The chlorobenzoate dioxygenase genes of *Burkholderia* sp. strain NK8 involved in the catabolism of chlorobenzoates

Perigio B. Francisco Jr, Naoto Ogawa, Katsuhisa Suzuki and Kiyotaka Miyashita

Author for correspondence: Kiyotaka Miyashita. Tel: +81 298 38 8256. Fax: +81 298 38 8199. e-mail: kmiyas@s.affrc.go.jp

Soil General Microbiology Laboratory, National Institute of Agro-Environmental Sciences, 3-1-1 Kannondai, Tsukuba City, Ibaraki 305-8604, Japan

*Burkholderia* sp. NK8 grows abundantly on 3-chlorobenzoate (3CB), 4-chlorobenzoate (4CB) and benzoate. The genes encoding the oxidation of (chloro)benzoates (*cbeABCD*) and catechol (*catA, catBC*), the LysR-type regulatory gene *cbeR* and the gene *cbeE* with unknown function, all of which form a single cluster in NK8, were cloned and analysed. The protein sequence of chlorobenzoate 1,2-dioxygenase (*CbeABC*) is 50–65% identical to the benzoate dioxygenase (*BenABC*) of *Acinetobacter* sp. ADP1, toluate dioxygenase (*XylXYZ*) of the TOL plasmid pWW0 and 2-halobenzoate dioxygenase (*CbdABC*) of *Burkholderia cepacia* 2CBS. Disruption of the *cbeA* gene resulted in the simultaneous loss of the ability to grow on benzoate and monochlorobenzoates, indicating the involvement of the *cbeABCD* genes in the degradation of these aromatics. The *cbeABCD* genes are preceded by *catA*, the gene for catechol dioxygenase. *lacZ* transcriptional fusion studies in *Pseudomonas putida* showed that *catA* and *cbeA* are co-expressed under the positive control of *cbeR*, a LysR-type transcriptional regulatory gene. The *cbeA::lacZ* transcriptional fusion studies showed that the inducers of the genes are 3CB, 4CB, benzoate and probably *cis,cis*-muconate. On the other hand, 2-chlorobenzoate (2CB) did not activate the expression of the genes. The chlorobenzoate dioxygenase was able to transform 2CB, 3CB, 4CB and benzoate at considerable rates. 2CB yielded both catechol and 3-chlorocatechol (3CC), and 3CB gave rise to 4-chlorocatechol and 3CC as the major and minor intermediate products, respectively, indicating that the NK8 dioxygenase lacks absolute regiospecificity. The absence of growth of NK8 on 2CB, despite its considerable degradation activity against 2CB, is apparently due to the inability of CbeR to recognize 2CB as an inducer of the expression of the *cbe* genes.

**Keywords:** *Burkholderia*, chlorobenzoate dioxygenase, *cbeABC*, *cbeR*, *catA*

**INTRODUCTION**

Chlorobenzoates are the major intermediate products of aerobic catabolism of polychlorinated biphenyls (PCBs).

In most PCB-degrading micro-organisms, however, chlorobenzoates are not further metabolized (Unterman, 1996). Degradation of chlorobenzoates, therefore, is important for the complete degradation of PCBs. Chlorobenzoate-degrading bacteria that possess aromatic-ring-hydroxylating dioxygenases involved in the initial steps of chlorobenzoate catabolism have been isolated from soil. Genes encoding these dioxygenases have been identified. These are the 3-chlorobenzoate 3,4-dioxygenase (*cbaAB*) and dehydrogenase (*cbaC*) genes of *Alcaligenes* sp. BR60 (Nakatsu & Wyndham, 1993; Nakatsu *et al*., 1995, 1997), the 2-halobenzoate...
1,2-dioxygenase genes (cbdABC) of Burkholderia cepacia 2CBS (Haak et al., 1995) and the ortho-halobenzoate 1,2-dioxygenase genes (ohbAB) of Pseudomonas aeruginosa 142 (Tsoi et al., 1999). CbABC generates protocatechuate and 5-chloroprotocatechuate from 3-chlorobenzoate (3CB), while CbdABC and OhbAB produce catechol from 2-chlorobenzoate (2CB) in the respective bacteria.

Bacterial degradation of chlorobenzoate via chlorocatechol is supposed to be one of the typical degradation pathways for chlorobenzoates. In this pathway, chlorobenzoates are converted to chlorocatechols by (chloro)benzoate dioxygenase and (chloro)benzoate-dihydrodiol dehydrogenase (Focht, 1996), and the chlorocatechols thus generated are transformed by the so-called ‘modified ortho pathway’ enzymes (Harwood & Parales, 1996; Reineke, 1998; van der Meer et al., 1992). The genes encoding these enzymes were apparently derived from the ‘ortho pathway’ genes for catechol degradation (Daubaras & Chakrabarty, 1992; Frantz & Chakrabarty, 1987; Reineke, 1998; van der Meer et al., 1992). While the structure and expression of the modified ortho pathway genes have been extensively studied (McFall et al., 1998; van der Meer et al., 1992), those genes for (chloro)benzoate dioxygenase in bacteria that transform chlorobenzoates to chlorocatechols have not been sufficiently examined. Analysis of (chloro)benzoate dioxygenase genes is essential for the elucidation of the molecular mechanism of chlorobenzoate degradation.

Burkholderia sp. NK8 is a soil isolate that shows broad specificity for chlorobenzoate degradation, being capable of growth on 3CB and 4-chlorobenzoate (4CB). The current study was conducted to examine the genes for chlorobenzoate dioxygenase(s) responsible for the broad substrate specificity of NK8 for chlorobenzoates. The chlorobenzoate dioxygenase genes of NK8 were cloned and analysed using various genetic and enzymic methods.

METHODS

Bacterial strains, plasmids and bacterial growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. Burkholderia sp. NK8, which was isolated in Japan from a 3CB-enriched soil sample, has a 16S rDNA sequence that is 97% and 95% identical to those of Burkholderia glathei (GenBank accession no. U69235) and Burkholderia andropogonis (accession no. X67037), respectively. The bacterial strains, plasmids and bacterial growth conditions are listed in Table 1.

DNA manipulation. Genomic DNAs were prepared as described by Ausubel et al. (1987) and plasmid DNA preparations were done using Qiagen spin mini- and midi-prep kits (Qiagen). Restriction enzyme digestions and DNA ligations were performed following the recommendations of the suppliers (Takara, Toyobo and NipponGene). Competent cells for transformations by electroporation in a Bio-Rad Gene Pulser or by 42 °C heat shock were prepared using the methods of Smith et al. (1990) and Hanahan (1985), respectively. All DNAs for ligations (vectors and inserts) were purified from agarose gels using GeneClean II (Bio101). Mobilizations were performed basically as described by Franklin (1985).

Cloning of the benzoate dioxygenase genes. Degenerate PCR primers were designed from highly homologous regions of benA of Acinetobacter sp. strain ADP1 and xylX of P. putida TOL plasmid pWW0 (Harayama et al., 1991). Using purified NK8 genomic DNA as template, PCR products were carried out with different degenerate primer combinations. The forward primer BAF1 [5'-GC/C/T)/CA/C/T)GA/G/AAGC-CAGATTCCC-3'] with the reverse primer BAR2 [5'-GGTGG-C(G/T)/GC/G/A)TAGTTCACGTG-3'] yielded an approximately 500 bp fragment, which was then cloned in the pCR2.1 TA cloning vector (Invitrogen) and sequenced with an ALfred DNA Sequencer (Pharmacia Biotech). The cloned PCR product was used to probe for the benzoate dioxygenase genes of Burkholderia sp. NK8. Purified NK8 genomic DNA was digested with various restriction endonucleases. Restriction fragments were separated on agarose gel by electrophoresis and then blotted onto Hybond-N+ nylon membrane (Amersham). The Southern blot was probed with the 500 bp PCR product labelled using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim). An approximately 5.3 kb EcoRI fragment was selected for cloning into pUC118 and pBluescript II KS(+). DNA fragments of about 5–5.5 kb recovered from the agarose gel were cloned and used to transform E. coli DH5α. Identification of positive clones was done by colony hybridization with the DIG-labelled PCR product. Putative clones were verified through direct colony PCR with the primers BAF1 and BAR2, followed by Southern blot analysis. The 568 bp EcoRI–PstI segment at the left end of the cloned 5.3 kb EcoRI fragment in Fig. 1 was excised, labelled and used to probe NK8 genomic DNA for overlapping upstream fragments. Among the positive bands, the 8.1 kb HindIII–PstI fragment was selected for cloning into pBluescript II KS(+).

Sequencing and sequence analysis. The restriction maps of the cloned NK8 DNA fragments were drawn for generating subclones for sequencing. Overlapping subcloned fragments were sequenced in an ABI 373S Automated Sequencer (Perkin-Elmer Applied Biosystems) with the ABI Dye Primer Ready Reaction Kit according to the manufacturer’s instructions. Sequences were generated from both DNA strands. Ambiguous portions of any sequence were verified by sequencing with the ABI Dye Terminator Ready Reaction Kit. Nucleotide sequence data were analysed using the GENETYX-Mac (version 10.1) and ATSEQ software (Software Development).

Determination of (chloro)benzoate dioxygenase activity. NK8 cells were grown on succinate, benzoate or 3CB liquid medium to late exponential phase, harvested by centrifugation and resuspended in 30 mM potassium phosphate buffer (pH 7.0) containing 2 mM MgCl2. The bacteria were broken by sonication and the cell-free extract was cleared by centrifugation. For the determination of (chloro)benzoate dioxygenase activity, it was resolved to 100 µg protein in a final volume of 250 µl. The reaction was initiated by the addition of 25 µl of (chloro)benzoate (0.5 mM) and was terminated after 2 h at 37 °C with 200 µl of 20% TCA. The precipitated proteins were removed by centrifugation. The absorbance of the supernatant at 550 nm was determined for the production of (chloro)benzoate dioxygenase.
**Table 1. Bacterial strains and plasmids**

Ap', ampicillin resistant; Cm', chloramphenicol resistant; Gm', gentamicin resistant; Hm', hygromycin resistant; Km', kanamycin resistant; Rp', rifampicin resistant; Sm', streptomycin resistant; Tc', tetracycline resistant; Ω, omega interposon.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties</th>
<th>Source/reference</th>
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<tr>
<td><strong>Burkholderia sp. strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK8</td>
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<td>This study</td>
</tr>
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<td>NDBA1</td>
<td>cbeA::ΩGm', megaplasmid⁻</td>
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<td>NCAD</td>
<td>catA::ΩGm'</td>
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<td>NCRD</td>
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<td>NBALZ</td>
<td>cbeA::lacZ–Km'</td>
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<td>PRS4020</td>
<td>cbeR::ΩGm', benzoate⁻</td>
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<td>DH5α</td>
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<td>K-12, Sm⁻</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
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<td>Vieira &amp; Messing (1987)</td>
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<td>Schweizer (1992)</td>
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<td>pMOB3</td>
<td>Km⁻ Cm⁺ ; source of MOB cassette</td>
<td>Schweizer (1992)</td>
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<td>pJRD215</td>
<td>Sm⁻ Km⁻ ; cosmid vector</td>
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<td>pET14b</td>
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<td>W. Lotz*</td>
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<td>pFJ50cbeRcatA'</td>
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<td>pFJ50cbeR’catA’</td>
<td>790 bp SalI–StuI cbeR’catA’ insert in pQF50, catA truncated at aa 77 out of 311, cbeR truncated at aa 128 out of 306</td>
<td>This study</td>
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</table>

* Friedrich-Alexander Universität, Erlangen, Germany.
fugation, washed three times with 20 mM potassium phosphate buffer (pH 7.5) and stored at −80 °C until used. The cells were thawed on ice, disrupted by sonication and ultracentrifuged at 164000 g for 40 min at 4 °C. The supernatants were evaluated for their ability to convert benzoate, 2CB, 3CB and 4CB, according to the method of Romanov & Hauseing (1994) except that the reaction mixture, with a total volume of 2 ml, contained 1 mM aromatic substrate and about 30–40 mg protein in addition to 5 mM Na-MES (pH 6.5), 10 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ 100 mM NADH and 2 µM FAD. Aliquots of 500 µl, which were taken at the initiation of the reaction and at 15 or 30 min thereafter, were immediately added to 86 µl 7 M trichloroacetic acid in microtubes to precipitate the proteins. Samples were prepared for quantitative HPLC analysis according to Fetzner et al. (1989) by adjusting the supernatant pH to about 6 with 5 M sodium hydroxide. After a second centrifugation, samples were diluted with 1 vol. HPLC solvent. (Chloro)benzene dioxygenase activity of the supernatant was determined by measuring substrate consumption in the supernatant by HPLC (HP1100; Hewlett Packard) on an Eclipse XDB-C18 (Agilent Technologies) reversed phase column, using acetonitrile: 10 mM H$_2$PO$_4$ (50:50, v/v) as the solvent at a flow rate of 1 ml min$^{-1}$. Authentic benzoate, 2CB, 3CB, 4CB and catechol (all purchased from Wako Pure Chemicals), and 3-chlorocatechol (3CC) and 4-chlorocatechol (4CC) (both purchased from Tokyo-Kasei) standards were run to verify their respective retention times.

**Construction of NK8 cbeA, cbeR and catA disruptant strains.** These disruptants were generated by omega (Ω) cassette interposon mutagenesis following the method of Schweizer (1992). In all gene disruptants, internal fragments of considerable length were excised, i.e. the 603 bp BstI–HincII fragment in cbeA, the 341 bp StuI–EcoRI fragment in catA and the 473 bp SphI–NruI fragment in cbeR, and replaced by the 1721 bp HindIII Ω gentamicin-resistance (Gm$^+$) cassette (Ωaac) of pH454aac, the 1773 bp SmaI Ωaac cassette of pH454aac and the 2267 bp HindIII Ω hygromycin-resistance (Hm$^+$) cassette (Ωhyg) of pH454hyg (Blondelet-Rouault et al., 1997), respectively. In all constructs, DNA fragments ranging from 1.5 to 2.2 kb flank the Ω cassette. The gene constructs and the MOB cassette of pMOB3 were sequentially cloned into pNOT322, which was used to transform E. coli strain S17-1 ppir. Conjugation of the transformed S17-1 ppir with NK8 cells was done according to Franklin (1985). Transconjugants were selected at 30 °C on DA agar plates containing gentamicin or hygromycin and were evaluated on DA agar plates with the appropriate antibiotic to separate double from single cross-overs. Allelic replacement of the wild-type genes by the Ω cassette-disrupted genes was verified by Southern hybridization analysis. Disruptants were evaluated for their ability to grow on benzoate, 3CB and 4CB containing the appropriate antibiotic. NK8 strains disrupted in their cbeA, catA and cbeR genes were named NDBA1, NCAD and NCRD, respectively.

**Complementation of the cbeA disruptant.** The 12.5 kb HindIII–EcoRI fragment of NK8 that carries the cbe–cat gene cluster was cloned into the broad-host-range plasmid vector pJR2125 (Davison et al., 1987) to generate plasmid pBAC1, which was utilized to transform E. coli S17-1. The E. coli S17-1 transformant was conjugated with the NK8 cbeA disruptant NDBA1. Transconjugants of NDBA1 were evaluated for their ability to utilize benzoate, 3CB or 4CB as the sole carbon source.

**Expression of NK8 cbeABCD in E. coli.** The NK8 cbeABCD gene cluster was amplified by PCR with the 77-mer forward primer 5′-CCCTCTAGAATAATTGTTAACTTTTAGAAGGGAGATATACCATGTCGCGCCTACCCGGACAAGCGAGTCGACGTCG-3′ and the 29-mer reverse primer 5′-CAGGATCCATAGCGAATCTTCTGTCGACAC-3′. The amplified fragment was digested with XbaI/BamHI and cloned into the XbaI/BamHI sites of PET14b to generate plasmid p14BEP. E. coli strain HMS174(DE3) was transformed with p14BEP carrying the correct fragment, as verified by sequencing. Transformants were cultured overnight at 30 °C in 2×YT medium (Sambrook et al., 1989) containing ampicillin. The cultures were diluted 50-fold with pre-warmed fresh 2×YT-ampicillin medium and again grown at 30 °C to an OD$_{600}$ of 0.4–0.6, at which point IPTG was added to a final concentration of 0.5 mM to induce expression of the cbeABCD genes. After 3 h of culture with the inducers, cells were harvested, washed three times with 20 mM potassium phosphate buffer (pH 7.5) and stored at −80 °C until used for the preparation of crude cell-free extracts for enzyme assay by HPLC.

**Determination of the products of cbeABCD.** Products generated from (chloro)benzoates by CbeABCD were determined using whole cells of E. coli HMS174(DE3) p14BEP. Cells freshly harvested from 150 ml culture grown as described above were washed with 1 vol. 20 mM potassium phosphate buffer (pH 7.5) and resuspended in 45 ml of the buffer. To aliquots of 10 ml, substrates were added to a final concentration of 2 mM and the reaction mixtures were incubated at 30 °C in a shaking water bath. Samples (1 ml) taken at selected time points were immediately centrifuged at 20000 g for 10 min at 4 °C. Aliquots (500 µl) of the supernatant were mixed with an equal volume of HPLC solvent, centrifuged and subjected to HPLC analysis at A$_{280}$.

**Transcriptional fusion studies.** Various DNA fragments from the NK8 cbe–cat region were ligated immediately upstream of the promoterless lacZ gene of the reporter plasmid pQF50 (Farinha & Kropinski, 1990) to generate several lacZ transcriptional fusion plasmids as shown in Fig. 3. The plasmid pFJS00cbeRcatAcbeA$^+$ contains the complete cbeR and catA genes and the truncated cbeA gene fused to lacZ; pFJS00cbeRcatAcbeA$^+$ differs from pFJS00cbeRcatAcbeA$^+$ in having its catA gene disrupted by the Ωhyg cassette; pFJS00cbeRcatAcbeA$^+$ is similar to pFJS00cbeRcatAcbeA$^+$ except that cbeR is incomplete; pFJS00cbeRcatA$^+$ and pFJS00cbeRcatA$^+$, the truncated catA fused to lacZ. These lacZ transcriptional fusion plasmids were introduced by electroporation into PRS4020, the catA knockout mutant of P. putida (Parales & Harwood, 1993). Transformed PRS4020 cells were assayed for β-galactosidase activity. Induction of the lacZ gene in PRS4020 transformants was performed by pre-culturing the cells overnight at 30 °C on LB medium containing gentamicin and carbenicillin. One hundred microtitre of the pre-culture was used to inoculate 10 ml basal synthetic medium (Aldrich et al., 1987) containing 10 mM glucose (BSMG), or BSGM supplemented with 5 mM benzoate, 3CB or 4CB, or with 0.1 or 0.05 mM catechol, or 5 mM cis,cis-muconate (Celgene), then grown for 17 h at 30 °C. β-Galactosidase activity was assayed according to the method of Miller (1972). All assays were done in triplicate. β-Galactosidase activity was expressed in Miller units [mmol nitrophenol generated min$^{-1}$ (mg protein)$^{-1}$].

**NK8 cbeA::lacZ–Km′ disruptant.** To determine the inducer(s) of expression of cbeA in NK8, the 473 kb lacZ–Km′ cassette of pKOK6.1, a derivative of pKOK6 (Kokotek & Lotz, 1989), was inserted into the EcoRV site of cbeA by allelic replacement.
benzoate activity as the standard. Data reported are means of three replicates. Relative activity was estimated using the same as those for the gene disruption by the \( \Omega \) interposon described above. In the final construct in pNOT322, to which the MOB cassette of pMOB3 was also inserted, the lacZ--\( \text{Km} \) cassette is flanked by 165 and 217 kb NK8 DNA fragments carrying the cbeA fragments to facilitate crossing over. Disruptants were verified by Southern hybridization analysis. NBALZ, the cbeA::lacZ--\( \text{Km} \) disruptant, was cultured in BSMG alone or in BSMG supplemented with 5 mM benzoate, 2CB, 3CB or 4CB, or with 1 mM catechol or 5 mM cis,cis-muconate. The \( \beta \)-galactosidase activity was measured as described above. Supernatants of aliquots of NBALZ cells grown with the various substrates were subjected to quantitative HPLC determination of possible degradation of the inducers before the lacZ assay was conducted.

RESULTS

Growth characteristics of NK8 on (chloro)benzoate media

*Burkholderia* sp. strain NK8 was isolated from a soil sample in Japan, under conditions in which 3CB was the sole source of carbon and energy. The strain grew abundantly on solid and liquid media containing 3CB, 4CB or benzoate as the sole carbon source. There was, however, no growth on 2CB.

At a concentration of 5 mM substrate in batch cultures, NK8 cells pre-cultured in 3CB grew fastest in 3CB medium, with a doubling time of approximately 4 h. Growth with 4CB and benzoate were slower, with doubling times of about 5.5 and 11 h, respectively. Interestingly, the lag phase with 4CB was rather long, reaching about 45 h in batch cultures inoculated with 3CB-grown cells. Corresponding values for benzoate and 3CB are 20 and 10 h, respectively. The reason for the long lag phase with 4CB is not clear. Even in batch cultures inoculated with 4CB-grown cells, the lag phase in 4CB was consistently longer than those in 3CB and benzoate media. Nevertheless, once the exponential phase commenced, NK8 cells grew rapidly, and as in 3CB medium, the transition to stationary phase in 4CB was clearly defined.

Degradation of chlorobenzoates

NK8 cells grown on succinate, benzoate or 3CB were disrupted by sonication and the supernatants were subjected to enzyme assay. The lysate of NK8 cells grown on succinate did not transform benzoate, 2CB, 3CB or 4CB (Table 2), indicating the absence of constitutive expression of the chlorobenzoate dioxygenase genes. Lysates of benzoate- or 3CB-grown cells, on the other hand, showed considerable conversion of the aromatic substrates. The relative activities for the chlorobenzoates are not remarkably different between benzoate- and 3CB-grown NK8 cells. It is noteworthy that the activity of NK8 cell lysate against 2CB is comparable to that against 3CB. These data suggest that NK8 has a (chloro)benzoate dioxygenase that possesses a broad substrate specificity.

Cloning and sequencing of the chlorobenzoate dioxygenase genes

The nucleotide sequence of the 500 bp PCR product of the primer pair BAf1/BAr2 on NK8 is similar to both xylX (67-8%) of the plasmid pWW0 and benA (59-4%) of *Acinetobacter* sp. ADP1. Probing NK8 genomic DNA for benzoate dioxygenase genes with this PCR product resulted in the cloning of a 5-3 kb EcoRI fragment (Fig. 1). Using the left end of this fragment as the probe, the overlapping 8-1 kb *HindIII*--PstI fragment was cloned. These two overlapping fragments span a 12-5 kb DNA region (Fig. 1).

**Table 2. Oxidation of (chloro)benzoates by *Burkholderia* sp. NK8 (chloro)benzoate dioxygenase**

NK8 cells were grown to late exponential phase on 5 mM succinate, benzoate or 3-chlorobenzoate while *E. coli* HMS174(DE3) cells harbouring the cbeABCD genes in pET14B were cultured on LB and induced with 0.5 mM IPTG. The cells were harvested by centrifugation, washed, sonicated and ultracentrifuged. The crude protein extracts were examined for their oxidation activity against 1 mM (chloro)benzoates. Specific activity, which is given only for benzoate, was based on the amount of substrate consumed as determined by HPLC. Relative activity was estimated using benzoate activity as the standard. Data reported are means ± SD of three replicates.

<table>
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<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Benzoate</th>
<th>2CB</th>
<th>3CB</th>
<th>4CB</th>
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<td>0</td>
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<td>34 ± 0.8</td>
<td>46 ± 2.7</td>
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<tr>
<td>NK8</td>
<td>3CB</td>
<td>100 (1-34 ± 0.01)*</td>
<td>34 ± 1.5</td>
<td>29 ± 2.1</td>
<td>61 ± 4.4</td>
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<td><em>E. coli</em>cbeABCD</td>
<td>LB/IPTG</td>
<td>100 (2-40 ± 0.05)*</td>
<td>14 ± 1.9</td>
<td>42 ± 3.0</td>
<td>76 ± 7.3</td>
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</table>

* Specific activity [nmol substrate oxidized (mg protein)^{-1} min^{-1}].
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Fig. 1. Organization of the chlorobenzoate (cbe) and catechol (cat) catabolic genes (a) clustered on the 12.5 kb HindIII–EcoRI genomic DNA region (b) of Burkholderia sp. strain NK8. Also shown in (a) are the approximate locations of the putative LysR-type transcriptional regulator binding sites and the length of each gene in the cluster. In (b), the two overlapping horizontal lines parallel to the restriction map represent the overlapping DNA fragments cloned in pBluescript II KS(›), heavy arrows indicate the points of insertion of the omega (Ω) and lacZ–Kmr interposon cassettes used for gene disruption and white segments on the restriction map represent gene fragments replaced by the Ω cassette.

Table 3. Identities (%) in deduced amino acid sequences between the NK8 CbeABCD proteins and their respective homologues in some class IB aromatic ring hydroxylases

<table>
<thead>
<tr>
<th>NK8 Cbe protein</th>
<th>Homologue*</th>
<th>Chd</th>
<th>Xyl</th>
<th>Ben</th>
<th>Ant</th>
<th>Tft</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbeA</td>
<td>Chi (ABC)</td>
<td>64-3</td>
<td>58-2</td>
<td>57-1</td>
<td>45-1</td>
<td>38-7</td>
</tr>
<tr>
<td>CbeB</td>
<td>BenABC (ABC)</td>
<td>56-2</td>
<td>60-5</td>
<td>52-2</td>
<td>39-9</td>
<td>38-9</td>
</tr>
<tr>
<td>CbeC</td>
<td>XylL (ABCD)</td>
<td>45-1</td>
<td>54-2</td>
<td>49-7</td>
<td>37-8</td>
<td>-</td>
</tr>
<tr>
<td>CbeD</td>
<td>-</td>
<td>-</td>
<td>57-0</td>
<td>56-4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Homologous proteins: CbdABC (2-halobenzoate 1,2-dioxygenase) of Burkholderia cepacia 2CBS (Haak et al., 1995), X79076; XylXYZ (toluate 1,2-dioxygenase) and XylL (toluate diole dehydrogenase) of Pseudomonas putida mt-2 TOL plasmid pWW0 (Harayama et al., 1991), M64747; BenABC (benzoate 1,2-dioxygenase) and BenD (DHBA dehydrogenase) of Acinetobacter sp. ADP1 (Neidle et al., 1987), AF009224; AntABC (anthranilate dioxygenase) of Acinetobacter sp. ADP1 (Bundy et al., 1998), AF071556; and TfrAB (2,4,5-trichlorophenoxyacetate dioxygenase) of Burkholderia cepacia AC1100 (Danganan et al., 1994), U11420. Chd, Ben and Xyl can oxidize benzoate, while Ant and Tft transform anthranilate and trichlorophenoxyacetate, respectively.

Nucleotide sequence analysis of this 12.5 kb DNA region revealed the presence of 9 ORFs. ORFs 5–9 are transcribed in the same direction (from left to right in Fig. 1), while ORFs 1–4 are transcribed divergently. After comparisons with related sequences in the database, ORFs 6–9 were designated cbeA, cbeB, cbeC and cbeD, respectively (Fig. 1). CbeA showed the highest amino acid sequence identity to CbdA (64%) (Table 3), the large subunit of the terminal oxygenase of the 2-halobenzoate 1,2-dioxygenase of B. cepacia 2CBS (Haak et al., 1995); CbeB to XylY (60%), the small subunit of the terminal oxygenase of toluate 1,2-dioxygenase of P. putida TOL plasmid pWW0 (Harayama et al., 1991); and CbeC to XylZ (54%), the reductase component of toluate 1,2-dioxygenase. Probably, CbeABC comprise an aromatic ring hydroxylase that belongs to group IB of Batie’s classification (Batie et al., 1992) as indicated by its closeness to the above-mentioned group IB hydroxylases. cbeD encodes a protein with an amino acid sequence that resembles those of the cis-diol dehydrogenase encoded by xylL on the TOL plasmid pWW0 (57% identity) and benD in Acinetobacter sp. ADP1 (56% identity).

The deduced amino acid sequence of ORF5 is most similar (82%) to the catA2 gene of Acinetobacter lwoffii
K24 (Kim et al., 1997). ORF5 was thus designated catA. ORF4 is a 935 bp gene divergently transcribed from catA. Its deduced amino acid sequence shares high identity with LysR-type transcriptional regulators involved in benzoate or catechol catabolism such as ORF$_R2$ (55%) of one of the two catechol gene clusters of Frateruria sp. ANA-18 (Murakami et al., 1999), catR of P. putida PRS2000 (50%) (Houghton et al., 1995) and RB1 (48%) (Rothmel et al., 1990), and benM (48%) and catM (45%) of ADP1 (Collier et al., 1998; Romero-Arroyo et al., 1995). NK8 ORF4 was named cbeR because subsequent results showed that the gene is involved in (chloro)benzoate catabolism, and that it responds to 3CB, 4CB and benzoate, as well as cis,cis-muconate.

The deduced amino acid sequences of ORFs 2 and 3 are similar to those of catC (63% identity) and catB (57% identity), respectively, of ADP1. Therefore, they were designated catC and catB, respectively. ORF1 encodes a protein with a deduced amino acid sequence that is 55% identical to that of benE of ADP1, the function of which is unknown (Collier et al., 1998). The gene was named cbeE.

### cbeA disruption and complementation

To ascertain the function of the cbeABCD gene cluster in chlorobenzoate catabolism, cbeA was disrupted by $\Omega$ interposon mutagenesis (Fig. 1). The disruption was confirmed by Southern hybridization. The disruptant strain NDBA1 failed to grow on 3CB, 4CB or benzoate, the substrates degraded by the wild-type strain NK8. This observation indicates that cbeA is involved in the catabolism of benzoate and monochlorobenzoates. The disruptant strain NDBA1 was complemented by the

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**Fig. 2.** Oxidation products of benzoate (b), 2CB (c), 3CB (d) and 4CB (e) resulting from the IPTG-induced expression of the NK8 (chloro)benzoate dioxygenase genes cbeABC in *E. coli*. Data were obtained from HPLC analysis at $A_{203}$ of the substrates subjected to conversion by LB-grown *E. coli* HMS174(DE3) cells harbouring the NK8 cbeABCD genes in the plasmid p14BEP. Initial substrate concentration was 2 mM. Data shown are for samples taken at the indicated times. Peaks visible in (a) are those for catechol (Cat), 3-chlorocatechol (3CC) and 4-chlorocatechol (4CC) standards.
12-kb HindIII–EcoRI fragment containing the cbe–cat gene cluster harboured by pBACI. The complemented disruptant (NDBA1/pBAC1) grew on benzoate, 3CB and 4CB (data not shown), indicating that the cbe–cat DNA region restored in the disruptant the ability to catabolize benzoate and monochlorobenzoates.

**Expression of cbeABCD in E. coli**

E. coli HMS174(DE3) transformed with p14BEP, a derivative of expression vector pET14b which carries the cbeABCD genes, was cultivated in the presence of IPTG to induce the expression of the genes. The cells were harvested, washed and disrupted by sonication. Cell-free extracts transformed 2CB, 3CB, 4CB and benzoate. Activity was greatest for benzoate followed by 4CB, 3CB and 2CB (Table 2). While the value for 2CB is relatively low compared to those of NK8 cell lysates, this result, together with that of the disruption of cbeA, confirms that the cbeABCD genes are involved in the oxidation of 2CB, 3CB, 4CB and benzoate in NK8.

Analysis of HPLC peaks emerging during enzyme reaction by whole cells of E. coli cbeABCD+ showed that benzoate generates catechol while 4CB produces 4CC. Oxidation of 3CB gives rise to 4CC and 3CC as the major and minor intermediate products of oxidation, respectively (Fig. 2), while 2CB yields not only 3CC but also catechol (apparently in equal amounts), suggesting that CbeABC lacks absolute regiospecificity.

**Disruption of cbeR and catA**

In the cbe–cat gene cluster, the single regulatory gene cbeR lies upstream of and divergently transcribed from catA. The function of cbeR in the transformation of benzoate and chlorobenzoates was determined by disrupting the gene through interposon mutagenesis with the Ωhyg cassette (Fig. 1). NCRD, the cbeR disruptant, simultaneously lost the ability to grow on 3CB, 4CB and benzoate (data not shown), suggesting that cbeR regulates the expression of the cbe genes. The catA disruptant strain NCAD, which was generated by interposon mutagenesis with the Ωlac cassette, likewise failed to grow on benzoate and chlorobenzoates. The probability that the disruption of catA prevented the transcription of the cbeABCD genes located downstream of catA, such that the cbeABCD genes were not expressed, was confirmed by later experiments (Figs 3 and 4).

**CbeR-mediated regulation of catA expression**

The function of cbeR in the expression of the cbe and catA genes was examined using various lacZ transcriptional fusion constructs in the reporter plasmid pQF50 (Fig. 3). PRS4020, the catR knock-out mutant of P. putida (Parales & Harwood, 1993), was used as the host for the lacZ reporter gene assay, since introduction of pQF50 derivatives into NK8 was unsuccessful. With PRS4020 as the host, the possibility of cross-activation of the genes from the host-encoded catR could be eliminated. In these PRS4020 transformants, β-galactosidase activity reflects the expression of cbeA or catA. When grown in BSMG medium supplemented with either benzoate, 3CB or 4CB, PRS4020 cells harbouring pFJ50cbeRcatAcbeA+ and pFJ50cbeRcatA+, both having an intact cbeR, exhibited β-galactosidase activity increased 60- to 100-fold compared to those grown in the absence of the inducers (Fig. 4). The cells harbouring plasmids with the truncated cbeR, i.e. pFJ50cbeRcatAcbeA– and pFJ50cbeRcatA–, did not elicit induction of β-galactosidase activity in the presence of 3CB, 4CB or benzoate. 2CB did not induce β-galactosidase activity regardless of the presence or absence of cbeR (data not shown). In a separate experiment involving the plasmids pFJ50cbeRcatAcbeA– and pFJ50cbeRcatAcbeA+, it was evident that catechol and cis,cis-muconate likewise induce considerable levels of β-galactosidase activity (data not shown). The above results indicate that benzoate, 3CB, 4CB, catechol and cis,cis-muconate can induce the expression of catA and cbeABCD genes in a P. putida PRS4020 background. Moreover, this evidence shows that CbeR is essential for the expression of catA and cbeA.

**Co-expression of the cbeA and catA genes**

In strain NK8, catA and cbeA are separated from each other by only 115 bp, and both lie upstream of and are transcribed divergently from cbeR (Fig. 1). Neither the −10 and −35 bacterial promoter-like sequences nor the consensus motif of LysR-type regulator recognition site exist in the catA–cbeA intergenic region. Thus, it is
likely that catA and cbeA are co-transcribed, a possibility that is further indicated by the inability of the catA disruptant NCAD to grow on (chloro)benzoates. To determine whether cbeA transcription is initiated from the catA–cbeA intergenic region, the \( \Omega \)hyg cassette was inserted into catA of the lacZ transcriptional fusion plasmid pFJ50cbeRcatAcbeA to generate pFJ50cbeRcatA::\( \Omega \)hygcebeA (Fig. 3), which was then introduced into PRS4020. There was no induction of \( \beta \)-galactosidase activity even in the presence of inducers (Fig. 4), indicating that cbeA is exclusively co-transcribed with catA.

Inducers of cbeA expression in NK8

A cbeA::lacZ transcriptional fusion construct was introduced into the NK8 wild-type genome by allelic replacement to generate strain NBALZ. HPLC analysis confirmed that the cbeA disruption had blocked the conversion of 3CB, 4CB and benzoate by NBALZ. Addition of 3CB, 4CB or benzoate to the BSMG medium increased \( \beta \)-galactosidase activity by more than 70-fold compared to those grown without these aromatics (Fig. 5). The addition of 2CB, on the other hand, did not increase the activity. cis,cis-Muconate induced \( \beta \)-galactosidase activity to a level comparable to those induced by benzoate, 3CB or 4CB. Catechol, added at a concentration low enough to allow NK8 growth, also induced \( \beta \)-galactosidase activity to a level almost half of those induced by benzoate, 3CB and 4CB. On the other hand, 3CC and 4CC did not induce \( \beta \)-galactosidase activity. These results are consistent with those obtained with PRS4020 harbouring the transcriptional fusion plasmid pQF50 derivatives (Fig. 4), and thus confirm 3CB, 4CB, benzoate and cis,cis-muconate as inducers of cbeA expression.

DISCUSSION

The NK8 chlorobenzoate CbeABC has a broad substrate specificity

The chlorobenzoate dioxygenase genes (cbeABC) involved in the transformation of benzoate, 3CB and 4CB were cloned from NK8 genomic DNA. The deduced amino acid sequence of NK8 chlorobenzoate dioxygenase (CbeABC) is 50–60% identical with those of the genetically characterized 2-halobenzoate dioxygenase (CbdABC) of \( P. \ cepacia \) 2CBS, benzoate dioxygenase (BenABC) of \( Acinetobacter \) sp. ADP1 and toluate dioxygenase (XylXYZ) of pWW0 (Table 3). Despite the similarities among these dioxygenases, the range of aromatics they oxidize is variable. The XylXYZ of \( P. \ putida \) PaW1, an \( m \)- and \( p \)-toluate degrader, shows a broad substrate specificity, being able to transform 3CB and 4CB, but not 2CB (Reineke, 1998). The CbdABC of \( B. \ cepacia \) 2CBS apparently has broader substrate specificity, but its activity against 4CB is negligible (Fetzner et al., 1992). BenABC of \( Acinetobacter \) sp.
ADP1, on the other hand, exhibits narrow substrate specificity, showing little or no oxidation of most substituted benzoates (Neidle et al., 1991). The 3CB degrader *Pseudomonas* sp. B13 grown on 3CB, and the benzoate degrader *Ralstonia eutropha* grown on benzoate, can degrade only 3CB in addition to benzoate (Reineke, 1998). The CbeABC of NK8 differs from these enzymes in that it can transform the three monochlorobenzoates almost equally well.

Similar or even greater ability to considerably transform all the monochlorinated benzoate isomers was previously reported for the toluate 1,2-dioxygenase of *B. cepacia* WR401, an o-toluolate degrader (Reineke, 1998). However, notable differences between this enzyme and NK8 (chloro)benzoate dioxygenase are evident. Relative to benzoate, NK8 CbeABC activities against the three isomeric monochlorobenzoates ranged from 28 to 61% (Table 2), while WR401 has activities estimated from Reineke (1998) of approximately 90, 110 and 50% for 2CB, 3CB and 4CB, respectively. Moreover, unlike WR401, which is a natural toluolate degrader, NK8 could not grow on o-, m- or p-toluolate medium. The clustering in NK8 of the *cbe* genes with the catechol degradation genes further justifies the classification of NK8 CbeABC as a (chloro)benzoate dioxygenase.

**The CbeABC has a relaxed regiospecificity**

Strain NK8 (chloro)benzoate dioxygenase appears to lack absolute regioselectivity as revealed by the species of intermediates it generates from the asymmetrical substrates 2CB and 3CB (Fig. 2). 2CB binds with NK8 CbeABC either as 2CB or ‘6CB’, apparently with equal affinity. The subsequent 1-2 dioxygenation of 2CB gives rise to the unstable intermediate 2-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (2-chloro-DHB), which spontaneously loses Cl\(^{-}\) to generate catechol. Dioxygenation of ‘6CB’ gives rise to 6-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (6-chloro-DHB), which spontaneously loses Cl\(^{-}\) to generate catechol. Dioxygenation of ‘6CB’ gives rise to 6-chloro-

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**Fig. 6.** Proposed degradation pathways for benzoate, 2CB, 3CB and 4CB in *Burkholderia* sp. strain NK8. In the conversion of the asymmetrical aromatics 2CB and 3CB, labelled arrows indicate steps in which 2CB binds with CbeR as 2CB (a) or ‘6CB’ (b), and in which 3CB binds as 3CB (c) or ‘5CB’ (d). The hypothetical 2-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid intermediate from 2CB (in square brackets) is presumed to spontaneously lose Cl\(^{-}\) to generate catechol (dotted arrow). The *catA* gene may also participate in the conversion of 4CC into 3-chloro-cis,cis-muconate. The gene responsible for the conversion of β-ketoadipate enol-lactone to β-ketoadipate has not been identified.
The preference of NK8 dioxygenase for 3CB that binds as ‘5CB’ (the Cl− substituent is distal to the dioxygenation point) rather than as 3CB, as indicated by the production of greater amount of 4CC than 3CC from 3CB, likewise demonstrates the lack of absolute regiospecificity of the NK8 dioxygenase. Low regiospecificity was also reported for other 2CB-oxidizing enzymes such as those of P. aeruginosa JB2 (Hickey & Focht, 1990) and Pseudomonas sp. 3CBS (Sylvestre et al., 1989), which generate 3CC and catechol from 2CB. In contrast, dioxygenases with absolute regiospecificity, as exemplified by the CbdABC from 2CB. In contrast, dioxygenases with absolute regiospecificity, as exemplified by the CbdABC of P. cepacia 2CBS (Fetzner et al., 1989) and by the ortho-halobenzoate 1,2-dioxygenase of P. aeruginosa 142 (Romanov & Hausinger, 1994; Tsoi et al., 1999), yield only catechol from the dioxygenation of 2CB.

3CB and 4CB are degraded via chlorocatechols in strain NK8

The complete degradation of benzoate and chlorobenzoates by NK8 apparently proceeds via separate routes as outlined in Fig. 6. Catechol, the CbeABCD-catalysed oxidation intermediate product from benzoate and 2CB, is likely to be converted by the ortho-cleavage pathway enzymes CatA, CatB and CatC, while the 3CC and 4CC generated from 3CB, 4CB and 2CB are processed via the modified ortho-cleavage pathway by the chlorocatechol-oxidizing enzymes. Substantiating this scheme are the following observations. NK8 mutants that are incapable of utilizing 3CB and 4CB, but could grow on benzoate, arose spontaneously after repeated subculture in LB. The chlorocatechol degradation genes of NK8, which have been cloned from its large plasmid, were shown by Southern hybridization to be absent in the mutants (unpublished data). Complementation of one of the spontaneous NK8 mutants (plasmid−, 3CB− and 4CB−) with either the Ralstonia eutropha NH9 chlorocatechol genes cbnRABCD in pEKC1 (Ogawa & Miyashita, 1999) or the plasmidborne NK8 chlorocatechol genes enabled the mutant to grow on 3CB and 4CB (unpublished data). These observations indicate that the plasmid-encoded chlorocatechol catabolic genes of the modified ortho pathway are involved in the transformation of 3CC and 4CC. Also, catA could take part in the transformation of 4CC to 3-chloro-cis,cis-muconate in NK8, as in other bacteria (Dorn & Knackmuss, 1978; Kim et al., 1997; Sauret-Ignazi et al., 1996).

The convergent organization of the cbe–cat gene cluster

The organization of the cbe and cat genes in NK8 is unique when compared with the corresponding genes of other Proteobacteria. Mapping studies indicated that both benzoate 1,2-dioxygenase genes (ben) and catechol 1,2-dioxygenase genes (cat) are contiguous but seemingly exist as distinct units in both the P. putida and P. aeruginosa chromosomes (Houghton et al., 1995; Jeffrey et al., 1992; Zhang et al., 1993a, b). In the well-characterized Acinetobacter sp. ADP1, a close physical association between the ben and cat gene clusters is evident, but the gene clusters are still distinctly separate (Collier et al., 1998; Neidle et al., 1987). Although the cbe and cat genes are also clustered in NK8, there are significant differences between ADP1 and NK8 in terms of the organization of the corresponding genes in the cluster. While the benABCD (and benE) genes exist as a distinct operon in ADP1, cbeA (and probably cbeBCD) forms an operon with catA in NK8 (Fig. 1). While benE is downstream of and cotranscribed with benABCD in ADP1, the corresponding gene cbeE of NK8 is downstream of catC. In ADP1, the ben and cat genes have their own LysR-type regulatory genes, i.e. benM and catM, respectively, catM regulating only the cat genes, while benM is able to regulate both ben and cat genes (Collier et al., 1998). In contrast, only the single LysR-type regulatory gene cbeR, which positively regulates the expression of catA and cbeA(BCD), and probably catBC, is present in the cbe–cat gene cluster of NK8 (Fig. 1). These organizational features of the NK8 cat and ben gene clusters, i.e. reshuffling, intermingling and co-regulation, might represent evolutionary events more recent than those that occurred in ADP1, P. aeruginosa or P. putida, in which the independently derived but metabolically related cat and ben gene clusters were brought physically close together by selection pressures (Harwood & Parales, 1996). The close proximity of catA and cbeABCD, coupled with their co-expression, further substantiates the perceived importance of benzoate as a favoured substrate that is funnelled into the catechol branch of the β-ketoadipate pathway (Harwood & Parales, 1996).

Chloro)benzoates are inducers of the transcriptional activation of the catA promoter

The transcriptional fusion study in P. putida PRS4020 showed that the transcription of catA and cbeA(BCD) is regulated by CbeR. Results of the β-galactosidase assay of NBALZ, the cbeA::lacZ–Km′ disruptant strain (Fig. 5), indicate that cbeA is induced by 3CB, 4CB, benzoate and cis,cis-muconate. In the well studied benzoate-degrading bacterium Acinetobacter sp. ADP1, cis,cis-muconate converted from catechol induces the expression of the benZ1 (ben) genes and catechol dioxygenase cat genes (Collier et al., 1998). In P. putida, cis,cis-muconate from catechol also activates the cat genes (Parsek et al., 1992). The amino acid residues in the putative binding region conserved among cis,cis-muconate-responsive regulatory proteins are also conserved in CbeR (from Ile-98 to Glu-152; data not shown). Although the possibility that the degradation product of cis,cis-muconate acts as an effector cannot be excluded, it is probable that cis,cis-muconate binds to CbeR and then activates the coupled transcription of catA and cbeA. The observed increase in β-galactosidase activity with catechol could be attributed to the cis,cis-muconate rapidly generated from catechol by CatA.
Quantitative HPLC analysis showed that benzoate and chlorobenzoates are not degraded by NBALZ. Therefore, the inducers of cbeA expression observed in lacZ assay of NBALZ are benzoate and chlorobenzoates themselves. BenM of Acinetobacter sp. ADP1 also responds to benzoate (Collier et al., 1998). However, the overall identity of CbeR with BenM (45%) is lower than that with CatR of P. putida RBS2000 (50%) and RB1 (48%), which respond to cis,cis-chloromuconate (Houghton et al., 1995; Parsek et al., 1992). NK8 CbeR appears to be the first example of a LysR-type regulator involved in the degradation of (chloro)benzoate that recognizes chlorobenzoates as inducers. The difference among the three monochlorobenzoate isomers in their ability to induce cbeA expression is obvious, 3CB and 4CB being as effective as benzoate and cis,cis-muconate, while 2CB is not an inducer (Fig. 5). The difference in their ability to support the growth of NK8 is also evident. NK8 grows well on 3CB and 4CB but does not grow on 2CB, notwithstanding the significant transformation by NK8 cell lysate of these three chlorobenzoate isomers (Table 2). The absence of growth of NK8 on 2CB may be due to the inability of CbeR to recognize 2CB as an effector. The induction of oxidative genes, in addition to the substrate specificity of the encoded enzymes, can be a bottleneck in the degradation of chlorobenzoate. Apparently, regulator recognition of effectors is essential in determining the substrate specificity of chlorobenzoate-degrading bacteria.

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