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

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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 Pseudo-
monas aeruginosa 142 (Tsoi et al., 1999). CbaABC
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 chloro-
catechol is supposed to be one of the typical degradation
pathways for chlorobenzoates. In this pathway, chloro-
benzoates 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 ap-
parently derived from the ‘ortho pathway’ genes for
catechol degradation (Dauraras & Chakrabarty, 1992;
Franz & 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 eluc-
dation 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. U69835) and Burkholderia	andropogonis (accession no. X67037), respectively. The
plasmids pUC118 and pBluescript II KS(›) (accession no. X67037) were used as plasmid hosts.

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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
Pulsor or by 42
°C heat shock were prepared using the methods
of Smith et al. (1990) and Hanahan (1985), respectively. All
DNAs for ligation (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'-GCT/C/T/ACA/C/T/GA/G/A/AGC-
CAGATTCCC-3'] with the reverse primer BAR2 [5'-GGTGGC-
G/C/G/T/AGTAGTCCAGTG-3'] yielded an approxi-
mately 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 prod-
uct 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 an agarose gel by electrophoresis
and then blotted onto Hybond-N+ nylon membrane
(Amersham). The Southern blot was probed with 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 analyses. 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. Ambi-
guous portions of any sequence were verified by resequencing with the ABI Dye Terminator Ready Reaction
Kit. Nucleotide sequence data were analysed using the
GENETYX-Mac (version 10.1) and ATSQ 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 centri-

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**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>
</tr>
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<tbody>
<tr>
<td><strong>Burkholderia sp. strains</strong></td>
<td></td>
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</tr>
<tr>
<td>NK8</td>
<td>Benzoate', 3CB', 4CB'</td>
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<tr>
<td>NDBA1</td>
<td><em>cheA</em>:ΩGm', megaplasmid</td>
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<td>NCAD</td>
<td><em>catA</em>:ΩGm'</td>
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<tr>
<td>NCRD</td>
<td><em>cheR</em>:ΩHm'</td>
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<td>NBALZ</td>
<td><em>cheA</em>:lacZ–Km'</td>
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<td><strong>P. putida strains</strong></td>
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<td>PRS4020</td>
<td><em>cheR</em>:ΩGm', benzoate'</td>
<td>Parales &amp; Harwood (1993)</td>
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<td><strong>E. coli strains</strong></td>
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<tr>
<td>DH5&lt;sup&gt;α&lt;/sup&gt;</td>
<td>supE44 lacU169(80lacZΔM15) bsdR17 recA1 endA1</td>
<td>Gibco BRL</td>
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<td>S17-1</td>
<td>C600::RP4 2-(Tc::Mu)(Km::Tn7) thi pro</td>
<td>Simon <em>et al.</em> (1983)</td>
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<tr>
<td>S17-1 ipir</td>
<td>K-12, Sm'</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
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<td>K-12 expression host, Rp'(E3)</td>
<td>Novagen</td>
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<td>Blondelet-Rouault <em>et al.</em> (1997)</td>
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<td>Schweizer (1992)</td>
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<td>pMOB3</td>
<td>Km' Cm'; source of MOB cassette</td>
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<td>pJRD215</td>
<td>Sm' Km'; cosmid vector</td>
<td>Davison <em>et al.</em> (1987)</td>
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<td>pET14b</td>
<td>Expression vector</td>
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<td><em>cheABCD</em> in pET14b</td>
<td>This study</td>
</tr>
<tr>
<td>pKOK6.1</td>
<td>Derivative of pKOK6 (Kokotek &amp; Lotz, 1989), source of lacZ–Km' cassette</td>
<td>W. Lotz*</td>
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<td>pFJ50cheRcatAcbeA'</td>
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<td>This study</td>
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<td>5.1 kb EcoRV <em>cheRcatA</em>:Ωhyg <em>cbeA'</em> insert in pQF50, <em>cheA</em> truncated at aa 35 out of 452</td>
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<tr>
<td>pFJ50cheRcatA'</td>
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<tr>
<td>pFJ50cheR'catA'</td>
<td>790 bp SalI–Stul <em>cheR'catA'</em> insert in pQF50, <em>catA</em> truncated at aa 77 out of 311, <em>cheR</em> truncated at aa 128 out of 306</td>
<td></td>
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</tbody>
</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 & Hausinger (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 Mg[NH₂]₂(SO₄)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 Fetner 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)benzoate 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 H₂PO₄ (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 PstI–HincII fragment in cbeA, the 341 bp SstI–EcoRI fragment in catar and the 473 bp SphI–NruI fragment in cber, and replaced by the 1721 bp HindIII Ω gentamicin-resistance (Gm') cassette (Ωaac) of pH445ac, the 1773 bp SmaI Ωac cassette of pH445aac and the 2267 bp HindIII Ω hygromycin-resistance (Hm') cassette (Ωhyg) of PHP45hyg (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 pir. Conjigation of the transformed S17-1 pir 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 crossovers. 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, catar 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′-CCCTCAAGAAATTTTTGTATACAG-3′ and the 29-mer reverse primer 5′-CAGGATCCATAGGCAATCAGTTCCTGACAG-3′. The amplified fragment was digested with XbaI/BamHI and cloned into the XbaI/BamHI sites of pET14b to generate plasmid pIK14E. E. coli strain HMS174(DE3) was transformed with pIK14E 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 OD₆₀₀ 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). 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 (300 μl) of the supernatant were mixed with an equal volume of HPLC solvent, centrifuged and subjected to HPLC analysis at A₂₅₄.

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 pF730cbecatAcbeA' contains the complete cbeR and catA genes and the truncated cbeA gene fused to lacZ; pF730cbecatA:: ΩhygcbeA differs from pF730cbecatAcbeA' in having its catA gene disrupted by the Ωhyg cassette; pF730cbecatAcbeA' is similar to pF730cbecatAcbeA in expressing its catA gene but disrupts the Ωhyg cassette; pF730cbecatAcbeA' is similar to pF730cbecatAcbeA' except that cber is incomplete; in pF730cbecatAcbeA' and pF730cbecatA', the truncated catA is fused to lacZ. These lacZ transcriptional fusion plasmids were introduced by electroporation into PRS4020, the catR 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 microlitres of the pre-culture was used to inoculate 10 ml basal synthetic medium (Aldrich et al., 1987) containing 10 mM glucose (BSMG), or BSMG 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 [nmol 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
(see Fig. 1) (Schweizer, 1992). The procedure was essentially the same as those for the gene disruption by the \( \Omega \) interposon described above. In the final construct in pNOT322, into which the MOB cassette of pMOB3 was also inserted, the \( lacZ-Km' \) 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 \( cbeA::lacZ-Km' \) disruptant, was cultured in BSMG alone or in BSMG supplemented with 5 mM benzoate, 2CB, 3CB or 4CB, or with 0.1 mM catechol or 3 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 benzoate- and 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>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Relative oxidation activity (%) against:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Benzoate</td>
<td>2CB</td>
</tr>
<tr>
<td>NK8</td>
<td>Succinate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NK8</td>
<td>Benzoate</td>
<td>28 ± 1.7</td>
<td>34 ± 0.8</td>
</tr>
<tr>
<td>NK8</td>
<td>3CB</td>
<td>34 ± 1.5</td>
<td>29 ± 2.1</td>
</tr>
<tr>
<td>( E. coli/cbeABCD )</td>
<td>LB/IPTG</td>
<td>14 ± 1.9</td>
<td>42 ± 3.0</td>
</tr>
</tbody>
</table>

* Specific activity [nmol substrate oxidized (mg protein)\(^{-1}\) min\(^{-1}\)].
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–Km 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 (ACB)</th>
<th>Homologue (XYZL)</th>
<th>Homologue (ABCD)</th>
<th>Homologue (ABC)</th>
<th>Homologue (AB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbeA</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>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>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>57.0</td>
<td>56.4</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*K 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 dioxygenase) of Pseudomonas putida mutant-2 TOL plasmid pWW0 (Harayama et al., 1991); M64747; BenABC (benzoate 1,2-dioxygenase) and BenD (DHBA dehydrogenase) of Acinetobacter sp. ADP1 (Norld 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. Cbd, Ben and Xyl can oxidize benzoate, while Ant and Tfr 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 hydroxy- lases. 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 (Fig. 1). Its deduced amino acid sequence shares high identity with LysR-type transcriptional regulators involved in benzoate or catechol catabolism such as ORF<sub>R2</sub>(55%) of one of the two catechol gene clusters of Frateuria 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 Ω 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
12.5 kb HindIII–EcoRI fragment containing the cbe–cat gene cluster harboured by pBAC1. 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 cultured 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 Ωtac 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 cat 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 pFJ30cbeRcatA, 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 or 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 cbe(BC) 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 Ωhyg cassette was inserted into catA of the lacZ transcriptional fusion plasmid pFJ50cbeRcatAcbeA′ to generate pFJ50cbeRcatA::ΩhygcebeA′ (Fig. 3), which was then introduced into PRS4020. There was no induction of β-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 β-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 β-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 β-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 β-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 *B. cepacia* 2CBS, benzoate dioxygenase (BenABC) of *Acinetobacter* sp. ADP1 and toluate dioxygenase (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.
**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.

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-*toluate 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 toluate degrader, NK8 could not grow on *o*-, *m*- or *p*-toluate 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-
DHB, which upon dehydrogenation by CbeD is converted into 3CC. 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 regioselectivity of the NK8 dioxygenase. Low regioselectivity 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 regioselectivity, 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 chlorocatehol 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 chlorocatehol genes cbrRABCD in pEKCI (Ogawa & Miyashita, 1999) or the plasmid-borne NK8 chlorocatehol genes enabled the mutant to grow on 3CB and 4CB (unpublished data). These observations indicate that the plasmid-encoded chlorocatehol 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-Ignazzi 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, while 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 cbeABC(D), 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 cbeABC(D), 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-ketoadiapate 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 cbeABC(D) 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 benzoate dioxygenase (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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