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# Degradation of substituted naphthalenesulfonic acids by *Sphingomonas xenophaga* BN6

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**_Sphingomonas xenophaga_ BN6 was isolated from the river Elbe as a member of a multispecies bacterial culture which mineralized 6-aminonaphthalene-2-sulfonate. Pure cultures of strain BN6 converted a wide range of amino- and hydroxynaphthalene-2-sulfonates via a catabolic pathway similar to that described for the metabolism of naphthalene to salicylate by _Pseudomonas putida_ NAH7 or _Pseudomonas_ sp NCIB 9816. In contrast to the naphthalene-degrading pseudomonads, _S. xenophaga_ BN6 only partially degraded the naphthalenesulfonates and excreted the resulting amino- and hydroxysalicylates in almost stoichiometric amounts. Enzymes that take part in the degradative pathway of the naphthalenesulfonates by strain BN6 were purified, characterized and compared with the isofunctional enzymes from the naphthalene-degrading pseudomonads. According to the enzyme structures and the catalytic constants, no fundamental differences were found between the 1,2-dihydroxynaphthalene dioxygenase or the 2′-hydroxybenzalpyruvate aldolase from strain BN6 and the isofunctional enzymes from the naphthalene-degrading pseudomonads. The limited available sequence information about the enzymes from strain BN6 suggests that they show about 40–60% sequence identity to the isofunctional enzymes from the pseudomonads. In addition to the gene for the 1,2-dihydroxynaphthalene dioxygenase, the genes for two other extradiol dioxygenases were cloned and sequenced from strain BN6 and the corresponding gene products were studied. _S. xenophaga_ BN6 has also been used as a model organism to study the mechanism of the non-specific reduction of azo dyes under anaerobic conditions and to establish combined anaerobic/aerobic treatment systems for the degradation of sulfonated azo dyes. Furthermore, the degradation of substituted naphthalenesulfonates by mixed cultures containing strain BN6 was studied in continuous cultures and was described by mathematical models.**

**Keywords:** *Sphingomonas*; biodegradation; dioxygenases; naphthalenesulfonic acids

## Introduction

Substituted naphthalenesulfonates are important products of the chemical industry and are utilized as dispersants and intermediates for the production of azo dyes, polymers and other chemicals [14,38,88]. They are highly water soluble and have been identified repeatedly as recalcitrant compounds in the environment [1,48,55,56,100,101]. Currently, several bacterial cultures are known to degrade (substituted) naphthalenesulfonates. *Comamonas testosteroni* A3 was obtained from a naphthalene-degrading consortium after continuous adaptation to degrade naphthalene-2-sulfonate. This strain grew on naphthalene-2-sulfonate, but not on substituted naphthalenesulfonates, as the sole source of carbon and energy [9,10]. Subsequently, enrichments were performed with 6-aminonaphthalene-2-sulfonate, 2-aminonaphthalene-1-sulfonate and naphthalene-2,6-disulfonate. These enrichments resulted in the isolation of taxonomically different Gram-negative bacteria which were originally classified as *Pseudomonas* sp or *Moraxella* sp [71–74,96]. One of these isolates, strain BN6, was intensively studied in our laboratory because it oxidized a wide range of substituted naphthalenesulfonates. Although it was originally identified as *Pseudomonas* sp, it was recently shown that strain BN6 is a member of the genus *Sphingomonas* [66,91].

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### *Enrichment of strain BN6*

Starting with an inoculum from the water of the river Elbe near Hamburg, Germany, enrichment with 6-aminonaphthalene-2-sulfonate (6A2NS) yielded a mixed bacterial culture which utilized 6A2NS as sole source of carbon, energy, nitrogen and sulfur. From this mixed culture, which consisted of at least ten different bacterial species, strain BN6 was isolated which was responsible for the conversion of 6A2NS. Surprisingly, the strain only converted 6A2NS to 5-aminosalicylate, which was excreted in almost stoichiometric amounts and was utilized by other members of the coculture [69].

### *Taxonomic classification of strain BN6*

Strain BN6 was originally identified as a *Pseudomonas* sp [69] but it was soon recognized that, according to the polyamine pattern and the presence of ubiquinone Q10, the strain belonged to the $\alpha$-subgroup of the *Proteobacteria* and thus was not an authentic *Pseudomonas* [11]. Sequencing the 16S rDNA clearly demonstrated that the strain is a member of the genus *Sphingomonas* [66]. A comparison with sequences from type strains demonstrated that the 16S rDNA sequence from strain BN6 showed the highest degree of identity (96%) from all type-strains with *Sphingomonas yanoikuyae*. Strain BN6 also differed from other known *Sphingomonas* species according to various physiological characteristics and the pattern of the polar lipids. It was therefore suggested that strain BN6, together with another bacterial isolate (strain N,N), which was isolated in Switzerland with N,N-dimethylaniline as sole source of carbon

and energy [84], belongs to a new species which was recently described as *S. xenophaga* [91]. Interestingly, a further 16S rDNA sequence in the data bank showed a high degree of similarity (99%) with the 16S rDNA sequence of strain BN6. This sequence belongs to an isolate (strain C7) which was isolated in the USA for its ability to decolorize aerobically sulfonated azo compounds [29]. This suggests that the ability to metabolize sulfonated aromatics may be found more widely spread within the genus *Sphingomonas*. Unfortunately, strain C7 seems to be no longer available, and therefore can not be studied in comparison to strain BN6.

There are in addition to *Sphingomonas* sp BN6 other bacterial isolates which are also able to grow with (substituted) naphthalenesulfonates [9,10,72–74,96]. Most of these strains have not been thoroughly investigated with respect to their taxonomic position. Only the naphthalene-2-sulfonate-degrading strain *Comamonas (Pseudomonas) testosteroni* A3 has been identified with sufficient accuracy as a *Comamonas* sp [9,10,12]. Thus it can be concluded that the ability to convert (substituted) naphthalenesulfonates is not a unique ability of members of the genus *Sphingomonas*.

### *The metabolic pathway for degradation of naphthalenesulfonates by strain BN6*

Resting cells of strain BN6 converted naphthalene-2-sulfonate (2NS) to salicylate (Figure 1), 5-hydroxynaphthalene-2-sulfonate to 6-hydroxysalicylate, 6-hydroxynaphthalene-2-sulfonate to 5-hydroxysalicylate (gentisate) and 7-amino- and 7-hydroxynaphthalene-2-sulfonate to the corresponding 3-substituted salicylates. The relative activities with different substituted naphthalenesulfonates were almost constant after growth in the presence of different substrates and inducers. It was therefore concluded that the initial attack on the sulfonated naphthalenes was catalysed by a highly non-specific enzyme, because a series of disubstituted naphthalene-2-sulfonates and naphthalene-1-sulfonate were also oxidized [70,71].

From the substitution pattern of the salicylates produced, it was concluded that strain BN6 initially attacked the (substituted) napthalene-2-sulfonates by a highly regioselective 1,2-dioxygenase, which resulted in a desulfonation of the naphthalenesulfonates and the formation of the corresponding (substituted) 1,2-dihydroxynaphthalene(s) (1,2-DHN). The intermediate formation of 1,2-DHN had already been described for the degradation of naphthalene by authentic *Pseudomonas* species [16]. It was therefore proposed that further metabolism of the (substituted) 1,2-DHN(s) by strain BN6 was analogous to the known degradative pathway of naphthalene [16,23,99]. Furthermore, different enzymes, which had been previously identified in the naphthalene degradative pathway [5,16,21,23]. (1,2-dihydroxynaphthalene dioxygenase, 2-hydroxychromene-2-carboxylate isomerase, 2′-hydroxybenzalpyruvate aldolase and salicylaldehyde dehydrogenase) (Figure 1) were also found in cell extracts or purified enzyme fractions from strain BN6 [52,54]. This suggested that the degradative pathways for naphthalenesulfonates by strain BN6 and the naphthalene pathway found in different *Pseudomonas* species converge with 1,2-DHN as the first common metabolite (Figure 1). In contrast to most naphthalene-degrading bacteria, strain BN6 utilized only the pyruvate which is released by action of the 2′-hydroxybenzalpyruvate aldolase and excreted the salicylates formed from the naphthalenesulfonates. A similar situation has been described for the degradation of chlorinated dibenzofurans by a bacterial consortium consisting of *Sphingomonas* sp RW16, *Pseudomonas putida* RW10, and some additional strains. In this coculture, the *Sphingomonas* strain converted the chlorinated dibenzofurans to chlorosalicylates which were subsequently mineralized by *Pseudomonas putida* RW10 [95]. Another *Sphingomonas* sp (strain BPSI-3), which was isolated from a biphenyl-degrading mixed culture, formed a co-culture with an *Alcaligenes* sp which was more efficient for the degradation of biphenyl [17].

In contrast to all other naphthalenesulfonates which were oxidized by strain BN6, 4-amino-, 4-hydroxy- and 5-aminonaphthalene-2-sulfonate were not converted to the corresponding salicylates (Figure 1). The turnover of 4-amino- and 4-hydroxynaphthalene-2-sulfonate resulted in accumulation of the corresponding naphthoquinones in the culture medium. Thus, degradation of 4-amino- and 4-hydroxy-naphthalene-2-sulfonates was restricted by rapid autoxidation of the substituted 1,2-dihydroxynaphthalenes formed as metabolites. The turnover of 5-aminonaphthalene-2-sulfonate resulted in the accumulation of 5-hydroxyquinoline-2-carboxylate, which was presumably formed by an intramolecular condensation reaction of the ring-fission product of 5-amino-1,2-dihydroxynaphthalene (Figure 1) [70,71].

### *1,2-Dihydroxynaphthalene dioxygenase*

The 1,2-dihydroxynaphthalene dioxygenase (DHNDO) of strain BN6 was purified to homogeneity, the amino terminal amino acid sequence was determined, and the enzyme was biochemically characterized and compared with the isofunctional enzymes from the naphthalene pathway of different *Pseudomonas* strains [54]. The enzyme was functionally and structurally a classical ferrous iron-requiring extradiol dioxygenase similar to those found in various other bacterial genera, including *Pseudomonas*, *Rhodococcus*, *Arthrobacter* and *Bacillus* [24]. Furthermore, it was shown that the enzyme was apparently not specifically adapted to the degradation of substituted 1,2-DHNs, because similar conversion rates for substituted 1,2-DHNs were also found with the DHNDOs from naphthalene-degrading pseudomonads. Only the aminoterminal amino acid sequence suggested some ‘*Sphingomonas*-specific traits’. The highest degree of homology was not found with the isofunctional DHNDO from *Pseudomonas* sp PpG7 (encoded on plasmid NAH7–14 of 29 amino acids identical), but with a suspected 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBPDO) from ‘*Pseudomonas paucimobilis*’ Q1 (22 of 29 amino acids identical) [54]. It was recently shown that, according to its 16S rDNA sequence, strain Q1 is a *Sphingomonas* which is closely related to *S. yanoikuyae* [43]. The 2,3-DHBPDO from strain Q1 also behaved according to its substrate specificity in a similar manner to the DHNDO from strain BN6 [53]. The gene for the DHNDO from strain BN6 was cloned and sequenced; the sequence was deposited in the data base of the National Center for Biotechnology Information (Bethesda, MD, USA; NCBI accession number U65001) [15]. A recent

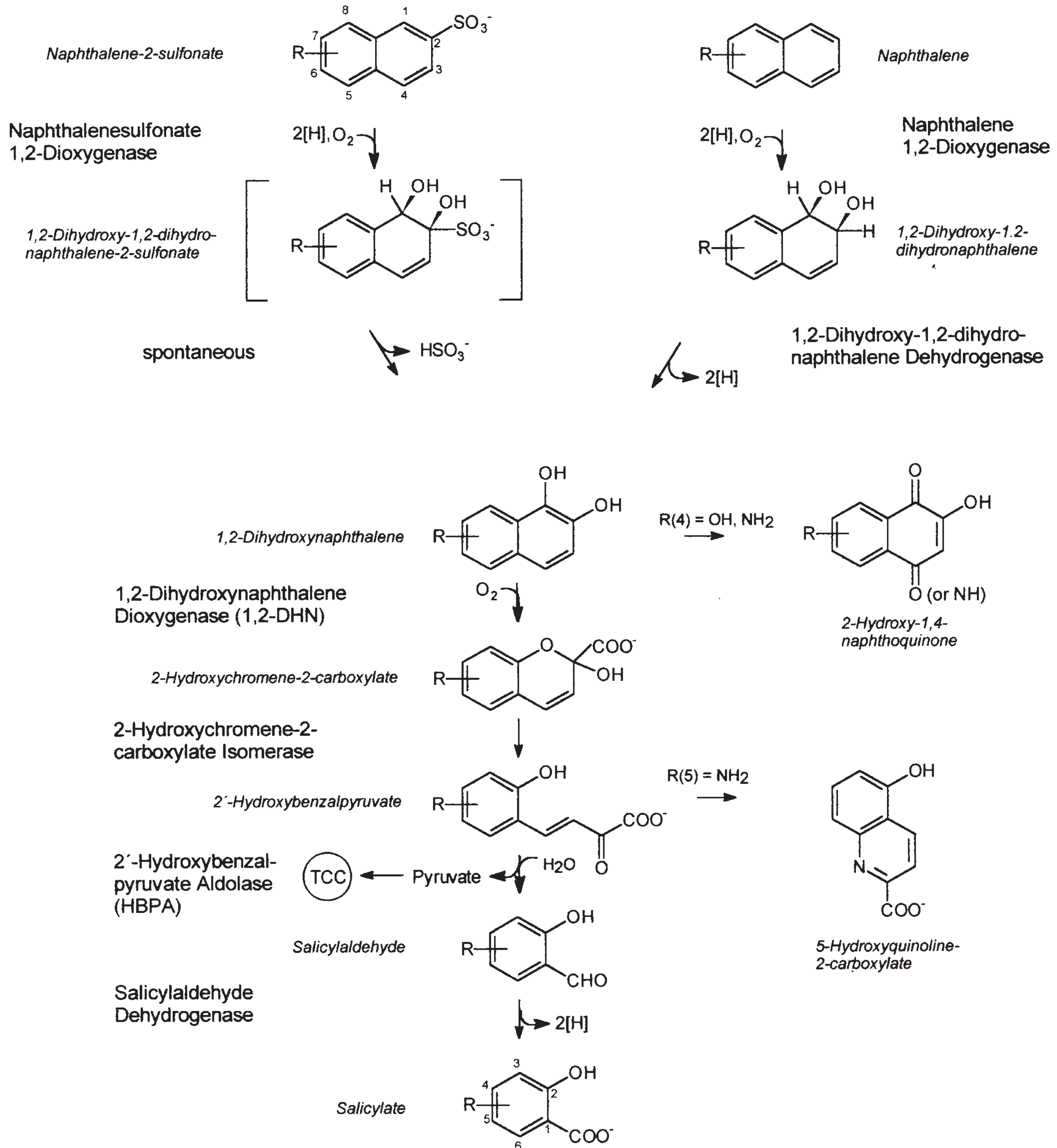


**Figure 1** Degradation of naphthalene by *Pseudomonas* sp NCIB 9816 and *Pseudomonas putida* NAH7 [5,16,23,99] and of substituted naphthalene-2-sulfonates by *Sphingomonas xenophaga* BN6 [51,52,54,69–71]. TCC, tricarboxylic acid cycle.

sequence comparison confirmed that the deduced amino acid sequence of the DHNDO from strain BN6 has the highest degree of similarity (86% identity) with the 2,3-DHBPDO from strain *Sphingomonas* sp Q1 (and also from *S. yanoikuyae* B1) among all sequences deposited to date in the data base. In contrast, only 59% identity was found between the deduced complete amino acid sequences of the DHNDOs from strain BN6 and *Pseudomonas putida* PpG7.

### *2′-Hydroxybenzalpyruvate aldolase*

The 2′-hydroxybenzalpyruvate (hydratase-)aldolase (HBPA) from strain BN6 converted 2′-hydroxybenzalpy-

ruvate to salicylaldehyde (2-hydroxybenzaldehyde) and pyruvate (Figure 1). The enzyme also converted 2′,4′- and 2′,6′-dihydroxybenzalpyruvate to pyruvate plus 2,4- and 2,6-dihydroxybenzaldehyde, respectively. The HBPAs from naphthalene-degrading authentic pseudomonads demonstrated a similar substrate specifity as the HBPA from strain BN6 [51]. Since the aminoterminal amino acid sequence of the HBPA (26 aa) was reported [51], the genes for (putative) HBPAs have been sequenced from *Pseudomonas putida* NAH7, three other *Pseudomonas* spp and from plasmid pNL1 from *Sphingomonas aromaticivorans* F199 [7,8,18,21,80]. A sequence comparison demonstrated that the relevant deduced amino acid sequences for the aminoterminal regions of the HBPAs, encoded by the *Pseudomonas* plasmids NAH7 and NPL1 and by strain *Pseudomonas* sp C18, are almost identical (24 of 26 aa conserved). This group of sequences showed a lower degree of homology (20 of 26 aa conserved) with a sequence obtained from *Pseudomonas stutzeri* AN10. The sequence obtained for the HBPA from strain BN6 differed significantly from the sequences determined for the *Pseudomonas* strains. In each case only 11 of 26 aa were conserved between the sequence of strain BN6 and those from the pseudomonads. The highest degree of homology (65% identity) was found between the sequence of the HBPA from strain BN6 and an open reading frame from plasmid pNL1 from *Sphingomonas aromaticivorans* F199.

### *Three different extradiol dioxygenases are encoded by strain BN6*

During our attempts to clone the gene for the DHNDO, the genes for two other extradiol dioxygenases were identified in a gene bank of strain BN6. The clones were identified by their ability to convert the substrate 2,3-dihydroxybiphenyl (2,3-DHBP) to the yellow ring-fission product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA). In addition to converting 2,3-DHBP, both enzymes converted various *ortho*-dihydroxybenzenes (eg catechol, 3-methyl-, 3-isopropyl-, and 4-chlorocatechol), but with lower activities. Both enzymes were therefore designated as 2,3-dihydroxybiphenyl-1,2-dioxygenases (DHBPDO) [36,37]. One of the extradiol dioxygenases (BphC1-BN6) belonged to a group of extraordinarily small dioxygenases which had been previously found only in *Rhodococcus globerulus* P6 and *Rhodococcus erythropolis* TA421 [3,57]. The corresponding gene was expressed in a T7 expression vector and BphC1-BN6 was purified from a recombinant *E. coli* strain. The protein was a dimer and oxidized, in addition to 2,3-DHBP, several other substituted catechols [37]. In contrast to almost all other extradiol dioxygenases, BphC1-BN6 converted 3-chlorocatechol by a newly discovered distal cleavage mechanism to 3-chloro-2-hydroxymuconic semialdehyde [78]. It was shown that the enzyme was rapidly inactivated not only during the oxidation of 3-chlorocatechol, but also during the turnover of other substituted catechols. This inactivation was apparently related to the loss of the weakly bound ferrous iron, which is the cofactor in the catalytic center [79].

A comparison of the deduced amino acid sequences of the three extradiol dioxygenases (NahC-BN6, BphC1-BN6, BphC2-BN6) with each other and with a representative collection of other extradiol dioxygenases suggested that they were only distantly related to each other and that in contrast to NahC-BN6 (see above), BphC1-BN6 and BphC2-BN6 showed the closest relationships to extradiol dioxygenases from other bacterial genera (Figure 2). This suggested that the extradiol dioxygenases from the genus *Sphingomonas* belong to the main groups of extradiol dioxygenases which are found in a wide range of Gram-negative and Gram-positive bacteria.

### *Plasmid transfer from strain BN6 to other bacterial genera and transfer of other plasmids to strain BN6*

Preliminary results have been obtained which suggest that the pathway for the degradation of the naphthalenesulfonates is encoded in strain BN6 on a 180-kb plasmid which may be visualized by pulse-field gel electrophoresis (J Klein, University of Stuttgart, personal communication). Plasmids of similar sizes have also been proposed to be involved in the degradation of toluene and naphthalene by *S. aromaticivorans* F199 [89] and fluoranthene by *Sphingomonas* sp strain EPA505 [6]. Attempts to transfer the ability to degrade naphthalenesulfonates by classical conjugation protocols from strain BN6 to different *Pseudomonas* strains have failed. This may indicate that *Sphingomonas* plasmids either do not establish in authentic pseudomonads or that the relevant genes are not expressed in this genetic background. Recently, we obtained some evidence that a transfer of the ability to degrade naphthalenesulfonates from strain BN6 to other bacteria is possible. After some years of cultivation of the 6A2NS-degrading mixed culture which contained *Sphingomonas xenophaga* BN6 and some accompanying bacteria, strain BN12, which completely degraded 6A2NS, was isolated from this culture. It was previously suggested that this strain evolved from a 5-aminosalicylate-degrading bacterium (strain BN11) which gained the ability to convert 6A2NS to 5-aminosalicylate from strain BN6 [68]. Recently, strain BN12 was taxonomically characterized and shown to be a member of the family *Rhizobiaceae* within the $\alpha$-subgroup of the *Proteobacteria* and identified as *Pseudoaminobacter salicylatoxidans* [42]. Although we were not able to repeat the conjugative transfer of the ability to degrade naphthalenesulfonates from strain BN6 to strain BN11 in the laboratory under controlled conditions, evidence for the transfer of the genes from strain BN6 into the new genetic background was obtained by PCR-amplification and sequencing of the DHNDO gene from strain BN12 (C Müller, University of Stuttgart, personal communication).

It was a surprising observation that strain BN6 did not convert salicylate or any of the substituted salicylates which were formed from (substituted) naphthalenesulfonate(s). It was therefore attempted to transfer the ability to degrade salicylate by conjugation from other bacteria to strain BN6 using plasmids NAH7, pWW60, SAL, pDTG11, pDTG13, pBS211 or pBS244 [83,99]. However, no transconjugants of strain BN6 were obtained which could mineralize salicylate or naphthalene-2-sulfonate. Surprisingly, the desired hybrid strains could readily be constructed if the genes for salicylate degradation were transferred into cloning vectors derived from the plasmid incompatibility groups IncP-1 (using pRK415) or IncP-4 (using pKT230) [81,82].

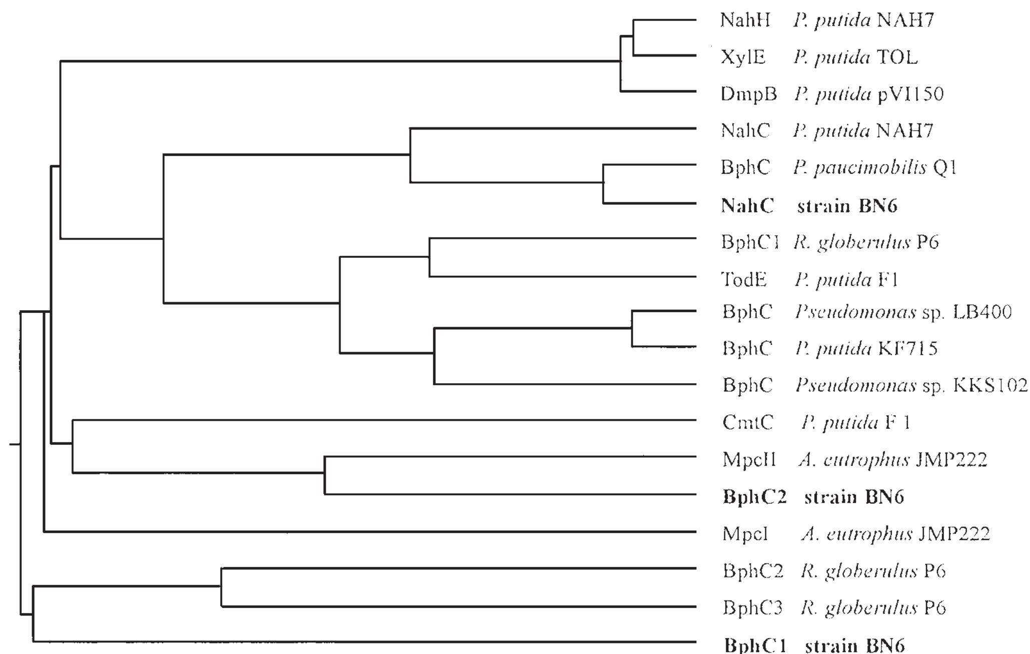


**Figure 2** Dendrogram resulting from pairwise alignments of amino acid sequences from various extradiol dioxgenases. Catechol 2,3-dioxygenases (NahH) from *P. putida* (NAH7) [32], *Pseudomonas putida* mt-2 (TOL) (XylE) [64], and *Pseudomonas* sp CF600 (pVI150) (DmpB) [87], 1,2-dihydroxynaphthalene dioxygenase (NahC) from *Pseudomonas putida* (NAH7) [30], 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) from '*Pseudomonas paucimobilis*' (= *Spingomonas* sp) Q1 [92], 1,2-dihydroxynaphthalene dioxygenase (NahC) from strain BN6 [15], 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC1) from *R. globerulus* P6 [3], catechol 2,3-dioxygenase (TodE) from *Pseudomonas putida* F1 [102], 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphC) from *Pseudomonas* sp LB 400 [39], *P. putida* KF715 [35], and *Pseudomonas* sp KKS102 [47], 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase (CmtC) from *Pseudomonas putida* F1 [22], catechol 2,3-dioxygenase (MpcII) from *Alcaligenes eutrophus* JMP222 [41], 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC2) from strain BN6 [36], catechol 2,3-dioxygenase (MpcI) from *Alcaligenes eutrophus* JMP222 [40], 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphC2, BphC3) from *R. globerulus* P6 [3], and strain BN6 (BphC1) [37].

We were also unable to transfer the ability to degrade toluate or 2,4-dichlorophenoxyacetate (2,4-D) by conjugation from *Pseudomonas putida* mt-2 or *Ralstonia eutropha* JMP134 to strain BN6 [92]. This strongly suggested that the typical degradative plasmids of other Gram-negative bacteria might not be transferred to *Sphingomonas xenophaga* BN6 but that the relevant genes from these plasmids can be expressed in strain BN6.

### *Utilization of strain BN6 to establish a process for the anaerobic/aerobic treatment of sulfonated azo dyes*

The 6A2NS-degrading mixed culture, containing strain BN6, was also used to study the possibility of an anaerobic/aerobic treatment of sulfonated azo dyes, because under anaerobic conditions cells of strain BN6 reduced sulfonated azo dyes to the corresponding amines [34,49]. This ability had been described for a number of other bacteria from different taxonomic groups [13,94]. A complete mineralization of sulfonated azo compounds could be achieved after the anaerobic cleavage of the azo bond because the mixed culture with strain BN6 mineralized a wide range of substituted naphthalenesulfonates after reaeration [34]. Recently, it was shown that the ability of strain BN6 to reduce azo dyes non-specifically under anaerobic conditions was enhanced by metabolites formed during aerobic metabolism of naphthalenesulfonates, which acted as redox mediators and shuttled redox equivalents from the cells to the extracellular azo dyes [44,50].

### *Degradation of naphthalenesulfonates in continuous culture by mixed bacterial cultures containing Sphingomonas xenophaga BN6*

The degradation of 6A2NS by the mixed culture containing strain BN6 was also investigated in continuous chemostat culture and steady-state data were experimentally determined for the mixed culture and calculated for strain BN6 [19,20]. It was shown that the 6A2NS-degrading mixed culture could be continuously operated with 6A2NS for more than 1.5 years in an airlift loop-reactor when the cells were immobilized on sand and that this system was rather stable to changes in temperature (12–35°C), pH-shock loadings and long-term oxygen defaults [19]. The kinetic constants for conversion of 6A2NS to 5-aminosalicylate were determined and a structured model regarding interspecies transfer of 5-aminosalicylate was developed. By these methods a low yield coefficient (0.044 $g_{BM}$ $mmol^{-1}$ 6A2NS) and a high maintenance coefficient (0.82 $mmol_{6A2NS}$ $h^{-1}$ $g_{BM}^{-1}$) were calculated. It was suggested that these values were due to the fact that strain BN6 only utilizes one mol of pyruvate per mol of 6A2NS converted [20].

### *Are there any general adaptation strategies which allow Sphingomonas strains to degrade a wide range of xenobiotic compounds?*

The genus *Sphingomonas* is attracting increasing interest because various xenobiotic-degrading bacterial isolates belong to this group of organisms. In addition to napthalenesulfonates, members of this genus are able to

degrade compounds such as biphenyl, naphthalene or pyrene [4,45,98], diphenylether and dibenzo-*p*-dioxin [61,85], herbicides and pesticides [26,62,65], polyethylenglycols [93], and chlorinated phenols [43,66,67].

Comparative studies between enzymes of the naphthalene pathway from different *Pseudomonas* sp and the isofunctional enzymes from the naphthalenesulfonic acid pathway from *Sphingomonas* sp BN6, as well as comparison of the extradiol dioxygenases from strain BN6 with those of different sources, suggest that there are no fundamental differences in the degradative pathways and the enzymes involved between the genus *Sphingomonas* and other genera of Gram-negative bacteria. Similar results have also been obtained for the extradiol dioxygenases which are involved in the degradation of compounds such as biphenyl, naphthalene and toluene by *Sphingomonas yanoikuyae* strain B1 [46].

Generally the degree of homology between the genes for degradative enzymes from sphingomonads and authentic pseudomonads seems to be lower than that within the genus *Sphingomonas* or the genus *Pseudomonas*. This becomes evident when hybridization studies are performed, even under conditions of low stringency. Thus no signal was obtained when a labeled probe of plasmid NAH7 isolated from *Pseudomonas putida* was hybridized with the total DNA from strain BN6 [90]. Furthermore, it has been found that among a collection of 2,4-dichlorophenoxyacetate (2,4-D)-degrading isolates, the *Sphingomonas* strains did not hybridize with different probes for genes of the 2,4-D-degradative pathways prepared from *Ralstonia eutropha* pJP4 [27]. Similarly, no signal was observed with a probe prepared from the gene of the 2,3-DHBPDO from *Sphingomonas* sp Q1 (formerly *Pseudomonas paucimobilis* Q1) in a hybridization experiment with DNAs prepared from a series of other biphenyl-degrading bacteria [28].

The hybridization studies and the limited comparative sequence information currently available suggest that there are some restrictions which limit the transfer of DNA from sphingomonads to other *Proteobacteria* and likewise the transfer of DNA from other bacteria to sphingomonads. One of the reasons for the differences between the genes encoding degradative abilities from sphingomonads and 'typical' xenobiotic-degrading isolates from the $\beta$- or $\gamma$-subgroup of the *Proteobacteria* (eg *Alcaligenes*, *Ralstonia* or *Pseudomonas*) is probably the host-range of the degradative plasmids present in these strains. As mentioned above we have repeatedly tried to transfer various degradative plasmids to strain BN6, but in no case were we able to establish the corresponding phenotype in strain BN6. Furthermore, we were also unable to transfer the ability to degrade naphthalenesulfonates from strain BN6 to other bacteria. Similarly, McGowan *et al* [59] were not able to transfer the ability to degrade 2,4-D from different *Sphingomonas* strains to *Burkholderia cepacia*. In contrast to these degradative plasmids, antibiotic resistance plasmids such as RP4 or derivatives thereof are transferred with high frequencies into strain BN6 [90] and may be able to cotransfer other genes with low frequencies to and out of strain BN6 and other sphingomonads.

Currently, the main obvious difference between *Sphingomonas* sp and other Gram-negative bacteria is the different organization of degradative genes. Previous studies with authentic pseudomonads suggested that the genes which encode catabolic enzymes are often organized in operons and are coordinately regulated. Classical examples for this are the *meta*-cleavage pathways from the TOL- or NAH-plasmids, the *ortho*-cleavage pathways for chlorocatechols from various plasmids or the chromosomally encoded $\beta$-ketoadipate pathway [25,31,33,99]. There is growing evidence that the genes for catabolic pathways in *Sphingomonas* strains are often organized so that they are physically separated from each other or are at least not organized in coordinately regulated operons. This appears to be the case for the genes involved in the degradation of $\gamma$-hexachlorocylohexane (lindane) by *S. paucimobilis* UT26 [60,63], protocatechuate by *S. paucimobilis* SYK-6 [58], naphthalene, biphenyl, and toluene by *S. yanoikuyae* B1 and *S. aromaticivorans* F199 [80,103], dibenzo-*p*-dioxin by *Sphingomonas* sp RW1 [2], and was recently observed for the genes involved in the metabolism of naphthalenesulfonic acids in strain BN6 (A Keck and J Klein, University of Stuttgart, personal communication).

In contrast to the examples mentioned above, there are also some 'traditional degradative operons' found in *Sphingomonas* spp. For example, the gene for BphC2-BN6 is apparently part of an operon, which also includes genes encoding a hydrolase and an isomerase or decarboxylase and which is accompanied by a regulatory gene, which is transcribed in the opposite direction [36; unpublished results]. Furthermore, the carbazole degradative pathway from *Sphingomonas* sp CB3 resembled, according to its gene arrangement, the biphenyl loci of different pseudomonads [86]. It could be hypothesized that in these cases, the relevant genes have only recently been transferred from other bacteria to the sphingomonads.

There is also some evidence that structures of the promoters of *Sphingomonas* genes are considerably different compared to those of other Gram-negative bacteria. Orser and coworkers analysed the start of transcription for three genes which encode proteins that are involved in the degradation of pentachlorophenol by *Sphingomonas chlorophenolica* (formerly *Flavobacterium* sp) ATCC 39723. They identified the transcriptional starting points at positions −67 to −91 in relation to the translational start sites and were usually not able to identify 'typical' promoter regions upstream of the transcriptional start sites [75–77,97]. This may explain why *Sphingomonas* genes often seem to be rarely or insignificantly expressed from their own promoters after their transfer to other genera.

Thus, the main difference between authentic pseudomonads, as the 'classical' xenobiotic-degrading *Proteobacteria*, and the sphingomonads identified today is the different organization of the participating genes. Presumably, these differences are also reflected in different regulatory mechanisms. A tempting hypothesis is that the organization of the genes in small units perhaps allows the *Sphingomonas* strains to reorganize the genes which together form a degradative pathway more rapidly than other bacterial strains and that therefore the sphingomonads are especially suitable for adaptation to new xenobiotic compounds in the environment.

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