Genes for Mn(II)-dependent NahC and Fe(II)-dependent NahH located in close proximity in the thermophilic naphthalene and PCB degrader, *Bacillus* sp. JF8: cloning and characterization

Daisuke Miyazawa,1 Gouri Mukerjee-Dhar,2 Minoru Shimura,2 Takashi Hatta3 and Kazuhide Kimbara1,2

Correspondence
Kazuhide Kimbara
kimbara@rtri.or.jp

1Department of Built Environment, Tokyo Institute of Technology, Yokohama 226-8502, Japan
2Biotechnology Laboratory, Railway Technical Research Institute, 2-8-38, Hikari-cho, Kokubunji, Tokyo 185-8540, Japan
3Research Institute of Technology, Okayama University of Science, Okayama 703-8232, Japan

Received 22 October 2003
Revised 22 December 2003
Accepted 2 January 2004

A 10 kb DNA fragment was isolated using a DNA probe derived from the N-terminal amino acid sequence of the extradiol dioxygenase purified from naphthalene-grown *Bacillus* sp. JF8, a thermophilic naphthalene and polychlorinated biphenyl degrader. The cloned DNA fragment had six open reading frames, designated *nahHLOMmocBnahC* based on sequence homology, of which the products NahH_JF8 and NahC_JF8 were extradiol dioxygenases. Although NahC_JF8 and NahH_JF8 exhibit low homology to known extradiol dioxygenases, the active-site residues and metal ion ligands are conserved. The presence of Mn(II) in culture medium was found to be essential for production of active recombinant NahC_JF8, while Fe(II) was necessary for active recombinant NahH_JF8. Inductively coupled plasma mass spectrometry analysis of active NahC_JF8 identified the cofactor to be manganese, indicating a Mn(II)-dependent extradiol dioxygenase. NahC_JF8 exhibited $K_m$ values of $32 \pm 5 \mu M$ for 1,2-dihydroxynaphthalene and $510 \pm 90 \mu M$ for 2,3-dihydroxybiphenyl at 60 °C. In cell-free extracts, NahH_JF8 exhibited a broad substrate range for 2,3-dihydroxybiphenyl, catechol, and 3- and 4-methylcatechol at 25 °C. Stability studies on the Mn(II)-dependent NahC_JF8 indicated that it was thermostable, retaining 50% activity after incubation at 80 °C for 20 min, and it exhibited resistance to EDTA and H$_2$O$_2$. Northern hybridization studies clarified that both NahC_JF8 and NahH_JF8 were induced by naphthalene; RT-PCR showed that *nahHLOMmocBnahC* is expressed as a single transcript.

INTRODUCTION

The degradation of environmental pollutants by soil bacteria has been widely reported and well studied. Aerobic bacteria initially hydroxylate the aromatic ring of a variety of common pollutants like benzene, toluene, biphenyl and naphthalene, producing catecholic metabolites possessing hydroxyl substituents on two adjacent carbon atoms (Axcell & Geary, 1975; Ensley et al., 1982; Haddock et al., 1993; Yeh et al., 1977). Extradiol dioxygenases act on these catecholic metabolites, cleaving *meta* to the hydroxyl substituents, opening up the aromatic ring and thus playing an important role in

the degradation of aromatic compounds. Sequence data indicate that there are at least two types of extradiol dioxygenases; the type I enzymes contain the PROSITE consensus sequence while the type II enzymes do not (Elitis & Bolin, 1996).

The degradation of aromatic compounds by thermophilic organisms and thermostable enzymes, especially when applied in biotechnology processes, could provide important advantages compared to mesophiles. The elevated temperatures would reduce the risk of contamination, and the bioavailability of the hydrophobic contaminants would increase. The improved stability of thermophilic enzymes compared to their mesophilic homologues is well documented (Sterner & Liebl, 2001; Vieille et al., 1996). Although thermophiles which can degrade compounds such as BTEX (benzene, toluene, ethylbenzene, xylene), phenol, cresol and naphthalene have been reported (Chen & Taylor, 1995; Buswell, 1974, 1975; Duffner & Muller, 1998; Duffner et al., 2000; Shimura et al., 1999), the metabolic pathways involved

Abbreviations: C23O, catechol 2,3-dioxygenase; DHBD, 2,3-dihydroxybiphenyl 1,2-dioxygenase; HPCD, homoprotocatechuate 2,3-dioxygenase; ICP-MS, inductively coupled plasma mass spectrometry; PCB, polychlorinated biphenyl.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB116258.
in the degradation of aromatic compounds have not been well studied in these organisms and there is very little information on the genes and proteins involved.

*Bacillus* sp. JF8 is a thermophilic polychlorinated biphenyl (PCB) degrader which can utilize biphenyl and naphthalene as the sole carbon and energy source (Shimura et al., 1999). The presence of separate pathways for the degradation of biphenyl and naphthalene was indicated by *Bacillus* sp. JF8N, a mutant of *Bacillus* sp. JF8 which had lost the ability to utilize biphenyl as a carbon source while retaining the ability to use naphthalene (Shimura et al., 1999). Further studies indicated that *Bacillus* sp. JF8 possessed a 40 kb plasmid which was absent in the mutant JF8N, hinting that the bph genes were borne on the plasmid while the nah genes were located on the chromosome (G. Mukerjee-Dhar, M. Shimura, T. Hatta & K. Kimbara, unpublished data). Hatta et al. (2003) isolated and characterized an atypical Mn(II)-dependent extradiol dioxygenase, BphC\_JF8, which was encoded on the plasmid.

Here we report the characterization of the two extradiol dioxygenases from *Bacillus* sp. JF8, both induced by naphthalene; one is thermostable and Mn(II)-dependent while the other is more thermolabile and Fe(II)-dependent. To the best of our knowledge, this is the first time Mn(II)- and Fe(II)-dependent extradiol dioxygenases have been isolated from the same organism and found to be transcribed in the same operon.

**METHODS**

**Bacterial strains and culture conditions.** *Bacillus* sp. JF8 and *Bacillus* sp. JF8N were cultured at 60 °C on 1.5 % agar plates of Castenholz D medium. Naphthalene and biphenyl were provided as vapour. Petri dishes were stacked in plastic containers with tight-fitting lids containing biphenyl and naphthalene as crystals. The composition of Castenholz D medium has been described previously (Shimura et al., 1999). bphC\_Q1 (encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Sphingomonas paucimobilis* Q1; Taira et al., 1988), a kind gift from Professor K. Furukawa of Kyushu University, was on a 2.6 kb *Xhol* fragment in pHSG396. *Escherichia coli* JM109, used for construction and maintenance of plasmids, was cultured at 37 °C on LB or M9 medium. The composition of the LB and M9 media has been described elsewhere (Sambrook et al., 1989). M9 medium was supplemented with 0.01 mM MnCl₂, Fe(NH₄)₂(SO₄)₃, MgSO₄, ZnSO₄ or CoCl₂, Ampicillin (100 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹), IPTG and X-Gal were used for selection of plasmids.

**Southern hybridization.** A DNA probe, spanning the determined N-terminal region of the extradiol dioxygenase that was isolated from naphthalene-grown *Bacillus* sp. JF8, was generated using PCR with the primers C1-F (5'-GGCATGCGGAATTTTGTCA-3') and C1-R (5'-TCTTCTACGATCGTTCGGG-3'). The probe was labelled with digoxigenin (DIG; Roche). Total DNA of *Bacillus* sp. JF8N was digested with BamHI, CiaI, EcoRI, HindIII and SalI, and fractionated by electrophoresis in 0.7 % agarose gel before transfer to Hybond-N+ nylon membranes (Amersham). For colony hybridization, the recombinant *E. coli* colonies were transferred to Hybond-N+ nylon membranes. Southern hybridization was performed at 60 °C with high-stringency washes at 60 °C (2 × 15 min, 0.1 × SSC+0.1 % SDS).

**Cloning and DNA sequencing.** SalI and BamHI gene banks (5 kb and 2 kb, respectively) of the chromosomal DNA of *Bacillus* sp. JF8N were constructed in pUC18 (Takara Biomedicals) and recombinant *E. coli* colonies probed with the DIG probe mentioned above. A clone containing a 5 kb SalI fragment designated pCS5 and a clone containing a 2 kb BamHI fragment designated pCBm2 gave a positive hybridization signal. DNA regions upstream of the 5 kb SalI fragment and downstream of the 2 kb BamHI fragment were isolated by inverse PCR as described elsewhere (Innis et al., 1990). PCR reactions were carried out with the primers up-F (5'-ACGGATCATCGATCTGCTGG-3'), up-R (5'-AACACTCTTCGTCTGTTGG-3') (for upstream), dw-F (5'-GGAACTCTCTGCCAGACC-3'), dw-R (5'-CGCAATGATGTCGACC-3') (for downstream). Conditions for amplification of the upstream region by inverse PCR were: 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by cooling to 4 °C. Conditions for amplification of the downstream region were: 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, followed by cooling to 4 °C. SplH-digested JF8N genomic DNA was used as template to isolate a 2 kb DNA fragment upstream of pCS5 while EcoRI-digested DNA was used to isolate a 3 kb fragment downstream of pCBm2. Deletion mutants were constructed by the Kilo-sequence Deletion kit (Takara), using the exon/mung system. Nucleotide sequences were determined using the CEQ DTCs-Quick Start Kit (Beckman Coulter) by the dyeoxy-chain-termination method, with a CEQ2000 DNA sequencer (Beckman Coulter).

**Sequence analysis.** DNA sequences were analysed by GENETYX software (Genetyx, Japan) and the deduced ORFs compared to those in the databases using BLAST and FASTA programs. Amino acid sequences exhibiting homology were retrieved from the protein database and aligned using CLUSTAL W (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbour-joining method and depicted using TreeView software (Page, 1996).

**Purification of the native and recombinant extradiol dioxygenases.** *Bacillus* sp. JF8 was grown on Castenholz D agar plates in the presence of naphthalene vapour. The cells were harvested, washed and resuspended in 20 mM phosphate buffer (pH 7.5) containing 2 mM β-mercaptoethanol (buffer A). The cell suspension was passed through a French press (Thermo IEC) and centrifuged at 17 000 g for 60 min and the supernatant applied to a DEAE-Toyopearl 650M (Toosoh, Japan) column. The enzyme was eluted with a 400 ml gradient of 0-0-0.4 M KCl. The enzyme activities of the eluted fractions were assayed against 2,3-dihydroxybiphenyl (as described below). The active fractions were collected, dialysed, and applied to a Phenyl Sepharose HP 26/10 column (Amersham) equilibrated with buffer A containing 1 M ammonium sulfate. The enzyme was eluted with a 400 ml gradient of 0-0-0.6 M ammonium sulfate. The enzyme activities of the eluted fractions were assayed against 2,3-dihydroxybiphenyl (as described below). The active fractions were collected and dialysed against buffer A and applied to a Mono Q HR 16/10 column (Amersham) equilibrated with buffer A. The enzyme was eluted with a 400 ml gradient of 0-1-0.35 M KCl. The fractions containing enzyme activity were pooled and dialysed against buffer A. The N-terminal sequence of the native enzyme was determined by automated Edman degradation on a model 492 protein sequencer (Applied Biosystems).

*E. coli* cells containing pCS2 [2 kb SalI–PstI fragment encoding nahH, the C-terminal region of *nahP1*, and part of *nahL* in pBlueScript II KS (+) (Stratagene)] and pCBm2 (2 kb BamHI fragment encoding *mocB* and *nahC* in pUC18) were cultivated at 37 °C in the presence of 1 mM IPTG in 400 ml LB medium containing 100 μg ampicillin ml⁻¹ (see Fig. 1 for plasmids). Harvested cells of the recombinant *E. coli* were suspended in buffer A, and sonicated with a homogenizer (Subsonic HMO-100; Iwaki, Japan) for 3 min. Cell debris was removed by centrifugation for 30 min at 18 000 g. The supernatant was referred to as the cell-free extract.
For NahC_JF8, the cell-free extract was incubated at 60 °C for 100 min, and denatured proteins were removed by centrifugation for 30 min at 18 000 g. (NH4)2SO4 was added to give 30% saturation and the precipitated protein was collected by centrifugation (20 min at 18 000 g) and dissolved in buffer A. To remove (NH4)2SO4 from the solution, desalting was carried out by Vivaspin-20 (Vivascience). The solution was loaded onto a HitTrap Q ion-exchange column (Amersham) equilibrated with buffer A. The enzyme activity was eluted with 160 ml gradient of 0–0–0–6 M NaCl and the active fractions eluted at around 0–25 M NaCl were concentrated by Vivaspin-20.

**Electrophoresis and activity staining of extradiol dioxygenases.** SDS-PAGE was carried out according to the method of Laemmli (1970) with Prestained Protein Marker Broad Range (New England Biolabs) and native nondenaturing PAGE was performed with the HMW Native Marker Kit (Amersham) using the same solutions without SDS. PAGE studies were done with purified recombinant NahC_JF8 and recombinant NahH_JF8 in cell-free extract. After electrophoresis, the native gels were assayed for extradiol dioxygenase activity by incubating the gels in 100 mM Tris/HCl (pH 8–0), 0–5 mM β-mercaptoethanol solution containing 2,3-dihydroxybiphenyl. Visual observation indicated formation of the yellow meta-cleavage product. Gels were stained with Coomassie brilliant blue R250 (Sandrock et al., 1989). The relative molecular masses were calculated from the mobilities of the marker proteins.

**Enzymic assays.** Extradiol dioxygenase activity was estimated by following the formation of the ring-fission products from the corresponding substrates using a DU-650 spectrophotometer (Beckman Coulter) at 25 °C. Reaction mixtures contained 50 mM sodium phosphate buffer (pH 7–5), substrate and enzyme solution. The absorption coefficients used for the ring-fission products of the substrates were as follows: catechol, λmax 375 nm, ε 33 M–1 cm–1 (Bayly et al., 1966); 3-methylcatechol, λmax 388 nm, ε 13 M–1 cm–1; 4-methylcatechol, λmax 375 nm, ε 17 M–1 cm–1; 3,4-dihydroxybiphenyl, λmax 343 nm, ε 13 M–1 cm–1; 4-chlorocatechol, λmax 379 nm, ε 40 M–1 cm–1 (Asturias & Timmis, 1993); homoproto catechuate, λmax 380 nm, ε 36 M–1 cm–1 (Miller & Lipscomb, 1996). 2,3-Dihydroxybiphenyl was used as a model substrate to assay activity against bicyclic compounds because of its occurrence in its use and ease of handling. Activity against 1,2-dihydroxynaphthalene was determined spectrophotometrically by the method of Kuhm et al. (1991b). Reactions were performed in 50 mM acetic acid/NaOH buffer (pH 5–5) and the initial rate of decrease of the absorbance at 331 nm was measured. A molar absorption coefficient (ε) for 1,2-dihydroxynaphthalene at 331 nm of 2-6 M–1 cm–1, as calculated by Kuhm et al. (1991b), was used in the enzyme activity calculations. Michaelis–Menten kinetics of reactions were verified by plotting reaction rates against substrate concentrations.

The range of substrate concentrations used in enzyme assays for determination of the kinetic parameters was: 1,2-dihydroxynaphthalene, 2–500 μM; 2,3-dihydroxybiphenyl, 10 μM–2 mM; 4-methyl catechol, 50 μM–10 mM; homoproto catechuate, 100 μM–10 mM; catechol, 100 μM–30 mM; 4-chlorocatechol, 100 μM–20 mM; 3-methylcatechol, 10 μM–5 mM.

The activation energy (Ea) was estimated using the Arrhenius equation for the temperature range of 30–80 °C. Enzyme reactions were performed in 50 mM phosphate buffer (pH 7–5) with 200 μM 1,2-dihydroxynaphthalene. The value of Ea was determined from the slope of the straight line that resulted when the logarithm of the reaction constant, k, was plotted against 1/T.

**Stability analysis.** To evaluate the temperature stability of the extradiol dioxygenases, 0–5 mg ml–1 of purified NahC_JF8 was incubated in 20 mM phosphate buffer (pH 7–5) at 60, 70 and 80 °C for up to 60 min. To study the influence of chelators and inhibitors on enzyme activity, 0–5 mg ml–1 of purified NahC_JF8 was incubated in 20 mM phosphate buffer (pH 7–5) with 5 mM and 25 mM EDTA, and 0–1 mM and 1 mM H2O2. For NahH_JF8, cell-free extract was used as enzyme solution. After incubation at different temperatures, removing enzyme activity was measured in 50 mM phosphate buffer (pH 7–5) with 1 mM 2,3-dihydroxybiphenyl at 25 °C.

**Metal analysis.** Metal content of the purified recombinant NahC_JF8 was determined by inductively coupled plasma mass spectrometry (ICP-MS) using a Seiko SPQ600 spectrometer. Samples for ICP-MS were prepared using acid-washed glassware. Sample and standards were prepared in 0.1% HNO3. Separate standard curves were routinely prepared for iron and manganese and samples measured in triplicate.

**RT-PCR.** RNA samples were treated with DNase I (Invitrogen). RT-PCR was carried out with the OneStep RT-PCR kit (Qiagen) using primers nH-F (5′-ATGATTTTTCGGCTTTGTG-3′), nC-R (5′-TCCAATTTTGGATGACTGGAC-3′) (for nahC), and nH-F (5′-ATGTTCACTG- GGAATTTT-3′), nH-R (5′-ACCCCTTGCGAAAAAGATTCA-3′) (for nahH) (Fig. 1). Detection was carried out using Anti-Digoxigenin-AP (Roche) and CSPD (Roche).

**Biotransformation assay.** Recombinant E. coli cells with nahC_JF8 or bphC_Q1 were cultured in LB medium with appropriate antibiotic. After 4 h of growth at 37 °C, 1 mM IPTG was added and the cells cultivated for a further 2 h. The cells were harvested, washed and suspended in 50 mM acetic acid/NaOH buffer (pH 5–5) at OD600 1. Five millilitres of the bacterial suspension was transferred to a 15 ml test tube and 1,2-dihydroxynaphthalene added at a concentration of 1 mM. The tubes were incubated at 37 °C for 2 h. The cell suspension was acidified by the addition of 50 μl concentrated HCl; an equal volume of ethyl acetate was added and mixed for 10 min. After centrifugation at 5000 g for 10 min to extract the transformatoms, the ethyl acetate was concentrated under a stream of nitrogen and trimethylsilylation done using BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) + TMCS (trimethylchlorosilane) (Sylon BFT kit, Supelco). The derivatized samples were analysed by gas chromatography (Hewlett Packard model 6890), equipped with an HP-5ms capillary column (50 m, 0.2 mm, 0.33 μm, Hewlett Packard) and a mass-selective detector (Hewlett Packard, model 5972A). The conditions for GC-MS have been described previously (Shimura et al., 1999). The products of biotransformation were analysed by operating the system in the scanning mode (50–700 m/z).
RESULTS

Sequencing of the nah cluster and sequence comparison

An extradiol dioxygenase was purified from naphthalene-grown cells of Bacillus sp. JF8 (result not shown) and the N-terminal sequence of the enzyme determined by Edman degradation to be MILRLGH-E-LFVTDLERAREFYVHILGFRENDSDKE. A DNA probe derived from the determined amino acid sequence was used to isolate and clone two overlapping fragments: a 5 kb SalI fragment and a 2 kb BamHI fragment. Sequencing the resulting contiguous 6.5 kb DNA fragment indicated it contained six ORFs, designated nahHLOMmocBnahC based on sequence homology (Fig. 1; GenBank accession no. AB116258). The deduced amino acid sequence of the N-terminal region of nahC was MILRLGHELFVTDLERAREFYVHILGFRENDSDKE, which is identical to the N-terminal amino acid sequence of the extradiol dioxygenase isolated from naphthalene-grown cells of Bacillus sp. JF8, as determined by Edman degradation. The deduced amino acid sequence of NahC_JF8 exhibits 40 % identity to homoproctocatechuate 2,3-dioxygenase (HPCD) from Brucella melitensis 16M, 33 % identity to catechol 2,3-dioxygenase (C23O) from Bacillus thermoleovorans, 16M, 33 % identity to catechol 2,3-dioxygenase (C23O) from Bacillus thermoleovorans A2, and 19 % identity to NahC_G7, the 1,2-dihydroxynaphthalene dioxygenase from Pseudomonas putida. NahH_JF8 exhibits 32 % identity to NahC_JF8 and 29 % identity to NahH_G7, the C23O from P. putida G7. The PROSITE extradiol dioxygenase fingerprint region, which spans 19–21 amino acid residues (including two active-site residues, H and Y, and a metal ligand, E), has the consensus pattern [GNTIV]–H–x(5,7)–[LIVMF]–Y–x(2)–[DENTA]–P–x–[GP]–x(2,3)–E, where x(n) indicates the presence of n residues of any type and brackets enclose residue types found at a position (Elitis & Bolin, 1996). In NahC_JF8, this fingerprint region is conserved, while in NahH_JF8 the consensus [LIVMF] is substituted by a Thr residue. NahL_JF8 has 46 % identity to 2-hydroxypenta-2,4-dienoate hydratase from Acinetobacter sp. YAA (accession no. BAA23558), NahO_JF8 has 59 % identity to acetaldehyde dehydrogenase from Bacillus thermoglucosidasius A7 (accession no. AA656653), while NahM_JF8 exhibits 55 % identity to the 4-hydroxy-2-oxovalerate aldolase from Pseudomonas stutzeri AN10 (accession no. AAD02153). The deduced amino acid sequence of the ORF downstream of nahM_JF8 exhibits 50 % identity to the monooxygenase, 4-nitrophenol hydroxylase component B from Rhodococcus sp. PN1 (accession no. BAB86379) and it was designated MocB (monooxygenase component B). The G+C contents of the genes are 40–50 mol%. Sequencing 2 kb upstream of nahH and 3 kb downstream of nahC did not locate any other genes or enzymes which could play a role in the degradation of naphthalene. Instead, two ORFs designated tnp1 and tnp2, were found flanking the nahHLOMmocBnahC operon (Fig. 1). Tnp1 exhibited 44 % identity to the transposase encoded by the gene BH0434 in Bacillus halodurans C-125 (accession no. NP_241300) and Tnp2 exhibited 28 % identity to the transposase encoded by the gene TTE2218 in Thermoanaerobacter tengcongensis MB4T (accession no. NP_623766).

In a phylogenetic tree of 27 extradiol dioxygenases, the enzymes cluster into three groups (Fig. 2). Ring-cleavage dioxygenases which exhibit a preference for monocyclic substrates cluster into one group (designated IB) while dioxygenases with a preference for bicyclic substrates form a second group (designated IC). The third group (designated

Fig. 1. Physical map of the 10 kb DNA fragment cloned from Bacillus sp. JF8 and location of RT-PCR amplicons. The two overlapping DNA fragments cloned are shown at the top and coding regions of the genes are indicated by boxes. The plasmid containing the 5 kb SalI fragment was designated pCSi5. The plasmid containing the 2 kb BamHI fragment was designated pCbm2. pCSP2 was constructed for expression of nahH_JF8. Primers used for construction of DNA probes and in RT-PCR are shown and their orientations indicated by arrows. DNA fragments amplified by RT-PCR are shown at the bottom. Enzymes: NahC, 1,2-dihydroxynaphthalene dioxygenase; NahH, catechol 2,3-dioxygenase (C23O); NahL, 2-hydroxypenta-2,4-dienoate hydratase; NahO, acetaldehyde dehydrogenase; NahM, 4-hydroxy-2-oxovalerate aldolase; MocB, monooxygenase component B; Tnp1, Tnp2, transposases.
IA) consists of the smaller extradiol dioxygenases which are single-domain enzymes, unlike the enzymes of the two previous groups, which have two identical domains. NahC_JF8 and NahH_JF8 cluster with group IB. The enzymes in this group appear to comprise three subgroups. While NahH_JF8 is in a subgroup by itself (IBa), NahC_JF8 and BphC_JF8 belong to the same subgroup (IBc). Subgroup IBc has several enzymes derived from extremophiles: HPDC_Oi (38% identity to NahC_JF8) derived from *Oceanobacillus iheyensis* HTE831, an extremely halotolerant and alkaliphilic deep-sea bacterium (Lu *et al.*, 2001); HPDC_R1 (35% identity), from *Deinococcus radiodurans*, a radioresistant bacterium (White *et al.*, 1999); C23O_Ss (31% identity), from *Sulfolobus solfataricus* P2, an extremely thermoacidophilic archaeon (She *et al.*, 2001); and PheB_FDTP3 (28% identity) from the thermophilic *Bacillus stearothermophilus* FDTP-3 (Dong *et al.*, 1992). MndD_CM2 (33% identity), a Mn(II)-dependent HPDC which exhibits high stability to metal chelators (Whiting *et al.*, 1996), and HPDC_Bf (38% identity), a stable Fe(II)-dependent HPDC which exhibits catalase and dioxygenase activity (Miller & Lipscomb, 1996), are also in this subgroup.

Fig. 3 shows a comparison of the amino acid sequences of NahC_JF8 and NahH_JF8 with NahC_G7 and NahH_G7 from *P. putida* G7, MPC_mt2, the C23O from *P. putida* mt-2, BphC_KKS102, the 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. KKS102, and BphC_LB400, the 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Burkholderia*.
cepacia LB400, for which crystal structures have been determined (Han et al., 1995; Kita et al., 1999; Senda et al., 1996). The metal ligands (His-152, His-211, Glu-264 in NahC_JF8 and His-145, His-206, Glu-257 in NahH_JF8) and active-site residues (His-197, Tyr-254 in NahC_JF8 and His-191, Tyr-247 in NahH_JF8) are conserved, as are the other strictly conserved residues (Gly-27, Leu-171, Pro-258 in NahC_JF8 and Gly-31, Leu-164, Pro-251 in NahH_JF8) which are thought to play a structural or folding role.

Metal ion dependence of the extradiol dioxygenases

Recombinant E. coli cells expressing NahC_JF8 and NahH_JF8 were grown in M9 mineral salts medium supplemented with different metal ions. Recombinant NahC_JF8 from E. coli cultured in M9 medium in the absence of metal ions did not exhibit meta-cleavage activity. Addition of Fe(II), Zn(II) and Co(II) to the M9 medium did not result in any enzymic activity, while the addition of Mn(II) resulted in meta-cleavage activity (Table 1). On the other hand, NahH_JF8 exhibited enzymic activity only when Fe(II) was added to LB medium, indicating that while NahC_JF8 is Mn(II)-dependent, NahH_JF8 is Fe(II)-dependent. The addition of 0.5 mM MnCl2 to LB medium resulted in a threefold increase in the specific activity of the recombinant NahC_JF8 in cell-free extract (0.025 ± 0.002 U mg⁻¹ in LB compared to 0.075 ± 0.003 U mg⁻¹ in LB with 0.5 mM MnCl2). Therefore, the LB medium was routinely supplemented with 0.5 mM MnCl2 when culturing recombinant E. coli for the production of NahC_JF8. However, addition of Fe(II) to LB medium did not increase the specific activity of the recombinant NahH_JF8.

ICP-MS analysis showed that the recombinant NahC_JF8 contained an average of 0.36 ± 0.004 g atom Mn per monomeric protein subunit. The Fe content was found to be consistently low at an average of 0.024 ± 0.002 g atom per enzyme subunit molecule.

Biochemical characterization of NahC_JF8 and NahH_JF8

Cell-free extracts from the recombinant E. coli cells expressing NahC_JF8 exhibited a specific activity of 0.06 U mg⁻¹.

Fig. 3. Structural comparison of extradiol dioxygenases. Alignment of NahC_JF8 and NahH_JF8 with catechol 2,3-dioxygenase (C23O) of Pseudomonas putida mt-2 (MPC_mt2), C23O of P. putida G7 (NahH_G7), 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) of Pseudomonas sp. KKS102 (BphC_KKS102), DHBD of Burkholderia cepacia LB400 (BphC_LB400), and 1,2-dihydroxynaphthalene dioxygenase of P. putida G7 (NahC_G7). The known secondary structures for MPC_mt2, BphC_KKS102 and BphC_LB400 are indicated by shading α-helices in dark grey and β-sheets in light grey. The metal ion ligands are indicated by asterisks (*) and the active site residues are indicated by plus signs (+).
Table 1. Metal ion dependence of recombinant NahC_JF8 and NahH_JF8

<table>
<thead>
<tr>
<th>Metal</th>
<th>Specific activity [mU mg⁻¹]</th>
<th>NahC_JF8</th>
<th>NahH_JF8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.09 ± 0.008</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>Fe(NH₄)₂(SO₄)₂</td>
<td></td>
<td>0.20 ± 0.09</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>MnCl₂</td>
<td></td>
<td>11 ± 2.6</td>
<td>0.14 ± 0.2</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td></td>
<td>0.04 ± 0.01</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>CoCl₂</td>
<td></td>
<td>0.02 ± 0.02</td>
<td>0.31 ± 0.1</td>
</tr>
</tbody>
</table>

Experiments were performed in 50 mM phosphate buffer (pH 7.5) at 25 °C, using 1 mM 2,3-dihydroxybiphenyl (for NahC_JF8) or catechol (for NahH_JF8) as substrate and cell-free extracts of recombinant E. coli cultured in M9 medium supplemented with 0.01 mM metal ion. Means ± SD from three experiments are shown.

with 2,3-dihydroxybiphenyl. NahC_JF8 could be purified 25-fold with an overall yield of 70% using a HiTrap Q ion-exchange column at room temperature, after thermal denaturation of the mesophilic proteins in the cell extract by treatment at 60 °C for 100 min (see Methods) (Table 2). In the case of recombinant NahH_JF8, the active enzyme could not be purified, although the heat treatment was omitted and the cell extract was directly loaded on to a HiTrap Q ion-exchange column at 4 °C. The addition of solvents such as ethanol, 2-propanol and acetone did not increase the stability of NahH_JF8, nor did addition of reducing agents such as β-mercaptoethanol and ascorbate, or changing the composition of phosphate buffer (20 mM to 100 mM). NahH_JF8 in cell-free extract retained more than 95% activity when incubated for 9 h at 4 °C. Therefore, the biochemical characterization of NahH_JF8 was done using cell-free extracts of recombinant E. coli.

The subunit molecular mass of recombinant NahC_JF8 and NahH_JF8 was determined by SDS-PAGE to be 35 kDa and 70 kDa, respectively. Purified recombinant NahC_JF8 exhibited a molecular mass of 120 kDa in native nondenaturing PAGE, indicating that the enzyme is a tetramer, whereas active recombinant NahH_JF8 in cell-free extract exhibited a molecular mass of 70 kDa in native non-denaturing PAGE, suggesting a dimeric structure. To ascertain that NahC_JF8 is functional as a tetramer and NahH_JF8 as a dimer, the nondenaturing polyacrylamide gel was assayed for meta-cleavage activity. Activity of the appropriate protein bands were visible on the gel as yellow-staining bands.

The $K_m$ value for 1,2-dihydroxynaphthalene of NahC_JF8 is 16 times lower than that for 2,3-dihydroxybiphenyl and 62 times lower than that for 4-methylcatechol (Table 3). The substrate preference of NahC_JF8 as based on $K_m$ value was in the order 1,2-dihydroxynaphthalene > 2,3-dihydroxybiphenyl > 4-methylcatechol > 3-methylcatechol > 4-chlorocatechol > homoprotocatechuate. Thus, the substrate preference of NahC_JF8 exhibited nearly the same trend as known 1,2-dihydroxynaphthalene dioxygenases (Patel & Barnsley, 1980; Hirose et al., 1994; Kuhm et al., 1991b). An increase in the temperature (from 25 °C to 60 °C) caused a decrease in the $K_m$ exhibited by NahC_JF8, while a substantial increase in the $V_{max}$ ensured that the specificity constants ($k_{cat}/K_m$) were higher at 60 °C (Table 3). The optimum temperature for NahC_JF8 under our assay conditions was determined to be 80 °C and the activation energy for the meta-cleavage of 1,2-dihydroxynaphthalene by NahC_JF8 was $11.6 ± 0.2$ kcal mol⁻¹ ($48.5 ± 0.8$ kJ mol⁻¹). NahH_JF8, in cell-free extract, exhibited a broad substrate preference (Table 4), with the $K_m$ ranging from 0.025 µM to 11 µM for 1,2-dihydroxynaphthalene, 2,3-dihydroxybiphenyl, catechol, 4-chlorocatechol, and 3- and 4-methylcatechol at 25 °C. NahH_JF8 exhibited activity against 1,2-dihydroxynaphthalene with a $K_m$ about 44-fold higher than that for 2,3-dihydroxybiphenyl.

Stability of NahC_JF8 and NahH_JF8

NahC_JF8 retained 60% of its activity after incubation at 70 °C for 60 min, and 50% of its activity after incubation at 80 °C for 20 min (Fig. 4), while retaining 90% activity after incubation at 60 °C for 12 h. Interestingly, the addition of 1 mM MnCl₂ to the enzyme solution at 70 °C prevented

Table 2. Purification of NahC_JF8 from recombinant E. coli

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol. (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)*</th>
<th>Total activity (U)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>20</td>
<td>176</td>
<td>0.058 ± 0.003</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>60 °C 100 min</td>
<td>17</td>
<td>70</td>
<td>0.21 ± 0.01</td>
<td>14</td>
<td>136†</td>
</tr>
<tr>
<td>30% (NH₄)₂SO₄</td>
<td>10</td>
<td>30</td>
<td>0.33 ± 0.01</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>HiTrapQ</td>
<td>6-0</td>
<td>4-8</td>
<td>1.5 ± 0.02</td>
<td>7-2</td>
<td>70</td>
</tr>
</tbody>
</table>

*Means ± SD from three experiments are shown.
†Apparent increase in yield probably due to denaturation of factors inhibiting enzyme activity.

http://mic.sgmjournals.org
H2O2 at 25 °C were incubated with different concentrations of EDTA or of NahC_JF8 and NahH_JF8 were studied. The enzymes were incubated with EDTA or H2O2, and NahC_JF8 lost 20% activity after 120 min incubation with 5 mM EDTA. EDTA at 5 mM and 25 mM did not increase thermostability. NahH_JF8, in cell-free extract, retained only 35% activity after incubation at 60 °C for 10 min, losing 95% activity after 30 min (data not shown).

The effects of metal chelators and inhibitors on the activity of NahC_JF8 and NahH_JF8 were studied. The enzymes were incubated with different concentrations of EDTA or H2O2 at 25 °C for up to 120 min. NahH_JF8 in cell-free extract lost 65% activity after incubation with 5 mM EDTA for 60 min (without EDTA there was no loss of activity) and lost 70% activity after incubation with 0.1 mM H2O2 for 10 min (without H2O2 there was no loss of activity). NahC_JF8 lost 20% activity after 120 min incubation with 0.1 mM H2O2; with 1 mM H2O2, NahC_JF8 lost 20% activity after 20 min incubation, 47% activity after 60 min and 67% activity after 120 min (in the absence of H2O2 there was no loss of activity). EDTA at 5 mM and 25 mM did not have an effect on NahC_JF8 activity up to 120 min. However, the addition of 5 mM EDTA at 70 °C resulted in 50% loss of activity in 20 min incubation (incubation at 70 °C for 20 min without EDTA caused 26% loss of activity), indicating that at the higher temperature the Mn(II) could be easily chelated from the enzyme.

### Table 3. Kinetic parameters of recombinant NahC_JF8

Abbreviations: DHNP, 1,2-dihydroxynaphthalene; DHBP, 2,3-dihydroxybiphenyl; 4MeCA, 4-methylcatechol; 3MeCA, 3-methylcatechol; 4ClCA, 4-chlorocatechol; HPCA, homoprotocatechuate; CA, catechol. Means ± SD from three experiments are shown.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>25 °C</th>
<th></th>
<th></th>
<th>60 °C</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_m (μM)</td>
<td>V_max (μmol min⁻¹ mg⁻¹)</td>
<td>k_cat (s⁻¹)</td>
<td>k_cat/K_m (μM⁻¹ s⁻¹)</td>
<td>K_m (μM)</td>
<td>V_max (μmol min⁻¹ mg⁻¹)</td>
</tr>
<tr>
<td>DHNP</td>
<td>18 ± 4</td>
<td>1.6 ± 0.3</td>
<td>84 ± 15</td>
<td>4.6 ± 0.2</td>
<td>32 ± 5</td>
<td>8.0 ± 0.4</td>
</tr>
<tr>
<td>DHBP</td>
<td>400 ± 25</td>
<td>2.4 ± 0.1</td>
<td>270 ± 10</td>
<td>0.68 ± 0.2</td>
<td>510 ± 90</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>4MeCA</td>
<td>890 ± 60</td>
<td>2.8 ± 0.2</td>
<td>320 ± 20</td>
<td>0.36 ± 0.2</td>
<td>2000 ± 100</td>
<td>8.7 ± 0.2</td>
</tr>
<tr>
<td>3MeCA</td>
<td>1300 ± 200</td>
<td>2.7 ± 0.2</td>
<td>160 ± 10</td>
<td>0.13 ± 0.01</td>
<td>2000 ± 60</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>4ClCA</td>
<td>1800 ± 100</td>
<td>0.62 ± 0.4</td>
<td>70 ± 5</td>
<td>0.038 ± 0.001</td>
<td>2200 ± 50</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>HPCA</td>
<td>2800 ± 600</td>
<td>0.75 ± 0.1</td>
<td>86 ± 10</td>
<td>0.031 ± 0.002</td>
<td>8200 ± 420</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>CA</td>
<td>9900 ± 900</td>
<td>2.3 ± 0.4</td>
<td>260 ± 40</td>
<td>0.027 ± 0.002</td>
<td>5100 ± 540</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

### Gene expression

To determine whether both naphthalene and biphenyl can induce NahC_JF8 and NahH_JF8, Bacillus sp. JF8 was grown on LB plates for 8 h and then incubated with naphthalene and biphenyl. Northern hybridization was done with probes for nahC_JF8 and nahH_JF8. As shown in Fig. 5(a), neither of the genes is constitutively expressed, while both genes are induced by naphthalene and not by biphenyl. RT-PCR indicated that nahHLOMmocBnahC is transcribed as a single unit (Fig. 1, Fig. 5b). As the genes appear to form an operon and are induced by naphthalene, they have been designated nah.

### Analysis of biotransformation products

The structural identity of the meta-cleavage product from 1,2-dihydroxynaphthalene by NahC_JF8 was confirmed by

[Image 339x153 to 509x305]

**Fig. 4.** Thermostability of NahC_JF8. Purified recombinant NahC_JF8 (0.5 mg ml⁻¹) in 20 mM phosphate buffer (pH 7.5) was incubated at 60 °C (○), 70 °C (△) and 80 °C (○), at 70 °C with 1 mM MnCl₂ (▲), at 70 °C with 5 mM EDTA (■), and at 80 °C with 1 mM MnCl₂ (●) for 60 min. After incubation, remaining enzyme activity was measured in 50 mM phosphate buffer (pH 7.5) with 1 mM 2,3-dihydroxybiphenyl at 25 °C. The data plotted are means ± SD (error bars) from three experiments.
DNA fragment from the thermophilic naphthalene degrader when the strain was grown on naphthalene, we cloned a 10 kb

Using a probe based on the extradiol dioxygenase produced

DISCUSSION

Interestingly, of the ten extradiol dioxygenases in subgroup IBc, six (including NahC_JF8 and BphC_JF8) are encoded by extremophiles while two more (MndD_CM2 and HPCD_Bf) are exceptionally stable extradiol dioxygenases (Boldt et al., 1995; Whiting et al., 1996; Miller & Lipscomb, 1996).

While analysing the evolutionary relationship between extradiol dioxygenases, Eltis & Bolin (1996) evaluated the functional significance of several conserved residues. They identified two residues Asn-243 and Asp-244 (numbering as in BphC_KKS102) in enzymes which preferentially cleave bicyclic substrates. Although NahC_JF8 preferentially cleaves a hydroxylated naphthalene ring, the Asn-Asp sequence is replaced by Leu-Ser. Similarly, in BphC_JF8, for which the preferred substrate is 2,3-dihydroxybiphenyl, the Asn-Asp residue is replaced with Ile-Ser (Hatta et al., 2003). Analysis of the amino acid sequence in the PROSITE extradiol dioxygenase fingerprint region showed that in NahH_JF8, the consensus [LIVMF] which represents residue types found at a position, is substituted by a Thr residue. As the adjacent Tyr (Y) is an active-site residue (Eltis & Bolin, 1996), the substitution of a hydrophobic residue (LIVMF) by a hydrophilic polar residue (T) might have had an effect on the enzyme activity. However, NahH_JF8 exhibited meta-cleavage activity against a wide range of substrates, and we propose that the consensus pattern be expanded to include the presence of a Thr residue as [LIVMFT].

Native nondenaturing PAGE of the purified recombinant NahC_JF8 and recombinant NahH_JF8 in cell-free extract appeared to indicate a tetrameric and dimeric structure, respectively, for the enzymes. BphC_LB400 and BphC_KKS102 were shown to be octamers while MPC_mt2 is a tetramer (Elsit et al., 1993; Senda et al., 1996; Kita et al., 1999). Kita et al. (1999) had opined that the long protruding loop at the end region of the N-terminal domain (Gly-130 to Trp-139) prevents the formation of an octameric structure in MPC_mt2. In the alignment of Fig. 3, a similar loop can be observed in NahC_JF8, which could explain its tetrameric

Bacillus sp. JF8N. Two extradiol dioxygenases, NahC_JF8 and NahH_JF8, were identified on the cloned fragment.

On a phylogenetic tree, NahC_JF8, NahH_JF8 and BphC_JF8 (Hatta et al., 2003) cluster with extradiol dioxygenases which exhibit a preference for monocyclic substrates (Fig. 2). This group of enzymes consists of three subgroups which are stable as evidenced by the bootstrap analysis, with NahH_JF8 as the sole member of subgroup IBa, and NahC_JF8 and BphC_JF8 as the members of subgroup IBc. When the relationship of the C-terminal domains, where the conserved active-site residues and metal ligands are located, was analysed, NahH_JF8 clustered with NahC_JF8 and BphC_JF8 (results not shown), indicating that the differences are mainly confined to the N-terminal domain. As the function of the N-terminal domain and the advantages it might confer on the extradiol dioxygenases are not clear, the implication of this observation is not evident.

Fig. 5. RNA analysis of nahC and nahH expression. (a) RNA dot-blotting analysis. Samples (1 mg) of total RNA from LB-grown cells (Ctrl), naphthalene-induced cells (NP) and biphenyl-induced cells (BP) were analysed. DIG-labelled DNA fragments carrying nahC_JF8 or nahH_JF8 were used as probes. (b) Agarose gel electrophoresis of RT-PCR products amplified from mRNA of naphthalene-induced Bacillus sp. JF8. The size of the DNA molecular mass markers in lane M (1 kb DNA Ladder, Bioneer) are indicated on the left. Lane 1, nH-F and nO-R (expected size, 2577 kb); lane 2, nO-F and nM-R (1951 kb); lane 3, nM-F and nC-R (2395 kb). No detectable products were obtained in control reactions from which the reverse transcription step had been omitted.

Using a probe based on the extradiol dioxygenase produced when the strain was grown on naphthalene, we cloned a 10 kb DNA fragment from the thermophilic naphthalene degrader Bacillus sp. JF8N. Two extradiol dioxygenases, NahC_JF8 and NahH_JF8, were identified on the cloned fragment.

GC-MS after trimethylsilylation, using the product of BphC_Q1 (which functions as 1,2-dihydroxynaphthalene dioxygenase; Kuhn et al., 1991a) as control. The mass spectra obtained with NahC_JF8 and BphC_Q1 were identical, showing a parent compound M+ with m/z 336 but the spectra did not exhibit the usually dominant (M−15)+ ion (loss of one methyl group from the trimethylsilyl moiety). The ion at m/z 219 is formed by the loss of [COOSi(CH3)3]+ (mass 117) and its abundance indicates it is relatively stable to further fragmentation. Ions at m/z 147 ([CH3]2SiOSi-(CH3)3]+ and m/z 73 ([CH3]2Si]+) were also present. As the biotransformation product of NahC_JF8 and BphC_Q1 from 1,2-dihydroxynaphthalene was identical, with a calculated molecular mass of 191, it corresponds to 2-hydroxychromene-2-carboxylate (Eaton & Chapman, 1992).
structure as deduced from the results of native non-denaturing PAGE.

To produce active recombinant NahC_JF8, the presence of Mn(II) ions in the medium was essential, indicating that the enzyme was a Mn(II)-dependent extradiol dioxygenase. Fe(II) was necessary for the production of active recombinant NahH_JF8, indicating that like most extradiol dioxygenases isolated so far, NahH_JF8 utilizes Fe(II). MndD_CM2 and BphC_JF8 are two Mn(II)-dependent extradiol dioxygenases which have been studied in detail (Boldt et al., 1995, 1997; Whiting et al., 1996; Hatta et al., 2003). They are distinct from Fe(II)-dependent extradiol dioxygenases like BphCII_P6 and BphC_LB400, which are completely inactivated by 0·1 mM H₂O₂ (Asturias et al., 1994). The presence of Mn(II) in NahC_JF8 was deduced by its stability towards H₂O₂ at 70 °C; the addition of 1 mM MnCl₂ prevented inactivation of NahC_JF8, indicating that the inactivation of the enzyme at 70 °C was most likely due to the loss of the metal cofactor. ICP-MS confirmed the presence of Mn(II) in NahC_JF8, although the result obtained of 1·45 ± 0·015 g atom Mn per homodimer indicates that it has less than stoichiometric metal content. It is possible that a partial loss of the metal cofactor occurred during the purification process.

The Kₘ of NahC_JF8 indicates its affinity for 1,2-dihydroxynaphthalene. An increase in temperature caused a decrease in the affinity of NahC_JF8 for its substrate; however, a significant increase in the Vₘₐₓ and kₐₚₜ compensated for the increase in Kₘ, actually increasing the catalytic efficiency of NahC_JF8 twofold at 60 °C. Observations of Vₘₐₓ increases keeping the kₐₚₜ/Kₘ value of thermophilic enzymes in a similar range at higher temperatures have been reported in other thermophiles ( Vieille et al., 1995). NahH_JF8, on the other hand, catalysed a broad range of substrates.

The thermostability of NahC_JF8 was comparable with that of BphC_JF8. Both enzymes are not inactivated at 60 °C and retain 50 % activity after 20 min incubation at 80 °C. In contrast, NahH_JF8, in cell-free extract, lost 65 % activity after 20 min at 60 °C. Although the thermostability of NahH_JF8 appears low in comparison with the two other extradiol dioxygenases from Bacillus sp. JF8, its thermostability is better than that of purified Fe(II)-dependent C23O from the thermophilic Bacillus thermoleovorans A2 (50 % loss of activity at 57 °C in 4·8 h and at 62 °C in 3·3 min; Milo et al., 1999) and mesophilic extradiol dioxygenases like BphC of P. putida OU83 (complete loss of activity in 10 min at 65 °C; Khan et al., 1996) and BphCII from Rhodococcus globulus P6 (90 % loss of activity at 50 °C in 10 min; Asturias et al., 1994). We were unable to purify recombinant NahH_JF8. It has been reported that C23Os are easily inactivated by various oxidizing reagents, such as air or H₂O₂ (Nozaki et al., 1968) and the inactivation is due to oxidation of the active-site Fe(II) to Fe(III). Purification of NahH_JF8 under anaerobic conditions might yield active NahH_JF8.

Although the in vivo role of the two extradiol dioxygenases could not be confirmed, as we were unable to transform Bacillus sp. JF8, Northern hybridization indicated that both NahC_JF8 and NahH_JF8 are induced in the presence of naphthalene and not biphenyl. RT-PCR studies showed that nahHLOMnocBnahC is transcribed as a single operon. In all nah or nah-like genes (pah, nag, nid) studied so far, the extradiol dioxygenase genes have been found coupled with the genes encoding the ring-hydroxylating dioxygenase, the dihydrodiol dehydrogenase and the hydrolytic enzyme which acts on the ring-cleavage product (Simon et al., 1993; Bosch et al., 1999; Kiyohara et al., 1994; Fuenmayor et al., 1998; Treadway et al., 1999). However, in JF8, the above-mentioned upper pathway genes were not found in association with nahC, although it is possible that some of the genes found in the operon could have activities similar to upper operon genes. Instead the operon was flanked by ORFs whose predicted amino acid sequences exhibited homology to transposases (Fig. 1). The presence of genes involved in the degradation of aromatic compounds on transposable elements has been noted before (Tsuda & Genka, 2001; Wyndham et al., 1994a) and the role of transposable elements in the horizontal transfer of genes is well established (Herrick et al., 1997; Wyndham et al., 1994b). Recruitment of catabolic transposable elements by bacteria would enhance the ability of the micro-organism to occupy new ecological niches. However, acquiring only a part of a metabolic pathway would not be useful, therefore the other genes and proteins which play a role in naphthalene metabolism must have been acquired separately and the chromosomally encoded upper pathway genes specific for naphthalene are present elsewhere in Bacillus sp. JF8.

REFERENCES


