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A WbpL mutant of *Pseudomonas putida* DOT-T1E strain, which lacks the O-antigenic side chain of lipopolysaccharides, is tolerant to organic solvent shocks

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Abstract Lipopolysaccharides (LPS) are major components of the outer membrane of gram-negative bacteria and are considered a defense barrier. To determine if LPS play a role in resistance to solvents in the solvent-tolerant *Pseudomonas putida* DOT-T1E strain, we have generated mutants unable to synthesize the O-antigen side chain of LPS. The *wbpL* gene, encoding the enzyme that begins the synthesis of the O-antigen side chain of LPS of the solvent-tolerant strain, was cloned, sequenced, and knocked out in vitro with a cassette encoding kanamycin resistance, and a mutant called WbpL0 of the DOT-T1E strain was generated in vivo by site-directed mutagenesis. The WbpL mutant was compared with the wild-type strain with regard to tolerance to a number of toxic compounds, including chelating agents, organic acids, detergents, and aromatic hydrocarbons. It was found that the mutant was as tolerant as the wild-type strain to organic acids and aromatic hydrocarbons and more sensitive to ethylenediaminetetraacetic acid and deoxycholate.

Key words Solvent tolerance · LPS · *Pseudomonas* · WbpL · Organic solvents

Introduction

Pseudomonas putida strains are gram-negative saprophytic bacteria that are found in most temperate aerobic and microaerobic habitats. The ubiquity of *P. putida* and its ability to exploit a wide range of nutritional opportunities reflect a highly developed degree of adaptation to changing environmental conditions. Lipopolysaccharides (LPS) of gram-negative bacteria are major components of the cell wall and play an important structural role while mediating interactions with the neighboring environment. LPS are composed of three parts: lipid A, embedded in the outer membrane; a core oligosaccharide, divided into an inner and outer core; and the variable O-antigen side chain, composed of repeated oligosaccharide units that extend out from the cell surface (Rocchetta et al. 1999). The location of these molecules in the outer leaflet of the outer membrane permits the interaction of LPS with the external milieu. As a result, research has focused on the role of LPS as a defense barrier. In *Escherichia coli*, *Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhimurium, Pseudomonas aeruginosa*, and *P. putida* strains, it has been suggested that lack of the O-antigen side chain of LPS leads to hypersensitivity to toxic compounds (Clarke et al. 1995; Meier-Dieter et al. 1992; Mouslim et al. 1998; Otha et al. 1991; Rocchetta et al. 1999; Rodríguez-Herva et al. 1999; Sturm et al. 1986). In the solvent-tolerant *P. putida* Idaho, it has been suggested that LPS may play a role in solvent tolerance (Pinkart and White 1997), but no genetic evidence for this suggestion was available.

The mechanism of O-antigen side-chain LPS synthesis begins through the activity of a transmembrane glycosyl transferase known as WbpL (WecA in *Escherichia coli*), which transfers *N*-acetylglucosamine-1-phosphate or *N*-acetylgalactosamine-1-phosphate and galactose-1-phosphate onto undecaprenyl-phosphate (Amer and Valvano 2000; Rocchetta et al. 1999). The WbpL enzyme has been identified in *P. aeruginosa*, and it has been found to be responsible for the initiation of the synthesis of A-band (homopolymer) LPS and B-band (heteropolymer) LPS (Burrows et al. 1996; Dasgupta and Lam 1995; Rocchetta et al. 1998). Following the initiation event catalyzed by WbpL, biosynthesis proceeds by specific enzymes catalyzing glycosidic linkage; the extent of O-unit polymerization is controlled by the Wzz protein (reviewed by Rocchetta et al. 1999).

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In *P. putida*, only B-band LPS are present. By random Tn5 mutagenesis, *P. putida* KT2440 mutants that failed to synthesize the O-antigen side chain of LPS have been described (Ramos-González 1993). The mutant gene was identified as *wbpL*. The corresponding *wbpL* gene of the solvent-tolerant DOT-T1E was rescued and inactivated, and the mutation was transferred, via homologous recombination, to the chromosome of DOT-T1E, and the constructed mutant was analyzed. This work shows that the WbpL mutant of *P. putida* DOT-T1E is deficient in O-antigen side-chain LPS; however, the WbpL mutant was as tolerant as the corresponding wild-type strain to aromatic hydrocarbons.

Materials and methods

Bacterial strains and plasmids

Pseudomonas putida strains were grown on Luria-Bertani (LB) medium or on modified M9 minimal medium with 10 mM benzoic acid as the sole carbon source (Abril et al. 1989). Cultures were incubated at 30°C in a rotary shaker operated at 150–200 strokes per minute. *P. putida* 5E-1 is a mini-Tn5-*phoA* derivative of the solvent-sensitive *P. putida* KT2440, which was not recognized by monoclonal antibody (mAb) 7.3B raised against the O antigen of LPS (Ramos-González et al. 1992). *P. putida* DOT-T1E is a solvent-tolerant strain that metabolizes toluene via the toluene-dioxygenase pathway and is able to thrive in the culture medium with >0.3% (v/v) toluene (Mosqueda et al. 1999). This strain is a spontaneous rifampicin-resistant mutant of *P. putida* DOT-T1 (Ramos et al. 1995).

The *Escherichia coli* strains used were HB101 (Boyer and Roulland-Dussoix 1969) and JM109 (Yanisch-Perron et al. 1985), for standard cloning, and *E. coli* CC118λ*pir* to replicate R6K origin of replication-based suicide plasmids (Herrero et al. 1990). *E. coli* cells were grown on LB medium at 37°C.

The following plasmids were used in this study: the cloning vector pUC18 (Yanisch-Perron et al. 1985) (pUC18Not is a pUC18 derivative with flanking *Not*I sites on both sides of the polylinker so that cloned fragments can be rescued as a *Not*I cassette if this site is not present within the cloned DNA fragment) (Herrero et al. 1990); pLAFR3, which is a tetracycline-resistant cosmid with the multiple cloning site from pUC8 (Staskawicz et al. 1987); pKNG101, a suicide vector encoding streptomycin resistance that is used for allelic exchange in *Pseudomonas* sp. (Kaniga et al. 1991); pHP45ΩKm, which carries a kanamycin resistance cassette flanked by translational stop signals (Fellay et al. 1987); plasmid pBBR1MCS5, a gentamicin-resistant mobilizable broad host range plasmid (Kovach et al. 1995); and plasmid pRK600, a chloramphenicol-resistant helper vector (Herrero et al. 1990).

Antibiotics were used when required at the following concentrations (in μg/ml); ampicillin (Ap), 100; chloramphenicol (Cm), 30; gentamicin (Gm), 10 (*E. coli*) or 200 (*P. putida*); kanamycin (Km), 25–50; and rifampicin (Rif), 20.

Analysis of LPS patterns

LPS were prepared from exponentially growing cultures of *P. putida* and the *P. putida* WbpL mutants as described by Ramos-González et al. (1992). They were separated by electrophoresis on sodium dodecyl sulfate- (SDS-) polyacrylamide gels as described by Ramos-González et al. (1992), and then stained by the silver nitrate method of Hichock and Brown (1983).

Recombinant DNA techniques

Plasmid DNA was transformed into *E. coli* JM109 as described by Herrero et al. (1990). Total DNA from *Pseudomonas* 5E-1 strain was prepared as described by Rodríguez-Herva et al. (1996). A *P. putida* 5E-1 total DNA library was prepared in *E. coli* HB101 host cells using cosmid pLAFR3, as previously described (Rodríguez-Herva et al. 1996). Different kanamycin-resistant *E. coli* HB101 clones were then isolated and one of them, harboring a cosmid called pLPS11, was selected for further analysis. A 5-kb *Kpn*I fragment from this cosmid was subcloned into pUC18 to give pLPS11KD. This plasmid harbored the complete minitransposon together with additional flanking sequences.

Polymerase chain reaction (PCR) was performed in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The reaction mixture contained 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 0.2 ng/μl *P. putida* DOT-T1E chromosomal DNA, 100 μM each dinucleoside triphosphate (dNTP), 0.5 μM each primer, and 0.025 U/μl Taq polymerase (Pharmacia Biotech, Uppsala, Sweden). The primers used for the amplification of the wild-type *wbpL* gene of the *P. putida* strains were WBPL0 (5′-TGCACATTGGAACAAGGAC-3′ [the first A corresponds to A-135 in the GenBank sequence AF209871]) and WBPM7 (5′-GGGAATTCTACCGCTTCGGGGTTG-3′ [the first C corresponds to C-1140 in the GenBank sequence AF209871]). The reaction mixture was subjected to the following cycles: 1 cycle at 94°C for 5 min; 33 cycles at 94°C for 15 s, 63°C for 15 s, and 72°C for 2 min; and a final extension at 72°C for 10 min.

Inactivation in vitro of the *P. putida wbpL* gene

The *wbpL* mutant was designed so that an internal deletion (65 bp) was introduced and an internal *Bam*HI site was incorporated to facilitate marker incorporation. We proceeded as follows. First, two PCR products were obtained: one was for the N-terminal region starting at base 2 (the T of the ATG start codon) and ending at +558 (556-bp product; for this PCR product, primers N1[W1-Hind], 5′-ATCAGAAGCTTTGATATGGCTGATTGTGCT-3′ and N2

[W1-BamH1], 5′-CCGGCATCGGATCCATATCGGCAG CCACGCTGCCTCG-3′ were used), and the other PCR product contained the C-terminal region of *wbpL* and extended from position +624 to +1,582 (the 958-bp PCR product obtained with primers C1[W1-BamS], 5′-CCGATATGGATCCGATGCCGGCAGTGGCTTTCT-GGGTAT-3′ and C2 (W1-SacS3), 5′-CCCCGAGCTCAC-GAACGGTATCTGATGGG-3′). Primers N1 and C2 contained a *Hin*dIII and *Sac*I restriction site, respectively. Primers N2 and C1 both contained a *Bam*HI site and were complementary at their 5′-end over 21 bp. Both PCR reaction products were purified, mixed, and used as templates with primers N1 and C2 in a second PCR reaction. The fusion product of 1,542 bp was gel purified, cut with enzymes *Sac*I and *Hin*dIII, ligated into a *Sac*I/*Hin*dIII-digested pUC18NotI vector, and transformed into *E. coli* DH5a. The resulting plasmid was called pUCWbpL.

A kanamycin-resistant gene of 2,269 bp, flanked by transcriptional stop signals on both sides, was excised from plasmid pHP45Ω-Km (Fellay et al. 1987) with *Bam*HI and ligated into the *Bam*HI site of pUCWbpL to yield pUCWbpLKm. The entire insert in pUCWbpL-Km (3,811 bp) was subcloned as a *Not*I fragment in the suicide vector pKNG101 (Kaniga et al. 1991), yielding pKNG101 wbpLKm, and transformed into *E. coli* CC118λ*pir*.

Nucleotide sequencing

Both DNA strands were sequenced using an Applied Biosystems (Madrid, Spain) model 373 STRECHT DNA sequencing unit. Sequencing reactions were performed using the DNA sequencing kit with AmpliTaq polymerase (Perkin-Elmer). The M13 universal and reverse primers and synthetic oligodeoxynucleotide primers were used for the sequencing. The *wbpL* DNA sequence flanking the mini-Tn5 O end was determined by using a synthetic primer (5′-CTTTCAAGCGACGTTCATTCA-3′).

Sequence analysis

Amino acid sequence similarities were detected using the BLAST program available at the NCBI network server, with the default settings. The FASTA3 program (Pearson 1990), available at the EMBL-EBI network server, was also used to detect similarities. Sequence alignment was carried out with the program Clustal W, available at the PBIL network server, using the default settings. Hydropathy plots were predicted by using a program based on the method of Kyte and Doolittle (1982). Predictions of membrane-spanning regions were made using the TMpred program, available at the ISREC network server.

Sensitivity testing of the bacterial strains

Sensitivity to ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), deoxycholate (DOC), acetate, and benzoate was tested in triplicate by the minimal inhibitory concentration (MIC) test as described by Amsterdam (1991).

GenBank accession number

The nucleotide sequence of the *P. putida wpbL* gene was submitted to GenBank under accession number AF209871.

Results

Rescue of the *wbpL* gene of *P. putida* DOT-T1E involved in O-antigen side-chain LPS biosynthesis

Pseudomonas putida DOT-T1E is a strain tolerant to toluene shocks (>0.3% toluene [v/v]) (Ramos et al. 1995). To address the role that the O-antigen side chain of LPS may play in tolerance to organic solvents in this strain, we took advantage of a mutant of *P. putida* KT2440 called 5E-1, which had been obtained previously after mini-Tn5 mutagenesis and that did not synthesize the O antigen of LPS (Ramos-González 1993). Based on the available sequence of the *wbpL* gene of KT2440, we designed primers WpbL0 and WbpM7 and obtained the *P. putida* DOT-T1E *wbpL* gene after PCR amplification. Examination of the sequence revealed the *wbpL* gene has 1,005 bp (the sequence was deposited in GenBank, accession number AF209871). This open reading frame (ORF) was found to contain 60.9% (mol) G+C, close to the average percentage of G+C of *P. putida* DNA (60% mol).

The codon usage of this ORF was very similar to the pattern of codon usage in this species. The predicted product of this ORF was a polypeptide of 334 amino acids, highly hydrophobic (Leu, Ala, and Val accounted for 42.3% of the total amino acids), and had eight putative transmembrane segments. As expected, comparison of the deduced amino acid sequence of this ORF with protein sequence databases (SWISS-PROT and translated sequences from the EMBL database) revealed high similarity to the WbpL proteins of *P. aeruginosa* strains PAO1 (serotype 05) and PA103 (serotype O11) (GenBank accession numbers PAU50396 and AF147795, respectively) (Dasgupta and Lam 1995; Dean et al. 1999; de Kievit et al. 1995). It showed 62.5% and 59.8% identity, respectively, with these two sequences. The *P. putida* polypeptide also showed similarity with the WecA (Rfe) proteins of other gram-negative bacteria (Amer and Valvano 2000; Burrows et al. 1996; Clarke et al. 1995; Meier-Dieter et al. 1992; Mouslim et al. 1998; Otha et al. 1991; Sturm et al. 1986; Vaara and Nurmier 1999).

The alignment of the WbpL proteins from different *Pseudomonas* strains showed an overall amino acid similarity (Fig. 1), although those of *P. aeruginosa* O5 WbpL were only 303 amino acids in length in contrast with those of PAO103 and *P. putida*, which were 334 amino acids long. The C-terminal truncation of $WbpL_{O5}$ could be the consequence of the deletion of a cytidine nucleotide at the 3′-end

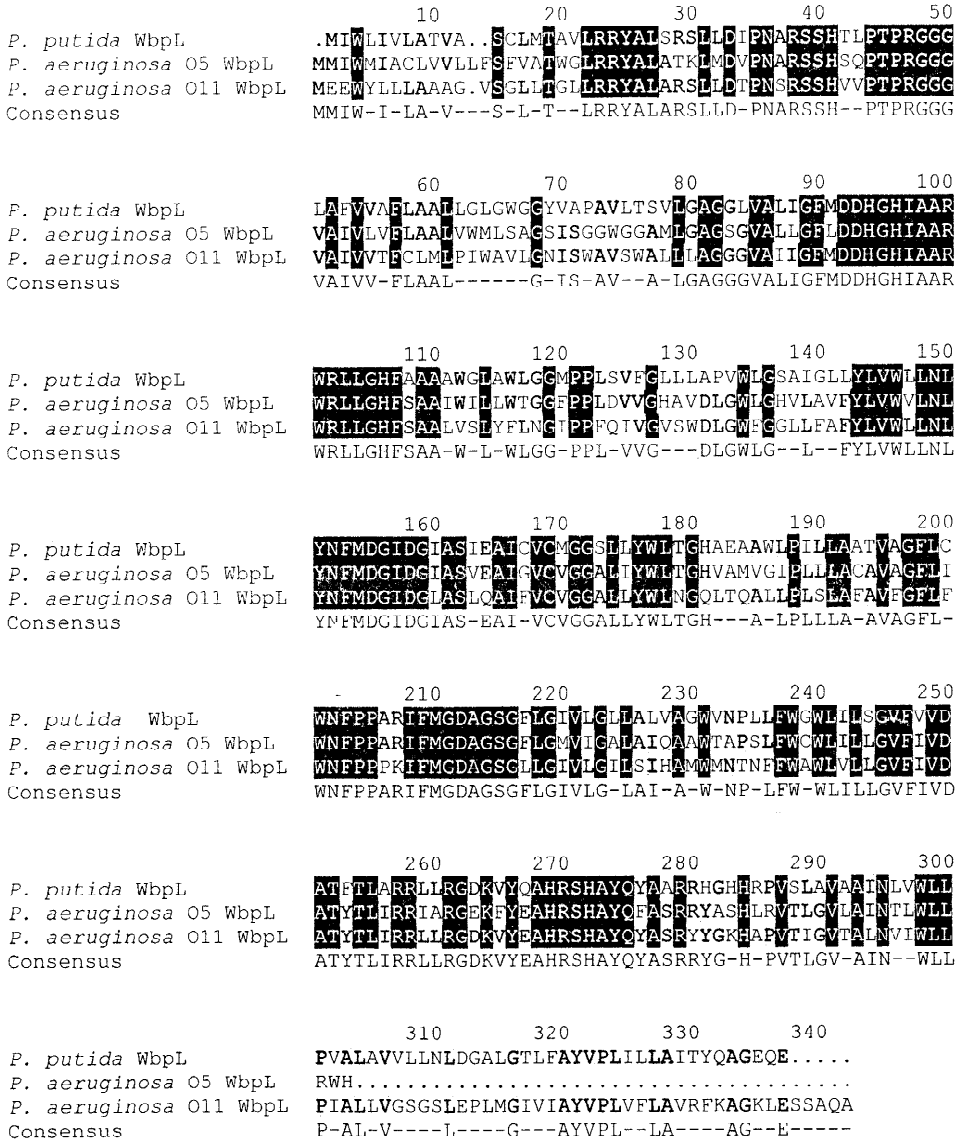

Fig. 1. Alignment of the deduced amino acid sequence of the putative *Pseudomonas putida* WbpL protein with *Pseudomonas aeruginosa* 05 and 011 WbpL proteins. Residues conserved in all the sequences are shaded in *black* and those present in two of the three positions are shaded in *gray*

of the gene, perhaps caused by chromosomal rearrangements following the insertion of IS*1209* after the *wbpL* gene in this strain (Rocchetta et al. 1999). It then follows that the last 30 C-terminal residues of WbpL are not important for protein stability or enzymatic activity in members of the genus.

Construction of a WbpL null mutant in *P. putida* DOT-T1E by homologous gene replacement

The *P. putida* DOT-T1E *wbpL* gene was inactivated in vitro with a kanamycin cassette as described in Materials and methods and used to generate a mutant in *P. putida* DOT-T1E by homologous recombination. To this end, the Sm- and Km-resistant suicide plasmid pKNG101wbpLKm was mobilized from *E. coli* CC118λ*pir* into the benzoate-utilizing recipient strain *P. putida* DOT-T1E (*E. coli* HB101 bearing the plasmid pRK600 was used as helper strain). The cells were plated on benzoate minimal medium containing kanamycin and streptomycin. This medium selects for the transconjugants of *P. putida* DOT-T1E bearing a cointegrate of the plasmid pKNG101wbpLKm in the chromosome, which was incorporated by a single homologous recombination event (Kaniga et al. 1991; Rodríguez-Herva and Ramos 1996). Because of the *sacB* genes in the pKNG101, transconjugants were unable to grow in the presence of 8% (w/v) sucrose in LB medium because of the synthesis of levans.

One colony of these cointegrates was picked and grown overnight in LB medium at 30°C deprived of antibiotics to select for the second recombination event, which would result in the loss of the wild-type gene, the Sm marker, and the *sacB* gene. The resulting *P. putida* DOT-T1E was expected to be sucrose tolerant, Km resistant, and Sm sensitive. More than 100 clones exhibiting these features were

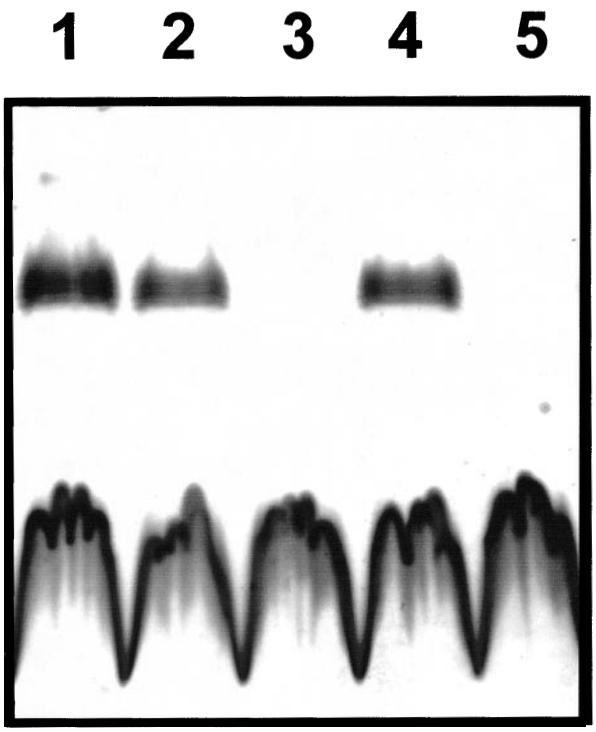

Fig. 2. Silver staining of the O-antigen side chain of wild-type *P. putida* strains and WbpL mutants. *Lane 1, P. putida* DOT-T1E; *lane 2, P. putida* DOT-T1EWbpL0 (pBBRWbpL); *lane 3, P. putida* DOT-T1EwbpL0; *lanes 4, 5*, controls; *lane 4*, *P. putida* KT2440; *lane 5*, *P. putida* 5E1

found, and one of them, *P. putida* strain DOT-T1EwbpL0, was retained for further studies. The successful cointegrate formation (insertion of the suicide plasmid construct pKNG101*wbpL*Km into the chromosome), which was obtained after the first recombination event, and the resolved cointegrate formation (the second recombination event in which the plasmid is excised from the chromosome) were confirmed in a Southern blot (not shown). In both cases, the wild-type band disappeared (sizes of bands in *Kpn*I digest: wild-type, 2.0 kb; cointegrate, 4.0 kb and 7.0 kb; resolved, 4.4 kb).

The absence of the O-antigen band of LPS in the *P. putida* DOT-T1E mutant strain was confirmed by SDS-polyacrylamide gel electrophoresis of LPS preparations (Fig. 2). The wild-type *wbpL* gene of *P. putida* DOT-T1E amplified by PCR as described earlier was subcloned in plasmid pBBR1MCS-5 to yield pBBRWbpL. This plasmid was transferred by conjugation to *P. putida* DOT-T1EwbpL0, and it was found that it restored the ability to produce O-antigen LPS (Fig. 2).

Phenotypic analysis of the *P. putida* DOT-T1E WbpL null mutant

In LB liquid medium, the wild-type *P. putida* DOT-T1E and the mutant DOT-T1EwbpL0 grew at the same rate (e.g., 42–48 min) and showed no difference in their growth behavior. We also tested whether the O-antigen side-chain LPS deficiency promoted cell clump formation or influenced the mutant strain motility, as observed for other *P. putida* mutants with altered outer membrane constituents (Rodríguez-Herva et al. 1996). No cell clump formation was observed in batch culture throughout all the growth phases with the mutant or wild-type strain. Motility was tested on 0.3% (w/v) soft agar LB plates; it was found that the mutant and the wild-type strain showed similar swarming behavior.

Table 1. Tolerance of *Pseudomonas putida* DOT-T1E and its *wbpL* mutant strain WbpL0 to toxic compounds

Compound	MIC (mM)	
	DOT-T1E	WbpL0
Benzoate	25	25
Acetate	100	100
EDTA	0.75	0.38
DOC	12.6	3.12

The assay conditions were described under Materials and methods
MIC, minimum inhibitory concentration; EDTA, ethylenediaminetetraacetic acid; DOC, deoxycholate

We have analyzed the tolerance of the WbpL mutant to compounds that, above a certain concentration, inhibit the growth of *Pseudomonas putida* DOT-T1E. For this series of assays, wild-type and mutant cells were grown on M9 minimal medium with glucose (0.5% w/v) as the sole C source; when a turbidity of about 1 at 660 nm was reached, 10 µl of the culture was used in MIC assays. The toxic compounds tested were benzoate, sodium acetate, EDTA, and deoxycholate (DOC). The wild type and the WbpL mutant were equally tolerant to benzoate and acetate, but the WbpL mutant was more sensitive to EDTA and DOC (Table 1). It was found that the wild type and the mutant strain grew in benzoate at concentrations of up to 25 mM and in acetate up to 100 mM (Table 1). *P. putida* DOT-T1E strain tolerated 12.5 mM DOC whereas the DOT-T1EwbpL0 mutant only tolerated 3.12 mM DOC. The DOT-T1E strain tolerated 0.75 mM EDTA, whereas 0.38 mM prevented growth of the WbpL0 mutant (Table 1).

The influence of O-antigen side-chain LPS in solvent tolerance in gram-negative bacteria has not yet been established, but some studies have suggested that LPS may affect the access of the solvent to the membrane (Inoue and Horikoshi 1989; Pinkart and White 1997; Ramos et al. 1995, 1997; Weber and de Bont 1996; Segura et al. 1999). We have tested, in LB liquid medium, tolerance to organic solvents of the wild-type DOT-T1E strain and the WbpL mutant. The assayed compounds were heptane (log P_{ow} 4.5), propylbenzene (log P_{ow} 3.5), *p*-xylene (log P_{ow} 3.2), octanol (log P_{ow} 2.8), and toluene (log P_{ow} 2.5). We found that DOT-T1E and WbpL0 grew in the presence of 0.3% (v/v) of all these solvents. We have also tested the response of the *P. putida*

Table 2. Survival of *P. putida* DOT-T1E and its WbpL mutant subjected to a sudden toluene shock

Strain	Growth conditions	
	Without toluene	With toluene
DOT-T1E	10^{-4}	0.8
WbpL0	10^{-4}	0.7

Cells were grown in Luria-Bertani (LB) medium in the absence and in the presence of toluene (supplied via the gas phase). When the turbidity of the culture at 660 nm was about 1, the number of viable cells was determined by plating serial dilutions on LB solid plates, and then toluene was added to reach a final concentration of 0.3% (v/v). Five minutes later, the number of viable cells was determined again. The number of viable cells is the ratio between the number of cells before and after the toluene shock

DOT-T1E wild-type strain and its isogenic WbpL0 strain to a sudden toluene shock (0.3% [v/v]) in cells growing in LB in the absence and in the presence of toluene in the gas phase. The behavior of the wild-type DOT-T1E and that of the mutant strain WbpL0 were similar (Table 2). We found, for cells that had not been pregrown with toluene, that toluene shock had a dramatic effect on cell survival; only about 0.01% of the initial cells survived the toluene shock. However, DOT-T1E and WbpL0 cells preinduced with toluene behaved as did those that are toluene tolerant; the survival rate was in the range 70%–80%. This evidence indicates that the O-antigen side chains of LPS are not important in solvent tolerance.

Discussion

It has been previously suggested that LPS could prevent access of the solvents to the membrane. This mechanism was suggested because different strains of *Pseudomonas putida* grew better in the presence of high Mg^{2+} or Ca^{2+} concentrations in the presence of solvents (Aono et al. 1994; Inoue and Horikoshi 1989; Inoue et al. 1991; Junker and Ramos 1999; Ramos et al. 1995). It was suggested that the divalent cations electrostatically linked adjacent polyanionic LPS chains and thus reduced charge repulsion (Ramos et al. 1995), allowing a denser packing of LPS molecules. Hence, the membrane becomes more hydrophilic and the permeability of the membrane to the solvents decreases. Furthermore, Pinkart and White (1997) observed the substitution of a higher LPS molecular weight band by a lower one when the solvent-resistant *P. putida* Idaho strain was grown in the presence of *o*-xylene. To test the potential role of LPS in solvent tolerance and tolerance to other toxic compounds in *P. putida* DOT-T1E, we generated a mutant impaired in O-antigen side-chain LPS biosynthesis.

The WbpL mutant of the *P. putida* DOT-T1E strain, which lacked the O-antigen side chain of LPS, was more sensitive than the wild-type strain to chelating agents and detergents, in accordance with previous observations in other microorganisms. In fact, *E. coli*, *P. aeruginosa*, and *P. putida* mutants lacking the O-antigen chain of LPS caused by mutations in WbpL (*P. aeruginosa*), WecA (*E. coli*), or Wzz (*P. putida*) show higher sensitivity toward hydrophobic antibiotics and chelating agents than does the wild type (Nurminen et al. 1997; Reeves 1993; Rocchetta et al. 1999; Rodríguez-Herva et al. 1999). In contrast with this increased sensitivity of the WbpL *P. putida* mutant to chelating agents and detergents, the WbpL mutant was as tolerant to aromatic hydrocarbons as the wild-type strain. This result suggested that the O-antigen side chains of LPS are not critical in solvent tolerance in this strain. In addition, it suggests that the positive effect exerted by divalent cations on solvent tolerance may be the result of induction of other, still unidentified, elements involved in solvent tolerance.

It is worth noting that in *P. putida* KT2440 a survey of the almost complete genome (www.tigr.com) revealed that there exists a large number of efflux pumps in *P. putida* strains that could be involved in the removal of drugs and solvents. This possibility seems to be the case with toluene and other aromatic compounds, because three efflux pumps have been found in DOT-T1E for the removal of toluene (Mosqueda and Ramos 2000; Ramos et al. 1998). Efflux pumps for removal of organic solvents have been identified in other gram-negative bacteria (Aono et al. 1988; Li and Poole 1998; Ma et al. 1993).

In short, our results indicate that WbpL is involved in the biosynthesis of LPS in the solvent-tolerant strain of *Pseudomonas putida*, and that the lack of the O-antigen side chain of LPS has no effect (or very little effect) regarding tolerance to different organic solvents.

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