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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

Abbreviations: 2CB, 2-chlorobenzoate; 3CB, 3-chlorobenzoate; 4CB, 4-chlorobenzoate; 3CC, 3-chlorocatechol; 4CC, 4-chlorocatechol; BSMG, basal synthetic medium + glucose; DA, Davis-adonitol medium; DHB, dihydrodihydroxybenzoate (3,5-cyclohexadiene-1,2-diol-1-carboxylic acid); PCBs, polychlorinated biphenyls.

The GenBank accession number for the sequence reported in this paper is AB024746.

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). 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 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. U96935) and *Burkholderia andropogonis* (accession no. X67037), respectively. The plasmids pUC118 and pBluescript II KS(+) were routinely used as cloning vectors while *Escherichia coli* strains DH5α, S17-1 and S17-1 λ*pir* were used as plasmid hosts. *E. coli* cells were grown on Luria–Bertani broth (LB) (Sambrook *et al*., 1989), *Pseudomonas putida* on LB or *Pseudomonas* isolation agar (Difco), while NK8 cells were cultured either on LB or on a basal salts medium (Ogawa & Miyashita, 1995) supplemented with benzoate, 2CB, 3CB or 4CB at a final concentration of 5 mM. Selection for transconjugants of NK8 and its derivatives was done on Davis-adonitol (DA) medium [per litre: 7 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2(SO_4)_2$, 0·1 g $MgSO_4.7H_2O$, 2 g adonitol]. As needed, antibiotics were added at the following concentrations (μg ml^{-1}): ampicillin (for *E. coli*), 50; gentamicin, 20; hygromycin, 50; kanamycin, 50; chloramphenicol, 25; and carbenicillin (for *P. putida*), 1000. *E. coli* cells were grown at 37 °C while *Pseudomonas* and *Burkholderia* strains were cultured at 30 °C.

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 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, PCRs were carried out with different degenerate primer combinations. The forward primer BAf1 [5′-GC(C/T)CA(C/T)GA(G/A)AGC-CAGATTCCC-3′] with the reverse primer BAr2 [5′-GGTGG-C(G/T)GC(G/A)TAGTTCCAGTG-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 an 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 *Eco*RI 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 868 bp *Eco*RI–*Pst*I segment at the left end of the cloned 5·3 kb *Eco*RI 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 *Hin*dIII–*Pst*I 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 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-

Table 1. Bacterial strains and plasmids

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

Strain/plasmid	Relevant properties	Source/reference
***Burkholderia* sp. strains**		
NK8	Benzoate$^+$, 3CB$^+$, 4CB$^+$	This study
NDBA1	*cbeA*::ΩGm^r, megaplasmid$^-$	This study
NCAD	*catA*::ΩGm^r	This study
NCRD	*cbeR*::ΩHm^r	This study
NBALZ	*cbeA*::*lacZ*–Km^r	This study
***P. putida* strains**		
PRS4020	*cbeR*::ΩGm^r, benzoate$^-$	Parales & Harwood (1993)
***E. coli* strains**		
DH5α	*supE44 lacU169*(f80*lacZφ*M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco BRL
S17-1	C600::RP4 2-(Tc::Mu)(Km::Tn*7*) *thi pro hsdR hsd*M$^+$ *rec*A	Simon *et al.* (1983)
S17-1 *λpir*	K-12, Sm^r	de Lorenzo & Timmis (1994)
HMS174(DE3)	K-12 expression host, Rp^r(E3)	Novagen
Plasmids		
pUC118	Ap^r; cloning vector	Vieira & Messing (1987)
pBlueScript II KS(+)	Ap^r; cloning vector	Stratagene
pCR2.1	TA cloning vector	Invitrogen
pHP45Ω*aac*	Ap^r; source of ΩGm^r cassette	Blondelet-Rouault *et al.* (1997)
pHP45Ω*hyg*	Hm^r; source of ΩHm^r cassette	Blondelet-Rouault *et al.* (1997)
pNOT 322	Ap^r Tc^r; cloning vector	Schweizer (1992)
pMOB3	Km^r Cm^r; source of MOB cassette	Schweizer (1992)
pJRD215	Sm^r Km^r; cosmid vector	Davison *et al.* (1987)
pBAC1	NK8 *cat–cbe* genes in pJRD215	This study
pET14b	Expression vector	Novagen
p14BEP	*cbeABCD* in pET14b	This study
pKOK6.1	Derivative of pKOK6 (Kokotek & Lotz, 1989), source of *lacZ*–Km^r cassette	W. Lotz*
pQF50	*lacZ* promoter probe vector, Ap^r	Farinha & Kropinski (1990)
pFJ50cbeRcatAcbeA′	2·8 kb *Eco*RV *cbeRcatAcbeA′* insert in pQF50, *cbeA* truncated at aa 35 out of 452	This study
pFJ50cbeRcatA::ΩHm^r cbeA′	5·1 kb *Eco*RV *cbeRcatA*::Ω*hyg* cbeA′ insert in pQF50, *cbeA* truncated at aa 35 out of 452	This study
pFJ50cbeR′catAcbeA′	1·7 kb *Sal*I–*Eco*RV *cbeR′catA-cbeA′* insert in pQF50, *cbeA* truncated at aa 35 out of 452, *cbeR* truncated at aa 128 out of 306	This study
pFJ50cbeRcatA′	1·9 kb *Eco*RV–*Stu*I *cbeRcatA′* insert in pQF50, *catA* truncated at aa 77 out of 311	This study
pFJ50cbeR′catA′	790 bp *Sal*I–*Stu*I *cbeR′catA′* insert in pQF50, *catA* truncated at aa 77 out of 311, *cbeR* truncated at aa 128 out of 306	This study

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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 $Fe(NH_4)_2(SO_4)_2$, 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)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 H_3PO_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 *Pst*I–*Hin*cII fragment in *cbeA*, the 341 bp *Stu*I–*Eco*RI fragment in *catA* and the 473 bp *Sph*I–*Nru*I fragment in *cbeR*, and replaced by the 1721 bp *Hin*dIII Ω gentamicin-resistance (Gm^r) cassette (Ωaac) of pHP45aac, the 1773 bp *Sma*I Ωaac cassette of pHP45aac and the 2267 bp *Hin*dIII Ω hygromycin-resistance (Hm^r) 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 λpir. Conjugation of the transformed S17-1 *λpir* 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 *Hin*dIII–*Eco*RI fragment of NK8 that carries the *cbe–cat* gene cluster was cloned into the broad-host-range plasmid vector pJRD215 (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′-CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGTCCGCCATCACCGACAAAGCCAGTCAGCTCG-3′ and the 29-mer reverse primer 5′-CAGGATCCATAGCGAATCTTCTCGTACAC-3′. The amplified fragment was digested with *Xba*I/*Bam*HI and cloned into the *Xba*I/*Bam*HI 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* HMS(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_{203}.

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 pFJ50cbeRcatAcbeA′ contains the complete *cbeR* and *catA* genes and the truncated *cbeA* gene fused to *lacZ*; pFJ50cbeRcatA :: ΩhygcbeA′ differs from pFJ50cbeRcatAcbeA′ in having its *catA* gene disrupted by the Ωhyg cassette; pFJ50cbeR′catAcbeA′ is similar to pFJ50cbeRcatAcbeA′ except that *cbeR* is incomplete; in pFJ50cbeR′catA′ and pFJ50cbeRcatA′, 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^r disruptant. To determine the inducer(s) of expression of *cbeA* in NK8, the 4·73 kb *lacZ*–Km^r cassette of pKOK6.1, a derivative of pKOK6 (Kokotek & Lotz, 1989), was inserted into the *Eco*RV 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 Ω interposon described above. In the final construct in pNOT322, into which the MOB cassette of pMOB3 was also inserted, the *lacZ*–Km^r cassette is flanked by 1·65 and 2·17 kb NK8 DNA fragments carrying the *cbeA* fragments to facilitate crossing over. Disruptants were verified by Southern hybridization analysis. NBALZ, the cbeA::*lacZ*–Km^r 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 5 mM *cis*,*cis*-muconate. The β-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 *Eco*RI fragment (Fig. 1). Using the left end of this fragment as the probe, the overlapping 8·1 kb *Hin*dIII–*Pst*I 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.

Strain	Growth medium	Relative oxidation activity (%) against:			
		Benzoate	2CB	3CB	4CB
NK8	Succinate	0	0	0	0
NK8	Benzoate	100 (3·18 ± 0·07)*	28 ± 1·7	34 ± 0·8	46 ± 2·7
NK8	3CB	100 (1·34 ± 0·01)*	34 ± 1·5	29 ± 2·1	61 ± 4·4
E. coli/*cbeABCD*	LB/IPTG	100 (2·40 ± 0·05)*	14 ± 1·9	42 ± 3·0	76 ± 7·3

*Specific activity [nmol substrate oxidized (mg protein)$^{-1}$ min^{-1}].

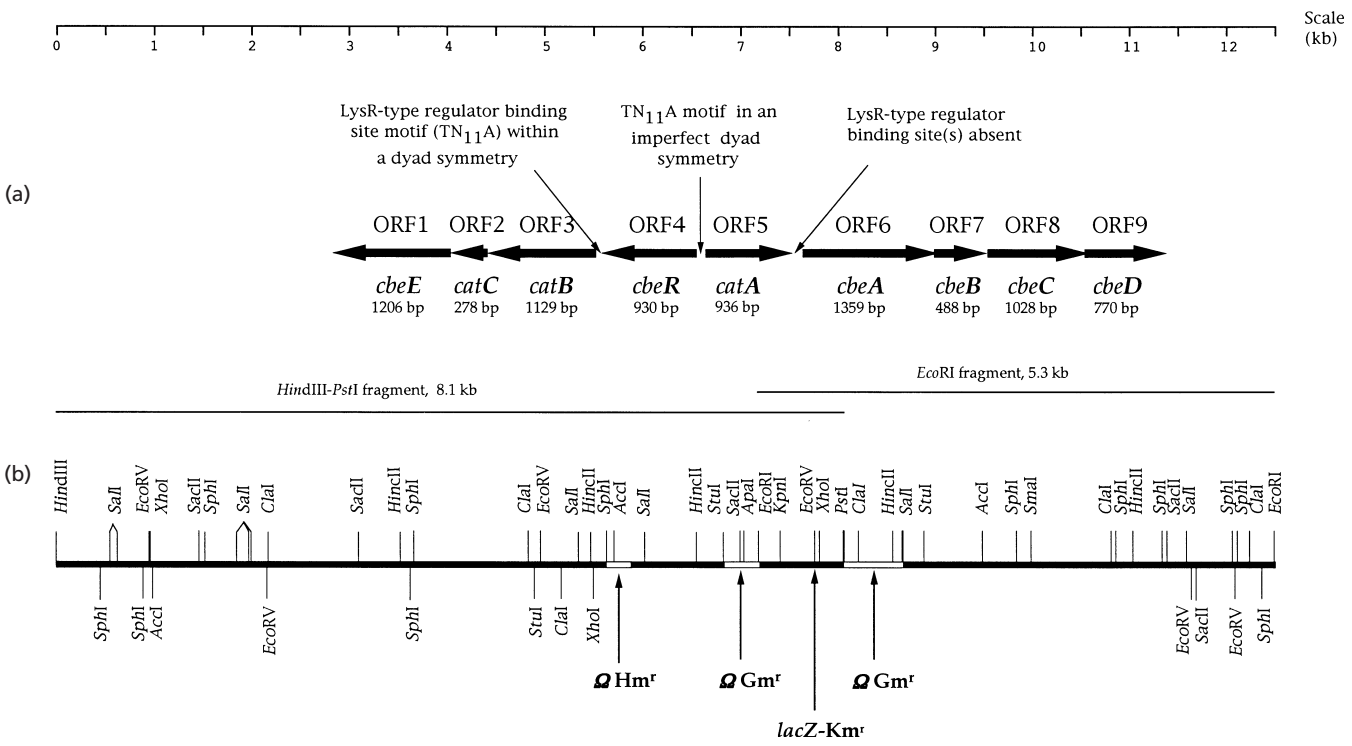

Fig. 1. Organization of the chlorobenzoate *(cbe)* and catechol *(cat)* catabolic genes (a) clustered on the 12·5 kb *Hin*dIII–*Eco*RI genomic DNA region (b) of *Burkholderia* sp. strain NK8. Also shown in (a) are the approximate locations of the putative LysR-type transcriptional regulator binding sites 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^r 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

NK8 Cbe protein	Homologue*				
	Cbd (ABC)	Xyl (XYZL)	Ben (ABCD)	Ant (ABC)	Tft (AB)
CbeA	64·3	58·2	57·1	45·1	38·7
CbeB	56·2	60·5	52·2	39·9	38·9
CbeC	45·1	54·2	49·7	37·8	–
CbeD	–	57·0	56·4	–	–

*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 diol dehydrogenase) of *Pseudomonas putida* mt-2 TOL plasmid pWW0 (Harayama *et al*., 1991), M64747; BenABC (benzoate 1,2-dioxygenase) and BenD (DHB dehydrogenase) of *Acinetobacter*, sp. ADP1 (Neidle *et al*., 1987), AF009224; AntABC (anthranilate dioxygenase) of *Acinetobacter* sp. ADP1 (Bundy *et al*., 1998), AF071556; and TftAB (2,4,5-trichlorophenoxyacetate oxygenase) of *Burkholderia cepacia* AC1100 (Danganan *et al*., 1994), U11420. Cbd, 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 *catA*2 gene of *Acinetobacter lwoffii*

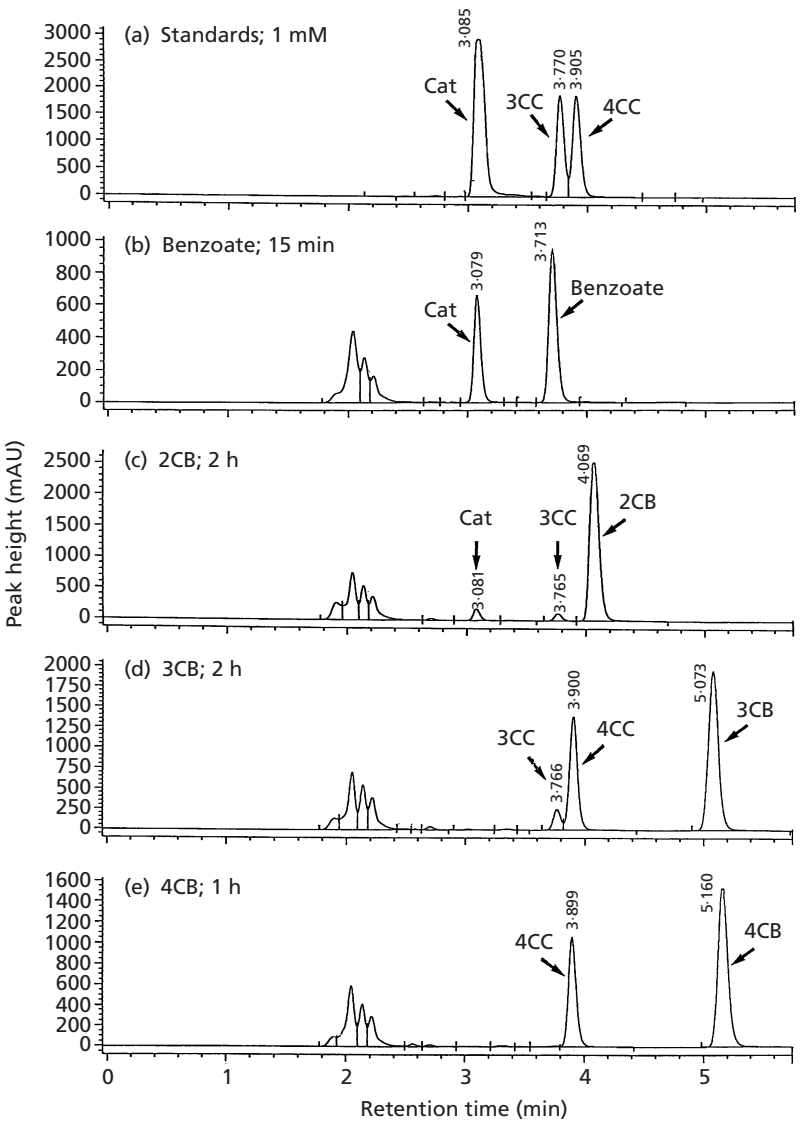

Fig. 2. Oxidation products of benzoate (b), 2CB (c), 3CB (d) and 4CB (e) resulting from the IPTG-induced expression of the NK8 (chloro)benzoate dioxygenase genes *cbeABC* in *E. coli*. Data were obtained from HPLC analysis at A_{203} of the substrates subjected to conversion by LB-grown *E. coli* HMS174(DE3) 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.

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_{R2} (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 *Hin*dIII–*Eco*RI 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 Ω*hyg* 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 Ω*aac* 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-meditated 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

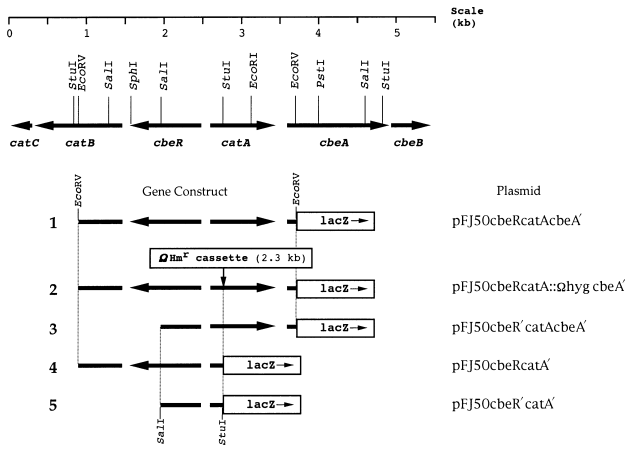

Fig. 3. Diagrams of the *cbe–cat lacZ* fusion constructs in pQF50. Shown are the NK8 DNA fragments cloned at the multicloning site of pQF50 immediately upstream of its promoterless *lacZ* gene. In the designated names of the pQF50-derived plasmids, the prime sign (′) indicates that the gene bearing it is truncated. In construct number 2, *catA* transcription was blocked by the insertion of the 2·3 kb Ω*hyg* cassette at the *Stu*I site of *catA*. The organization of the *cat–cbe* genes is given for reference. Only the NK8 genes are drawn to scale.

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. pFJ50cbeR′catAcbeA′ or pFJ50cbeR′catA′, 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 pFJ50cbeR′catAcbeA′, 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 *cbeA*(*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

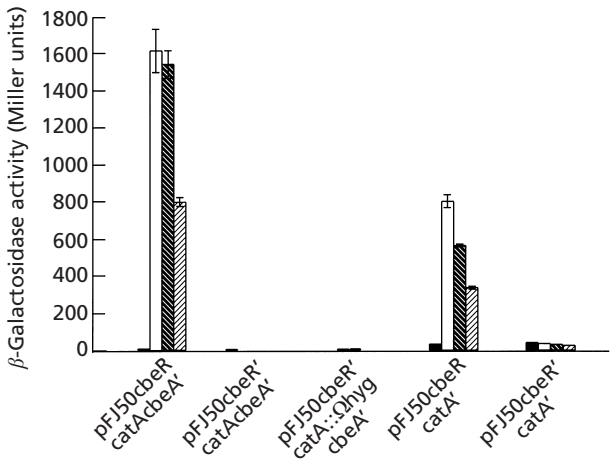

Fig. 4. *β*-Galactosidase activities resulting from the expression of the pQF50-derived NK8 *cbe–cat lacZ* fusion plasmid constructs in the *catR* knock-out mutant PRS4020 of *P. putida*. The cells were grown for 17 h in BSMG medium supplemented with inducers (black bars, no inducer; white bars, 5 mM benzoate; black bars with white hatching, 5 mM 3CB; white bars with black hatching, 5 mM 4CB). Bars represent means of three replicates. Vertical lines on top of the bars indicate standard deviations from the means. See Fig. 3 for the details on the insert of each plasmid.

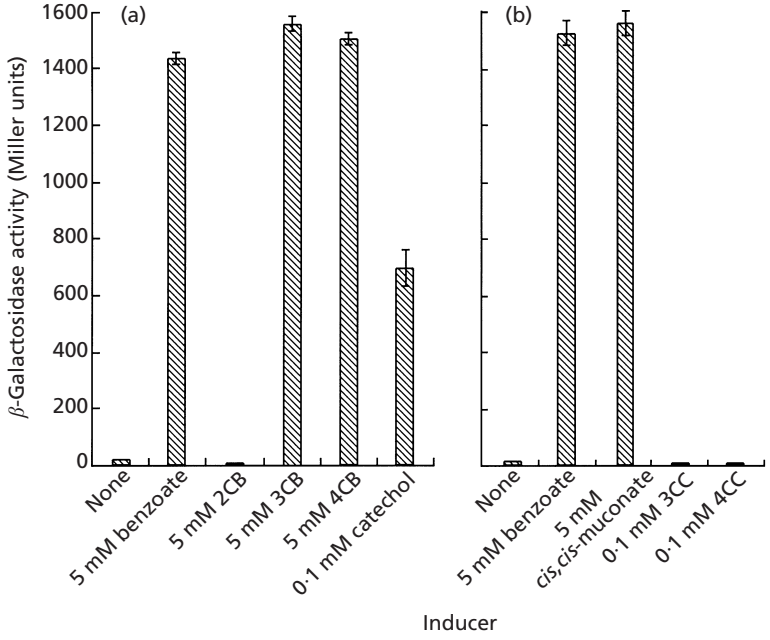

Fig. 5. *β*-Galactosidase activity of the NK8 *cbeA*::*lacZ*–Km^r disruptant. Cells were grown for 17 h in BSMG medium supplemented with the indicated inducers. Bars represent means of three replicates. Error bars represent standard deviations from the means. (a) and (b) are separate experiments.

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 Ω*hyg* cassette was inserted into *catA* of the *lacZ* transcriptional fusion plasmid pFJ50cbeRcatAcbeA′ to generate pFJ50cbeRcatA::ΩhygcbeA′ (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 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.

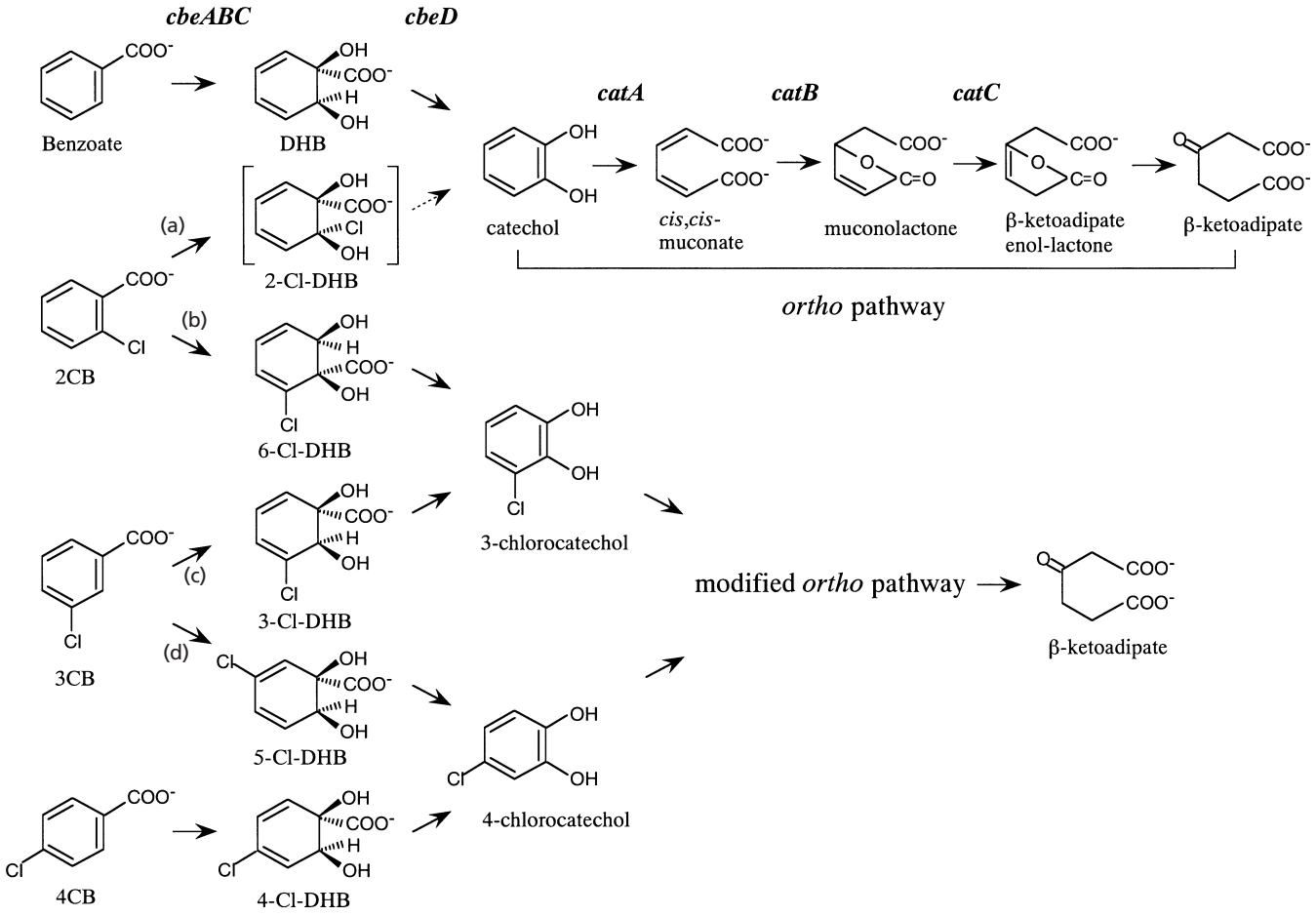

Fig. 6. Proposed degradation pathways for benzoate, 2CB, 3CB and 4CB in *Burkholderia* sp. strain NK8. In the conversion of the asymmetrical aromatics 2CB and 3CB, labelled arrows indicate steps in which 2CB binds with CbeR as 2CB (a) or '6CB' (b), and in which 3CB binds as 3CB (c) or '5CB' (d). The 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 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 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 plasmid-borne 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^r 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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