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Mutations in Genes Involved in the Flagellar Export Apparatus of the Solvent-Tolerant *Pseudomonas putida* DOT-T1E Strain Impair Motility and Lead to Hypersensitivity to Toluene Shocks

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Pseudomonas putida **DOT-T1E is a solvent-tolerant strain able to grow in the presence of 1% (vol/vol) toluene in the culture medium. Random mutagenesis with mini-Tn*5-′phoA*-Km allowed us to isolate a mutant strain (DOT-T1E-42) that formed blue colonies on Luria-Bertani medium supplemented with 5-bromo-4-chloro-3-indolylphosphate and that, in contrast to the wild-type strain, was unable to tolerate toluene shocks (0.3%, vol/vol). The mutant strain exhibited patterns of tolerance or sensitivity to a number of antibiotics, detergents, and chelating agents similar to those of the wild-type strain. The mutation in this strain therefore seemed to specifically affect toluene tolerance. Cloning and sequencing of the mutation revealed that the mini-Tn*5-′phoA*-Km was inserted within the *fliP* gene, which is part of the *fliLMNOPQRflhBA* cluster, a set of genes that encode flagellar structure components. FliP is involved in the export of flagellar proteins, and in fact, the *P. putida fliP* mutant was nonmotile. The finding that, after replacing the mutant allele with the wild-type one, the strain recovered the wild-type pattern of toluene tolerance and motility unequivocally assigned FliP a function in solvent resistance. An *flhB* knockout mutant, another gene component of the flagellar export apparatus, was also nonmotile and hypersensitive to toluene. In contrast, a nonpolar mutation at the *fliL* gene, which encodes a cytoplasmic membrane protein associated with the flagellar basal body, yielded a nonmotile yet toluene-resistant strain. The results are discussed regarding a possible role of the flagellar export apparatus in the transport of one or more proteins necessary for toluene tolerance in *P. putida* DOT-T1E to the periplasm.**

Organic solvents are extremely toxic for living organisms because they partition in the cell membranes and disorganize them by removing lipids and proteins, which eventually leads to cell death (6, 46). Following Inoue and Horikoshi's report (15) on the isolation of a *Pseudomonas* sp. strain able to grow on liquid medium with up to 50% (vol/vol) toluene, a number of *Pseudomonas* sp. strains have been isolated as able to grow in the presence of highly toxic organic solvents such as toluene (partition coefficient in an octanol-water mixture [$\log P_{ow}$] = 2.5), styrene ($\log P_{ow}$ = 2.9), and xylenes ($\log P_{ow}$ = 3.4) (5–7, 42, 48). One of these isolates, named *Pseudomonas putida* DOT-T1E, has been shown to be not only toluene tolerant and able to grow in liquid culture medium in the presence of a second phase of these aromatic hydrocarbons, but also capable of using toluene, ethylbenzene, and other compounds as the sole C source.

The mechanisms underlying solvent tolerance are not fully understood yet, although a number of factors are claimed to be involved in this process. Several laboratories have recently shown that efflux pumps of the resistance-nodulation-cell division family are involved in the removal of toluene and other toxic compounds from the cell membranes (16, 21, 24, 34, 40, 41). In *P. putida* DOT-T1E, the *ttgABC, ttgDEF*, and *ttgGHI* operons encode efflux pumps that have been found to be involved in toluene tolerance: *ttgB, ttgD*, and *ttgG* mutants exhibited increased solvent sensitivity compared to the parental strain (34, 41, 42a). The *ttgB* and the *ttgH* mutants, but not the *ttgD* mutant, also showed increased sensitivity to antibiotics in comparison with the parental strain, suggesting that the TtgABC and TtgGHI pumps may exhibit a wider substrate specificity than the TtgDEF one. The TtgABC, TtgDEF, and TtgGHI pumps showed a high degree of similarity to the antibiotic efflux pumps MexAB/OprM, MexCD/OprJ, and MexEF/OprN of *Pseudomonas aeruginosa* PAO1 (25, 37, 38) and the AcrAB/TolC pump of *Escherichia coli* (1, 10, 28, 49). Although all these pumps were known to expel antibiotics, it has recently been shown that they are also able to remove organic solvents, although neither *P. aeruginosa* nor *E. coli* is able to withstand a second phase of toluene or ethylbenzene in liquid medium (1, 27, 49).

The finding that microbes such as *P. aeruginosa* with operational pumps are toluene sensitive suggests either that other as yet unidentified elements are involved in toluene tolerance or that the expression level and regulation of the pumps—a relatively unexplored research area—are also of importance for toluene tolerance in *Pseudomonas* spp. (22, 34). Other elements that have been proposed to be involved in solvent tolerance are the *cis→trans* isomerization of lipids and surface lipopolysaccharides (LPS). Our group and others have found a positive correlation between the degree of *trans* isomers of the C16:1,9 and C18:1,9 fatty acids and bacterial growth in the presence of toluene and other aromatic hydrocarbons (7, 13, 14, 18, 36, 40). Indeed, *P. putida* DOT-T1E cells growing in the absence of toluene exhibited a high proportion of *cis* isomers

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TABLE 1. Strains used in this study

Strain	Relevant characteristics	Source or reference
P. putida		
DOT-T1E	Rif^r Tol^r	41
DOT-T1E-42	Rif^r Tol^s Km^r *fliP*::Km	This study
DOT-T1E-PS23	Rif^r Tol^r *fliL*::Km	This study
DOT-T1E-PS50	Rif^r Tol^s *flhB*::Km	This study
DOT-T1E-PS21	Rif^r Tol^r	This study
E. coli		
CC118λ*pir*	Rif^r, host strain to replicate plasmids bearing the R6K origin of replication	40
HB101	Sm^r, host for plasmid pRK600	40
JM109	*recA*, used in cloning experiments	2

of fatty acids (*cis*/*trans* ≅ 7.5), whereas in the presence of toluene, the *cis* and *trans* isomers were equally abundant (*cis*/*trans* ≅ 1). The *cti* gene encoding the *P. putida cis,trans*-isomerase has been cloned (14, 18), and a *cti* knockout mutant of *P. putida* DOT-T1E was isolated and characterized (18). Growth of this mutant was delayed in the presence of organic solvents (18). It has also been suggested that LPS are a barrier to the entry of aromatic compounds through the cell membrane (36). We have generated LPS mutants of the solvent-tolerant *P. putida* DOT-T1E strain and found that LPS is not critical for solvent tolerance (19).

To further elucidate the process of solvent tolerance, we mutagenized *P. putida* DOT-T1E with a mini-Tn*5*-*'phoA*. Among the mutants, we looked for toluene-sensitive ones that conserved the wild-type pattern of lipids and resistance or sensitivity to antibiotics under different growth conditions. A mutant was found that exhibited a transposon insertion at the *fliP* gene, whose gene product is involved in flagellar assembly; as a consequence of this insertion, the cells were nonmotile. This unexpected finding led us to generate mutations in genes whose products are involved in flagellum biosynthesis. An *flhB* knockout mutant showed hypersensitivity to toluene and was also nonmotile. In contrast, the nonmotile mutant lacking the FliL protein, associated with the flagellar basal body, was toluene tolerant. These results indicated that the proteins of the flagellar export apparatus are neccesary for toluene tolerance.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture medium, and growth conditions. The bacterial strains used in this study are shown in Table 1. Plasmid pUT-*'phoA*-Km has the R6K origin of replication and encodes resistance to ampicillin and kanamycin. The latter marker together with *'phoA* is part of the mini-Tn*5* borne on this plasmid (41). Plasmid pRK600 was used as a helper; it encodes resistance to chloramphenicol and provides the *tra* functions for the mobilization of the pUT plasmid (41). For site-directed mutagenesis of the chromosomal *fliL* and *flhB* genes, plasmid pUNϕ18, bearing a knockout *fliL*::Ω-Km gene, and plasmid pUC18, bearing a knockout *flhB*::Ω-Km, were used.

Bacterial strains were routinely grown on liquid Luria-Bertani (LB) medium (2). Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per min. Growth was usually determined as CFU on LB solid medium supplemented with appropriate antibiotics. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml for *E. coli* and 300 μg/ml for *P. putida*; carbenicillin (Cb), 300 μg/ml; chloramphenicol (Cm), 90 μg/ml; kanamycin (Km), 50 μg/ml; piperacillin (Pi), 100 μg/ml; rifampin (Rif), 20 μg/ml; and tetracycline, 15 μg/ml.

Isolation of toluene-sensitive Tn*5*-*'phoA* mutants of *P. putida* DOT-T1E. About 5,000 mini-Tn*5* transconjugants of *P. putida* DOT-T1E were obtained after triparental mating of the strain with *E. coli* CC118λ*pir*(pUT-*'phoA*-Km) and *E. coli* HB101(pRK600). About 10% of the Km-resistant clones appeared as blue colonies in plates supplemented with 5-bromo-4-chloro-3-indolylphosphate (BCIP). Each individual Km-resistant blue transconjugant was tested for its ability to grow on LB medium supplemented with either 1% (vol/vol) toluene or 1% (vol/vol) octanol. One clone that failed to grow in the medium with 1% (vol/vol) toluene but that did grow in the presence of the same amount of octanol was called *P. putida* DOT-T1E-42 and was retained for further studies.

Cloning of mutation in *P. putida* DOT-T1E-42 and analysis of surrounding DNA sequence. To clone the mutation in this strain, total DNA was digested with *Sph*I and ligated to pUC18. Two Km-resistant colonies were obtained, both containing an identical plasmid carrying an *Sph*I insert of about 3 kb. The resulting plasmid (pANA1) contained 1.7 kb of the mini-Tn*5* plus 1.1 kb of chromosomal DNA. The DNA was sequenced by the dideoxy sequencing termination method and a primer located at the "O" end of the mini-Tn*5*. This made it possible to read outside the Km resistance gene and within the chromosomal insert. Based on the DNA sequence obtained, specific 20-mer primers were designed for further DNA walking. DNA was sequenced on both strands.

Rescue of wild-type *P. putida* DOT-T1E *fli* genes from a gene bank. Wild-type genes were rescued from a DOT-T1E gene bank previously generated in our laboratory (41). The 1.1-kb *Sph*I-*Not*I fragment of pANA1 was labeled with digoxigenin by standard procedures and used to screen the library. A single clone bearing a 10.5-kb *P. putida* DOT-T1E insert was found. The plasmid was called pANA9, its insert was sequenced on both strands, and the sequence was deposited in Genbank under accession number AF031418.

Generation of a *P. putida* DOT-T1E *fliL*::Ω-Km mutant. Plasmid pANA9 was cut with *Sfi*I, an enzyme that cuts within the *fliL* gene (position 4081 of the insert in sequence AF031418). The Klenow fragment and the four deoxynucleoside triphosphates (dNTPs) were used to fill in the ends and make them blunt (2). The 2-kb Ω-Km cassette of plasmid pHP45ΩKm (9) was obtained after digestion with *Eco*RI, and these ends were made blunt, as above, and ligated to the linearized pANA9 plasmid. The ligation was transformed into *E. coli* JM109, and cells were selected on LB plates supplemented with ampicillin and Km. After analysis by restriction enzymes, a clone carrying the correct plasmid, called pANA61, was selected. Electroporation was used to transfer pANA61 to *P. putida* DOT-T1E, whose *colE* origin of replication is not recognized in *P. putida* and which behaves like a suicide vector. However, because of identical sequences, pANA61 integration into the host chromosome via homologous recombination can be selected on LB solid medium with Rif, Km, and Pi. A merodiploid clone was grown overnight on LB to allow a second recombination event in which the wild-type gene was replaced by the mutant allele. For this selection, colonies were plated again on LB with Rif and Km. Among these colonies, those that did not grow in the presence of piperacillin were selected as putative resolved merodiploids. These mutants were checked again by Southern blot hybridization, cutting the chromosomal DNA with *Bam*HI and using the 1,3-kb *Pst*I fragment (positions 2901 to 4192) containing part of the *fliK* and *fliL* genes as a probe. One of the clones that exhibited the correct mutation was called *P. putida* strain DOT-T1E-PS23 and kept for further studies.

Generation of a *P. putida* DOT-T1E *flhB*::Ω-Km mutant. Plasmid pANA9 (10.3 kb) was cut with *Eco*RI, an enzyme that cuts in position 1 within the pUC18 polylinker and at position 5610 of the insert in sequence AF031418. Plasmid pANA50 (7.6 kb) is the result of the religation of the pANA9 fragment. Plasmid pANA50 contains a 4.7-kb insert encompassing the *fliNOPQRflhB* genes. pANA50 was cut with *Bpu*AI, an enzyme that cuts at the 5′ end of the *flhB* gene (position 8229 in sequence AF031418). The Klenow fragment and the four dNTPs were used to fill in the ends and make them blunt (2). The 2-kb Ω-Km cassette of plasmid pHP45ΩKm (9) was obtained and treated as above. The Ω-Km fragment was ligated to the linearized pANA50 plasmid and transformed into *E. coli* JM109. The resulting Ap^r Km^r transconjugants were analyzed by digestion with restriction enzymes, and a clone carrying the correct plasmid, called pANA72, was selected. Plasmid pANA72 was introduced into *P. putida* DOT-T1E as above, and transformants incorporating the plasmid on the chromosome were selected on LB solid medium with Rif, Km, Cb, and Pi. One of the merodiploid clones was grown on LB for at least 20 generations to allow the second recombination event to occur. These clones were expected to be able to grow on LB plus Rif and Km but not in the presence of Cb and Pi. Seven clones were found as putative resolved merodiploids. These clones were checked by Southern blot hybridization, cutting the chromosomal DNA with *Bam*HI and using a PCR probe labeled with digoxigenin, that contained 500 bp of the 3′ end of *fliR* and the first 500 bp of *flhB*. One of the clones that exhibited the correct mutation was called *P. putida* strain DOT-T1E-PS50 and kept for further studies.

RT-PCR. *P. putida* DOT-T1E cells growing exponentially on LB medium were harvested by centrifugation (5,000 × *g*, 10 min). RNA from *P. putida* DOT-T1E was isolated with the RNeasy Total RNA kit (Qiagen). This RNA was treated with RNase-free Dnase I in the presence of an RNase inhibitor (Boehringer

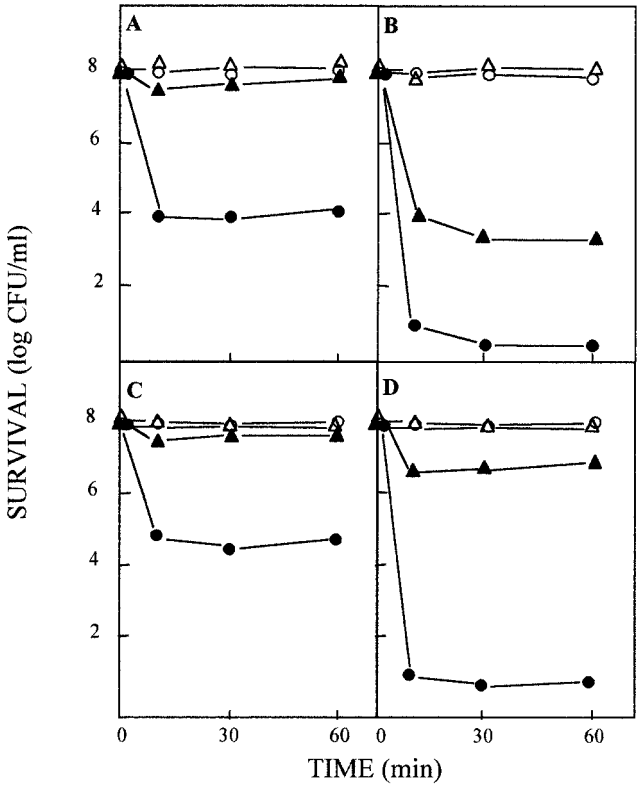


FIG. 1. Survival in response to toluene shocks of the wild-type *P. putida* DOT-T1E and a mutant derivative lacking the FliP, FliL, or FlhB protein. Cells were grown in 30 ml of LB (circles) or LB with toluene in the gas phase (triangles) until the culture reached an optical density at 660 nm of about 1. These cultures contained about 10^8 CFU/ml. The cultures were divided in two halves; to one we added 0.3% (vol/vol) toluene (solid symbols), and the other was kept as a control (open symbols). The number of viable cells was determined before toluene was added and 15, 30, and 60 min later. (A) *P. putida* DOT-T1E; (B) *P. putida* DOT-T1E-42; (C) *P. putida* DOT-T1E-PS23; (D) *P. putida* DOT-T1E-PS50.

Mannheim) to avoid DNA contamination and RNA degradation. Reverse transcription (RT)-PCR was performed with the Titan OneTube RT-PCR system according to the manufacturer's instructions (Boehringer Mannheim). The annealing temperature used in the PCR experiments was 60°C, and the cycling conditions were as follows: 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. After 30 cycles, the sample was incubated at 68°C for 10 min. Positive and negative controls were included in all assays. The approximate locations of the primers used for RT-PCR are indicated in Fig. 2A. Primer sequences are available on request.

Computer analysis. DNA primary sequences were analyzed with several programs included in the DNA Strider 1.1. package. Homology searches were performed with the BLAST database search program.

RESULTS

Tolerance of *P. putida* DOT-T1E and DOT-T1E-42 to organic solvents, detergents, chelating agents, and antibiotics. *P. putida* DOT-T1E-42 was isolated as a Km-resistant toluene-sensitive clone upon miniTn*5-'phoA* mutagenesis, although it resembled the wild-type strain in tolerance to heptane, propylbenzene, *m*-xylene, and octanol. The wild type and this mutant were able to form colonies on LB plates supplemented with chloramphenicol (90 μg/ml) or Cb (250 μg/ml). This finding contrasts with those reported previously for another toluene-sensitive derivative of DOT-T1E, called DOT-T1E-18, which did not form colonies in the presence of these high concentrations of chloramphenicol and Cb (41). This phenotypic difference clearly established that DOT-T1E-42 represented a distinct class of toluene-sensitive mutants.

P. putida DOT-T1E was shown before to exhibit differential toluene tolerance depending on the growth conditions. In fact, whereas most cells tolerated a 0.3% (vol/vol) toluene shock upon growth on LB with toluene in the gas phase, only a low proportion (0.01%) of cells tolerated this shock when they were grown in the absence of toluene in the gas phase (41), (Fig. 1A). The mutant DOT-T1E-42 strain did not tolerate sudden exposure to 0.3% (vol/vol) toluene when grown on LB, and very small numbers of cells (0.01%) survived the shock even when grown with toluene in the gas phase (Fig. 1B).

It was previously shown that *P. putida* DOT-T1E grows on LB solid medium supplemented with 1 mM EDTA, 100 mM deoxycholate, 3% (wt/vol) Triton X-100, 1.5% (wt/vol) sodium dodecyl sulfate (SDS), or 15 g of *p*-hydroxybenzoate per liter. *P. putida* DOT-T1E-42 also grew on LB plates with one of these chemicals at the indicated concentrations. These results suggest that *P. putida* DOT-T1E-42 is sensitive only to toluene.

Functionality of the *cis,trans*-isomerase and efflux pumps in *P. putida* DOT-T1E-42. Two elements are critical for solvent tolerance: *cis*→*trans* isomeration of unsaturated fatty acids, and the ability to extrude aromatic hydrocarbons (reviewed by Segura et al. [45]). The pattern of lipid composition of wild-type and DOT-T1E-42 cells grown on LB and LB plus 1% (vol/vol) heptane and toluene (supplied via the gas phase) was examined in cells growing exponentially. The results obtained were similar to those reported before for the wild-type strain (40) and suggest that DOT-T1E-42 did not exhibit any apparent damage with regard to fatty acid biosynthesis (not shown).

P. putida DOT-T1E was shown before to be able to extrude [^{14}C]toluene from the cell membrane when cells were grown on either LB or LB plus toluene supplied via the gas phase, while the solvent-sensitive strain DOT-T1E-18 showed impaired aromatic hydrocarbon extrusion (41). We tested the extrusion of [^{14}C]toluene in DOT-T1E-42 cells grown on LB that were exposed or not to toluene in the gas phase. Mutant cells extruded [^{14}C]toluene less efficiently than the wild-type cells (Table 2). However, they still extrude part of the aromatic

TABLE 2. Accumulation of [^{14}C]toluene in cells of *P. putida* DOT-T1E and its solvent-sensitive mutant DOT-T1E-42[a]

Growth conditions	Amt of ^{14}C in the cells (dpm/OD_{660})	
	Wild type	DOT-T1E-42
LB	5,300 ± 200	8,400 ± 400
LB + toluene	2,900 ± 500	5,500 ± 300

[a] *P. putida* DOT-T1E and DOT-T1E-42 cells were grown on LB medium in the absence and in the presence of toluene supplied via the gas phase. Exponentially growing cells were harvested by centrifugation, washed with 10 volumes of LB, and suspended in 1.5 ml of LB to an optical density at 660 nm (OD_{660}) of about 1. The cells were then incubated for 10 min at 30°C and exposed to 2 μCi of [^{14}C]toluene (39). After 10 min, when the equilibrium level had been achieved, 250 μl of the cell suspension was filtered through a 0.45-μm Millipore filter and washed with 2 ml of LB medium. The filters were dried, and the amount of ^{14}C associated with the cell pellet (disintegrations per minute) was determined in a Packard radiochemical detector. The data are the averages of three independent determinations.

hydrocarbon, because in cells treated with 200 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) the amount of ^{14}C accumulated in the cell membranes was three- to fivefold higher. These results indicated that in the mutant strain, functioning of the pump systems was compromised (21, 34, 40, 41, 45).

Cloning of the mutation in *P. putida* DOT-T1E-42. We cloned the mutation in DOT-T1E-42 and determined the nature of the mutated gene. The strategy is described in Materials and Methods. This approach yielded plasmid pANA1, which contained 1.7 kb of the mini-Tn*5-'phoA*-Km plus about 1.1 kb of the adjacent chromosomal DNA. Sequence analysis revealed that the *'phoA*-Km cassette was inserted within a putative open reading frame homologous to *fliP*, a protein necessary for flagellar biosynthesis described in several species (4, 8, 26, 31, 32, 33, 35). Like *fliP* mutants of enterobacteria, the *P. putida fliP* mutant was nonmotile, in contrast to the wild-type strain. *P. putida* DOT-T1E showed a tuft of three to four polar flagella, whereas the DOT-T1E-42 mutant was nonflagellated (data not shown).

To unequivocally establish the nature of the *fliP* gene, the wild-type gene was rescued in pANA9. The sequence of the 10.5-kb *Bam*HI insert of pANA9 was deposited in GenBank under accession number AF031418. Sequence analysis revealed the presence of 11 putative open reading frames, whose translated sequences were compared with those stored in several databanks. All of them showed high homology with different proteins related to flagella and chemotaxis in *P. aeruginosa* and enterobacteria. The gene order was found to be *orf563 fliKLMNOPQRflhBA* (Fig. 2a).

Transcriptional organization of *fli* genes: genes downstream of Tn*5-'phoA* insertion in *P. putida* DOT-T1E-42 are expressed. Given the cluster structure of the *fli* genes and the lack of information on the transcriptional organization of these genes in *P. putida*, we could not rule out the possibility that the Tn*5-'phoA* insertion exerted a polar effect on downstream genes. As a way to establish the transcriptional organization of the *fli* genes in this strain, we isolated total RNA from *P. putida* DOT-T1E and DOT-T1E-42 and determined mRNA contiguity by RT-PCR. The primers used are given in Materials and Methods and are based on the 3′ and 5′ ends of two adjacent genes. The results obtained are shown in Fig. 2b, c, and d.

In the wild-type strain, the *fli* genes may be organized in at least two transcriptional units. RT-PCR yielded negative results when primers based on *fliK* and *fliL* were used for amplification. Internal primers within *fliK* and *fliL* gave positive results with the same sample; thus, the negative result cannot be attributed to the lack of mRNA. Thus, we concluded that *fliK* and *fliL* belong to different transcriptional units, with *fliK* transcribed presumably as a monicistronic mRNA. Because all other tested combinations of primers based on adjacent genes yielded positive results, we assumed that the other unit involved the *fliLMNOPQRflhB* genes. With mRNA isolated from DOT-T1E-42, we confirmed that the *fliK* gene constituted a transcriptional unit independent of the other *fli* genes. As expected, amplification with *fliL* and *fliM, fliM* and *fliN* and *fliO* primers (genes located upstream from *fliP*) with RT-PCR yielded positive results (Fig. 2b). Surprisingly, we also found RT-PCR products when we used *fliP*/*fliQ* and *fliR*/*flhB* primers (Fig. 2c), which suggests either that the mini-Tn*5-'phoA* insertion at *fliP* does not exert a drastic polar effect on genes located downstream or that multiple promoters are involved in expression of the *fliQRflhBA* genes. Although fine transcriptional analysis is needed to resolve the gene expression pattern, for the purpose of our study, the above results suggest that *fliP* is the gene responsible for the observed phenotypes of lack of motility plus sensitivity to solvents in *P. putida* DOT-T1E.

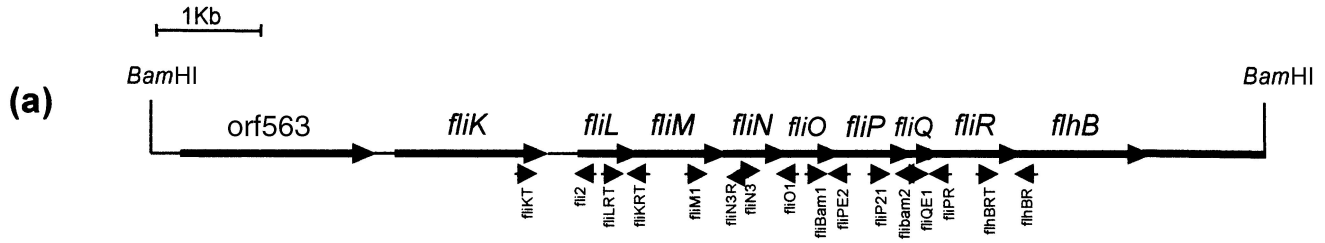
Replacement of mutant allele in *P. putida* DOT-T1E-42 with wild-type allele. The suicide plasmid pANA9, bearing a 10.5-kb insert, was electroporated into *P. putida* DOT-T1E-42, and Pi-resistant clones were selected. These clones resulted from a single recombination event due to the integration of pANA9 within the host chromosome. After repetitive growth on LB medium, we spread cells on LB plates and searched for Km-sensitive clones. These clones were expected to result from the replacement of the mutant allele with the wild-type one. One such clone was found and called DOT-T1E-PS21. Southern blot was used to confirm the nature of the replacement (not shown). We found that DOT-T1E-PS21 cells had recovered motility and the ability to tolerate toluene shocks (not shown).

Are motility and solvent tolerance linked traits in *P. putida* DOT-T1E? To answer this question, we generated mutations in *fliL* and *flhB* by inserting an Ω-Km interposon as described in Materials and Methods. We chose these genes because FliK, FliO, FliQ, FliR, FlhB, and FlhA, together with FliP, have been suggested to be part of the flagellar export apparatus, whereas FliL has been suggested to be a cytoplasmatic protein associated with the basal body (23, 44). The in vivo construction of the knockouts in *fliL* and *flhB* yielded DOT-T1E-PS23 and DOT-T1E-PS50 mutants, bearing an *fliL*::Ω-Km and *flhB*::Ω-Km insertion, respectively. Southern blots revealed the successful replacement of the wild-type *fliL* or *flhB* gene with the mutant *fliL*::Ω-Km or *flhB*::Ω-Km in DOT-T1E-PS23 and DOT-T1E-PS50, respectively (not shown). RT-PCR assays revealed that in DOT-T1E-PS23, in which the insertion was at the 5′ end of the *fliL* gene cluster, did not prevent the expression of the genes downstream of the Ω-Km insertion, as revealed by successful amplification with appropriate primers (Fig. 2b).

The *fliL*::Ω-Km and *flhB*::Ω-Km mutants were nonmotile in soft agar plates (0.3%, wt/vol). Electron microscopy showed that the *fliL* mutant was nonflagellated, whereas the *flhB* mutant had lophotrichous flagella (data not shown). Their behavior with regard to toluene tolerance was different. In fact, while mutant DOT-T1E-PS23 was able to grow on 0.3% (vol/vol) toluene and behaved like the wild-type strain in response to sudden shocks with organic solvents, including toluene (Fig. 1C), the DOT-T1E-PS50 mutant was hypersensitive to sudden toluene shocks (Fig. 1D) and was unable to grow in LB in the presence of 0.3% (vol/vol) toluene. These results suggest that motility and toluene tolerance are not linked themselves but that the flagellar transport system is involved in toluene tolerance.

DISCUSSION

Our initial search for clones exhibiting sensitivity to toluene with a pattern of resistance to antibiotics similar to that of the wild type resulted in the isolation of one mutant out of 5,000 transconjugants analyzed. The analysis of this mutant led us to



(b)

	fliK-fliL	*fliL-fliM*	*fliM-fliN*	*fliN-fliO*	*fliO-fliP*	*fliP-fliQ*	*fliQ-fliR*	*fliR-flhB*
DOT-T1E	-	+	+	+	+	+	+	+
DOT-T1E-42	-	+	+	+	n.d.	+	n.d.	+
DOT-T1E-PS23	-	-	+	+	n.d.	+	n.d.	+

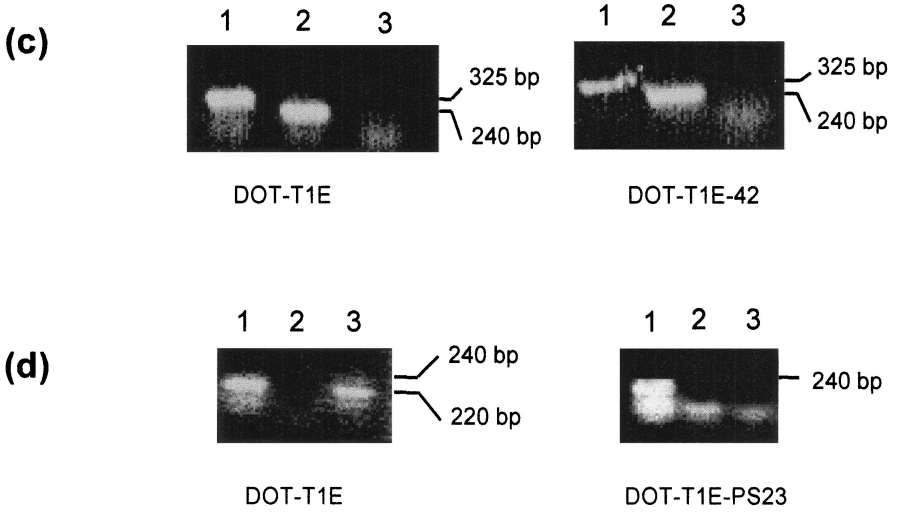


FIG. 2. Physical map of *fli-flh* gene cluster in the solvent-tolerant *P. putida* DOT-T1E strain. (a) Gene order. The arrows indicate the direction of transcription. Small arrows indicate the positions of the primers used in RT-PCR. (b) Summary of RT-PCR results obtained with total RNA isolated from the indicated strains growing on LB. Symbols: +, RT-PCR yielded a fragment of the expected size when primers based on the indicated genes were used; −, RT-PCR failed to amplify RNA with the indicated primers; n.d., not determined. (c) Specific RT-PCR results with primers based on *fliP* and *fliQ* and on *fliR* and *flhB*. Total RNA was isolated from *P. putida* DOT-T1E and DOT-T1E-42, and RT-PCRs were done with primers based on *fliP* and *fliQ* that yielded a 325-nucleotide (nt) band (lane 1) and *fliR* and *flhB* that yielded a 240-nt band (lane 2). Lane 3, negative control using primers based on *fliP* and *fliQ* but in the absence of reverse transcriptase. (d) Specific RT-PCR with primers based on *fliR* and *flhB* and on *fliL* and *fliM*. RNA was isolated from *P. putida* DOT-T1E and DOT-T1-PS23 and RT-PCRs were done with primers based on *fliR* and *flhB* that yielded a 240-nt band (lane 1) and *fliL* and *fliM* that yielded a 220-nt band (lane 3). Lane 2, negative control using primers based on *fliR* and *flhB* but in the absence of reverse transcriptase.

an unexpected finding: the mini-Tn*5-'phoA* insertion in *P. putida* DOT-T1E-42 occurred at the *fliP* gene, whose gene product seems to be a component of the flagellar export apparatus (32). Like *fliP* mutants of other microorganisms (4, 8, 20), the *P. putida fliP* mutant lost motility, suggesting that the *P. putida fli* genes are related to motility. However, the novel phenotype of solvent sensitivity indicates an unexpected connection between the FliP protein and toluene tolerance.

Our study shows that the *fliP* gene is located within a cluster of genes involved in flagellum biosynthesis and chemotaxis. At least two transcriptional units have been identified, one consisting of *fliK* and the other of *fliLMNOPQRflhBA*. RT-PCR assays revealed that in DOT-T1E-42 the genes downstream from *fliP* were expressed in spite of the insertion of a mini-Tn*5-'phoA*. This could be due to the existence of multiple promoters in the gene cluster, as described for *E. coli* (29, 31). The fact that in DOT-T1E-42 the genes upstream and downstream of *fliP* were expressed (Fig. 2b) suggests that the *'phoA-*

Km insertion does not exert important polar effects and that there is a specific role for the FliP protein in toluene tolerance in *P. putida* DOT-T1E. The role of FliP in solvent tolerance was further confirmed when replacement of the *fliP*::Tn*5-'phoA* allele with the wild-type gene resulted in recovery of motility and toluene tolerance.

The FliP protein has been found associated with the basal body MS ring, and its role seems to be facilitating the export of proteins from the cytoplasmic compartment to a compartment on the other side of the membrane, either the periplasm (as in the case of the flagellar rod proteins) or the cell exterior (as in the case of the hook and filament proteins). To determine whether the toluene sensitivity in the *fliP* mutant was specific for toluene tolerance or whether it resulted from loss of the flagellum, we generated *fliL* and *flhB* mutants. The FliL and FlhB proteins play different roles in flagellar structure. In fact, while FlhB is a 39-kDa cytoplasmic membrane protein involved in substrate specificity switching and part of the flagellar export apparatus system (33), the FliL protein is associated with the flagellar basal body (17, 39, 44). The *P. putida* DOT-T1E-PS23 (FliL mutant) and DOT-T1E-PS50 (FlhB mutant) mutants were nonmotile, but DOT-T1E-PS23 was as tolerant to sudden toluene shocks as the wild type, whereas DOT-T1E-PS50 was toluene sensitive. This indicates that solvent sensitivity in DOT-T1E-42 arises not from the loss of the flagellum itself (DOT-T1E-42 is not flagellated, whereas DOT-T1E-PS50 is), but from the absence of FliP. Therefore, it follows that FliP and FlhB play a direct or an indirect role in toluene tolerance, suggesting that an intact flagellar export system is required for toluene tolerance.

The higher toluene tolerance of DOT-T1E-42 and DOT-T1E-PS50 in induced cells versus uninduced cells could be because the expression of the *ttgDEF* and *ttgGHI* pump genes increased in response to solvents (34, 42a). Differences in solvent sensitivity between DOT-T1E-42 and DOT-T1E-PS50 may be due to altered stoichiometry of the flagellar transport components in the two mutants, although this remains to be tested.

Flagellar transport system proteins have homologues in type III transport systems, responsible for the export of virulence factors such as SpaP and SpaR proteins in *Salmonella* spp. (12), Spa24 and Spa29 in *Shigella flexneri* (43), Ysc proteins in *Yersinia* spp. (3), and Hrp in *Pseudomonas solanacearum* (47). Therefore, it is possible that the role of FliP and other flagellar transport proteins in toluene tolerance is to facilitate the transfer to the periplasmic space or to the outer membrane of a protein(s) involved in solvent exclusion. Because the *P. putida* FliP mutant was less efficient in toluene extrusion than the wild type, it is possible that FliP is involved in the transfer of the efflux pump components located in the periplasmic space (i.e., the TtgA, TtgD, and TtgG elements of the TtgABC, TtgDEF, and TtgGHI pumps [34, 41]) and/or in the outer membrane (i.e., TtgC, TtgF, and TtgI proteins). If this were the case, it would mean that the export of these proteins somehow utilizes the flagellum export system. This unusual case has, however, two precedents. One of them is the export to the outer medium of one of the virulence factors in *Yersinia enterocolitica*, a phospholipase (50). Mutants of this *Yersinia* sp. damaged in the flagellar export apparatus failed to export phospholipase and were less virulent than the wild type. In the second case, motility and pathogenicity have also been linked in *Xenorhabdus*, a bacterium symbiotically associated with nematodes of the steinernematide family. Cells with mutations in the flagellar master operon *flhDC* of *Xenorhabdus nematophilus* were nonmotile and exhibited reduced virulence due to decreased export of lipases and hemolysin (11). However, the authors did not investigate whether or not export in *Xenorhabdus* of these virulence proteins required the flagellar export apparatus.

Our results, together with the studies of virulence in *Yersinia* (50) and *Xenorhabdbus* (11), show that the flagellum system is coupled through unknown mechanisms to major networks involving bacterial physiology as well as motility.

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