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Purification and Characterization of a 1,2-Dihydroxynaphthalene Dioxygenase from a Bacterium That Degrades Naphthalenesulfonic Acids

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1,2-Dihydroxynaphthalene dioxygenase was purified to homogeneity from a bacterium that degrades naphthalenesulfonic acids (strain BN6). The enzyme requires Fe^{2+} for maximal activity and consists of eight identical subunits with a molecular weight of about 33,000. Analysis of the NH_2-terminal amino acid sequence revealed a high degree of homology (22 of 29 amino acids) with the NH_2-terminal amino acid sequence of 2,3-dihydroxybiphenyl dioxygenase from strain *Pseudomonas paucimobilis* Q1. 1,2-Dihydroxynaphthalene dioxygenase from strain BN6 shows a wide substrate specificity and also cleaves 5-, 6-, and 7-hydroxy-1,2-dihydroxynaphthalene, 2,3- and 3,4-dihydroxybiphenyl, catechol, and 3-methyl- and 4-methylcatechol. Similar activities against the hydroxy-1,2-dihydroxynaphthalenes were also found in cell extracts from naphthalene-degrading bacteria.

Amino- and hydroxynaphthalenesulfonic acids (ANS and HNS, respectively) serve as building blocks for the large-scale synthesis of azo dyes. Since arylsulfonates are very rare among natural compounds (21), naphthalenesulfonic acids are referred to as xenobiotics. Arylsulfonates are major pollutants of the environment, and the contamination of the Rhine River by sulfur-organic compounds is largely due to this class of compounds (25). Nevertheless, bacteria which degrade naphthalenesulfonic acids have repeatedly been isolated (4, 5, 29–31, 41, 43). Brilon et al. (4, 5) selected pseudomonads which degraded naphthalene-2-sulfonic acid (2NS) and naphthalene-1-sulfonic acid (1NS). The initial reaction in the degradation of 1NS and 2NS is catalyzed by a 1,2-dioxygenase which weakens the carbon-sulfur bond. Spontaneous elimination of sulfite and rearomatization of the dihydrodiol intermediate generate 1,2-dihydroxynaphthalene (1,2-DHN). This compound is further metabolized via the normal degradative pathway of naphthalene (see Fig. 1) (4, 5, 11).

Recently, the enrichment of a mixed bacterial community growing with 6-aminonaphthalene-2-sulfonic acid (6A2NS) has been reported by this laboratory (29). The complete mineralization of 6A2NS was basically described as mutual interaction of two bacterial strains. Strain BN6 effected the initial conversion of 6A2NS to 5-aminosalicylic acid in quantitative amounts, whereas the partner strain BN9 could grow with 5-aminosalicylic acid but not with 6A2NS or naphthalene. Strain BN6 converted a wide range of isomeric ANS and HNS and excreted stoichiometric amounts of the corresponding amino- or hydroxysalicylic acids (29).

Funnelling of substituted naphthalenesulfonic acids into the naphthalene pathway requires a unique desulfonating 1,2-dioxygenase. However, it is still an open question whether further degradation of amino- and hydroxysubstituted naphthalenes requires a special set of enzymes or whether ordinary enzymes of the naphthalene pathway can take on this function. Purification of the 1,2-DHN dioxygenase from strain BN6 and comparison with the corresponding enzymes from bacteria degrading naphthalene should answer this question.

MATERIALS AND METHODS

Bacterial strains. The isolation and characterization of strains BN6 and BN9 have been reported by Nörtemann et al. (29). Strain BN6 was originally assumed to belong to the genus *Pseudomonas*. Recent investigations showed "*Chromobacterium folium*" NCTC 10591 to be the next phylogenetic neighbor of strain BN6 (12). *Pseudomonas* sp. strain NCIB 9816 and *Pseudomonas* sp. strain NCIB 10535 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Great Britain. *Pseudomonas putida* KT2442 NAH7 was kindly provided by S. Harayama, Geneva, Switzerland.

Media. Mineral medium was prepared as described by Dorn et al. (13). Strain BN6 was grown in 0.7 liter of a mineral medium containing 10 mM glucose. To obtain cells induced for the catabolism of naphthalenesulfonic acids, 0.5 mM 2NS was added during early exponential growth and cells were harvested about 12 h later (A_{546} = 0.8).

For growth of strains, *Pseudomonas* sp. strain NCIB 9816, *Pseudomonas* sp. strain NCIB 10535, and *P. putida* KT2442 NAH7 on naphthalene culture media were prepared as described by Shamsuzzaman and Barnsley (36).

Preparation of cell extracts. Cell suspensions in 50 mM Na-K phosphate buffer, pH 7.3, were disrupted by using a French press (Aminco, Silver Spring, Md.) at 80 MPa. Cell debris was removed by centrifugation at 100,000 × *g* for 30 min at 4°C.

Protein was determined by the method of Bradford (3), with bovine serum albumin as standard.

Enzyme assays. One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min.

Naphthalene dioxygenase was assayed spectrophotometrically with whole cells as described by Shamsuzzaman and Barnsley (36). Activity of 2NS-dioxygenase was determined

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with whole cells. Strain BN6 was harvested in the late exponential growth phase, washed, and resuspended in Na-K phosphate buffer (50 mM; pH 7.3) to an optical density at A_{546} of about 5. The cell suspension (10 ml) was incubated at 30°C in 100-ml Erlenmeyer flasks, 2NS (1 mM) was added, and the suspension was vigorously shaken. Every 10 min, a sample was taken and the cells were removed by centrifugation; the concentration of 2NS was determined by high-pressure liquid chromatography (HPLC). 1,2-Dihydroxy-1,2-dihydronaphthalene dehydrogenase was measured by the method of Patel and Gibson (33). The activity of 1,2-DHN dioxygenase was measured by a modification of the spectrophotometric assay described by Shamsuzzaman and Barnsley (37). The reaction mixture contained (in 1 ml) 50 μmol of acetic acid-NaOH buffer (pH 5.5) and 1 to 50 μg of protein. The reaction was started by the addition of 0.4 μmol of 1,2-DHN in 10 μl of tetrahydrofuran.

In aqueous solution, 1,2-DHN is subject to rapid autoxidation, yielding 1,2-naphthoquinone (1,2-NQ). This reaction is accompanied by a characteristic change in the absorption spectrum (11, 33). In acetic acid-NaOH buffer, the absorption maxima of 1,2-DHN (λ_{max} = 228, 286, and 332 nm) are replaced by the maxima of 1,2-NQ (λ_{max} = 249, 338, and 408 nm). These changes of the absorption spectrum are characterized by an isosbestic point at 331 nm (see Fig. 2). The cell extract of strain BN6 showed no activity with 1,2-NQ under the test conditions described above. Therefore, 1,2-DHN dioxygenase was routinely determined at λ = 331 nm. From the extinction difference between 1,2-DHN (or 1,2-NQ) and the reaction product 2-hydroxychromene-2-carboxylic acid, a molar reaction coefficient (ε) of 2.60 mM^{-1} cm^{-1} was calculated. This method allowed the determination of 1,2-DHN dioxygenase activity without being falsified by autoxidation of 1,2-DHN. Enzymatic activity with 1,2,5-trihydroxynaphthalene (1,2,5-THN) was determined at 273 nm (ε = 5.10 mM^{-1} cm^{-1}), with 1,2,6-THN at 342 nm (ε = 1.56 mM^{-1} cm^{-1}), and with 1,2,7-THN at 287 nm (ε = 7.55 mM^{-1} cm^{-1}); these were the corresponding isosbestic points found during autoxidation of these substrates.

To determine enzyme activities during the purification procedure, it was necessary to reactivate the enzyme with Fe^{2+} ions. The maximal reactivation was achieved after about 6 h of incubation of the enzyme with Fe^{2+} and L-ascorbic acid. Prior to assaying enzyme activity, samples from column chromatography were incubated routinely with 2 mM $(NH_4)_2Fe(SO_4)_2$ and 5 mM L-ascorbic acid for 30 min, which restored about 30% of maximum activity. *cis*-2'-hydroxybenzalpyruvate aldolase was measured by a modification of the method given by Barnsley (1). Cell extract was added to a solution (final volume, 1 ml) containing 0.1 M Na-K phosphate buffer (pH 7.0) and 0.1 μmol of *cis*-2'-hydroxybenzalpyruvate. The decrease of absorption at 296 nm was measured photometrically. Reaction rates were calculated by using an extinction coefficient of 12.8 mM^{-1} cm^{-1} (1). Salicylaldehyde dehydrogenase was determined by the method of Shamsuzzaman and Barnsley (37).

Enzyme purification. Protein was purified at room temperature by use of a fast-performance liquid chromatography system consisting of a LCC 500 controller, pump 500, UV-1 monitor, REC-482 recorder, and FRAC autosampler from Pharmacia (Uppsala, Sweden).

Crude extract was incubated with 1 M ammonium sulfate for 15 min and filtered. The filtrate was transferred to a phenyl-Sepharose fast-flow CL 4B-gel column (HR 16/10; Pharmacia) and eluted with 200 ml of a linear gradient of Tris-HCl (50 mM, pH 7.5) plus 500 mM $(NH_4)_2SO_4$ into Tris-HCl (50 mM, pH 7.5) at a flow rate of 2 ml/min. Fractions (3.5 ml each) were collected, and the activities of 1,2-DHN dioxygenase were determined as described above. Fractions containing the enzyme were pooled and concentrated by ultrafiltration (ultrafiltration unit 8050, filter PM10; Amicon, Danvers, Mass.).

The concentrated sample (220 μl) was applied to a Superose-6 column (HR 10/30; Pharmacia) and eluted with 30 ml of Tris-HCl (50 mM, pH 7.5) containing 100 mM NaCl at a flow rate of 0.3 ml/min. Fractions (0.5 ml each) were collected, and the activities were determined. Fractions containing 1,2-DHN dioxygenase were pooled and applied to a Mono-Q column (HR 5/5; Pharmacia). Protein was eluted with 50 ml of a linear gradient of Tris-HCl (50 mM, pH 7.5) into Tris-HCl (50 mM, pH 7.5) plus 500 mM NaCl at a flow rate of 1 ml/min. Fractions (0.7 ml each) were collected and enzyme activity was determined as before. Fractions containing 1,2-DHN dioxygenase were pooled.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (24). Gels were silver stained by the method of Merril (26) with the Bio-Rad (Bio-Rad, Richmond, Calif.) silver stain kit.

NH_2-terminal amino acid sequencing. The NH_2-terminal amino acid sequence of the protein was determined by automated Edman degradation on an Applied Biosystems model 477A pulsed liquid phase sequencer with model 120A on-line PTH analyzer.

HPLC. Naphthalenesulfonic acids, DHN, and THNs and their corresponding NQs were analyzed by HPLC (pump model 510, WISP model 712; Waters, Milford, Mass.), with a programmable UV-VIS-detector (Spectroflow model 783; Kratos, Ramsey, N.J.) and a D-2000 Chromato-Integrator E. Merck AG, Darmstadt, Federal Republic of Germany). A C_8 reverse-phase column (125 by 4.6 mm [internal diameter]; Bischoff, Leonberg, Federal Republic of Germany) with Lichrosorb RP8 5-μm particles (Merck) was used to separate individual compounds which were detected spectrophotometrically (at 210 nm).

Autoxidation of THNs. 1,2-DHN and 1,2,5- and 1,2,6-THN and their respective NQs were separated by HPLC. The mobile phases used are described in Table 1. The THNs (0.4 mM) were dissolved at different pH values and vigorously stirred, and samples were taken at short time intervals (1.5 to 2.0 min) and immediately analyzed by HPLC. Although the retention times of the compounds analyzed were higher than the intervals of sample injection (Table 1), the signals of the overlapping chromatograms were easily correlated with the corresponding sample injection by the retention times of the THNs. 1,2,7-THN was not separated from the corresponding NQ. The rate of autoxidation of 1,2,7-THN was therefore determined spectrophotometrically. A molar reaction coefficient at 270 nm was calculated from the extinction difference of 1,2,7-THN and 7-H-1,2-NQ at different pH values (ε = 19.97 mM^{-1} cm^{-1} at pH 5.5 to 6.5 and ε = 17.72 mM^{-1} cm^{-1} at pH 7.0). Various concentrations of the THN (0.04 to 0.2 mM) were dissolved at different pH values, and the increase in absorbance was measured at 270 nm within 1 min.

For each THN and each pH value, the determinations were done twice; the average values are given. The decrease of the concentrations of THNs followed first-order kinetics. From these data, the half-lives were calculated.

Relative activities of the enzyme preparations with (substituted) catechols and dihydroxybiphenyls. For determination of the relative activities, the following reaction coefficients

TABLE 1. Solvent systems as mobile phases for HPLC of naphthalene, substituted naphthalenes, and related compounds[a]

Compound	Mobile phase (% [vol/vol])		Retention time[d] (min)
	A[b]	B[c]	
1,2-DHN	30	70	21.8
1,2,5-THN	30	70	7.3
1,2,6-THN	30	70	7.3
1,2,7-THN	30	70	7.3
1,2-NQ	30	70	9.8
5-H-1,2-NQ	30	70	15.2
6-H-1,2-NQ	30	70	12.7
7-H-1,2-NQ	30	70	7.4
Naphthalene	70	30	5.5
2-NS	45	55	2.5
2,3-DHBP[e]	60	40	4.8
3,4-DHBP	60	40	4.3

[a] For experimental conditions, see text. The mobile phase was composed from stock solutions by thorough mixing.
[b] A was methanol containing 0.1% (vol/vol) H_3PO_4.
[c] B was H_2O with 0.1% (vol/vol) H_3PO_4.
[d] Retention times were determined at a flow rate of 1 ml/min.
[e] DHBP, dihydroxybiphenyl.

were used (50 mM Na-K phosphate, pH 7.0): 2,3-dihydroxybiphenyl, λ_{max} = 435 nm, ε = 22 mM^{-1} cm^{-1} (18); catechol, λ_{max} = 375 nm, ε = 36 mM^{-1} cm^{-1}; 3-methylcatechol, λ_{max} = 382 nm, ε = 32 mM^{-1} cm^{-1}; 4-methylcatechol, λ_{max} = 388 nm, ε = 17 mM^{-1} cm^{-1}; and 4-chlorocatechol, λ_{max} = 379 nm, ε = 40 mM^{-1} cm^{-1} (34).

Chemicals. 5-, 6-, and 7-H-1,2-NQs were prepared by the method of Teuber and Götz (39) by treatment of 1,5-, 2,6-, or 2,7-DHN with potassium nitrosodisulfonate. The synthesized NQs were pure as judged by HPLC analysis. Absorption maxima and mass spectroscopic data were recorded as follows:

(i) 5-H-1,2-NQ gave red-brown crystals and showed absorption maxima (in dioxan) at 258 nm, (336 nm), 428 nm (literature values [lit.], 250 nm, 428 nm). *m/z* (190), 174, 146, 118, 89; relative intensities (1.5): 24.4: 100: 67.5: 31.9.

(ii) 6-H-1,2-NQ gave brick-red needles with (in chloroform) λ_{max} = 238 nm, 275 nm, 369 nm (lit. 280 nm, 370 nm). *m/z* 174, 146, 118, 89; relative intensities 26.9: 100: 49.2: 20.1.

(iii) 7-H-1,2-NQ gave dark violet crystals and showed absorption maxima (in chloroform) at 267 nm, 340 nm, 473 nm (lit. 265 nm, 335 nm, 455 nm). *m/z* (189), 174, 146, 118, 89; relative intensities (2.5): 14.4: 100: 42.6: 19.8.

(iv) 1,2,5-, 1,2,6-, and 1,2,7-THNs were prepared by reducing the corresponding hydroxy-1,2-NQs (9). Melting points for the THNs were found as follows: 1,2,5-THN, 170 to 180°C; 1,2,6-THN, 172 to 180°C (lit. 188°C); 1,2,7-THN, 175 to 185°C (lit. 197°C).

2-Hydroxychromene-2-carboxylic acid was prepared enzymatically from 1,2-DHN by crude extract of strain BN6 and was isolated as the chromenium perchlorate according to the description of Davies and Evans (11). The perchlorate was hydrolyzed with NaOH at pH 11 for about 1 h (1), yielding *cis*-2'-hydroxybenzalpyruvate. It showed the same absorption spectra in 6 N HCl or 0.1 N NaOH or at pH 7 as given for the authentic compound by Barnsley (1).

1,2-DHN was purchased from Aldrich (Steinheim, Federal Republic of Germany), 2,3-dihydroxybiphenyl was obtained from Wako Chemicals (Neuss, Federal Republic of Germany), and 3,4-dihydroxybiphenyl was from Promochem (Wesel, Federal Republic of Germany). 1,2-Dihydroxy-1,2-dihydronaphthalene was kindly provided by V. Strubel.

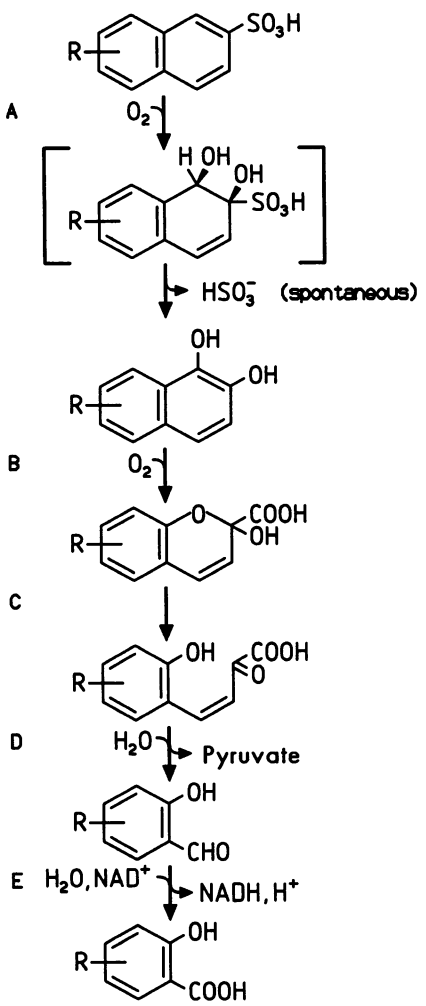

FIG. 1. Proposed pathway for partial degradation of A2NS and H2NS by strain BN6 (29). (A) Naphthalenesulfonic acid dioxygenase. (B) 1,2-DHN dioxygenase. (C) 2-Hydroxychromene-2-carboxylic acid isomerase. (D) *cis*-2'-hydroxybenzalpyruvate aldolase. (E) Salicylaldehyde dehydrogenase. R, NH_2, OH.

RESULTS

Induction of enzymes. Strain BN6 uses a catabolic pathway for the degradation of 2NS analogous to the reactions involved in the bacterial metabolism of naphthalene (29) (Fig. 1). Resting cells of strain BN6 converted a wide range of ANS and HNS to the corresponding salicylates. In pure culture, however, strain BN6 grew poorly with naphthalenesulfonic acids, presumably because of the accumulation of toxic salicylates in the medium. 1,2-DHN dioxygenase (DHNDO), *cis*-2'-hydroxybenzalpyruvate aldolase, and salicylaldehyde dehydrogenase were detected after growth of strain BN6 in the presence of 2NS (Table 2). The DHNDO and the *cis*-2'-hydroxybenzalpyruvate aldolase were induced in the presence of 2NS, while the salicylaldehyde dehydrogenase was present at a constitutive level. The strain did not synthesize a 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase, which is induced in *Pseudomonas* sp. strain

TABLE 2. Specific enzyme activities for the catabolism of 2NS from strain BN6 grown on glucose or glucose plus 2NS

Enzyme	Specific activity[a] (U/g of protein) after growth with:	
	Glucose	Glucose + 2NS
2NS dioxygenase (whole cells)	29	319
1,2-DHN dioxygenase	793	5,660
cis-2'-hydroxybenzalpyruvate aldolase	54	765
Salicylaldehyde dehydrogenase	530	447

[a] Enzyme activities were determined as described in Materials and Methods.

NCIB 9816 during growth with naphthalene. Growth of strain BN6 in the presence of 2NS induced the 2NS dioxygenase (Table 2). These cells did not convert naphthalene. 2NS (1 mM) was also converted in the presence of naphthalene (0.2 to 0.3 mM) but with reduced relative activities (43 to 29% as opposed to 100% activity in the absence of naphthalene).

Purification of the 1,2-DHN dioxygenase. To determine optimal conditions for storage of the 1,2-DHN dioxygenase during purification, crude extracts of strain BN6 were incubated in different buffer systems, and the remaining activity was assayed. The enzyme was very stable in Na-K phosphate buffer, pH 7.5. After 1 week of storage (2.4 mg of protein per ml) at 4°C, 100% of the original activity was recovered. In general, the enzyme was more stable at slightly alkaline pH values and better preserved in Na-K phosphate buffer than in Tris-HCl or glycine-NaOH. After storage for 1 week at 4°C in the presence of 10% (vol/vol) ethanol or acetone, the enzyme was completely inactivated.

For purification of 1,2-DHN dioxygenase, the procedure shown in Table 3 was used. With the exception of the crude extract, it was necessary to activate all enzyme preparations by Fe^{2+} treatment prior to assay (see Materials and Methods).

The enzyme was purified 54-fold, giving a specific activity of 83.4 U/mg of protein. The overall yield was 7% of the activity present in cell extracts and 0.14% of its total proteins. The purified enzyme gave a single band by SDS-gel electrophoresis (2 to 4 μg of enzyme) as well as by gel filtration.

Molecular weight and subunit structure. The molecular weight of the purified 1,2-DHN dioxygenase as determined by gel filtration was estimated to be 290,000. The molecular weight of one subunit was determined by SDS-gel electrophoresis to be 33,000. Therefore, it can be assumed that the enzyme consists of eight subunits of identical size.

Determination of the NH_2-terminal amino acid sequence. The NH_2-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation and compared with the NH_2-terminal sequences of other extradiol cleaving dioxygenases (Table 4). The DHNDO from strain BN6 showed a high sequence homology to the 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas paucimobilis* Q1 (22 of 29 amino acids were identical). Surprisingly the NH_2-terminal amino acid sequence of 1,2-DHN dioxygenase from strain BN6 was only 48% homologous with that of the isofunctional enzyme encoded by the NAH7 plasmid of *Pseudomonas putida* (20).

TABLE 4. Comparison of the NH_2-terminal amino acid sequence of 1,2-DHN dioxygenase from strain BN6 with those of 2,3-dihydroxybiphenyl dioxygenases and catechol 2,3-dioxygenases

Dioxygenase[a]	Sequence[b]
	* * *
NahC1	SSVSELGYLGMSVTDLDAWRAYAAEVAGM
BphC1	VAVTELGYLGLTVTNLDAWRSYAAEVAGM
NahC2	AAVIELGYMGISVKDPDAWKSFAMNMLGL
BphC2	SIRSLGYMGFAVSDVAAWRSFLTQKLGL
BphC3	SIERLGYLGFAVKDVPAWDHFLTKSVGL
TodE	SIQRLGYLGFEVADVRSWRTFATTRLGM
Xyl E	KGVMRPGHVQLRVLDMSKALEHYVELLGLI

[a] NahC1, 1,2-DHN dioxygenase from strain BN6; BphC1, 2,3-dihydroxybiphenyl dioxygenase from *P. paucimobilis* Q1 (38); NahC2, 1,2-DHN dioxygenase from plasmid NAH7 (20); BphC2, 2,3-dihydroxybiphenyl dioxygenase from *P. pseudoalcaligenes* KF707 (17); BphC3, 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. strain KKS102 (23); TodE, catechol 2,3-dioxygenase from *P. putida* F1 (44); XylE, catechol 2,3-dioxygenase encoded by the TOL plasmid pWWO (28).

[b] * and boldfaced type represent amino acids which are conserved among the seven dioxygenases; underlined amino acids are conserved in six of the seven dioxygenases.

Autoxidation of the THNs. 1,2-DHN is known to be rapidly autoxidized in aqueous solution (11). It was therefore necessary to analyze the spontaneous reactions of the THNs in aqueous solutions before analyzing the enzymatic turnover of these substrates. In acetic acid-NaOH buffer the freshly dissolved THNs exhibited the following absorption maxima: 1,2,5-THN, λ_{max} = 227, 300, and 335 nm; 1,2,6-THN, λ_{max} = 225 and 349 nm; and 1,2,7-THN, λ_{max} = 232 nm. In acetic acid-NaOH buffer (pH 5.5), spectrophotometric analysis revealed that THNs reacted in a similar way to 1,2-DHN (Fig. 2). Generally, the A_{225} to A_{232} due to the trihydroxy compounds decreased, whereas the absorbance at 250 to 275 nm increased. In addition, broad absorption maxima in the visible region of the spectrum which were identical with the long wavelength absorbances of the corresponding NQs were as follows: 1,2-NQ, λ_{max} = 249, 338, and 408 nm; 5-H-1, 2-NQ, λ_{max} = 252 and 462 nm; 6-H-1, 2-NQ, λ_{max} = 272 and 380 nm; and 7-H-1, 2-NQ, λ_{max} = 269 and 471 nm. The identity of the autoxidation products with the corresponding quinones was confirmed by HPLC. When the

TABLE 3. Purification of 1,2-DHN dioxygenase from induced cells of strain BN6[a]

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/g)	Recovery (%)	Purification factor
Crude extract	10.0	112.2	174.0	1,550	100	1.0
Hydrophobic-interaction-chromatography eluate	10.5	3.0	33.1	11,070	19	7.1
Gel-filtration-chromatography eluate	1.5	0.26	13.8	52,970	8	34.2
Ion-exchange-chromatography eluate	2.1	0.16	12.9	83,360	7	53.7

[a] Experimental details are given in Materials and Methods.

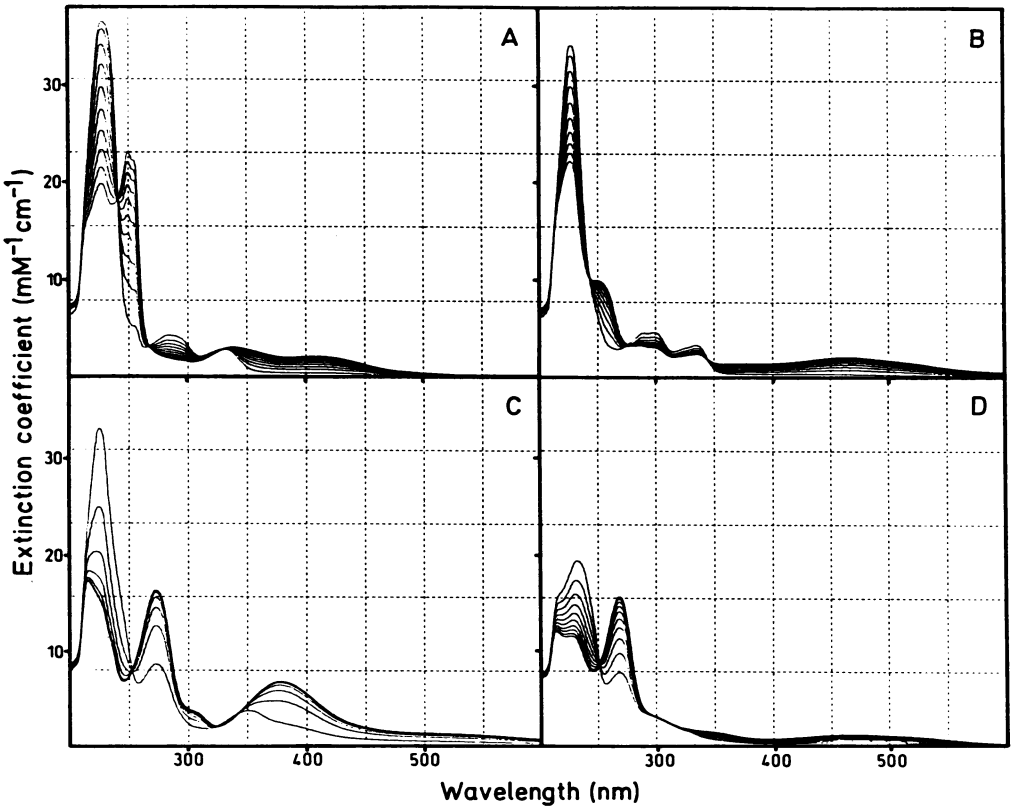

FIG. 2. Spectral changes observed during autoxidation of substituted 1,2-DHNs. Substituted 1,2-DHNs (0.08 μmol in 20 μl of tetrahydrofurane) were added to 1 ml of Na acetate buffer (50 mM, pH 5.5), and the spectra were recorded at intervals of 3 min. (A) 1,2-DHN. (B) 1,2,5-THN. (C) 1,2,6-THN. (D) 1,2,7-THN.

THNs were incubated in well-aerated solutions at pH 5.5, the signals of the THNs were replaced by those of the corresponding NQs. The rates of spontaneous oxidations were determined (Table 5). Half-lives of 1,2,5-THN and 1,2,7-THN were comparable to those of 1,2-DHN, whereas 1,2,6-THN was oxidized more readily.

Substrate specificity of the DHNDO. Different aromatic 1,2-diols were incubated with the purified enzyme, and changes of the absorption spectra were recorded spectrophotometrically. The following substrates were cleaved by DHNDO: 1,2-DHN, 1,2,5-, 1,2,6-, and 1,2,7-THN, 2,3- and 3,4-dihydroxybiphenyl, catechol, and 3- and 4-methylcatechol (Table 6). No activity with 3-chloro-, 3,5-dichloro-, and 4-aminocatechol, alizarin, 1,3 and 2,3-DHN, and pyrogallol was observed.

The K_m value for 1,2-DHN was calculated to be 34 μM.

TABLE 5. Rates of autoxidation of substituted DHNs at various pH values

Compound	Half-life[a] (min) at pH:			
	5.5	6.0	6.5	7.0
1,2-DHN	16.5	6.6	3.5	2.7
1,2,5-THN	19.1	7.1	3.8	2.9
1,2,6-THN	3.9	1.5	0.7	ND[b]
1,2,7-THN	11.7	5.9	3.3	1.8

[a] The half-lives were determined as described in Materials and Methods.
[b] ND, could not be determined by the present method.

Purified DHNDO from strain BN6 converted 1,2-DHN into a compound with the spectral characteristics of 2-hydroxychromene-2-carboxylic acid (λ_{max} at pH 5.5 = 254 and 299 nm). Under alkaline conditions (pH 11), the absorption maxima were replaced by a new absorption maximum at 420 nm within 30 min. This reaction was due to the formation of *cis*-2'-hydroxybenzalpyruvate (1).

THNs were incubated with the DHNDO from strain BN6. The spectral changes observed were different from those during the autoxidation of THNs. The reaction products were separated from protein by ultrafiltration (ultrafiltration unit 8050, filter PM10; Amicon) and the UV spectra were recorded at different pH values (Fig. 3).

At pH 5.5, the reaction product of 1,2,5-THN showed a λ_{max} = 265 nm and a shoulder at 300 nm. The absorption maxima changed under acidic conditions (λ_{max} = 283, 345, and 496 nm) and alkaline conditions (λ_{max} = 392 nm over a period of 20 min) in a way similar to that described for the enzymatic product of 1,2-DHN. At pH 5.5 and at pH 1, the spectra obtained from the product of the enzymatic reaction of 1,2,6-THN resembled those found for 2-hydroxychromene-2-carboxylic acid (Fig. 3C). At pH 5.5, the compound showed a λ_{max} = 260 nm and a shoulder at 302 nm. This absorption maximum was replaced in 6 M HCl by strong absorption maxima at 271 and 360 nm. In 0.1 M NaOH, a new absorption maximum was found at 510 nm, which was replaced within a few minutes by λ_{max} = 381 nm. In contrast to the other THN products, the reaction product of 1,2,7-THN showed absorption maxima at 303 and 352 nm

TABLE 6. Relative ring cleavage activities of the purified DHNDO from strain BN6 and cell extracts from different organisms with various substrates

Substrate	Purified DHNDO	Crude extracts of strain[a]			
		BN6	9816	10535	NAH7
1,2-DHN	100	100 (4,960)	100 (3,650)	100 (3,950)	100 (2,770)
1,2,5-THN	41	45	26	19	19
1,2,6-THN	41	43	40	29	45
1,2,7-THN	13	15	69	65	84
2,3-DHBP[b]	6	7	4	4	9
3,4-DHBP	4	7	1	1	2
Catechol	3	2	ND	ND	ND
3-Methylcatechol	3	4	ND	ND	ND
4-Methylcatechol	6	6	ND	ND	ND

[a] 9816, *P. putida* NCIB 9816; 10535, *P. putida* NCIB 10535; NAH7, *P. putida* KT2442 NAH7; ND, not determined. The reaction rates are expressed as percentages of that for DHN taken as 100%. Absolute specific activities (units per gram of protein) are given in parentheses for the relative rates reported as 100%.
[b] DHBP, dihydroxybiphenyl.

(pH 5.5). Under alkaline conditions, the UV spectrum changed immediately to λ_{max} = 443 nm. The maxima at 303 and 352 nm were restored instantaneously by neutralization.

The product from 2,3-dihydroxybiphenyl showed an absorption maximum in Na-K phosphate buffer (pH 7.0) at 434 nm, which changed at pH 1 to λ_{max} = 332 nm. These spectral properties are characteristic of the *meta* cleavage product of 2,3-dihydroxybiphenyl, which was identified as 2-hydroxy-6-oxo-phenylhexa-2,4-dienoate (8).

Unexpectedly, the purified DHNDO from strain BN6 also attacked 3,4-dihydroxybiphenyl. Taira et al. (38) maintained that this compound was metabolized neither by the 2,3-dihydroxybiphenyl dioxygenases from *P. pseudoalcaligenes* KF707 nor from *P. paucimobilis* Q1. The reaction product of 3,4-dihydroxybiphenyl showed an absorbance maximum at 380 nm and a shoulder at 324 nm (pH 7.0), which changed to λ_{max} = 328 nm at pH 2. To determine the relative activities, 2,3- and 3,4-dihydroxybiphenyl were incubated with the

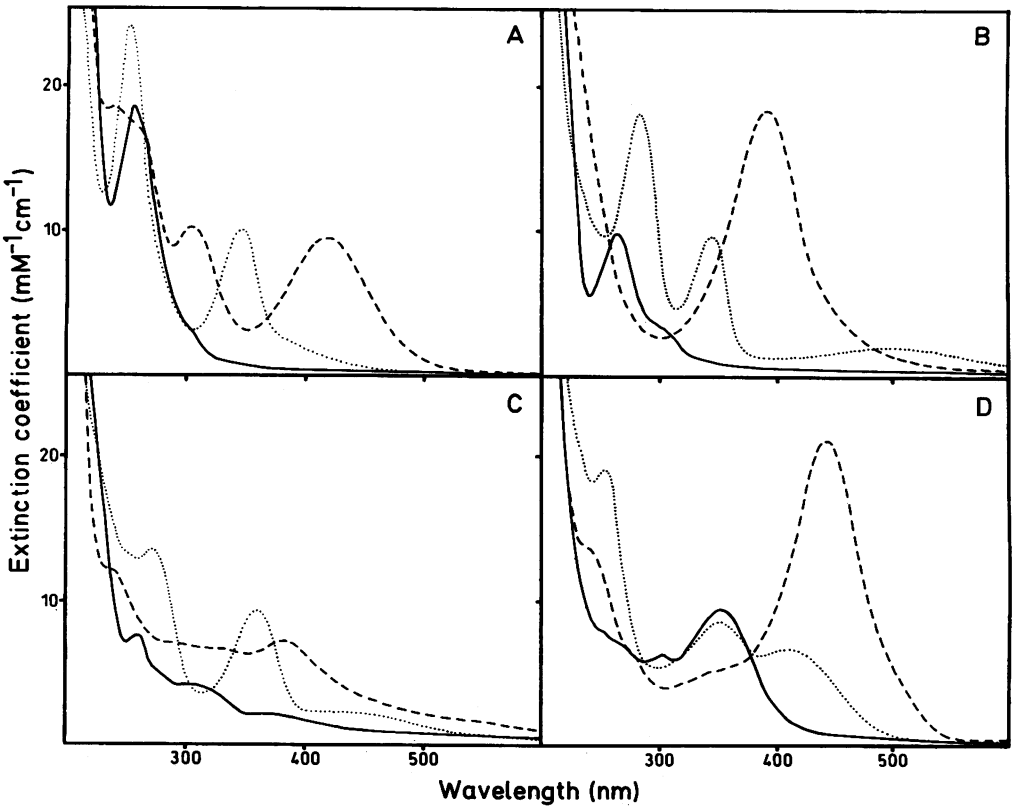

FIG. 3. Absorption spectra of the ring fission products of the THNs. The THNs (0.4 mM each in 50 mM Na acetate, pH 5.5) were transformed by the purified DHNDO from strain BN6. To avoid autoxidation of the THNs, sufficient dioxygenase activity was added to complete the enzymatic reaction within 1 min. After total conversion, the solution was deproteinized by ultrafiltration. The solutions were diluted (1:5) with Na acetate (pH 5.5) (——), 0.1 N NaOH (– – –), or 6 N HCl (· · ·). Reaction products of (A) 1,2-DHN, (B) 1,2,5-THN, (C) 1,2,6-THN, and (D) 1,2,7-THN.

purified DHNDO from strain BN6 (each 0.2 mM in Na-K phosphate buffer, pH 7.0), and the reaction which followed was analyzed by HPLC. The specific activities were 1.03 U/mg of protein with 2,3-dihydroxybiphenyl and 0.91 U/mg of protein with 3,4-dihydroxybiphenyl.

The products of the reactions with catechol (λ_{max} = 375 nm), 3-methylcatechol (λ_{max} = 388 nm), 4-methylcatechol (λ_{max} = 382 nm), and 4-chlorocatechol (λ_{max} = 379 nm) showed the characteristic properties of the *meta* cleavage products of these compounds.

Crude extracts from 2NS-induced cells of strain BN6 showed almost identical activities with the above substrates as the purified DHNDO (Table 6). It was therefore concluded that the DHNDO was the only enzyme in the crude extract from strain BN6 which was responsible for the ring cleavage of these aromatic diols.

Conversion of THNs by cell extracts from naphthalene-degrading bacteria. It was of interest to find out whether strain BN6 is unique in its ability to oxidize THNs. Therefore, cell extracts from naphthalene-degrading organisms such as *P. putida* NCIB 9816, NCIB 10535, or KT2442 NAH7 were incubated with 1,2,5-, 1,2,6- or 1,2,7-THN. In all cases, the same spectral changes were found as those observed with the purified DHNDO from strain BN6. The relative activities for the individual substrates were also very similar to those found for DHNDO from strain BN6 (Table 6).

Localization of the genes involved in the breakdown of naphthalenesulfonic acids by strain BN6. The genetic information for the degradation of naphthalene by bacteria is often carried by plasmids (2, 6, 7, 10, 14, 15, 39). We therefore attempted to examine the genetic basis for the metabolism of naphthalenesulfonic acids in strain BN6. Several established methods for the detection and isolation of catabolic plasmids were used (10, 16, 19, 22), but no plasmids were found in lysates from strain BN6. No curing of the 6A2NS$^+$ phenotype could be accomplished when strain BN6 was grown in liquid culture for about 30 generations under nonselective conditions and in the presence of mitomycin C. The NS$^+$ phenotype could not be transferred by conjugation to *P. putida* KT2442 or a derivative thereof containing plasmid NAH7 (frequency, $\leq 10^{-7}$). These results indicate that the relevant genes are localized on the bacterial chromosome in strain BN6.

DISCUSSION

The results presented confirm that the pathways for the degradation of naphthalenesulfonic acids and naphthalene are analogous (5, 11). Nevertheless, some distinct differences between the two pathways exist. First, cells from strain BN6 did not oxidize naphthalene. Therefore, the initial enzyme of naphthalenesulfonic acid catabolism has to be considered a unique naphthalenesulfonic acid dioxygenase which is different from ordinary types of naphthalene dioxygenases. It requires as substrates naphthalenes with acidic functional groups at C-2, such as SO_3H or CO_2H and hydroxylates these in 1,2 position (29). On the other hand, the naphthalenesulfonic acid dioxygenase from strain BN6 was inhibited by naphthalene and therefore seems to exhibit some affinity for this compound. Second, strain BN6 did not synthesize a 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase. This agrees with the proposed mechanism for desulfonation of 2NS, which includes the spontaneous elimination of sulfite and rearomatization of the dihydrodiol to 1,2-DHN (Fig. 1) (5). Therefore, degradation of naphthalenesulfonic acids does not require a 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase. The third difference we noticed was a constitutive synthesis of the salicylaldehyde dehydrogenase by strain BN6, while the naphthalenesulfonic acid dioxygenase, DHNDO, and *cis*-2′-hydroxybenzalpyruvate aldolase were inducible. This is in contrast to the situation in *P. putida* PpG7 and NCIB 9816, in which the salicylaldehyde dehydrogenase belongs to the upper naphthalene operon together with the DHNDO and *cis*-2′-hydroxybenzalpyruvate aldolase (37, 42).

In accordance with the enzyme from *P. putida* NCIB 9816 (32, the purified 1,2-DHN dioxygenase of strain BN6 required Fe^{2+} for catalytic activity but was significantly different in several respects. The subunits of the DHNDO from *P. putida* NCIB 9816 had a molecular weight of 19,000, and that of the holoenzyme is reported to be higher than 275,000 (32). Both DHNDOs converted 3- and 4-methylcatechol, whereas unsubstituted catechol was attacked only by the BN6 enzyme at a significant rate. From the nucleotide sequence of the *nahC* gene of plasmid NAH7, a molecular weight of the subunits of 33,882 was calculated (20). This plasmid and the plasmids isolated from *P. putida* NCIB 9816 (NAH2, pDTG1) showed significant homology (10, 35). Therefore, the DHNDOs from both plasmids are expected to be similar in molecular mass. Remarkably, the purified 2,3-dihydroxybiphenyl dioxygenases from *P. pseudoalcaligenes* KF707 and *P. paucimobilis* Q1 resembled the DHNDO from strain BN6 with respect to molecular weight (ca. 260,000) and subunit structure (octamers with a molecular weight of the subunits of about 33,000 [38]). Generally higher sequence similarity seems to exist between enzymes converting substituted biphenyls or naphthalenes compared with the classical metapyrocatechases (Table 4), which always appear to be tetrameric proteins.

All DHNDOs from bacteria which degrade naphthalene and the enzyme from strain BN6 oxidized hydroxy-substituted 1,2-DHNs. A relaxed substrate specificity seems to be a general property of these enzymes since several bacteria which degrade naphthalene are known to convert 2-chloro- and 2-methylnaphthalene to the corresponding 4-substituted salicylates (27, 40). Therefore, the DHNDOs from these strains must convert at least 7-substituted 1,2-DHNs.

The enzymatic activity of the DHNDO from strain BN6 with 2,3-dihydroxybiphenyl and the homology of the NH_2-terminal amino acid sequence of the DHNDO from strain BN6 with the *bphC* gene product from *P. paucimobilis* Q1 suggest a close relationship between the degradative pathways of naphthalene and biphenyl. This corresponds to the observation that the 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase from *P. putida* NP also converted 2,3-dihydroxy-2,3-dihydrobiphenyl (33).

Obviously, the DHNDO from strain BN6 oxidized all substrates by extradiol cleavage. Dioxygenation of 1,2-DHN resulted in a compound with the same spectral characteristics as described for 2-hydroxychromene-2-carboxylic acid (1). Similar products were generated by ring fission of 1,2,5- and 1,2,6-THN. Probably extradiol dioxygenation of DHN and 1,2,5- and 1,2,6-THN generate nonaromatic primary products which instantaneously form 2-hydroxychromene-2-carboxylic acids by isomerization. For unknown reasons, chromene formation with 1,2,7-THN as substrate was not observed. In this case, the only detectable product of ring cleavage appears to be a substituted benzalpyruvate. To elucidate this mechanism in detail, it will be necessary to purify the subsequent enzymes of the naphthalenesulfonic acid pathway from strain BN6.

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