Identification and functional analysis of the genes for naphthalenesulfonate catabolism by *Sphingomonas xenophaga* BN6

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Sphingomonas xenophaga BN6 degrades various (substituted) naphthalenesulfonates to the corresponding (substituted) salicylates. A gene cluster was identified on the plasmid pBN6 which coded for several enzymes participating in the degradative pathway for naphthalenesulfonates. A DNA fragment of 16 915 bp was sequenced which contained 17 ORFs. The genes encoding the 1,2-dihydroxynaphthalene dioxygenase, 2-hydroxychromene-2-carboxylate isomerase, and 2'-hydroxybenzalpyruvate aldolase of the naphthalenesulfonate pathway were identified on the DNA fragment and the encoded proteins heterologously expressed in *Escherichia coli*. Also, the genes encoding the ferredoxin and ferredoxin reductase of a multi-component, ring-hydroxylating naphthalenesulfonate dioxygenase were identified by insertional inactivation. The identified genes generally demonstrated the highest degree of homology to enzymes encoded by the phenanthrene-degrading organism *Sphingomonas* sp. P2, or the megaplasmid pNL1 of the naphthalene- and biphenyl-degrading strain *Sphingomonas aromaticivorans* F199. The genes of *S. xenophaga* BN6 participating in the degradation of naphthalenesulfonates also shared the same organization in three different transcriptional units as the genes involved in the degradation of naphthalene, biphenyl, and phenanthrene previously found in *Sphingomonas* sp. P2 and *S. aromaticivorans* F199. The genes were flanked in *S. xenophaga* BN6 by ORFs which specify proteins that show the highest homologies to proteins of mobile genetic elements.

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INTRODUCTION

Substituted naphthalenesulfonic acids (NSAs) are commonly used as detergents, dispersants and building blocks for the synthesis of azo dyes and various other products of the chemical industry (Tully, 1997). Since arylsulfonates are very rarely found in nature, NSAs are referred to as xenobiotics. Naphthalenesulfonates are only slowly degraded in the environment and have been repeatedly isolated from contaminated environments (Wellens, 1990; Alonso *et al.*, 1999; Riediker *et al.*, 2000; Ruckstuhl *et al.*, 2002).

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Abbreviations: DHNDO, 1,2-dihydroxynaphthalene dioxygenase; 2NSA, naphthalene-2-sulfonic acid; NSA, naphthalenesulfonic acid; NsaA, naphthalenesulfonate dioxygenase; NsaC, 1,2-dihydroxynaphthalene dioxygenase; NsaD, 2-hydroxychromene-2-carboxylate isomerase; NsaE, 2'-hydroxybenzalpyruvate aldolase; NsaF, salicylaldehyde dehydrogenase.

Nevertheless, several bacterial strains have been isolated which convert different naphthalenesulfonates (Brilon *et al.*, 1981a, b; Ohe & Watanabe, 1986, 1988; Wittich *et al.*, 1988; Zürrer *et al.*, 1987). The most intensively studied bacterial strain with the ability to degrade naphthalenesulfonates is *Sphingomonas xenophaga* BN6, which degrades various amino- and hydroxynaphthalenesulfonates to the corresponding amino- or hydroxysalicylic acids (Nörtemann *et al.*, 1986, 1994; Stolz *et al.*, 2000). The catabolism of the naphthalenesulfonates by *S. xenophaga* BN6 is initiated by an oxygenolytic desulfonation of the substrates in connection with a spontaneous rearomatization to the corresponding (substituted) 1,2-dihydroxynaphthalenes (Fig. 1). The following enzymic steps are identical in the degradative pathways of naphthalene and naphthalenesulfonates. The (substituted) 1,2-dihydroxynaphthalenes are cleaved by a 1,2-dihydroxynaphthalene dioxygenase (NsaC) to 2-hydroxychromene-2-carboxylates, which are converted by a 2-hydroxychromene-2-carboxylate isomerase (NsaD) to *trans*-2'-hydroxybenzalpyruvates, which are finally converted by 2'-hydroxybenzalpyruvate aldolase/hydratases (NsaEs) to pyruvate and (substituted) salicylaldehydes. Several of these key enzymes of the naphthalenesulfonate

Fig. 1. Pathway for naphthalenesulfonate degradation by *S. xenophaga* BN6.

degradative pathway have been studied in *S. xenophaga* (Kuhm *et al.*, 1991a; Stolz, 1999). Three of the relevant enzymes, NsaC, NsaD and NsaE, have been purified and biochemically characterized. Furthermore, the N-terminal amino acid sequences of NsaC and NsaE have been determined (Kuhm *et al.*, 1991a, 1992, 1993).

The genes for two extradiol dioxygenases (BphC1 and BphC2) have been cloned from *S. xenophaga* BN6 by screening of a gene bank for 2,3-dihydroxybiphenyl dioxygenase activity (Heiss *et al.*, 1995, 1997), but sequence and biochemical data show that the two extradiol dioxygenases are not involved in the degradation of NSAs. Recently, the genes for the degradation of NSAs have been localized on a 180 kb megaplasmid in *S. xenophaga* BN6 (Basta *et al.*,

2004). Therefore, in the present study, the relevant gene cluster was analysed in detail.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used and their relevant characteristics are listed in Table 1. The isolation and characterization of *S. xenophaga* BN6 has been described previously (Nörtemann *et al.*, 1986; Stolz *et al.*, 2000). The strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as DSM 6383. Strain BN6 was either grown in nutrient broth or a mineral medium (Dorn *et al.*, 1974) supplemented with glucose (MMG, 25 mM), naphthalene-2-sulfonic acid (2NSA, 5 mM), glucose (25 mM) and 2NSA (1 mM), or 6-aminonaphthalene-2-sulfonic acid (5 mM).

All cloning experiments and plasmid preparations were carried out in *Escherichia coli* JM109 (Viera & Messing, 1982). *E. coli* strains carrying different plasmids were grown at 37 °C in 2 × yeast tryptone (YT) liquid medium or on 2 × YT agar plates (Sambrook *et al.*, 1989). The antibiotics ampicillin, kanamycin and tetracycline were used at final concentrations of 100, 50 and 20 µg ml⁻¹, respectively.

DNA manipulation and preparation, cell transformation, and conjugation.

Small-scale plasmids were prepared by the method of Kieser (1984). Genomic DNA was isolated and all manipulations were carried out as described by Sambrook *et al.* (1989). All enzymes were purchased from Roche Diagnostics and used according to the manufacturer's suggestions. *E. coli* was transformed with plasmid DNA by the method of Chung *et al.* (1989). The conjugation experiments were performed as described by Keck *et al.* (1997).

PCR amplification of the 1,2-dihydroxynaphthalene dioxygenase (DHND) gene fragment.

The PCR reaction mixtures contained, in a volume of 40 µl, 10–100 ng genomic DNA, 0.5 µM of each forward and reverse primer (MWG-Biotech), 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs (Pharmacia Biotech) and 2.5 U *Taq* DNA polymerase (Pharmacia Biotech). The amino acid sequences GYLGM and WRAYAA found at the N terminus of DHND (Kuhm *et al.*, 1991a) were used to design the primers 5'-GGY-TAY-CTS-GGY-ATG-3' (DHND01, coding strand) and 5'-GC-SGC-RTA-SGC-SCG-CCA-3' (DHND02, non-coding strand). The *Pseudomonas* codon usage was used (Wada *et al.*, 1992). The mixtures were placed in a thermal cycler (PTC-200; MJ Research). The first step consisted of denaturation for 1 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 92 °C, annealing of primers for 1 min at 35 °C and extension for 2 min at 72 °C, with extension for 5 min during the last cycle. The PCR fragments were separated by electrophoresis through 1 % agarose gels at 10 V cm⁻¹ and stained by ethidium bromide.

Nucleotide sequence analysis. The DNA sequence was determined by the dideoxy chain-termination method in an automated DNA sequencer (ALF-Sequencer; Pharmacia Biotech) by use of reverse primers, sequencing primers or primers which were derived from known sequences. Database searches were done online with the programs BLASTX, BLASTP and BLASTN provided by the BLAST E-mail server (Altschul *et al.*, 1990; Gish & States, 1993). CLUSTALW (Thomson *et al.*, 1994) was used to align the amino acid sequences. All parameters were set at their default values. The sequence alignments were edited and analysed by using the multiple sequence alignment editor and shading utility GeneDoc (version 1.1.004) (Nicholas & Nicholas, 1996).

Isolation of the genomic DNA region. The 1.3 kb PCR fragment was treated with the Klenow fragment of DNA polymerase I

Table 1. Bacterial strains and plasmids

SOE, splicing by overlap extension.

Strain or plasmid	Relevant characteristics*	Source or reference
Strains		
<i>S. xenophaga</i> BN6		Stolz <i>et al.</i> (2000)
Wild-type (DSM 6383)	NSA ⁺	Nörtemann <i>et al.</i> (1986)
AKE2	ΔORF6:: <i>nptII</i> (Δ <i>nsaA1</i>), Km ^r	This study
AKE3	ΔORF9:: <i>nptII</i> (Δ <i>nsaA2</i>), Km ^r	This study
AKE4	ΔORF8:: <i>nptII</i> (Δ <i>nsaA3</i>), Km ^r , NSA ⁻	This study
AKE5	ΔORF16:: <i>nptII</i> (Δ <i>nsaA4</i>), Km ^r , NSA ⁻	This study
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsdR17 relA1 supE44 Δ(lac-proAB)</i> F'[traD36 <i>proAB lacI^q lacZΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
S17-1	<i>recA pro hsdR hsdM⁺ RP4</i> (Tc::Mu, Km::Tn7, Tp ^r , Sm ^r)	Simon <i>et al.</i> (1983)
Plasmids		
pAKE16	ORF5 in pJOE2702; Amp ^r	This study
pAKE17	ORF13 in pJOE2702; Amp ^r	This study
pAKE18	ORF12 in pJOE2702; Amp ^r	This study
pAKE3-5	RTS1, 12 kb genomic insert of strain BN6	This study
pAKE35.1	<i>sacB</i> in pMHS298	This study
pAKE36	SOE fragment of ORF6 in pAKE35.1; Tc ^r Km ^r	This study
pAKE37	SOE fragment of ORF8 in pAKE35.1; Tc ^r Km ^r	This study
pAKE39	SOE fragment of ORF9 in pAKE35.1; Tc ^r Km ^r	This study
pAKE41	SOE fragment of ORF16 in pAKE35.1; Tc ^r Km ^r	This study
pJKS325	ORF7 in pJOE2702; Amp ^r	This study
pJOE2702	P _{rha} Amp ^r	Volff <i>et al.</i> (1996)
pJOE890	Positive selection vector	Altenbuchner <i>et al.</i> (1992)
pJKS240	1.3 kb fragment in pJOE890	This study
pMHS298	Mobilizable plasmid; Amp ^r Tc ^r	Hils (1998)

*NSA⁺, degradation of naphthalenesulfonate; NSA⁻, mutant of strain BN6 which had lost NSA degradation activity.

(Boehringer Mannheim) and inserted into *EcoRV*-cut pJOE890 (Altenbuchner *et al.*, 1992) to yield pJKS240. A λ RES vector, with a built-in Tn1721-encoded excision system (Altenbuchner, 1993), was used to construct a genomic library of the wild-type *S. xenophaga* BN6. The PCR fragment was DIG-labelled to identify the phage carrying the relevant genomic region. One of the identified phages was converted into the RTS1-derived pAKE3-5 (Altenbuchner, 1993).

Hybridization of DNA. Total genomic DNA was isolated and digested with restriction endonucleases. After electrophoresis, the DNA was transferred onto a positively charged nylon membrane (Roche Diagnostics). Probe DNA was DIG-labelled by use of a DNA labelling and detection kit (Roche Diagnostics). Hybridization was carried out at 68 °C in hybridization buffer, as described by the manufacturer. The Southern blots were developed using chemiluminescent detection, according to the protocol of the DNA labelling and detection kit.

Inverse PCR. Inverse PCR was performed, as described by Ochman *et al.* (1988), to isolate the 5' and 3' flanking sequences of the genomic sequence fragment of pAKE3-5. To isolate the 5' flanking sequence, genomic DNA of strain BN6 was cut with *HindIII* and hybridized against the chromosomal fragment of pAKE3-5. The hybridization signal corresponded to a fragment of 3.5 kb for the additional 5' sequence. The DNA of strain BN6 was cut with *HindIII*, ligated and used for PCR studies (forward primer S1659, 5'-GAC-GAT-GAG-CAG-GTA-GAA-CT-3'; reverse primer S1660,

5'-ACC-TTC-ATC-ATG-TCG-TCT-TC-3'). To isolate the 3' flanking sequence, genomic DNA of *S. xenophaga* BN6 was cut with *PstI* and hybridized against the 12 kb chromosomal insert of pAKE3-5. The hybridization signal corresponded to a fragment of ~1.5 kb for the additional 3' sequence. Thus, genomic DNA of strain BN6 was cut with *PstI* and ligated in different concentrations (100 ng, 1 µg). The ligated DNA was used for PCR with the primer pair S2124 (5'-GCA-AAT-CGT-CGT-CAG-GAT-TA-3') and S2125 (5'-GCG-CCG-ACT-GTT-ATC-TTC-TT-3'). The 5' (3.5 kb) and 3' (1.5 kb) flanking fragments of the genomic DNA region were cloned into *EcoRV*-cut pUC18 and sequenced.

Subcloning of ORF5, ORF7, ORF12 and ORF13 into pJOE2702. The primers which were used for the subcloning experiments are summarized in Table 2. The forward primer S1817 and the reverse primer S1818 were used to amplify a 1519 bp fragment containing the ORF5 gene. The PCR was performed as described for the DHND0 gene fragment. The primer pairs S1252/S1352, S1821/S1822 and S1823/S1824 were used to generate PCR fragments of ORF7 (916 bp), ORF12 (607 bp) and ORF13 (1006 bp), respectively. To facilitate cloning of the PCR products, *NdeI* and *BamHI* restriction sites were added to the primers. The appropriate PCR products were cut with *NdeI* and *BamHI* and inserted into *NdeI*/*BamHI*-cleaved pJOE2702 (Volff *et al.*, 1996) to give pAKE16 (ORF5), pJKS325 (ORF7), pAKE18 (ORF12) and pAKE17 (ORF13). *E. coli* strains JM109(pAKE16), JM109(pJKS325), JM109(pAKE18)

Table 2. Oligonucleotides used in this study for the cloning of the gene encoding NsaC, and the construction of expression vectors and hybrid genes by splicing by overlap extension.

Primer	Sequence*	Target region
DHND01	GG(C/T)TA(C/T)CT(G/C)GG(C/T)ATG	<i>nsaC</i>
DHND02	GC(G/C)GC(G/A)TA(G/C)GC(G/C)CGCCA	<i>nsaC</i>
S1252	aataattcatATGTCATCGGTCAGCGAACTT	5' end of ORF7
S1352	tataggatccCTAGAGAGGAACATCCAT	3' end of ORF7
S1821	aataattcatATGACGAAAACGATCGATTCTA	5' end of ORF12
S1822	tataggatccTTAATCTAGAGATAACTTTCAAG	3' end of ORF12
S1823	aataattcatATGGCCCGTACACTCATGAAACC	5' end of ORF13
S1824	tataggatccTCATTTTTTCGGCTGCGAGCG	3' end of ORF13
S1817	aataattcatATGGCTACACAGTTGAAAAGTGG	5' end of ORF5
S1818	tataggatccTTACTGATCGAACATCCCGG	3' end of ORF5
S2051	tatatctagaACATCAATCTGACGCTCGCG	5' flanking sequence of ORF6
S2052	cttgctgtTACCTGTCTCTCCCGC	Fusion of 5' end <i>nptII</i> end and 5' flanking sequence of ORF6
S2053	gacaggttaACAGCAAGCGAACC GG	Fusion of 5' flanking sequence of ORF6 and 5' <i>nptII</i> end
S2054	gacgtcatTCAGAAGAAGCTCGTCA	Fusion of 3' flanking sequence of ORF6 and 3' <i>nptII</i> end
S2055	tcttctgaATGACGTCGATGCAAA	Fusion of 3' <i>nptII</i> end and 3' flanking sequence of ORF6
S2056	aataattcatatgAAGCACAACCGTGTTACCCG	3' flanking sequence of ORF6
S2057	tatatctagaCGAGCATGCTGGACACAGCG	5' flanking sequence of ORF8
S2058	cttgctgtCGTGCTTCGTAGTCTCT	Fusion of 5' <i>nptII</i> end and 5' flanking sequence of ORF8
S2059	gaagcagcACAGCAAGCGAACC GG	Fusion of 5' flanking sequence of ORF3 and 5' <i>nptII</i> end
S2060	gatccatTCAGAAGAAGCTCGTCA	Fusion of 3' flanking sequence of ORF8 and 3' <i>nptII</i> end
S2061	tcttctgaGATGGATCCGAACATT	Fusion of 3' <i>nptII</i> end and 3' flanking sequence of ORF8
S2062	aataattcatatgCGCCAATGCTTTCCGCACGG	3' flanking sequence of ORF8
S2087	tatatctagaTAGCAGTGACTGTGGCGAGC	5' flanking sequence of ORF9
S2088	cttgctgtCTCAGGCCAATCCTTC	Fusion of 5' <i>nptII</i> end and 5' flanking sequence of ORF9
S2089	ggcctgagACAGCAAGCGAACC GG	Fusion of 5' flanking sequence of ORF9 and 5' <i>nptII</i> end
S2090	caatcggtTCAGAAGAAGCTCGTCA	Fusion of 3' flanking sequence of ORF9 and 3' <i>nptII</i> end
S2091	tcttctgaGCCGATTGTGAGATTT	Fusion of 3' <i>nptII</i> end and 3' flanking sequence of ORF9
S2092	aataattcatatgCAGGGTGTTCTGAATCTGCT	3' flanking sequence of ORF9
S2155	tatatctagaATCCTGACGACGATTGCCA	5' flanking sequence of ORF16
S2082	cttgctgtCCGCTAGATTGGCGCC	Fusion of 5' <i>nptII</i> end and 5' flanking sequence of ORF16
S2083	tctagcggACAGCAAGCGAACC GG	Fusion of 5' flanking sequence of ORF16 and 5' <i>nptII</i> end
S2084	ccgacggtTCAGAAGAAGCTCGTCA	Fusion of 3' flanking sequence of ORF16 and 3' <i>nptII</i> end
S2205	tcttctgaTTATGCAGTCTGCTCG	Fusion of 3' <i>nptII</i> end and 3' flanking sequence of ORF16
S2189	aataattcatatgGGACGGTTGCAGATCAATTC	3' flanking sequence of ORF16

*Relevant recognition sequences for the restriction enzymes *NdeI*, *BamHI* and *XbaI* are underlined. Lower-case type indicates the sequences which are not homologous to the template DNA.

and JM109(pAKE17) were each grown to the early exponential growth phase (OD₆₀₀ 0.3) and induced for 4 h with 0.2% (w/v) rhamnose. The cell extracts were analysed by 12% (w/v) SDS-PAGE.

Insertional inactivation of ORF6, ORF8, ORF9 and ORF16 in *S. xenophaga* BN6. The DNA fragments to be joined were amplified in separate PCRs, cut with *XbaI*/*NdeI* and inserted into the *XbaI*/*NdeI*-cut mobilizable pAKE35.1 (Table 1). The ORFs were deleted and replaced by the kanamycin-resistance gene (*nptII*) of Tn5. The chromosomal regions for homologous recombination of the 5' and 3' flanking regions of the inactivated gene, which were ligated to the *nptII* gene, were set to ~1 kb. This resulted in pAKE36 (for ORF6), pAKE37 (for ORF8), pAKE39 (for ORF9) and pAKE41 (for ORF16). The plasmids were transferred separately from *E. coli* S17-1 (Simon *et al.*, 1983) to *S. xenophaga* BN6 by conjugation. Since pAKE35.1 (Tc^r) does not replicate in strain BN6, integration

mutants were selected on MMG agar plates supplemented with tetracycline. To isolate integration mutants which had lost the vector fragment by homologous recombination, the strains were selected on MMG supplemented with sucrose (4%) and kanamycin. Genomic DNA was isolated, digested with different restriction enzymes and hybridized against the deleted gene, the *nptII* gene and the 5' and 3' flanking fragments of the deleted gene, to verify the integration of the *nptII* gene into the corresponding gene. The primers used are listed in Table 2, and the procedure was performed as described previously (Keck *et al.*, 2002).

Preparation of cell extracts. Cells were harvested by centrifugation at 6000 g, washed with 5 ml and resuspended in 1 ml 50 mM sodium/potassium phosphate buffer (pH 7.3). The cells were disrupted by sonication (Ultrasonics Sonicator W-385; Heat Systems-Ultrasonics; Microtip, 2 × 30 s, duty cycle 50% s⁻¹). Cell debris was

removed by centrifugation for 30 min at 100 000 g. Protein concentrations were determined according to the method of Bradford (1976), by use of the Bio-Rad protein assay.

Enzyme assays. The naphthalenesulfonate dioxygenase (NsaA) activity was quantified, as described by Nörtemann *et al.* (1986), using resting cells of strain BN6 incubated with 2NSA (1 mM), and is given as nanomoles of 2NSA transformed per minute per milligram of protein present in the crude extract (Nörtemann *et al.*, 1994). The NSA degradation pathway was induced by the addition of 0.5 mM salicylate to the exponentially growing cells and the culture was further incubated at 30 °C for 3 h.

NsaC and salicylaldehyde dehydrogenase (NsaF) activity were measured as described by Kuhm *et al.* (1991a, b). NsaE and NsaD were assayed spectrophotometrically, as described by Kuhm *et al.* (1992, 1993).

SDS-PAGE. The proteins were separated on a 12 % (w/v) denaturing polyacrylamide gel by use of the discontinuous buffer system described by Laemmli (1970) and stained with Coomassie blue R250.

HPLC analysis. The degradation of NSA was monitored by HPLC (Lichograph L-6200 gradient pump equipped with a L-4200 UV-VIS detector; Merck). A reversed-phase column (250 × 4 mm internal diameter; GROM), filled with 5 µm diameter particles of Lichrospher RP18 (Merck), was used to identify individual compounds which were detected spectrophotometrically at 210 nm. The mobile phase consisted of 50 % (v/v) methanol, 49.75 % (v/v) H₂O and 0.25 % (v/v) H₃PO₄, and the flow rate was 1 ml min⁻¹.

Chemicals. NSAs were obtained from Bayer. 2'-Hydroxybenzalpyruvate was kindly provided by A. Kuhm, Institut für Mikrobiologie, Stuttgart. 2-Hydroxychromene-2-carboxylate was prepared enzymically with whole cells of strain BN6, as described by Kuhm *et al.* (1993). All other chemicals were obtained from Sigma-Aldrich or Merck. Biochemicals were from Boehringer Mannheim.

RESULTS

Cloning of the gene encoding NsaC

The NsaC of strain BN6 was previously purified and characterized, and the N-terminal amino acid sequence determined (Kuhm *et al.*, 1991a). The primers DHNDO1 and DHNDO2 (Table 2) were derived from the N-terminal amino acid sequence and used for the attempted amplification of the corresponding part of the gene (~60 bp) encoding NsaC. Surprisingly, amplification of a 1.3 kb DNA fragment was achieved from the genomic DNA of strain BN6 with primer DHNDO1 alone, using a low annealing temperature (35 °C). The 1.3 kb PCR fragment was cloned into pUC18 and sequenced. Database searches demonstrated a significant homology of the encoded peptide to different bacterial 2,3-dihydroxybiphenyl-1,2-dioxygenases and NsaCs. Furthermore, these sequence comparisons demonstrated that the encoded protein was different from two other extradiol dioxygenases (BphC1 and BphC2) which were previously cloned from strain BN6 (Heiss *et al.*, 1995, 1997).

Isolation of the DNA region surrounding the gene encoding the NsaC

A genomic library of the wild-type *S. xenophaga* BN6 was constructed using a λRES vector (Altenbuchner, 1993). The PCR fragment encoding the NsaC was labelled and used to screen the library. pAKE3-5 was identified, which carries the genomic context of the *nsaC* gene. Using inverse PCR, 3.5 kb upstream and 1.5 kb downstream of the genomic insert of pAKE35.1 were identified and sequenced. This resulted in a total continuous DNA sequence of 16 915 bp. The G+C content of this DNA sequence was 57.7 %, a value which is lower than the total G+C content of the genomic DNA of *S. xenophaga* BN6 (62.1 ± 0.2 %) (Stolz *et al.*, 2000). Computer analysis revealed 15 complete (ORF2–ORF13, ORF15–ORF17), one partial (ORF1) and one disrupted (ORF14) ORFs. ORF10 and ORF11 were homologous to the N- and C-terminal parts, respectively, of the large component of an oxygenase. This indicated that ORF10 and ORF11 are inactive fragments generated by an amber mutation (Q→stop) from the gene encoding the large component of a ring-hydroxylating aromatic oxygenase (Fig. 2). The deduced protein sizes of all identified proteins are summarized in Table 3. The identified genes are presumably organized in at least six different clusters. Two of the putative transcriptional units are separated by a putative rho-independent transcription terminator (<http://www.bioinfo.rpi.edu/applications/mfold>; Fig. 2).

Construction of deletion mutants in the genes supposed to encode the initial NsaA

S. xenophaga BN6 degrades (substituted) naphthalene-sulfonates after an initial desulfonation via a modified naphthalene pathway with (substituted) 1,2-dihydroxynaphthalene(s) as initial metabolite(s) (Nörtemann *et al.*, 1986; Kuhm *et al.*, 1991a). Thus, the initial desulfonating, ring-hydroxylating dioxygenase is of central importance in this pathway. Analysis of the 17 kb genomic sequence revealed several ORFs which shared significant homologies with different proteins of multicomponent dioxygenases. Thus, ORF6, ORF8, ORF9 and ORF16 demonstrated sequence similarities with the α, ferredoxin, small and reductase components of ring-hydroxylating dioxygenases (Table 3). To analyse if these ORFs encode (parts of) the initial naphthalenesulfonate dioxygenase or different multicomponent dioxygenases, the corresponding genes were each deleted by site-specific integration mutagenesis and replaced by the kanamycin-resistance gene (*nptII*) of Tn5. The integration cassettes were constructed via gene splicing by overlap extension (Horton *et al.*, 1989). The strains which were further analysed were designated AKE2 (ΔORF6), AKE3 (ΔORF9), AKE4 (ΔORF8) and AKE5 (ΔORF16). The integration of the *nptII* gene into each ORF was verified by Southern blot analysis, as described previously (Keck *et al.*, 2002).

Fig. 2. (a) Genetic map of the 17 kb DNA fragment from *S. xenophaga* BN6. The positions and orientations of the identified ORFs are indicated by filled arrows. The putative α and β subunits of a multicomponent dioxygenase are shaded in grey. ORF14, which is disrupted by ORF15, is indicated by a dotted outline. ORF10 and ORF11 are truncated genes of a dioxygenase and are shown as a single, non-functional ORF. The genes encoding proteins which have been shown to be involved in the degradation of naphthalenesulfonates (*nsa* genes) are displayed in italic type. Relevant restriction sites are shown. A rho-independent terminator-like structure (T) is indicated between ORF12 and ORF13. (b) In comparison to the genes of the BN6 locus, homologous proteins which are encoded on three different, non-contiguous loci of pNL1 from *S. aromaticivorans* F199 are displayed. Homologous genes from strain F199 which encode proteins with functions similar to those of the *nsa* genes are shaded.

Biochemical analysis of the deletion mutants in the genes presumably encoding NsaA

The conversion of 2NSA by cell suspensions of the integration mutants AKE2 (Δ ORF6::*nptII*), AKE3 (Δ ORF9::*nptII*), AKE4 (Δ ORF8::*nptII*) and AKE5 (Δ ORF16::*nptII*) was tested. Resting cells of these strains were analysed for NsaA activity and the corresponding cell extracts were analysed for NsaC, NsaE and NsaF activity. The addition of salicylate induced NsaC, NsaE and NsaF activities in the mutant and wild-type strains (Table 4). NsaA activity was only detected with resting cells of strains AKE2, AKE3 and the wild-type strain. Strains AKE4 and AKE5 did not show any measurable NsaA activity, irrespective of the presence of salicylate. These results strongly support the view that ORF8 and ORF16 encode the ferredoxin (*nsaA3*) and reductase (*nsaA4*) components of NsaA, whereas ORF6 and ORF9 might encode the oxygenase component of a not-yet-identified ring-hydroxylating dioxygenase.

In silico identification of the genes encoding the NsaC, NsaD, NsaE and NsaF involved in the degradation of naphthalenesulfonates

NsaC and NsaE from *S. xenophaga* BN6 have previously been purified and their N-terminal amino acid sequences determined (Kuhm *et al.*, 1991a, 1992). This allowed the identification of ORF7 and ORF13 as the genes encoding the NsaC and NsaE of the naphthalenesulfonate pathway,

because the N-terminal 29 or 26 aa of the predicted protein sequences were identical to the data obtained experimentally by protein sequencing. Furthermore, the predicted molecular masses of the proteins encoded by ORF7 (33 301 Da) and ORF13 (35 459 Da) were in good agreement with the values of 33 000 and 38 500 Da determined experimentally for the purified NsaC and NsaE (Kuhm *et al.*, 1991a, 1992).

The amino acid sequence of ORF12 shared significant homologies to different NsaDs (Table 3), such as NahD from *S. aromaticivorans* F199 and *Sphingomonas* sp. CHY-1 (76–77% identical residues). ORF5 shared the highest degree of homology with different presumed benzaldehyde or salicylaldehyde dehydrogenases from different sphingomonads (Table 3). Thus, it appeared that all enzymes necessary for the conversion of (substituted) 1,2-dihydroxynaphthalene(s) to (substituted) salicylate(s) were encoded on the analysed DNA fragment.

Experimental identification of the genes encoding the NsaC, NsaD, NsaE and NsaF involved in the degradation of naphthalenesulfonates

The genes encoding the putative NsaC, NsaD, NsaE and NsaF were amplified from genomic DNA of strain BN6 via PCR (Table 2). The genes were integrated into the expression plasmid pJOE2702 (Volff *et al.*, 1996) to give pAKE16 (ORF5), pJKS325 (ORF7), pAKE18 (ORF12) and pAKE17

Table 3. Identified genes and gene products of the sequenced 17 kb DNA fragment

ORF	Position (bp) (accession no. U65001)	Calculated molecular mass (Da)	Putative function	Homologous proteins	Amino acid identity (%)	GenBank accession no.
ORF1 (incomplete)	1–423	> 15 683	Transposon-associated ATP-binding protein	Y4sD of <i>Rhizobium</i> sp. NGR234	78	NP_444057.1
				Putative transposase from <i>Bradyrhizobium japonicum</i> USDA 110	79	NP_771639.1
ORF2	498–1424 C*	35 229	Transposon-associated ATP-binding protein	Putative insertion sequence ATP-binding protein from <i>Rhizobium etli</i>	77	AAM54812.1
				Putative site-specific recombinase from <i>Sinorhizobium meliloti</i> 1021	44	NP_435669.1
ORF3	1929–2606	25 518	Transposase	Putative transposase of <i>Burkholderia cepacia</i> R1808	66	ZP_00219685.1
ORF4	3035–3836 C	27 972	1,2-Dihydrodiol-1,2-dihydroxydehydrogenase	Transposase of <i>Janthinobacterium</i> sp. J3	63	BAC56751.1
				BphB of <i>S. aromaticivorans</i> F199	75	AF079317
ORF5	3875–5374 C	54 223	NsaF	Short-chain alcohol dehydrogenase of <i>S. aromaticivorans</i> DSM 12444	69	ZP_00302761.1
				Benzaldehyde dehydrogenase of <i>S. aromaticivorans</i> DSM 12444	84	ZP_00302762.1
				Benzaldehyde dehydrogenase of <i>Sphingomonas chungbukensis</i>	81	AAD03838.2
ORF6	5755–7167 C	52 713	Multicomponent dioxygenase α component	XylX of <i>Sphingomonas</i> sp. P2	80	BAC65430
ORF7	7467–8363	33 301	NsaC	XylX of <i>S. aromaticivorans</i> F199	68	AF079317
				BphC of <i>Sphingomonas</i> sp. P2	85	BAC65429.1
ORF8	8420–8746	11 595	Ferredoxin component of a dioxygenase	BphC of ' <i>Sphingomonas paucimobilis</i> ' Q1	85	M20640
				BphA3 of <i>Sphingomonas</i> sp. P2	82	BAC65428.1
ORF9	8748–9236	18 724	β component of a dioxygenase	BphA3 of <i>S. chungbukensis</i> CHY-1	78	CAG17580.1
				BphA2 of <i>Sphingomonas</i> sp. P2	82	BAC65427.1
				Small component of a phenylpropionate dioxygenase of <i>S. aromaticivorans</i> DSM 12444	66	ZP_00302780.1
ORF10 (5' end of a truncated gene)	9258–9656	14 864	Large component of a dioxygenase	dioxygenase of <i>S. aromaticivorans</i> DSM 12444	78	BAC65426.1
				Large component of an oxygenase from <i>Sphingomonas</i> sp. P2	77	AAD04009.1
ORF11 (3' end of a truncated gene)	9663–10520	31 739	Large component of a dioxygenase	Large component of an oxygenase from <i>S. aromaticivorans</i> F199 (aa 1–132)	77	AAD04009.1
				Large component of an oxygenase from <i>Sphingomonas</i> sp. P2	88	BAC65426.1
				Large component of a phenylpropionate dioxygenase from <i>S. aromaticivorans</i> DSM 12444 (aa 135–420)	84	ZP_00302781.1
ORF12	10517–11104	22 064	NsaD	NahD of <i>S. aromaticivorans</i> DSM 12444	77	ZP_00302782.1
ORF13	11462–12448 C	35 459	2'-Hydroxybenzalpyruvate aldolase	NahD of <i>Sphingomonas</i> sp. CHY-1	76	CAG17583.1
				NahE of <i>Sphingomonas</i> sp. P2	89	BAC65452.1

Table 3. cont.

ORF	Position (bp) (accession no. U65001)	Calculated molecular mass (Da)	Putative function	Homologous proteins	Amino acid identity (%)	GenBank accession no.
ORF14 (interrupted by ORF15)	12614–13800	25 400	Diol dehydrogenase	NahE of <i>S. aromaticivorans</i> DSM 12444 XylL of <i>Sphingomonas aromaticivorans</i> F199	82 62	ZP_00302750.1 AF07931
ORF15	13081–13551 C	16 760	Transposase	BenD of <i>Acinetobacter calcoaceticus</i> Transposase from <i>Mesorhizobium loti</i> /transposase C terminus from <i>S. aromaticivorans</i> F199	36 60	P07772 NP_085609.1
ORF16	13823–15052 C	44 135	Ferredoxin reductase, part of NSA dioxygenase	Ferredoxin reductase from <i>Sphingomonas</i> sp. P2	54 79	AAD03945.1 BAC65450.1
ORF17	15381–16383 C	37 987	Resolvase	Ferredoxin reductase from <i>S. aromaticivorans</i> DSM 12444 Putative resolvase-like protein of <i>M. loti</i> Resolvase-like protein of <i>Acidithiobacillus ferrooxidans</i>	72 48 48	ZP_00302752.1 NP_106849.1 AAC80184.1

*C, complementary DNA strand.

(ORF13) (Table 5). The corresponding strains *E. coli* JM109(pAKE16), JM109(pJKS325), JM109(pAKE18) and JM109(pAKE17) were each grown to the early exponential growth phase and induced by the addition of rhamnose (see Methods). The cell extracts were analysed via SDS-PAGE. Additional proteins of the predicted sizes were only present in cell extracts of the rhamnose-induced strains (Fig. 3). The corresponding enzyme activity was analysed in comparison to cell extracts of the uninduced strain *E. coli* JM109(pJOE2702). As summarized in Table 5, the putative enzyme functions were confirmed for ORF7 (NsaC), ORF12 (NsaD), ORF13 (NsaE) and ORF5 (NsaF). The cell extracts of the uninduced strains and the control strain JM109(pJOE2702) did not show any NsaC, NsaD, NsaE or NsaF activity.

DISCUSSION

The results of the present study of the genes encoding the degradative pathway for naphthalenesulfonates by *S. xenophaga* BN6, in combination with those of previous studies of the molecular organization of the genes responsible for the degradation of naphthalene and biphenyl by *S. aromaticivorans* F199 (Romine *et al.*, 1999) and phenanthrene by *Sphingomonas* sp. P2 (Pinyakong *et al.*, 2003a), clearly demonstrate a unique conserved organization of the genes involved in the degradation of bicyclic and polycyclic aromatic compounds by sphingomonads. Thus, in all three strains, three conserved gene clusters were identified, which shared significant nucleotide sequence identities ranging from 60 to 90 %. One of these gene clusters has also recently been identified in the chrysene-degrading strain *Sphingomonas* sp. CHY-1 (Demanèche *et al.*, 2004), and a similar genetic organization also seems to be present in the biphenyl-degrading strain *Sphingomonas yanoikuyae* B1 (Kim & Zylstra, 1999), although unfortunately the relevant gene sequence has not yet been deposited in a public database. Furthermore, some recent PCR experiments, using primers derived from conserved sequences within these gene clusters, suggest that these gene clusters are also present in other aromatic-degrading sphingomonads (although in different arrangements and at different distances from each other) (Basta *et al.*, 2005). The conservation of these gene clusters among different sphingomonads is also reflected by the results of the amino acid sequence comparisons of the proteins of the *S. xenophaga* BN6 aromatic degradation pathway and the sequences deposited at the NCBI database. These alignments always resulted in the identification of genes from other sphingomonads as the most closely related sequences. This clearly demonstrates that, although the relevant genes are encoded on plasmids in *S. aromaticivorans* F199 and *S. xenophaga* BN6 (Romine *et al.*, 1999; Basta *et al.*, 2004), some restrictions seem to occur which prevent a broad dissemination of the relevant genetic information among bacterial strains which do not belong to the sphingomonads. This was also indicated by our failure to transfer *Sphingomonas* plasmids labelled with antibiotic resistance markers to bacterial strains not belonging to the

Table 4. Biochemical characterization of different mutations in the genes putatively encoding NsaA

The strains were cultivated, and induced by addition of salicylate (0.5 mM), as described in Methods. sal, salicylate.

Strain	Inactivated gene	Putative component of NsaA	Enzyme activity			
			NsaA [nmol 2NSA min ⁻¹ (mg protein) ⁻¹]	NsaC (U g ⁻¹)	NsaE (U g ⁻¹)	NsaF (U g ⁻¹)
AKE2	ORF6	α	6.1	0	18	67
AKE2 + sal			52	913	100	83
AKE3	ORF9	β	8.3	0	20	63
AKE3 + sal			61	548	167	85
AKE4	ORF8	Ferredoxin	0	0	44	85
AKE4 + sal			0	753	198	87
AKE5	ORF16	Ferredoxin reductase	0	0	41	106
AKE5 + sal			0	567	194	79
BN6*			5.9	0	31	84
BN6 + sal			50	850	258	114

*The wild-type of *S. xenophaga* BN6 was used as positive control.

Sphingomonadaceae (Basta *et al.*, 2004). Thus, the organization of the genes and the sequence comparisons of the individual genes both suggest some unique lines of evolution for the degradation of aromatics among the sphingomonads.

The previous work with *S. aromaticivorans* F199 and *Sphingomonas* sp. P2 was mainly concerned with the sequencing of the relevant parts of the bacterial genomes, and the putative functions of the identified genes were almost exclusively deduced from their homologies with genes of known function from the databases. In contrast to this situation, for *S. xenophaga* BN6, much more biochemical data were available from previous studies. This, together with the extensive generation of mutants performed in the course of the present study, allowed an unequivocal identification of the *nsaC* and *nsaE* genes participating in the degradation of the naphthalenesulfonates.

Table 5. Rhamnose-inducible expression of the genes ORF5, ORF7, ORF12 and ORF13 using pJOE2702 (Volff *et al.*, 1996)

The enzyme activities of the corresponding cell extracts are summarized. ND, Not determined.

ORF	Plasmid	Enzyme activity (U mg ⁻¹)			
		NsaF	NsaC	NsaD	NsaE
ORF5	pAKE16	2	ND	ND	ND
ORF7	pJKS327	ND	39	ND	ND
ORF12	pAKE18	ND	ND	5.4	ND
ORF13	pAKE17	ND	ND	ND	1

This furnished the final experimental proof that these two genes are indeed not part of the same operon in various sphingomonads.

The phenotypic analysis of the mutants clearly suggested that ORF8 and ORF16 encode the ferredoxin (*nsaA3*) and ferredoxin reductase (*nsaA4*), respectively, of the initial desulfonating NsaA, because these mutants no longer

Fig. 3. SDS-PAGE (12%) of crude extracts of strains JM109(pAKE16) (lanes 2 and 3), JM109(pAKE17) (lanes 4 and 5), JM109(pAKE18) (lanes 6 and 7) and JM109(pJKS325) (lanes 8 and 9) from rhamnose-induced (+) or uninduced (-) cells. Molecular mass standard (S) was obtained from Bio-Rad. The sizes and positions of the marker proteins are indicated to the left and right. The positions of the ORF5-, ORF7-, ORF12- and ORF13-encoded proteins are indicated by arrows.

converted 2NSA. This demonstrated that the genes encoding the ferredoxin and ferredoxin reductase also belong to different gene clusters. In contrast, the mutations in ORF6 and ORF9 (which presumably encode the large and small components, respectively, of a hydroxylating oxygenase) clearly demonstrated that these ORFs either do not encode the initial desulfonating activity or can be replaced by some other (redundant) activity. A similar observation was made when the putative oxygenase gene of a ring-hydroxylating dioxygenase from *S. yanoikuyae* B1 was deleted, but no influence on the ability of the strain to oxidize naphthalene or biphenyl was found. The analysis of the relevant gene sequence from *S. yanoikuyae* B1 demonstrated the presence of at least six oxygenase components of ring-hydroxylating dioxygenases (XylXY, BphA1a2a, BphA1b2b, BphA1c, BphA1d and BphA1e). On the other hand, there is only one copy of the genes specifying the ferredoxin (*bphA3*) and ferredoxin reductase (*bphA4*) (Zylstra & Kim, 1997). Thus, analysis of the relevant genomic regions of *S. aromaticivorans* F199 (and also of *Sphingomonas* sp. P2) suggests that various terminal oxygenases can be synthesized by these strains (Romine *et al.*, 1999; Pinyakong *et al.*, 2003a). These findings indicate that it is a general concept in the metabolism of aromatic compounds by sphingomonads to couple a unique electron transport system (consisting of a ferredoxin and a ferredoxin reductase) with multiple oxygenase components (presumably with different substrate specificities). Thus, it is probable that the genes for the large and small components of the NsaA are located in *S. xenophaga* BN6 at a different position.

The analysed DNA fragment from *S. xenophaga* BN6 encoded, in addition to ORF6 and ORF9, which were analysed by deletion mutagenesis, two more ORFs (ORF10 and ORF11) which demonstrated homology to large components of ring-hydroxylating oxygenases. The sequence comparisons suggested that the proteins encoded by ORF10 and ORF11 are significantly truncated in comparison to classical, functional, large components of ring-hydroxylating oxygenases. Thus, ORF10 may encode only a protein of 132 aa and ORF11 a protein of 285 aa. The sequence alignments suggested that ORF10 and ORF11 are homologous to the N- and C-terminal parts, respectively, of the large component of an oxygenase. This indicated that ORF10 and ORF11 are inactive fragments generated by an amber mutation (Q→stop) from the gene encoding the large component of a ring-hydroxylating aromatic oxygenase. The BLAST searches indicated that both ORF10 and ORF11 show the highest degree of sequence identity with AhdA1c from *Sphingomonas* sp. P2. Furthermore, we found that ORF9 from strain BN6 showed the highest degree of homology to the small component (AhdA2c) of the same oxygenase from *Sphingomonas* sp. P2. This indicated that *S. xenophaga* BN6 only possesses an inactivated derivative of the enzyme from *Sphingomonas* sp. P2. Interestingly, it has recently been shown that AhdA1c and AhdA2c encode a monooxygenase in *Sphingomonas* sp. P2 which converts (substituted) salicylate(s) to (substituted) catechol(s)

(Pinyakong *et al.*, 2003b). Thus, the amber mutation between ORF10 and ORF11 might be responsible for the inability of *S. xenophaga* BN6 to oxidize (substituted) salicylate(s) which are formed from (substituted) naphthalenesulfonate(s) (Nörtemann *et al.*, 1986, 1994). This is of central importance for the ability of this strain to establish stable mixed bacterial cultures with salicylate-degrading bacteria and, presumably, is also responsible for the ability of strain BN6 to convert a wide range of substituted naphthalenesulfonates.

It was previously demonstrated that the genes responsible for the degradation of naphthalenesulfonates are encoded on a 180 kb plasmid (pBN6) in *S. xenophaga* BN6 (Basta *et al.*, 2004). The sequence analysis performed during the present work suggested that the genes encoding the degradation of naphthalenesulfonates are flanked by genes (ORF1, ORF2, ORF3 and ORF17) specifying proteins with homologies to proteins encoded by transposons or insertion elements (Table 4). The presence of such genes suggests that the identified *nsa* genes may be part of functional or non-functional mobile genetic elements. Similar genetic arrangements might also be responsible for the previous observations that the genes encoding the degradative pathway(s) for naphthalene (and biphenyl) are either found on the chromosome or on large plasmids of certain sphingomonads (Kim *et al.*, 1996). The pronounced rearrangements which have been observed after the transfer of plasmids among sphingomonads might be due to these special structural features and homologies (Feng & Ogram, 1997; Basta *et al.*, 2004).

REFERENCES

- Alonso, M. C., Castillo, M. & Barceló, D. (1999). Solid-phase extraction procedure of polar benzene- and naphthalenesulfonates in industrial effluents followed by unequivocal determination with ion-pair chromatography/electrospray-mass spectrometry. *Anal Chem* **71**, 2586–2593.
- Altenbuchner, J. (1993). A new λ RES vector with a built-in Tn1721-encoded excision system. *Gene* **123**, 63–68.
- Altenbuchner, J., Vieill, P. & Pelletier, I. (1992). Positive selection vectors based on palindromic DNA sequences. *Methods Enzymol* **216**, 457–466.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipmann, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Basta, T., Keck, A., Klein, J. & Stolz, A. (2004). Detection and characterization of conjugative degradative plasmids in xenobiotics-degrading *Sphingomonas* strains. *J Bacteriol* **186**, 3862–3872.
- Basta, T., Bürger, S. & Stolz, A. (2005). Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. *Microbiology* **151**, 2025–2037.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brilon, C., Beckmann, W. & Knackmuss, H. J. (1981a). Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. *Appl Environ Microbiol* **42**, 44–55.

- Brilon, C., Beckmann, W., Hellwig, M. & Knackmuss, H. J. (1981b). Enrichment and isolation of naphthalenesulfonic acid-utilizing pseudomonads. *Appl Environ Microbiol* **42**, 39–43.
- Chung, C. T., Niemela, S. L. & Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci U S A* **86**, 2172–2175.
- Demanèche, S., Meyer, C., Micoud, J., Louwagie, M., Willison, J. C. & Jouanneau, Y. (2004). Identification and functional analysis of two aromatic-ring-hydroxylating dioxygenases from a *Sphingomonas* strain that degrades various polycyclic aromatic hydrocarbons. *Appl Environ Microbiol* **70**, 6714–6725.
- Dorn, E., Hellwig, M., Reinecke, W. & Knackmuss, H.-J. (1974). Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. *Arch Microbiol* **99**, 61–70.
- Feng, X. & Ogram, A. (1997). Plasmid mediated mineralization of carbofuran by *Sphingomonas* sp. strain CF06. *Appl Environ Microbiol* **63**, 1332–1337.
- Gish, W. & States, D. J. (1993). Identification of protein coding regions by database similarity search. *Nat Genet* **3**, 266–272.
- Heiss, G., Stolz, A., Kuhm, A. E., Müller, C., Klein, J., Altenbuchner, J. & Knackmuss, H.-J. (1995). Characterization of a 2,3-dihydroxy-biphenyl dioxygenase from the naphthalenesulfonate-degrading bacterium strain BN6. *J Bacteriol* **177**, 5865–5871.
- Heiss, G., Müller, C., Altenbuchner, J. & Stolz, A. (1997). Analysis of a new dimeric extradiol dioxygenase from a naphthalenesulfonate-degrading sphingomonad. *Microbiology* **143**, 1691–1699.
- Hils, M. (1998). *Mutanten der D-Carbamoylase zur Bildung aktiven Enzyms bei Expression des Gens in Escherichia coli und Analyse eines Genclusters für die Enzyme des Hydantoin-Abbaus aus Agrobacterium sp. I-671*. PhD thesis, University of Stuttgart.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.
- Keck, A., Klein, J., Kudlich, M., Stolz, A., Knackmuss, H.-J. & Mattes, R. (1997). Reduction of azo dyes by redox mediators originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas* sp. BN6. *Appl Environ Microbiol* **63**, 3684–3690.
- Keck, A., Rau, J., Reemtsma, T., Mattes, R., Stolz, A. & Klein, J. (2002). Identification of quinoide redox mediators that are formed during the degradation of naphthalene-2-sulfonate by *Sphingomonas xenophaga* BN6. *Appl Environ Microbiol* **68**, 4341–4349.
- Kieser, T. (1984). Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 19–36.
- Kim, E. & Zylstra, G. J. (1999). Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and m-xylene degradation by *Sphingomonas yanoikuyae* B1. *J Ind Microbiol Biotechnol* **23**, 294–302.
- Kim, E., Aversano, P. J., Romine, M. F., Schneider, R. P. & Zylstra, G. J. (1996). Homology between genes for aromatic hydrocarbon degradation in surface and deep-subsurface *Sphingomonas* strains. *Appl Environ Microbiol* **62**, 1467–1470.
- Kuhm, A. E., Stolz, A. & Knackmuss, H.-J. (1991a). Metabolism of naphthalene by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1. *Biodegradation* **2**, 115–120.
- Kuhm, A. E., Stolz, A., Ngai, K.-L. & Knackmuss, H.-J. (1991b). Purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acids. *J Bacteriol* **173**, 3795–3802.
- Kuhm, A. E., Knackmuss, H.-J. & Stolz, A. (1992). Purification and properties of 2'-hydroxybenzalpyruvate aldolase from a bacterium that degrades naphthalenesulfonates. *J Biol Chem* **268**, 9484–9489.
- Kuhm, A. E., Knackmuss, H.-J. & Stolz, A. (1993). 2-Hydroxy-chromene-2-carboxylate isomerase from bacteria that degrade naphthalenesulfonates. *Biodegradation* **4**, 155–162.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Nicholas, K. B. & Nicholas, H. B., Jr (1996). GENEDOC: a tool for editing and annotating multiple sequence alignments. Distributed by the author per anonymous ftp (<http://www.psc.edu/biomed/genedoc>).
- Nörtemann, B., Baumgarten, J., Rast, H. G. & Knackmuss, H.-J. (1986). Bacterial communities degrading amino- and hydroxy-naphthalene-2-sulfonates. *Appl Environ Microbiol* **52**, 1195–1201.
- Nörtemann, B., Kuhm, A. E., Knackmuss, H.-J. & Stolz, A. (1994). Conversion of substituted naphthalenesulfonates by *Pseudomonas* sp. BN6. *Arch Microbiol* **161**, 320–327.
- Ochman, H., Gerber, A. S. & Hartl, D. L. (1988). Genetic applications of inverse polymerase chain reaction. *Genetics* **120**, 621–623.
- Ohe, T. & Watanabe, Y. (1986). Degradation of 2-naphthylamine-1-sulfonic acid by *Pseudomonas* strain TA-1. *Agric Biol Chem* **50**, 1419–1425.
- Ohe, T. & Watanabe, Y. (1988). Microbial degradation of 1,6- and 2,6-naphthalenedisulfonic acid by *Pseudomonas* sp. DS-1. *Agric Biol Chem* **52**, 2409–2414.
- Pinyakong, O., Habe, H. & Omori, T. (2003a). The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). *J Gen Appl Microbiol* **49**, 1–19.
- Pinyakong, O., Habe, H., Yoshida, T., Nojiri, H. & Omori, T. (2003b). Identification of three isofunctional novel salicylate 1-hydroxylases involved in the phenanthrene degradation of *Sphingobium* sp. strain P2. *Biochem Biophys Res Commun* **201**, 350–357.
- Riediker, S., Suter, M. J.-F. & Giger, W. (2000). Benzene- and naphthalenesulfonates in leachates and plumes of landfills. *Wat Res* **34**, 2069–2079.
- Romine, M. F., Stillwell, L. C., Wong, K. K., Thurston, S. J., Sisk, E. C., Sensen, C., Gaasterland, T., Fredrickson, J. K. & Saffer, J. D. (1999). Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. *J Bacteriol* **181**, 1585–1602.
- Ruckstuhl, S. M., Suter, J.-F., Kohler, H.-P. E. & Giger, W. (2002). Leaching and primary biodegradation of sulfonated naphthalenes and their formaldehyde condensates from concrete superplasticizers in groundwater affected by tunnel construction. *Environ Sci Technol* **36**, 3284–3289.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad-host-range mobilization system for in vivo genetic engineering and transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**, 784–791.
- Stolz, A. (1999). Degradation of substituted naphthalenesulfonic acids by *Sphingomonas xenophaga* BN6. *J Ind Microbiol Biotechnol* **23**, 391–399.
- Stolz, A., Schmidt-Maag, C., Denner, E. B. M., Busse, H.-J., Egli, T. & Kämpfer, P. (2000). Description of *Sphingomonas xenophaga* sp. nov. for strains BN6^T and N,N which degrade xenobiotic aromatic compounds. *Int J Syst Bacteriol* **50**, 35–41.
- Thomson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tully, P. S. (1997). Sulfonic acids. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th edn, vol. 23, pp. 194–217. New York: Wiley.

- Vieira, J. & Messing, J. (1982).** The pUC plasmids and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- Volff, J.-N., Eichenseer, C., Viell, P., Piendl, W. & Altenbuchner, J. (1996).** Nucleotide sequence and role in DNA amplification of the direct repeats composing the amplifiable element AUD1 of *Streptomyces lividans* 66. *Mol Microbiol* **21**, 1037–1047.
- Wada, K., Wada, Y., Ishibashi, F., Gojobori, T. & Ikemura, T. (1992).** Codon usage tabulated from the Genbank genetic sequence data. *Nucleic Acids Res* **20**, 2111–2118.
- Wellens, H. (1990).** Zur biologischen Abbaubarkeit mono- und disubstituierter Benzolderivate. *Z Wasser-Abwasser Forsch* **23**, 85–98.
- Wittich, R. M., Rast, H. G. & Knackmuss, H.-J. (1988).** Degradation of naphthalene-2,6- and naphthalene-1,6-disulfonic acid by a *Moraxella* sp. *Appl Environ Microbiol* **54**, 1842–1847.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. *Gene* **33**, 103–119.
- Zürcher, D., Cook, A. M. & Leisinger, T. (1987).** Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. *Appl Environ Microbiol* **53**, 1459–1463.
- Zylstra, G. J. & Kim, E. (1997).** Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. *J Ind Microbiol Biotechnol* **19**, 408–414.