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**Ana Segura · Antonia Rojas · Ana Hurtado
María-José Huertas · Juan L. Ramos**

Comparative genomic analysis of solvent extrusion pumps in *Pseudomonas* strains exhibiting different degrees of solvent tolerance

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Abstract Organic solvents are inherently toxic for microorganisms. Their effects depend not only on the nature of the compound, but also on the intrinsic tolerance of the bacterial species and strains. Three efflux pumps belonging to the RND (resistance-nodulation-cell division) family of multidrug extrusion pumps are the main factor involved in the high intrinsic tolerance to toluene of *Pseudomonas putida* DOT-T1E. We have analyzed the tolerance to toluene shocks [0.1% and 0.3% (v/v)] of a number of strains belonging to different species of the genus *Pseudomonas* upon growth in the absence and in the presence of sublethal concentrations of toluene. The strains can be grouped in three categories: (1) highly resistant strains, in which almost 100% of the cells precultured in the presence of sublethal concentrations of toluene withstood a 0.3% (v/v) toluene shock, (2) moderately resistant strains, in which only a fraction (10^{-4}–1) of the cells withstood a 0.1% (v/v) toluene shock, but fewer than 1 in 10^7 cells survived a sudden 0.3% (v/v) toluene shock regardless of the growth conditions, and (3) sensitive strains, in which regardless of the growth conditions fewer than 10^{-5} cells survived a 0.1% (v/v) toluene shock. We also studied the number and type of efflux pumps in different strains in comparison with the *P. putida* DOT-T1E strain.

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A. Segura (✉) · A. Rojas · A. Hurtado · J. L. Ramos
Consejo Superior de Investigaciones Cientificas,
Departamento de Bioquímica y Biología Molecular y
Celular de Plantas, Estación Experimental del Zaidín,
C/Profesor Albareda 1, 18008 Granada, Spain
E-mail: ansegura@eez.csic.es
Tel.: +34-958-121011
Fax: +34-958-129600

M.-J. Huertas
Departamento de Bioquímica y Biología Molecular,
Edificio Severo Ochoa pta.1. Campus Universitario de Rabanales,
Córdoba, Spain

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Introduction

Organic solvents with a $\log P_{ow}$ value (i.e., the logarithm of the partition coefficient of the target compound in a mixture of octanol and water) between 1.5 and 3 are extremely toxic for microorganisms (de Smet et al. 1978; Sikkema et al. 1995). These compounds accumulate in the bacterial membrane, impairing vital functions (de Smet et al. 1978). Their toxic effects are dependent not only on the inherent toxicity of the solvent, but also on the intrinsic tolerance of the microorganisms (Aono and Kobayashi 1997).

Gram-negative bacteria can extrude a large variety of structurally unrelated compounds to the medium through multidrug efflux pumps belonging to different families (Paulsen et al. 1996; Poole and Srikumar 2001). The active efflux of organic solvents by members of the RND (resistance-nodulation-cell division) family of efflux pumps is a common mechanism that different bacteria of the genus *Pseudomonas* have developed to protect themselves against these toxic chemicals (Segura et al. 1999; Ramos et al. 2002). These efflux pumps are characterized by the broad range of substrates they transport, and most of them are able to extrude not only several organic solvents, but also different antibiotics (Ramos et al. 1998; Duque et al. 2001; Rojas et al. 2001).

The RND efflux pumps are made up of three proteins that traverse both the inner and the outer membrane (Koronakis et al. 2000). Each pump is formed by a transporter located in the cytoplasmic membrane, an outer membrane channel that expands into the periplasm and probably contacts the inner membrane transporter, and an inner membrane-anchored lipoprotein that expands into the periplasm and which is thought to clump the other two components (Zgurskaya

and Nikaido 1999; Koronakis et al. 2000). This structural organization allows the extrusion of substrates directly into the external medium, bypassing the periplasm (Koronakis et al. 2000).

The presence of several efflux pumps with overlapping substrate specificities in a single strain is apparently a general characteristic in different *Pseudomonas* species. Some of these pumps have a relatively high level of constitutive expression, and confer the so-called ''intrinsic resistance'', whereas others are induced by the product to be transported and confer ''induced resistance''. In *Pseudomonas putida* DOT-T1E, a strain that tolerates high concentrations of aromatic compounds with a $\log P_{ow} > 2.4$, three different efflux pumps of the RND family have been described as involved in intrinsic and acquired tolerance to toluene (Rojas et al. 2001). The TtgABC pump is constitutively expressed at a high level, and extrudes aromatic hydrocarbons such as toluene, ethylbenzene, and xylenes as well as several antibiotics including tetracycline, ampicillin, and chloramphenicol (Ramos et al. 1998; Duque et al. 2001; Rojas et al. 2001). The second efflux pump, TtgDEF, expels toluene and styrene but not antibiotics (Mosqueda and Ramos 2000). The *ttgDEF* operon is induced in response to the presence in the culture medium of these solvents. Recently, a third efflux pump, TtgGHI, which extrudes toluene, styrene, and different antibiotics, has been described (Rojas et al. 2001). The *ttgGHI* operon is expressed at a relatively high level in the absence of toluene, and its expression increases in the presence of organic solvents (Rojas et al. 2001).

Four efflux pumps with overlapping specificity belonging to the RND family have also been described in *P. aeruginosa* as involved in resistance to antibiotics. The MexAB-OprM pump (Poole et al. 1993) extrudes a wide range of antibiotics, including tetracycline and chloramphenicol. The MexCD-OprJ (Gotoh et al. 1998) and MexEF-OprN pumps (Kohler et al. 1997) exhibit overlapping specificity with some of the compounds they extrude, but are also specific to some antibiotics. Recently, the MexXY efflux system has also been described as involved in the extrusion of several antibiotics (Masuda et al. 2000). Mutations that lead to the overexpression of the *mexAB-oprM*, *mexCD-oprJ*, and *mexEF-oprN* operons resulted in increased tolerance to organic solvents such as *n*-hexane and *p*-xylene (Li et al. 1998).

In this study we examined the organic solvent tolerance of different strains belonging to different species of the genus *Pseudomonas*. Our results allowed us to group the strains into three categories: highly tolerant, moderately tolerant, and sensitive, depending on their survival rate to a sudden shock of 0.3% and 0.1% (v/v) toluene ($\log P_{ow} = 2.69$). We searched for the presence of the three efflux pumps described in *P. putida* DOT-T1E in different *Pseudomonas* sp. strains. Only the *P. putida* strains are equipped with efflux pumps highly similar to those in *P. putida* DOT-T1E. We show that tolerance in these *P. putida* strains correlates with the number and type of efflux pumps that they bear.

Materials and methods

Bacterial strains

Bacterial strains were grown according to routine procedures in liquid Luria-Bertani (LB) medium. Cultures were shaken on an orbital platform operating at 200 strokes per min at 30 °C. All the strains used in this study have been described previously: *P. putida* DOT-T1E (Ramos et al. 1995), *P. putida* MTB6 and *P. putida* MTB5 (Huertas et al. 2000), *P. putida* KT2440 (Franklin et al. 1981), *P. putida* S12 (Weber et al. 1994), *P. putida* SM0116 (Møller et al. 1996), *P. putida* F1 (Gibson et al. 1970), *P. putida* JLR11 (Esteve-Nuñez et al. 2000), *P. putida* OUS82 (Kiyohara et al. 1994), and *P. mendocina* KR1 (White and Gibson 1991). All other *Pseudomonas* strains used in this work were obtained from the Pseudomonas Reference Culture Collection established at the Estación Experimental del Zaidín in Granada, and were a gift of Dr. Estrella Duque.

Survival in response of toluene shocks

Cells were grown in 75 ml LB medium with or without toluene in the gas phase overnight. To supply toluene through the gas phase, we used 250-ml capacity conical culture flasks with a central vessel in which 0.3 ml toluene was introduced. Flasks containing toluene were closed tightly with a Teflon screw top. On the following day the cultures were diluted 1:100 and grown under the same conditions until the culture reached a turbidity of about 0.8 at 660 nm. These cultures contained about 10^8 viable cells per ml and were divided in three aliquots: to two of them we added toluene to a final concentration in the culture medium of 0.1% (v/v) or 0.3% (v/v), and the third one was kept as a control. The number of viable cells was determined by drop plating serial dilutions on LB medium before toluene was added, and 10 min later.

DNA techniques

Preparation of chromosomal DNA, digestion with restriction enzymes, electrophoresis, and Southern blotting were carried out with standard methods (Sambrook et al. 1989; Ausubel et al. 1991). Southern hybridizations were done against total DNA of different strains digested with *Bam*HI. The hybridization probes were based on the *ttgB*, *ttgE*, and *ttgH* genes. Probes were obtained by PCR and labeled with digoxigenin. The primers used in the PCR reactions were: 5′-CAACGGTGTCGAAGCGATGG-3′ and 5′-CCCAGCCTCATAACGTGG-3′ to amplify a 697-bp fragment of the 3′-end of the *P. putida* DOT-T1E *ttgB* gene (2450–3147 in the *ttgB* gene), primers 5′-CAGCCAGTATCCCAACATTGCTGC-3′ and 5′-ATCCTGACCTCGGTATTTGGCGG-3′ amplified a 760-bp fragment of the 5′ end of the *P. putida* DOT-T1E *ttgE* gene (from position 96 to 855 in the *ttgE* sequence), and primers 5′-GCGCTTGTCCACCGGCGGCAATC-3′ and 5′-CCCTCGGTACGTTCAGCGG-3′ amplified an 891-bp fragment located in the middle of the *P. putida* DOT-T1E *ttgH* gene (position 872–1763). For PCR amplification of homologous *P. putida* DOT-T1E *ttgB*, *ttgE*, and *ttgH* genes in other *Pseudomonas* strains, the above primers were used and the PCR reaction procedure was as follows: after an initial step of 5′ at 94 °C, 30 cycles of 1′ at 94 °C, 1′ at 60 or 50 °C, and 1′ at 72 °C were run. Amplification products were visualized on 0.8% (w/v) agarose gels stained with ethidium bromide, and the corresponding band was extracted with the QUIAEX II Gel Extraction kit (Qiagen). The PCR products were sequenced using an ABI-PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq DNA polymerase in an automatic DNA sequencer (model ABI-PRISM 3100; Applied Biosystems, USA). The *ttgABC*, *ttgDEF*, and *ttgGHI* sequences have been deposited in GenBank under accession numbers AF031417, Y19106, and AF299253, respectively.

Results and discussion

Survival rate of different *Pseudomonas* sp. strains upon sudden toluene shock

Nineteen different strains belonging to six different species of the genus *Pseudomonas* (see Table 1) were studied to determine their intrinsic and induced tolerance to toluene. The bacteria were grown in LB medium in the absence (non-induced) and in the presence of toluene in the vapor phase (induced), as described in Materials and methods, until they reached the late logarithmic growth phase. Then the cultures were challenged with 0.1% or 0.3% (v/v) toluene and the fraction of cells surviving the solvent shock was determined 10 min later.

Nearly 100% of the cells of *P. putida* DOT-T1E, *P. putida* MTB6, and *P. putida* S12 survived a sudden shock with 0.1% (v/v) toluene regardless of the growth conditions (Table 1); however, there was a significant difference in the survival rate of these three strains between the preinduced and non-preinduced cultures when the concentration of toluene was 0.3% (v/v). In this case only a fraction of the cells (10^{-4} to 10^{-6}) survived when not preinduced, but the fraction of cells that survived the 0.3% (v/v) toluene shock increased considerably when the cells were preinduced (survival rate between 10^{-3} and 1). Regardless of the growth conditions the survival rate of *P. putida* S12 upon sudden toluene shock was significantly lower than that of *P. putida* DOT-T1E or *P. putida* MTB6. These two latter strains are considered highly tolerant to toluene, whereas *P. putida* S12 is a medium- to highly tolerant strain.

All the other *Pseudomonas* sp. strains tested had survival rates equal to or below 10^{-7} when they received a sudden shock of 0.3% (v/v) toluene, regardless of the growth conditions. To identify different degrees of tolerance between them, we decreased the amount of toluene to 0.1% (v/v). When non-induced cells were assayed under these conditions, the survival rate of *P. putida* MTB5, *P. putida* SMO116, *P. putida* F1, *P. putida* 43, *P. putida* JLR11, *P. aeruginosa* PA01162, *P. aeruginosa* PAO1162RK, and *P. fluorescens* PF11 was $\geq 10^{-4}$. We considered these strains as moderately tolerant to toluene (Table 1). In this group we could distinguish two subgroups, namely, one constituted by strains whose survival when pregrown on toluene was higher than when not preinduced, i.e., *P. putida* MTB5, *P. putida* SMO116, and *P. putida* F1, and another subgroup including strains *P. putida* 43, *P. putida* JLR11, *P. aeruginosa* PAO1162 and PAO1162RK, and *P. fluorescens* PF11, whose survival upon a sudden 0.1% (v/v) shock was lower when pregrown in the presence of toluene (Table 1). We suggest that bacteria belonging to the first subgroup are equipped with one or more inducible mechanisms to achieve increased levels of tolerance to toluene when pre-exposed to sublethal concentrations of this aromatic hydrocarbon (see also below). In contrast, the decreased viability of the strains in the second group may reflect the already harmful effects of sublethal concentrations of the organic solvent in the cultures.

Table 1 Survival rate of the different *Pseudomonas* strains upon sudden toluene shocks. Bacterial strains were grown in the absence (*NI*) or in the presence of toluene (*I*). When the turbidity of the cultures was 0.8–1, the cultures were split into three aliquots, and two of them received toluene to a final concentration of 0.1% (v/v) and 0.3% (v/v), respectively. The third one was used as a control. The number of viable cells was determined 10 min after addition of the solvent. The values given are the ratio between the number of cells in the control flask and those that survived the shock when the solvent was added. Data are the average of at least three independent determinations with standard deviation below 5% of the given values. The *last column* indicates the degree of tolerance of the strain to toluene. (*H* Highly resistant, *M* moderately resistant, *S* sensitive)

Strain	0.1% Toluene		0.3% Toluene		Toluene tolerance
	NI	I	NI	I	
P. putida DOT-T1E	1	1	10^{-4}–10^{-5}	0.5–1	H
P. putida MTB6	1	1	10^{-4}–10^{-5}	10^{-1}	H
P. putida S12	1	1	10^{-6}	10^{-2}–10^{-3}	M/H
P. putida MTB5	10^{-4}	1	$<10^{-8}$	10^{-6}	M
P. putida SMO116	10^{-4}–10^{-5}	10^{-1}	$<10^{-8}$	$<10^{-8}$	M
P. putida F1	10^{-4}	10^{-3}	$<10^{-8}$	10^{-7}	M
P. putida 43	10^{-3}–10^{-4}	10^{-5}–10^{-6}	$<10^{-8}$	$<10^{-8}$	M
P. putida JLR11	10^{-3}–10^{-4}	10^{-4}–10^{-5}	$<10^{-8}$	$<10^{-8}$	M
P. putida 2440	10^{-5}	10^{-6}	$<10^{-8}$	$<10^{-8}$	S
P. putida OUS82	10^{-5}	10^{-5}	$<10^{-8}$	$<10^{-8}$	S
P. mendocina KR1	10^{-6}	10^{-8}	$<10^{-8}$	$<10^{-8}$	S
P. aeruginosa SSS1	10^{-7}	10^{-7}	$<10^{-8}$	$<10^{-8}$	S
P. aeruginosa 7NSK2	10^{-6}	10^{-7}	$<10^{-8}$	$<10^{-8}$	S
P. aeruginosa PAO1162	1	10^{-4}	$<10^{-8}$	$<10^{-8}$	M
P. aeruginosa 1162RK	1	10^{-2}	$<10^{-8}$	$<10^{-8}$	M
P. fluorescens PF11	1	10^{-1}	10^{-7}	10^{-7}	M
P. fluorescens EEZ23	10^{-6}	10^{-6}	$<10^{-8}$	$<10^{-8}$	S
P. stutzeri	10^{-6}	10^{-4}	$<10^{-8}$	$<10^{-8}$	S
P. syringae pv. *syringae*	10^{-5}	10^{-5}	$<10^{-8}$	$<10^{-8}$	S

Finally, those strains in which 10^{-5} cells or fewer survived the shock with 0.1% (v/v) toluene were classified as toluene-sensitive (Table 1). In this category we included *P. putida* KT2440, *P. putida* OUS82, *P. mendocina* KR1, *P. aeruginosa* SSS1, *P. aeruginosa* 7NSK2, *P. fluorescens* EEZ23, *P. stutzeri*, and *P. syringae* pv. *syringae*.

Identification of efflux pumps homologous to those described in the *Pseudomonas putida* DOT-T1E in the other *Pseudomonas* sp. strains

To determine the presence or absence of efflux pumps homologous to those found in *P. putida* DOT-T1E, we prepared chromosomal DNA of all strains listed above, and upon total digestion with *Bam*HI, DNA fragments were separated in an agarose gel and transferred to a nylon membrane. Southern hybridizations were carried out against the *P. putida ttgB*, *ttgE*, and *ttgH* genes. High-stringency conditions (65 °C) were used to avoid crosshybridization among the different efflux pump genes within the same strain. The result of one of such blots against the *ttgB* gene is shown in Fig. 1. It can be observed that chromosomal DNA prepared from the different *P. putida* strains hybridized this probe. In contrast, with the *P. putida* DOT-T1E *ttgE* and *ttgH* probes only chromosomal DNA from certain *P. putida* strains revealed hybridization band(s).

Table 2 summarizes the results obtained with the different *P. putida* strains with the three available efflux pump gene probes. Using the set of primers listed in Materials and methods, we amplified part of the *ttgB*, *ttgE*, and *ttgH* genes in the different *P. putida* strains. The PCR-amplified fragments were sequenced, and the results revealed identity greater than 98% in most cases (Table 3).

Although we detected a weak hybridization band with DNA of *P. aeruginosa* PAO1162, *P. mendocina* KR1, and *P. aeruginosa* PAO1162RK with the *ttgB* probe (Fig. 1), we could not recover a PCR band using the corresponding DNA as templates and with primers based on the *P. putida* DOT-T1E *ttgB* gene. This suggests that the identity of the hybridizing band was not sufficiently high as to allow us to recover the gene by PCR amplification. We did not detect any hybridization band when *ttgE* and *ttgH* were used as probes against chromosomal DNA prepared from strains other than *P. putida*.

The results presented in Tables 2 and 3 show that only the three most resistant strains (DOT-T1E, MTB6, and S12) hybridized against the *ttgH* probe. This is consistent with our previous evidence that the TtgGHI efflux pump is the most relevant efflux pump involved in solvent tolerance in *P. putida* DOT-T1E. A *P. putida* DOT-T1E mutant strain in which the *ttgH* gene had been knocked out was unable to survive a 0.3% (v/v) toluene shock regardless of the growth conditions (Rojas et al. 2001). The TtgGHI efflux pump in *P. putida* S12

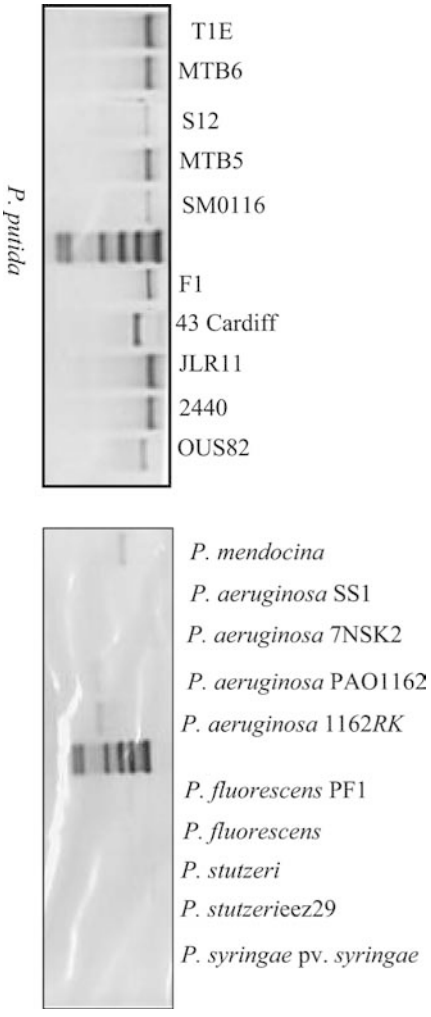

Fig. 1 Southern hybridization of chromosomal DNA of different *Pseudomonas* sp. strains against the *P. putida ttgB* gene probe. Chromosomal DNA was digested with *Bam*HI, size-fractionated in an agarose gel, and hybridized under high-stringency conditions against a digoxigenin-labeled *ttgB* probe prepared by PCR. Development of the hybrid was as recommended by Boehringer Mannheim

Table 2 Identification of homologous efflux pump genes in different *P. putida* strains. Hybridization conditions were as described in Materials and methods. The probes used were *ttgB*, *ttgE*, or *ttgH*. The + symbol indicates that a single hybridization band (or two bands in the case of *ttgE* was(were) found, and that using the primers reported in Materials and methods, a PCR amplificant was obtained suitable for DNA sequencing. The − symbol indicates the absence of hybridization band(s)

Strains	Tolerance	*ttgABC*	*ttgDEF*	*ttgGHI*
P. putida DOT-T1E	H	+	+	+
P. putida MTB6	H	+	+	+
P. putida S12	H	+	−	+
P. putida F1	M	+	+	−
P. putida MTB5	M	+	+	−
P. putida SMO116	M	+	+	−
P. putida 43	M	+	−	−
P. putida JLR11	M	+	−	−
P. putida 2440	S	+	−	−
P. putida OUS82	S	+	−	−

Table 3 Identity between *P. putida* DOT-T1E *ttgB*, *ttgE*, and *ttgH* and the corresponding gene in other *P. putida* strains. Primers used for amplification are listed in Materials and methods. The size of the amplificant was about 400 nucleotides, and the degree of identity refers to the corresponding regions. (*n.a.* Not applicable)

Strain	*ttgB*	*ttgE*	*ttgH*
P. putida DOT-T1E	100%	100%	100%
P. putida MTB6	100%	99.7%	100%
P. putida S12	98%	n.a.	99.8%
P. putida MTB5	99.5%	100%	n.a.
P. putida SMO116	98.5%	100%	n.a.
P. putida F1	99.5%	100%	n.a.
P. putida 43	90.7%	n.a.	n.a.
P. putida JLR11	98.5%	n.a.	n.a.
P. putida 2440	98.2%	n.a.	n.a.
P. putida OUS82	93.9%	n.a.	n.a.

(named SrpABC) had been previously identified by de Bont's group (Kieboom et al. 1998), and shown to be the major element responsible for solvent tolerance in this strain (Wery et al. 2001).

Given that the TtgGHI efflux pump is present in all three strains able to survive a 0.3% (v/v) toluene shock, we concluded that the presence of this efflux pump is a key factor in the high level of resistance to toluene in these strains. The difference in survival of the three strains to a sudden 0.3% (v/v) toluene shock as noted above may be dictated by the different degree of expression of the pump in each strain, or by the presence in *P. putida* DOT-T1E and MTB6 of the TtgDEF efflux pump, which is not present in S12.

Pseudomonas putida MTB5, SMO116, and F1 showed hybridization bands with the *ttgABC* and *ttgDEF* efflux pumps. The presence of these two efflux pumps also correlates with their levels of resistance, as these three strains were moderately resistant to sudden toluene shocks and showed a better survival to high toluene concentrations when preinduced with sublethal concentrations of solvent (Table 1). All three strains degrade toluene through the toluene dioxygenase pathway (Huertas et al. 2000), and although toluene degradation may decrease the product's toxicity (Mosqueda et al. 1999), their capability to survive a sudden toluene shock is most likely linked to the presence of the *ttgDEF* operon, which is inducible by toluene and extrudes this aromatic hydrocarbon from the cell membranes. It was previously shown that a *todC1* knocked-out mutant of *P. putida* DOT-T1E, unable to grow with toluene as the only carbon source, was as resistant as the wild type to toluene shocks (Mosqueda et al. 1999).

On the basis of their survival rates, we classified *P. putida* 43 and *P. putida* JLR11 as moderately resistant to toluene, but without an inducible response to toluene. This is in agreement with the fact that they only exhibit the *ttgABC* operon, which is expressed constitutively. Two other *P. putida* strains bearing the *ttgABC* genes were considered as sensitive to toluene. This apparent discrepancy may be due to different levels of expression of this pump in the different strains, or to different degrees of efficiency of the pump in each strain in the extrusion of toluene. In this connection it should be noted that the *ttgB* of *P. putida* OUS82 exhibits only 94% identity with that of *P. putida* DOT-T1E. On the other hand, mechanisms different from efflux pumps, such as *cis-trans* isomerization of the unsaturated fatty acids in the membranes (Junker and Ramos 1999; Junker et al. 2001; Cronan 2002), the rate of lipid biosynthesis (Pinkart and White 1997), and membrane hydrophobicity, also influence solvent tolerance (Aono and Kobayashi 1997). These factors may also account for the differences observed in toluene tolerance between the strains studied here.

In conclusion, our results suggest that solvent tolerance in different strains of *Pseudomonas* sp. correlates with the number and type of efflux pumps that the strains possess. Given that solvent-tolerant strains are envisaged as powerful tools for the biotransformation of toxic chemicals into others of added value (Bühler et al. 2002), and for the biodegradation of toxic solvents (Ramos et al. 1995), transfer of the *ttg* genes to different strains with potential applications in biotransformation and bioremediation may result in increased solvent tolerance, and consequently enhanced performance of these strains.

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