Cloning and Expression of the Transposable Chlorobenzoate-3,4-Dioxygenase Genes of Alcaligenes sp. Strain BR60

CINDY H. NAKATSU AND R. CAMPBELL WYNDHAM*

Institute of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

Received 15 March 1993/Accepted 26 July 1993

Growth on 3-chlorobenzoate was found to induce the enzymes of the protocatechuate meta ring fission pathway in Alcaligenes sp. strain BR60. The chlorobenzoate catabolic genes, designated cba, were localized to a 3.7-kb NotI-EcoRI fragment within the nonrepeated region of the composite transposon Tn5271. The cba genes were cloned onto two broad-host-range vectors and expressed in Escherichia coli and Alcaligenes sp. strain BR6024. In E. coli, expression of the cba genes with the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible tac promoter of the IncQ vector pMMB66HE resulted in the production of protocatechuate and chlorodihydroxybenzoate metabolites of 3-chlorobenzoate. Expression of this construct in one orientation resulted in the formation of two polypeptides 51 and 42 kDa in size. This result was confirmed by subcloning into pGEM3zf and then incorporating 1-25S-methionine into newly synthesized proteins, using the thermally regulated T7 polymerase-promoter system. Introduction of the NotI-EcoRI fragment into Alcaligenes sp. strain BR6024 (Cba+) lacked any functional 3-chlorobenzoate-degradative phenotype and resulted in the accumulation of protocatechuate and chlorodihydroxybenzoate intermediates. The data indicate that a two-component dioxygenase specified by Tn5271 oxidizes 3-chlorobenzoate at the 3,4- or 4,5-positions. This activity extends the range of pathways for chloroaromatic compounds known to be functional in the environment. The new pathway avoids the toxicity attributed to the accumulation of chlorocatechol metabolites in bacteria degrading chlorobenzoate.

The metabolism of chlorinated aromatic compounds by bacteria has been a subject of investigation for many years. The benefits of this work are beginning to be realized in bioremediation efforts worldwide. However, as we learn more about the metabolic diversity of bacteria, we are coming to the understanding that the biodegradation types for specific pollutants that have been well characterized represent only a small fraction of the genetic diversity that is to be found in the environment. This becomes evident when nucleic acid probes derived from specific catabolic operons are applied to collections of isolates from the environment. The 2,4-dichlorophenoxyacetate degradative genes provide a good example. The tdflA (2,4-dichlorophenoxyacetate monooxygenase) and tdflB (2,4-dichlorophenol hydroxylase) genes detected a significant fraction of the 2,4-dichlorophenoxyacetate-degrading bacteria in soils exposed to this herbicide, while probes prepared from the tdflCDEF (dichlorocatechol ring fission) operon failed to detect these bacteria (15). Similarly, the diversity of bacteria involved in chlorobiphenyl degradation in soils was not reflected in the number of isolates hybridizing with either the narrow- or the broad-substrate-range cpcb (2,3-dihydroxybiphenyl dioxygenase) genes of Pseudomonas putida (33). Findings such as these support the need for continuing basic research on the diversity of metabolic pathways in environmental isolates, combined with studies to assess the distribution and abundance of these pathways in natural and industrial environments.

When natural metabolic diversity fails to remove chemical contaminants, there is a need to develop bacterial consortia containing engineered strains (19). Genetic manipulation that uses both in vivo and in vitro techniques to expand the substrate range of bacteria requires a greater pool of cloned catabolic genes than is currently available. For example, one of the factors limiting the usefulness of chlorobiphenyl-degrading Comamonas testosterone B-356 and other polychlorinated biphenyl-degrading strains is the accumulation of the chlorobenzoate metabolites chlorocatechol and chloroanisole semialdehyde (27). These metabolites inhibit 2,3-dihydroxybiphenyl-1,2-dioxygenase, thereby reducing the rate of chlorobiphenyl metabolism. This constraint could be overcome by substituting an alternative pathway for chlorobenzoate metabolism in these organisms.

The enzymes involved in 3-chlorobenzoate degradation in P. putida have been well characterized, and their genetic organization within the cldaB operon has been determined (7, 25). However, when DNA from 3-chlorobenzoate-degrading Alcaligenes species and C. acidovorans isolates from a chemical landfill runoff was hybridized to a genetic probe derived from the cldaB operon of P. putida, no similarity was detected even at low stringency (37). This observation prompted our further investigations into the structure of plasmid pBRC60 that carries the 3-chlorobenzoate catabolic genes and our characterization of the catabolic transposon, Tn5271, in these isolates (22). We have also investigated the conjugal transfer and expression of the catabolic genes on this plasmid in a natural freshwater environment (8, 9).

In this report, we describe the subcloning of the dioxygenase genes of Tn5271, designated cba, onto mobilizable, broad-host-range expression vectors. When expressed in Escherichia coli, the cba genes give rise to two proteins. The cba dioxygenase directs oxygen to the 3,4- or 4,5-carbon positions of 3-chlorobenzoate. The significance of this new pathway is discussed with respect to chlorobenzoate and chlorobiphenyl degradation in nature.

* Corresponding author.
MATERIALS AND METHODS

Bacterial strains and growth conditions. *Alcaligenes* sp. strains BR60, BR40, and BR6024 and their growth conditions have been described previously (8, 37). *Alcaligenes* sp. strain BR6024 (rup, Cm 3Cba 3Ba ) was used as a recipient for mating experiments. *E. coli* strains and their plasmids have been described as follows: DH5α (13); CB1273(pMMB66EH) and CB1274(pMMB66HE) (10); HB101(pGS65) (29); CAG82(pGPl-2) (32); DH5α(pGM3ZI) (Promega Corp.); MM294(pRK2013) (6). *E. coli* strains were grown in TYE (1% tryptone, 1% yeast extract, 0.5% NaCl) with the addition of required antibiotics, ampicillin (50 µg·ml−1), chloramphenicol (30 µg·ml−1), kanamycin (50 µg·ml−1), and/or tetracycline (15 µg·ml−1), at 37°C. The temperature-sensitive *E. coli* CAG82(pGP1-2) host was grown at 30°C.

Analytical methods and chemicals. Oxygen uptake rates into whole cells were determined with an Orion polarographic oxygen electrode as described before (38), with 1 mM concentrations of substrates and a cell optical density of 0.5 at 600 nm. Whole-cell protein content was determined by the method of Bradford (3). Chemicals were supplied as follows: 3-chlorobenzoate, 3,4-dichlorobenzoate, and 3-chloro-4-hydroxybenzoate (Aldrich Chemical Co.); hydroxy- and dihydroxybenzoates (Sigma Chemical Co.); 3-chlorocatechol and 4-chlorocatechol (Helix Biotech Inc., Richmond, B.C., Canada).

For extraction of metabolic intermediates, *Alcaligenes* sp. strains BR60(pBRC60) and BR6024(pBRCN13a) were grown to late exponential phase on 3-chlorobenzoate, and the cells were centrifuged, washed in minimal medium A, and then resuspended in minimal medium A containing 1 mM 3-chlorobenzoate. *E. coli* DH5α(pBRCN13a) was grown on 100 ml of TYE-ampicillin medium containing 1 mM 3-chlorobenzoate. IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM final concentration) was added to cultures of strains carrying pBRCN13a in order to induce the genes. Cultures were incubated for 4 h and centrifuged, and the supernatants were extracted for gas chromatography-mass spectrometry (GC-MS) analysis. The medium was acidified to pH 2.5 and then extracted with an equal volume of ethyl acetate, dried with anhydrous sodium sulfate, and evaporated under dry N2, using a Büchi 461 rotary evaporating unit (Brinkmann Instruments, Westbury, N.Y.). The sample was treated with excess N,O-bis-trimethylsilyl-acetamide to generate trimethylsilyl derivatives for GC-MS analysis. A GC (Hewlett-Packard 5890 series II) with a 30m J&W DB5 column was operated at 120°C for 2 min, increasing to 300°C at 10°C/min. The GC was linked to an MS (Concept 2H; Kratos Analytical, Manchester, England) operated at 70-eV energy accelerating at 8 kV.

High-pressure liquid chromatography (HPLC) was used to measure the concentrations of 3-chlorobenzoate and selected metabolites for which standards were available, as described previously (38).

The formation of 2-hydroxy-4-carboxymuconic acid semialdehyde via the protocatechuate meta ring fission pathway in *Alcaligenes* strains was determined spectrophotometrically (30).

Construction of vector pBW13. Vector pBW13 was constructed from the IncP mobilizable plasmid pGS65 (29). The vector was modified by the removal of the EcoRI site and kanamycin resistance gene and the addition of a multiple cloning site (Fig. 1). The EcoRI site present on pGS65 was first eliminated by restriction with EcoRI and treatment with mung bean nuclease (Boehringer Mannheim) in order to create blunt ends and then religated with T4 DNA ligase (Boehringer Mannheim) by the methods outlined in Sambrook et al. (26). Single-stranded synthetic oligonucleotides were made on a Cyclone DNA synthesizer (Biosearch Inc.). The two oligonucleotides were complementary and contained the nucleotide sequence for the restriction enzyme sites in the following order: HindIII, BamHI, SacI, HpaI, EcoRI, PstI, BglII, and XhoI.

![FIG. 1. Construction of the IncP broad-host-range, mobilizable plasmid pBW13 from pGS65 (29) by deletion of the EcoRI site and addition of a synthetic polylinker. The nucleotide sequences of the complementary oligonucleotide strands used to deletion of the multiple cloning site are as follows: 5'-AGCTTGGATCCGAGCTCTTAAACGTATTGCGAGATC-3' and 3'-ACCTAGTCTGACGTTGCTAAAAGCCTGAGCT-5'. The duplex codes for the restriction enzyme sites HindIII, BamHI, SacI, HpaI, EcoRI, PstI, BglII, and XhoI.](http://aem.asm.org/.../FIG. 1. Construction of the IncP broad-host-range, mobilizable plasmid pBW13 from pGS65 (29) by deletion of the EcoRI site and addition of a synthetic polylinker. The nucleotide sequences of the complementary oligonucleotide strands used to deletion of the multiple cloning site are as follows: 5'-AGCTTGGATCCGAGCTCTTAAACGTATTGCGAGATC-3' and 3'-ACCTAGTCTGACGTTGCTAAAAGCCTGAGCT-5'. The duplex codes for the restriction enzyme sites HindIII, BamHI, SacI, HpaI, EcoRI, PstI, BglII, and XhoI.)
the vectors necessitated the conversion of the NotI end of the 5.2-kb NotI-HindIII fragment of Tn5271 into a HindIII end, using a HindIII synthetic linker (Boehringer Mannheim), by the method outlined in Ausubel et al. (1). Nested deletions of the constructed plasmid pBRCN5 containing the PsrI-HindIII fragment of Tn5271 were created by digestion from the HindIII site, using exonuclease III (Bethesda Research Laboratories), following the procedure of Sambrook et al. (26). The extent of the deletion was determined by restriction enzyme mapping of the clones.

Broad-host-range constructs in E. coli DH5α were mobilized into Alcaligenes sp. strain BR6024, using triparental mating of overnight cultures on filters supported on TYE agar plates. E. coli MM294 carrying the helper plasmid pRK2013 was mixed with equal volumes of the recipient and donor cells. Transconjugants were selected by dilution plating onto minimal medium A (38) with the addition of 4 mM 3-chlorobenzoate or 10 mM succinate and 0.1 mM tetrycline and the antibiotics tetracycline and chloramphenicol at the concentrations indicated above. To confirm growth and degradation of 3-chlorobenzoate by transconjugants, the increase in optical density at 600 nm of cultures grown with 3-chlorobenzoate as sole carbon source was measured, and substrate disappearance was determined by HPLC analysis as described above.

Gene expression. The expression of the catabolic genes of Tn5271 was tested by using the tac promoter system (10) and the T7 RNA polymerase-promoter system (32). In the first instance, restriction fragments from the norrepeated region of Tn5271 were cloned into pMMB66HE (10) for in vivo experiments in E. coli. Proteins expressed following induction with IPTG (1 mM final concentration) were resolved on sodium dodecyl sulfate (SDS)--polyacrylamide (7.5 and 12%) denaturing gels by the method of Laemmli (18). The gels were stained with 0.25% Coomassie brilliant blue 250 (U.S. Biochemicals Co.) in 40% methanol--10% glacial acetic acid, destained in the same solvent, and photographed.

Dioxygenase activity following in vivo expression in E. coli DH5α was determined with the pMMB66HE construct pBRCN13a that contained the HindIII-modified NotI-HindIII fragment in one orientation. One milliliter of overnight culture was added to two flasks containing 1 mM 3-chlorobenzoate in 100 ml of TYE--ampicillin medium. IPTG (1 mM final concentration) was added to one flask in order to induce the genes. Cultures were grown for 4 h and centrifuged, and the supernatant was analyzed by HPLC and then extracted for GC-MS analysis as described above.

For expression with the temperature-sensitive T7 RNA polymerase-promoter system, restriction fragments were cloned into pGEM3Zf (Promega Biotech Corp.) and then transformed into E. coli CA682/pGP1-2 (32). Transformants were labelled in vivo with 35S-methionine (50 μCi) in the presence of rifampin (150 μg·ml−1) to inhibit host transcription following a temperature shift from 30 to 42°C. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and stained as described above; then they were dried and exposed to X-ray film to distinguish 35S-labelled protein bands. Sizes of the protein bands were determined by comparison with protein standards of known molecular weight.

Expression of these genes in Alcaligenes strain BR6024 was carried out in transconjugants containing the nested deletion constructs pBRCN8 to -12. Cultures were first grown in medium A plus succinate, tryptophan, tetracycline, and chloramphenicol as indicated above and then inoculated into fresh medium with 1 mM 3-chlorobenzoate. The supernatant was analyzed by HPLC and then extracted for analysis by GC-MS as described above.

**RESULTS**

Oxygen uptake by Alcaligenes sp. strains BR60 and BR40. Oxygen uptake experiments with whole-cell suspensions of Alcaligenes sp. strain BR60 (Table 1) showed equivalent respiration rates when resting cells initially grown on 3-chlorobenzoate were exposed to 3-chlorobenzoate or protocatechuate (3,4-dihydroxybenzoate). Similar activities were observed when BR60 was grown on 3,4-dichlorobenzoate. Oxygen was taken up by cell suspensions exposed to 3- or 4-hydroxybenzoate at less than half the rate observed when cells were exposed to protocatechuate. There was no uptake of oxygen in the presence of either 3-chlorocatechol or 4-chlorocatechol. These results point to metabolic pathways involving either successive oxidations at the 3- and 4-positions or direct dioxygenase attack at the 3- and 4-positions. Low oxygen uptake rates for 3-chloro-4-hydroxybenzoate suggested that 3-chlorobenzoate was not subject to an initial hydroxylation in the para position and that 3,4-dichlorobenzoate was unlikely to be subject to dehalogenation in the para position (28). The absence of oxygen uptake by chlorobenzoate-grown cells in the presence of gentisate (2,5-dihydroxybenzoate) suggested that an initial hydroxylation catalyzed by Tn5271-encoded enzymes did not occur at the meta position (16). In contrast, cells of benzoate-grown strains BR60 and BR40 (a Tn5271 deletion derivative of BR60 [22]) respired oxygen in the presence of both gentisate and protocatechuate, suggesting that this pathways coexist in Alcaligenes sp. strain BR60, as they do in C. acidovorans (34). Respiration measurements therefore suggest that the host expresses both gentisate and protocatechuate pathways but that Tn5271 codes for the direct dioxygenation of chlorobenzoate to yield protocatechuate.

**TABLE 1. Oxygen uptake rates by Alcaligenes sp. strains BR60 and BR40 with various carbon sources**

<table>
<thead>
<tr>
<th>Test substrate</th>
<th>O2 uptake (μmol min−1 mg of protein)</th>
<th>BR60</th>
<th>BR40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>17</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>3-Hydroxybenzoate</td>
<td>7</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>4-Hydroxybenzoate</td>
<td>4</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Gentisate</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Protocatechuate</td>
<td>3</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>3-Chlorobenzoate</td>
<td>0</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>3,4-Dichlorobenzoate</td>
<td>0</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4-Chlorocatechol</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3-Chloro-4-hydroxybenzoate</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

* No oxygen uptake occurred in the presence of 2-chlorobenzoate, 2,3-, 2,4-, 2,6-, and 3,5-dihydroxybenzoate, or 2,3-, 2,4-, 2,5-, 2,6-, and 3,5-dichlorobenzoate.

* Growth substrates are benzoate (Ba), 3-chlorobenzoate (3Cba), and 3,4-dichlorobenzoate, (3,4Dcb).  
* BR40 is a BR60 derivative lacking Tn5271 (22).

2,3-Dihydrobenzoate.

3,4-Dihydroxybenzoate.
sufficient to restore 3-chlorobenzoate degradation abilities to the 3-chlorobenzoate-deficient mutant BR6024. The smallest restriction enzyme fragment containing genes necessary for 3-chlorobenzoate degradation was the 5.2-kb NotI-HindIII fragment cloned in pBRCN6. When the internal Scal fragment was removed from the PstI-HindIII clone pBRCN5 and religated to give pBRCN7, the clone was no longer able to restore 3-chlorobenzoate degradation. Therefore, part or all of the DNA within the Scal fragment was required. The absence of unique restriction sites on the right side of

![Diagram](http://aem.asm.org/)

**FIG. 2.** Cloning of the 3-chlorobenzoate catabolic genes of Tn5271. (A) Physical map of Tn5271 showing restriction enzyme sites that were used in subcloning: BsuEII (B), EcoRI (E), EcoRV (Ec), HindIII (H), HpaI (Hp), NotI (N), and PstI (P). The locations of the IS1071 inverted repeats marking the extent of nonrepeated DNA within the transposon are indicated by filled triangles. The kilobase scale begins at the first nucleotide in the left IS1071 copy (22). (B) Restricted Tn5271 fragments were cloned onto pBW13 and mated into Alcaligenes sp. strain BR6024 in order to determine restoration of 3-chlorobenzoate degradation (+) or no growth (-). The pBRCN6 NotI-HindIII fragment was modified for cloning by the conversion of the NotI site into a HindIII site by the addition of a synthetic polynucleotide. This fragment was also cloned in both orientations into the expression vector pMMB66HE, creating pBRCN13a and -13b, and into pGEM3Zf, creating pBRCN16a and -16b. (C) Clones were created by manipulating the internal Scal fragment to create pBRCN7 or by nested deletions from the HindIII site to create pBRCN8 to -12. The Scal-HindIII fragment was cloned onto pGEM3Zf to create clone pBRCN17.
pBRCN6 made it necessary to employ nested deletions in order to localize the coding regions. Deletions of DNA to the right of the EcoRI site at the 8.1-kb position enabled growth (Fig. 2C). However, the colonies initially formed on selection plates after mating turned black. Repeated subculturing of these clones restored the normal off-white colony color and normal growth when 3-chlorobenzoate was used. Growth experiments and HPLC analyses confirmed that the clones containing constructs pBRCN1, -2, -5, -6, -8, -9, and -10 (Fig. 2B and C) completely utilized 3-chlorobenzoate for growth. Clones of cutbacks into the EcoRI fragment between the 7- and 8.1-kb region of Tn5271 (pBRCN11 and -12; Fig. 2C) were not able to degrade 3-chlorobenzoate.

**Protein expression.** Two plasmids, pBRCN13a and -b, were constructed by ligating the HindIII linker-modified NotI-HindIII fragment (4.6 to 9.8 kb on Tn5271) from pBRCN6 into the expression vector pMMB66HE in opposite orientations. Following IPTG induction of the pBRCN13a clone, two unique protein products approximately 51,000 and 42,000 in molecular weight were resolved in an SDS-PAGE (7.5%) gel (Fig. 3a). No unique proteins were resolved in IPTG-induced clones carrying plasmid pBRCN14 (with the EcoRI-NotI fragment from 8.1 to 10.5 kb cloned in the same orientation as pBRCN13a) or pBRCN13b (with the NotI-HindIII fragment from 4.6 to 9.8 kb in the opposite orientation).

Because these cells contained many additional soluble proteins which may have obscured products expressed from the tac promoter, the results were confirmed with the T7 RNA polymerase-promoter expression system (32). Plasmids pBRCN16a and -b were constructed by ligating the HindIII linker-modified NotI-HindIII fragment (4.6- to 9.8-kb positions) in opposite orientations into pGEM3Zf. The ScaI-HindIII fragment (5.7- to 9.8-kb positions of Tn5271) was also cloned into pGEM3Zf to give pBRCN17. These constructs were then transformed into CAG82 (pGPl-2) at 30°C, and expression was induced at 42°C. The pBRCN16a construct produced two unique 35S-methionine-labelled protein products approximately 51 and 42 kDa which were resolved in an SDS-PAGE (7.5%) gel (Fig. 3b). No unique proteins were observed in lysates of temperature-induced clones transformed with the vector pGEM3Zf alone or with pBRCN16b with the NotI-HindIII fragment cloned in the opposite orientation. The pBRCN17 clone carrying the ScaI-HindIII fragment from the right side of the coding region (5.7- to 9.8-kb positions of Tn5271) revealed a single protein product 42 kDa in size.

Restriction mapping of all plasmids expressing the 51- or 42-kDa protein or both (pBRCN13a, pBRCN16a, and pBRCN17) indicated that translation proceeded from left to right in the orientation of Tn5271, as shown in Fig. 2A.

**In vivo expression and degradation of 3-chlorobenzoate.** The presence of intermediates of 3-chlorobenzoate metabolism in *Alcaligenes sp.* strain BR60 and subclones of the *cba* genes was determined by GC-MS (Fig. 4). Protocatechuate (compound II) was found in the culture medium of 3-chlorobenzoate-grown BR60. Also present were intermediates with mass and fragmentation patterns equivalent to those of 1-carboxy-3-chloro-3,4-dihydroxybenzena-1,5-diene (compound I) and 1-carboxy-3-chloro-4,5-dihydroxybenzena-2,6-diene (compound III). In the absence of standards, mass spectra were assigned to these two metabolites on the basis of the expected stability of the chlorine substituent. Compound III would be expected to retain the chlorine atom isotope signature during fragmentation. This was observed for the ions at m/e 406, 389, 301, 289, 257, and 227, as shown in the assigned spectrum (Fig. 4b, compound III). Compound I should be very unstable, exhibiting a fragmentation pattern showing the loss of the chlorine isotope signature. This was observed for all major ions, as shown in the assigned spectrum (Fig. 4b, compound I).

The metabolites formed in *Alcaligenes sp.* strain BR6024 carrying clones pBRCN5, pBRCN9, and pBRCN10 (Fig. 2C) revealed similar results. When cells induced for 3-chlorobenzoate metabolism were exposed to 1 mM 3-chlorobenzoate, the substrate was completely metabolized. Metabolites that accumulated in the supernatants of these clones had mass...
FIG. 4. Proposed metabolic pathway encoded by the cba genes of TnS271 and identification of intermediates by GC-MS. (a) Proposed metabolic pathway for 3-chlorobenzoate degradation by *Alcaligenes* sp. strain BR60(pBRC60[TnS271]). The substrate was oxygenated in the 3,4-carbon positions to yield 1-carboxy-3-chloro-3,4-dihydroxycyclohexa-1,5-diene (compound I) or in the 4,5-carbon positions to yield 1-carboxy-3-chloro-4,5-dihydroxycyclohexa-2,6-diene (compound III). Dehydrogenase activity results in either the loss of HCl and re-aromatization to protocatechuate (compound II) or loss of H2O and re-aromatization to chloroprotocatechuate (compound IV). Final degradation of protocatechuate proceeded via the meta ring fission pathway, as indicated by the formation of 2-hydroxy-4-carboxymuconic semialdehyde from protocatechuate (30). The chloroprotocatechuate metabolite (compound IV) was metabolized further; however, the nature of the metabolites is unknown. (b) Mass spectra of the trimethylsilyl derivatives of compounds I, II, III, and IV. The metabolites were extracted from culture supernatants following the incubation of *Alcaligenes* sp. strain BR60(pBRC60), *Alcaligenes* sp. strain BR6024(pBRCN13a), or *E. coli* DH5α(pBRCN13a) with 3-chlorobenzoate.
The protocatechuate meta ring fission pathway enzyme 3,4-dihydroxybenzoate-4,5-dioxygenase was induced in each of the above Alcaligenes sp. strain BR6024 clones following growth on 3-chlorobenzoate.

The pBRCN11-containing clone did not grow when 3-chlorobenzoate was the sole carbon source (Fig. 2C), but 3-chlorobenzoate was completely degraded. The major metabolite formed had a fragmentation pattern identical to that of the chlorodihydrobenzoate metabolite formed by the pBRCN8, pBRCN9, and pBRCN10 clones. In addition, metabolites with mass spectra identical to those of the chlorodihydrodiols (Fig. 4, compounds I and III) were formed. There was no degradation of 3-chlorobenzoate by Alcaligenes sp. strain BR6024 carrying the pBRCN12 clone.

E. coli clones containing the construct pBRCN13a accumulated a purple-black metabolite when induced with IPTG in the presence of 3-chlorobenzoate. This color did not form in the absence of IPTG or in clone pBRCN13b containing the construct in the opposite orientation. GC-MS analysis revealed that 3-chlorobenzoate was converted almost stoichiometrically to chlorodihydroxybenzoate (Fig. 4, compound IV), with minor amounts of protocatechuate and chlorohydroxybenzoate formed.

**DISCUSSION**

The results for oxygen uptake by 3-chlorobenzoate-grown Alcaligenes sp. strain BR60 and the GC-MS analysis of metabolites formed during metabolism indicate that degradation occurs by means of a dioxygenase activity directed at distal (3,4 or 4,5) carbon positions of 3-chlorobenzoate. The four key intermediates expected for this pathway (Fig. 4) were detected in culture supernatants of either the parent strain or the subclones. Since the natural host is able to grow on both benzoate and protocatechuate but not chlorobenzoate, in the absence of Tn5271, the cba degradative genes coding for chlorobenzoate dioxygenase must have been carried on the transposon. This was confirmed by subcloning DNA fragments from the nonrepeated region of Tn5271 onto broad-host-range, mobilizable vectors in order to restore the 3Cbα+ phenotype to a 3Cbα− Alcaligenes host.

The data presented here localize the genes required for 3-chlorobenzoate degradation to a 3.7-kb region of Tn5271 flanked by NotI and EcoRI sites (Fig. 2A). No other phenotype has been associated with Tn5271, so the function, if any, of the remaining 7 kb of DNA in the nonrepeated region of the transposon remains unknown.

Expression of the genes encoded on the Tn5271 NotI-HindIII fragment, using the construct pBRCN13a in E. coli, caused the accumulation of a major product with the mass spectrum of a chlorodihydrobenzoate. Chlorodihydrobenzoate isomers were not available as commercial standards so the absolute regiochemistry of the metabolite could not be determined. The observation that the medium of clones accumulating this metabolite turned purple and then black indicates that the metabolite is a vicinal diol subject to autooxidation.

When the NotI-HindIII fragment of Tn5271 was expressed in Alcaligenes sp. strain BR6024, using broad-host-range pBRCN5 and -6 clones (Fig. 2B), both protocatechuate and chlorodihydroxybenzoate intermediates were formed in the medium (Fig. 4, compounds II and IV). However, the chlorodihydroxybenzoate metabolite (compound IV) was not detected in the natural host, BR60, suggesting that either there is a difference in the activity of the dioxygenase when it is expressed from subclones in E. coli and Alcaligenes sp. strain BR6024, compared with its expression in the wild-type Alcaligenes sp. strain BR60, or, more likely, the activity is similar in these hosts but the chlorodihydrobenzoate intermediate is rapidly metabolized in the Alcaligenes sp. strain BR60 host. The literature on the regioselectivity of dioxygenases suggests that a degree of relaxed specificity is to be expected. This is true of the activity of Pseudomonas sp. strain B13 and Alcaligenes eutrophus B9 benzoate-1,2-dioxygenases acting on 2-fluorobenzoate (4) and also of the 2-chlorobenzoate-1,2-dioxygenase of Pseudomonas sp. strain B300 (31). The cloned dioxygenase of Tn5271 most probably introduces oxygen at either the 3,4-carbons or the 4,5-carbons of 3-chlorobenzoate (Fig. 4). We speculate that 5-chloroprotocatechuate (compound IV) is the identity of the major metabolite in E. coli.

The formation of protocatechuate in the host Alcaligenes sp. strain BR60 and in the Tn5271 subclones induces the meta ring fission pathway for this intermediate. The key enzyme in this pathway, protocatechuate-4,5-dioxygenase, is expressed from a chromosomal gene, as indicated by the growth of Alcaligenes sp. strains lacking pBRC60 and Tn5271 on p-hydroxybenzoate and protocatechuate and accumulation of the meta ring fission product. The chlorodihydroxybenzoate metabolite is also further metabolized since it accumulates only transiently in BR6024 carrying the cloned dioxygenase genes. Kersten et al. (17) showed that the meta ring fission enzyme protocatechuate-4,5-dioxygenase isolated from C. testosteroni is functional on substituted protocatechuates containing chlorine or methyl groups in the 5-carbon position. Alcaligenes sp. strain BR60 is closely related to members of the family Comamonadaceae (35) and expresses a similar enzyme activity following induction by 3-chloro- or 3,4-dichlorobenzoate. Therefore, it is probable that the protocatechuate meta ring fission enzyme in Alcaligenes sp. strain BR60 opens the ring of 5-chloroprotocatechuate (Fig. 4). Until the coding region is isolated, we cannot say how active this pathway is in Alcaligenes sp. strain BR60 or what the fate of the chlorine substituent is.

All prokaryotic dioxygenase reactions so far examined yield cis-dihydriodiol intermediates, and it is known that these intermediates are unstable (12). Spontaneous loss of either H₂O or HCl from a chlorodihydriodiol structure can occur, resulting in a return to aromaticity. We speculate that the major chlorodihydroxybenzoate product formed in the subclones accumulates as the result of chemical dehydration of the dihydriodiol intermediate(s) during ethyl acetate extraction of metabolites from the culture supernatants at acidic pH. Others have observed spontaneous dehydration under acidic conditions of chlorodihydriodiol and dihydriodiol metabolites to yield phenols (11, 14). In vivo, cis-diol structures have been found to re-aromatize both enzymatically and spontaneously. Enzymatic re-aromatization of cis-diol structures to catechol is catalyzed by specific dehydrogenases during benzoate and 3-chlorobenzoate degradation by P. putida and Acinetobacter calcoaceticus (23). However, the conversion of 4-sulfobenzoate to protocatechuate following the action of a 4-sulfobenzoate-3,4-dioxygenase is reported to occur spontaneously in C. testosteroni (20). The cba genes evidently encode a dehydrogenase in the region of...
Tn5271 around the EcoRI site at 8 kb (Fig. 2A) that is required for re-aromatization of the dihydrodiols to protocatechuic and 5-chloroprotocatechuic. Our evidence for this comes from the expression of these genes in clone BR6024(pBRCN11). This clone was not able to grow on 3-chlorobenzoate but produced a metabolite with a mass spectrum equivalent to that of a chlorohydrodiol of cyclohexadiene-1,4-carboxylate (compound I or III), the expected product of a chlorobenzoate dioxygenase activity directed at the 3,4- or 4,5-carbon positions of 3-chlorobenzoate. The major metabolite accumulating in the medium of the pBRCN11 clone was a chlorohydroxybenzoate. As discussed above, this is the expected product of spontaneous dehydration of the intermediate chlorohydroxydiols during extraction.

The clone carrying pBRCN12 also did not grow on 3-chlorobenzoate, and in this clone 3-chlorobenzoate remained unchanged. This suggests that information in the 7-kb region is essential for dioxygenase activity.

Two unique protein bands corresponding to 51 and 42 kDa were distinguished in the pBRCN13a and pBRCN16a clones by SDS-PAGE (Fig. 3). The pBRCN17 clone produced only the 42-kDa protein, indicating that the gene producing the 51-kDa protein is transcribed first, in the region between 4.5 and 5.7 kb. Transcription proceeds in the left-to-right direction for the orientation of Tn5271, as shown in Fig. 2A. Previous studies have demonstrated that dioxygenases that form cis-diols are composed of two or three different polypeptides that make up a short electron transport chain with a flavoprotein reductase, a ferredoxin-like unit (with these two fused in two-component systems), and a terminal non-heme iron dioxygenase (5, 12, 24). The two-component dioxygenase systems for 4-sulfobenzoate-3,4-dioxygenase (20) and 4-chlorophenylacetate-3,4-dioxygenase (21) appear to have some similarity to the cba gene products in both polypeptide size and regioselectivity. Both are composed of approximately 50-kDa oxygenases and 36-kDa reductases, and both introduce oxygen at the distal 3,4-carbon positions of aromatic rings containing carboxyl groups linked to the 1-carbon position. We speculate that the 51- and 42-kDa polypeptides expressed from the cba genes are involved in a two-component dioxygenase system. However, this observation leaves us with an interesting problem. The results with cutback clones pBRCN11 and pBRCN12 suggest that a 42-kDa dihydrodiol dehydrogenase is coded in the 8-kb region of Tn5271 and that this gene is required for growth on 3-chlorobenzoate. This leaves only the 51-kDa protein as a dioxygenase component. Since the smallest dioxygenase complexes contain two components, either this dioxygenase is unusual or a component is missing in our protein analysis. Further studies are in progress to determine the nucleotide sequence of Tn5271 and the number of proteins encoded in this region.

During the course of these investigations, we found that Alcaligenes sp. strain BR60 is capable of growth on 3,4-dichlorobenzoate (Table 1). This compound was not used previously for enrichment. The removal of two chlorine substituents during the degradation of 3,4-dichlorobenzoate is unlikely to occur in a concerted dioxygenase attack at the 3,4-carbons. Instead, we speculate that non-specificity in the regioselectivity of dioxygenase attack could account for growth on 3,4-dichlorobenzoate. Initial attack at the 4,5-carbon positions and elimination of HCl during dehydrogenase-catalyzed re-aromatization would form 5-chloroprotocatechuic (compound IV), with subsequent removal of the remaining chlorine substituent occurring during ring fission. We are currently investigating this possibility.

The regioselectivity of the Tn5271 chlorobenzoate dioxygenase is distal to the carboxyl group and is therefore unlike the chlorobenzoate dioxygenases that initiate chlorobenzoate metabolism via chlorocatechols in the fluorescent pseudomonads and A. eutrophus. This new pathway has some potential for use in extending the chlorobenzoate catabolic activity of chlorobenzenyl-degrading bacteria. The reason for this is twofold. First, many chlorobenzenyl-degrading bacteria contain broad-substrate-specificity benzoate-1,2-dioxygenases that convert chlorobenzoate to chlorocatechols. These metabolites, and the chloromuconate semialdehydes formed from them, are known to inhibit the activity of chlorobenzenyl-degrading bacteria (27). It may be possible to avoid inhibition by eliminating the benzoate-1,2-dioxygenase genes of these bacteria by mutagenesis, replacing them with the cloned cba genes of Tn5271. Second, chlorobenzenyl congeners substituted in the 3,4- and 3',4'-carbon positions (meta and para) are known to be difficult to degrade compared with congeners substituted in the ortho and para positions (2). One reason for this, aside from regioselectivity in biphenyl dioxygenases, may be the inhibitory effects of 3,4-dichlorobenzoate metabolites of these meta- and para-substituted biphenyls. The cba genes of Tn5271 may be useful in extending the activity and substrate range of biphenyl degraders.

In this report, we demonstrate that Tn5271 carries genes for 3-chlorobenzoate-3,4-(4,5)-dioxygenase within a 3.7-kb Nosi-EcoRI fragment. These genes are now cloned onto the IncP and IncQ mobilizable, broad-host-range vectors pBW13 and pMMB66HE, respectively. Dioxygenase activity can be expressed in E. coli under the control of tac or T7 promoters. The new pathway extends the range of known chlorinated aromatic dioxygenases and may be a useful addition to the repertoire of cloned aromatic pathways for bioremediation.

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REFERENCES

CLONING OF cba GENES OF ALCALIGENES SP.


