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Bacterial Communities Degrading Amino- and Hydroxynaphthalene-2-Sulfonates

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A 6-aminonaphthalene-2-sulfonic acid (6A2NS)-degrading mixed bacterial community was isolated from a sample of river Elbe water. The complete degradation of this xenobiotic compound may be described by a mutualistic interaction of two *Pseudomonas* strains isolated from this culture. One strain, BN6, could also grow on 6A2NS in monoculture, however, with accumulation of black polymers. This organism effected the initial conversion of 6A2NS into 5-aminosalicylate (5AS) through regioselective attack of the naphthalene skeleton in the 1,2-position. 5AS was totally degraded by another member of the community, strain BN9. After prolonged adaptation of strain BN6 to growth on 6A2NS, this organism readily converted all naphthalene-2-sulfonates with OH- or NH_2-substituents in the 5-, 6-, 7-, or 8-position. The corresponding hydroxy- or aminosalicylates were excreted in stoichiometric amounts, with the exception that the metabolite from 5A2NS oxidation was not identical with 6AS.

Amino- and hydroxynaphthalenesulfonic acids (ANSs and HNSs) are important building blocks for the large-scale synthesis of azo dyes. The amino and hydroxy substituents are indispensable as auxochromic groups, while the water solubility of this class of reactive dyes is essentially based on the sulfonic acid group. Since arylsulfonates are very rare among natural compounds (8), the sulfonic acid group confers a xenobiotic character to this class of chemicals. Consequently, unadapted microbial populations of activated sludge do not degrade sulfonated naphthalenes or degrade them incompletely. Previous work from this laboratory (3, 4) has shown that naphthalene-degrading bacteria with low substrate specificities but high regioselectivities can be selected by continuous enrichment with naphthalene-2-sulfonic acid (2NS) or naphthalene-1-sulfonic acid (1NS) as the sole carbon source. These xenobiotic compounds were totally degraded via the known catabolic sequence of naphthalene (5) after oxygenolytic cleavage of the carbon-sulfur bond, i.e., 1,2-dioxygenation, and spontaneous rearomatization with elimination of sulfite.

The 2NS- or 1NS-degrading organisms readily cooxidize some isomeric amino- or hydroxynaphthalene-2-sulfonic acids, with accumulation of amino- or hydroxysalicylates. In part, these metabolites are subject to autoxidation or cooxidation, yielding toxic intermediates and finally dark products (unpublished results). Phenomenologically, the situation resembles the incomplete degradation of ANSs or HNSs by activated sludge, in which brown-black polymers are generated and bacterial growth is strongly inhibited.

These observations prompted us to carry out enrichment experiments with ANSs as selective substrates. Preliminary experiments had shown that, of all the ANSs, 6-aminonaphthalene-2-sulfonic acid (6A2NS) was cooxidized at the highest rate. 5-Aminosalicylate (5AS) was identified as a metabolite (R. M. Wittich, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1984) which is a structural analog of the natural compound gentisate.

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The present paper describes mixed cultures which degrade 6A2NS by interspecies transfer of 5AS.

MATERIALS AND METHODS

Media. Media were prepared in a mineral base described by Brilon et al. (3). Heat-labile and autoxidable substrates (such as vitamin solutions and 5AS) were sterilized by membrane filtration (pore size, 0.2 μm; Sartorious, Göttingen, Federal Republic of Germany); all other substrates were added to the medium and autoclaved in situ at 121°C. For determination of ammonium concentration in the culture fluid during growth on 5AS, the mineral base was modified to contain, per liter, 12 g of $Na_2HPO_4 \cdot 2H_2O$, 2 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 25 mg of $CaCl_3 \cdot 6H_2O$, 5 mg of $FeCl_3 \cdot 6H_2O$, and 1 ml of a trace solution described by Pfennig and Lippert (11); however, it did not contain iron salt and EDTA.

Solid media were prepared by addition of 1.5% of Ionagar no. 2 (Oxoid Ltd., London, England) to the mineral medium containing the appropriate carbon sources. When naphthalene was used as a carbon source, mineral agar plates were incubated upside down with a few crystals of naphthalene in the lid of the petri dish.

Isolation of microorganisms and culture conditions. A bacterial mixed culture able to grow on 6A2NS was isolated by direct enrichment in stationary culture in Erlenmeyer flasks with baffles (1,000 ml with 200 ml of medium). Cultures were incubated at 30°C on a rotary shaker at 120 rpm. For catabolism experiments, cells were harvested by centrifugation and washed in 0.05 M phosphate buffer (pH 7.3). Stock cultures of new isolates were subcultured monthly and stored at 4°C. *Pseudomonas* sp. strains BN6 and BN9 were maintained as separate streaks on the same 6A2NS (10 mM) agar plate. Other isolates were maintained on ASPG medium, which contained 6 mM 6A2NS, 10 mM succinate, 10 mM pyruvate, and 5 mM gentisate, supplemented by a vitamin solution described by Genthner et al. (7). The same medium was used for viable counts.

Chemicals. Naphthalene, napthalenesulfonates, and 3AS were obtained from Bayer AG, Leverkusen, Federal Republic of Germany. 5AS, 3-hydroxysalicylate, and 5-hydroxy-

salicylate were obtained from EGA-Chemie, Steinheim, Federal Republic of Germany. Cycloheximide, salicylate, 4-hydroxysalicylate, and 6-hydroxysalicylate were purchased from Fluka, Buchs, Switzerland. 4AS was purchased from Serva, Heidelberg, Federal Republic of Germany. Napthalene-2-carboxylic acid and all other chemicals used for mineral salts media and the buffer solution were supplied from E. Merck AG, Darmstadt, Federal Republic of Germany.

Identification of bacteria. For identification of organisms, the methods of Stanier et al. (13) were used. Moreover, the API 20E system (API-System S.A., La Balme les Grottes, France) for identification of members of the family *Enterobacteriaceae* and other gram-negative bacteria was applied. A *Pseudomonas putida* strain (DSM Göttingen 241) was used as a reference.

Measurement of growth. Growth of the culture was monitored spectrophotometrically by measuring the optical density at 546 nm with a Kontron Uvikon 820 spectrophotometer (Kontron, Eching, Federal Republic of Germany).

Protein estimation and enzyme assays. The protein content of whole cells was determined by the method of Schmidt et al. (12). Cell extracts were prepared by using a French press at 140 mPa and 0°C (Aminco, Silver Spring, Md.). The protein content of extracts was determined by the method of Bradford (2); bovine serum albumin was used as a standard. Specific activities are expressed as micromoles of substrate utilized per minute per gram of protein at 25°C. For measurement of the gentisate 1,2-dioxygenase reaction, the procedure of Wheelis et al. (14) was modified and used for 5AS oxygenation. Instead of monitoring the reaction at 340 nm, the UV spectra (210 to 400 nm) were recorded at intervals.

Analytical methods. For the determination of sulfate ion concentration in the culture fluid the method of Bertolacini and Barney (1) was modified as follows. The culture supernatant fluid (1 ml) was diluted with distilled water to a final volume of 15 ml. Ethanol (25 ml) was added, and the solution was acidified with citric acid (1 M) to pH 4. After admixture of barium chloroanilate (150 mg), the sample was diluted again with distilled water to a final volume of 50 ml and shaken for 10 min. Undissolved particles were removed by centrifugation. Photometric measurements were carried out at 530 nm. A sulfate-free probe of mineral medium was treated in a similar way and used as a standard. The calibration curve was established by using standard solutions of sodium sulfate.

The sulfite concentration in the culture fluid was measured by the method of Johnston et al. (10). Ammonia was determined with glutamate dehydrogenase by the method of Da Fonseca-Wollheim et al. (6). The reaction mixture was slightly modified and contained, in a final volume of 1 ml, 50 μmol of imidazole (pH 7.9), 10 μmol of 2-oxoglutarate, 1 μmol of EDTA, 2 μmol of ADP, 0.14 μmol of NADH, and 0.01 ml of glutamate dehydrogenase in glycerol (Boehringer GmbH, Mannheim, Federal Republic of Germany). The reaction was started by the addition of 5 to 25 μl of cell-free culture fluid or standard solution of ammonium (0 to 20 mM). The decrease in NADH concentration was measured at 366 nm (ε = 3,400 M^{-1} cm^{-1}).

Substituted naphthalenes and metabolites were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) (HPLC chromatograph with M-660 solvent programmer and WISP 710 B autosampler; Waters Associates, Inc., Milford, Mass.) equipped with a Schoeffel GM 770 detector (Kratos GmbH). A C_8 reverse-phase column (125 mm by 4.6 mm [internal diameter]; Bischoff, Leonberg, Federal Republic of Germany) Licrosorb RP8 with 5-μm particles (Merck) was used to separate individual compounds which were detected spectrophotometrically at 215 nm. The following solvent systems were used as mobile phases: solvent A, containing acetonitrile (22%), water (78%), and H_3PO_4 (0.1%) and solvent B, containing methanol (30%), water (70%), isopropanol (0.25%), and H_3PO_4 (0.1%). Samples (1 to 10 μl) of culture fluids were injected after cells had been removed by centrifugation in 1-ml microtest tubes for 3 min at 20,000 rpm (Mikro-Hämokrit; Heraeus Christ, Osterode, Federal Republic of Germany). Metabolites were compared with authentic salicylates by their retention times and in situ scanning of the UV spectra after the flow had been stopped. Further identification with authentic chemicals was carried out by thin-layer chromatography with precoated thin-layer chromatography plates, silica gel 60 F_{254} (Merck), and a solvent system of (i) diisopropylether-formic acid-water (200:7:3), or (ii) isopropanol-methanol-chloroform (65:25:10). Dissolved organic carbon was assayed with a Beckman TOC Analyzer 915-B (Beckman Instruments, Fullerton, Calif.). Samples (50 μl) of culture fluid were injected after removal of cells and particles by centrifugation.

RESULTS

Enrichment of 6A2NS-degrading cultures. A water sample taken from the river Elbe near Hamburg was supplemented with mineral salts medium containing 6A2NS and 2NS so that the final concentrations of the sulfonic acids were 8 and 2 mM, respectively. HPLC analysis revealed that both substrates disappeared after the culture had been incubated at 30°C on a rotary shaker for 6 days. During subculturing on mineral salts medium with 10 mM 6A2NS as the sole carbon source, the growth of eucaryotic cells was suppressed by repeated exposure to cycloheximide (10 mg/liter). Initially the culture grew very slowly (doubling time [t_d] ≥ 15 h), and the medium discolored to a brownish red, indicating incomplete degradation of the xenobiotic compound. During subculture over 6 months the culture fluid brightened and bacterial growth became homogeneous. Simultaneously, the doubling time of the culture decreased to 8 h. Finally, after 12 months of adaptation, t_d was 3.3 h and the inoculum from an exponentially growing culture (optical density at 546 nm, ca. 2) could be reduced to ≤1% (vol/vol). The culture could degrade 6A2NS even at high concentrations (≤30 mM) without the accumulation of dark polymers. If nitrogen and sulfur sources were omitted from the mineral salts medium, 6A2NS was also utilized as the sole source of nitrogen and sulfur. Under these conditions a longer doubling time (8.4 h instead of 4.5 h with 6A2NS and an external nitrogen source added) was observed.

Isolation and characterization of strains. When streaked on 6A2NS (2 to 10 mM)–mineral salts agar, the adapted mixed culture formed coherent cell masses without developing individual colonies. Different selective and nonselective media were used to isolate single strains from the mixed culture. Ten isolates, which all grew well on solid ASPG medium could be distinguished by the morphology of their colonies.

One of these isolates, BN6, generated a strong red pigment, which partly diffused into the agar and identified this isolate as a 6A2NS oxidizer. Isolate BN6 readily lost this 6A2NS-degrading activity during growth on nutrient broth agar. It formed very small colonies on pure 6A2NS-agar plates, generating the reddish coloration, which turned in-

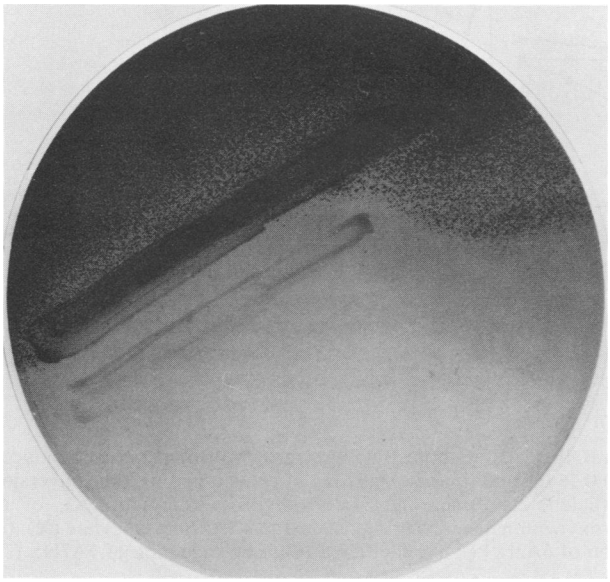

FIG. 1. Growth of *Pseudomonas* sp. strain BN6 and BN9 on mineral salts agar with 6A2NS as the sole source of carbon and energy. The biomass of strain BN6 is discernible as the long dark streak in the upper part of the plate. Incomplete degradation of 6A2NS by this strain is indicated by precipitation of dark autoxidation products from a metabolite. Accumulation of these products is avoided in the lower part of the plate, where shorter parallel streaks of strain BN9 were inoculated.

creasingly brown until black flocs were precipitated in the medium. If strain BN6 was mixed with each of the other isolates, some of these two-species cultures (e.g., that with isolate BN9) developed strong growth of colorless organisms on 6A2NS agar plates. In this respect these cultures were indistinguishable from the original mixed culture.

If isolate BN6 and one of the complementary organisms, such as isolate BN9, were inoculated as separate streaks, interspecies transfer of a metabolite could be demonstrated (Fig. 1). Obviously, on the side of the BN6 streak where both organisms developed in close proximity, strong growth was observed without discoloration of the medium. However, precipitation of dark polymers appeared on the side where BN9 was not streaked. Under optimum conditions of cocultivation on solid medium, growth of strain BN9 was always stronger than that of BN6.

When 6A2NS-grown mixed cultures (defined or undefined) were plated on the ASPG medium, relatively few colonies from BN6 (usually less than 10% of the total cell number) were counted. From microscopic observations it became evident that BN6 tends to form filaments which cannot be broken up by whirl mixing or drastic treatments such as ultrasonication and exposure to detergents or NaOH.

The BN6 colonies were small (1 to 2 mm in diameter), round, smooth, and intense yellow which turned brown during growth on mineral salts-ASPG medium. No growth was observed at 4 or 41°C. Cells were gram-negative rods with rounded ends (length, 1.8 to 4.0 μm; diameter, 1.1 to 1.2 μm) and, when grown on glucose, became motile by the formation of a polar flagellum. Older cells formed capsules. Oxidase and catalase reactions were positive. Arginine dihydrolase, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, urease, and tryptophan desaminase activities were not detectable. With the exception of glucose and maltose, sugars were not oxidized. The Voges-Proskauer reaction was positive. Gelatin was not liquified. The BN6 colonies could not produce H_2S from thiosulfate.

During prolonged adaptation of the bacterial community to growth on 6A2NS, cells of strain BN9 became more and more dominant (from 30% initially to almost 80% of the total cell number). Strain BN9 exhibited small (diameter, 1 to 3 mm), round, and smooth brownish-beige colonies on ASPG medium. The gram-negative cells were motile by the possession of a polar flagellum. The bacterium grew at 41°C but not at 4°C. Catalase and oxidase reactions were positive. No sugars were oxidized. Pure cultures of BN9 required the addition of a vitamin solution (7). Strains BN6 and BN9 exhibited most of the phenotypic characters of the genus *Pseudomonas* according to the diagnostic key of Stanier et al. (13).

After 40 weeks of subculture on 6A2NS mineral medium, more than 85% of the cells of the mixed culture were strains BN6 and BN9. All other isolates obtained from ASPG medium were found to be minor members of the community. Obviously, these organisms were not important for the major catabolic activity of the culture, because total degradation of 6A2NS could essentially be described by mutualistic interaction of strains BN6 and BN9 (see below).

Catabolism of 6A2NS. Interspecies metabolite transfer between *Pseudomonas* sp. strain BN6 and *Pseudomonas* sp. strain BN9 is indicated by the experiment shown in Fig. 1. To analyze this combined catabolic mechanism in greater detail, 6A2NS-induced cells of *Pseudomonas* sp. strain BN6 had to be incubated with naphthalenesulfonates. However, strain BN6 grew poorly in liquid culture with 6A2NS as the sole carbon source. Furthermore, the accumulation of brownish-black polymers hindered the determination of protein and the turbidity measurements. On the other hand, induction of the degradative enzymes was very slow in acetate-, succinate-, or nutrient broth-grown cells. Therefore, *Pseudomonas* sp. strain BN6 was grown with acetate (≤20 mM) in the presence of 2NS and 6A2NS. Preliminary experiments had shown that relative specific activities for the oxidation of differently substituted 2NSs were not altered, irrespective of whether strain BN6 was grown in the presence of 2NS or 6A2NS. The optimum substrate concentration was found to be ≤1 mM each, which induced sufficient naphthalenesulfonate turnover activity without causing the accumulation of toxic levels of metabolites.

2NS–6A2NS-induced cells of *Pseudomonas* sp. strain BN6 converted 2NSs quantitatively into salicylates (Fig. 2), which were not further metabolized. These metabolites were indistinguishable from authentic compounds by both HPLC and thin-layer chromatography with different solvent systems (see Materials and Methods). They were further identified by in situ scanning of the UV spectra during stopped-flow analysis. An exception is the cooxidation of 5A2NS, in which the reaction stopped before the substrate was consumed. Nevertheless, the relative increase in the peak intensity of the product always corresponded to the relative amount of 5A2NS consumed. The UV absorption spectrum (hydrochloride salts in CH_3OH) of this metabolite (λ_{max} = 260 nm) was not identical to that described for 6AS (λ_{max} = 234 nm) by Hillis and Gould (9). Identification of this metabolite and the reason for the incomplete metabolism will be described elsewhere. Activities for the cooxidation of 2NSs with OH or NH_2 at C-8 were very low, so that yields of the corresponding salicylates could not be determined (Table 1).

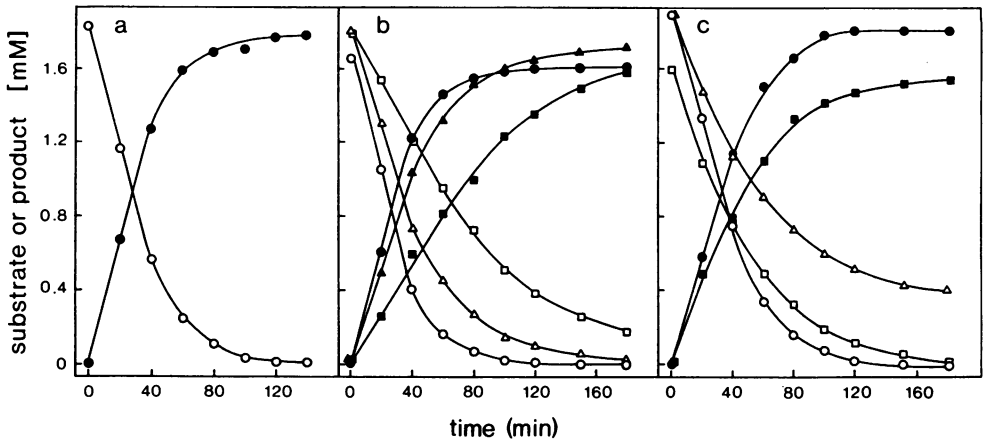

FIG. 2. Conversion of 2NSs into salicylates by *Pseudomonas* sp. strain BN6. Cells of strain BN6 were grown with acetate and induced by 2NS-6A2NS as described in the text. The cell suspensions contained (a) 0.16 mg and (b and c) 0.32 mg of protein per ml. (a) Conversion of 2NS (○) into salicylate (●). (b) Conversion of 5-hydroxy-naphthalene-2-sulfonate (△) into 6-hydroxysalicylate (▲), of 6-hydroxynaphthalene-2-sulfonate (○) into 5-hydroxysalicylate (●), of 7-hydroxynaphthalene-2-sulfonate (□) into 4-hydroxysalicylate (■). (c) The reaction product of 5A2NS (△) was not identified (see text). Oxidation of 6A2NS (○) yielded 5AS (●), and oxidation of 7A2NS (□) generated 4AS (■).

Desulfonation as initial catabolic reaction. To make a comparison with previous observations of Brilon et al. (3, 4), it was of interest to see whether 2NS and 6A2NS were attacked by the same initial catabolic mechanism. Inhibition of 6A2NS turnover was reversible, and the catabolic activity was fully restored after consumption of 2NS (Fig. 3). Obviously, both substrates competed for the same catabolic enzyme. During growth with 2NSs, sulfite was always detected in the culture fluid. This metabolite was readily oxidized to sulfate, so that its maximum concentration at the late-exponential growth phase never exceeded 0.02 mM. Finally, all of the organic sulfur was converted to sulfate.

The structural analog of 2NS, naphthalene-2-carboxylate (2NC), was quantitatively converted into 1,2-dihydroxy-1,2-dihydronaphthalene-2-carboxylate by 2NS–6A2NS-induced cells. 4-Hydroxybenzoate-grown cells of *Pseudomonas* sp. strain BN6 were induced for the metabolism of 2NC and naphthalene-2-sulfonates, with relative turnover activities of 15 to 60% compared with those observed with 2NS-induced cells. However, when *Pseudomonas* sp. strain BN6 was grown with 4-hydroxybenzoate and incubated with 6A2NS or 8A2NS, a rapid and irreversible cell inactivation was frequently observed. In these cases HPLC analysis revealed significant, hitherto unobserved, metabolites which were missing when 6A2NS- or 8A2NS-induced cells were used.

Degradation of 5AS. From the 6A2NS-utilizing mixed culture, three strains were isolated which could grow on 5AS as the sole source of carbon and nitrogen.

Cells of strain BN9 exhibited the highest specific turnover rates (165 μmol · g of protein^{-1} · min^{-1}) compared with isolates BN2 and BN3 (87 and 96 μmol · g of protein^{-1} · min^{-1}, respectively). Consequently, strain BN9 clearly dom-

TABLE 1. Oxidation of 2NSs by 2NS–6A2NS–induced cells of *Pseudomonas* sp. strain BN6[a]

Substrate	Sp. act[b]	Product	Yield (%)
2Ns	165	Salicylate	98
5A2NS	64	–[c]	
6A2NS	95	5AS	93
7A2NS	78	4AS	99
8A2NS	0.1	3AS	–[d]
5H2NS	87	6-Hydroxysalicylate	97
6H2NS	100	5-Hydroxysalicylate	98
7H2NS	47	4-Hydroxysalicylate	95
8H2NS	1	3-Hydroxysalicylate	–[d]

[a] The experimental conditions were the same as those described in Fig. 2. The initial substrate concentrations were approximately 1.8 mM. Samples were taken at intervals of 5 to 30 min.

[b] Specific turnover rates were expressed as micromoles per minute per gram of protein. Yields of products were calculated on the basis of the amounts of substrate converted. Loss of product through autoxidation was not considered, so that the actual yields may be higher than those given.

[c] –, Not identified (see text).

[d] –, Not determined.

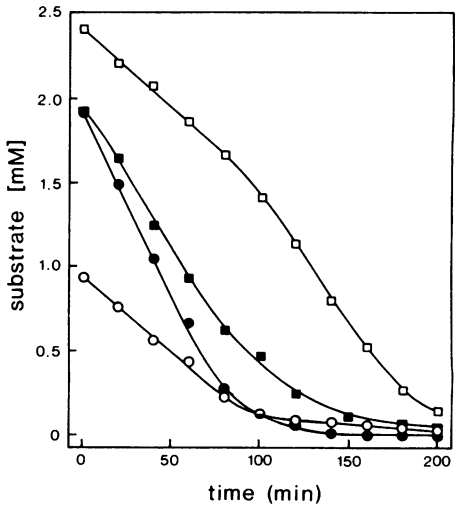

FIG. 3. Turnover of 6A2NS by *Pseudomonas* sp. strain BN6 in the presence of 2NS. The experimental conditions were the same as those described for Fig. 2. The cell suspension (0.13 mg of protein per ml) was incubated with a mixture of 6A2NS and 2NS (initial concentrations, 2.5 and 1.0 mM, respectively). The disappearance of 6A2NS (□) and 2NS (○) from the culture fluid was followed by HPLC. The turnover of 6A2NS (■) and 2NS (●) alone was given as control. Of the 2NS, 97% was converted to salicylate and of 6A2NS, at least 90% was recovered as 5AS (data not presented).

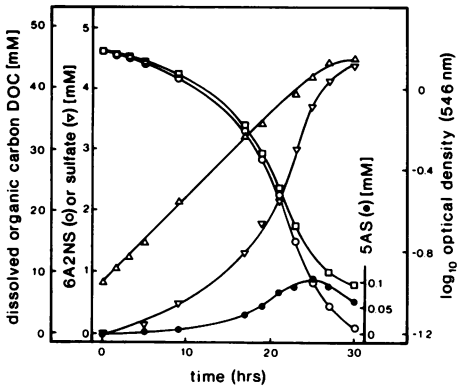

FIG. 4. Growth of a two-species culture with 6A2NS. A mineral salts medium containing 6A2NS as sole source of carbon was inoculated with an exponentially growing preculture of *Pseudomonas* sp. strains BN6 and BN9. Increase in cell density (△) was determined photometrically at 546 nm. The concentration of 6A2NS (○) and 5AS (●) was measured by HPLC. Sulfate (▽) was quantified by the method of Bertolacini and Barney (1), and dissolved organic carbon (□) was determined by a TOC analyzer.

inated in the mixed culture after 40 weeks of acclimatization to growth with 6A2NS as sole carbon source.

The doubling time of strain BN9 with 5mM 5AS as sole source of carbon and nitrogen was 2.9 h. After all the 5AS had been consumed, 1.4 mM ammonia was detected in the culture fluid. The concentration of dissolved organic carbon was still 100 mg/liter (from an initial value of 420 mg/liter), and its further decrease to 30 mg/liter required longer incubation periods (≥6 h).

5AS oxidation was completely inhibited by the addition of 2,2′-bipyridyl or 1,10′-phenanthroline (0.8 to 1.0 mM). In crude extracts of 5AS-grown cells of *Pseudomonas* sp. strain BN9, the turnover of 5AS caused the same spectral changes as observed for gentisate. In both cases bathochromic shifts of the absorption maxima (from 318 to 330 nm for gentisate and from 330 to 352 nm for 5AS) were noticed, together with a considerable increase of the peak intensity. However, maleylpyruvate (from gentisate oxidation) was further transformed by the crude extract, whereas the product of 5AS oxidation remained unchanged. Further metabolism was observed only when reduced glutathione or dithiothreitol was added. The enzyme which catalyzed the turnover of 5AS was not gentisate 1,2-dioxygenase, because gentisate-grown cells did not oxidize 5AS, although 5AS-grown cells readily metabolized gentisate. Turnover of 5AS but not of gentisate was inhibited by the oxidation product of 5AS. Therefore, the metabolite could not yet be generated in sufficient amounts to be identified.

Degradation of 6A2NS by a two-species community. When 2NS–6A2NS-induced cells of *Pseudomonas* sp. strain BN6 and 5AS-grown cells of *Pseudomonas* sp. strain BN9 were mixed in a 6A2NS mineral salts medium, the culture fluid turned red, even if cells of the aminosalicylate-degrading strain BN9 were present in large excess. After the cells had been transferred several times into fresh medium, growth rates increased and autoxidation products were avoided. Actually, this two-member community grew with 6A2NS as the sole source of carbon (Fig. 4). At the end of exponential growth a relatively high concentration of dissolved organic carbon (ca. 100 ppm) was still present in the culture fluid. This explains why these cultures were especially susceptible to contamination. Notably, the dissolved organic carbon concentration decreased considerably more rapidly if the two-species culture had been cultivated under nonsterile conditions for a longer period. Contaminants seem to stabilize the growth of strains BN6 and BN9, even though these "escorting" strains (≤15% of the total cell number) do not attack 6A2NS or 5AS. With respect to catabolic efficiency and stability, these cultures resemble the original mixed 6A2NS-degrading community, and the results are similar to older observations with 2NS and 1NS-degrading cultures (3, 4).

Adaptation to growth on 8A2NS. The undefined 6A2NS-degrading mixed culture was repeatedly subcultured on a fresh mineral medium containing 4 mM 6A2NS and 4 mM 8A2NS. Initially, only small amounts (≤5%) of 8A2NS were converted to 3-aminosalicylate (3AS). Growth was increasingly inhibited and stopped completely as soon as 6A2NS was consumed. The growth yield was strictly proportional to the amount of 6A2NS converted.

After 10 to 12 months of adaptation to growth with the substrate mixture, the culture generated a dark-brown coloration while the maximum cell density remained low (optical density at 546 nm, 0.9 to 1.2). HPLC analysis indicated that the substrate mixture was completely consumed and that 3AS accumulated at concentrations close to 3.6 mM. Finally, after 20 months of adaptation, the culture fluid only slightly discolored during growth on the isomeric ANSs, and the final cell density increased considerably (optical density of 546 nm, 2.2 to 2.3).

A typical growth curve is shown in Fig. 5a, which demonstrates that 6A2NS was initially broken down at a higher rate than was 8A2NS, whereas the latter substrate was preferentially metabolized at lower substrate concentrations. Consequently, 6A2NS was still detectable in the culture fluid after all the 8A2NS had been consumed. The initial doubling time of the culture was 4.0 h. Surprisingly, 3AS was only found at very low concentrations (≤0.03 mM), whereas high concentrations of 5AS (ca. 0.2 mM) were transiently excreted into the medium.

Another undefined bacterial community, derived from the same inoculum, was cultured for 26 months with 6A2NS as the sole carbon source. In general, this culture showed a similar growth characteristic when exposed to a mixture of 6A2NS and 8A2NS (Fig. 5b). However, 3AS was not degraded and consequently accumulated in stoichiometric amounts. The initial doubling time was 4.1 h instead of 2.9 h when the culture was grown with 6A2NS as the sole substrate (data not presented).

Surprisingly, the ability to metabolize 8A2NS was also acquired during cultivation of the mixed culture with 6A2NS as the only selective substrate. Therefore, this phenomenon was studied further with a pure culture of *Pseudomonas* sp. strain BN6, which initially (after 10 months of subculture with 6A2NS as the sole carbon source) exhibited very low turnover rates with 8A2NS as substrate (0.03% that of 6A2NS). 8A2NS, however, effectively inhibited the turnover of 6A2NS (Fig. 6a). The turnover rate of 1.9 mM 6A2NS was reduced to 57% by the presence of 1.0 mM 8A2NS. Corresponding to the observations with the undefined mixed culture, after 26 months of subculture of *Pseudomonas* sp. strain BN6 with 6A2NS, the turnover rate of 8A2NS (4-hydroxybenzoate-grown and 8A2NS-induced cells) drastically increased to 88% that of 6A2NS (Fig. 6b).

DISCUSSION

A mixed culture of a water sample from the river Elbe was enriched by selection with 6A2NS. Degradation of this

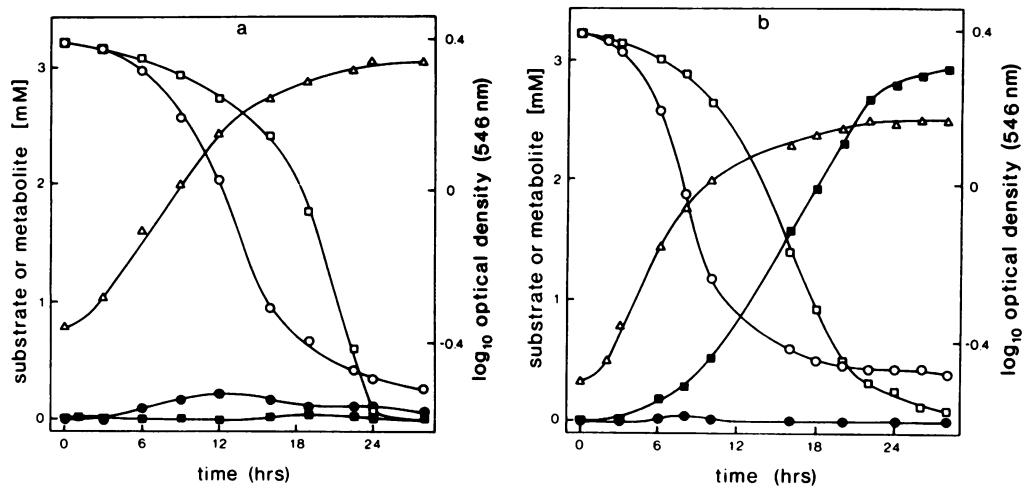

FIG. 5. Utilization of a mixture of 6A2NS and 8A2NS by differently adapted undefined mixed cultures. Media containing equimolar concentrations of 6A2NS (○) and 8A2NS (□) were inoculated with differently adapted precultures. (a) The inoculum was derived from a culture grown for 6 months with 6A2NS and then for 20 months with a mixture of 6A2NS and 8A2NS. (b) The same initial bacterial community as for panel (a) was grown for 26 months with 6A2NS alone. Increase in cell density (△) was determined photometrically at 546 nm. Concentration of ANSs, 5AS (●), and 3AS (■) was measured by HPLC.

xenobiotic compound by the undefined culture can be described by a defined culture of two *Pseudomonas* strains which were the major members of the community after prolonged subcultivation (ca. 12 months). One of the strains, BN6, partially degraded the naphthalene skeleton and utilized carbon atoms-1, -2, and -3. The appearance of 5AS as a metabolite in a pure culture of strain BN6 corresponded mole for mole with the amount of 6A2NS converted. In the mixed culture, the 5AS from 6A2NS oxidation by strain BN6 was totally degraded by the other strain, BN9. Obviously, a mutualistic interaction exists beween these two organisms. On the one hand, strain BN6 generates the growth substrate 5AS for strain BN9. On the other hand, accumulation of 5AS, which causes the precipitation of brown-black polymers (Fig. 1) and, at higher concentrations, inhibits the growth of strain BN6, is avoided as a result of utilization by strain BN9.

Cooxidation of naphthalene-2-carboxylic acid (2NC) by strain BN6 yielded 1,2-dihydroxy-1,2-dihydronaphthalene-2-carboxylate in stoichiometric amounts. This, together with the formation of equimolar amounts of salicylates plus sulfate from different substituted naphthalenesulfonates, indicates that strain BN6 harbors a naphthalene 1,2-dioxygenase with very low substrate specificity but high regioselectivity. As demonstrated for *Pseudomonas testosteroni* A3 or C22, this highly selective mode of 1,2-dioxygenation of naphthalenes occurs if acidic groups such as CO_2H, CH_2CO_2H, or SO_3H are present as substituents in the 1 or 2 position (3,4). 1,2-Dioxygenation of naphthalenesulfonates makes the C—S bond labile, so that sulfite is eliminated spontaneously. As expected for such a mechanism, small amounts of SO_3 were transiently accumulated in the culture fluid of the two-species culture when 6A2NS was used as substrate. Compared with the corresponding activity in *P. testosteroni* A3, the naphthalene dioxygenase of strain BN6 is even less specific because it readily converts a

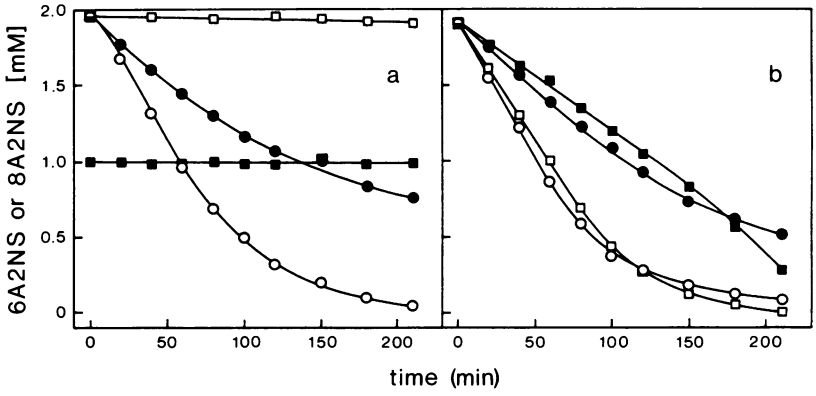

FIG. 6. Inhibition of 6A2NS oxidation by 8A2NS. Exponentially growing cells of *Pseudomonas* sp. strain BN6 were harvested, washed, suspended in phosphate buffer (pH 7.2), and incubated with a mixture of 6A2NS (●) and 8A2NS (■). Turnover of 6A2NS alone (○) and 8A2NS alone (□) was monitored as a control. (a) Strain BN6 subcultured for 10 months with 6A2NS was grown with acetate and induced with 2NS-6A2NS; cell suspensions contained 0.21 mg of protein per ml. (b) Strain BN6, after being subcultured for 25 months with 6A2NS, was grown with 4-hydroxybenzoate and induced with 8A2NS; cell suspensions contained 0.20 mg of protein per ml. 5AS and 3AS were excreted in stoichiometric amounts (data not presented).

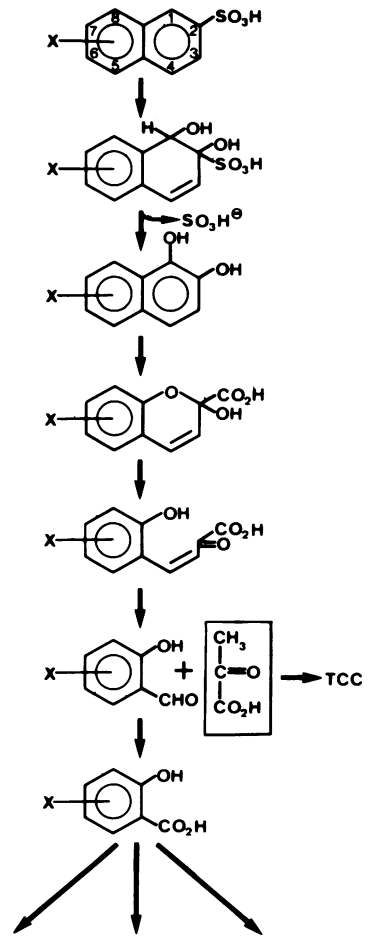

FIG. 7. Degradation of 5-, 6-, 7-, or 8-substituted ANSs or HNSs by *Pseudomonas* sp. strain BN6 and assimilation of salicylates through other bacteria harboring complementary catabolic sequences. X represents —NH_2 or —OH.

broader spectrum of naphthalenesulfonates with OH or NH_2 substituents in the 5, 6, 7, or, after prolonged adaptation to growth with 6A2NS, 8 position. In particular, the 8-substituted compounds were not attacked by strain A3. Since strain A3 had been selected from a naphthalene-degrading population, it could still grow with the unsubstituted parent compound. In contrast, for the adapted strain BN6 (phenotype $6A2NS^+$ $8A2NS^+$) naphthalene is neither a growth substrate nor an inhibitor of naphthalenesulfonate oxidation. Nevertheless, mutual inhibitory effects between differently substituted naphthalenesulfonates and accumulation of equimolar amounts of salicylates from these xenobiotic compounds clearly demonstrate that all these congeners were degraded through the same catabolic sequence (Fig. 7). In principle, this pathway for strain BN6 has already been described as the initial sequence for the catabolism of 2NS (X = H) by *P. testosteroni* A3 (3, 4).

Another important property distinguishes strain BN6 from strain A3. The latter organism totally degraded the carbon skeleton of naphthalene and 2NS via catechol and predominantly via gentisate. These metabolites are formed from salicylate 1-hydroxylase or a salicylate 5-hydroxylase. Both these activities and gentisate 1,2-dioxygenase activity are totally missing in strain BN6, so that naphthalenesulfonates with OH or NH_2 groups in the 6-position conferred an exceptional selective advantage to this organism if 5-hydroxysalicylate or 5AS degraders were coenriched. The inability of strain A3 to grow on 6A2NS and to form stable communities with 5AS degraders is readily explained through cooxidation of amino- or hydroxysalicylates. Fortuitous hydroxylation of these metabolites by salicylate hydroxylases obviously generated hydroxylated products, which are subject to rapid autoxidation. Since salicylate hydroxylases are absent in strain BN6, hydroxy- and aminosalicylates are not further oxidized. Thus, during selection with 6A2NS, *Pseudomonas* sp. strain BN6 can form stable communities with 5AS degraders such as *Pseudomonas* sp. strain BN9.

Strain BN9 can grow with 5-hydroxysalicylate (gentisate) or 5AS as sole carbon source. Although gentisate 1,2-dioxygenase is found in 5AS-grown cells, this enzyme has no activity with 5AS. In contrast, cell extracts of 5AS-grown cells readily convert both 5AS and gentisate, exhibiting similar spectral changes in the photometric test. A gentisate-analogous dioxygenation product from 5AS could not yet be isolated, so that the fate of this metabolite remains to be elucidated.

The original 6A2NS-degrading culture exhibited only marginal activity with 8A2NS. However, during subculture with 6A2NS the growth rate of the culture increased not only for 6A2NS but also for 8A2NS. A similar change was observed with strain BN6. Obviously, turnover rates for 6A2NS and 8A2NS increased simultaneously. Whether this is due to gene-enzyme modification or activation of cryptic genes encoding altered but still isofunctional naphthalene-2-sulfonate 1,2-dioxygenases must await further investigation (see below).

The present data indicate that strain BN6 is rather multifunctional with respect to the degradation of differently substituted naphthalenesulfonates. It converts 2NSs with OH or NH_2 substitutents in the 5-, 6-, 7-, or 8-position into the corresponding salicylates (Fig. 7). An exception is the oxidation of 5A2NS, for which the product was not identical to 6AS (9). Growth through assimilation of pyruvate from C-1, C-2, and C-3 is, for unknown reasons, restricted to 2NS, 6A2NS, 7A2NS, 8A2NS, 5H2NS, and 6H2NS. Total degradation of sulfonated naphthalenes by mixed communities with strain BN6 as a salicylate-generating member is feasible not only for 6A2NS but also for 2NS and 6H2NS, for which salicylate and gentisate as readily degradable metabolites are generated. Since 3AS and 6HS degraders can be isolated from natural habitats (unpublished data), 8A2NS and 5H2NS can totally be degraded by synergistic bacterial communities.

Loss of salicylates by autoxidation during interspecies transfer may be a critical reaction, particularly under practical conditions of biological wastewater treatment. To obviate these uncertainties, immobilization of mixed communities with complementary catabolic routes or construction of hybrid organisms which carry complete catabolic sequences for HNSs and ANSs is under investigation.

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