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Identification and Characterization of a Transmissible Linear Plasmid from *Rhodococcus erythropolis* BD2 That Encodes Isopropylbenzene and Trichloroethene Catabolism

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***Rhodococcus erythropolis* BD2, which is able to utilize isopropylbenzene as a sole carbon and energy source, was shown to contain a conjugative linear plasmid, pBD2. The estimated size of pBD2 is 208 to 212 kb. Linear plasmid-deficient strains had lost both the isopropylbenzene degradation and trichloroethene degradation characteristics, as well as the arsenite resistance and mercury resistance phenotypes. Reintroduction of pBD2 restored all four characteristics. Conjugational transfer of pBD2 to a plasmidless mutant of strain BD2 and other *R. erythropolis* strains occurred at frequencies between 3.5×10^{-5} and 2.6×10^{-3} transconjugants per recipient. *R. erythropolis* BD2 degrades isopropylbenzene via 3-isopropylcatechol and 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate. Both isopropylbenzene-oxidizing and *meta*-cleavage activities were shown to correspond with the presence of pBD2. Southern hybridizations with DNA encoding the toluene dioxygenase structural genes (*todC1C2BA*) from *Pseudomonas putida* F1 revealed homology to linear plasmid DNA. These results indicate that the isopropylbenzene degradation pathway encoded by linear plasmid pBD2 is initiated by an isopropylbenzene dioxygenase analogous to toluene dioxygenase.**

Over the past 10 years the genus *Rhodococcus* has been shown to comprise a group of gram-positive soil bacteria known for their ability to degrade a wide variety of xenobiotic compounds, including phenols (36), insecticides (17), herbicides (5), acrylamides (25), anilines, and halogenated alkanes (30).

Recently, we described the isolation of *Rhodococcus erythropolis* BD1 in enrichment cultures containing isopropylbenzene (IPB) as a sole carbon and energy source. IPB was found to be a new inducing substrate for trichloroethene (TCE) oxidation by strain BD1 (10) and by strain BD2, a phenotypically identical derivative lacking the typical clotting phenomenon observed in BD1 cultures. Monoalkylbenzenes, such as IPB, ethylbenzene, and toluene, are known to be growth substrates for many *Pseudomonas* species, and the five different pathways currently known for toluene degradation have been summarized in a recent review (40). IPB metabolism has been studied in detail with *Pseudomonas putida* F1 (21) and RE204 (13, 14), and it has been shown that in the IPB metabolism pathway the side chain is not oxidized before *meta* cleavage (Fig. 1). Furthermore, the IPB-catabolic pathway in *P. putida* F1 was found to be chromosomally encoded, whereas IPB metabolism in *P. putida* RE204 was found to be specified by a covalently closed circular (cccDNA) plasmid.

The instability of the IPB degradation pathway in *R. erythropolis* BD2 and the transferability of the IPB characteristic to spontaneous IPB^- mutants of the same strain indicated that the IPB^+ characteristic of *R. erythropolis* BD2 is plasmid encoded. As all our attempts to detect cccDNA plasmids in wild-type strain BD2 failed, we searched for linear plasmids.

In this paper we describe the isolation and characterization of transmissible linear catabolic plasmid pBD2, which is responsible for the IPB, propylbenzene, ethylbenzene, toluene, and TCE degradation characteristics, as well as arsenite resistance and mercury resistance, in *R. erythropolis* BD2.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Rhodococcus* strains used in this study are listed in Table 1. *Escherichia coli* JM109 harboring pKK223-3-based recombinant plasmid pDTG601 was kindly provided by D. T. Gibson, University of Iowa. Plasmid pDTG601 contains the *todC1C2BA* genes, which encode the four subunits of the toluene dioxygenase, which catalyzes the first step in the oxidative degradation of toluene by *P. putida* F1 (39). *Rhizobium meliloti* MV II-1 was obtained from H. Poth, GBF, Braunschweig, Germany.

The *Rhodococcus* strains were grown in a mineral salt medium (M3) containing (per liter of distilled water) 1.0 g of NH_4NO_3, 0.82 g of $MgSO_4 \cdot 7H_2O$, 1.87 g of KH_2PO_4, 6.4 g of $Na_2HPO_4 \cdot 2H_2O$, 1 ml of a trace element solution (37), and 1 ml of a vitamin solution (8); the final pH was adjusted to 7.0 with 1 M H_2SO_4. For solid media, Bacto Agar (Difco Laboratories, Detroit, Mich.) was added to a concentration of 1.5% (wt/vol). For cultivation on solid media at the expense of volatile aromatic compounds, such as IPB and toluene, 60-μl portions of substrate were added on filter disks (inside diameter, 6 mm) which were placed in the lids of petri dishes. During growth in liquid media the volatile alkylbenzenes were supplied in the vapor phase. For mating experiments *n*-hexadecane was used as the carbon source; 250 μl of *n*-hexadecane on filter paper was added to each M3 agar plate and, therefore, was available via the gas phase. The dishes were incubated upside down in closed gas-tight jars. The complex media used were tryptic soybean medium (Difco) and fructose-tryptose-yeast extract medium (32). Antibiotics were used at the following concentrations: streptomycin sulfate, 300 μg/ml; and rifampin, 30 μg/ml. In all cases cultures were incubated at 30°C.

E. coli JM109 carrying recombinant plasmid pDTG601 was

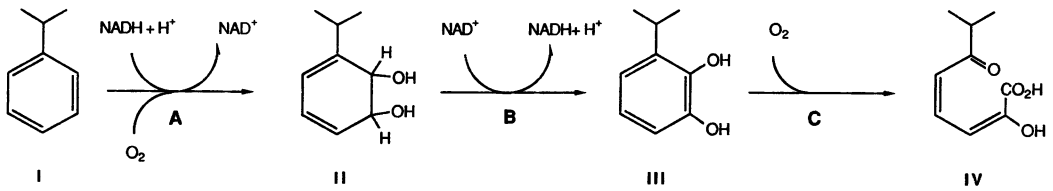

FIG. 1. Tentative pathway for oxidation of IPB to HOMODA by *R. erythropolis* BD2. Compound I, IPB; compound II, 2,3-dihydro-2,3-dihydroxyisopropylbenzene; compound III, 3-isopropylcatechol; compound IV, HOMODA; enzyme A, IPB dioxygenase; enzyme B, 2,3-dihydro-2,3-dihydroxyisopropylbenzene dehydrogenase; enzyme C, 3-isopropylcatechol dioxygenase.

grown at 37°C on Luria-Bertani medium supplemented with 50 μg of ampicillin per ml.

Screening for indole oxidation and *meta*-cleavage activity. Indole oxidation activity and *meta*-cleavage activity (3-isopropylcatechol 2,3-dioxygenase activity) could be induced by growing *R. erythropolis* wild-type strain BD2 (IPB^+) on IPB. To test the corresponding enzyme activities of a spontaneous plasmidless (IPB^-) mutant of *R. erythropolis* BD2, strain BD2.101, another method for enzyme induction first was tested with wild-type cells and subsequently was used for induction of spontaneous IPB^- mutants. Cells were plated onto M3 medium supplemented with 10 mM succinate and were incubated until visible growth of single colonies occurred. Further incubation of cells in the presence of IPB for 12 to 24 h was found to cause expression of the IPB degradation activities in question.

Enzymes were detected by activity staining on agar plates. Therefore, 10 μl of 2 M indole dissolved in methanol was placed near a single colony, and after 20 to 30 min of incubation at room temperature the colony turned blue because of the formation of indigo (16). To detect 3-isopropylcatechol *meta*-cleavage activity, 10 μl of a 100 mM 3-isopropylcatechol solution in dimethylformamide was added to an IPB-induced colony, which became yellow within a few minutes if the reaction was positive. The yellow compound was identified as the *meta*-cleavage product 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate (HOMODA) by its typical absorption spectra in an acid (λ_{max}, 323 nm) and a base (λ_{max}, 393 nm) (13).

TCE degradation assays. TCE degradation assays were performed with resting cells as described previously (10), except that the cells were grown on M3 medium supplemented with 10 mM succinate as the carbon source. After 24 h of growth the cells were fed 4 mM IPB; this was followed by another 4 h of incubation before the cells were harvested. The protein content of each washed cell suspension was 400 μg/ml.

Mating experiments. Plasmid pBD2 was transferred from donor strain *R. erythropolis* BD2.1 to various acceptor strains by a filter mating technique (32). Filters containing a mixture of donor and recipient strains at a 1:50 ratio were incubated for 96 h at 30°C on the surface of *n*-hexadecane agar. Then the cells were suspended in Triton X-100–saline, which contained (per liter) 9 g of NaCl, 2 g of $MgSO_4 \cdot 7H_2O$, and 1 ml of Triton X-100, and the appropriate dilutions were plated onto selective media (see below). To differentiate between potential spontaneous IPB^+ revertants and IPB^+ transconjugants, spontaneous rifampin-resistant mutants of wild-type strain BD2 were selected and used as donor cells. Spontaneous streptomycin-resistant (Str^r) mutants were selected and used as recipients during mating experiments. IPB^+ Str^r transconjugatns were selected by directly plating the mating mixtures on streptomycin-containing mineral agar supplemented with IPB as the sole growth substrate. In every case we confirmed that the purified transconjugants were rifampin sensitive.

TABLE 1. Bacterial strains and plasmids used

Strain	Relevant properties[a]	Size of linear plasmid (kb)	Size(s) of cccDNA plasmid(s) (kb)	Source or reference[b]
Rhodococcus erythropolis				
BD1	IPB^+ Str^S Rif^S	~200		10
BD2	IPB^+ Str^S Rif^S	~200		This study
BD2.1	IPB^+ Str^S Rif^r	~200		This study
BD2.101	IPB^- Str^r Rif^S			This study
cE2	IPB^- Str^r Rif^S	~420	~90	Reh
Ce3	IPB^- Str^r Rif^S		~90	Reh
MS14	IPB^- Str^r Rif^S		~90	DSM 772
Rhodococcus rhodochrous MS18	IPB^- Str^r Rif^S		~90	DSM 363
Rhodococcus sp.				
MR152	IPB^- Str^r Rif^S		~140	DSM 364
MR2226	IPB^- Str^r Rif^S			32
Escherichia coli JM109(pDTG601)	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*ZΔM15*), *todC1C2BA*			39
Rhizobium meliloti MV II-1			57.6, 86.4, 144, 166	Poth

[a] IPB^+, ability to degrade IPB; Str^r, streptomycin resistance; Str^S, streptomycin sensitivity; Rif^r, rifampin resistance; Rif^S, rifampin sensitivity; *todC1C2BA*, toluene dioxygenase subunits.

[b] Reh, M. Reh, Institut für Mikrobiologie, Georg-August-Universität Göttingen, Göttingen, Germany; H. Poth, GBF, Braunschweig, Germany.

To eliminate the possibility that transformation was responsible for transfer of IPB degradation ability, filter matings were done in the presence of DNase. Matings were performed as described above, except that the donor-recipient mixture contained 1 mg of DNase I (Boehringer Mannheim) per ml. We found that DNase activity was still detectable on the filters after 96 h of incubation. The test was performed as follows. The donor-recipient mixture was washed from the filter as described above, and an aliquot (7 μl) of the resulting suspension was added to 310 ng of λ DNA. After incubation for 2 h at 37°C, this probe was analyzed by conventional agarose gel electrophoresis. Disappearance of the DNA showed that DNase still was active.

Preparation of cccDNA. cccDNAs from *Rhodococcus* strains were prepared by a modified alkaline lysis procedure (32). Large circular plasmids from *Rhizobium meliloti* MV II-1 were isolated by using the procedure of Wheatcroft and Williams (38). A standard alkaline lysis technique was used to isolate recombinant plasmid DNA from *E. coli* JM109.

Preparation of DNA for detection of linear megaplasmids. Cultures of *Rhodococcus* strains were grown and treated with sucrose (14%, wt/vol) and glycine (2%, wt/vol) before the cells were harvested and resuspended in 15 μl of EET [0.1 M EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 mM Tris; pH 8.0] per mg (wet weight). To minimize shearing forces during DNA preparation, the cells (1 mg [wet weight] in 15 μl of EET buffer) were embedded in 15 μl of agarose (2% wt/vol) and treated with lysozyme, sodium dodecyl sulfate (SDS), and proteinase K (28). Agarose plugs (length, 5 mm; diameter, 1 mm) were used for contour-clamped homogenous field electrophoresis (CHEFE) analysis.

Electrophoresis. Conventional electrophoresis was performed by standard methods (31). CHEFE was performed by using a Pulsaphor system obtained from Pharmacia LKB, Freiburg, Germany; 1% agarose slab gels (15 by 15 by 0.4 cm) in 0.5× TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA; pH 8.5) were used for all separations. The gels were run in 0.5× TBE electrophoresis buffer at 15°C for 15 h at 170 V. During electrophoresis the pulse times were increased in the following manner: first hour, 0.1 s; second hour, 1 s; and during the next 13 h the pulse time was steadily increased from 1 to 30 s. Concatemers of λ DNA were used as high-molecular-mass DNA standards (1).

Isolation and restriction digestion of linear megaplasmid DNA. To isolate linear plasmid DNA, agarose plugs were prepared as described above, except that a 15-fold greater amount of *R. erythropolis* BD2 cells was embedded in agarose. Linear plasmid DNA was obtained by electroelution by using a HSB-Elutor apparatus obtained from Biometra, Göttingen, Germany. After electroelution the DNA was concentrated by ethanol precipitation and then dialyzed against water.

Single digestions with restriction enzymes were performed in the restriction buffer recommended by the supplier (GIBCO/BRL, Eggenstein, Germany), whereas double digestions were performed in one buffer according to the instructions of the manufacturer (Pharmacia LKB GmbH, Freiburg, Germany). The restriction fragments were analyzed by CHEFE.

DNA restriction fragments were isolated from agarose gels by using a Geneclean kit according to the instructions of the supplier (Bio 101, La Jolla, Calif.).

Southern hybridization. Linear plasmid DNA from *R. erythropolis* BD2 was digested with restriction enzymes and separated by CHEFE. Southern blotting (35) was performed with nylon membranes (GeneScreen Plus; Dupont, NEN Research Products, Dreieich, Germany) under the conditions described by Anderson and Young (2). Hybridizations were carried out under not very stringent conditions at 37°C in the presence of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filters were washed twice in 2× SSC containing 0.1% SDS for 10 min at 60°C and subjected to autoradiography. The DNA probes were radiolabeled with [α-^{32}P]dATP (Amersham Buchler GmbH, Braunschweig, Germany) by using a nick translation kit obtained from GIBCO/BRL.

Determination of resistance characteristics. To test for various heavy metal resistance characteristics, 0.1 ml of a BD2 or BD2.101 preculture was mixed with 3 ml of Luria-Bertani soft agar (0.9%, wt/vol) and immediately plated onto 25 ml of Luria-Bertani agar. Then 30 μl of a heavy metal stock solution (0.1 mM $HgCl_2$ or 3.3 M $NaAsO_2$) was added on a filter (diameter, 0.9 mm; Schleicher & Schüll, Dassel, Germany) which was placed on top of the soft agar. After 48 h of incubation at 30°C, the diameter of the inhibition zone was measured. The levels of resistance were determined in 2.5-ml liquid cultures (M3 medium containing succinate) containing different concentrations of mercury chloride (0.2 to 5 μM) and sodium arsenite (1 to 10 mM). After inoculation with either BD2 or BD2.101 the cultures were incubated for 48 h at 30°C on a rotary shaker. The turbidity was measured at 600 nm with a Bausch & Lomb photometer.

RESULTS

Characterization of the IPB degradation pathway. *R. erythropolis* BD1 and BD2 were isolated in our laboratory by using IPB as a carbon and energy source (10). These two strains have identical phenotypes except that strain BD2 does not have the cell-clotting phenomenon characteristic of strain BD1; therefore, strain BD2 was used in all further experiments.

To characterize the IPB degradation pathway of strain BD2, IPB metabolism was examined by measuring substrate-dependent oxygen uptake rates, by performing a spectral analysis of IPB degradation intermediates, and by performing inhibitor studies with induced and uninduced cells. The results below are consistent with the degradation of IPB by an analogous *P. putida* RE204 pathway (14, 15) via 3-isopropylcatechol and *meta* (extradiol) fission to 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate (HOMODA). (i) 3-Isopropylcatechol is oxidized at a high rate (300 nmol/min/mg of protein) by IPB-grown cells but not by succinate-grown cells; (ii) *o*-, *m*-, *p*-cresols, protocatechuate, and gentisic acid do not serve as growth substrates and cannot be oxidized by IPB-induced cells; (iii) HOMODA could be identified spectrophotometrically as an intermediate (λ_{max} at pH 7, 325 nm; λ_{max} at pH 12, 393 nm); and (iv) in the presence of the *meta* fission inhibitor 3-chlorocatechol the oxidation of 3-isopropylcatechol by resting cells of strain BD2 was completely inhibited (data not shown).

Instability of the IPB degradation phenotype. When we examined IPB degradation, we observed spontaneous instability of the IPB$^+$ phenotype of *R. erythropolis* BD2. After growth for 30 generations under nonselective conditions, 0.8% of the cells had lost the IPB$^+$ phenotype and after 130 generations 1.7% of the cells were incapable of growing on IPB. Revertants from IPB$^-$ to IPB$^+$ were not observed (revertant rate, $<10^{-9}$). These results strongly suggested that the genes responsible for IPB degradation are organized on a plasmid.

Isolation of linear plasmid pBD2 and restriction map. Our attempts to isolate cccDNA from IPB-grown *R. erythropolis* BD2 wild-type cells by a modified alkaline lysis method failed. Examination of strains BD2 and BD1 for the presence of linear DNA molecules by releasing DNA from cells embedded in agarose and analyzing agarose plugs by CHEFE gave rise to

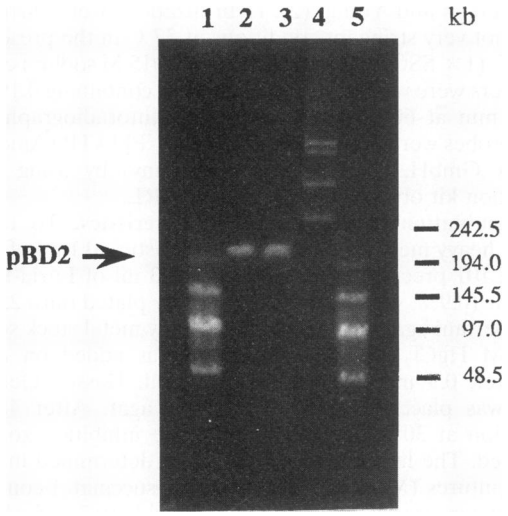

FIG. 2. Detection and separation of linear DNA molecules in lysed cells of *R. erythropolis* by CHEFE. Lanes 1 and 5, λ ladder; lane 2, BD1; lane 3, BD2; lane 4, *Rhizobium meliloti* MV II-1 57.6-, 86.4-, 144-, and 166-kb cccDNA plasmids.

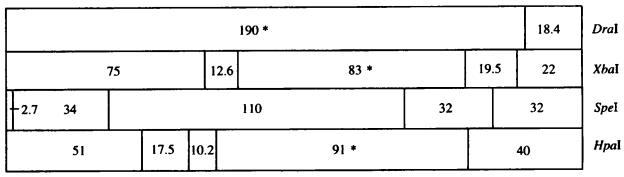

FIG. 3. Restriction map of linear plasmid pBD2. Isolated plasmid pBD2 was analyzed by single and double digestions with restriction endonucleases. The asterisks indicate restriction fragments of pBD2 that hybridize with recombinant plasmid pDTG601 carrying the *P. putida* F1 toluene dioxygenase genes (*todC1C2BA*). The small difference in size between the 34-kb *Spe*I fragment and the two 32-kb *Spe*I fragments prevented the identification of a distinct *Spe*I fragment that hybridized with the *todC1C1BA* DNA probe.

one discrete DNA band (Fig. 2, lane 2 and 3). On the basis of the results of a comparison with linear DNA standards, such as concatemers of λ DNA, a size of approximately 210 kb was estimated, whereas when cccDNA plasmid standards from *Rhizobium meliloti* MV II-1 were used (Fig. 2, lane 4), a size of only 35 kb was calculated for DNA segment pBD2. Therefore, information concerning the real size of pBD2 was necessary to answer the question of whether pBD2 is a linear molecule or a circular molecule. A restriction analysis of pBD2 DNA after electroelution gave rise to five fragments when endonucleases *Xba*I, *Hpa*I, and *Spe*I were used and two fragments when *Dra*I was used. On the basis of the sizes of the pBD2 restriction fragments and the sizes of the fragments obtained by double restriction with *Dra*I plus *Hpa*I, *Spe*I plus *Hpa*I, and *Spe*I plus *Dra*I, we derived a restriction map (Fig. 3). A total size of 208 to 212 kb was calculated for pBD2; this size is consistent with the linear character of this plasmid.

A total of 20 isolated IPB^- mutants were tested for the presence of linear extrachromosomal DNA. None of these carried linear plasmid pBD2 or a pBD2 derivative of a different size (Fig. 4, lane 3). These observations strongly suggest that either part of the IPB degradation pathway in *R. erythropolis* BD2 or even the complete pathway is encoded by linear plasmid pBD2.

Conjugative transfer of the IPB^+ phenotype. To assay for conjugative gene transfer, a spontaneous Rif^r mutant of IPB^+ wild-type strain BD2 (designated BD2.1) was isolated as donor strain, and a spontaneous Str^r mutant of a plasmidless IPB^- derivative of strain BD2 (designated BD2.101) was isolated as a receptor strain. In mating experiments freshly cultivated BD2.101 (IPB^- Str^r) and *R. erythropolis* cE2, Ce3, and MS14, as well as strains of other *Rhodococcus* species, were mixed with donor BD2.1 cells (IPB^+ Rif^r). Transconjugants were screened for an IPB^+ Str^r phenotype on IPB-containing mineral agar supplemented with streptomycin. Using various *R. erythropolis* recipient strains, we obtained IPB^+ Str^r colonies at a frequencies of 3.5×10^{-5} to 2.6×10^{-3} transconjugant per recipient and 2.8×10^{-4} to 7.3×10^{-2} transconjugant per donor, whereas with other *Rhodococcus* strains, such as *Rhodococcus* sp. strains MR152 and MR2226 and *Rhodococcus rhodochrous* MS18, no transconjugants were detected (Table 2). The *R. erythropolis* transconjugants were confirmed to be Rif^S and to contain linear plasmid pBD2, as shown by CHEFE (Fig. 4), and they were able to transfer pBD2 to plasmidless strain BD2.101, yielding IPB^+ Str^r transconjugants. The DNase sensitivity of plasmid transfer from BD2.1 to BD2.101 was tested. An effect on the transfer frequency was not observed, so the possibility that transformation was a mechanism of transfer can be eliminated.

Physiological characterization of linear plasmid pBD2. The growth substrates of *R. erythropolis* wild-type strain BD2 and plasmidless IPB^- mutant strain BD2.101 were compared. Strain BD2.101 did not grow on IPB, toluene, ethylbenzene, or propylbenzene. This suggests that key genes of the alkylbenzene degradation pathway are encoded by linear plasmid pBD2. However, degradation of phenol and degradation of isobutyrate were not affected by the spontaneous loss of pBD2.

In addition, the plasmidless mutants were tested for their activity in the initial IPB oxygenation step, as well as in the 3-isopropylcatechol *meta*-cleavage step, by using activity staining. Bacteria that are able to oxidize aromatic hydrocarbons to *cis*-dihydrodiols with aromatic dioxygenases usually oxidize indole to indigo (13, 16). Consistent with this, we detected IPB-induced indigo formation from indole in BD2 wild-type cells. Inducible indigo formation from indole was also found after growth of BD2 with succinate and subsequent induction with IPB. However, all induced plasmidless mutants of BD2, including BD2.101, gave negative reactions. Furthermore, the plasmidless spontaneous mutants did not produce the characteristic yellow color due to the chromogenic *meta*-cleavage product HOMODA when they were incubated with 3-isopropylcatechol. In addition BD2.101 did not exhibit IPB- or 3-isopropylcatechol-dependent oxygen uptake. A determination of TCE oxidation activity revealed that BD2.101 had also lost this ability. Taken together, these findings suggest that the information for the IPB dioxygenase, the information for the 3-isopropylcatechol *meta*-cleavage enzyme, and the information for TCE oxidation are encoded by linear plasmid pBD2.

Hybridization of pBD2 with *tod* genes. In order to confirm the role of pBD2 in IPB degradation, a Southern hybridization was performed with the heterologous toluene dioxygenase genes from *P. putida* F1 carried on plasmid pDTG601 (40). The 3.5-kb *Eco*RI-*Bgl*II fragment of pDTG601 carries the genes encoding all four subunits of the toluene dioxygenase (*todC1C2BA*) except the last 50 bp of the reductase carboxy terminus (*todA*). The hybridization profile observed with *Nde*I- or *Not*I-digested pBD2 DNA with the pDTG601 DNA probe revealed a 26-kb *Nde*I hybridization signal and a 15-kb *Not*I

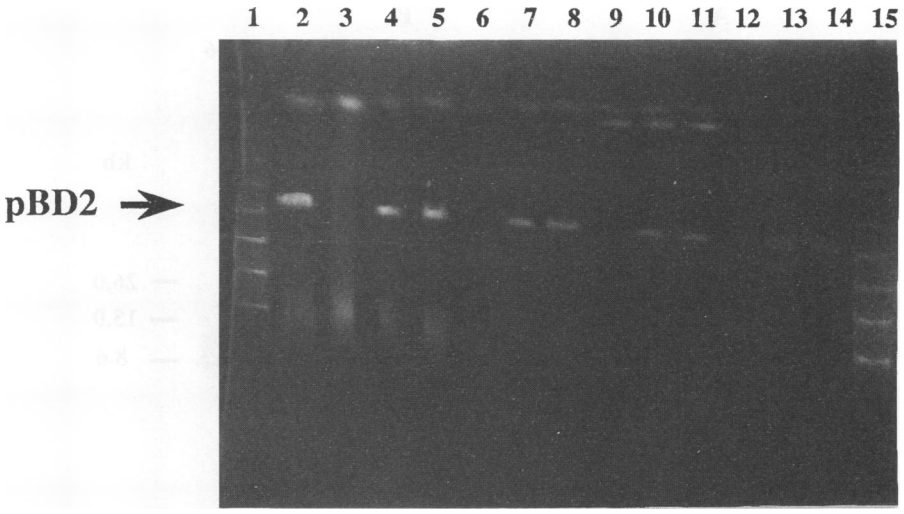

FIG. 4. CHEFE separation of lysates of donor, recipient, and transconjugant *Rhodococcus* strains. Lanes 1 and 15, λ ladder; lane 2, BD2.1 (donor); lane 3, BD2.101; lanes 4 and 5, transconjugants of BD2.101; lane 6, Ce3; lanes 7 and 8, transconjugants of Ce3; lane 9, cE2; lanes 10 and 11, transconjugants of cE2; lane 12, MS14; lanes 13 and 14, transconjugants of MS14.

hybridization signal (Fig. 5B, lane 3 and 4). The specific hybridization signal between the heterologous probe and pBD2 DNA was repeatedly confirmed. These results provide strong evidence that the IPB dioxygenase gene is located on linear plasmid pBD2.

Phenotypic changes resulting from the loss of pBD2. Various antibiotic resistance characteristics and heavy metal resistance characteristics of wild-type strain BD2 were compared with the characteristics of plasmidless mutant BD2.101. A reproducible difference in the growth responses of the two strains was observed in agar diffusion tests performed with sodium arsenite, as well as in tests performed with mercury chloride. BD2 wild-type cells were resistant to arsenite at concentrations up to 16 mM (Fig. 6A) and to mercury at concentrations up to 3 μM (Fig. 6B), whereas plasmidless strain BD2.101 did not grow in the presence of 6 mM sodium arsenite and 0.9 μM mercury chloride. The loss of pBD2 was accompanied by a 2.7-fold decrease in the level of arsenite resistance and a 3.3-fold decrease in the level of mercury resistance. Plasmid transfer experiments performed with BD2 wild-type cells and plasmidless strain BD2.101 revealed that both resistance characteristics, as well as the IPB^+ and TCE^+ characteristics, were simultaneously transmissible to BD2.101. A comparison of the arsenite and mercury resistance characteristics of BD2 wild-type cells and BD2.101 transconjugants revealed identical levels of resistance.

DISCUSSION

Gram-negative bacteria, such as members of the genera *Pseudomonas* and *Alcaligenes*, are well known as degraders of hydrocarbons and a large variety of aromatic compounds (19, 20). The genetic information for the catabolic pathways required is often encoded by transmissible circular plasmids. However, gram-positive bacteria, such as *Rhodococcus* spp., exhibit a broad substrate spectrum that includes hydrophobic compounds in particular (18). Not much is known about the role of plasmids in this context; most of the plasmids detected in gram-positive organisms confer resistance to antibiotics or other drugs. However, nicotine degradation in *Arthrobacter oxidans* and atrazine degradation in *Rhodococcus* strains are plasmid mediated (5, 6). In this paper we present evidence that in *R. erythropolis* BD2 the information for degradation of IPB and TCE, as well as the information for mercury resistance and arsenite resistance, resides on a transmissible linear plasmid that is 208 to 212 kb long. The experimental evidence is summarized below. (i) Although the ability to degrade IPB is unstable in *R. erythropolis*, circular plasmids were not detectable. (ii) Pulsed-field electrophoresis of cell lysates yielded a 210-kb DNA fragment which was not present in IPB^- strains but was present in IPB^+ transconjugants. (iii) The sum of the fragment sizes obtained by digestion of pBD2 with various restriction endonucleases was 208 to 212 kb and was consistent with the linear character of pBD2. Thus, pBD2 had properties similar to, for instance, plasmid pHG201, which encodes the genetic information for lithoautotrophic growth in *Nocardia opaca* MR11 (24). Experiments to identify terminal proteins in pBD2 have not been performed so far.

The mating experiments with *R. erythropolis* BD2 and other *R. erythropolis* strains proved that pBD2 is transmissible within one species. The lack of any transconjugants when other *Rhodococcus* strains were used as recipients could be explained in several ways, including no plasmid transfer from BD2 to these strains, the inability of other *Rhodococcus* strains to express the BD2 genes of the IBP degradation pathway, and a lack of growth of recipients on the downstream products generated by the plasmid-encoded enzymes.

TABLE 2. Conjugative transfer of plasmid pBD2 from *R. erythropolis* BD2.1 to various *R. erythropolis* strains

R. erythropolis recipient strain	No. of transconjugants per recipient[a]	No. of transconjugants per donor
BD2.101	3.8×10^{-5}	1.1×10^{-3}
cE2	3.5×10^{-5}	2.8×10^{-4}
Ce3	7.4×10^{-4}	1.2×10^{-2}
MS14	2.6×10^{-3}	7.3×10^{-2}

[a] Transconjugants were selected on streptomycin-containing mineral agar supplemented with IPB as the sole growth substrate. The streptomycin-sensitive donor spontaneously mutated to Str^r at a frequency of 1×10^{-8}.

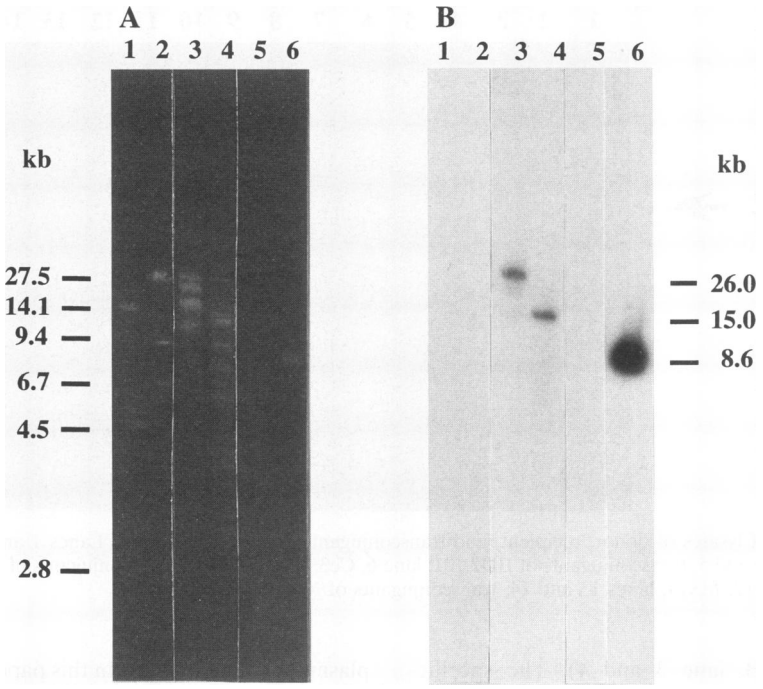

FIG. 5. (A) CHEFE of restriction endonuclease digests of pBD2. (B) Southern blot analysis performed with the radiolabeled 3.54-kb *Eco*RI-*Bgl*II fragment of pDTG601 carrying the toluene dioxygenase genes (*todC1C2BA*) of *P. putida* F1. Hybridization was performed under low-stringency conditions. Lanes 1, λ DNA digested with *Pst*I; lanes 2, λ DNA digested with *Hin*dIII; lanes 3, pBD2 digested with *Nde*I; lanes 4, pBD2 digested with *Not*I; lanes 5, pKK223-3 (negative control); lanes 6, *Eco*RI-linearized pDTG601 (positive control).

The spontaneous loss of pBD2 was accompanied by the loss of the IPB$^+$ characteristic of *R. erythropolis* BD2, and retransfer of pBD2 reconstituted this phenotype. This indicates that the IPB$^+$ trait of *R. erythropolis* BD2 is conferred by linear plasmid pBD2. On the basis of spectral, enzyme activity, inhibitor study, and hybridization data, we determined that the upper pathway of IPB degradation is initiated by an IPB dioxygenase, which gives 3-isopropylcatechol. Analogous to the oxidative breakdown of various alkylcatechols (15), 3-isopropylcatechol subsequently undergoes a *meta*-cleavage reaction by a 3-isopropylcatechol dioxygenase (Fig. 1). Thus, we suggest that the IPB pathway in *R. erythropolis* BD2 is analogous to pathways proposed for the degradation of IPB in *P. putida* RE204 (13, 14), as well as the breakdown of toluene (21), 2-phenylbutane (4), *t*-butylbenzene (7), and biphenyl (22).

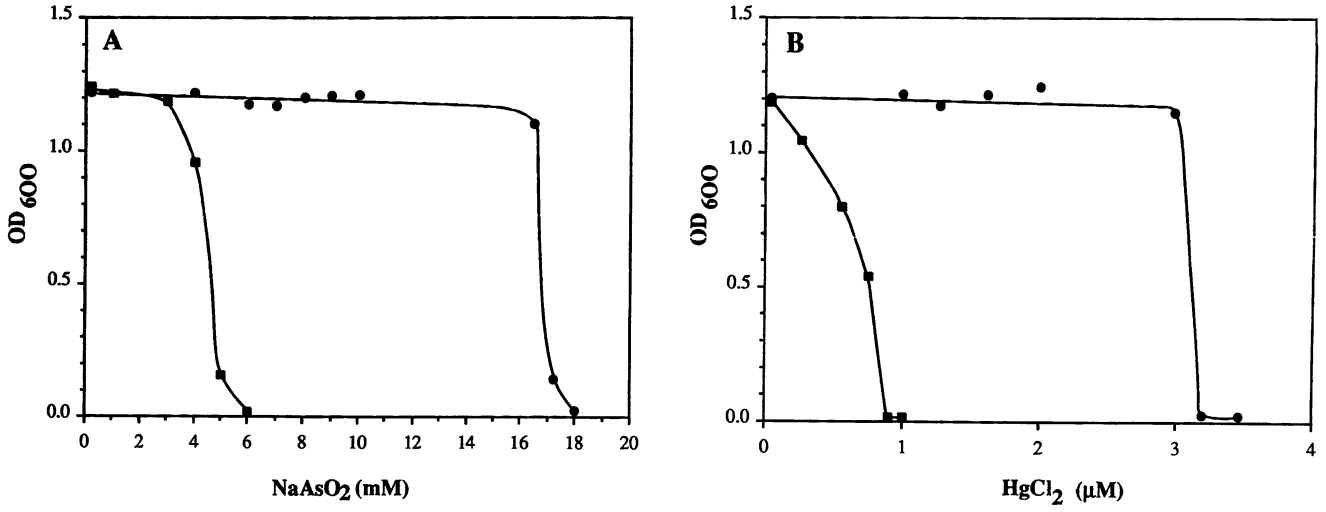

FIG. 6. Growth responses in liquid media of *R. erythropolis* wild-type strain BD2 (●) and spontaneous IPB$^-$ mutant strain BD2.101 (■). Aliquots (25 μl) of overnight cultures of the organisms were inoculated into 2.5-ml portions of mineral medium containing various concentrations of $NaAsO_2$ (A) or $HgCl_2$ (B). After incubation for 48 h at 30°C, the optical density at 600 nm (OD_{600}) was measured.

Plasmid-deficient mutants of strain BD2 were still able to grow on isobutyrate, and, therefore, we assumed that only the upper part of the IPB pathway, including the information required for the HOMODA-hydrolyzing activity that yields isobutyrate, is encoded by pBD2. The isobutyrate metabolism of BD2 has not been studied yet but may be similar to the metabolism of isobutyryl coenzyme A in *P. putida* (26).

Aromatic ring dioxygenases, such as toluene, naphthalene, phthalate, and benzene dioxygenases in gram-negative bacteria, all contain the same type of redox components and are functionally and evolutionary related (23, 27). As Southern hybridization of pBD2 with the toluene dioxygenase gene from *P. putida* F1 revealed a specific signal, we expect that the oxygenase initiating the IPB breakdown in *R. erythropolis* BD2 exhibits significant homology to the multicomponent toluene dioxygenase of *P. putida* F1. The homology to *P. putida* F1 genes is interesting, because no hybridization homology was found when three *meta*-cleavage dioxygenase genes of *Rhodococcus globerulus* P6 and the gene of the *meta*-cleavage enzyme of *P. putida* F1 were examined (3).

Native plasmids that mediate heavy metal characteristics, resistance such as arsenate, arsenite, cadmium, and thallium resistance characteristics, have been described in several nocardioform actinomycetes, including *Rhodococcus fascians* (11), *Rhodococcus* sp. strain H13-A (34), *R. erythropolis* ATCC 12674 (formerly *Nocardia aurantia*) (9), and two *Rhodococcus* sp. strains (formerly *N. opaca* MR11 and *N. opaca* MR22) (12, 24). The mechanism of cccDNA plasmid-encoded arsenite resistance has been studied intensively in *Staphylococcus aureus* and *E. coli* on a biochemical level as well as a molecular level and has been found to result from an ATPase-mediated accelerated efflux of arsenite (33). Recent investigations of the arsenite resistance operons in two *Staphylococcus* strains, however, revealed the absence of the *arsA* gene, which is known to encode an ATPase subunit with an ATP-binding motif of arsenite efflux ATPases (29). This led to an alternative hypothesis for energy-dependent arsenic efflux in gram-positive bacteria either without ArsA or with an *ars* system functioning with a chromosomally determined ArsA-ATPase subunit. Arsenite resistance in BD2 has not been investigated yet, but a *Staphylococcus* type of energy-dependent arsenic efflux mediated by a member of the proposed new family of ATPases has to be considered.

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