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Cloning, Nucleotide Sequence, and Expression of the Plasmid-Encoded Genes for the Two-Component 2-Halobenzoate 1,2-Dioxygenase from *Pseudomonas cepacia* 2CBS

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The two-component nonheme iron dioxygenase system 2-halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS catalyzes the double hydroxylation of 2-halobenzoates with concomitant release of halogenide and carbon dioxide, yielding catechol. The gene cluster encoding this enzyme, *cbdABC*, was localized on a 70-kbp conjugative plasmid designated pBAH1. The nucleotide sequences of *cbdABC* and flanking regions were determined. In the deduced amino acid sequence of the large subunit of the terminal oxygenase component (CbdA), a conserved motif proposed to bind the Rieske-type [2Fe-2S] cluster was identified. In the NADH: acceptor reductase component (CbdC), a putative binding site for a chloroplast-type [2Fe-2S] center and possible flavin adenine dinucleotide- and NAD-binding domains were identified. The *cbdABC* sequences show significant homology to *benABC*, which encode benzoate 1,2-dioxygenase from *Acinetobacter calcoaceticus* (52% identity at the deduced amino acid level), and to *xylXYZ*, which encode toluate 1,2-dioxygenase from *Pseudomonas putida* mt-2 (51% amino acid identity). Recombinant pKT231 harboring *cbdABC* and flanking regions complemented a plasmid-free mutant of wild-type *P. cepacia* 2CBS for growth on 2-chlorobenzoate, and it also allowed recombinant *P. putida* KT2440 to metabolize 2-chlorobenzoate. Functional NADH:acceptor reductase and oxygenase components of 2-halobenzoate 1,2-dioxygenase were enriched from recombinant *Pseudomonas* clones.

The initial step in the degradation of aromatic compounds by aerobic bacteria frequently is a dihydroxylation of the substrate, catalyzed by a multicomponent nonheme iron dioxygenase system. These dioxygenases consist of two or three soluble proteins that constitute an electron transport chain, transferring electrons from NADH via flavin and [2Fe-2S] redox centers to the site of dioxygen activation (15, 29). 2-Halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS is a two-component enzyme which catalyzes the double hydroxylation of 2-halobenzoates with concomitant release of halogenide and carbon dioxide, yielding catechol (11, 12) (Fig. 1). It consists of an NADH:acceptor reductase component containing both flavin adenine dinucleotide (FAD) and chloroplast-type [2Fe-2S] ferredoxin and a Rieske-type [2Fe-2S]-containing oxygenase component (12). Thus, 2-halobenzoate 1,2-dioxygenase belongs to the oxygenase system class IB as defined by Batie et al. (2).

Two isofunctional class IB dioxygenases that have been investigated thoroughly are the chromosomally encoded benzoate 1,2-dioxygenase and the TOL plasmid pWW0-encoded isofunctional toluate 1,2-dioxygenase. These enzyme systems were shown to be highly homologous (16, 33, 34). Our studies of the biochemical properties of 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS suggested a putative similarity of this enzyme system to benzoate- and toluate 1,2-dioxygenase (12). 2-Halobenzoate 1,2-dioxygenase from strain 2CBS exhibited a very broad substrate specificity, but benzoate analogs with electron-withdrawing substituents at the *ortho* position were transformed preferentially. The recently described three-component *ortho*-halobenzoate 1,2-dioxygenase from *Pseudomonas aeruginosa* 142 also oxidized a wide spectrum of halosubstituted benzoates. However, contrary to the results for the two-component enzyme from *P. cepacia* 2CBS, the enzymic rates increased with increasing size and decreasing electronegativity of the substituent (44). In contrast to these two distinct halobenzoate 1,2-dioxygenases, benzoate 1,2-dioxygenases show narrow substrate specificity, with little, if any, oxidation of substituted benzoates (42, 63). The isofunctional toluate 1,2-dioxygenase, although possessing a much broader substrate specificity, forming *cis*-dihydrodiols from many *meta*- and *para*-substituted benzoates, also does not transform 2-halobenzoates (17, 62).

Genes encoding enzyme systems that catalyze the conversion of benzoate to cyclohexadiene 1,2-diol-1-carboxylic acid have been isolated from the chromosomes of *Acinetobacter calcoaceticus* (*benABC*) (34, 35), *P. aeruginosa* PAO1 (20), and *Pseudomonas putida* PPO200 (20) and from the TOL plasmid pWW0 of *P. putida* mt-2 (*xylXYZ*) (16, 17). In order to compare 2-halobenzoate 1,2-dioxygenase with other dioxygenase systems, especially with benzoate and toluate 1,2-dioxygenases, we cloned and sequenced the three genes (*cbdABC*) encoding the two subunits of the terminal oxygenase and the NADH: acceptor reductase component. The conjugatability of the degradative plasmid (designated pBAH1) that was found to carry the *cbdABC* genes was examined, and the nucleotide sequences of the structural genes *cbdABC* and flanking regions were determined. Putative cofactor-binding domains were identified in the deduced amino acid sequences of CbdABC, and the relatedness of CbdABC, BenABC, and XylXYZ was investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. cepacia* 2CBS, which had been isolated by selective enrichment on 2-chlorobenzoate (2-cba) as the sole source of carbon and energy, has been described previously (11). *P. cepacia* 2CBSM4 is a plasmid-free mutant of strain 2CBS (this work). *P. cepacia* 2CBSM1 Rif^r, a plasmid-free

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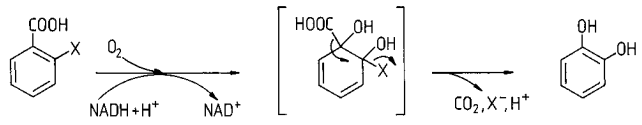

FIG. 1. Reactions catalyzed by 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS. X represents F, Cl, Br, or I.

mutant of strain 2CBS (this work), *P. putida* KT2440 Rif^r, and a Rif^r mutant of the 4-chlorophenylacetate (4-cpa)-degrading *Pseudomonas* sp. strain CBS3 (27) were used as recipient strains for mating experiments. *Escherichia coli* TG1 (14), *E. coli* JM105 (64), and *E. coli* MV1190 (60) and *P. putida* KT2440 (1) were used as host strains. *E. coli* S17-1 contains a modified chromosomally integrated RP4 plasmid with the ability to mobilize RSF1010-based vectors to other hosts (52).

Cloning vectors pUC18, pUC19, M13mp18, and M13mp19 (38, 59), expression vector pKK223-3 (4), and broad-host-range vector pKT231 (1) have been described previously.

pBAH1 is a 70-kbp degradative plasmid from *P. cepacia* 2CBS (this work).

Media and growth conditions. *P. cepacia* 2CBS was routinely grown at 30°C in mineral salts medium, with 2-cba as the sole carbon source, as described previously (12). For the preparation of plasmid DNA, an overnight culture (30°C) of strain 2CBS in Luria-Bertani (LB) medium (45) was used. Recombinant *E. coli* MV1190 clones were routinely grown at 37°C in 2× TY medium or H broth (32). Ampicillin, kanamycin, and rifampin were added to the media at final concentrations of 100, 50, and 300 μg/ml, respectively.

Isotopes, enzymes, and chemicals. α-^{35}S-dATP was obtained from Amersham (Braunschweig, Germany). Restriction endonucleases, T4 DNA ligase, shrimp alkaline phosphatase, the T7 sequencing kit, and Deaza T7 sequencing mixes were from Pharmacia (Freiburg, Germany). The GenecleanII kit was from Dianova (Hamburg, Germany), and the digoxigenin (DIG) oligonucleotide 3′-end labeling kit and DIG luminescent detection kit were purchased from Boehringer (Mannheim, Germany).

DNA isolation and DNA manipulations. The degradative plasmid pBAH1 from wild-type *P. cepacia* 2CBS and recombinant plasmid DNA from *Pseudomonas* clones were isolated by the method of Kieser (23). When necessary, pBAH1 DNA was purified by CsCl-ethidium bromide gradient centrifugation. For analytical purposes, recombinant plasmid DNA from *E. coli* clones was isolated by the alkaline lysis method (45). Genomic DNA was prepared as described by Davis et al. (7). Agarose gel electrophoresis, DNA restriction, treatment with alkaline phosphatase, and DNA ligation were done by standard procedures (45). DNA fragments were isolated from agarose gels with the GenecleanII kit as recommended by the supplier. DNA was transferred from agarose gels to charged nylon membranes (Hybond-N; Amersham) as described by the manufacturer.

Elimination of plasmid pBAH1. *P. cepacia* 2CBS was subcultured repeatedly in LB medium. Prior to each transfer, cells were grown to the stationary phase of growth. After the 10th transfer, aliquots of the subculture were spread onto LB agar plates. Individual colonies were transferred onto mineral salts medium containing 3.5 mM 2-cba as the sole carbon source and onto LB plates. Mutants which were unable to use 2-cba as the sole carbon source were examined for the presence of plasmid pBAH1.

Hybridization. On the basis of the N-terminal amino acid sequence of the reductase component of the 2-halobenzoate 1,2-dioxygenase system (12), the mixed oligonucleotide TTC GAG(A) GAC(T) GAC(T) GTC(G) ACC(G) TAC TTC ATC was synthesized. Labeling of the oligonucleotide with the DIG oligonucleotide 3′-end labeling kit was performed as described by the supplier. Hybridizations were carried out at 54°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (45). Blots were stringently washed twice in 2× SSC containing 0.1% SDS at 54°C for 5 min and then once in 0.5× SSC containing 0.1% SDS at 30°C for 5 min. Immunological detection was performed with the DIG luminescent detection kit.

Transformation and conjugation. *E. coli* host strains were transformed with recombinant plasmid DNA as described by Mandel and Higa (26). Mating experiments and plasmid mobilization from *E. coli* S17-1 donors to *Pseudomonas* recipients were performed by standard procedures (3, 13).

DNA sequencing and sequence analysis. The strategy of DNA sequencing is shown in Fig. 2. Procedures described in the [α^{35}S]T7 Sequencing Kit instruction manual (Pharmacia) were followed for the transfection of *E. coli* MV1190 with M13 phage DNA and for the preparation of single-stranded DNA sequencing templates from selected phage plaques. All coding regions were sequenced in both directions by the dideoxy chain termination method (46). Sequencing reaction mixtures were electrophoretically separated on 6% polyacrylamide gels with 42% urea in Tris-borate-EDTA buffer. Computer-assisted comparative sequence analysis was done with GENMON (Gesellschaft für Biologische Che-

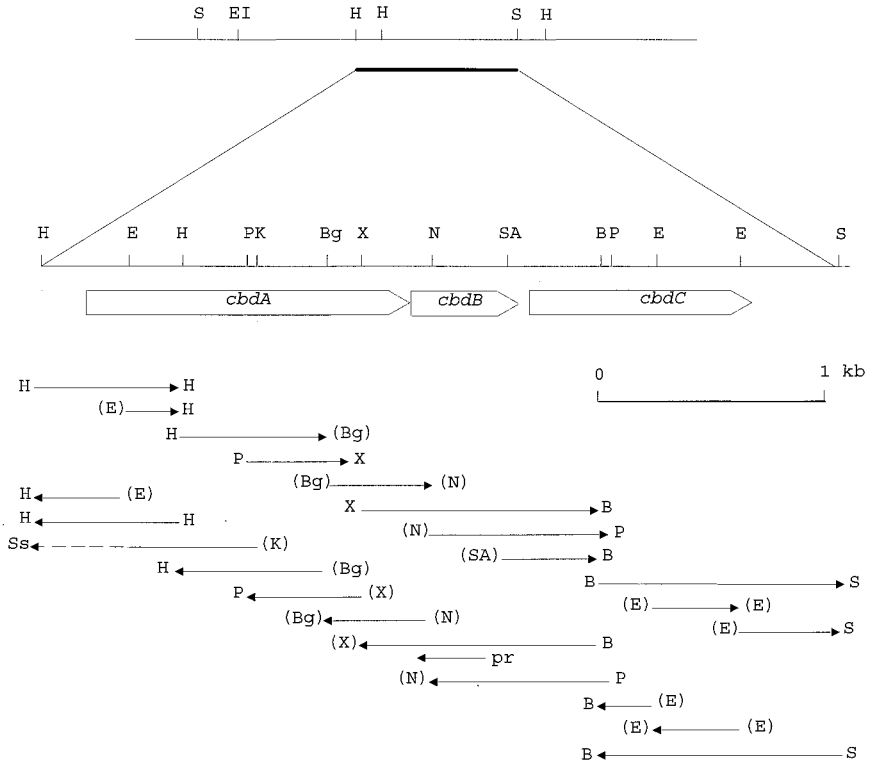

FIG. 2. Partial restriction map for the *cbdABC* region of pBAH1 and restriction fragments used to construct subclones for sequence analysis. pr, synthetic primer. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RV; EI, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; P, *Pst*I; S, *Sma*I; SA, *Sau*3A; Ss, *Sst*I; X, *Xho*I. The restriction sites in parentheses were deleted in the M13mp subclones.

mie, Braunschweig, Germany) and HUSAR (Heidelberg Unix Sequence Analysis Resources) software.

Analytical methods. The concentration of chloride ions in culture broth was determined with a Chlor-o-counter from Marius Instrumenten, Utrecht, The Netherlands. Metabolite monitoring of culture supernatant was performed by high-pressure liquid chromatography (HPLC) on a Lichrospher RP18 column (250 by 4.6 mm; 5-μm particle size). A mixture of 10 mM H_3PO_4 and CH_3OH (equal volumes) was used as an eluent.

Enrichment of 2-halobenzoate 1,2-dioxygenase components. Recombinant *P. putida* KT2440 and recombinant *P. cepacia* 2CBSM4 harboring the genes of the 2-halobenzoate 1,2-dioxygenase system on a 6.0-kbp *Eco*RI insert in pKT231 were grown on 2-cba as the sole source of carbon and energy. Cells were harvested by centrifugation and subjected to ultrasonic treatment as described previously (12). Crude cell extract from 15 g (wet weight) of cells was applied to a DEAE-cellulose DE52 column (2.5 by 7.5 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.9). The column was washed with the same buffer, and bound proteins were eluted with a linear gradient of K_2SO_4 (0 to 0.25 M in 20 mM sodium phosphate buffer [pH 6.9]; gradient volume, 200 ml). Fractions were assayed for protein at 280 nm and tested for the presence of 2-halobenzoate 1,2-dioxygenase components. The red-brown oxygenase component was detected by measuring the A_{460}. The activity of the reductase component was measured as NADH-dependent reduction of 2,6-dichlorophenol indophenol at 600 nm.

Enzyme assay. The activity of the 2-halobenzoate 1,2-dioxygenase system was assayed spectrophotometrically by measuring 2-cba-dependent NADH consumption at 340 nm as described previously (12).

PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Schägger and von Jagow (47), using a 10% T–3% C separating gel with a 4% T–3% C stacking gel. Protein bands were stained with Coomassie brilliant blue R-250 as described previously (12).

Nucleotide sequence accession number. The DNA sequence presented in this report, for the 2-halobenzoate 1,2-dioxygenase structural genes *cbdABC* of plasmid pBAH1 of *P. cepacia* 2CBS, has been deposited with the EMBL Data Library, Heidelberg, Germany, under accession no. X79076.

RESULTS

Conjugative plasmid pBAH1 encodes the 2-halobenzoate 1,2-dioxygenase system. Prolonged cultivation of *P. cepacia* 2CBS in a high-nutrient broth lacking 2-cba led to a spontaneous loss of the ability to grow on 2-cba. However, the mutants that were unable to utilize 2-cba as the sole carbon source (2-Cba$^-$ mutants) retained the ability to grow on catechol. Spraying of catechol-grown 2-Cba$^-$ mutants with a solution of 5 mM catechol–10 mM Fe_2SO_4 in 20 mM potassium phosphate buffer resulted in a yellow coloration of the colonies. Thus, we assume that the 2-Cba$^-$ mutants still possess a catechol 2,3-dioxygenase, which catalyzes the formation of 2-hydroxy-*cis*, *cis*-muconic acid semialdehyde from catechol. Wild-type *P. cepacia* 2CBS was previously shown to also degrade catechol mainly via the *meta*-cleavage pathway (11).

By the method of Kieser (23), a plasmid was isolated from wild-type *P. cepacia* 2CBS. This plasmid, designated pBAH1, was about 70 kbp in size, as estimated by agarose gel electrophoresis of restriction endonuclease digests. The spontaneous 2-Cba$^-$ mutants of *P. cepacia* 2CBS lacked plasmid pBAH1. In order to investigate the possibility of integration of pBAH1 DNA into the chromosome of *P. cepacia* 2CBS, the DIG-labeled oligonucleotide probe which corresponds to a stretch of DNA encoding the N terminus of the reductase component was hybridized with genomic DNA isolated from 2-Cba$^-$ mutants. Whereas the oligonucleotide probe clearly hybridized with genomic DNA from wild-type *P. cepacia* 2CBS as well as with purified pBAH1 DNA, there was no hybridization with genomic DNA from any 2-Cba$^-$ mutant. Thus, the possibility of insertion into the chromosome and inactivation of the *cbdABC* gene cluster was excluded. However, since the plasmid-free 2-Cba$^-$ mutants still possessed catechol 2,3-dioxygenase activity, there appears to be a chromosomally encoded *meta*-cleavage enzyme in *P. cepacia* 2CBS.

The 2-Cba$^-$ mutant *P. cepacia* 2CBSM1 Rif^r as well as *P. putida* KT2440 Rif^r acquired the ability to utilize 2-cba as the sole carbon source upon conjugation with wild-type *P. cepacia* 2CBS. Mating of a Rif^r mutant of the 4-cpa-degrading (4-Cpa$^+$) *Pseudomonas* sp. strain CBS3 with wild-type *P. cepacia* 2CBS resulted in the transconjugant *Pseudomonas* sp. strain CBS3 Rif^r 2-Cba$^+$ 4-Cpa$^+$. 4-Chlorophenylacetate 3,4-dioxygenase, which is a two-component enzyme system catalyzing the initial step of 4-cpa degradation (28, 48), is encoded on the chromosome of *Pseudomonas* sp. strain CBS3 (49).

Cloning of the *cbdABC* genes encoding 2-halobenzoate 1,2-dioxygenase. 2-Halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS is a two-component nonheme iron dioxygenase consisting of a terminal oxygenase and an NADH:acceptor reductase component (12). The DIG-labeled oligonucleotide probe which corresponds to a stretch of DNA encoding the N terminus of the reductase component was hybridized with restriction endonuclease fragments of purified pBAH1 DNA. The probe hybridized with a 1.7-kbp *Pst*I fragment, a 3.8-kbp *Hin*dIII fragment, a 7.0-kbp *Sma*I fragment, an 8.0-kbp *Bam*HI fragment, and a 13.5-kbp *Eco*RI fragment. By using pUC18 as a vector, these fragments were cloned in *E. coli* TG1. The restriction map of the *cbdABC* coding area and flanking regions is shown in Fig. 2.

Nucleotide sequence of *cbdABC* and flanking regions. The nucleotide sequence of the 3,548-bp fragment of pBAH1 carrying the *cbdABC* genes is shown in Fig. 3. It contained three open reading frames (ORFs). The first ORF (1,395 bp; nucleotides 288 to 1682) encoded a protein product of 52.4 kDa, which corresponds to the size of the large subunit of the oxygenase component of 2-halobenzoate 1,2-dioxygenase as determined by SDS-PAGE (52 kDa [12]). The ATG translational start codon of the first ORF (designated *cbdA*) was preceded by a putative ribosome binding sequence, 5′-GGAGG-3′ (50).

The second ORF, designated *cbdB*, started at nucleotide 1682. The deduced molecular mass of the CbdB protein (19.5 kDa) corresponded to the molecular mass of the small subunit of the oxygenase component as determined by SDS-PAGE (12). The putative ribosome binding sequence 5′-GAGGT-3′ preceded the initiation codon ATG. The ATG start codon of *cbdB* overlapped with the TGA stop codon of *cbdA* (Fig. 3). The same arrangement has been found for *benA* and *benB*, which encode the two subunits of the oxygenase component of the chromosomally encoded benzoate 1,2-dioxygenase from *A. calcoaceticus* (34), whereas the corresponding genes *xylX* and *xylY* of the TOL plasmid-encoded toluate 1,2-dioxygenase do not overlap (16). Such overlaps, which have been observed with further catabolic genes such as *bph* (10) and *tod* (66) genes, have been suggested to be involved in translational coupling (37).

The third ORF (*cbdC*) started at position 2202. The translation initiation codon ATG was preceded by the putative ribosome binding site 5′-AGGTG-3′. The deduced molecular mass of the *cbdC* product, 37.1 kDa, was in agreement with the result of previous analysis by SDS-PAGE of the NADH:acceptor reductase component of 2-halobenzoate 1,2-dioxygenase (37.5 kDa [12]).

Comparison of the deduced N-terminal amino acid sequences of *cbdABC* with the N-terminal amino acid sequences of the (subunits of) the purified protein components of the 2-halobenzoate 1,2-dioxygenase system described previously (12) confirmed that *cbdA*, *cbdB*, and *cbdC* encode the large subunit of the oxygenase component, the small subunit of the oxygenase component, and the NADH:acceptor reductase component, respectively.

The nucleotide sequence was also analyzed for potential regulatory signals. A sequence homologous to the consensus sequence TTGACA-$N_{(15-21)}$-TATAAT of canonical −35/−10 *E. coli* promoters was identified 59 bp upstream of *cbdABC*

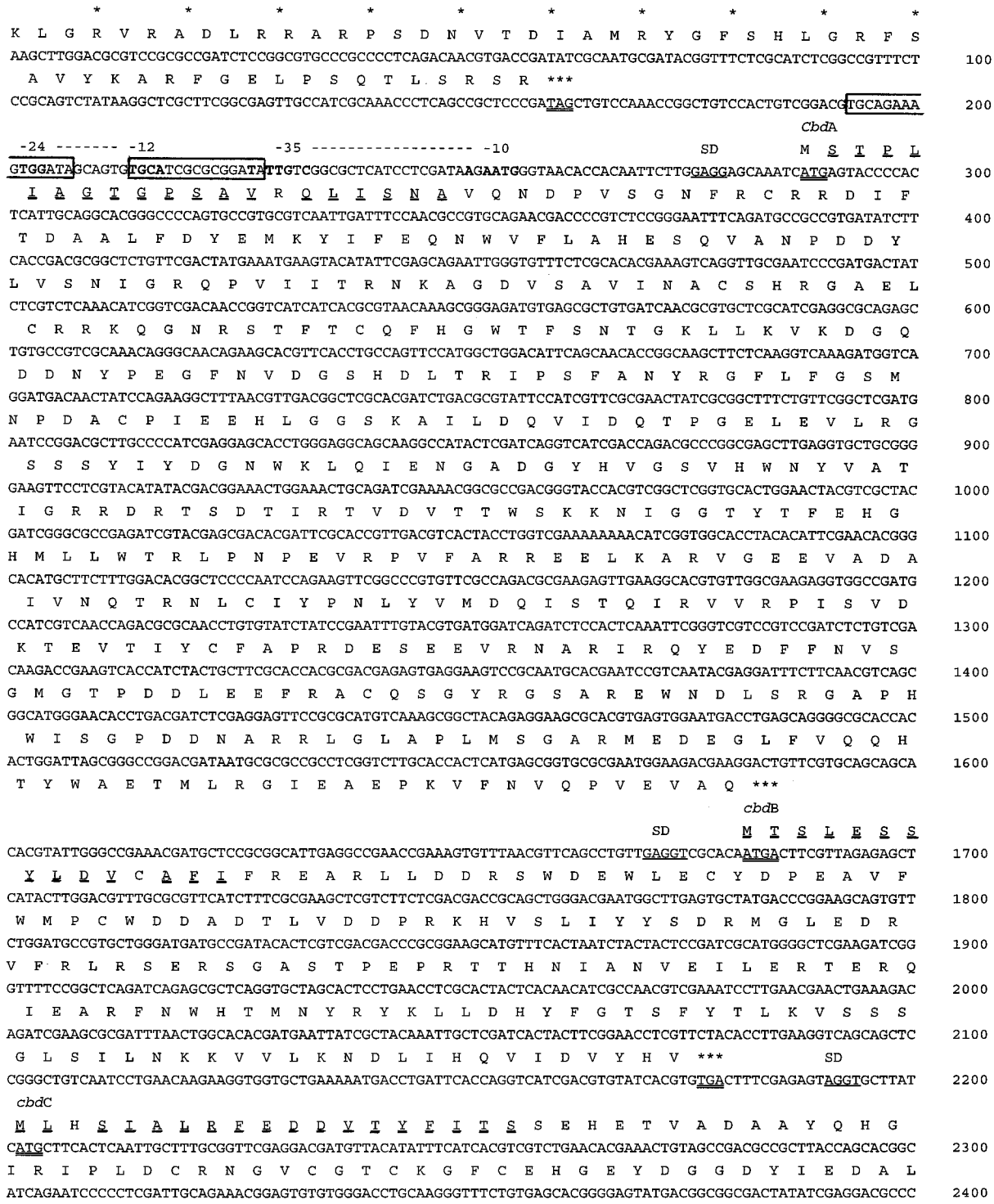

FIG. 3. Nucleotide sequence of the *cbdABC* structural genes and flanking regions on pBAH1 from *P. cepacia* 2CBS. The deduced amino acid sequences of CbdA, CbdB, and CbdC are shown above the DNA sequence. Underlined amino acids are identical to those determined by automated Edman degradation (12). Potential ribosomal binding sites (SD; underlined) are shown. A possible −24(GG)/−12(GC) promoter and a putative *E. coli* promoter (−10. . .−35) upstream of the *cbdABC* coding region are indicated (boldface). Two direct repeats which are homologous to Om_L and Om_R of the *Pm* (*meta* pathway operon) promoter of TOL plasmid pWW0 (boxes), putative stop codons (three asterisks), and initiation codons of *cbdABC* (double underlines) are indicated. Upstream of the *cbdABC* promoter region, part of another ORF which shows homology to a corresponding stretch of *xylS* is indicated. Downstream of the *cbdABC* structural genes, an inverted repeat possibly representing a signal for transcription termination is indicated (arrows).

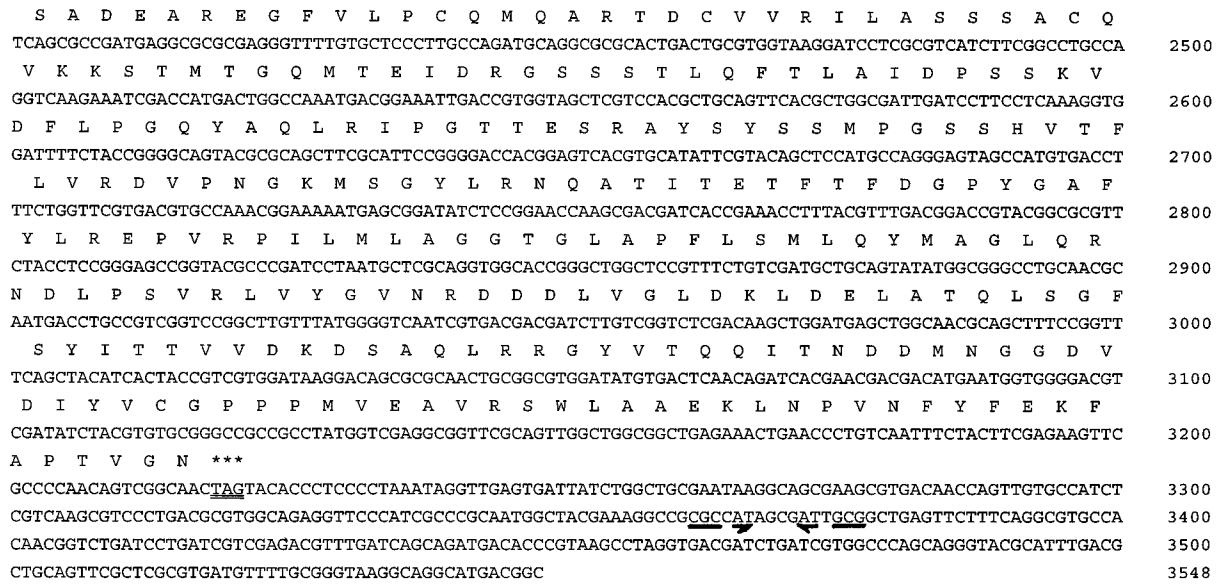

FIG. 3—*Continued.*

(Fig. 3) (30, 65). Another possible promoter, showing homologies to some *Pseudomonas* promoters, e.g, the *xylCAB* and *xylS* promoters of TOL plasmid pWW0, was found 86 bp upstream of the *cbdA* translational start. These promoters possess the −24(GG)/−12(GC) conserved core structure of promoters recognized by a minor form of RNA polymerase containing subunit sigma[54] (25, 58).

The promoter region upstream of *cbdABC* was found to contain two direct repeats which show homology to the *Om* operator region of the *Pm* (*meta* operon) promoter of TOL plasmid pWW0 (21, 22) (Fig. 3). There was no region with homology to the consensus sequence TNTNAN(x)NTNANA of the proposed symmetrical recognition site for *E. coli* transcriptional regulators (9).

An inverted repeat sequence which might represent a putative signal for transcription termination (41) was detected in the 3′-flanking region downstream of the termination codon of *cbdC* (Fig. 3).

Downstream of the *cbdABC* coding region, there was no further potential ORF within the next 327 nucleotides.

Upstream of *cbdABC*, part of another potential ORF, which ended 125 bp in front of the *cbdA* start codon, was identified (Fig. 3). This stretch of DNA was homologous to a corresponding stretch of the *xylS* gene of TOL plasmid pWW0 of *P. putida* mt-2 (47% amino acid identity). *xylS* of TOL plasmid pWW0 encodes a transcriptional activator (18, 31, 53).

The G+C content of the *cbdABC* coding region was 55.9%. *cbdA*, *cbdB*, and *cbdC* showed G+C contents of 56.4, 51.2, and 57.9%, respectively. These values are significantly lower than the G+C content of 67.4% reported for the *P. cepacia* genome (40), indicating the possibility of acquisition of the *cbdABC* genes by horizontal gene transfer. The codon usage in the *cbdABC* genes showed preferential usage of G and C in the third position.

Expression of *cbdABC* in *P. putida* KT2440 and in *P. cepacia* 2CBSM4. The 4.4-kbp *Hin*dIII fragment of pBAH1 (consisting of the 0.6- and 3.8-kbp *Hin*dIII fragments; see Fig. 2) were cloned into the *tac* promoter vector pKK223-3. *E. coli* JM105 was used as the recipient strain. Recombinant *E. coli* clones were incubated at 30°C in diluted (1:2) LB medium (without NaCl) containing 2-cba. In order to induce gene expression, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and chloride release from 2-cba was investigated. Recombinant *E. coli* clones were also grown in LB medium, harvested by centrifugation, and resuspended in chloride-free mineral salts medium containing 2-cba. However, chloride release from 2-cba was not detected in either the LB broth or the mineral salts medium of *E. coli* clones harboring the *cbdABC* genes in pKK223-3. Expression from the promoter(s) located upstream of the *cbdABC* genes on the *Hin*dIII fragment was not induced by 2-cba in the *E. coli* clones, and there was also no expression from the *tac* promoter of pKK223-3.

In order to clone the *cbdABC* genes into the broad-host-range vector pKT231, a 7.0-kbp *Sma*I fragment (Fig. 2, top) was cloned into pUC18, from which a hybrid 6.0-kbp *Eco*RI fragment was subcloned into pKT231. *E. coli* S17-1 clones which were transformed with recombinant pKT231 were not able to release chloride from 2-cba. Recombinant pKT231 was then mobilized from *E. coli* S17-1 to *P. putida* KT2440 and to *P. cepacia* 2CBSM4. *P. putida* KT2440 and *P. cepacia* 2CBSM4, harboring the 6.0-kbp *Eco*RI fragment of pBAH1 on recombinant plasmid pKT231, utilized 2-cba as the sole source of carbon and energy. The concentrations of 2-cba in the culture supernatants of wild-type strain 2CBS and recombinant KT 2440 and 2CBSM4 clones were monitored by HPLC. The rate of 2-cba removal by the recombinant *Pseudomonas* clones was comparable to that of fully induced wild-type *P. cepacia* 2CBS growing on 2-cba (11). The transcription of the *cbdABC* structural genes in the recombinant *Pseudomonas* clones apparently originated from a promoter of the DNA insert, which obviously was functional only in the *Pseudomonas* host strains.

Enrichment of 2-halobenzoate 1,2-dioxygenase from recombinant *P. putida* KT2440 and from recombinant *P. cepacia* 2CBSM4. From crude extracts of recombinant *P. putida* KT2440 and *P. cepacia* 2CBSM4 grown on 2-cba, a red-brown protein component and a protein showing NADH:dichlorophenol indophenol reductase activity were enriched by means of anion-exchange chromatography. The proteins eluted with the linear gradient at K_2SO_4 concentrations of about 120 mM (red-brown protein) and 160 mM (NADH:acceptor reductase),

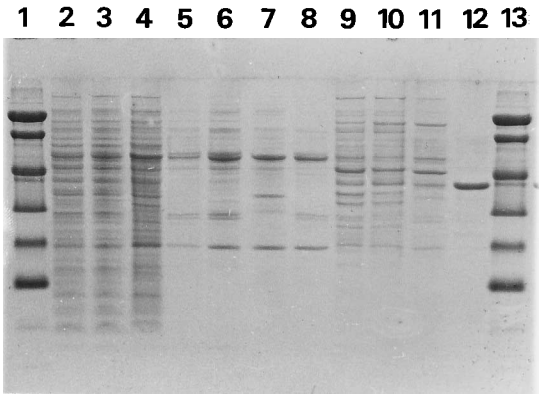

FIG. 4. SDS-PAGE of preparations of oxygenase and NADH:acceptor reductase components of 2-halobenzoate 1,2-dioxygenase enriched from recombinant *Pseudomonas* clones and from wild-type *P. cepacia* 2CBS. Lanes 1 and 13, molecular mass marker proteins phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (39 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa); lanes 2 through 4, crude extracts; lanes 5 through 7, preparations of the oxygenase component after anion-exchange chromatography; lane 8, oxygenase component from wild-type *P. cepacia* 2CBS (12); lanes 9 through 11, preparations of the NADH:acceptor reductase component after anion-exchange chromatography; lane 12, NADH:acceptor reductase component purified from wild-type *P. cepacia* 2CBS (12). Wild-type *P. cepacia* 2CBS (lanes 2, 5, and 9), recombinant *P. cepacia* 2CBSM4 (lanes 3, 6, and 10), and recombinant *P. putida* KT2440 (lanes 4, 7, and 11) were used.

which corresponded to the elution characteristics on DEAE-cellulose of the oxygenase and reductase components of the 2-halobenzoate 1,2-dioxygenase as described previously (11). When crude extracts from the host strains *P. putida* KT2440 and *P. cepacia* 2CBSM4 were applied to the anion-exchange column, these two proteins were not detected in the elution profiles. SDS-PAGE of aliquots of the fractions containing the red-brown protein revealed two predominant peptide bands with molecular masses of 52 and 19.5 kDa, corresponding in size to the α and β subunits, respectively, of the oxygenase component purified from wild-type *P. cepacia* 2CBS (Fig. 4). The fractions showing NADH:dichlorophenol indophenol reductase activity were also subjected to SDS-PAGE. A 37-kDa protein corresponding in size to the NADH:acceptor reductase component purified from wild-type strain 2CBS was detected (Fig. 4). The results of the SDS-PAGE confirmed that the two protein pools obtained by means of the anion-exchange chromatography were likely to contain the oxygenase and reductase components of the 2-halobenzoate 1,2-dioxygenase system.

When aliquots of the two pools containing the putative enzyme components were combined, 2-cba-dependent consumption of NADH was detected in the enzyme assay. In contrast, neither the combined eluate of the chromatography of crude extract from host strain *P. putida* KT2440 nor the combined eluate of the chromatography of crude extract from *P. cepacia* 2CBSM4 showed any activity. Thus, recombinant *Pseudomonas* clones harboring *cbdABC* were shown to produce functionally active 2-halobenzoate 1,2-dioxygenase. The red-brown oxygenase component enriched from recombinant *Pseudomonas* KT2440 and 2CBSM4 clones was active with the reductase component purified from wild-type *P. cepacia* 2CBS, and the NADH:dichlorophenol indophenol reductase enriched from both recombinant *Pseudomonas* clones also was active with the oxygenase component isolated from wild-type strain 2CBS.

DISCUSSION

A number of pathways specifying catabolism of aromatic compounds by pseudomonads are encoded on degradative plasmids. The genes encoding the two-component nonheme iron dioxygenase 2-halobenzoate 1,2-dioxygenase from *P. cepacia* 2CBS were localized on a 70-kbp plasmid designated pBAH1. pBAH1 was transmissible to *P. putida* KT2440 and to *Pseudomonas* sp. strain CBS3 by means of conjugation.

The structural genes *cbdABC*, which encode the large (CbdA) and small (CbdB) subunits of the oxygenase component and the NADH:acceptor reductase component (CbdC), were clustered on pBAH1, forming an operon.

Two potential promoter sequences upstream of the translational start of the *cbdABC* structural genes were identified. The first putative promoter, located 59 bp upstream of the *cbdA* translational start, was homologous to the −35 TTGACA −10 TATAAT consensus sequence of canonical *E. coli* promoters (30) (Fig. 3). However, despite the presence of this putative *E. coli* promoter, the *E. coli* S17-1 clones harboring recombinant plasmid pKT231 were not able to metabolize 2-cba. A second possible promoter structure localized 86 bp upstream of the *cbdA* start codon showed homology to *nif*- and *ntr*-like promoters, which contain the invariant −24(GG)/−12(GC) core typical for a DNA region recognized by a minor form of RNA polymerase utilizing the alternative sigma^{54} factor (25, 58). However, nucleotide sequence comparisons suggested similar organizations of the promoter region of *cbdABC* and the *Pm* (*meta* pathway operon) promoter of TOL plasmid pWW0: two direct repeats showing homologies to the Om_L and Om_R half-sites of *Pm* (21, 22) were identified (Fig. 3). Since the phenotypic expression of the *cbdABC* genes was achieved in both *P. putida* KT2440 and *P. cepacia* clones but not in recombinant *E. coli* S17-1, we assume that transcription of *cbdABC* originates from the *Pseudomonas* promoter.

Upstream of the *cbdABC* gene cluster, part of another putative ORF (tentatively designated *cbdS*) (Fig. 3), which was homologous to a corresponding stretch of the *xylS* gene of TOL plasmid pWW0 (18, 31, 53), was identified. However, determination of whether the putative ORF upstream of *cbdABC* encodes a XylS-like regulator which interacts with a specific operator sequence will have to await further studies.

The deduced amino acid sequences of the protein components CbdABC of 2-halobenzoate 1,2-dioxygenase were compared with those of the chromosomally encoded benzoate 1,2-dioxygenase from *A. calcoaceticus* (BenABC) (34) and with those of the TOL plasmid pWW0-encoded toluate 1,2-dioxygenase from *P. putida* mt-2 (XylXYZ) (16). The overall amino acid identity of CbdABC, XylXYZ, and BenABC was 42.2%. CbdABC and XylXYZ showed 51.3% identical amino acids, and CbdABC and BenABC showed 52.1% identical amino acids. Comparisons of CbdA and XylX, CbdA and BenA, CbdB and XylY, CbdB and BenB, CbdC and XylZ, and CbdC and BenC revealed amino acid sequence identities of 52.9, 53.3, 55.2, 52.1, 47.2, and 50.4%, respectively. These overall homologies indicate that *cbdABC*, *xylXYZ*, and *benABC* genes were derived from a common ancestral DNA fragment.

The deduced amino acid sequence of the *cbdABC* coding region also was analyzed for potential cofactor binding sites. On the basis of biochemical studies of the purified protein components of 2-halobenzoate 1,2-dioxygenase, the oxygenase component had been suggested to be a Rieske-type iron-sulfur protein, and the NADH:acceptor reductase had been proposed to contain FAD and a [2Fe-2S] cluster (12).

[2Fe-2S] clusters are divided into two distinct groups. Whereas binding of iron-sulfur centers is mediated by four

cysteine residues in chloroplast-type [2Fe-2S] ferredoxins, coordination of Rieske-type [2Fe-2S] clusters has been proposed to be mediated by two cysteine and two histidine residues (29). In CbdA as well as in BenA and XylX, the motif $CxH(x)_{17}CxxH$ may coordinate the Rieske-type iron-sulfur cluster. Chloroplast-type [2Fe-2S] ferredoxins and Rieske-type [2Fe-2S] clusters show distinct electron paramagnetic resonance spectra. Electron paramagnetic resonance, electron spin echo envelope modulation, and electron nuclear double resonance studies of 2-halobenzoate 1,2-dioxygenase (43) confirmed the determination of the oxygenase component as a Rieske-type protein. The motif $CxH(x)_{15-17}CxxH$ is also conserved among the (large subunits of the) oxygenases of other multicomponent enzyme systems, such as phthalate 3,4-dioxygenase (Pht3 [36]; class IA), vanillate demethylase (VanA [5]; class IA), toluene dioxygenase (TodC1 [66]; class IIB), biphenyl dioxygenase (BphA and BphA1 [10, 55]; class IIB), benzene dioxygenase (P1 and BedC1 [19, 57]; class IIB), naphthalene dioxygenase (DoxB, NdoB, and NahAc [8, 24, 51]; class III), and polycyclic aromatic hydrocarbon dioxygenase (PahAc [56]; class III). The small iron-sulfur proteins (ferredoxin components) of the three-component systems belonging to classes IIB and III as defined by Batie et al. (2) also possess the conserved cysteine and histidine residues which are proposed to bind the Rieske-type [2Fe-2S] cluster (2, 29).

The small (β) subunits of the oxygenase components do not appear to possess any putative cofactor-binding domains. The oxygenase component of the 2-halobenzoate 1,2-dioxygenase system was previously characterized as a Rieske-type iron-sulfur protein, containing three [2Fe-2S] clusters per $\alpha_3\beta_3$ oligomer, which apparently are localized on the large (α) subunit, CbdA. The small subunits of the oxygenase components of both toluate 1,2-dioxygenase from *P. putida* mt-2 and benzoate 1,2-dioxygenase from *A. calcoaceticus* have been proposed to be important for the determination of substrate specificity of the enzyme system (17, 34). However, sequence comparison of CbdAB, BenAB, and XylXY did not shed light on a putatively important amino acid domain(s) determining substrate specificity. The construction of hybrid gene clusters combining the genes encoding large and small subunits of the oxygenase components of benzoate, toluate, and 2-halobenzoate dioxygenases might be an approach to investigate whether the β subunit is indeed crucial for the different substrate specificities of these isofunctional dioxygenases.

The N-terminal region of CbdC was found to resemble the sequences of chloroplast-type ferredoxins. Residues important for ferredoxin structure and function were conserved, including the CxxxxCxxC binding site (39). Homologous binding sites for [2Fe-2S] clusters with the conserved motif $CxxGxCGxC(x)_6G(x)_{8-13}L(x)_{8-13}C$ also have been found in the NADH:acceptor reductases of phthalate dioxygenase (PDR and Pht2 [6, 36]; class IA), vanillate demethylase (VanB [5]; class IA), naphthalene dioxygenase (NahAa [51]; class III), polycyclic aromatic hydrocarbon dioxygenase (PahAa [56]; class III), and xylene methyl-monooxygenase (XylA [54]). In the C-terminal region of CbdC, sequences homologous to possible FAD- and NAD-binding domains as allocated by Correll et al. (6) and Neidle et al. (34) were identified. The domains showing the amino acid fingerprints proposed to be involved in flavin and pyridine nucleotide binding are indicated in Fig. 5. The amino acid residues typical of the proposed fingerprints are conserved in the reductase components of benzoate and toluate 1,2-dioxygenases (class IB) (Fig. 5), phthalate dioxygenase (PDR and Pht2 [6, 36]; class IA), vanillate demethylase (VanB [5]; class IA), naphthalene dioxygenase (NahAa [51]; class III), polycyclic hydrocarbon dioxygenase (PahAa [56]; class III), and xylene methyl-monooxygenase (XylA [54]). However, the orientations of the chloroplast-type [2Fe-2S]-binding domain and the flavin mononucleotide- or FAD-isoalloxazine- and NAD-ribose-binding domains are reversed in the class IA reductases VanB and Pht2 compared with those of the class IB NADH:acceptor reductases, a fact which has been attributed to alternative fusions of the distinct modular domains (6). The amino acid sequences of the FAD-containing reductase components of the class IIB enzymes benzene dioxygenase (P4 [19]), toluene dioxygenase (TodA [66]), and biphenyl dioxygenase (BphG [10]) are dissimilar to the flavin- and NAD-binding domains discussed above. These reductases possess amino acid regions which match the consensus sequence (61) for the βαβ-fold secondary structures likely to be involved in FAD and NAD binding. Thus, there are different classes of reductase components, which differ not only in cofactor composition but also in the modular organization of their cofactor-binding domains.

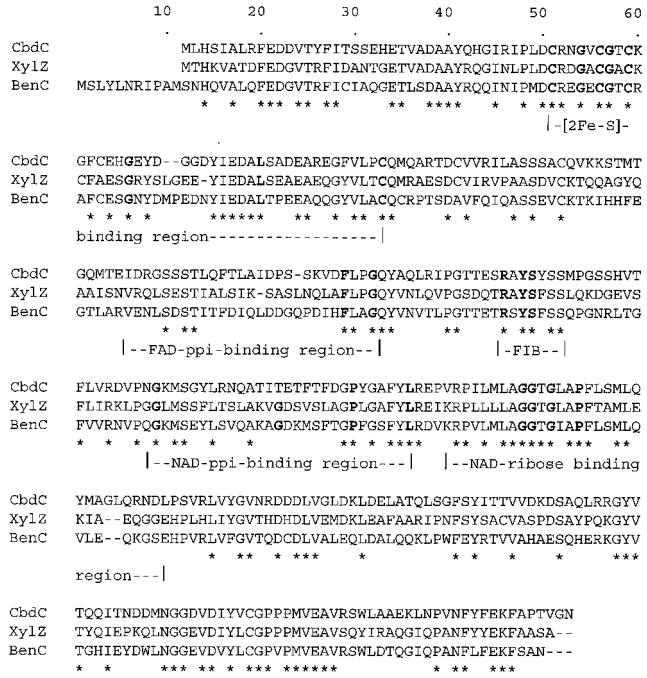

FIG. 5. Alignment of the deduced amino acid sequences of CbdC with the published sequences of BenC (34) and XylZ (16). Conserved amino acid residues are indicated (asterisks). The secondary structures of possible cofactor domains are shown as allocated by Correll et al. (6) and Neidle et al. (34). FIB, FAD-isoalloxazine ring-binding domain.

The chromosomally encoded benzoate 1,2-dioxygenase BenABC, the TOL plasmid pWW0-encoded toluate 1,2-dioxygenase XylXYZ, and the plasmid pBAH1-encoded 2-halobenzoate 1,2-dioxygenase CbdABC were shown to be evolutionarily related. Further comparative analyses of these class IB enzymes, which additionally should consider the recently described three-component (class II?) *ortho*-halobenzoate 1,2-dioxygenase from *P. aeruginosa* (44), are required to gain more insight into the evolution of catabolic enzymes, their regulatory proteins, and changes associated with substrate specificity.

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