10^{-8} células por generación y conduce a la pérdida de tolerancia a tolueno en *P. putida* DOT-T1E debido a que se pierde el principal determinante de tolerancia a tolueno de esta cepa (*ttgGHI*).

8- La pérdida de tolerancia a tolueno también puede tener lugar mediante la deleción espontánea de un fragmento de 50 kb aproximadamente del plásmido pGRT1, en el que se encuentra el operón *ttgGHI*. Este evento es aun más raro que la pérdida del plásmido completo.

9- El transportador *ttg2ABCDE* está implicado en la respuesta a altas concentraciones de tolueno en la cepa *P. putida* DOT-T1E y participa en la tolerancia de esta cepa a muchos otros compuestos tóxicos.

10- El transportador Bcr está implicado en la tolerancia a tolueno.

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