The TOL Plasmid pWW0-161 Gene Product from Pseudomonas putida Is Involved in m-Xylene Uptake

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TOL plasmids in Pseudomonas putida encode the metabolic pathways for the degradation of toluene, xylenes, and their alcohol and carboxylic acid derivatives (1). The TOL degradation pathways generally consist of two parts: an upper pathway that converts toluene and xylenes to their carboxylic acid derivatives (11) and a lower (or meta-cleavage) pathway that transforms the carboxylic acids to the precursors of Krebs cycle intermediates (12). Genetic studies have shown that the genes for these catabolic enzymes are organized into two operons, one encoding enzymes for the upper pathway (the upper operon) and the other encoding enzymes for the meta-cleavage pathway (the meta operon) (6, 9, 10, 23).

The upper operon of the TOL plasmid pWW0 of Pseudomonas putida encodes a set of enzymes involved in the conversion of toluene and xylenes to their carboxylic acid derivatives. The last gene of the upper operon, xylN, encodes a 465-amino-acid polypeptide which exhibits significant sequence similarity to FadL, an outer membrane protein involved in fatty acid transport in Escherichia coli. To analyze the role of the xylN gene product, xylN on TOL plasmid pWW0 was disrupted by inserting a kanamycin resistance gene, and the phenotypes of P. putida harboring the wild-type and xylN mutant TOL plasmids were characterized. The growth of P. putida harboring the wild-type TOL plasmid was inhibited by a high concentration of m-xylene, while that of P. putida harboring the xylN mutant TOL plasmid was not. The apparent \( K_m \) value for the oxidation of m-xylene in intact cells of the xylN mutant was fourfold higher than that of the wild-type strain, although the TOL catabolic enzyme activities in cell extracts from the two strains were almost identical. We therefore presume that the xylN gene product is a porin involved in the transport of m-xylene and its analogues across the outer membrane. Western blot analysis confirmed the localization of XylN in the outer membrane.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. pWW0-161 is a Tn401 insertion derivative of TOL plasmid pWW0 and confers resistance to ampicillin on Escherichia coli hosts (6). The Tn401 insertion occurs outside the catabolic genes in pWW0-161. In this study, an xylN::Km\(^m\) mutant of pWW0-161 was isolated and named pWW0-161-xylN. E. coli JM110 was used for routine genetic manipulations. E. coli cells containing recombinant plasmids were maintained on Luria-Bertani plates (29) supplemented with appropriate antibiotics. The concentrations of the antibiotics used were 50 \( \mu \)g/ml for ampicillin and streptomycin and 20 \( \mu \)g/ml for chloramphenicol, while the kanamycin concentration was either 50 \( \mu \)g/ml for E. coli harboring a high-copy-number kanamycin resistance plasmid or 20 \( \mu \)g/ml for E. coli harboring pWW0-161-xylN. P. putida PaW94 (42) harboring pWW0-161 was maintained on M9 minimal medium (29) containing 5 mM m-toluate, while P. putida PaW94 harboring pWW0-161-xylN was maintained on M9 medium containing 5 mM m-toluate and 50 \( \mu \)g of kanamycin/ml. To induce the upper and meta operons on pWW0-161, cells of PaW94 harboring pWW0-161 or those harboring pWW0-116-xylN (hereafter referred to as the wild type) or those harboring pWW0-116-xylN (hereafter referred to as the xylN mutant) were grown for 16 h at 30°C with shaking in 100 ml of M9 minimal medium containing 5 mM benzyl alcohol. m-Xylene vapor was supplemented for the cultures when required. The simultaneous addition of benzyl alcohol and m-xylene vapor to the culture assured the reproducible induction of TOL catabolic enzymes.

DNA sequence analysis. DNA sequencing with double-stranded DNA was carried out by using a dye terminator cycle-sequencing kit (Applied Biosystems) according to the manufacturer's instructions, and the products were analyzed with a model 377 DNA sequencer (Applied Biosystems). The computer-assisted sequence analysis was made with the PC/GENE software package (IntelliGenet).

Fractionation of membranes. Cells of the wild-type strain were induced as described above, harvested by centrifugation, washed with 50 mM sodium phosphate buffer (pH 8.6), and resuspended in the same buffer. The cells were disrupted on ice using a sonifier (model 250; Branson) at 20-W output by three 30-s bursts of sonication interspersed with 30-s cooling. Nondisrupted bacteria and large cell debris were removed by centrifugation at 8,000 \( g \) for 30 min. The supernatant was removed and centrifuged at 138,000 \( g \) for 1 h. After this step, the supernatant was used as the soluble cytoplasmic fraction. The inner and outer membranes were prepared as described by Feilmeier et al. (5) with slight modifications. Cells were suspended in 20% (wt/vol) sucrose in 10 mM Tris (pH 8.0) and disrupted by passing the cell suspension through a French pressure cell (Ohtake) at 137 MPa. After nonlysed bacteria and cell debris were removed, the clarified lysate was layered onto a 40 to 60% (wt/vol) sucrose gradient and centrifuged at 247,000 \( g \) for 18 h. The inner membrane fraction was recovered at the top of the 60% (wt/vol) sucrose layer, while the outer membrane fraction was collected at the top of the 70% (wt/vol) sucrose layer. The recovered samples were diluted threefold with distilled water. Each fraction was layered on the second 60 to 70% (wt/vol) discontinuous sucrose gradient and centrifuged as described above. The membrane fractions were again diluted as described above and centrifuged at 100,000 \( g \) for 2 h to pellet the membranes. The pellets were washed once with 1 M KCl followed by an additional centrif-
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. putida</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KT2440</td>
<td>PAW94</td>
<td>Benzoate-1,2-dioxygenase-negative mutant</td>
<td>10</td>
</tr>
<tr>
<td>E. coli</td>
<td>JM110</td>
<td>pWW0-161</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pWW0-161-xylN</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pUC4K</td>
<td>Amp' Km'</td>
</tr>
</tbody>
</table>

a Tol' indicates that the cells grow on m-xylene and toluene. Nal, Str, and Rif' stand for sensitivity to nalidixic acid, streptomycin, and rifampicin, while Ap', Cm', and Km' stand for resistance to ampicillin, chloramphenicol, and kanamycin, respectively.

Plasmids

pFC121: xylB' N'; ColE1 replicon
pSHG398: Cm'; ColE1 replicon
pWW0-161: Tol' Ap' (Trp401) 5
pWW0-161-xylN: Tol' Ap' (Trp401) xylN::Km' 5
pXN960: Cm' 3
pXN961: Cm' Km' 3
pUC4K: Amp' Km' 34

RESULTS AND DISCUSSION

Nucleotide sequence analysis of xylN. Hayaraya et al. (11) have reported that xylN was the last gene of the upper operon on the TOL plasmid pWW0 and that its product was a 52-kDa protein which was processed to a 47-kDa polypeptide. In this study, the nucleotide sequence of xylN was determined. The gene encoded a 465-amino-acid-long polypeptide with an estimated molecular mass of 49 kDa. A comparison of the deduced amino acid sequence of XylN with translated sequences from the GenBank database suggested that XylN had significant homology with a group of proteins in hydrocarbon-degrading bacteria, including PorA from Pseudomonas sp. strain Y2 (37), CumH from Pseudomonas fluorescens IP01 (8), IpBH from P. putida RE204 (GenBank accession no. AF006691), CymD from P. putida F1 (4), TodX from P. putida F1 (38), TbxU from Ralstonia pickettii PK01 (17), and PhX from Ralstonia eutropha JMP134 (GenBank accession no. AF065899) (Table 2). These proteins are homologous with FadL from E. coli (2), which is involved in the transport of long-chain fatty acids across the outer membrane (19).

Location of the xylN gene in the outer membrane of P. putida cells. The signal sequence was predicted at the N-
terminal region of XylN by using both SignalP (version 1.1; Center for Biological Sequence Analysis [http://www.cbs.dtu.dk]) (24) and PSORT (http://psort.nibb.ac.jp) (22). These programs suggested the predicted cleavage site to be at position 24, 25, or 26, and the estimated molecular mass of the mature polypeptide was 47 kDa. This result agrees with that of the previous study: a 52-kDa XylN product was processed to a 47-kDa polypeptide (11). PSORT (22) predicted XylN to be an outer membrane protein. The computer program for predicting bacterial outer membrane β-strand proteins (Faculty of Biology, University of Konstanz [http://loop8.biologie.uni-konstanz.de]) (3) also produced this result. The existence of the signal sequence together with the sequence similarity to the outer membrane protein FadL suggested XylN is an outer membrane protein.

To identify the localization of XylN, cells of the wild-type strain were cultivated under conditions to induce the upper operon and fractionated into cytoplasm, inner membrane, and outer membrane. Western blot analysis detected XylN in the outer membrane (Fig. 1). No XylN was detected among cytoplasmic proteins and inner membrane proteins.

**Construction of an xylN mutant of pWW0-161.** To analyze the possible role of the xylN product, PaW94 harboring pWW0-161-xylN was constructed by inserting the DNA fragment containing a kanamycin resistance gene in xylN. To confirm the mutation in this plasmid, total DNAs were extracted from the wild-type strain and the xylN mutant, and partial xylN fragments were amplified from the total DNAs by PCR. As expected, a 1.5-kb-long DNA fragment was amplified from the DNA that had been isolated from the xylN mutant, while a 0.3-kb-long fragment was amplified from the wild-type strain.

**Growth inhibition of P. putida by m-xylene.** Growth inhibition of the wild-type strain but not the xylN mutant was observed with 2.5 mM m-xylene as described below. Cells grown in the presence of benzyl alcohol and m-xylene vapor were used to inoculate M9 medium containing 5 mM benzyl alcohol with or without 2.5 mM m-xylene in tightly stoppered bottles. They were cultivated at 30°C with shaking, and their growth was monitored by determining the turbidity of the cultures at 600 nm (Fig. 2). When the wild-type strain and the xylN mutant were grown on M9 medium containing 5 mM benzyl alcohol without m-xylene, the exponential growth was observed without an apparent lag phase, and the specific growth rate constants (μ) for these two strains were almost the same, namely, 0.20 and 0.19 h⁻¹, respectively. However, when 2.5 mM m-xylene was added to the culture of the wild-type strain, a lag phase of about 40 h followed by the exponential growth phase (μ = 0.12 h⁻¹) was observed. On the other hand, the growth of the xylN mutant occurred without significant lag, and the growth rate was similar to that in the absence of m-xylene (μ = 0.19 h⁻¹). The same results were obtained when o-xylene was added to the cultures (data not shown). Our results with the wild-type TOL plasmid agree with a previous observation: it has been reported that the direct addition of aromatic hydrocarbons to a culture medium prevented the growth of P. putida harboring the TOL plasmid while growth of this strain was observed when these compounds were supplied in the vapor phase (7, 41).

**FIG. 1.** Localization of XylN in the outer membrane. The cytoplasmic, inner membrane, and outer membrane fractions were prepared from cells of the wild-type strain grown under conditions to induce the upper operon. XylN was immunodetected with antibodies raised against XylN. Lanes: 1, cytoplasmic fraction; 2, inner membrane fraction; 3, outer membrane fraction. The positions of the molecular mass markers are shown.

**FIG. 2.** Effect of m-xylene on the growth of the wild-type strain (circles) and the xylN mutant (squares). Cells were grown in M9 medium containing 5 mM benzyl alcohol at 30°C with (solid) and without (open) 2.5 mM m-xylene. Growth was determined by monitoring the turbidity at 600 nm. The values are averages of three independent experiments. The standard deviation was less than 35% of the averages.

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**TABLE 2.** Membrane-associated proteins sharing homology with XylN

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<tr>
<td>CumH</td>
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<td>IpBH</td>
<td><em>P. putida</em> RE204</td>
<td>49</td>
<td>GenBank accession no. AF006691</td>
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<td>PorA</td>
<td><em>Pseudomonas</em> sp. strain Y2</td>
<td>50.9</td>
<td>35</td>
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<td>41.8</td>
<td>16</td>
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<td>FadL</td>
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Xylenes, like toluene, are toxic to microorganisms, even at a subsaturated concentration in water (30). It has been suggested that the accumulation of such organic solvents as toluene and xylenes in the cytoplasmic membrane disturbs its structural and functional properties (30). Accumulated aromatic compounds may reduce the integrity of the cytoplasmic membrane (16, 25, 28) or due to solvent-impermeable outer membranes (13, 27, 28, 39). Considering the structural and functional properties (30). Accumulated aromatic compounds may reduce the integrity of the cytoplasmic membrane (16, 25, 28) or due to solvent-impermeable outer membranes (13, 27, 28, 39). Considering the location of XyN in the outer membrane, we propose that the resistance to xylenes of the wild-type strain was due to a reduction in the permeability of xylene across the outer membrane, which was caused by the inactivation of the XyN outer membrane porin.  

**m-Xylene-dependent oxygen consumption by whole cells.** Cells of the wild-type strain and the xylN mutant were grown under inducing conditions and resuspended in a 50 mM potassium phosphate buffer (pH 7.4) with the turbidity of each suspension adjusted to 1.0 at 600 nm. The suspensions were incubated for 5 h at 30°C to reduce the endogenous energy sources. The stimulation of oxygen consumption by whole cells upon the addition of various concentrations of m-xylene was then determined (Fig. 3). The apparent $K$ value for $m$-xylene oxidation in the xylN mutant (6.7 ± 1.9 μM) was four-fold higher than that of the wild-type strain (1.7 ± 1.2 μM), while the apparent $V_{max}$ value for the xylN mutant (0.12 ± 0.02 μmol s$^{-1}$ g of dry cells$^{-1}$) was 70% of that for the wild-type strain (0.17 ± 0.05 μmol s$^{-1}$ g of dry cells$^{-1}$).

To confirm that the upper and meta operons of TOL plasmids pWW0-161 and pWW0-161-xylN had been induced at the same level, the activities of benzyl-alcohol dehydrogenase and catechol 2,3-dioxygenase in PaW94 harboring these two plasmids were determined. The activities of benzyl-alcohol dehydrogenase in these two strains were 31 ± 11 and 32 ± 10 nmol min$^{-1}$ mg$^{-1}$, respectively, while the activities of catechol 2,3-

**FIG. 3.** $m$-Xylene-dependent oxygen consumption by the wild-type strain (open squares) and the xylN mutant (solid squares). The values are averages of three independent experiments. Each vertical bar represents one standard deviation. The bars appear only above the average values of the data for the wild-type strain to make the graph clearer.

**REFERENCES**


