

Role of *Pseudomonas putida* *tol-oprL* Gene Products in Uptake of Solutes through the Cytoplasmic Membrane

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Proteins of the Tol-Pal (Tol-OprL) system play a key role in the maintenance of outer membrane integrity and cell morphology in gram-negative bacteria. Here we describe an additional role for this system in the transport of various carbon sources across the cytoplasmic membrane. Growth of *Pseudomonas putida tol-oprL* mutant strains in minimal medium with glycerol, fructose, or arginine was impaired, and the growth rate with succinate, proline, or sucrose as the carbon source was lower than the growth rate of the parental strain. Assays with radiolabeled substrates revealed that the rates of uptake of these compounds by mutant cells were lower than the rates of uptake by the wild-type strain. The pattern and amount of outer membrane protein in the *P. putida tol-oprL* mutants were not changed, suggesting that the transport defect was not in the outer membrane. Consistently, the uptake of radiolabeled glucose and glycerol in spheroplasts was defective in the *P. putida tol-oprL* mutant strains, suggesting that there was a defect at the cytoplasmic membrane level. Generation of a proton motive force appeared to be unaffected in these mutants. To rule out the possibility that the uptake defect was due to a lack of specific transporter proteins, the PutP symporter was overproduced, but this overproduction did not enhance proline uptake in the *tol-oprL* mutants. These results suggest that the Tol-OprL system is necessary for appropriate functioning of certain uptake systems at the level of the cytoplasmic membrane.

Two membranes separate the cytoplasm from the external environment in gram-negative bacteria. The outermost membrane contains pore-forming proteins that allow the entry of small molecules by passive diffusion (38), whereas the cytoplasmic membrane houses proteins that link across the periplasmic space with the outer membrane to provide the energy required for the uptake across the outer membrane of certain nutrients, such as iron siderophores and vitamin B₁₂ (7). The cytoplasmic membrane also houses the transport systems that promote the entry of compounds from the periplasm to the cytoplasm, in addition to energy-generating systems. In *Escherichia coli* and *Pseudomonas* sp. maintenance of the structure of the cellular envelope involves the products of the *tol-pal* (*tol-oprL* in *Pseudomonas*) operons (4, 27, 32, 43). The transcriptional organization of the *tol-oprL* gene cluster of *Pseudomonas putida* is shown in Fig. 1 (33). The Tol-OprL (or Tol-Pal [peptidoglycan-associated lipoprotein]) system is made up of seven proteins. The *E. coli* Tol-Pal system is organized into two protein complexes: a cytoplasmic membrane complex composed of the

TolQ, TolR, and TolA proteins, which interact with each other via their transmembrane domains (12, 17, 18, 24, 25); and an outer membrane complex made up of TolB and Pal, which also interact with Lpp, OmpA, and the peptidoglycan layer (6, 10, 25).

TolQ is an integral cytoplasmic membrane protein that contains three transmembrane domains. The TolR and TolA proteins are anchored to the cytoplasmic membrane by a single transmembrane-spanning segment near the N terminus, which leaves most of the protein exposed to the periplasm (31, 37).

TolB is a periplasmic protein (23), whereas Pal is an outer membrane peptidoglycan-associated lipoprotein (30). The latter protein is anchored to the outer membrane by its N-terminal lipid moiety and strongly interacts with the peptidoglycan layer through its C-terminal region (5, 27). The C-terminal region of Pal also interacts with TolB (41). Interactions of Pal with TolB and the peptidoglycan appear to be mutually exclusive since the TolB-Pal complex is not strongly associated with the peptidoglycan (5, 10).

The cytoplasmic membrane TolQRA and outer membrane TolB-Pal complexes have been shown to be associated through the interactions of TolA with Pal (9) and TolB (14, 52). The TolA-Pal interactions require the proton motive force and the TolQ and TolR proteins (9). These interactions imply that the complexes form a link between the cytoplasmic and outer membranes, confirming the previous observation which indicated that Tol proteins are preferentially located in the contact regions between the cytoplasmic and outer membranes (19).

How the Tol-Pal (Tol-OprL) system plays its role in the

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FIG. 1. Physical and transcriptional organization of the *tol-oprL* cluster of *P. putida* KT2440. The solid arrows represent the different *tol* genes, their relative sizes, and the directions of transcription. The positions of the P_1 and P_L promoters are indicated (33).

maintenance of outer membrane integrity is unknown. It has been hypothesized that TolA could be involved in the energy-dependent passage of synthesized outer membrane components across the periplasm (34). This hypothesis is consistent with the observed *in vitro* interactions of TolB and TolA with trimeric outer membrane porins (13, 42). Furthermore, TolA was found to be required for surface expression of the O-antigen-containing lipopolysaccharide (16).

In the present study we identified an unexpected new role for the Tol-Pal (Tol-OprL) system. We found that *tol-oprL* mutants of *P. putida* do not grow on a number of carbon sources because of limited uptake of these nutrients across the cytoplasmic membrane. This observation was corroborated for the well-defined *tol* mutants of *E. coli* and hypothetical *tol* mutants of *Pseudomonas aeruginosa*. In this paper we discuss the hypothesis that the Tol-OprL system is required for proper functioning of certain transport systems in the cytoplasmic membrane.

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 grown by using routine methods in liquid Luria-Bertani (LB) medium or in M9 minimal medium with benzoic acid (5 mM) as the sole carbon source (44). To isolate outer membranes, cells were grown in LB medium or BM2 minimal medium with glucose as a carbon source (20). *Pseudomonas putida* was incubated at 30°C, and *P. aeruginosa* and *E. coli* strains were incubated at 37°C on an orbital platform operating at 200 strokes per min.

When required, antibiotics were used at the following final concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; chloramphenicol, 30 $\mu\text{g ml}^{-1}$; gentamicin, 10 $\mu\text{g ml}^{-1}$ (for *E. coli*) or 30 $\mu\text{g ml}^{-1}$ (for *Pseudomonas* sp.); kanamycin, 25 $\mu\text{g ml}^{-1}$ (for *E. coli*), 50 $\mu\text{g ml}^{-1}$ (for *P. putida*), or 300 $\mu\text{g ml}^{-1}$ (for *P. aeruginosa*); and streptomycin, 50 $\mu\text{g ml}^{-1}$ (for *E. coli*), 100 $\mu\text{g ml}^{-1}$ (for *P. putida*), or 500 $\mu\text{g ml}^{-1}$ (for *P. aeruginosa*).

General molecular biology methods. Standard molecular biology techniques were used for DNA manipulations (44). Southern blot analyses, PCR, and nucleotide sequencing were done as previously described (32, 33).

Construction of a *P. putida oprB* mutant strain. A mutant strain bearing an inactivated chromosomal *oprB* gene was constructed as follows. Plasmid pCHESI Ω Km is a pUC18 derivative containing the *oriT* origin of transfer of RP4 and the Ω -Km interposon of plasmid pHP45 Ω Km (15) cloned as a *Hind*III fragment (Fig. 2A). To generate the *oprB* mutation, a 338-bp fragment of the *P. putida oprB* gene was amplified by PCR by using primers with *Eco*RI sites and was subsequently cloned in the *Eco*RI site of pCHESI Ω Km in the same transcriptional direction as the P_{lac} promoter. The resulting plasmid, pCHESI-B (Fig. 2B), was mobilized from *E. coli* DH5 α into *P. putida* KT2440 by triparental mating by using the *E. coli* HB101(pRK600) helper strain (32). *P. putida* transconjugants bearing a cointegrate of the plasmid in the host chromosome were selected on M9 minimal medium with benzoic acid (10 mM) as the sole C source and 50 μg of kanamycin per ml. A few Km^r clones were chosen for Southern blot hybridization to confirm that pCHESI-B integrated into and disrupted the *oprB* gene. All of the clones analyzed contained an inactivated *oprB* gene, and a single random clone was chosen and designated *P. putida oprB*.

Outer membrane isolation and immunodetection. Outer membranes were prepared from cultures grown to a turbidity (measured at 660 nm [OD₆₆₀]) of ~0.8 by sucrose gradient density centrifugation (20). Protein profiles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). Immunoblotting procedures were performed as previously described (32, 40).

Uptake assays. (i) Assays with whole cells. Cells of the wild-type strain *P. putida* KT2440 and different mutant strains were grown to an OD₆₆₀ of ~0.5 to 0.7 in M9 minimal medium supplemented with the appropriate carbon source. Cells were washed once and resuspended in substrate-free M9 medium to an OD₆₆₀ of ~0.3. This cell density ensured that uptake remained linear throughout the experiment. Radiolabeled substrates, added at a final concentration of 250 μM , contained the radiolabeled compound at a concentration of 1 μM . The specific activity was 160 mCi/mmol for [¹⁴C]glycerol, 306 mCi/mmol for [¹⁴C]glucose, and 242 mCi/mmol for [¹⁴C]proline. The cells were incubated at 30°C. After radiolabeled substrates were added, samples were taken at 0, 10, 20, and 30 min. One milliliter was removed, filtered through 0.45- μm -pore-size filters (Millipore) by using a Millipore filter manifold, and washed twice with 3 ml of 0.1 M LiCl. Background radioactivity due to unspecific binding to bacterial cells and filters was assessed by using cells incubated on ice. Filters were placed in scintillation vials, and 4 ml of scintillation liquid was added. Samples were allowed to equilibrate for 12 h before scintillation counting.

(ii) Assays with spheroplasts. Cells were converted to spheroplasts by treatment with lysozyme and EDTA. The cells were grown to an OD₆₆₀ of ~0.5 to 0.7 in M9 minimal medium supplemented with the appropriate carbon source. Cultures (50 to 100 ml) were centrifuged (10,000 $\times g$, 5 min, 4°C), and the cells were resuspended in 6.3 ml of 0.75 M sucrose–10 mM Tris-HCl (pH 7.8) and treated with 0.1 mg of lysozyme per ml for 2 to 5 min on ice. Then 13.2 ml of a 1.5 mM EDTA (pH 7.5) solution was added. The cell suspension was incubated for 15 min at 30°C and observed under a phase-contrast microscope. More than 95% of the cells appeared to be spheroplasts. For uptake assays, the OD₆₆₀ of the sample was adjusted to ~0.4, and for the uptake of radiolabeled substrates we used the procedure described above.

Determination of $\Delta\psi$. Generation of a membrane potential ($\Delta\psi$) in spheroplasts of the wild-type strain and *tol-oprL* mutants was monitored by using the cationic dye 3,3'-diethylthiadicarbocyanine iodide [DiSC₂(5)], which translocates into the lipid bilayer of hyperpolarized membranes, resulting in quenching of its fluorescence. Each reaction mixture (total volume, 1 ml) contained buffer A (125 mM HEPES, 0.9% [wt/vol] NaCl, 1 mM KCl, 1 mM MgCl₂, 0.4% [wt/vol] glucose), about 10⁶ spheroplasts, and 0.08 μM DiSC₂(5). The fluorescence emitted by DiSC₂(5) was measured at 670 nm with excitation at 650 nm by using an SLM Aminco SPF-500C fluorimeter. To dissipate the $\Delta\psi$, valinomycin (final concentration, 2 μM) was added to the reaction mixture. The $\Delta\psi$ was determined by determining the difference between the fluorescence quenching of DiSC₂(5) when it accumulated in the cytoplasmic membrane and the fluorescence of DiSC₂(5) when it was released upon addition of valinomycin.

RESULTS

Growth of *P. putida tol-oprL* mutants on different carbon sources. The role of the proteins of the Tol-OprL system in maintaining the integrity of the outer membrane is well established. However, the involvement of these proteins in other cellular processes is less clear. We observed that the turbidity of overnight cultures of *P. putida tol-oprL* mutants grown on M9 minimal medium with glucose was significantly lower (OD₆₆₀, ~0.5) than that of the wild-type strain (OD₆₆₀, >3) (Table 2). This prompted us to analyze the growth of *P. putida* KT2440 and the different *tol-oprL* mutant strains in M9 minimal medium with different carbon sources, including arginine (10 mM), benzoate (10 mM), fructose (10 mM), glucose (10 mM), glycerol (20 mM), proline (10 mM), succinate (10 mM), and sucrose (10 mM). All *P. putida tol-oprL* mutants grew as well as the wild-type strain in minimal medium with benzoate

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference(s) or source
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR1 recA1 endA1 gyrA96 thi1 relA1</i>	44
HB101	<i>supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	44
1292	<i>supE hsdS met gal lac Y fhuA</i>	4, 6, 23
JC7782	1292 <i>tolA</i> (stop after codon 40)	4
JC7752	1292 <i>tolB</i> (stop after codon 363) Δ <i>pal</i>	6
JC3417	1292 <i>tolB</i> (stop after codon 329)	4, 23
JC8031	1292 Δ <i>tolRA</i>	12
GM1	<i>ara</i> Δ (<i>lac-pro</i>) <i>thi/F'</i> [<i>lac-pro</i>]	48
TPS13	GM1 <i>tolQ</i> (stop after codon 36)	48, 49
TPS300	GM1 <i>tolR::Cm^r</i>	49
<i>P. aeruginosa</i> strains		
H103	Wild-type PAO1	20
H636	H103 <i>oprF::ΩSm^r</i>	55
H729	H103 <i>oprD::Km^r</i>	22
PAO1403	PAO1 <i>tol-1</i> , aeruginocin-tolerant mutant, requires tryptophan	21
PAO1408	PAO1 <i>tol-2</i> , aeruginocin-tolerant mutant, requires tryptophan	21
PAO1419	PAO1 <i>tol-4</i> , aeruginocin-tolerant mutant, requires methionine	21
PAO1654	PAO1, <i>tol-6</i> , aeruginocin-tolerant mutant	21
<i>P. putida</i> strains		
KT2440	<i>hsdR1</i>	32
AX	KT2440 <i>tolA::xylE</i>	32
A Ω	KT2440 <i>tolA::ΩKm</i>	32
BX	KT2440 <i>tolB::xylE</i>	32
B Ω	KT2440 <i>tolB::ΩKm</i>	43
CRR216	KT2440 <i>putP</i>	Our laboratory
DOT-OX2	KT2440 <i>oprL::xylE</i>	32
<i>oprB</i>	KT2440 with plasmid pCHESI-B inserted into the <i>oprB</i> gene (insertion after codon 112), Km ^r	This study
QX	KT2440 <i>tolQ::xylE</i>	32
Q Ω	KT2440 <i>tolQ::ΩKm</i>	32
RX	KT2440 <i>tolR::xylE</i>	32
R Ω	KT2440 <i>tolR::ΩKm</i>	32
Plasmids		
pBBR1MCS-5	Gm ^r , <i>oriT</i> RK2	26
pBBRPutP	pBBR1MCS-5 carrying a 1.5-kb chromosomal fragment containing the <i>P. putida</i> KT2440 <i>putP</i> gene obtained by PCR as an <i>Hind</i> III- <i>Xba</i> I fragment	This study
pCHESI Ω km	Ap ^r Km ^r , pUN ϕ 18 with the <i>Hind</i> III insert from pHP45 Ω km (Ω -Km interposon) at the <i>Hind</i> III site, <i>oriT</i> RP4	Rodríguez-Herva and Marqués ^b
pCHESI-B	pCHESI Ω km carrying, at the <i>Eco</i> RI site, a 338-bp chromosomal fragment from the <i>P. putida</i> KT2440 <i>oprB</i> gene obtained by PCR as an <i>Eco</i> RI- <i>Eco</i> RI fragment	This study
pHP45 Ω -Km	Ap ^r Km ^r , <i>ori</i> ColE1, source of the Ω -Km interposon	15
pRK600	Cm ^r , helper plasmid, <i>ori</i> ColE1 <i>mobRK2 traRK2</i>	32
pUN ϕ 18	Ap ^r , pUC18 with <i>oriT</i> RP4 to allow replication in <i>Pseudomonas</i>	35

^a Ap^r, Cm^r, Gm^r, Km^r, and Sm^r, resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, and streptomycin, respectively.

^b J. J. Rodríguez-Herva and S. Marqués, unpublished data.

(Table 2); however, the turbidities of *tol* mutant cultures were significantly lower with the other carbon sources. In particular, growth of the *tol* mutants was negligible with arginine, fructose, and glycerol (Table 2). When proline was used as the sole carbon source, we observed that any polar mutation in the *tolQRAB* cluster (strains Q Ω , R Ω , A Ω , and B Ω) impeded or seriously limited growth (Table 2). In contrast, mutant strains with nonpolar mutations in the *tolQ* (strain QX), *tolR* (strain RX), and *tolA* (strain AX) genes grew relatively well (the lack of a polar effect in *xylE* was confirmed by reverse transcription-PCR assays performed with the appropriate primers [data not shown]), although inactivation of *tolB*, the last gene in the cluster, produced a mutant which showed limited growth on proline. Given that the *P. putida tolQRAB* genes form an operon (33), insertion of the Ω -Km interposon into *tolQ*, *tolR*,

or *tolA* seems to exert a polar effect on expression of the *tolB* gene. The inability of these polar mutants and of the BX mutant, in which the *tolB* gene is inactivated by an *xylE* insertion, to grow on M9 minimal medium with proline seems to be mainly due to the lack of the TolB protein in the cell envelope. In marked contrast, growth on succinate was compromised in all mutants except those that lacked the *tolB* gene product (the BX and B Ω mutants), and with sucrose as the sole carbon source, growth of all mutants was slower than growth of the parental strain.

Growth limitation of the *tol* mutants of *P. putida* is not due to defects in the synthesis of porins. The Tol-Pal protein complex of *E. coli* has been proposed to be involved in various steps in the biogenesis of porins and in their assembly in the outer membrane (27, 34). Thus, the growth deficiencies in the *P.*

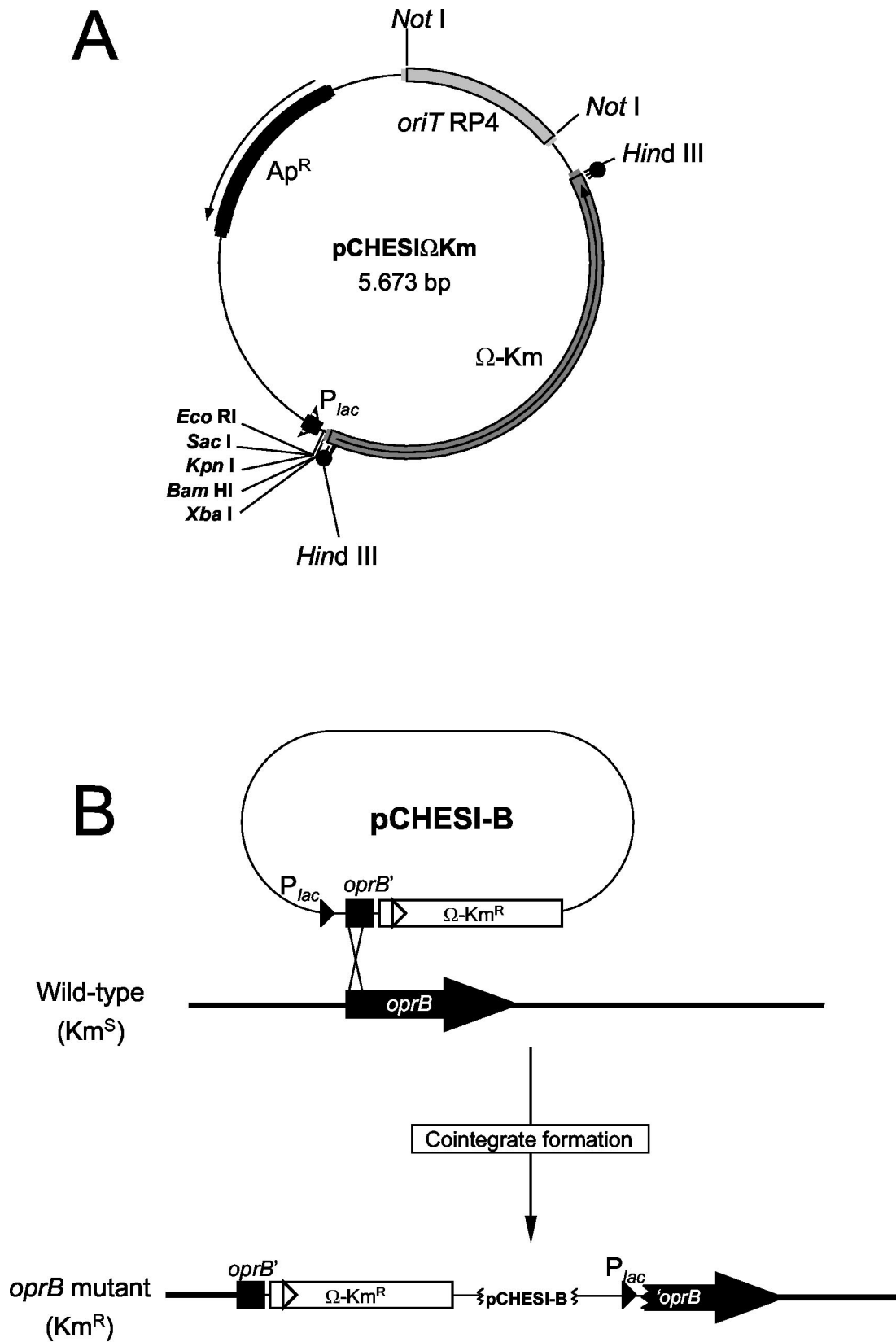


FIG. 2. Strategy used to construct an *oprB* mutant of *P. putida*. (A) Physical map of pCHESIΩKm. Single restriction sites are indicated by boldface type. (B) Physical map of pCHESI-B and cointegration into the host chromosome to obtain an *oprB* mutant. Details of the cloning and cointegration procedures are described in the text.

TABLE 2. Growth of the *P. putida tol-oprL* mutants with different carbon sources

Carbon source	Growth of <i>P. putida</i> strains ^a									
	KT2440	QΩ	RΩ	AΩ	BΩ	QX	RX	AX	BX	DOT-OX2
Arginine	++	-	-	-	-	-	-	-	-	-
Benzoate	++	++	++	++	++	++	++	++	++	++
Fructose	+++	-	-	-	-	-	-	-	-	-
Glucose	+++	+	+	+	+	+	++	++	+	++
Glycerol	+++	-	-	-	-	-	-	-	-	-
Proline	+++	-	-	+	+	++	++	++	+	++
Succinate	++	+	-	+	++	+	+	+	++	+
Sucrose	++	+	-	+	+	-	-	+	+	+

^a Culture cell density was determined after 23 h of incubation at 30°C in M9 minimal medium supplemented with the different carbon sources. +++, OD₆₆₀ ≥ 2; ++, 0.7 ≤ OD₆₆₀ < 2; +, 0.2 ≤ OD₆₆₀ < 0.7; -, OD₆₆₀ < 0.2. The initial OD₆₆₀ of the cultures were 0.06 to 0.1.

putida tol-oprL mutants may be due to defects in the assembly or synthesis of porins involved in the entry of the substrates tested into the periplasm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that the pattern of outer membrane proteins in the *P. putida tol-oprL* mutants was similar to that in the wild-type strain (Fig. 3A)

In *P. aeruginosa*, OprD has been shown to facilitate the diffusion into the periplasm of arginine and small peptides containing basic amino acids (50). The OprB porin was shown to contain a binding site for glucose and other compounds, such as glycerol and fructose (1, 56). We analyzed the presence of these two outer membrane porins in *P. putida tol-oprL* strains by Western blotting. Cells were grown in BM2 minimal medium with glucose to induce OprB expression (45) or in LB

medium to detect OprD. The amounts of OprD and OprB in the outer membranes of all mutant strains were similar to the amounts in the wild-type strain (data not shown).

We examined growth of the wild-type *P. putida* strain and the *oprB* mutant with glucose, benzoate, glycerol, or fructose as the sole carbon source, and we found that the doubling times in the exponential phase of the mutant strain with these carbon sources were similar to those of the wild-type strain with the same carbon sources (data not shown). Together, these results suggest that the poor growth of the *P. putida tol-oprL* mutants on certain carbon sources is not the result of defective assembly of the porins for these compounds in the outer membrane.

Uptake of glucose, glycerol, and proline in intact cells and spheroplasts. To determine whether transport of glucose, glyc-

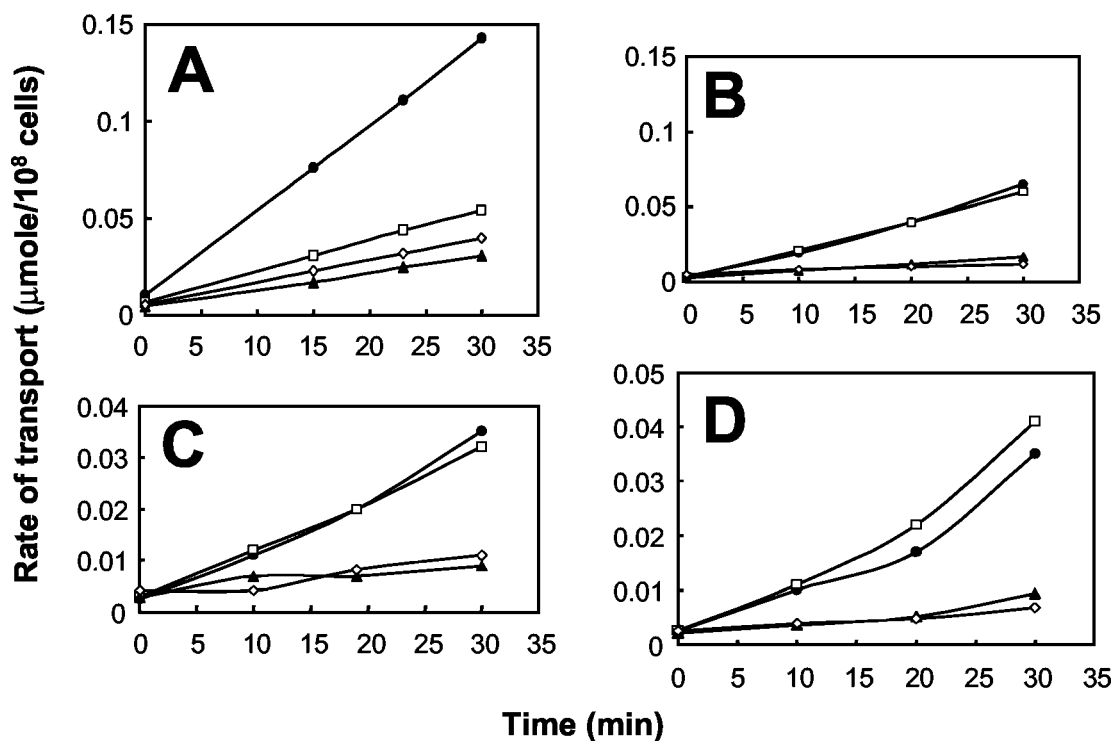


FIG. 3. Uptake of different carbon sources by *P. putida* KT2440 and its isogenic mutants. Uptake of glucose (A and B) and glycerol transport (C and D) by whole cells (A and C) or spheroplasts (B and D) of the *P. putida* wild-type strain (●) and *P. putida* QX (*tolQ* mutant) (◇), BX (*tolB* mutant) (▲), and *oprB* (□) mutant strains. Cells were grown in minimal glucose medium until the exponential growth phase was reached, and uptake was analyzed as described in Materials and Methods.

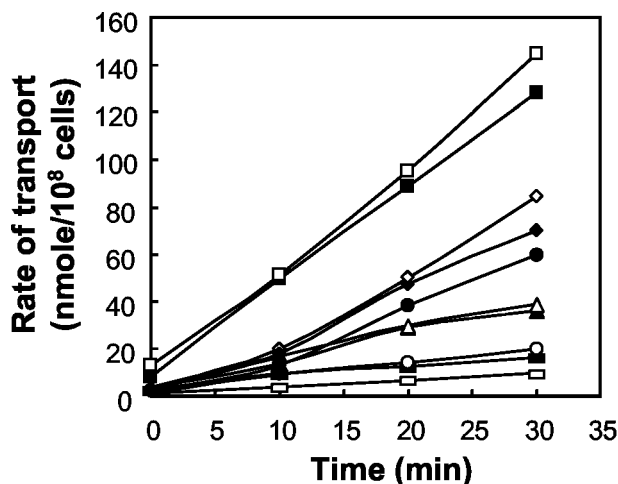


FIG. 4. Uptake of proline by whole cells of different strains of *P. putida*. The strains used were *P. putida* KT2440 (squares), *P. putida* CRR216 (*putP* mutant) (circles), *P. putida* BX (*tolB* mutant) (triangles), *P. putida* QX (*tolQ* mutant) (diamonds), and *P. putida* QΩ (*tolQ* mutant) (rectangles) bearing pBBR1MCS-5 as a negative control (open symbols) or pBBRPutP (solid symbols). Cells were grown in LB medium until an OD₆₆₀ of ~0.5 was reached, washed once, resuspended in M9 minimal medium, and incubated for 3 h. Uptake was measured as described in Materials and Methods.

erol, and proline through the cell envelope was affected in the *P. putida tol-oprL* mutants, we used radiolabeled substrates to analyze the uptake of these compounds by intact cells. For the glucose and glycerol uptake assays, cells were grown on M9 minimal medium with glucose, whereas for the proline uptake assays, cells were grown on LB medium until an OD₆₆₀ of ~0.5 was reached, washed once, and incubated in minimal medium with proline for 3 h. The rates of glucose, glycerol, and proline uptake across the cell envelope in the *tolQ* and *tolB* mutants were between 5- and 10-fold lower than the rates of uptake in the wild-type strain (Fig. 3A and C and 4). In contrast, the loss of OprB resulted in a decrease in glucose transport across the cell envelope (Fig. 3A) but not in a decrease in glycerol transport (Fig. 3C).

Since the amounts of outer membrane proteins appeared to be unaffected in the *tol-oprL* mutants, the reduced uptake of various carbon sources might have been due to a defect in transport across the cytoplasmic membrane. To test this possibility, the uptake of glucose and glycerol was measured in spheroplasts, in which the outer membrane barrier had been lost. The rates of uptake of these compounds by spheroplasts of the wild-type strain and the *oprB* mutants were similar (Fig. 3B and D). This result was expected, since the transport defect of the *oprB* mutant was related to outer membrane transport. The incorporation rates in these spheroplasts were between 10- and 20-fold higher than the rates determined for spheroplasts of the *tolQ* and *tolB* mutants (Fig. 3B and D). These results therefore suggest that in the *tol* mutants, glucose and glycerol uptake is affected at the level of cytoplasmic membrane transport.

Proline transport in cells that overproduce the PutP cytoplasmic membrane protein. The reduced uptake of certain carbon sources across the cytoplasmic membrane of the *P.*

putida tol-oprL mutants might have been a result of the absence of or reduced amounts of specific transport proteins in the cytoplasmic membrane. To test this hypothesis, we investigated whether the *tol* mutations could be complemented by overproduction of these transporters from a constitutive promoter. The uptake of proline in *P. putida* is mediated by the cytoplasmic membrane-located PutP protein, which functions as a Na⁺ symporter. The wild-type *putP* gene of *P. putida* KT2440 (51) was subcloned into plasmid pBBR1MCS-5 to obtain pBBRPutP. In this plasmid, the *putP* gene is expressed from the P_{lac} promoter, which is constitutively expressed in *Pseudomonas* sp.; thus, all cells contain PutP. The rate of proline uptake was analyzed in *P. putida* KT2440 and in QX (*tolQ* mutant), QΩ (*tolQ* mutant), BX (*tolB* mutant), and *putP* mutant strains bearing or not bearing pBBRPutP. Figure 4 shows that overproduction of the PutP symporter partially restored the ability of the *P. putida putP* mutant strain to transport proline, which shows that the *putP* gene was functionally expressed. However, plasmid pBBRPutP did not enhance the uptake of proline in the QX, QΩ, and BX mutant strains. The rates of transport of proline in the *P. putida* BX strain, which had a nonpolar mutation in the *tolB* gene, and the *P. putida* QΩ strain, which had a polar mutation in the *tolQ* gene, were lower than the rate of transport of proline in the *P. putida* QX strain, which had a nonpolar mutation in the *tolQ* gene (Fig. 4). This finding is in agreement with the fact that the *P. putida tolQRA* polar mutants, as well as the *P. putida* BX and BΩ mutants, were unable to grow with proline as the carbon source, whereas leaky growth of the *P. putida tolQRA* nonpolar mutants was observed.

Determination of ΔΨ in *P. putida tol-oprL* mutant strains. The reduced activities of certain cytoplasmic membrane transporters in the *P. putida tol-oprL* mutants might have been due to a reduction in the proton motive force. To test this possibility, we measured ΔΨ in the *P. putida tol-oprL* mutant strains using the fluorochrome DiSC₂(5). Cells were grown on LB medium, and generation of the proton motive force in spheroplasts was monitored by monitoring the fluorescence quenching of DiSC₂(5). The proton motive forces in the *P. putida* AX (*tolA* mutant), BX (*tolB* mutant), and DOT-OX2 (*oprL* mutant) strains were similar (about 93.7, 83.4, and 87.5%, respectively) to the proton motive force produced by the wild-type strain (Fig. 5). Although the ΔΨ was found to be lower in the *P. putida* QX (*tolQ* mutant) and RX (*tolR* mutant) strains, it was not completely dissipated (Fig. 5), and therefore, it seems highly unlikely that the reduced uptake of various carbon sources in the *tol* mutants could have been a result of the reduced proton motive force.

Growth of *P. aeruginosa* and *E. coli tol-oprL (tol-pal)* mutants with different carbon sources. The reduced uptake of various carbon sources observed in the *P. putida* mutants represents a new phenotype for *tol-oprL* mutations. Previously, a number of *P. aeruginosa* and *E. coli* mutants with mutations in the *tol-pal (tol-oprL)* system were isolated (Table 1), but their growth on various carbon sources was not investigated systematically. Therefore, we decided to determine whether these mutants exhibited limited growth with different carbon sources. To do this, growth of the wild-type and *tol* mutant strains was assayed in M9 minimal medium supplemented with arginine (10 mM), fructose (10 mM), glycerol (20 mM), glucose (10 mM), and

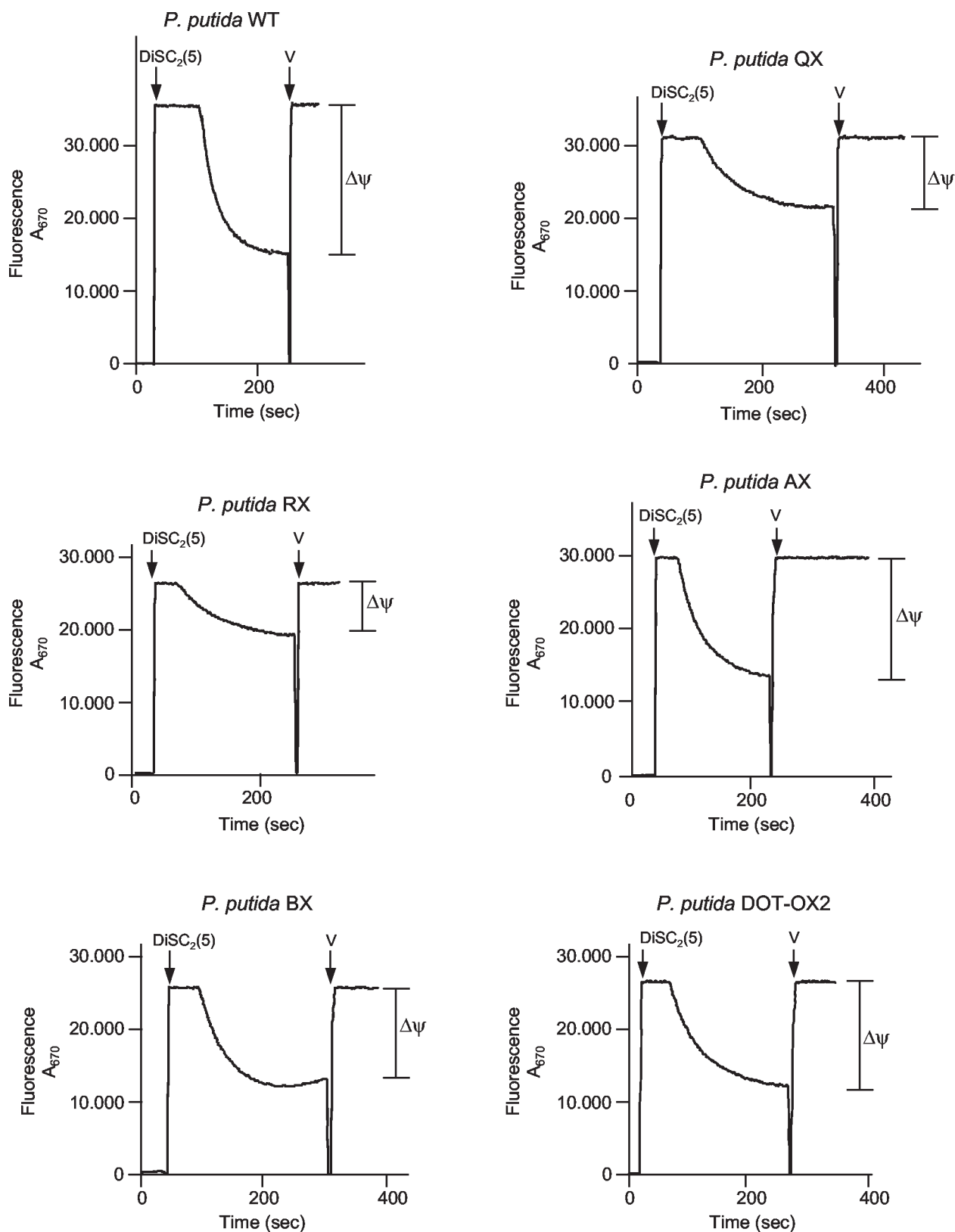


FIG. 5. Determination of $\Delta\psi$ in *P. putida* KT2440 (WT) and *tol::xylE* mutant strains (*P. putida* QX, RX, AX, BX, and DOT-OX2). Cells were grown in LB medium, harvested in the exponential phase of growth (OD_{660} , ~ 0.4), and converted to spheroplasts as described in Materials and Methods. Generation of the proton motive force by spheroplasts was monitored by the fluorescence quenching of DiSC₂(5) as described in Materials and Methods. The arrows indicate the times when the fluorescence probe and valinomycin (V) were added.

TABLE 3. Growth of the *P. aeruginosa tol* mutants with different carbon sources

Carbon source	Growth of <i>P. aeruginosa</i> strains				
	H103	<i>tol-1</i>	<i>tol-2</i>	<i>tol-4</i>	<i>tol-6</i>
Arginine	++	-	-	+	+
Glucose	+++	-	+	+	++
Glycerol	++	+	-	+	++
Proline	++	-	+	++	++
Succinate	++	-	+	++	++

^aCulture cell density was determined after 20 h of incubation at 37°C in M9 minimal medium supplemented with different carbon sources. +++, OD₆₆₀ ≥ 2; ++, 0.7 ≤ OD₆₆₀ < 2; +, 0.2 ≤ OD₆₆₀ < 0.7; -, OD₆₆₀ < 0.2. The initial OD₆₆₀ of the cultures were 0.06 to 0.1.

proline (10 mM) in the case of *P. aeruginosa* and with glucose (10 mM), glycerol (20 mM), and maltose (10 mM) in the case of *E. coli*. *P. aeruginosa tol-1* and *tol-2* mutant strains were not able to grow or grew poorly with arginine, glycerol, proline, or succinate (Table 3). Growth of the *P. aeruginosa tol-4* mutant was compromised with arginine, glucose, and glycerol and was less notably compromised with proline and succinate (Table 3). Growth of the *P. aeruginosa tol-6* mutant was affected only with arginine and glucose as the carbon sources (Table 3).

For *E. coli* we used two parental strains, GM1 and 1292, and their corresponding isogenic mutants. Growth of GM1 and growth of the isogenic *tolQ* TPS13 and *tolR* TPS300 mutants were similar in glucose and glycerol; however, in maltose growth of the *E. coli* mutant strains was seriously compromised (Table 4). Growth of a *tolB* mutant derivative of *E. coli* 1292 (i.e., strain JC3417) in glucose was similar to growth of the wild-type strain, but its growth was compromised in glycerol and maltose (Table 4). *E. coli* JC7782 ($\Delta tolA$), JC8031 ($\Delta tolRA$), and JC7752 ($\Delta tolBpal$) were not able to grow or grew very poorly with glucose, glycerol, or maltose as the sole carbon source. Hence, reduced uptake of certain carbon sources, first observed for the *P. putida tol-oprL* mutants, appears to be a characteristic of many, although not all, *P. aeruginosa* and *E. coli tol-oprL* (*tol-pal*) mutants.

DISCUSSION

The results presented here demonstrate that mutations in the *tol-oprL* (*tol-pal*) genes of *P. putida*, *P. aeruginosa*, and *E. coli* lead to organisms that are defective in the utilization of a number of carbon sources and support the hypothesis that the Tol system of *P. putida* influences the transport of certain carbon sources across the cytoplasmic membrane, although the molecular mechanisms behind this phenotype(s) is still unknown. These results are surprising because functions previ-

ously associated with the Tol-Pal (Tol-OprL) system were all related to the correct biogenesis of the outer membrane, the assembly of porins, and transfer of lipopolysaccharide.

Lazzaroni et al. (28) found that the level of expression of *E. coli* OmpF and LamB outer membrane porins was lower in an *E. coli tolA* mutant and suggested that the TolA protein exerted positive indirect control at the transcriptional level over the *ompF* and *lamB* genes. In contrast, our immunodetection analysis of *P. putida* demonstrated that the levels of the OprD, OprB, and OprF porins of the outer membrane in the *tol-oprL* mutants were similar to the levels in the wild-type strain. Furthermore, immunofluorescence assays showed that at least the OprF protein seemed to be correctly exposed on the surface of the outer membrane of the *P. putida tol-oprL* mutants (data not shown). This is in agreement with the fact that in the *E. coli tol-pal* mutants, the outer membrane porins are correctly processed and assembled in the outer membrane (11, 27). These results suggest that the *P. putida* Tol-OprL system is not involved in the export or assembly of the porins in the outer membrane in this microorganism. However, we cannot rule out the possibility that some components of the outer membrane are not functional in the *tol-oprL* mutants, since the stability of the membrane is seriously affected in these strains (32).

It was surprising to find that the carbon sources that are not taken up by the *P. putida tol-pal* mutants are transported by transport systems that are very different. Thus, glycerol uptake involves facilitated diffusion down a concentration gradient (53, 54), whereas the uptake of proline is mediated by the PutP protein, which is an energy-dependent Na⁺ symporter (8, 51). For the transport of glucose there are two separate inducible systems in *P. aeruginosa* (36); one is a low-affinity transport system which involves the extracellular oxidation of glucose to gluconate or to 2-ketogluconate prior to transport into the cytoplasm, and the second is a high-affinity system which transports glucose directly into the cytoplasm via a periplasmic binding protein-dependent ABC transport system that requires ATP as an energy source (2). The existence in *P. putida* of a periplasmic glucose binding protein is suspected because (i) the rate of glucose transport across the spheroplasts was lower than the rate of glucose transport across whole cells of the wild-type strain (Fig. 3A and B) and (ii) a polyclonal antibody against the *P. aeruginosa* glucose binding protein cross-reacted with a protein of *P. putida* (data not shown). We identified in the *P. putida* KT2440 genome a gene similar to the *P. aeruginosa gltK* gene (1) which encodes one of the cytoplasmic membrane components of the glucose ABC transport system. In *P. aeruginosa*, the uptake of arginine is also mediated by a periplasmic binding protein-dependent ABC transport system (39). Although the transport of glucose and arginine might

TABLE 4. Growth of the *E. coli tol-pal* mutants with different carbon sources

Carbon source	Growth of <i>E. coli</i> strains ^a							
	GM1	TPS13 (<i>tolQ</i>)	TPS300 (<i>tolR</i>)	1292	JC3417 (<i>tolB</i>)	JC7752 ($\Delta tolBpal$)	JC7782 (<i>tolA</i>)	JC8031 ($\Delta tolRA$)
Glucose	++	++	++	++	++	+	+	+
Glycerol	++	++	++	++	+	-	-	-
Maltose	+++	+	+	+++	-	-	-	-

^a Culture cell density was determined after 20 h of incubation at 37°C in M9 minimal medium supplemented with the different carbon sources. +++, OD₆₆₀ ≥ 2; ++, 0.7 ≤ OD₆₆₀ < 2; +, 0.2 ≤ OD₆₆₀ < 0.7; -, OD₆₆₀ < 0.2. The initial OD₆₆₀ of the cultures were 0.06 to 0.1.

have been influenced by the generation of a proton motive force, the *tol-pal* mutants of *P. putida* were not particularly defective in the generation of a proton motive force, which eliminated the possibility that the reduced transport rates in the *tol-oprL* mutants were due to a low proton motive force. Consistently, the transport of glycerol, which was taken up through a facilitated diffusion process, was also affected in the *P. putida tol-oprL* mutants. In *E. coli*, the transport and catabolism of glycerol are mediated by the components of the *glp* regulon. The *glpFK* operon encodes a membrane diffusion facilitator for glycerol and a cytoplasmic glycerol kinase (53). In *P. aeruginosa*, glycerol was also found to be transported by a facilitated diffusion system (54) that involved a GlpFK system which was 80% identical to the *E. coli* glycerol diffusion facilitator and to cytoplasmic glycerol kinase (46, 47). Our results with *P. putida* suggest that glycerol transport is not associated with a periplasmic binding protein, since the rates of glycerol transport were similar in whole cells and spheroplasts (Fig. 3C and D). Furthermore, the *P. putida* KT2440 genome contains the *glpFK* operon, and the proteins exhibit strong homology to the *E. coli* and *P. aeruginosa* GlpF and GlpK proteins. The data available for *P. aeruginosa* and *P. putida* are consistent with transport of glycerol by a facilitated diffusion system, which does not require energy. The rate of glycerol uptake by the *P. putida tol-oprL* mutants is as low as the rate of glycerol uptake by a mutant lacking the *glpFK* genes that encode the transport system (46). This reinforces the hypothesis that the Tol system influences glycerol uptake at the cytoplasmic membrane level.

Why are *tol-oprL* mutants defective in the uptake of certain carbon sources? One possibility is that the Tol-OprL system can directly or indirectly influence the correct insertion or functioning of certain transport systems in the cytoplasmic membrane. To our knowledge, this is the first time that such a role has been attributed to the Tol-Pal (Tol-OprL) system, although reduced utilization of some carbon sources has been reported previously for *E. coli tol-pal* mutants. This phenotype in *E. coli* was ascribed to loss of the periplasmic binding proteins of the corresponding transporters (3, 29). However, in this work we demonstrated that this hypothesis is not correct since the *P. putida tol-oprL* mutants are affected in transport at the level of the cytoplasmic membrane. Although the *P. putida tol-oprL* mutants release periplasmic proteins into the extracellular medium (32), the amounts of periplasmic glucose binding protein that remain in the periplasm of the mutants are similar to the amount found in the wild-type strain (data not shown). Moreover, not only the transport systems that do not depend on a periplasmic binding protein are affected in these mutants, as described above.

In *P. putida* the polar mutations in *tolQRAB* had more dramatic effects on the range and levels of utilization of different carbon sources than individual knockouts, as assessed by culture turbidity (Table 2). In this regard, the *tol-1* and *tol-2* mutants of *P. aeruginosa* exhibited very limited or no growth with several carbon sources, whereas utilization of carbon sources was less affected in the *tol-4* and *tol-6* mutants. It would therefore be interesting to determine the specific nature of the mutations in these *P. aeruginosa tol* mutants to establish a closer link with the mutations in *P. putida*. Our results with *P. putida* suggest that while TolB is the most critical protein affecting proline transport, this protein is not essential for

growth on succinate, which is affected more in mutants lacking the other components of the Tol system. On the other hand, in *E. coli*, growth defects were more evident with maltose than with any other sugar, and double mutants showed greater impairment than single mutants. The growth defects with maltose could be partially due to the known leakiness of the outer membrane and the loss of MalE from the periplasm, although it should be noted that not only periplasmic binding protein-dependent transport systems are affected in the *E. coli tol-pal* mutants since these mutants are also unable to grow in glycerol. The defects in growth of these mutants seem to be similar to that of *P. putida*, for which a cytoplasmic membrane transport defect has been demonstrated. The weak phenotype of the *tolQ* and *tolR* mutants of *E. coli* could be explained by the presence of the ExbB and ExbD proteins, which might take over the functions of TolQ and TolR to some extent (27).

In summary, the results presented here suggest a new physiological role for the Tol-Pal (Tol-OprL) system, namely, an effect on the uptake of a number of carbon sources at the level of the cytoplasmic membrane.

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