Isolation and Characterization of Toluene-Sensitive Mutants from the Toluene-Resistant Bacterium Pseudomonas putida GM73

KWANG KIM, SUNGJIN LEE, KYUNGHEE LEE, AND DONGBIN LIM*
Department of Microbiology, Gyeongsang National University, Gazwadong, Chinju 660-701, Korea

Received 17 November 1997/Accepted 14 May 1998

To understand the mechanism underlying toluene resistance of a toluene-tolerant bacterium, Pseudomonas putida GM73, we carried out Tn5 mutagenesis and isolated eight toluene-sensitive mutants. None of the mutants grew in the presence of 20% (vol/vol) toluene in growth medium but exhibited differential sensitivity to toluene. When wild-type cells were treated with toluene (1% [vol/vol]) for 5 min, about 2% of the cells could form colonies. In the mutants Ttg1, Ttg2, Ttg3, and Ttg8, the same treatment killed more than 99.9999% of cells (survival rate, <10−6). In Ttg4, Ttg5, Ttg6, and Ttg7, about 0.02% of cells formed colonies. We cloned the Tn5-inserted genes, and the DNA sequence flanking Tn5 was determined. From comparison with a sequence database, putative protein products encoded by ttg genes were identified as follows. Ttg1 and Ttg2 are ATP binding cassette (ABC) transporter homologs; Ttg3 is a periplasmic link protein of a toluene efflux pump; both Ttg4 and Ttg7 are pyruvate dehydrogenase; Ttg5 is a dihydrolipoamide acetyltransferase; and Ttg7 is the negative regulator of the phosphate regulon. The sequences deduced from tgg8 did not show a significant similarity to any DNA or proteins in sequence databases. Characterization of these mutants and identification of mutant genes suggested that active efflux mechanism and efficient repair of damaged membranes were important in toluene resistance.

Organic solvent partition preferentially in the cell membrane, and this accumulation causes expansion of the membrane and loss of membrane integrity (2, 25). This results in inhibition of membrane protein functions, disruption of proton motive force, and ensuing lysis and cell death. Organic solvents with a low log P_w value (logarithm of the partition coefficient of the target compound in a mixture of n-octanol and water) are particularly toxic. Nevertheless, bacteria that are able to tolerate high concentrations of organic solvents in their culture medium do exist (1, 9, 10, 21). These bacteria have potential applications in bioremediation of contaminated sites and in biocconversion of water-insoluble compounds dissolved in appropriate solvents.

It was observed that some bacteria could adapt to high concentrations of toxic solvents (27). Alteration of the cell envelope structure was observed as the bacterium was exposed to organic solvents. Weber et al. observed an increase of trans-unsaturated fatty acid contents in cells grown with toluene (26). It was suggested that this isomerization of cis- into trans-unsaturated fatty acids plays an important role in solvent tolerance in bacteria (7, 22). To support this, a mutant lacking the cis—trans isomerization activity was sensitive to toluene (22). Pinkart et al. observed a modification of lipopolysaccharide and an increase in total fatty acids in solvent-treated cells in addition to the increase in trans-unsaturated fatty acid content (19). They suggested that these envelope modifications aid in bacterial survival at high concentrations of organic solvents. The presence of an active efflux system for toluene in solvent-resistant bacteria was also demonstrated (11), and this energy-dependent export system was shown to be important in toluene resistance (12). Studies by Ramos et al. showed that the increased cell membrane rigidity resulting from changes in fatty acid and phospholipid compositions, exclusion of toluene from the cell membrane, and removal of intracellular toluene by degradation all contribute to the toluene resistance of Pseudomonas putida DOT-T1 (22).

In this study, we took a molecular genetic approach in investigating genes functioning in the toluene tolerance of P. putida GM73, a field isolate resistant to high concentrations of toluene and other organic solvents. We carried out transposon mutagenesis with Tn5 and isolated eight toluene-sensitive mutants. Characterization of these mutants and identification of mutant genes suggested that an active efflux mechanism and efficient repair of damaged membranes were important in the toluene resistance of P. putida GM73.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Escherichia coli JM109 and E. coli JM83 were used as hosts for cloning and sequencing. E. coli C600(pGSP:Tn5) was used as a Tn5 donor in transposon mutagenesis (5). E. coli HB101 (pRK2013) was a helper in triparental mating (5, 23). P. putida ATCC 12633 and three toluene-resistant isolates, P. putida GM62, P. putida GM73, and Pseudomonas sp. strain GM80, isolated as described below, were grown in Luria-Bertani (LB) medium at 30°C. LB medium supplemented with 10 mM MgCl2 (LBmg) was used when these bacteria were cultivated in the presence of toluene (10). To test toluene tolerance, cells were streaked on LBmg agar plate and plates were overlaid with toluene to a depth of at least 5 mm.

Isolation of toluene-resistant bacteria. Toluene-resistant bacteria were isolated from various soil samples collected from southern Korea. Drops of samples were directly inoculated into LBmg broth with 10% (vol/vol) toluene. The samples were cultured for 72 h at 30°C. In 3 out of 400 samples, bacterial growth was found. A single colony from each culture was isolated on LBmg agar plates overlaid with toluene. Colonies that appeared after 48 h of incubation at 30°C were purified and stored. For identification (24), the isolates were cultured on tryptic soy agar medium at 28°C for 48 h. Cells were harvested from the plates by scraping with a sterile glass loop and used for fatty acid methyl ester analysis. Saponification, methylation, and extraction were performed by using the procedures described in the MIDI manual (Microbial Identification, Inc.) (24).

Isolation of P. putida GM730, a mutant strain to which MNNG-treated cells were grown as a single pool to

* Corresponding author. Mailing address: Department of Microbiology, Gyeongsang National University, Gazwadong, Chinju 660-701, Korea. Phone: 82-591-751-5946. Fax: 82-591-750-0187. E-mail: dblim@nongae.gnsu.ac.kr.

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an optical density at 600 nm (OD<sub>600</sub>) of 0.8. One milliliter of culture was transferred to a microcentrifuge tube and centrifuged. Cells were washed twice with saline and resuspended in 300 μl of saline. E. coli C600(pLAFR3) (23) and E. coli HB101(pKK2013), a plasmid donor and a helper, respectively, were cultivated and washed with saline as described above. They were resuspended in 300 μl of saline. Triparental mating was carried out by placing 30 μl of each strain with a micropipette onto LB agar plates. The plates were dried and incubated at 30°C. After 8 h of incubation, cells were collected by scraping and transconjugants were selected on LB plates containing tetracycline (30 μg/ml) for selection of plasmid pLAFR3 and ampicillin (50 μg/ml) for counterselection. From transconjugants, strains lacking plasmid pLAFR3 were isolated by replica plating cells grown overnight without tetracycline. Plasmid-free tetracycline-sensitive cells were picked and tested for tolune resistance. By performing subsequent mating experiments, we found that plasmids can be efficiently transferred by conjugation to these mutants. One of the mutants, P. putida GM730, was chosen for transposon mutagenesis. Transposon Tn5 mutagenesis. E. coli C600(pGS9::Tn5) (5) and P. putida GM730 were grown to an OD<sub>600</sub> of 0.8. They were washed and mated on an agar plate as described in the previous section. Transconjugants were selected on plates containing ampicillin (50 μg/ml) and kanamycin (60 μg/ml). Colonies were picked and replica plated. Toluene was overlaid onto one of the plates, and tolune-sensitive mutants were screened. Colonies which could not grow in the plates overlaid with toluene were purified, and their tolune sensitivity was reconfirmed. It was confirmed, by Southern hybridization with Tn5 DNA frag ment as a probe, that these tolune-sensitive cells were derived from P. putida GM730 by a single Tn5 insertion. Tolune sensitivity of ttg mutants. Five milliliters of LBMg medium was inoculated with 50 μl of overnight culture, and cells were grown at 30°C until an OD<sub>600</sub> of ca. 0.6 was reached. Fifty microliters of culture was taken and plated on LBm agar with serial dilution. To the remaining culture, 50 μl of tolune was added and the mixture was incubated with agitation for 5 min. Cells were plated on LB agar, and colonies were counted after 24 h of incubation. Survival rates were calculated from the number of colonies present before and after tolune treatment. Identification of ttg genes. DNA was isolated from mutant strains, digested with restriction enzyme EcoRI, which did not cut Tn5, and ligated to plasmid vector pTZ19R (New England Biolabs). Colonies that appeared on the plates containing both kanamycin and ampicillin were collected, and the DNA sequence flanking Tn5 was determined by using a synthetic primer (5′-CATGGA AGTCAGATCCT-3′) complementary to the distal end of Tn5. The obtained sequence was translated; amino acid sequences inferred from each open reading frame as a probe showed a single hybridized band in all mutants, indicating that the probe was complementary to the distal end of Tn5 and its growth was compared with that of the parent strain. The toluene-sensitive cells were affected in toluene tolerance genes, and they were selected by replica plating on the plates overlaid with tolune. From a screening of about 10,000 colonies, we isolated eight mutants which did not grow in the tolune-overlaid plates. These mutants should be affected in tolune tolerance genes, and they were called ttg mutants. Southern hybridization with a Tn5 fragment as a probe showed a single hybridized band in all mutants, revealing that they were single transposon insertion mutants (Fig. 1). Characterization of ttg mutants. All ttg mutants, especially Ttg4, Ttg5, and Ttg7, grew more slowly than the parent in LB medium (Fig. 2). Unlike the parental strain or other mutants, strains Ttg4, Ttg5, and Ttg7 did not grow in minimal medium with glucose, but they grew fine in medium with succinate. With 20% (vol/vol) tolune added to LBm medium, no growth was observed for mutants Ttg1, Ttg2, Ttg3, Ttg4, Ttg5, Ttg7, and Ttg8, but Ttg6 grew after a long lag phase (15 h) (Fig. 2). In spite of this long lag phase, the growth rate of Ttg6 in medium with added tolune was similar to that of the wild type. Colonies isolated from the culture were no longer sensitive to tolune. Thus, we considered them revertants. All ttg mutants could grow in LBm plate overlaid with p-xylene and styrene (13). Sensitivity to tolune was examined by measuring the fraction of cells surviving after a short treatment with tolune. Cells cultivated in LBm medium to log phase were treated with 1% tolune for 0.5 min. They were plated on LBm agar medium with serial dilution, and the number of colonies that appeared was counted. For P. putida GM730, about 2% of cells survived after such treatment (Table 1). For mutants Ttg1, Ttg2, Ttg3, and Ttg8, no colonies were obtained, indicating that more than 99,999% of the cells were killed. For mutants Ttg4, Ttg5, Ttg6, and Ttg7, about 0.02 to 0.05% of the cells survived. In the control experiment with tolune-sensitive P.
putida ATCC 12633, no colonies appeared in the plates, indicating that more than 99.9999% of the cells were killed. It should be mentioned that 99 and 99.9999% of toluene-tolerant P. putida DOT-T1 and toluene-sensitive P. putida mt-2, respectively, were killed with similar treatments (22).

**Identification of ttg genes.** To elucidate possible functions of ttg genes, we cloned the Tn5-inserted genes, and the DNA sequence flanking the transposon was determined as described in Materials and Methods. The possible function of each ttg gene was inferred from a comparison of the translated amino acid sequence with protein sequences in a database.

Cloning and sequencing of ttg1 and ttg2 showed that their sequences at and around the Tn5 insertion sites were identical but that the transposon orientations were opposite, showing that Tn5 insertion occurred at the exact same positions. The amino acid sequence deduced from the DNA sequence of ttg1 or ttg2 had significant similarity to the sequences of a group of genes, which have a high sequence homology with pyruvate dehydrogenase from various bacteria (8). From this observation, it was concluded that the toluene-sensitive phenotype of three mutants (Ttg4, Ttg5, and Ttg7) is due to the lack of pyruvate dehydrogenase activity.

Sequence comparison of Ttg6 showed that it is phoU homolog (Fig. 3) (16). The gene product of phoU is a negative regulator of the pho regulon, and the E. coli phoU mutant constitutively produces alkaline phosphatase (16, 17). We found that alkaline phosphatase was constitutively expressed in the Ttg6 mutant (13), confirming that it is a phoU mutant of P. putida GM73. We do not know the function of Ttg8 since its deduced amino acid sequence did not show any significant similarity with any DNA or protein sequence in the database.

**DISCUSSION**

Here we report the isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium P. putida GM73. We isolated eight toluene-sensitive mutants, and sequence analysis showed that two were identical mutants and another two were mutants of the same gene. Therefore, we
identified six genes which may play a role in toluene resistance of *P. putida* GM73.

Of eight mutants, three were found to be defective in the pyruvate dehydrogenase complex (Ttg4, Ttg5, and Ttg7). Pyruvate dehydrogenase catalyzes oxidative decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA), which is a central enzyme in glucose metabolism. We found that Ttg4 and Ttg7 could not utilize glucose as a carbon, probably due to the lack of pyruvate dehydrogenase activity. It is reasonable to think that the lack of pyruvate dehydrogenase activity would lower the intracellular level of acetyl-CoA, a building block of fatty acid, and consequently this may affect membrane biosynthesis. Recently, Pinkart and White found an increase in phospholipid content and increased phospholipid turnover rate after exposure of bacteria to xylene (18). They suggest that solvent-resistant bacteria have a greater ability than solvent-sensitive bacteria to repair damaged membranes through efficient turnover and increased phospholipid biosynthesis. Because of the inadequate amount of acetyl-CoA in our mutants, the ability to repair damaged membranes is probably lower in our three mutants than in the wild type, and this may weaken the membrane rigidity and lower the permeability barrier. It should be noted that they could not grow in the medium containing 20% (vol/vol) toluene but that they still have some toluene tolerance as revealed by short-term treatment (Table 1).

Isken and de Bont and Ramos et al. reported that an energy-dependent efflux system is responsible for the resistance to toluene in *P. putida* S12 and DOT-T1 (11, 22). Recently the genes for the efflux system were cloned, and the efflux system was found to be a three-component pump with a striking resemblance to a multidrug efflux pump (12). Our sequence obtained from the *ttg3* gene is almost identical to that of *srpA*, a gene for the periplasmic linker protein of this efflux pump. Thus, strain Ttg3 is a mutant lacking the toluene efflux pump. The absolute lack of survival of Ttg3 cells after short-term treatment showed that this pump plays an important role in toluene resistance in our strain (Table 1).

The *ttg2* gene encodes a transporter protein containing an ATP-binding cassette (ABC transporter). The ABC transporter participates in the transportation of widely different substances (3). We found that Ttg2 is very sensitive to short-term treatment with toluene, suggesting the importance of this transporter in toluene resistance. At present, it is not clear whether this gene encodes a protein acting as a toluene pump.

FIG. 3. Sequence analysis of *ttg* genes. The nucleotide sequence of each *ttg* gene flanking Tn5 was determined, and the deduced amino acid sequence was compared with database sequences. Tn5 insertion sites are underlined. The symbols I and : indicate identical and similar amino acids, respectively.
There may exist two efflux pumps that participate in the toluene resistance of \textit{P. putida} GM73. Alternatively, the gene may encode a transporter protein functioning in outer membrane synthesis, which is an important barrier to penetration by growth inhibitors (6).

Like mutant strains T4g, T4g5, and T4g7, some fraction of T4g6 cells could survive after toluene treatment (Table 1). In T4g6, alkaline phosphatase was constitutively expressed, indicating a phosphate deficiency in the cells. It is not clear whether the sensitivity to toluene is a direct effect of the phosphate deficiency in the cells or is an effect of physiological changes caused by the phosphate deficiency. When an outer membrane protein profile of the T4g6 mutant was compared with that of the wild type, we found that a 44-kDa protein was overproduced in T4g6 (13). It is possible that this 44-kDa protein forms an outer membrane channel for toluene, and its overexpression could result in the lower membrane permeability barrier observed in T4g6, although Li et al. proposed that the 38-kDa protein OprF was a channel for toluene in \textit{P. aeruginosa} (14). Alternatively, phosphate deficiency may affect phospholipid synthesis and thus alter membrane structure. This may lower the permeability barrier. The diffusion rate of ethidium bromide through the membrane into cytoplasm as measured with a fluorometer was found to be much greater in T4g6 than in the wild type (13), suggesting that the permeability barrier of the membrane was lowered.

Since the T4g8 mutant is very sensitive to toluene, as shown in Table 1, the mutated gene should encode a protein that plays an important role in toluene tolerance. Sequence analysis did not show a significant similarity between the deduced amino acid sequence of \textit{ttg8} and any protein sequence in the database. Ramos et al. reported that a mutant lacking the \textit{trans} isomers of the unsaturated C16:1 and C18:1 vaccenic fatty acids was sensitive to toluene, but it is not clear whether \textit{ttg8} encodes \textit{cis-trans} isomerase or not.

On the basis of these results, we are beginning to understand the general mechanism of toluene tolerance of \textit{P. putida} GM73. Our analysis of T4g mutants and other studies showed that three factors are important in the toluene resistance of \textit{P. putida} GM73, namely, an active efflux pump(s), permeability barriers, and efficient repair of membrane damaged by solvent.

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