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Transcriptional Phase Variation at the *flhB* Gene of *Pseudomonas putida* DOT-T1E Is Involved in Response to Environmental Changes and Suggests the Participation of the Flagellar Export System in Solvent Tolerance

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Frameshift mutations in a poly(G) track at the *flhB* gene of *Pseudomonas putida* DOT-T1E are responsible for the diminished swimming of this strain on semisolid medium, which contrasts with the high swimming ability of *P. putida* KT2440, which does not exhibit a poly(G) track at the *flhB* gene. We previously showed that a mutant lacking FlhB was more sensitive to solvents than the wild-type strain (Segura et al., J. Bacteriol., 183:4127-4133, 2001). In this study, we show that swimming ability correlates with solvent tolerance in *P. putida* DOT-T1E, so that growth conditions favoring a functional *flhB* gene (growth on semisolid medium) resulted in increased innate tolerance to a sudden toluene shock.

The flagellar biosynthetic pathway in gram-negative bacteria has been extensively studied (1, 14). It involves the sequential expression, localization, and assembly of subunits leading to a mature flagellum. Control of at least 50 genes, including those encoding the chemosensory apparatus, is required for flagellum formation (15). These motility genes are controlled at the transcriptional level in a hierarchical fashion, allowing bacteria to stringently control the production and assembly of flagellum subunits in response to environmental signals and to sense organelle structural intermediates (10). The flagellar export apparatus is involved mainly in flagellum assembly, but it has recently been reported that this system is parasitized to export proteins that are unrelated to flagellum assembly, i.e., export of a phospholipase to the periplasmic space or the outer medium in *Yersinia enterocolitica* (27) and export of lipases and hemolysin in *Xenorhabdus nematophilus* (9).

In *Pseudomonas putida* DOT-T1E and *P. putida* S12, flagellar genes have been shown to be involved in organic solvent tolerance, although the role of their proteins in solvent tolerance remains unclear (13, 24). Organic solvents with a $\log P_{ow}$ (logarithm of its partition coefficient in *n*-octanol and water) between 1.5 and 4.0 are extremely toxic for microorganisms and other living cells because they partition preferentially in the cytoplasmic membrane, disorganizing its structure and impairing vital functions. Several elements have been suggested to be involved in the response to these toxic chemicals. Some responses, such as increased rigidity of the cell membrane via the isomerization of *cis*-unsaturated fatty acids to the corresponding *trans* isomers, occur very rapidly (the reaction has been observed just 2 min after the exposure of the cell to organic solvents), whereas others, such as the increase in the content of phospholipids, can take place up to 30 min after addition of the solvent (12, 20). Although changes in phospholipid fatty acids are not essential for solvent tolerance, they probably represent a first response that allows the cells to gain time for de novo biosynthesis of other components involved in tolerance.

The efflux of the organic solvents by efflux pumps of the resistance, nodulation, and cell division (RND) family is probably the most important mechanism of solvent tolerance in gram-negative bacteria (22). In *P. putida* DOT-T1E, three efflux pumps are mainly involved in solvent tolerance (17, 21, 23). The TtgABC and TtgGHI pumps are expressed at a certain level in the absence of toluene. Expression of the TtgDEF and TtgGHI efflux pumps is increased when toluene is present in the culture medium (6, 17, 21, 23). Therefore, the TtgABC and TtgGHI efflux pumps are involved in the innate tolerance response, and TtgGHI is also involved in induced resistance. TtgDEF, on the other hand, seems to be involved only in induced tolerance.

Given that mutated FliP, FlhB, and other proteins involved in flagellum export were sensitive to solvents, we hypothesized that the flagellar export machinery could be involved in the possible translocation of toluene tolerance proteins (such as some components of the efflux pumps) to the periplasmic space (13, 24).

In the course of our investigation, reversible frameshift mutations in a short homopolymeric tract of guanine residues located at the 5′ region of the *flhB* gene were found in *P. putida* DOT-T1E. In this study, we show that phase variation in the *P. putida* DOT-T1E *flhB* gene influences its swimming ability and its tolerance to toluene shocks. Translational variation caused by frameshift mutations has been shown to be a widespread mechanism for adaptation to new environments.

***P. putida* DOT-T1E presented retarded motility in soft-agar plates with respect to *P. putida* KT2440.** *P. putida* KT2440 (8) and *P. putida* DOT-T1E (19) cells were pregrown on Luria-Bertani (LB) liquid or solid medium and inoculated as a single spot in the center of a semisolid LB plate whose agar concentration was 0.3% (wt/vol). *P. putida* KT2440 showed a swim-

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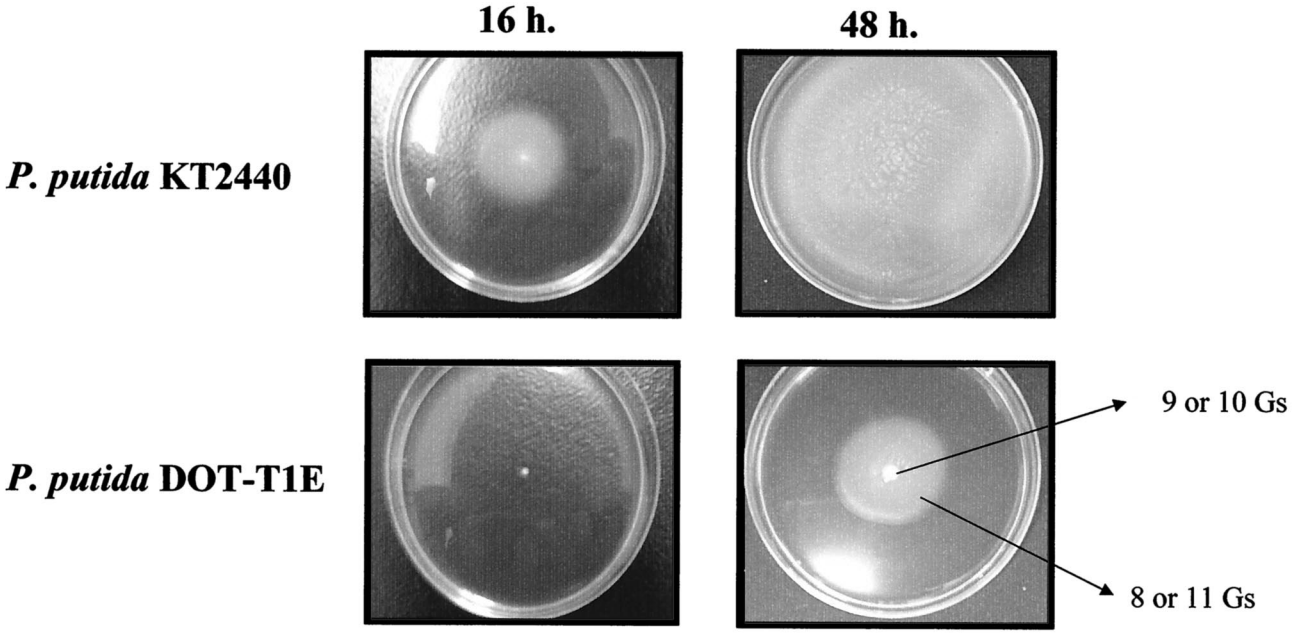

FIG. 1. Morphology of *P. putida* DOT-T1E and *P. putida* KT2440 colonies in soft-agar plates.

ming halo of around 4 cm in diameter after 16 h, whereas DOT-T1E cells needed about 48 h to achieve a similar-sized swimming halo (Fig. 1). Given that the growth rate of both strains is similar in liquid LB medium, 60 ± 1 min for *P. putida* DOT-T1E versus 66 ± 2 min for *P. putida* KT2440, the above results suggested that *P. putida* DOT-T1E has to adapt to the new medium. If this were the case, the adapted cells would have an increased capability for swimming. When *P. putida* DOT-T1E cells pregrown on LB soft-agar plates for 48 h were used to inoculate in a single spot a new soft-agar plate, the 4-cm swarming halo was observed 16 h after the inoculation (data not shown). These results indicate that in LB liquid or solid medium *P. putida* DOT-T1E cells do not have the ability to swim and suggest that change(s) needs to occur in the population in response to the new environment. In fact, when we observed the cells from the liquid and soft-agar medium under the microscope, we detected two different cell morphologies in the liquid culture: large nonmotile cells, and smaller motile cells. Cultures from soft-agar plates contained only small motile cells.

The *flhB* gene of *P. putida* DOT-T1E is translationally out of frame when the cells are grown on LB liquid or solid medium but not in soft-agar medium. Some of the flagellar genes, *fliLMNOPQR* and *flhBA*, had previously been cloned and identified in our laboratory (24). In other bacteria, these genes are involved in the formation of the flagellar export apparatus (16). Sequence analysis of the *P. putida* DOT-T1E flagellar operon (GenBank accession number AF031418) revealed a run of eight G's at the 5′ end of the *flhB* gene (Fig. 2A). Phase variation due to a high frequency of reversible frameshift mutations in poly(A), poly(G), or poly(T) stretches have been described in several bacterial genes as response mechanisms to different environmental conditions (3, 4, 5, 11, 18, 25, 26, 28). To analyze whether the stretch of G's within the *flhB* gene was involved in the retarded swimming of *P. putida* DOT-T1E, PCR and appropriate primers (available upon request) were used to amplify a DNA fragment (2) within this flagellar region by using as a template chromosomal DNA from several independent DOT-T1E cultures grown on either liquid or solid LB medium as well as from cells pregrown on soft-agar plates for 48 h. When the inoculum came indistinctly from liquid or solid LB medium, three different sequences were found that differed only in the number of G's (10, 9, or 7). In all cases, the translated FlhB protein was truncated shortly after the run of G's (Fig. 2B). The *flhB* sequence obtained from cells growing on soft-agar plates contained a string of 8 or 11 G's, and the corresponding open reading frame extended over 380 amino acids in the first case and 381 amino acids in the latter, approximately the length of other FlhB proteins described for several microorganisms, i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Rhodobacter* sp., and *Campylobacter* sp. (GenBank sequences NP416394, AAG04838, D32203, and D90830).

Moreover, when we sequenced the DNA extracted from the cells of the inner part of the soft-agar colony, the *flhB* gene contained 9 or 10 G's, and its translation yielded a truncated FlhB peptide. When the DNA came from the cells from the border of the colony, the corresponding G stretch in the *flhB* gene contained 8 or 11 G's encoding a full-length protein. These results explain why when the cells used to inoculate the soft-agar plates came from the swimming halo they produced a swimming halo of 4 cm after only 16 h (as in *P. putida* KT2440).

A frameshift mutation in a poly(T) strip within the *flhA* gene was reported for *Campylobacter coli*. The authors of that study suggested that these frameshift mutations allowed the cell to save energy under circumstances where motility was not necessary (18), which is clearly the case for *P. putida* DOT-T1E growing on liquid medium in which the nutrients are homogenously distributed.

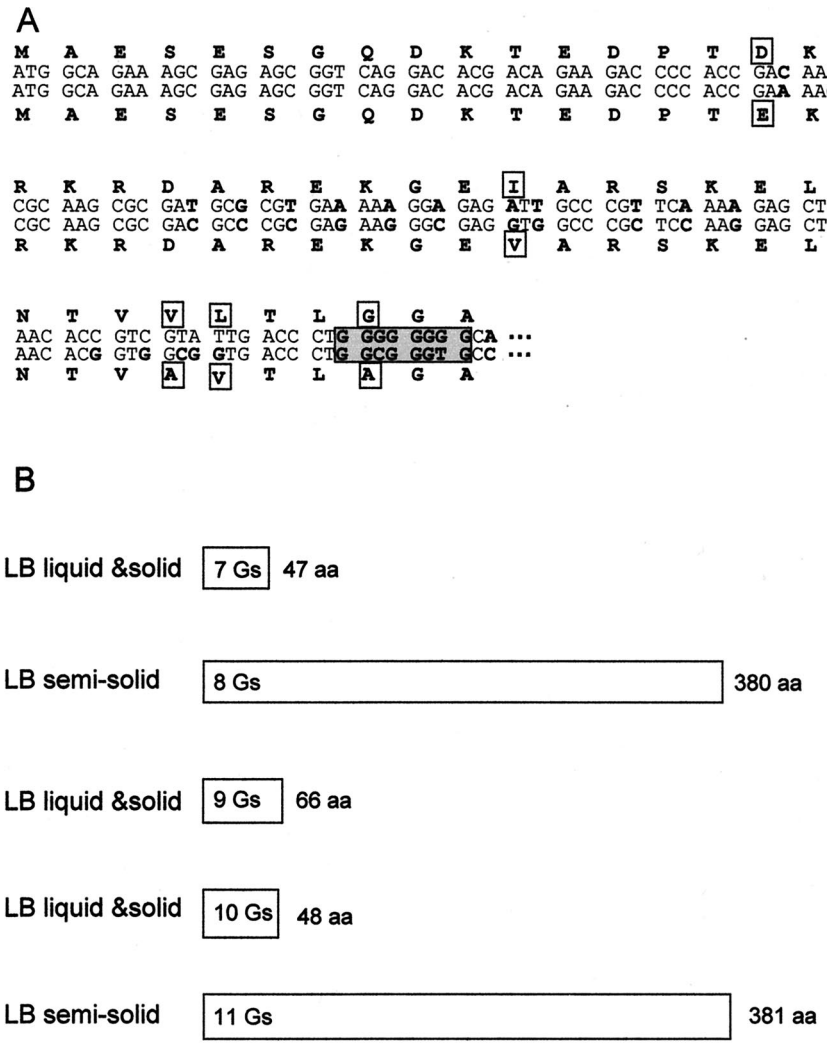

FIG. 2. (A) Alignment of the 5′ ends of *P. putida* DOT-T1E (upper sequence) and *P. putida* KT2440 (lower sequence) *flhB* genes. The deduced protein sequences are also shown. Amino acid differences between the two strains are shown inside squares. Changes in nucleotides are in bold. The eight Gs in *P. putida* DOT-T1E and the corresponding sequence in *P. putida* KT2440 are shown in the shaded box. (B) Schematic representation of the sizes of five different polypeptides deduced from the sequences obtained. The length of each deduced protein is shown on the right. The number of Gs within the *flhB* gene is shown inside the protein representation. aa, amino acids.

Since *P. putida* KT2440 did not exhibit the adaptive behavior described above for the DOT-T1E strain, we expected not to find the strip of G's within the *flhB* gene of this strain. Using an appropriate set of primers, we amplified and sequenced the *flhB* gene from *P. putida* KT2440 chromosomal DNA. The nucleotide sequence was 75% identical over the whole length of the genes from both strains. In the *flhB* sequence of *P. putida* KT2440, we found the 5′-GGCGGGTG-3′ sequence instead of the eight-G strip (Fig. 2A).

Swimming motility in *P. putida* DOT-T1E can be improved by introducing the *P. putida* KT2440 *flhB* gene in *trans*. We cloned the *P. putida* KT2440 *flhB* gene in the pBRR1MCS5 vector to yield plasmid pED17, which was electroporated into *P. putida* DOT-T1E as previously described (7). *P. putida* DOT-T1E(pED17) exhibited swimming ability similar to that of *P. putida* KT2440; i.e., it produced a 4-cm halo in soft-agar plates when inoculated as a spot by using cells grown on liquid LB medium. In a similar way, we cloned the *flhB* gene that carries eight G's in pBRR1MCS5 (plasmid pANA98), and we introduced it by electroporation into the *P. putida* DOT-T1E strain. However, the recombinant cells were not able to form the 4-cm swimming halo after 16 h. When we sequenced the *flhB* gene of the plasmid extracted from these cells, nine G's were present in the gene, indicating that the frameshift variation also took place in the plasmid.

Implications of the frameshift mutations for solvent tolerance in *P. putida* DOT-T1E. *P. putida* DOT-T1E is a toluene-tolerant microorganism, but tolerance is influenced by growth conditions. In fact, only 1 out of 10^4 cells survives a sudden toluene shock (19, 21). However, preinduction of the cells through their exposure to low toluene concentrations led to the survival of almost 100% of the cells after a sudden 0.3% (vol/vol) toluene shock. Innate and inducible tolerance to toluene in *P. putida* DOT-T1E is compromised by mutations in a series

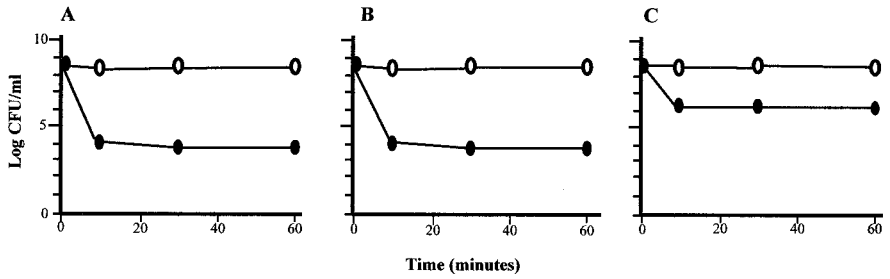

FIG. 3. Survival rate of *P. putida* DOT-T1E pregrown in liquid LB medium (A), solid LB medium (B), and soft-agar medium (C). Cells were collected very carefully by using a loop, letting them stand for 10 min on LB, and then centrifuging the culture at 2,000 rpm (Desaga MC-2; Sarstedt-Gruppe) for 1 min to pellet the soft agar. Cells were suspended in LB until they reached a density of around 10^9 CFU per ml. The sample was divided into two halves; one was kept as a control (open symbols), and the other was treated with 0.3% (vol/vol) toluene (closed symbols). At the indicated time, the number of CFU/ml was determined.

of toluene efflux pumps (17, 21, 23) of proteins involved in phospholipid turnover (Segura et al., unpublished data) and mutations in the flagellar export apparatus (24). Interestingly, it has been reported that the solvent-tolerant *P. putida* DOT-T1E strains with knockout mutations in either the *fliP* or *flhB* gene are more sensitive to toluene than the parental strain. An *flhB*::ΩKm mutant is unable to resist a sudden addition of 0.3% (vol/vol) toluene when cultures are not preinduced (24).

In light of the new findings described above, we hypothesized that only part of the *P. putida* DOT-T1E cells grown on liquid culture make the FlhB protein, and that most of the cells within the population have a truncated FlhB protein. If the FlhB protein is necessary for solvent tolerance (as shown previously), all the cells in the liquid culture that did not carry the in-frame protein should be toluene sensitive. This frameshift mutation could be a plausible explanation for the fact that only 1 in 10,000 cells of a wild-type liquid culture survived the toluene shock (Fig. 3A).

As mentioned above, reiterative growth of *P. putida* DOT-T1E cells on soft agar led to a high swimming ability and to the synthesis of a functional FlhB protein. To test whether the presence of a complete FlhB protein improved toluene tolerance, we recovered *P. putida* DOT-T1E cells from soft-agar plates and LB solid plates and studied their survival rate after toluene shocks. We found that cells pregrown on soft-agar plates were more resistant (about 1 out of 10^2 cells survived) to sudden toluene shocks than those pregrown on LB solid plates (1 out of 10^4) or on liquid medium (Fig. 3). These results suggest that, in *P. putida* DOT-T1E, the presence of an intact FlhB protein helps the cell to overcome the toxic effect of toluene.

Based on the above results, we even expected differential survival of DOT-T1E growing on a soft-agar plate, depending on where the cells had been taken from, since cells from the inner part of the colonies showed 9 or 10 Gs and did not produce FlhB, whereas cells from the border of the swimming halo had 8 or 11 Gs and made a functional FlhB protein. Cells from different parts of a colony in a soft-agar plate were suspended in LB liquid medium, and the survival rate after the addition of 0.3% (vol/vol) toluene was determined. We found that cells from the border of the colony were relatively tolerant to toluene (survival rate was 1 out of 10^2 cells), whereas cells from the inner part of the colony were less resistant (survival rate was 1 out of 10^4).

In support of the finding that a functional FlhB protein increased solvent tolerance is the finding that *P. putida* DOT-T1E(pED17) exhibited behavior similar to that of *P. putida* DOT-T1E taken from the border of the colony.

In conclusion, the results presented in this study reveal a clear phenomenon of phase variation related to motility in *P. putida* DOT-T1E. This phenotype correlates with mutations in a G strip that either yielded or did not yield a functional FlhB protein. Early studies by Ramos et al. (21) revealed that *P. putida* DOT-T1E with innate solvent tolerance also showed traits related to phase variation; i.e., 1 out of 10^4 cells survived a sudden toluene shock. Our group and other groups had previously found that mutations in the flagellar export machinery led to solvent sensitivity in *P. putida* DOT-T1E (24) and S12 (13). Interestingly, we have now found in *P. putida* DOT-T1E a phase variation associated with flagellum biosynthesis linked to the *flhB* gene, which correlates with the innate solvent tolerance of this strain. Although our results should not be interpreted as meaning that the FlhB protein is sufficient per se for solvent resistance, they support the finding that the presence of intact flagellar machinery is critical for the immediate and innate solvent-tolerant response in this strain. While it is clear that the lack of FlhB directly influences the swimming ability of DOT-T1E, we cannot ascertain whether the lack of FlhB is directly responsible for solvent sensitivity or whether it is simply a side effect.

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