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Mutations in Each of the *tol* Genes of *Pseudomonas putida* Reveal that They Are Critical for Maintenance of Outer Membrane Stability

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The outer membrane of gram-negative bacteria functions as a permeability barrier that protects cells against a large number of antibacterial agents. OprL protein of *Pseudomonas putida* has been shown to be crucial to maintain the stability of this cell component (J. J. Rodríguez-Herva, M.-I. Ramos-González, and J. L. Ramos. J. Bacteriol. 178:1699–1706, 1996). In the present study we cloned and mutagenized the *orf1*, *tolQ*, *tolR*, *tolA*, and *tolB* genes from *P. putida* KT2440, which were located upstream of the *oprL* gene. Polar and nonpolar mutations of the *P. putida tolQ*, *tolR*, *tolA*, and *tolB* genes were generated in vitro by using the Ω-Km^r interposon, which carries two transcriptional stop signals, or a promoterless *xylE* cassette, lacking any transcriptional stop signal, respectively. The mutant constructs were used to inactivate, by reverse genetics procedures, the corresponding chromosomal copies of the genes. The phenotype of each mutant strain was analyzed and compared with those of the wild-type strain and the previously characterized *P. putida oprL*::*xylE* mutant. All mutant strains exhibited a similar phenotype: altered cell morphology, bleb formation at the cell surface, release of periplasmic and outer membrane proteins to the extracellular medium, increased sensitivity to a variety of compounds (i.e., EDTA, sodium dodecyl sulfate, deoxycholate, and some antibiotics), filament formation, and severely reduced cell motility. Altogether, these results demonstrate the importance of the Tol-OprL system for the maintenance of outer membrane integrity in *P. putida* and suggest a possible role of these proteins in assembling outer membrane components.

Among other functions, the outer membrane of gram-negative bacteria plays a major role as an exclusion barrier against a number of potentially harmful compounds, as well as acting as a selective permeability barrier to other solutes (20, 47). Bacterial outer membrane consists of a lipid bilayer which significantly differs from most biological membranes because of its asymmetric structure and distinctive composition. While the inner leaflet of the outer membrane is composed of phospholipids (mainly with phosphatidylethanolamine as the head group), its outer monolayer consists of negatively charged lipopolysaccharide (LPS) molecules strongly associated with each other through divalent cation cross bridging (24, 50). All major outer membrane proteins studied so far have also been found to interact with LPS (21, 31). The stability of these associations constitutes the primary basis for the exclusion ability of the outer membrane (20, 45). In addition, the molecular sieving properties of this membrane are due to the presence of a number of proteins which form water-filled pores (43, 44).

Our current knowledge about the structure and functioning of the bacterial outer membrane is mainly based on studies with *Escherichia coli* (38, 45, 46), while the number of studies available for other bacteria is rather small. In *E. coli*, a number of mutants have been isolated which exhibit altered outer membrane organization. Among these mutants, just a few were found to be affected in structural genes involved in maintenance of outer membrane structure, namely, *lpp* mutants lacking the Braun lipoprotein (18), *ompA* mutants (47), and *tol-pal* mutants (5). Of these mutants, the *tol-pal* mutants exhibit the most severe alterations in outer membrane integrity. Their pleiotropic phenotype includes release of periplasmic proteins into the extracellular medium, hypersensitivity to some drugs and detergents, and formation of outer membrane vesicles (33). In *E. coli*, the Tol-PAL system consists of seven proteins: three inner membrane proteins (TolQ, TolR, and TolA), whose topologies have been extensively studied; two periplasmic proteins (TolB and Orf2), one outer membrane lipoprotein (PAL), and one cytoplasmic protein (Orf1). The genes encoding these proteins in *E. coli* are transcribed from two adjacent operons, one composed of the *orf1*, *tolQ*, *tolR*, and *tolA* genes and the other comprising *tolB*, *pal*, and *orf2* (62). The Tol-PAL system is organized into two protein complexes: an inner membrane complex that consists of the TolQ, TolR, and TolA proteins, which interact with each other via their transmembrane domains, and another complex, associated with the outer membrane and composed of TolB and PAL, which also interact with Lpp, OmpA, and the peptidoglycan (for recent reviews, see references 33 and 34). Both *orf1* and *orf2* encode proteins of unknown function.

In addition to their structural role, TolQ, TolR, TolA, and TolB proteins are required for the uptake of most group A colicins and of single-stranded DNA from some filamentous phages (32, 33). Although it has been proposed that the Tol-PAL system could be involved in porin and/or LPS translocation or assembly, these hypotheses still lack solid experimental evidence (33).

The importance of the Tol-PAL system for cell architecture is supported by the fact that homologues of the *tol-pal* genes have been found in many gram-negative bacteria, such as *Brucella abortus* (60), *Haemophilus influenzae* (12, 56), *Pseudomo-*

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nas aeruginosa (13, 37), *Pseudomonas putida* (53), and many others. However, the effect of mutations in the *tol-pal* system has been studied only in *E. coli* and recently in *Vibrio cholerae* (23), since attempts to construct *tol-pal* mutants in other bacteria (including *P. aeruginosa*) have been unsuccessful (13, 57). This fact has considerably limited understanding of the Tol-PAL complex function in other gram-negative bacteria.

In a previous work, we constructed and characterized an *oprL* (*pal*) null mutant of *P. putida* (52). In the present study, the *tol* genes of *P. putida*, located upstream of the *oprL* gene, were cloned, sequenced, and mutagenized in vitro. Then, each *tol* mutation was transferred to the *P. putida* host chromosome, and the resulting *tol* mutants were characterized in detail. Our results revealed that these mutants show an altered cell morphology, exhibiting bleb formation at their cell surface and increased sensitivity to a number of drugs. The mutants also released periplasmic and outer membrane proteins to the extracellular medium and formed filaments, which showed reduced cell motility.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Bacterial strains were routinely grown in liquid Luria-Bertani (LB) medium (55) or in M9 minimal medium with benzoic acid (5 mM) as the sole carbon source (1). *P. putida* was usually incubated at 30°C, and *E. coli* strains were incubated at 37°C. When required, antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin, 100; chloramphenicol, 30; kanamycin, 25 or 50; and streptomycin, 50 or 100.

Construction of the *P. putida tol* mutants. The different *tol* mutant strains were constructed by reverse genetic procedures. All the plasmids used for allelic replacement were based on the pKNG101 suicide vector and are listed in Table 1. Plasmids pKQΩKm, pKRΩKm, pKAΩKm, and pKBΩKm were used to construct polar mutations in the *tolQ*, *tolR*, *tolA*, and *tolB* genes, respectively. For the construction of the *tolQ*, *tolR*, *tolA*, and *tolB* nonpolar mutant derivatives, plasmids pKSmaIQxylE, pKSmaIRxylE, pKSmaIAxylE, and pKSmaIBxylE were used, respectively. Each of these pKNG101 derivatives was transferred from *E. coli* CC118λ*pir* to *P. putida* KT2440 by triparental mating using the helper strain *E. coli* HB101(pRK600), and the allelic exchange was carried out as previously described (52).

Sensitivity to different chemical compounds. To determine the bacterial sensitivity to deoxycholate (DOC), sodium dodecyl sulfate (SDS), and EDTA, overnight cultures of each strain were diluted in fresh LB medium containing 2% (wt/vol) DOC, 0.5% (wt/vol) SDS, or 0.5 mM EDTA, respectively, to reach an optical density at 660 nm (OD_{660}) of ~0.1. After 4 h of incubation at 30°C with agitation, the numbers of CFU per milliliter in the different cultures were determined by spreading suitable dilutions on LB plates. As a control, CFU per milliliter in cultures without any added agent was also determined. Cell survival was calculated as the ratio of the CFU per milliliter in the cultures supplemented with the tested compound to the CFU per milliliter in the unsupplemented cultures. MICs of antibiotics were determined by the microtiter broth dilution method (3).

Microscopy studies. *P. putida* cells grown in LB medium were harvested in the logarithmic growth phase and subjected to microscopy analysis. For transmission electron microscopy (TEM), samples were prepared and observed as previously described (53). For scanning electron microscopy (SEM) studies, 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 a Zeiss DSM950 scanning electron microscope.

Leakage of proteins into the extracellular medium. *P. putida* strains bearing the plasmid pJB3Km1, which encodes the periplasmic enzyme β-lactamase, were grown in LB medium to reach an OD_{660} of ~0.5. Cultures were then centrifuged (15,000 × *g*, 7 min, 25°C), and the resulting supernatant fraction was centrifuged again under the same conditions. The pellet fraction from the first centrifugation was solubilized in Laemmli sample buffer (30) treated with Benzonase (Merck) for 10 min to degrade the DNA and heated for 5 min at 95°C. The resulting sample was designated whole-cell lysate. Part of the supernatant fraction from the second centrifugation step was precipitated by incubation for 30 min at 4°C with 10% (wt/vol) trichloroacetic acid. After 30 min of centrifugation (15,000 × *g*) at 4°C and washing with acetone, the pellet (designated the supernatant fraction) was suspended in Laemmli sample buffer and treated as mentioned above. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and silver stained (63) or electrotransferred onto nitrocellulose and immunodetected with an anti-β-lactamase polyclonal antibody, with the monoclonal antibody MA7-2 raised against the *P. aeruginosa* OprF protein (39), or with the monoclonal antibody MA1-6 raised against the *P. aeruginosa* OprL protein (42). SDS-PAGE and Western immunoblotting analyses were performed as described previously (30, 61). The *P. putida* RpoS protein, used as a cytoplasmic marker, was detected with a polyclonal antibody raised against the *E. coli* RpoS protein.

Other methods. Standard molecular biology techniques were used for DNA manipulations (55). Southern blot analyses, PCR amplifications, and nucleotide sequencing were performed as previously described (54). Amino acid sequence similarities were detected using the BLAST program (2) available at the National Center for Biology Information network server, with the default settings.

Nucleotide sequence accession number. The nucleotide sequence corresponding to the *P. putida* chromosomal DNA fragment shown in Fig. 1 (7,577 bp) has been deposited in GenBank under accession number X74218. This sequence is identical to the *P. putida* KT2440 sequence deposited in The Institute for Genomic Research (TIGR) Microbial Database (http://www.tigr.org/tdb/mdb/mdb.html).

RESULTS

Cloning and sequencing of the *P. putida tol* genes. We previously reported the cloning and sequencing of the *P. putida* KT2440 *oprL* and *orf2* genes (53) (note that, in the genus *Pseudomonas*, the *pal* gene is called *oprL* according to nomenclature recommendations for this genus [22]). These genes were located in the 2.3-kb *Sph*I fragment of plasmid pPRO200 (Table 1). This fragment was used as a probe to search, in cosmids pPRO50 and pPRO6, for chromosomal regions flanking *oprL* and *orf2*. The different DNA fragments obtained from these cosmids were cloned and sequenced to complete a total of 7,577 bp. The sequenced region was predicted to contain nine complete open reading frames (ORFs), which included the *oprL* and *orf2* genes, and one truncated ORF at the 5′ end (Fig. 1). This partial ORF was 516 bp, encoding the 171 carboxyl-terminal amino acids of a polypeptide which showed high similarity with the RuvB proteins from a number of microorganisms, such as *P. aeruginosa*, *H. influenzae*, and *E. coli* (89.4, 74.4, and 71.8% identity, respectively). Five ORFs were found between *′ruvB* and *oprL*, whose predicted products exhibited high similarity with the *orf1*, *tolQ*, *tolR*, *tolA*, and *tolB* genes of the *tol-pal* system from various gram-negative bacteria (Fig. 1). The secondary structures predicted for these proteins were very similar to those determined for the *E. coli* Tol proteins. Separated from *orf2* by 168 bp and from each other by 17 bp, two additional ORFs were found, called *orf3* and *orf4* (Fig. 1), whose hypothetical products showed similarity with the ExsD (28.6% identity) and ExsB (39.2% identity) proteins, respectively, from *Rhizobium meliloti*. In *R. meliloti*, the *exsB* gene is located in megaplasmid 2, adjacent to the *exo* genes, which are involved in the biosynthesis of the exopolysaccharide succinoglycan. ExsB has been proposed to function as a negative regulator of the synthesis of this polymer, although it does not act at the transcriptional level (4).

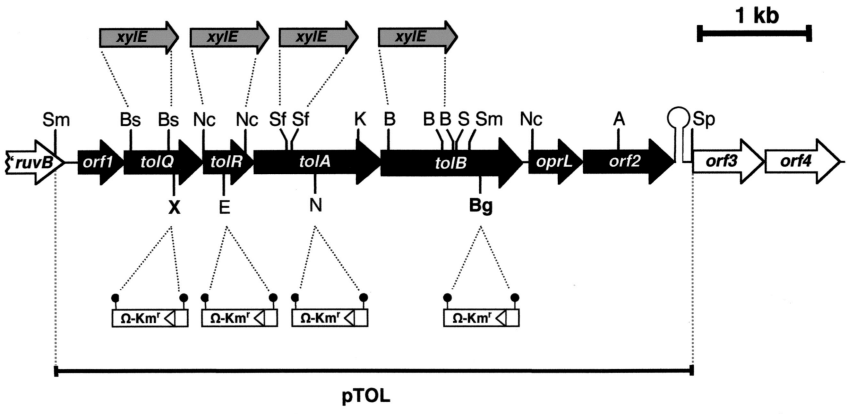
Construction of *P. putida tol* mutant strains. To analyze the function of the Tol proteins in *P. putida*, different mutant strains bearing an inactivated chromosomal copy of the *tolQ*, *tolR*, *tolA*, or *tolB* gene were constructed by allelic exchange as described in Materials and Methods. Two types of mutant derivatives were designed: polar mutants, containing an Ω-Km^r interposon insertion in the corresponding *tol* gene (designated *tol*::ΩKm mutants) (Table 1; Fig. 1), and nonpolar mutants, in which different internal fragments of each *tol* gene were deleted and replaced by a promoterless *xylE* cassette lacking any transcriptional stop signals downstream of its stop codon (designated *tol*::*xylE* mutants) (Table 1; Fig. 1). These *P. putida tol* mutant strains were basically constructed as previously described for the *P. putida oprL* mutant (52), although with some modifications. First, the sucrose concentration in the selective media was increased to 10% (wt/vol) to improve the killing

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1*, lysogenized with λ*pir*	25
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	9
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	55
P. putida		
KT2440	*hsdR1*	17
AX	KT2440 *tolA*::*xylE* (TolA shortened to 94 amino acids)	This study
AΩ	KT2440 *tolA*::ΩKm (insertion after codon 180)	This study
BX	KT2440 *tolB*::*xylE* (TolB shortened to 29 amino acids)	This study
BΩ	KT2440 *tolB*::ΩKm (insertion after codon 314)	This study
DOT-OX2	KT2440 *oprL*::*xylE* (OprL shortened to 16 amino acids)	52
QX	KT2440 *tolQ*::*xylE* (TolQ shortened to 17 amino acids)	This study
QΩ	KT2440 *tolQ*::ΩKm (insertion after codon 129)	This study
RX	KT2440 *tolR*::*xylE* (TolR completely removed)	This study
RΩ	KT2440 *tolR*::ΩKm (insertion after codon 46)	This study
Plasmids		
pHP45ΩKm	Ap^r, Km^r; *ori*ColE1, source of the Ω-Km^r interposon	15
pJB3Km1	Ap^r, Km^r; *oriV* RK2, *trfA*, *oriT*RK2, α-*lacZ*	8
pKNG101	Sm^r; gene replacement vector, *ori*R6K, *oriT*RK2, *sacB*	28
pRK600	Cm^r; helper plasmid, *ori*ColE1, *mob*RK2, *tra*RK2	29
pUC18	Ap^r; cloning vector, *ori*ColE1, *rop* mutant, α-*lacZ*	48
pUC18Not	Ap^r; identical to pUC18 but with *Not*I sites flanking the polylinker of pUC18	25
pXYLE10	Km^r; source of the promoterless *xylE* cassette	58
pKAΩKm	pKNG101 with the *Not*I insert from pNotAΩKm at the *Not*I site	This study
pKBΩKm	pKNG101 carrying, at the *Sma*I site, a 2.5-kb *Kpn*I-*Asp*700 fragment from pTOL with an Ω-Km^r cassette inserted at the *Bgl*II site of *tolB*	This study
pKQΩKm	pKNG101 with the *Not*I insert from pNotQΩKm at the *Not*I site	This study
pKRΩKm	pKNG101 with the *Not*I insert from pNotRΩKm at the *Not*I site	This study
pKSmaIAxylE	pKNG101 carrying, at the *Sma*I site, a 2.6-kb *Xho*I-*Stu*I fragment from pTOL with a *xylE* cassette replacing a 45-bp *Sfi*I fragment internal to *tolA*	This study
pKSmaIBxylE	pKNG101 carrying, at the *Sma*I site, a 2.6-kb *Nco*I fragment from pTOL with a *xylE* cassette replacing a 633-bp *Bst*EII fragment internal to *tolB*	This study
pKSmaIQxylE	pKNG101 carrying, at the *Sma*I site, a 2.1-kb *Sma*I-*Sfi*I fragment from pTOL with a *xylE* cassette replacing a 294-bp *Bst*XI fragment internal to *tolQ*	This study
pKSmaIRxylE	pKNG101 carrying, at the *Sma*I site, a 3.8-kb *Sma*I fragment from pTOL with a *xylE* cassette replacing a 393-bp *Nco*I fragment internal to *tolR*	This study
pNotAΩKm	pUC18Not carrying a 2.6-kb *Xho*I-*Stu*I fragment from pTOL with the Ω-Km^r cassette in the *Not*I site of *tolA*	This study
pNotQΩKm	pUC18Not carrying a 2.7-kb *Sma*I-*Kpn*I fragment from pTOL with the Ω-Km^r cassette in the *Xho*I site of *tolQ*	This study
pNotRΩKm	pUC18Not carrying a 2.7-kb *Sma*I-*Kpn*I fragment from pTOL with the Ω-Km^r cassette in the *Eco*NI site of *tolR*	This study
pPRO200	Ap^r; pUC18 with a 2.3-kb *Sph*I insert from pPRO50 carrying the *oprL* and *orf2* genes	53
pPRO50	Tc^r; pLAFR3 carrying a ~29-kb chromosomal fragment from *P. putida* KT2440 with the *oprL* gene	53
pPRO6	Tc^r, Km^r; pLAFR3 carrying a ~24-kb chromosomal fragment from *P. putida* 14G-3 with the *oprL*::*phoA* mutant gene	53
pTOL	pUC18 carrying a 5.7-kb *Sma*I-*Sph*I chromosomal fragment from *P. putida* KT2440 (*orf1 tolQ tolR tolA tolB oprL orf2*)	This study

[a] Ap^r, Cm^r, Km^r, Sm^r, and Tc^r, resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, and tetracycline, respectively.

efficiency of the *sacB* gene encoded by the pKNG101 vector. Second, we observed that *sacB*-induced cell lysis of the *P. putida* clones bearing the pKNG101 cointegrate was more effective when bacteria were incubated at temperatures lower than 30°C. Consequently, to select for sucrose-resistant (Suc^r) colonies, plates were incubated overnight at 22°C. In all cases the successful allelic exchange was checked by PCR and by Southern blot hybridization (data not shown). The complete excision of the pKNG101 vector from the host chromosome was also confirmed by Southern blot hybridization using the pKNG101 vector as a probe (data not shown). Figure 1 shows the insertion positions of the Ω-Km^r and *xylE* cassettes in each of the *tol* genes. All mutant strains were viable, although on LB plates, colonies of the mutants were more translucent than those of the parental strain. The viability of all *P. putida tol* mutants demonstrates that these genes are not essential for the survival of this microorganism, as was also the case for the *P. putida oprL* (*pal*) mutant and for the *E. coli tol* mutants (6, 52, 59), but in contrast with the essential role proposed for *tolQ* and *tolA* in *P. aeruginosa* (13).



	Orf1	TolQ	TolR	TolA	TolB	OprL	Orf2
Predicted aa length:	150	231	150	372	433	166	268
% identity with *E. coli*:	48.0	55.0	34.5	37.8	43.4	39.8	32.0

FIG. 1. Schematic map of the 7,577-bp chromosomal DNA fragment containing the *tol-oprL* gene cluster of *P. putida*. The arrows indicate the different ORFs and their transcriptional direction. Closed arrows indicate the genes of the *tol-oprL* system; open arrows indicate adjacent ORFs (*ruvB* was only partially sequenced); shaded arrows represent the promoterless *xylE* cassette; and open rectangles represent the Ω-Km^r interposon (triangles in the open rectangles indicate the transcription direction, and the closed circles represent bacteriophage T4 transcriptional termination signals). A putative hairpin structure ($\Delta G° = -19.2$ kcal) was found 25 bp downstream of the *orf2* stop codon and proposed to function as a Rho-independent transcription termination signal (53). The predicted amino acid (aa) lengths of the *P. putida* Tol-OprL proteins and their percentages of identity to the *E. coli* Tol-PAL proteins are indicated below the chart. The long horizontal bar indicates the 5,745-bp *Sma*I-*Sph*I insert carried by the pTOL plasmid. In vitro mutations were generated in the pTOL plasmid or its derivatives. For the *P. putida* QΩ, RΩ, and AΩ mutants, the Ω-Km^r cassette was excised from pHP45ΩKm as a 2,243-bp *Bam*HI fragment, filled in with the Klenow fragment of *E. coli* DNA polymerase I, and inserted at the indicated sites, which had been blunt-ended before ligation. For *P. putida* BΩ, the *Bam*HI Ω-Km^r cassette was cloned into the *Bgl*II site. The mutations were transferred to the host chromosome, and the position of the Ω-Km^r interposon in the different *tol*::ΩKm mutants is shown below the ORF map. For construction of the *tol*::*xylE* mutations, the *xylE* cassette was obtained from pXYLE10 as a 962-bp *Sma*I fragment. DNA fragments internal to the different *tol* genes were excised, and the resulting cohesive ends were blunt-ended by treatment with the Klenow fragment of *E. coli* DNA polymerase I or with T4 DNA polymerase. The *xylE* cassette was then cloned, replacing the deleted fragments. The position of the *xylE* cassette in the *tol*::*xylE* mutants is shown above the ORF map. Only relevant restriction sites are shown. Unique sites in the fragment are indicated in boldface type. The remaining sites are also present in other positions (not shown) in the fragment. Restriction sites are as follows: A, *Asp*700; B, *Bst*EII; Bg, *Bgl*II; Bs, *Bst*XI; E, *Eco*NI; K, *Kpn*I; N, *Not*I; Nc, *Nco*I; S, *Stu*I; Sf, *Sfi*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I.

Morphological and physiological characterization of the *P. putida tol* mutants. *P. putida* KT2440 and the different *tol* mutant strains were grown in LB medium; the doubling times of all *tol* mutant strains in the exponential phase (40 ± 2 min, $n = 6$) were similar to that of the wild-type strain (doubling time, 37 ± 1 min; $n = 4$). Growth rates of the *P. putida tolB*::ΩKm and *tolR* mutants were slightly lower (doubling times around 44 and 48 min, respectively). The bacterial cultures exhibited high turbidities ($OD_{660} > 2.5$) when they reached the stationary phase, although all mutant strains showed a tendency to produce clumps at this phase, probably due to the adhesion of cell debris derived from lysed bacteria.

Cell cultures of the *P. putida* DOT-OX2 (*oprL*) and *P. putida tol* mutant strains were harvested in the exponential phase of growth and observed by phase-contrast microscopy. Cells of the wild-type strain presented the typical appearance of the members of the family *Pseudomonaceae*, whereas the *tol-oprL* mutant cells seemed to be shorter than the parental ones, and most of them grew by forming filaments frequently composed of 10 or more cell units (not shown). Motility of these cellular filaments seemed to be very reduced when compared with that of the individual cells. Swarm assays, carried out on LB plates containing 0.3% (wt/vol) agar, confirmed these results; while the wild type formed a 30- ± 2-mm-diameter growth halo after 16 h of incubation at 30°C, the halo of the mutant strains was basically restricted to the inoculation spot, indicating a severe deficiency in motility for the *tol-oprL* mutants.

Wild-type and *tol* mutant cells in the exponential phase of growth were also examined by SEM. Wild-type cells appeared as well-defined rod-shaped bacteria. Most of them were found in pairs, in different phases of the cell division process (Fig. 2A). However, all the *P. putida tol-oprL* mutants grew as chains, corroborating the optical-microscopy observations. In addition, cells within a chain were often shorter than wild-type cells (compare for instance wild-type cells [Fig. 2A] versus those of the different *tol* mutants [Fig. 2B to F]). Within chains, division septa were usually easily distinguishable, and many of them seemed to be in an incomplete but advanced stage of the cell division process (Fig. 2B to F). A difference found between *P. putida tolQ*, *tolR*, and *tolA* cells and *P. putida* BX or DOT-OX2 cells was the frequent presence of big blebs at the cell surface of the former (Fig. 2B to D), whereas blebs appeared only occasionally in the latter (Fig. 2E and F).

Under TEM, the wild-type *P. putida* cells exhibited the typical appearance of bacteria of the genus *Pseudomonas* (Fig. 3A). The appearances of all *P. putida tol* strains harvested in the exponential phase of growth were similar to that of the parental strain except for the presence of filaments when several adjacent cells within a chain coincided with the plane of the section (Fig. 3B). A detailed examination of a number of cells from different filaments at their division sites confirmed that, in many cases, the division process was at a very advanced stage, as suggested by the previous SEM observations (Fig. 3C). Furthermore, outer and inner membranes, as well as the periplasm, of mutant cells appeared well defined with no appreciable structural alterations (Fig. 3C).

The patterns of antibiotic resistance and sensitivity of the *tol* mutants were assayed by the MIC method with a number of antibiotics. The polar and nonpolar mutant strains were used in these assays. The results obtained with both types of mutants

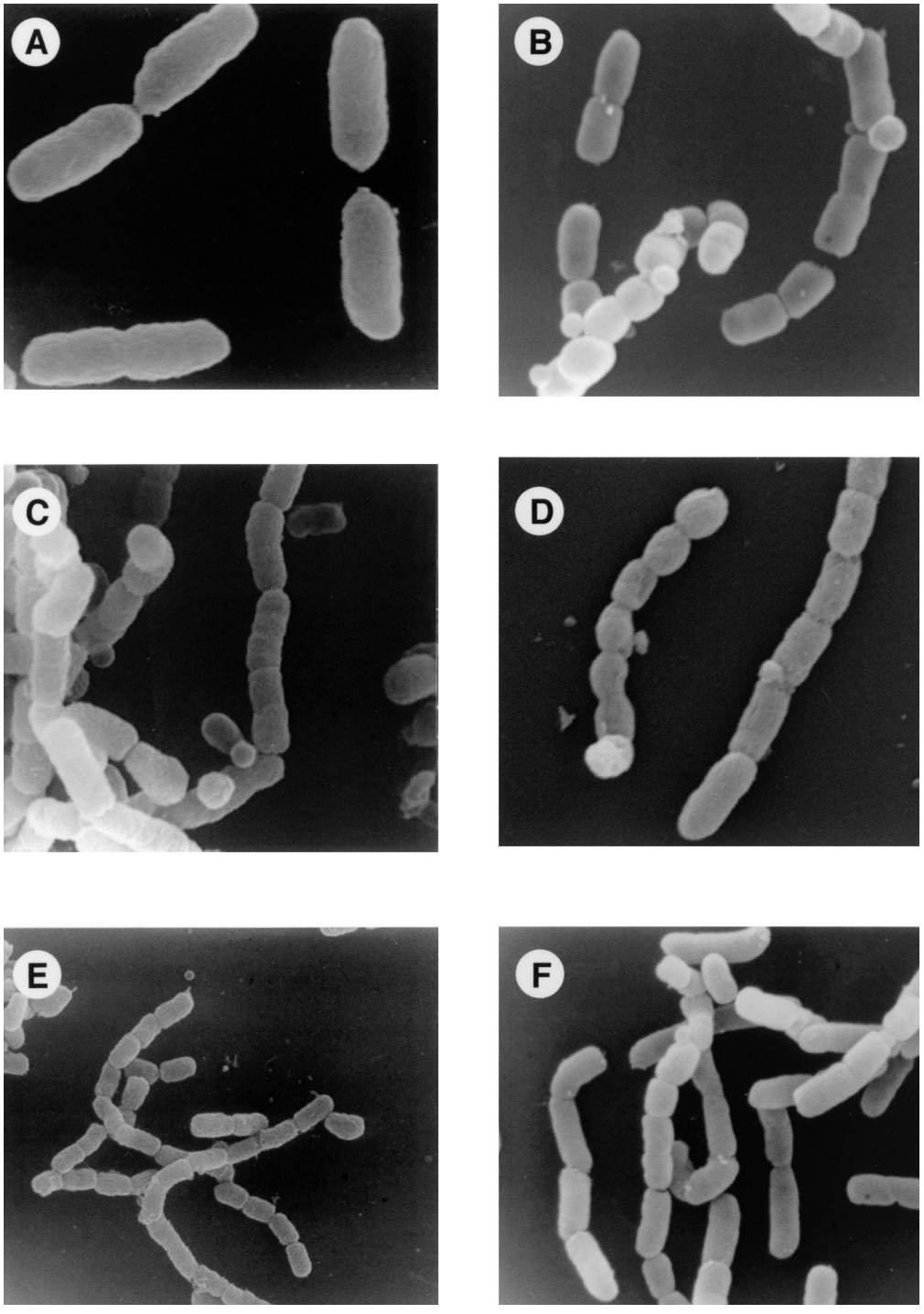


FIG. 2. SEM of *P. putida* KT2440 and some of the *P. putida tol* mutants. Cells were grown on LB medium, harvested in the exponential phase of growth, and treated for SEM as described in Materials and Methods. (A) Strain KT2440. Magnification, ×15,000. (B) Strain QΩ. Magnification, ×10,000. (C) Strain RΩ. Magnification, ×10,000. (D) Strain AΩ. Magnification, ×10,000. (E) Strain BX. Magnification, ×5,000. (F) Strain DOT-OX2. Magnification, ×7,000.

were similar, and Table 2 shows those obtained with the *tol*::*xylE* mutants. The *tol*::*xylE* strains were more sensitive than the KT2440 strain to the hydrophobic antibiotics fusidic acid, novobiocin, and, particularly, rifampin (Table 2). They also exhibited increased sensitivity to some aminoglycosides (such as gentamicin and streptomycin), some β-lactams (such as cefepime and piperacillin, but not imipenem), and to nalidixic acid. Mutants were also more sensitive to chloramphenicol and slightly more sensitive to tetracycline (both antibiotics are usually removed from the cell by active efflux mechanisms). In general, the most susceptible *tol*::*xylE* strains were *P. putida* BX and DOT-OX2.

The degrees of resistance or sensitivity of the mutant strains to the detergents DOC and SDS and to the chelating agent

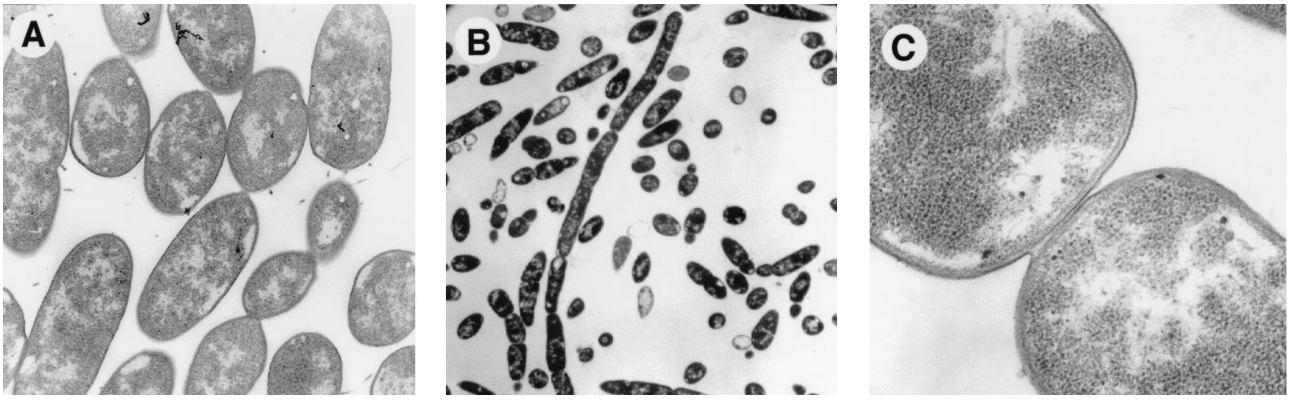


FIG. 3. TEM of *P. putida* KT2440 and some of the *P. putida tol*::*xylE* mutants. Cells were grown on LB medium, harvested in the exponential phase of growth, and then processed for TEM as described in Materials and Methods. (A) Strain KT2440. Magnification, ×15,200. (B) Strain QX. Magnification, ×2,850. (C) Detail of the division septum between two DOT-OX2 cells which belong to a longer filament. Magnification, ×47,500.

EDTA were also analyzed. Mutant cells were incubated for 4 h at 30°C in LB medium and in LB medium supplemented with 2% (wt/vol) DOC, 0.5% (wt/vol) SDS, or 0.5 mM EDTA, and the survival rates were determined as described in Materials and Methods. While survival of the wild-type cells was in all cases in the range of 70% to 99%, for the *tol* mutants, survival ranged from 0.1% to 0.6% for SDS and EDTA and from 0.004% to 0.1% for DOC (data not shown). Among the mutants, the *P. putida tolR* strains were the most sensitive, particularly to DOC (with a survival of 0.006%). *P. putida* DOT-OX2 was also very sensitive to this compound (survival of 0.004%).

In summary, all the above results clearly indicate that the permeability barrier functions of the outer membrane were significantly altered in the *P. putida tol* mutant strains.

Leakage of periplasmic β-lactamase. Since many *E. coli tol* mutants were originally isolated as strains that released periplasmic proteins into the extracellular medium (16, 35), we decided to study whether the *P. putida tol* strains exhibited this phenotype. As a model protein we chose the periplasmic enzyme β-lactamase. First, plasmid pJB3Km1, which carries the *bla* gene encoding β-lactamase, was transferred to *P. putida* KT2440 and to the *tol* strains. Then the presence of β-lactamase in the supernatants of the different *P. putida* cultures was analyzed by Western blot. Samples were harvested from exponentially growing cultures to avoid possible interference with lysed cells, which could appear in a late growth phase. Analysis of the different culture supernatants by SDS-PAGE and silver staining showed the presence in the supernatant fractions of the *tol* mutants of numerous protein products (varying within a wide range of electrophoretic mobilities) that were absent from the *P. putida* wild-type extracellular fraction (Fig. 4A). The proteins were transferred onto a nitrocellulose membrane, and the presence of β-lactamase was analyzed by using a polyclonal antibody raised against this protein. The amounts of β-lactamase in the culture supernatant fractions of all mutant strains were similar (Fig. 4B). The periplasmic enzyme was not detected in the extracellular fraction of the parental strain. Immunodetection with an anti-RpoS (σ^{38}) antibody did not show any detectable amounts of this protein, used as a cytoplasmic marker, in the culture supernatants (data not shown). However, this protein was present in considerable amounts in the whole-cell lysates of these strains, and it was also proven to be stable enough to be immunodetected after its release into the external medium (data not shown). On the other hand, it has been previously shown that, in addition to periplasmic proteins, the *tol-pal* mutants of *E. coli* released outer membrane vesicles into the extracellular medium which contained outer membrane proteins (5). Immunodetection of the supernatant fractions of the *P. putida tol-oprL* cultures with a monoclonal antibody raised against the *P. aeruginosa* OprF protein (cross-reacting with *P. putida* OprF) revealed the presence in these fractions of a product with an apparent molecular mass of 42 kDa, which would correspond to the *P. putida* OprF protein (Fig. 4C). However, in contrast with the *E. coli tol-pal* mutants, the OprL protein could not be immunodetected in the supernatant fractions of the *P. putida tol-oprL* mutants with the monoclonal antibody MA1-6, which cross-reacts with *P. putida* OprL (data not shown). From these results, it can be concluded that mutations in any of the *P. putida tol* genes lead to a significant leakage of periplasmic and outer membrane proteins.

TABLE 2. MICs of antibiotics for *P. putida* KT2440 and the *P. putida tol*::*xylE* mutants[a]

Antibiotics	MIC (μg ml^{-1}) for strain					
	WT[b]	QX	RX	AX	BX	DOT-OX2
Cefepime	2	0.5	0.25	0.5	0.12	0.12
Chloramphenicol	128	16	16	16	16	16
Fusidic acid	512	128	128	64	64	128
Gentamicin	2	0.25	0.5	0.5	0.12	0.12
Imipenem	0.12	0.12	0.12	0.12	0.12	0.12
Nalidixic acid	32	8	8	8	4	4
Novobiocin	256	64	64	32	64	64
Piperacillin	16	16	4	4	4	2
Rifampin	8	2	1	4	0.5	0.5
Streptomycin	8	1	4	2	2	0.5
Tetracycline	2	1	0.5	1	1	2

[a] Antibiotics were assayed following serial twofold dilutions. Data are the averages of three independent experiments.
[b] WT, wild type.

DISCUSSION

Sequence and functional similarities between the Tol-PAL (OprL) systems of *E. coli* and *P. putida*. Previously we identified the *oprL* and *orf2* genes of the *P. putida tol-oprL* system (53). In this study, we cloned and sequenced the remaining genes of the *P. putida tol-oprL* gene cluster. Among the different *P. putida* Tol-OprL proteins, TolQ and Orf1 were the best

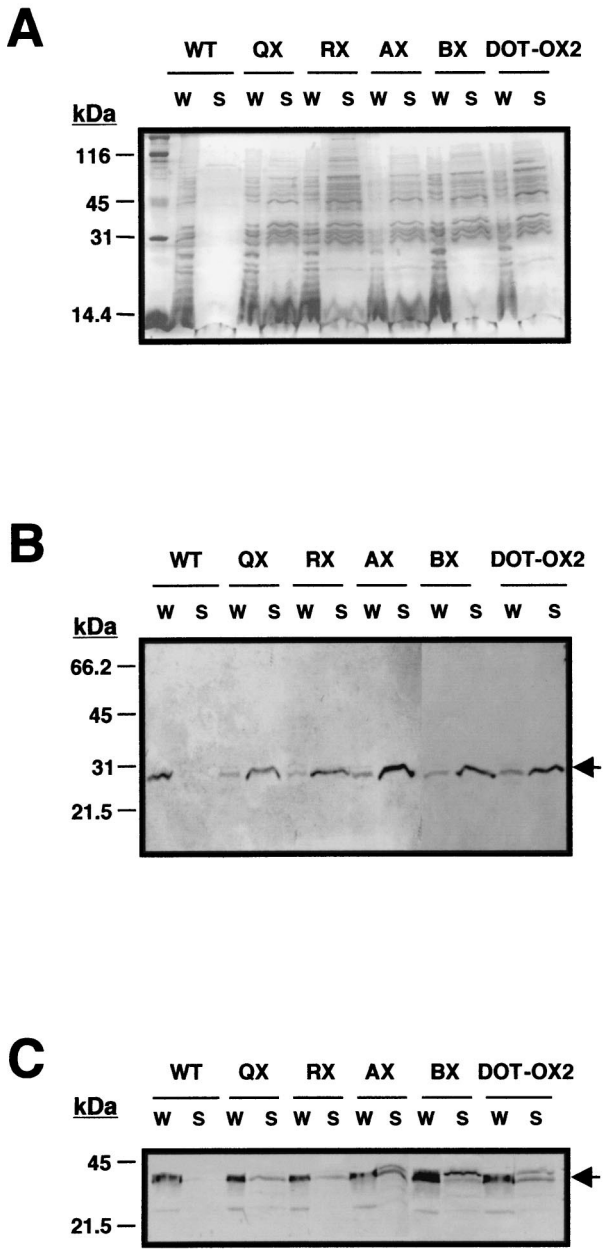


FIG. 4. Immunodetection of β-lactamase in the supernatant fractions (S) and whole-cell lysates (W) of *P. putida* KT2440 (WT) and the different *tol*::*xylE* mutants (QX, RX, AX, BX, and DOT-OX2), bearing the plasmid pJB3Km1. About 1×10^8 cells (3×10^6 for the silver staining) or the equivalent of the supernatant of 2×10^8 cells were loaded on the gel. The different fractions were prepared as described in Materials and Methods. Proteins were separated on SDS-polyacrylamide (12.5%, wt/vol) gel electrophoresis and silver stained (A), or they were transferred onto nitrocellulose and immunodetected with an anti-β-lactamase polyclonal antibody (B) or with the anti-OprF antibody MA7-2 (C). The Western blot was developed using the peroxidase colorimetric method (55). The arrows show the positions of the β-lactamase (B) or the OprF proteins (C). The positions of the molecular size markers are indicated on the left.

conserved (Fig. 1). The high degree of conservation of TolQ probably reflects its key role in the assembly of the TolQRA inner membrane complex, since TolQ is involved in a significant number of interactions within this protein complex (14, 19, 36). On the other hand, the conservation of Orf1 suggests that this protein could play an important role in the cell. However, *E. coli orf1* chromosomal mutants did not show a Tol phenotype or any other differential phenotypes compared with the wild-type strain (11, 59), and consequently the function of Orf1 remains unknown. We are currently constructing *P. putida orf1* mutants in order to elucidate the physiological role of this protein. *P. putida* TolR was one of the least conserved proteins with respect to the *E. coli* Tol proteins (Fig. 1). In spite of its relatively low degree of sequence similarity, *P. putida tolR* was able to complement the *tolR* mutant strain *E. coli* TPS300 (59) in terms of colicin A and colicin E3 tolerance and sensitivity, which demonstrated the existence of a high degree of functional similarity between both proteins in these bacteria (51). These results seem to contrast with those obtained by Dennis and coworkers (13) with the *P. aeruginosa* TolR protein (82% identical to *P. putida* TolR). These authors reported no complementation of the *E. coli* TPS300 strain with the *P. aeruginosa tolR* gene in terms of colicin E1 tolerance or sensitivity. However, the choice of colicin E1 to carry out these complementation studies is inappropriate since the *E. coli* TolR protein is not involved in the translocation of this colicin (27, 32). Nonetheless, we cannot discard the possibility that *P. aeruginosa* TolR could differ from *P. putida* TolR in some residue(s) critical for the complementation of the *E. coli tolR* mutation.

On the other hand, the existence of similarity between the *E. coli* TolQ-TolR-TolA and TonB-ExbB-ExbD protein complexes is well known (33). *E. coli* TolQ and TolR are in fact structurally and functionally homologous to ExbB and ExbD, respectively, and they are able to partially cross complement each other (10). As expected, *P. putida* TolQ and TolR were also very similar to *P. putida* ExbB and ExbD, respectively. Furthermore, in *P. putida* both systems were also similar in gene organization: *tolQ-tolR-tolA* (this work) and *exbB-exbD-tonB* (7). This supports the idea that these systems probably derive from a common ancestor. It has been reported that *P. aeruginosa tolQ* was able to complement an *E. coli exbB* mutant but not a *P. putida exbB* mutant (13). Perhaps this could be because *P. putida* ExbB (329 amino acids) is larger than *E. coli* ExbB protein (244 amino acids) and *P. aeruginosa* TolQ protein (231 amino acids). A relevant point concerning the possible role(s) of the Tol-PAL system is the recent finding that *E. coli* TolA could undergo conformational changes depending on TolQ, TolR, and the proton motive force (34). This would definitively confirm the relationship between the TolQ-TolR-TolA and the TonB-ExbB-ExbD complexes and could open new possibilities in the field of energy transduction between membranes.

Outer membrane integrity is altered in *P. putida tol* mutants. In this study we constructed *P. putida* polar (by insertion of an interposon flanked by the transcriptional terminator of the phage T4 gene 32) and nonpolar mutations in each of the *tol* genes. The terminator activity of this T4 sequence has been previously demonstrated both in vivo and in vitro by other authors (49). We are currently studying the transcriptional organization of the *P. putida tol-oprL* gene cluster by different approaches (by primer extension analysis, measuring the catechol-2,3-dioxygenase activity in the *tol*::*xylE* mutants, and by Western blot analysis), and preliminary results strongly support the idea that, while the *xylE* cassette does not affect gene transcription, the Ω-Km^r interposon indeed acts as a transcriptional terminator in our system. On the other hand, we have also tried to complement the *tol* mutations by using different (medium- and low-copy-number) plasmids vectors bearing the whole gene cluster, the *orf1*, *tolQ*, and *tolR* genes, or the *tolB* gene alone, but we have been unable to obtain transconjugants (or transformants) which maintained these plasmids, even with

the wild-type strain. These results suggest that even slight overexpression of the *tol* proteins in *P. putida* could be very toxic, and they are in agreement with other authors who suggest that the stoichiometry of the Tol complex is essential for its stability (5, 33). Our results could also explain why all our mutants (polar and nonpolar) exhibited similar phenotypes, since the lack of any component of the Tol system would cause an equivalent destabilization of this protein complex.

All mutant strains were viable although their survival during short-term storage (on LB plates at 4°C) and long-term storage (at −80°C) was reduced compared with that of the wild-type strain. Whereas on plates at 4°C the wild-type strain is viable for 3 months and for several years at −80°C, none of the *tol* mutants generated in this study survived longer than a month at 4°C on LB plates, and none were viable after 1 year at −80°C. This reduced viability could be related to alterations in the cell envelope of these mutants. Under SEM, mutant cells presented blebs at their cell surface. It should be noted that, when the mutant cells were visualized by TEM, the blebs were not present, which is probably due to the mechanical rupture of the blebs in the centrifugation steps used to collect and wash the cells during the fixation, as has been previously reported in the case of the *E. coli lpo* mutants (18). Blebbing was more frequent in the *P. putida tolQ*, *tolR*, and *tolA* than in the *tolB* and *oprL* mutants, which is in agreement with previous observations made in *E. coli*, where the *tolQ*, *tolR*, and *tolA* mutants presented a higher level of vesicle formation than the *tolB* and *pal* ones (5).

We analyzed the patterns of resistance and sensitivity of the different *P. putida tol-oprL* mutants to a variety of antibiotics and other chemical agents (SDS, DOC, and EDTA). All mutants were sensitive to a variety of compounds, although the *P. putida* BX (*tolB*) and *P. putida* DOT-OX2 (*oprL*) strains were the most susceptible to these drugs. Mills and Holloway (41) described a putative *P. aeruginosa tol* strain (selected by its tolerance to pyocin AP41) that showed specific hypersensitivity to aminoglycosides but not to other drugs. In *P. putida*, the *tol-oprL* mutations produced a wider antibiotic sensitivity pattern. The increased sensitivity to drugs of the *P. putida tol-oprL* mutants isolated in this study should not be specifically attributed to a defect in the hydrophobic barrier function of the outer membrane, to the inactivation of efflux pumps, or to any other particular deficiency in these strains, but rather it would seem that the *tol-oprL* mutants show a quite complex global permeability alteration.

Another striking phenotype exhibited by all the *P. putida tol* mutants was cell filamentation. This characteristic was also found in a presumed *P. aeruginosa tol* strain (selected as a spontaneous mutant tolerant to pyocin AP41) and in the *V. cholerae tol* mutants (23, 26). Meury and Devilliers (40) have reported that an *E. coli tolA* mutant showed cell filamentation when it was grown in conditions of low or high osmolarity. Within the filaments they observed the presence of oblique septa and numerous anucleate cells. Based on these results it was suggested that TolA could play a role in positioning the division sites. Our analysis of the *P. putida tolA* mutant cells under TEM did not reveal the above features. In fact, all *P. putida tol* strains formed relatively short filaments where the cells seemed to be in an advanced state of cell division. Hence, the *P. putida* Tol-OprL proteins could be directly or indirectly involved in the late stages of the cell division process, although they seemed not to be essential to complete this process.

The *P. putida tol-oprL* mutant strains also showed a leaky phenotype, releasing periplasmic and outer membrane proteins into the extracellular medium. In *E. coli* this phenotype was also observed with *tol-pal* strains (5). However, we found that *P. putida* did not release the OprL protein, while the homologous PAL protein was found in the outer membrane vesicles released by *E. coli tol* mutants (5). In short, our results show that strains with mutations in each of the *tol-oprL* genes of *P. putida* are viable although they exhibit severe defects in cell morphology and altered outer membrane structure and function.

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