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NOTES

In Vivo Construction of a Hybrid Pathway for Metabolism of 4-Nitrotoluene in *Pseudomonas fluorescens*

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***Pseudomonas fluorescens* 410PR grows on 4-nitrobenzoate but does not metabolize 4-nitrotoluene. The TOL pWW0Δpm plasmid converts 4-nitrotoluene into 4-nitrobenzoate through its upper pathway, but it does not metabolize 4-nitrobenzoate. *P. fluorescens* 410PR(pWW0Δpm) transconjugants were isolated and found to be able to grow on 4-nitrotoluene. This phenotype was stable after growth for at least 300 generations without any selective pressure. *P. fluorescens* 410PR(pWW0Δpm) converted 4-nitrotoluene into 4-nitrobenzoate via 4-nitrobenzylalcohol and 4-nitrobenzaldehyde. 4-Nitrobenzoate was metabolized via 4-hydroxylaminobenzoate and finally yielded NH_4^+ and 3,4-dihydroxybenzoate, which was mineralized.**

Most nitroaromatic compounds in the biosphere are produced by the manufacture of products such as explosives, dyes, drugs, and solvents. The presence of the nitro group in the aromatic ring increases the toxicity and mutagenicity of the benzene aromatic ring (17). Therefore, the mineralization of nitroaromatic compounds has become a high-priority objective in bioremediation.

Nitroarenes are generally refractory to microbial attack, mainly because of the electron-withdrawing capacity of the nitro group, which prevents electrophilic attack by oxygenases (1). Polar nitroarenes, such as nitrophenols and nitrobenzoates, are usually less recalcitrant than nonpolar compounds, such as nitrotoluenes and nitrobenzenes. Also, the position of the nitro group on the aromatic ring (11) influences the metabolism of these compounds. Bacteria able to degrade 2- and 4-nitrotoluene have been previously described (8, 9, 16). In our laboratory, several 2- and 4-nitrotoluene degraders have been isolated (6) but their characterization has been impeded by two facts: the strains isolated were refractory to genetic analysis, and the catabolic character was unstable, even under selective pressure, in particular, when bacteria were grown under continuous culture close to their maximum growth rates.

To increase the predictability of nitrotoluene degraders, we decided to construct a hybrid pathway for the metabolism of 4-nitrotoluene by assembling the 4-nitrobenzoate-stable pathway from *Pseudomonas fluorescens* 410P and the upper pathway from the *P. putida* TOL pWW0Δpm plasmid, which allows biotransformation of 4-nitrotoluene to 4-nitrobenzoate (4).

Construction of a stable 4-nitrotoluene degrader strain, *P. fluorescens* 410PR(pWW0Δpm). *P. fluorescens* 410P uses 4-nitrobenzoate as the sole C and N source (2). This strain did not grow on any of the other nitroarenes tested (2-, 3-, or 4-nitrotoluene; 2- or 3-nitrobenzoate; or 2,4- or 3,5-dinitrobenzoate). *P. fluorescens* 410PR is a rifampin-resistant derivative which grows in M8 minimal medium (4) supplemented with 5 mM 4-nitrobenzoate with a generation time of about 120 min during the exponential phase (Table 1). One hundred percent of the cells kept the ability to grow on minimal medium supplemented with 4-nitrobenzoate as the sole C and N source after growth in a nonselective medium for more than 300 generations.

P. putida 2440(pWW0) is an *hsdR* derivative of *P. putida* mt-2 (18) in which the pWW0 TOL plasmid encodes the enzymes for the metabolism of toluene and xylenes to Krebs cycle intermediates. Delgado and coworkers (4) showed in vitro that the enzymes from the pWW0 upper pathway are able to convert 4-nitrotoluene to 4-nitrobenzoate via 4-nitrobenzylalcohol and 4-nitrobenzaldehyde. When growing with glucose as the C source, *P. putida*(pWW0) biotransformed 4-nitrotoluene into 4-nitrobenzoate in vivo (12). This finding also confirmed that 4-nitrotoluene was an effector of XylR, the positive transcriptional regulator of the TOL upper-pathway operon (3). On the basis of this knowledge, we undertook the construction of a 4-nitrotoluene degrader strain by introducing pWW0 via conjugation into *P. fluorescens* 410PR. Transconjugants able to grow on M9 minimal medium (2) supplemented with toluene and rifampin were selected at a frequency of 10^{-4} per recipient. All of them were able to grow on 4-nitrobenzoate as the sole C and N source but were unable to grow on 4-nitrotoluene, which was unexpected.

In the construction of hybrid pathways for chloroaromatic metabolism, it was noted that misrouting of chlorobenzoates through the TOL *meta*-cleavage pathway resulted in blockage of the metabolism of these compounds (15). We then considered the following hypothesis: 4-nitrobenzoate could be transformed to a certain extent by the pWW0 *meta* pathway enzymes, and some of the products may interfere with the

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TABLE 1. Growth of *P. fluorescens* 410PR and 410PR(pWW0Δpm) on minimal medium supplemented with different carbon sources and their aromatic-dependent rates of oxygen consumption[a]

Strain and substrate	Doubling time (min)	Oxygen consumption[b] (μmol of O_2 × mg of protein^{-1} × min^{-1})
410PR		
4-NB	120	47
4-NT	NG	ND
3,4-DOHB	68	49
410PR(pWW0Δpm)		
4-NB	120	35
4-NT	450	ND
3,4-DOHB	72	53
4-AB	NG	1
4-NHOHB	ND	38

[a] Cells were grown on M8 minimal medium with 4-nitrotoluene (4-NT) supplied in the gas phase, with 5 mM 4-nitrobenzoate (4-NB), with 5 mM 4-aminobenzoate (4-AB), with 5 mM 3,4-dihydroxybenzoate (3,4-DOHB) plus 10 mM NH_4Cl, or with 4-hydroxylaminobenzoate (4-NHOHB). Doubling times were estimated in cultures growing exponentially. NG, no growth; ND, not determined.

[b] To test oxygen consumption with a Clark electrode, 4-nitrobenzoate-grown cells were harvested, washed, and suspended in 50 mM phosphate buffer. The oxygen consumption rate is the increase in the rate of endogenous oxygen consumption upon addition of the indicated substrate at 0.5 mM.

catabolism of 4-nitrotoluene. To test this, we filter mated *P. putida* bearing pWW0Δpm, a streptomycin-resistant pWW0 derivative in which the *meta* pathway promoter is replaced by the omega interposon (10), with *P. fluorescens* 410PR. Transconjugants able to grow on minimal medium supplemented with rifampin, 4-nitrobenzoate, and streptomycin were obtained at a frequency of 10^{-4} per recipient. All of these transconjugants were able to grow on 4-nitrotoluene, supplied in the vapor phase, on both liquid and solid media and kept the ability to grow on 4-nitrobenzoate (Table 1).

The 4-nitrobenzoate catabolic pathway of *P. fluorescens* is non-self-transmissible (12); however, Ramos-González and coworkers (14) reported that the TOL plasmid was able to mobilize chromosomal markers among pseudomonads. To confirm that the transconjugants able to grow on 4-nitrotoluene were true *P. fluorescens* 410P(pWW0Δpm) rather than *P. putida* retrotransconjugants, we performed a series of metabolic tests available in the commercial kit API 2ONE (BioMérieux). These analyses identified all of the transconjugants as *P. fluorescens.*

A random clone was then chosen for further characterization and called *P. fluorescens* 410PR(pWW0Δpm). To examine the stability of the 4-nitrotoluene degradation character, *P. fluorescens* 410PR(pWW0Δpm) was grown on rich medium with and without streptomycin for at least 300 generations and then spread on minimal medium with streptomycin and 4-nitrotoluene as the sole C and N source. One hundred percent of the cells kept the ability to grow on 4-nitrotoluene, and they also kept the pWW0Δpm plasmid (data not shown). This result agrees with the high stability of pWW0 previously described in other fluorescens pseudomonads (13, 18). Cells of *P. fluorescens* 410PR(pWW0Δpm) on M8 minimal medium supplied with 4-nitrotoluene in the vapor phase as the only C and N source grew exponentially to an optical density at 660 nm of about 0.5. The doubling time in this phase was 450 ± 30 min (Table 1). Growth was also monitored by measuring the total protein of the culture and the number of viable cells. All of the data obtained were consistent with the results presented in Table 1.

To measure the rate of consumption of 4-nitrotoluene by *P. fluorescens* 410P(pWW0Δpm), cells growing exponentially on 4-nitrotoluene as the sole C and N source were washed twice in M8 without any C or N source and resuspended in this M8 medium supplemented with 0.5 mM 4-nitrotoluene. Samples were removed periodically, and after centrifugation, supernatants were analyzed by high-performance liquid chromatography with a Hypersil C_{18} (octyldecyl silane) column. Aromatic compounds were detected at 254 nm with a Hewlett-Packard UV-visible light detector. The rate of uptake was approximately 15 nmol of 4-nitrotoluene × min^{-1} × mg of protein^{-1}.

Characterization of the 410PR(pWW0Δpm) 4-nitrotoluene degradation pathway. Culture supernatants of *P. fluorescens* 410PR(pWW0Δpm) cells growing on 4-nitrotoluene were analyzed by extracting them with ethyl acetate to isolate intermediates of the pathway. The extracts were concentrated to dryness, and the solid residue was collected for ^1H nuclear magnetic resonance analysis or coupled gas chromatography-mass spectrometry (GC-MS) analysis with a Hewlett-Packard 8590 chromatograph and a Hewlett-Packard mass detector. No intermediates or side products resulting from the metabolism of this compound were detected. Because 4-nitrotoluene is converted to 4-nitrobenzoate via the TOL upper pathway, which has already been characterized (see above and reference 4), we studied the catabolism of 4-nitrobenzoate by *P. fluorescens* 410PR.

In culture supernatants of *P. fluorescens* 410PR cells grown on 4-nitrobenzoate, no nitrite was found; however, up to 2 mM NH_4^+ accumulated. These results suggest that *P. fluorescens* 410P metabolized 4-nitrobenzoate via a reductive pathway. Two reductive pathways for 4-nitrobenzoate metabolism have been described (5, 7). In one of them, 4-nitrobenzoate is reduced to 4-aminobenzoate, and in the other, reduction to 4-hydroxylaminobenzoate takes place (7). To determine which pathway was used, respirometric assays were performed with 4-nitrobenzoate-grown *P. fluorescens* cells. These cells oxidized 4-nitrobenzoate, 4-hydroxylaminobenzoate, and 3,4-dihydroxybenzoate at similar rates (35 to 50 μmol of O_2 × mg of protein^{-1} × min^{-1}) (Table 1) but did not oxidize 4-aminobenzoate or 4-hydroxybenzoate. This indicates that *P. fluorescens* 410PR uses the hydroxylamino pathway for 4-nitrobenzoate metabolism. This was confirmed by the finding that under oxygen-limiting conditions *P. fluorescens* 410PR biotransformed 4-nitrobenzoate into 3,4-dihydroxybenzoate. On the basis of these data, we propose the pathway shown in Fig. 1. Cells grown on 3,4-dihydroxybenzoate and NH_4^+ oxidized this compound but not 4-nitrobenzoate, suggesting that 4-nitrobenzoate acted as an inducer of the catabolic segment involved in the early metabolism of this nitroarene. Culture supernatants of 410RP grown in 4-nitrobenzoate became yellow with time. Analyses of the supernatant by high-performance liquid chromatography and GC-MS revealed that approximately 2% (wt/wt) of the 4-nitrobenzoate was transformed into side products. Four different compounds were purified and subjected to GC-MS and ^1H nuclear magnetic resonance analyses. They were identified as 3-hydroxy-4-acetamide benzoate, 4-acetamide benzoate, 4-aminobenzoate, and 4,4′-azoxybenzoate.

When *P. fluorescens* 410PR(pWW0Δpm) cells were grown on 4-nitrotoluene, analysis of supernatants showed no accumulation of metabolites and, obviously, no yellow color developed in the culture. Therefore, it seems that 4-nitrotoluene was fully consumed and converted into Krebs cycle intermediates without accumulation of undesirable compounds. The absence of side product accumulation when cells were grown on 4-nitrotoluene, in contrast to cells grown on 4-nitrobenzoate, may well be due to the fact that the growth rate of *P. fluorescens* 410PR in 4-nitrobenzoate is much faster (doubling time, 2 h)

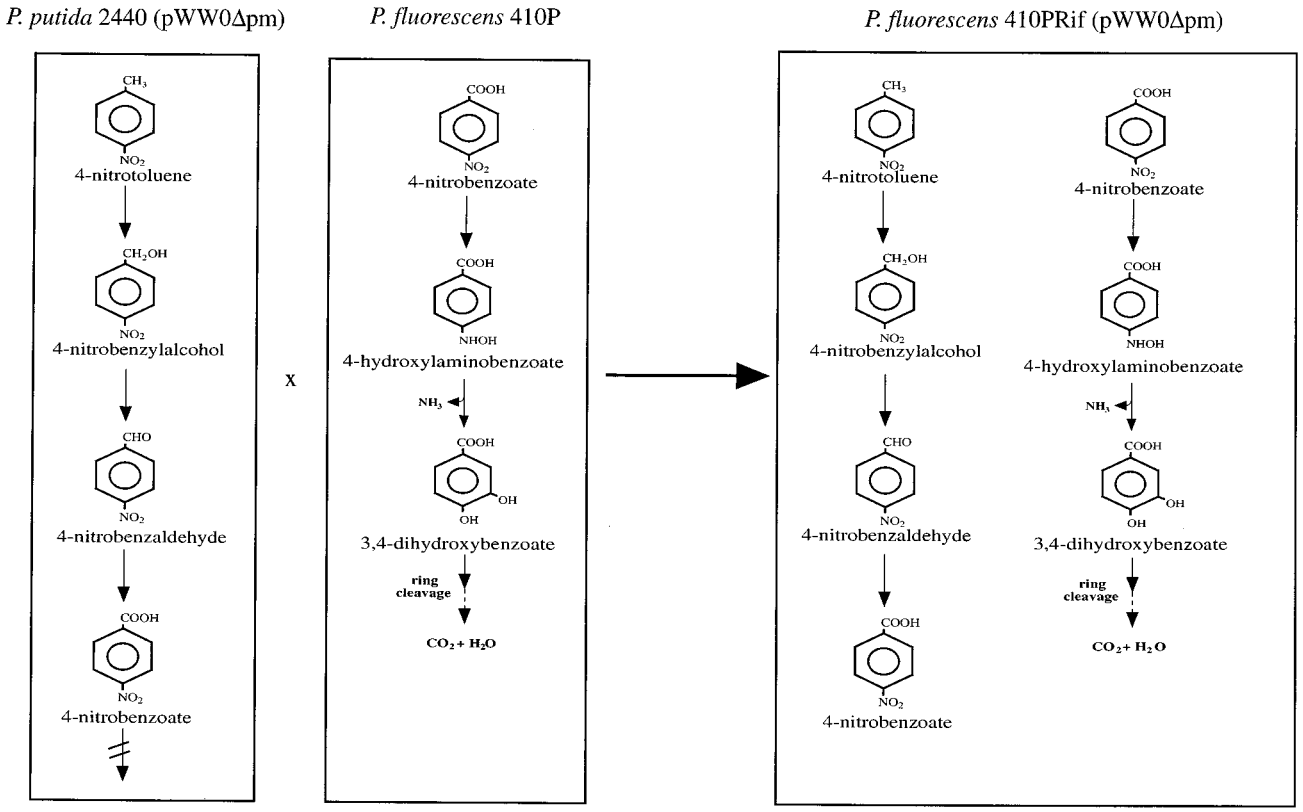

FIG. 1. Proposed pathway for 4-nitrotoluene catabolism in *P. fluorescens* 410PR(pWW0Δpm).

than that of *P. fluorescens* 410PR(pWW0Δpm) in 4-nitrotoluene (doubling time, 7.5 h). If this is the case, the bottleneck of the hybrid pathway is likely to be located in the conversion of 4-nitrotoluene to 4-nitrobenzoate.

In summary, by combining the ability of *P. fluorescens* 410P to degrade 4-nitrobenzoate and the TOL pWW0 upper pathway for oxidation of 4-nitrotoluene into 4-nitrobenzoate, we bioengineered a new 4-nitrotoluene degrader strain, called *P. fluorescens* 410PR(pWW0Δpm). Two characteristics make this strain suitable for use in the removal of 4-nitrotoluene from polluted sites: (i) the 4-nitrotoluene degradation character is stable in the absence of selective pressure, and (ii) *P. fluorescens* 410PR(pWW0Δpm) removed 4-nitrotoluene until its level was negligible without producing undesirable side products.

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