Identification and Functional Characterization of CbaR, a MarR-Like Modulator of the cbaABC-Encoded Chlorobenzoate Catabolism Pathway

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In Comamonas testosteroni BR60 (formerly Alcaligenes sp. strain BR60), catabolism of the pollutant 3-chlorobenzoate (3CBA) is initiated by enzymes encoded by cbaABC, an operon found on composite transposon Tn5271 of plasmid pBR60. The cbaABC gene product CbaABC converts 3CBA to protocatechuate (PCA) and 5-Cl-PCA, which are then metabolized by the chromosomal PCA meta (extradiol) ring fission pathway. In this study, cbaA was found to possess a ω-type promoter. O2 uptake experiments with whole cells and expression studies with cbaA-lacZ constructs showed that cbaABC was induced by 3CBA. Benzoate, which is not a substrate of the 3CBA pathway, was a gratuitous inducer, and CbaR, a MarR family repressor coded for by a divergently transcribed gene upstream of cbaABC, could modulate induction mediated by benzoate. Purified CbaR bound specifically to two regions of the cbaA promoter (PcbaA): site I, a high-affinity site, is between the transcriptional start point (position +1) and the start codon of cbaA, while site II, a lower-affinity site, overlaps position +1. 3CBA at concentrations as low as 40 μM interfered with binding to PcbaA. PCA also interfered with binding, while benzoate only weakly disrupted binding. Unexpectedly, benzoate with a hydroxyl or carboxyl at position 3 improved CbaR binding. Data are also presented that suggest that an unidentified regulator is encoded on the chromosome that induces cbaABC in response to benzoate and 3CBA.

The chlorinated benzoic acids (CBA) are a common class of pollutants that occur in the environment as a result of intentional introduction (e.g., in the form of herbicides) or incomplete bacterial metabolism of some accidentally released chemicals (e.g., polychlorinated biphenyls) (46). Bacteria possess a remarkable assortment of metabolic pathways for biodegradation of CBA, and the innate ability of bacteria to degrade CBA has been exploited for bioremediation of contaminated sites (47). Several aerobic degradation pathways have been characterized at the biochemical and genetic levels. The most intensively studied pathway is encoded by the cba genes of Pseudomonas putida that specify intradiol ring fission of 3-chlorocatechol, a metabolite generated by nonspecific activity of benzoate or toluene dioxygenases with 3-chlorobenzoate (3CBA) (19). In contrast, the cba-encoded pathway involves a dioxygenase and a dehydrogenase that convert 3CBA, 4CBA, or 3,4-dichlorobenzoate to the vicinal diol intermediates protocatechuate (PCA) and 5-Cl-PCA (Fig. 1A) (40, 41). Other CBA degradation operons include the cbaABC operon of Burkholderia cepacia (22) and the cbaABC operon of Alcaligenes sp. strain BR60, which specify dioxygenase-mediated conversion of 3CBA to catechol, the fcb pathway of Arthrobacter globiformis for conversion of 4CBA to 4-hydroxybenzoate by a coenzyme A ligase and a hydrolase (61); and an Alcaligenes sp. pathway that converts 3CBA to 3-hydroxybenzoate (31, 32). Proven or putative regulatory factors for these pathways are encoded by genes closely linked to the catabolic operons. These factors include ClcR, a LysR-like regulator for clc (9); CbdR, an AraC-like regulator for cbaA (22); and OhbR, an IcI-like regulator for ohb (60).

The cbaABC operon is located in composite transposon Tn5271 (Fig. 1A) found on the self-transmissible IncPβ plasmid pBR60 (38, 37). Despite the potential for being mobilized to members of various subclasses of the class Proteobacteria (14, 20, 21, 47, 65), the natural host range of this plasmid includes primarily members of the β subclass of the Proteobacteria, particularly strains that degrade PCA, the product of the cbaABC-mediated catabolism of CBA, by an extradiol pathway (39). From an ecological point of view, little is known about factors that limit the horizontal spread of this mobile pathway. We therefore sought to better understand regulation of cbaABC expression, not only because horizontal transfer in contaminated environments brings this operon into a variety of genetic backgrounds and we are curious about whether the capacity to effectively regulate cbaABC plays a role in determining the host range of these genes, but also because cbaABC encodes an additional pathway by which bacteria metabolize CBA and thus has potential applications in bioremediation of contaminated sites. We therefore characterized induction of cbaABC in the original host, and in this report we show that a cis-encoded MarR-like regulator, CbaR, plays a role in modulating expression of cbaABC. We also present evidence that a chromosomally encoded protein may be involved in regulating this operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. Escherichia coli was routinely grown at 37°C in Luria-Bertani medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5%
(wt/vol) NaCl) containing ampicillin (250 mg liter\(^{-1}\)), kanamycin (40 mg liter\(^{-1}\)), or chloramphenicol (50 mg liter\(^{-1}\)), as required. *Comamonas testosteroni* strains (formerly *Alcaligenes* sp. strains [see below]) were grown as described below in minimal medium A (64) containing 10 mM succinate, aromatic substrate at a concentration of 4 mM, and chloramphenicol (50 to 100 mg liter\(^{-1}\)), as required. All chemicals and antibiotics were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). When necessary, growth media were solidified by adding agar to a final concentration of 1.6% (wt/vol).

**DNA sequencing and sequence analysis.** To add to the phenotypic characteristics previously determined for 3CBA-degrading strain BR60(pBRC60) (67), both strands of the first 523 bases of the 16S rRNA gene were sequenced by MIDI Labs (Newark, Del.) by using primers that anneal beginning at positions 5 and 531 in the 16S rRNA gene of *E. coli*. The sequences of both strands of Tn\(^{5271}\) DNA between IS\(^{1071L}\) and \(cbaA\) (Fig. 1A) were determined by the chain-terminating dideoxy method with an ABI Prism automated sequencer (Biotechnology Research Institute, University of Ottawa, Ottawa, Ontario, Canada) using appropriate primers and pBRE11 (Table 1) as the template. This DNA was analyzed for similarities to entries in the GenBank nonredundant database by using the BLAST (5) network service of the National Center for Biotechnology Information, Bethesda, Md. (http://www.ncbi.nlm.nih.gov). A divergently transcribed open reading frame (ORF) upstream of \(cbaA\) was identified and designated \(cbaR\). Because the putative product of this ORF showed similarity to MarR and various other transcriptional regulators that are known to respond to aromatic compounds or control aromatic catabolism genes (see below), this region was taken into consideration when we designed the various \(lacZ\) expression constructs described below. DNA between \(cbaC\) and IS\(^{1071R}\) (Fig. 1A) had been sequenced previously and was not taken into consideration as it contains ORFs whose products show similarity to proteins involved in uptake of aromatic compounds (unpublished results) and a truncated aryl coenzyme A ligase (14).

**Determination of \(cbaA\) transcriptional start site and reverse transcription (RT)-PCR assay.** The transcriptional start of \(cbaA\) was determined by primer extension analysis as described elsewhere (53). Briefly, *C. testosteroni* BR60(pBRC60) was grown to the mid-log phase on minimal medium A containing 3CBA or succinate (a noninducing substrate as determined by O\(_2\) uptake analysis [see below]), and DNA-free total RNA was extracted with an E.Z.N.A bacterial RNA kit (PeqLab, Erlangen, Germany) according to the manufacturer’s instructions. CBAAR was annealed to equal amounts of RNA from each source and reverse transcribed by

![FIG. 1. (A) Physical and restriction map of 3CBA catabolism transposon Tn5271 found on plasmid pBRC60 of *C. testosteroni* BR60. The solid arrows indicate various genes, including *cbaABC*, which encode the dioxygenase (*CbaAB*) and dehydrogenase (*CbaC*) that convert 3CBA to PCA or 5-Cl-PCA; *cbaR*, which codes for a regulatory protein (this study); the transposase *tnpA* gene of the two copies of IS\(^{1071}\); and an ORF that codes for a truncated aryl coenzyme A ligase-like product (ORF 8). Flanking *tnpA* are the left and right inverted repeats of IS\(^{1071L}\) (open triangles). (B) Restriction map of EcoRI fragment 11 (E11) from pBRC60 and schematic diagrams of *lacZ* constructs made to study expression of *cbaA* and *cbaR*. Note that *lacZ* (grey boxes) is not drawn to scale.
TABLE 1. Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant phenotypic and/or genotypic characteristics* | Reference or source |
|-----------------------------|-----------------------------------------------------|---------------------|
| C. testosteroni strains     |                                                     |                     |
| BR60(pBRC60)               | Wild type; Tn5271 proficient                       | 66                  |
| BR6020                      | BR60 cured of pBRC60; Tn5271 deficient              | 66                  |
| BR6020(pBRC60)             | Trp<sup>+</sup> and Cmr<sup>-</sup> strain derived from BR6020; Tn5271 proficient; used to conjugate pBRC60 into BR6020 strains harboring lacZ expression constructs | 42                  |
| E. coli strains             |                                                     |                     |
| XL-1 Blue                   | Used during cloning and DNA manipulation; α-lacZ complementing strain | Stratagene          |
| CC18:ApI                   | Host for pUTCm and pUTCm-based suicide delivery vectors | 12                  |
| M15                         | Host for pQE30, pQE30-based expression vectors, and pREP4 | Qiagen              |
| Source plasmids             |                                                     |                     |
| pCRScript SK+               | Amp<sup>+</sup>; α-lacZ MCS; general-purpose cloning vector | Stratagene          |
| pUC18                       | Amp<sup>+</sup>; α-lacZ MCS; general-purpose cloning vector | 68                  |
| pUC18Not                    | Amp<sup>+</sup>; α-lacZ MCS; general-purpose cloning vector | 13                  |
| pUC128                      | Amp<sup>+</sup>; α-lacZ MCS; general-purpose cloning vector | 27                  |
| pUTCm                       | Amp<sup>+</sup> Cmr<sup>-</sup>; Tn5<sup>-</sup>-based suicide delivery vector | 12                  |
| pUTCmMCS.3                  | Amp<sup>+</sup> Cmr<sup>-</sup>; pUTCm with oligonucleotide containing additional restriction sites cloned into NorI site<sup>+</sup> | This study          |
| pRK2013                     | Km<sup>-</sup> RK2<sup>-</sup> tru; used in triparental mating to mobilize pUTCm-based plasmids | 18                  |
| pBW30::Tnphao<sup>-</sup>4  | Amp<sup>+</sup> Km<sup>-</sup>; vector used as source of lacZ gene; removed either as a BamHI-HindIII fragment or a BamHI-BglII fragment, which releases a promoterless lacZ gene lacking DNA encoding the first nine amino acids | 63                  |
| pMP10.1                     | Amp<sup>+</sup>; lacZ cloned as BamHI-HindIII fragment into pUC18Not | This study          |
| pMP28.1                     | Amp<sup>+</sup>; lacZ cloned as a BamHI-BglII fragment into pUC18Not digested with BamHI | This study          |
| pBRE11                      | Amp<sup>+</sup>; EcoRI fragment 11 from pBRC60 cloned into pUC18 | 38                  |
| pQE30                       | Amp<sup>+</sup>; IPTG-inducible expression vector for adding a six-histidine affinity tag to the N terminus of proteins cloned into the MCS | Qiagen              |
| pREP4                       | Km<sup>-</sup>; codes for Lac<sup>+</sup>, which represses expression from pQE30 promoter | Qiagen              |
| LacZ expression constructs  |                                                     |                     |
| pBRCW34                     | Amp<sup>+</sup>; Nfr1-ScaI fragment from pBRE11 cloned into SphI site of pCR-Script SK (+) so that the ScaI end (i.e., cbaA') is adjacent to BamHI | This study          |
| pBRCW34L                    | Amp<sup>+</sup>; lacZ cloned as a BamHI-HindIII fragment into pBRCW34, placing it in frame with cbaA' | This study          |
| pMP68.3                     | Amp<sup>+</sup>; EcoRI fragment from pBRE11 cloned into pBRCW34L digested with SacI (blunt ended) and NotI; DNA coding for cbaR, a divergently transcribed ORF between cbaA and IS107/1L, is placed upstream of cbaA' | This study          |
| pMP70.5                     | Amp<sup>+</sup>; Cmr<sup>-</sup>; blunt-ended NorI-HindIII fragment from pMP68.3 containing construct cbaRA'−lacZ cloned into pUTCm digested with NotI and also blunt ended | This study          |
| pMP14.10                    | Amp<sup>+</sup>; BglII fragment from pBRCW34L cloned into pUC18Not digested with BamHI | This study          |
| pMP17.6                     | Amp<sup>+</sup> Cmr<sup>-</sup>; NorI fragment from pMP14.10 containing construct cbaA'−lacZI cloned into pUTCm | This study          |
| pBRCW27                     | Amp<sup>+</sup>; Nfr1-ScaI fragment from pBRE11 cloned into SphI site of pCRScript SK (+) so that the Nfr1 end (i.e., cbaR) is adjacent to BamHI | This study          |
| pBRCW27L                    | Amp<sup>+</sup>; lacZ cloned as a BamHI-HindIII fragment into pBRCW27, placing it in frame with cbaR' | This study          |
| pMP105.1                    | Amp<sup>+</sup> SacI fragment from pBRCW27L cloned into pMP28.1 | This study          |
| pMP123.2                    | Amp<sup>+</sup>; SacI-XbaI fragment from pMP105.1 cloned into pUC128 | This study          |
| pMP125.1                    | Amp<sup>+</sup> Cmr<sup>-</sup>; NorI-NotI fragment from pMP123.2 containing construct cbaR'−lacZI cloned into pUTCmMCS.3 | This study          |
| pMP106.1                    | Amp<sup>+</sup> Cmr<sup>-</sup>; NorI fragment from pMP105.1 containing construct cbaR'−lacZI cloned into pUTCm | This study          |
| pMP50.5                     | Amp<sup>+</sup>; BglII fragment from pBRCW27L cloned into pUC18Not digested with BamHI | This study          |
| pMP54.9                     | Amp<sup>+</sup> Cmr<sup>+</sup>; NorI fragment from pMP50.5 containing construct cbaR−lacZ3 cloned into pUTCm | This study          |
| pMP19.9                     | Amp<sup>+</sup> Cmr<sup>+</sup>; NorI fragment from pMP10.1 containing control expression construct (promoterless lacZ) cloned into pUTCm | This study          |
| Affinity-tagged CbaR plasmids|                                                     |                     |
| pMP130.4                    | Amp<sup>+</sup>; PCR-amplified cbaR cloned into SmaI site of pCRScript SK (+) | This study          |
| pQE30ebaR                   | Amp<sup>+</sup>; cbaR from pMP130.4 subcloned into pQE30 as a BamHI-SacI fragment | This study          |

* Abbreviations: MCS, multiple cloning site; Amp<sup>+</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Km<sup>r</sup>, kanamycin resistance.

b BR6020 strains harboring chromosomal inserts of the various lacZ expression constructs (see Materials and Methods) are not listed because of space considerations.

The sequence of the modified cloning site in pUTCmMCS.3 is 5'−GGCGCCGGAA GTTGGATGC CTGCAGGTCG ACTCTTAGAG GTTCCCCGGG GGCTTAAAT ATGCATCGGC GC−3'. This sequence contains restriction sites for NotI, HindIII, SphI, PstI, XbaI, BamHI, Smal, SwaI, NorI, and EagI. Only the restriction sites for NotI, SmaI, and NorI are unique to this vector. The 5' end is proximal to the Cm<sup>r</sup> gene.
Cloning and overexpression of cbaR. Affinity-purified CbaR for gel shift studies and DNase I protection assays (described below) was obtained as follows. The gene coding for CbaR was PCR amplified by using forward primer CBA3958 (5'-cgggattctgcaagagattcctgca-3') and reverse primer CBA3440 (5'-aggaattcttacagtgtct-3') (lowercase nucleotides are not native to cbaR, and the underlined nucleotides in the two sequences are restriction sites for BamHI and SacI, respectively). CBA3958 is complementary to the portion of cbaR coding for the N terminus but lacks the native start codon, while CBA3440 is complementary to the C-terminal coding portion of cbaR up to and including the native stop codon. The amplification product was initially cloned into pCRScript SK(+) digested with Smal (resulting in pCM3958), and the insert was sequenced to confirm that it had not introduced mutations. CbaR was then placed downstream from and in frame with DNA coding for the N-terminal affinity tag MRSHG6, GS digested by pMP132.4 with BamHI and SacI and cloning the ~550-bp fragment that was generated into pQE30 digested with the same enzymes, which resulted in pQE30cbaR. CbaR was then overexpressed and affinity purified with an Ni-nitrilotriacetic acid column as recommended by the manufacturer (Qiagen, Mississauga, Ontario, Canada), dialyzed twice for ~18 h against glycerol storage buffer (300 mM NaCl, 50 mM NaH2PO4, 50% [vol/vol] glycerol; pH 8.0) by using a Pierce Slide-A-Lyzer cassette (10-kDa molecular mass cutoff; MJ BioLynx, Brockville, Ontario, Canada), and stored at −20°C. The protein concentration was determined by using the Bradford reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada) with bovine serum albumin as the standard. Based on the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the purified extract contained traces of contaminating proteins or degradation products (see Results). 3A. Therefore, as a control to confirm that CbaR caused the effects observed in gel shift assays (see below), an extract was prepared as described above from cells harboring the expression construct with no insert. Gel shift assays. Binding of CbaR to DNA containing the cbaA or cbaR transcriptional start site was characterized by gel shift assays using three DNA templates, shown schematically in Fig. 3B. In the initial assays we tested interactions with two templates, template 1 and 2. Template 1 putatively contained the transcriptional start site of cbaR, which was roughly mapped in expression studies (see below), and spanned nucleotides (nt) 4096 (BglII site) to 4446 (NolI site). Template 2 contained the transcriptional start site of cbaR (determined by primer extension analysis [see below]), as well as sufficient upstream DNA so that cbaA expression was normal (as determined in expression studies with construct cbaA-lacZ [see below]). The latter sequence spanned nt 4446 (NolI site) to 4705 within cbaA (SacI site). Templates 1 and 2 were obtained by digesting pMP14.10 with NolI and BamHI and gel purifying the ~400- and ~260-bp fragments, respectively. For more detailed gel shift studies we used a 186-bp fragment (molecular weight, 114,000) that was similar to template 2 but slightly shorter at both ends (Fig. 3B). The third template of this gel shift series, template 3, is shown in Fig. 2B, and it was generated by PCR with primers PCB4480 (5'-gctgggttaaacacctagaa-3') and CBAA6655 (5'-ttcggacccacatcgtttt-3') (lowercase and italicized nucleotides are not native to cbaR). The amplification product was agarose gel purified and quantified by UV spectroscopy. All templates were end labeled with digoxigenin-11-dUTP by using terminal transferase as recommended in the manual (Boehringer Mannheim). Shift assay (Roche Molecular Biochemicals, Laval, Québec, Canada). Templates were separated from unincorporated label by spin column chromatography (Qiagen) and treated as described below with respect to binding. DNA binding assays were performed in a total volume of 10 μl, and the reaction mixtures contained 2 μl of 5% binding buffer [100 mM HEPES (pH 7.6), 5 mM EDTA, 50 mM (NH4)2SO4, 5 mM dithiothreitol, 1% (w/vol) Tween 20, 150 mM KCl], 0.5 μl of poly(dI-dC) (1 μg/μl), 0.5 μl of poly-l-lysine (0.1 μg/μl; Roche Molecular Biochemicals), 4 μg of digoxigenin-labeled template DNA, various amounts of amendments depending on the experiment (see below), and 1 to 3 μl of CbaR in glycerol storage buffer. Glycerol storage buffer containing no CbaR was added as required so that the final concentration of glycerol was 15% (vol/vol), and sterile distilled H2O was added as required to adjust the volume of the reaction mixture. The DNA-binding reactions were stopped by the addition of 1 μl of glycerol in the binding reaction mixtures made loading buffer for subsequent electrophoresis unnecessary. Samples were kept at room temperature for 15 to 30 min and placed on ice for at least 5 min, and then a 4- to 5-μl aliquot of each binding reaction mixture was applied to a nondenaturing polyacrylamide gel (10 by 10 by 0.08 cm) at 4°C that had been prerun for 1 to 2 h at a constant current in a running buffer containing Tris base, boric acid, and EDTA (TBE) (final concentrations, 22.25, 22.25, and 0.5 mM, respectively) (pH 8.0). The first one-third of the gel (stacking gel) contained 3.5% (w/vol) polyacrylamide, and the last two-thirds (separating gel) contained 12% (w/vol) polyacrylamide; both parts of the gel contained TBE at the same concentration as the running buffer.
CBAA were identical to the 186-bp gel shift template described above, Aldrich was added, and template DNA was digested at 22°C as follows. For corresponding portion of the 16S rRNA gene of the gene. This portion of the 16S rRNA gene was identical to the we therefore sequenced a 523-nt portion of the 16S rRNA (see below) as described above for the gel shift assays; the only differences were amounts of CbaR with and without 4 mM 3CBA or 4 mM 3-carboxybenzoate polynucleotide kinase so that only the noncoding strand (\( {\text{32P}} \)PCBA) or the genetic and metabolic similarities to other C. testosteroni m mend by the manufacturer (Roche Molecular Biochemicals). Detected by chemiluminescence with an antidigoxigenin system used as recom-
Semi-Dry electroblotter (Owl Separation Systems, Portsmouth, N.H.). DNA was transferred to a positively charged nylon membrane (Roche Molecular Bio-
Samples were resolved by electrophoresis at 10 V/cm for 3 to 4 h and then transferred to a positively charged nylon membrane (Roche Molecular Bio-
chemicals) by electrophoblotting with TBE for 60 to 90 min at 300 mA in a Panther Semi-Dry electrophoblotter (Owl Separation Systems, Portsmouth, N.H.). DNA was detected by chemiluminescence with an antidigoxigenin system used as recom-
mended by the manufacturer (Roche Molecular Biochemicals).

DNase I protection assays. The specific areas bound by CbaR were deter-
mained by DNase I protection assays. The two templates (\( {\text{32P}} \)PCBA and \( {\text{32P}} \)CBAA) were identical to the 186-bp gel shift template described above, except that one of the primers used during amplification had been previously radioactively end labeled using [\( {\gamma}^{32} \)P]ATP (Amersham Canada Ltd.) and T4 polynucleotide kinase so that only the noncoding strand (\( {\text{32P}} \)PCBA) or the coding strand (\( {\text{32P}} \)CBAA) was phosphorylated. Following amplification, PCR products were separated from unincorporated label by spin column chromatography (Qiagen). \( {\text{32P}} \)P-labeled DNA templates (\( \sim \)6 ng) were bound to various amounts of CbaR with and without 4 mM 3CBA or 4 mM 3-carboxybenzoate (see below) as described above for the gel shift assays; the only differences were that the binding buffer contained 5 mM MgCl\(_2\) and 2.5 mM CaCl\(_2\), and the reaction volume was 12 \( \mu \)l. After binding, 2.5 \( \times \) 10\(^{-3}\) U of DNase I (Sigma-
Aldrich) was added, and template DNA was digested at 22°C as follows. For \( {\text{32P}} \)PCBA, 4-\( \mu \)l samples were removed from the reaction mixture at 30, 60, and 120 s and combined into one tube containing 1.5 \( \mu \)l of stop solution (500 mM EDTA); for \( {\text{32P}} \)CBAA, 1.5 \( \mu \)l of stop solution was added after 60 s of digestion. Three microliters of a glycerol loading buffer was then added, and samples were placed in a boiling water bath for 3 min. A 3-\( \mu \)l aliquot was applied to a denaturing sequencing gel (8% [wt/vol] polyacrylamide, 8 M urea), and sequencing ladders (generated with the appropriate primer using mPM14.10 as the template) were included in adjacent lanes. Fragments were resolved electrophoretically and visualized by autoradiography.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers U18133 (for CbaR) and AF345907 (for 16S ribosomal DNA).

**RESULTS**

Classification of strain BR60(pBRC60). The identity of 3CBA-degrading strain BR60(pBRC60), originally described as an *Alcaligenes* sp. strain (67), was not firmly established, and we therefore sequenced a 523-nt portion of the 16S rRNA gene. This portion of the 16S rRNA gene was identical to the corresponding portion of the 16S rRNA gene of the *C. testosteroni* type strain, and because BR60(pBRC60) has several genetic and metabolic similarities to other *C. testosteroni* strains (see below), *Alcaligenes* sp. strain BR60 is reclassified as *C. testosteroni* BR60.

**Identification of cbaR and determination of cbaA transcriptional start.** BLAST analysis of DNA upstream of Tn5271 revealed the presence of a divergently oriented ORF beginning 667 bp upstream of *cbaA* (Fig. 1). This ORF was designated *cbaR* and codes for a 19.4-kDa protein that exhibits identity to various members of the MarR family of transcriptional regulators (Table 2). Because many proteins belonging to this family respond to specific aromatic compounds or are involved in regulating genes for metabolism of aromatic compounds, we focused on the effect of CbaR on expression of *cbaA* (see below).

The transcriptional start site of *cbaA* was determined in order to better characterize the promoter of this gene. As determined by primer extension analysis with DNA from 3CBA-grown cells, this transcriptional start site is an adenine 99 nt upstream of the putative start codon (Fig. 2A). A hexam er starting at position −12, TATAAT, is similar to the consensus enterobacterial −10 hexamer for \( \sigma^{34} \)-dependent genes (TATAAT), indicating that *cbaA* possesses a \( \sigma^{34} \)-like promoter. However, no sequence similar to TTGACA, the consensus enterobacterial −35 hexamer, was detected. An RT-PCR assay was conducted to compare levels of *cbaA* mRNA in uninduced cells (succinate grown) and induced cells (3CBA grown), and the intensity of the signal obtained with the former cells was much lower than the intensity obtained with the latter cells (Fig. 2B), indicating that growth on 3CBA leads to an increase in *cbaA* transcription. This experiment also showed that CBAAR, the primer used in the RT step of these two assays, was specific for *cbaA* mRNA.

**Induction of 3CBA degradation activity and expression of cbaA.** In order to determine which growth substrates induced 3CBA degradation activity, we initially investigated control of 3CBA catabolism in *C. testosteroni* BR60(pBRC60) by performing O\(_2\) uptake assays with whole cells, and the results are shown in Table 3. As expected, maximal 3CBA degradation

**TABLE 2. Proteins in the GenBank database showing identity to CbaR\(^\text{a}\)**

| Protein | Accession no. | % Identity to CbaR | Organism | Function and effectors, if known | Reference(s) |
|---------|-------------|-----------------|---------|---------------------------------|-------------|
| BadR    | U75363      | NA\(^b\)        | *Rhodopseudomonas palustris* CGA009 | Activates genes required for anaerobic growth on benzolate; may respond to benzoyl coenzyme A | 15, 16       |
| CinR    | U64802      | NA              | *Batyribrio fibrisolvens* E14    | Represses genes for cinnamoyl ester hydrolyase; responds to feruloyl-containing sugars | 11          |
| EmrR    | U19993      | 20              | *Escherichia coli*               | Represses multidrug resistance pump genes; responds to 2,4-dinitrophenol | 7, 34        |
| HpcR    | S56952      | 19              | *Escherichia coli* C             | Represses homoprotocatechuate catabolic genes | 25, 52       |
| MarR    | M96235      | 28              | *Escherichia coli*               | Represses multiple antibiotic resistance genes; responds to 2-hydroxybenzoate and 2,4- dinitrophenol | 2, 6, 56     |
| MexR    | U23763      | 28              | *Pseudomonas aeruginosa*         | Represses multiple antibiotic resistance genes; may respond to chloramphenicol | 17, 48, 57   |
| NhhD    | D67027      | NA              | *Rhodococcus rhodochrous* JI     | Activates nitrite hydratase genes | 30          |
| PecS    | X74409      | 22              | *Erwinia chrysanthemi*           | Represses pectinase and cellulase genes | 51          |
| SlyA    | U30884      | 25              | *Salmonella enterica* serovar    | Activates genes required for hemolysis and infection of and survival in mouse macrophages | 8, 33, 43 |

\(^{a}\) Various uncharacterized ORFs showing identity to CbaR were also identified, but they were not included as they had no known function.

\(^{b}\) NA, not applicable. A BLASTP search with CbaR as the query did not identify this protein as a CbaR homologue. However, the protein is included because it is a MarR family member based on sequence similarity determined by using homologues of CbaR as the query in a second round of BLASTP analysis.
activity was induced by 3CBA. Benzoate, which is not a substrate of this pathway and is not channeled via the PCA extradiol pathway (50), gratuitously induced 3CBA degradation activity (∼29% of the maximum activity). Similarly, benzoate degradation activity was gratuitously induced by 3CBA (∼22% of the maximum activity observed following growth on benzoate). Succinate, a nonaromatic carbon source, did not induce 3CBA activity, nor did 3-hydroxybenzoate, 4-hydroxybenzoate, and PCA, three aromatic carbon sources that are channeled through the PCA extradiol ring fission pathway, suggesting that the cbaA pathway, at least with respect to 3CBA, responds to the same substrate but not to downstream metabolites that are common to these degradative pathways (i.e., PCA or the ring fission products). We observed good reproducibility between trials for all cultures except those grown on benzoate. For unknown reasons, cells exhibited variable lag phases (18 to 36 h) in the three trials before the onset of logarithmic growth. This apparently affected O₂ uptake rates with benzoate but not with the other carbon sources (data not shown). The results presented here are the results for the trial in which the log phase was achieved within 18 h, the time necessary for the other growth substrates.

Control of cbaABC-mediated catabolism of 3CBA was further characterized by studying cbaA expression in the presence and absence of CbaR. Expression levels were measured with strain BR6020 (Table 1) harboring the constructs cbaA′-lacZ1 and cbaRA′-lacZ (Fig. 1B) and grown on 3CBA or the gratuitous inducer benzoate (see above). The levels of expression were compared to those observed following growth on the noninducing substrate succinate (see above). We initially hypothesized that CbaR was a repressor and that in its absence we would observe constitutive (i.e., maximal) cbaA expression. The Ts5271-deficient genetic background provided by BR6020 allowed us to specifically test its role by eliminating trans-encoded CbaR, while reintroduction of pBRC60, which created a Ts5271-proficient background, restored all functions necessary for growth on 3CBA and created a strain that was essentially identical to the parent strain, BR60(pBRC60). The results are summarized in Fig. 3. In Ts5271-deficient cells, LacZ activity under noninducing conditions was roughly the same with both constructs. For benzoate-grown cells, the induction ratios compared to succinate-grown cells were 3.2- and 7.4-fold in BR6020 harboring cbaRA′-lacZ and BR6020 harboring cbaA′-lacZ1, respectively. From these data, it appeared that control of cbaA was not due to simple repression by CbaR and that CbaR might be a modulator of benzoate-induced activity. In addition, because cbaA was induced by benzoate in the absence of any pBRC60/Ts5271-encoded functions, our data suggested that there is a benzoate-responsive chromosomally encoded regulator.

In a Ts5271-proficient background, the LacZ activities under noninducing conditions were roughly the same with both constructs, and compared to succinate-grown cells, the induction ratios for benzoate-grown cells (4.1- to 4.2-fold) and 3CBA-grown cells (5.5- to 5.8-fold) were virtually the same with both constructs (Fig. 3). After pBRC60 was introduced to create a Ts5271-proficient background, the levels of cbaA expression were compared to succinate-grown cells were 3.2- and 7.4-fold in BR6020 harboring cbaRA′-lacZ and BR6020 harboring cbaA′-lacZ1, respectively. From these data, it appeared that control of cbaA was not due to simple repression by CbaR and that CbaR might be a modulator of benzoate-induced activity. In addition, because cbaA was induced by benzoate in the absence of any pBRC60/Ts5271-encoded functions, our data suggested that there is a benzoate-responsive chromosomally encoded regulator.
In an effort to determine if cbaR coded for a functional DNA binding protein, CbaR was tagged at the N terminus, overexpressed in *E. coli*, and affinity purified. As determined by SDS-PAGE analysis, affinity-purified extracts of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cultures of M15(pQE30cbaR, pREP4) contained large amounts of a protein that comigrated with the 20.1-kDa size standard (Fig. 4A), which is very similar to the predicted molecular mass of CbaR with the six-histidine tag (20.7 kDa). Initial gel shift assays with purified CbaR were then conducted to determine if CbaR bound DNA containing either the transcriptional start site of *cbad* (template 1) or *cbaf* (template 2), and the results are shown in Fig. 4C. CbaR did not affect migration of template 1 but retarded migration of template 2. With template 2, two CbaR-DNA complexes that moved more slowly were observed; these complexes were designated C1 and C2, and addition of 4 mM 3CBA strongly disrupted their formation. If unlabeled template 2 was added as competitor DNA to a binding reaction mixture containing template 2, a shift towards less retarded DNA was observed (data not shown). As a control, the effect of an affinity-purified extract from *E. coli* containing the expression vector with no insert on migration of the two DNA templates was tested, and no change was observed (data not shown), indicating that the effects elicited by the extract were caused by CbaR.

More detailed gel shift assays to study interactions between CbaR and the *cbaf* promoter (P_{cbaf}) were then performed by using template 3 (Fig. 4B). Experiments with a range of 3CBA concentrations revealed that significant disruption occurred with 40 μM 3CBA (Fig. 4D). The stoichiometry of C1 and C2 formation was also studied, and the results are shown in Fig. 4E. Small amounts of C1 appeared when CbaR and P_{cbaf} DNA were present at approximately equimolar concentrations, and equal amounts of unbound DNA and C1 resulted when there was a 10-fold molar excess of CbaR. Small amounts of C2 were already apparent by this point, but equal amounts of C1 and C2 did not appear until there was a 250-fold molar excess of CbaR. C2 did not predominate until there was at least a 2,500-fold molar excess of CbaR. Because the template used in these assays contained two CbaR binding sites (see below), we were not able to unambiguously determine binding constants for P_{cbaf} DNA. A final set of gel shift assays was conducted to determine whether other aromatic compounds disrupted the CbaR-P_{cbaf} DNA complexes. We tested the effect of benzoate or one of the three hydroxylated benzoates used in the O_{2} uptake experiments (see above), and the results are presented in Fig. 4F. Benzoate was a weak disrupter (i.e., it caused a small shift of DNA towards less retarded forms); 4-hydroxybenzoate had no effect; PCA was a strong disrupter (i.e., it interfered with binding at levels comparable to the 3CBA levels); and unexpectedly, 3-hydroxybenzoate improved CbaR binding (i.e., it caused DNA to shift towards more retarded forms). The latter effect was also elicited by 3-carboxybenzoate (see below).

DNase I protection assays were then performed to determine the specific binding sites of CbaR on P_{cbaf} and the results are presented in Fig. 5A. Approximately 6 ng of DNA was used per reaction mixture, and according to gel shift data presented in Fig. 4E, the smallest amount of CbaR tested (10 ng or a ~9-fold molar excess compared to the amount of P_{cbaf} DNA) resulted mostly in C1 and very little C2, while the largest

expression compared to those in Tn5271-deficient cells tended to decrease with both constructs (Fig. 3). For succinate-grown cells the effect was insignificant to moderate (1.1- to 1.6-fold decreases), but for benzoate-grown cells harboring *cbaf-lacZ* the effect was relatively strong (2-fold decrease), probably because of *trans*-encoded CbaR from pBR6060. For benzoate-grown cells harboring *cbaf-lacZ*, the effect was slight (1.2-fold), presumably because a *cis*-encoded CbaR was already provided by the construct. In BR6020 harboring the control construct (promoterless lacZ) and grown as described above in a Tn5271-deficient or Tn5271-proficient background, LacZ activity was negligible (~1 Miller unit).

Expression of cbaR and gel shift and DNase I footprinting assays with affinity-purified CbaR. In order to better understand the gene coding for CbaR, we investigated expression of *cbaf* with various LacZ constructs, not only to determine whether the levels of *cbaf* expression were affected by growth conditions and genetic backgrounds that affected *cbaf* expression but also to roughly map the transcription starting point. LacZ activity in BR6020 harboring *cbaf-lacZ* and *cbaf-lacZZ* was very low (~20 Miller Units), but compared to the control (see above) the activity was significant. These levels of expression were observed in Tn5271-deficient cells grown on succinate or benzoate and in Tn5271-proficient cells grown on succinate or 3CBA, implying that Tn5271-encoded functions, particularly CbaR, did not have an effect on *cbaf* expression. With BR6020 harboring *cbaf-lacZ* in both genetic backgrounds and grown under the same conditions, the activity was negligible (~1 Miller unit). This implied that *cbaf* transcription began before nt 4098 (i.e., the BglII site defining the end of construct *cbaf-lacZ* but after nt 4446 (i.e., the NotI site defining the end of construct *cbaf-lacZZ*, in which expression was observed) (Fig. 1B).

FIG. 3. Levels of *cbaf* expression in *C. testosteroni* BR6020 harboring different lacZ constructs. Expression was measured with a growth substrate that did not induce 3CBA degradation activity (succinate) or did induce this activity (benzoate or 3CBA) in Tn5271-deficient cells (−pBR6060) and Tn5271-proficient cells (+pBR6060). The data are averages based on three independent trials; the error bars indicate standard errors. 3Cba, 3-chlorobenzoate.
amount (600 ng or an ~800-fold molar excess) resulted in approximately equal amounts of C1 and C2. Two protected regions were identified: site I (a higher-affinity site), which was clearly evident with 10 ng of CbaR, and site II (a lower-affinity site), which became apparent only with larger amounts of CbaR. If 3CBA was included in the binding reaction mixture, neither site was protected from DNase I digestion. If the binding enhancer 3-carboxybenzoate was included, protection of both sites was improved ~60-fold (i.e., 10 ng of CbaR in the presence of 3-carboxybenzoate protected sites I and II as strongly as 600 ng of CbaR in the absence of 3-carboxybenzoate). Furthermore, it allowed us to more clearly define nucleotides protected within site II.

Site II overlaps the transcription starting point (position +1) of cbaA, and site I is approximately 40 nt downstream (Fig. 5B). Site I possesses a 4-nt inverted repeat (IR) separated by 6 or 9 nt (5′-GTGG[N]_6/9CAAC-3′). Modified forms of the two IRs are present in the lower strand of site II (5′-GTGG[N]_7T AAC-3′ or 5′-GTAG[N]_9TAAC-3′).

**DISCUSSION**

**Classification of strain BR60(pBRC60).** We reclassified Alcaligenes sp. strain BR60(pBRC60) as C. testosteroni BR60 (pBRC60) based on several metabolic and genetic similarities between this bacterium and various other strains of the β-proteobacterium C. testosteroni (59, 62). The metabolic similarities include an inability to grow on various carbohydrates (67); induction of the PCA meta ring fission enzymes by growth on 3-hydroxybenzoate, 4-hydroxybenzoate, benzoate, or PCA (41); and a lower growth rate with benzoate than with hydroxylated benzoates (62; Providenti, unpublished data). The genetic similarities are the presence of the same partial 16S ribosomal DNA sequence (see above) and highly homologous genes for PCA meta ring fission pathway enzymes (J. Mampel, M. A. Providenti, R. C. Wyndham, and A. M. Cook, unpublished data).

**Control of 3CBA degradation activity.** We initially hypothesized that cbaABC was negatively regulated by CbaR, which was consistent with the predominant role of the MarR family of proteins (Table 2) (see below), but studies with the cbaA expression constructs implied that the major role of CbaR may be to modulate gratuitous induction by compounds like benzoate (see above). Gel shift experiments showing that benzoate only weakly disrupted CbaR binding to P_{cbaA} DNA supported this model (Fig. 4F). Furthermore, previous work that showed that the cbaR region is not required for cbaABC-mediated growth on 3CBA (41) suggested that CbaR is not essential for cbaA expression. Because studies with Tn5271-deficient cells harboring construct cbaA-lacZ1 showed that growth on benzoate (Fig. 3) or growth on succinate in the presence of 3CBA (data not shown) induced cbaA expression, we propose that there is a chromosomally encoded regulator that, along with CbaR, controls cbaABC. 3CBA and benzoate gratuitously activate each other's pathways and exhibit similar induction spectra with respect to 3-hydroxybenzoate, 4-hydroxybenzoate, and PCA (Table 3), and we therefore hypothesize that there is a shared regulator for the two pathways. The cbaABC regulatory system may thus have similarities to CatR activation of both the chromosomally encoded catBCA operon for catechol metabolism and the plasmid-encoded pheBA operon for phenol metabolism.
metabolism (26, 44). Alternatively, we may have observed a cross-activation phenomenon, like induction of the lower pathway of the plasmid-encoded xyl genes in response to benzoate despite the absence of XylS, the normal activator (10, 24, 28), or CatR- and ClcR-mediated cross-activation of the clc- and cat-encoded catabolic pathways (37, 45).

**CbaR: expression of the gene and functional characterization.** The gene that encodes CbaR appears to be expressed at a very low level (see above), and neither benzoate nor 3CBA, two growth substrates that increased cbaA expression, altered the level of cbaR expression. In addition, CbaR itself does not appear to exert control over expression of its own gene. This conclusion is suggested by the observation that the levels of cbaR expression were the same regardless of the presence of Tn5271, which provided a trans-encoded CbaR (see above), and the observation that CbaR did not bind to DNA spanning the putative −10 hexamer; and the start codon of *cbaA* (ATG). Inverted repeats found in site I and modified versions of these repeats in site II are indicated by arrows, and bases present in both sites are indicated by boldface type.

![DNase I protection assay](image)

**FIG. 5.** (A) DNase I protection assay to determine specific regions of P$_{cbaA}$ DNA protected by CbaR. Template DNA was end labeled with $^{32}$P$_{PO_4}$ on the noncoding strand ($^{32}$P-PCBA) or the coding strand ($^{32}$P-CBAA). Various amounts of CbaR were added, and the effect of 4 mM 3CBA (3Cba) or 4 mM 3-carboxybenzoate (3Crba) on CbaR protection of two regions (sites I and II) was determined. A sequencing reaction conducted with the appropriate primers was included during electrophoretic resolution of DNase I-digested samples. (B) Sequences of both strands of the template DNA used for the assay, indicating the positions of sites I and II relative to the transcriptional start site of *cbaA* (position +1); the putative −10 hexamer; and the start codon of *cbaA* (ATG). Inverted repeats found in site I and modified versions of these repeats in site II are indicated by arrows, and bases present in both sites are indicated by boldface type.
the region putatively containing the cbaR transcription start site (see above) (Fig. 4C). Furthermore, no sequences similar to the two binding sites of CbaR were detected when the complete region upstream of cbaA was analyzed.

CbaR bound the cbaA promoter at two sites and exhibited different affinities for these sites, protecting site I strongly and site II less strongly (Fig. 5B). Sites I and II have many identical bases, and IR structures were detected in site I (Fig. 5B). Although a direct role for the IR structures has not been determined, it is noteworthy that many of the sequence differences between sites I and II occur in the possible modified forms of the IR structures in site II (Fig. 5B), which may explain the different binding affinities. By correlating gel shift stoichiometry data (Fig. 4F) with DNase I protection results (Fig. 5A), it was shown that C1 represents CbaR bound to site I while C2 represents CbaR bound to both site I and site II. Presumably, independent CbaR proteins bind to each site, but this remains to be shown. When binding inhibitors are present, binding to site II is affected first, as disappearance of C2 always precedes disappearance of C1 (Fig. 4D), presumably because the lower affinity of CbaR for site II makes it more susceptible to binding disrupters. This may allow CbaR-mediated repression at site II, which overlaps the transcription start site of cbaA (Fig. 5A), to be overcome more easily and is probably an adaptation that increases the sensitivity of the cba pathway to potential substrates and allows it to respond to the concentrations of CBA (which are expressed in parts per million) typically encountered in contaminated environments (67). Our gel shift assays showing that 40 μM 3CBA (−6 ppm) disrupted binding (Fig. 4D) support this view.

A brief survey of the effects of other aromatic compounds on CbaR binding identified PCA as a strong disrupter of the CbaR-P$_{bad}$ complex (Fig. 4F), suggesting that CbaR evolved so that it responds to both 3CBA, the substrate of the cba pathway, and at least one downstream metabolite. This may be a positive feedback mechanism that ensures derepression of cbaABC under conditions in which PCA is being produced because of CBA metabolism. Unexpectedly, two meta-substituted benzoates (3-hydroxybenzoate and 3-carboxybenzoate) improved CbaR binding to F$_{chaA}$ DNA (Fig. 4F and 5A). To our knowledge, this is the first time that this phenomenon has been reported for a MarR-like protein, and we are currently exploring the effect in greater detail and studying its physiological significance. Conceivably, improved binding by CbaR could lead to increased repression of cbaA, a phenomenon observed with MarR mutant proteins that bind more strongly to their cognate operator and superrepress marRAB (3).

As a member of the MarR family of regulators, CbaR belongs to a diverse group of proteins that control a variety of microbial functions, including antibiotic resistance, catabolism of various substrates, plant pathogenicity, and animal virulence (Table 3). On a functional level, CbaR exhibits some similarities to MarR, PecS, and MexR, which form two or three protein-DNA complexes in a concentration-dependent fashion when they are combined with DNA containing the appropriate cognate promoter; the number of complexes reflects the number of binding sites (17, 35, 36, 49, 56). Furthermore, the binding sites of MexR (17) contain an inverted repeat structure (5′-GTTG[A][N]$_n$TC AAC-3′) that is extremely similar to one of the possible inverted repeats detected in the higher-affinity binding site of CbaR (see above). Otherwise, the binding sites for CbaR, MarR (36), PecS (49), and MexR (17) differ with respect to sequence, size, spacing relative to each other, and position relative to the transcriptional start site and start codons for target genes. For the most part, regulators belonging to the MarR family are repressors, but BadR, NhdR, and SlyA are activators of the operons that they control (15, 30, 43). The organizations of genes that encode MarR family proteins relative to the genes that they control and the methods by which expression of the gene that encodes a MarR family protein is regulated vary substantially. Interesting contrasts include genes that encode MarR-like proteins that are divergently transcribed from their target genes, including hpcR, mexR, nhdD, and cbaR (29, 48, 52; this study); that are part of the same operon, including emrR and marR (4, 34); or that are part of a different operon but are transcribed in the same direction, including badR (15). Expression of the genes that encode some MarR-like regulators is subject to induction by other regulators; these genes include nhdD, which is induced by NhhC (29), and marR, which is induced by MarA (4). In addition, autorepression has been demonstrated for emrR, mexR, and marR (4, 34, 48). For the most part, MarR-like proteins appear to act alone on target genes, but in some cases they act in concert with other regulators. An example of this is BadR, which works together with AadR to fully activate the bad genes of Rhodopseudomonas palustris (15). Similarly, PecS is part of an intricate regulatory network which, in concert with a variety of other regulators (both repressors and activators), controls or attenuates expression of a host of physically unlinked operons spread over the Erwinia chrysanthemi chromosome (23, 54). Despite the diversity of the MarR-like proteins, shared features of these proteins include an ability to respond to aromatic compounds (Table 3) and a conserved motif in the center of the protein (58) whose function may be to bind DNA (1).

In conclusion, we found that the cba catabolic pathway of C. testosteroni BR60(pBR60) is controlled in part by CbaR, a cis-encoded 3CBA-responsive MarR-like regulator whose major role may be to modulate gratuitous induction by benzoate or compounds like benzoate. There also appears to be an unidentified, chromosomally encoded benzoate-3CBA-responsive regulator that induces cbaABC, and we are currently attempting to isolate this protein in order to better understand factors that control cbaA expression.

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