APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1988, p. 1842–1847
0099-2240/88/071842-06$02.00/0

Vol. 54, No. 7

Degradation of Naphthalene-2,6- and Naphthalene-1,6-Disulfonic Acid by a *Moraxella* sp.

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Received 1 February 1988/Accepted 18 April 1988

A naphthalene-2,6-disulfonic acid (2,6NDS)-degrading *Moraxella* strain was isolated from an industrial sewage plant. This culture could also be adapted to naphthalene-1,6-disulfonic acid as growth substrate. Regioselective 1,2-dioxygenation effected desulfonation and catabolism to 5-sulfosalicylic acid (5SS), which also could be used as the sole carbon source. 5SS-grown cells exhibited high gentisate 1,2-dioxygenase activity. Neither 5SS- nor gentisate-grown cells oxidized 2,6NDS; therefore, 2,6NDS or an early metabolite must serve as an inducer of the initial catabolic enzyme(s).

Naphthalenesulfonic acids are intermediates for the large-scale production of azo dyes, wetting agents, dispersants, etc. This versatile field of application is based, in part, on the sulfonic acid group as a structural element which is introduced by sulfonation and which can be replaced by OH through alkali melt. Furthermore, nitration and subsequent reduction of naphthalenesulfonic acids yield naphthylaminesulfonic acids.

It has been shown that bacteria can be selected from naphthalene-degrading populations which make the carbon-sulfur bond labile through regioselective oxygenation in the 1,2-position (3, 4). Subsequent spontaneous elimination of sulfite removes the xenobiotic character of the naphthalenesulfonic acid. Thus, 1,2-dihydroxynaphthalene was generated from naphthalene-1- or 2-sulfonic acid (1NS or 2NS) and assimilation via the classical naphthalene pathway. Very recently, it has been demonstrated that amino- and hydroxynaphthalene sulfonic acids are also subject to total degradation by mixed cultures harboring complementary catabolic sequences (8). One member of this community was shown to desulfonate amino- or hydroxynaphthalene sulfonic acids by 1,2-dioxygenation and to use carbon atoms 1, 2, and 3 as pyruvates corresponding to the catabolic sequences described for 2NS (4). Amino- or hydroxysalicylates were excreted as dead-end metabolites in stoichiometric amounts and assimilated by other members of the mixed community.

Compounds with an even more xenobiotic character are naphthalene disulfonic acids, which are extremely polar and resistant to biodegradation by nonacclimated microbial populations. The present paper shows that naphthalene-2,6-disulfonic acid (2,6NDS) and naphthalene-1,6-disulfonic acid (1,6NDS) can be totally degraded by a single organism. This is a unique catabolic property because two desulfonation reactions are required. The first desulfonation occurs again by regioselective 1,2-dioxygenation, and the second occurs during hydroxylation of 5-sulfosalicylic acid (5SS). Thus, naphthalene disulfonates are channeled into the gentisate pathway, which has also been found in naphthalene monosulfonic acid catabolism (4).

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MATERIALS AND METHODS

Media. Media were prepared in a mineral base containing per liter: 14 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 50 mg of $Ca(NO_3) \cdot 4H_2O$, 20 mg of ammonium ferric citrate, and 1 ml of a trace solution described by Pfennig and Lippert (9) without iron salts and EDTA. Solid media were prepared by adding 15 g of Ion agar no. 2 (Oxoid Ltd., London, England) to the mineral medium containing the appropriate carbon sources.

Isolation of microorganisms and culture conditions. 2,6NDS-degrading bacteria were selected and grown in batch culture in Erlenmeyer flasks with baffles (1,000 ml with 200 ml of medium). Cultures were incubated at 28°C on a rotary shaker at 150 rpm (type RC 106; Infors AG, Basel, Switzerland). Larger-scale growth of biomass was carried out in 2-liter fermentors (Biolafitte, Maison-Lafitte, France) containing 1 to 1.5 liters of medium. The fermentor was stirred at 200 to 400 rpm and aerated at a rate of 0.2 to 0.3 liter/min. For cooxidation of naphthalene-2,6-dicarboxylic acid (2,6NDC), cells were grown in a 10-liter fermentor (Biostat; Braun, Melsungen, Federal Republic of Germany).

Continuous cultures were grown in 250-ml chemostats. Fresh medium was added continuously by means of a peristaltic pump (model 12,000; Varioperpex; LKB, Bromma, Sweden). Polyethylene glycol at a final concentration of 0.001% (vol/vol) was added to the medium as an antifoaming agent.

Measurement of growth. Growth of the culture was measured spectrophotometrically at 546 nm.

Protein estimation and enzyme assays. The protein content of whole cells was determined by the method of Schmidt et al. (10). Cell extracts and enzyme assays were prepared as described previously (4). Bovine serum albumin was used as a standard for the determination of soluble protein by the method of Bradford (2).

Analytical methods. Sulfate ion concentration in the culture was determined by the method of Bertolacini and Barney (1) which was modified as described recently (8). A standard solution of sodium sulfate in 80 mM phosphate buffer (pH 7.0) was used for calibration. The sulfite concentration in the culture fluid was measured by the method of Johnston et al. (7).

Naphthalenesulfonic acids and metabolites were analyzed by high-pressure liquid chromatography (HPLC) (chromatograph, model 4200; Varian Aerograph, Palo Alto, Calif.). The eluate was examined with a Schoeffel GM 770 detector (Schoeffel, Westwood, N.Y.). Signals were analyzed by use of a computing integrator (Autolab System I; Spectra Physics, Santa Clara, Calif.). A HIBAR reverse-phase column (internal diameter, 250 by 4 mm, octadecylsilane chemically bonded to LiChrosorb 10-μm particles; E. Merck AG, Darmstadt, Federal Republic of Germany) was used to separate individual compounds. Solvent A, containing H_3PO_4 (0.01 M) and propanol-2 (1%, vol/vol) in degassed distilled water, was used as the mobile phase. Solvent B contained methanol (90%) and solvent A (10%, vol/vol). The elution rate was adjusted to 40 ml/h. Samples of 5 μl of culture fluid were injected after cells had been removed by centrifugation for 3 min at 16,000 × *g*. Metabolites were identified with authentic compounds by their retention time and in situ scanning of the UV spectra after the flow had been stopped.

UV spectra, enzyme assays, and absorption measurements were made on a Uvikon 820 spectrophotometer (Kontron AG, Zurich, Switzerland). Metabolites were isolated, purified, methylated, and identified by infrared, nuclear magnetic resonance, and low- and high-resolution mass spectra as described by Brilon et al. (4).

Chemicals. Substituted naphthalenes were obtained from Bayer AG, Leverkusen, Federal Republic of Germany. 4-Hydroxyisophthalic acid was purchased from ICN Pharmaceuticals, Inc., Plainview, N.Y. Mitomycin C was supplied by Sigma Chemical Co., St. Louis, Mo. 2,6NDC was derived from its dimethyl ester (EGA Chemie, Steinheim, Federal Republic of Germany) by saponification in alkaline methanolic solution. All other chemicals used for mineral salts media and buffer solutions, such as silica gel type 60PF 254 for preparative thin-layer chromatography, were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS AND DISCUSSION

Enrichment of 2,6NDS-degrading cultures. 2,6NDS-degrading bacterial cultures were isolated by direct enrichment in stationary culture from activated sludge taken from the sewage treatment plant of Bayer Leverkusen, Federal Republic of Germany. One of the isolates, strain ASL4, was identified as a *Moraxella* species on the basis of the following morphological and biochemical properties. It formed nonmotile, oxidase- and catalase-positive, gram-negative plump rods (1.5 μm wide by 2.5 μm in length) usually occurring in pairs. No spores were formed. The organism was highly sensitive to penicillin (≥10 U/ml). Carbohydrates, amino acids, and most metabolites of glycolysis and the tricarboxylic acid cycle, except fumarate, were not utilized. A number of aromatic substrates, such as 2,6NDS, gentisate, protocatechuate, 5-carboxysalicylate, 5SS, and 3-hydroxbenzoate, were accepted as sole carbon sources. The organism could not grow on complex media like nutrient broth or peptone.

Growth of *Moraxella* strain ASL4 with 2,6NDS and 1,6NDS. *Moraxella* strain ASL4 grew in a mineral salts medium with 2,6NDS as the sole source of carbon and energy (Fig. 1). In the course of the exponential growth phase (t_d, 7.5 h), sulfite was detected in small amounts. This ion was rapidly oxidized to sulfate. Finally, after consumption of 2,6NDS, both sulfonic groups of the substrate were present in the medium as sulfate in quantitative amounts. Strain ASL4 could also be adapted to utilize the isomeric compound 1,6NDS as the sole carbon source by stepwise replacement of 2,6NDS by 1,6NDS as the growth substrate. After an adaptation period of 15 weeks (approximately 250 generations), the culture could grow with 1,6NDS as the only carbon source. The growth curve was very similar to that observed with 2,6NDS (Fig. 1). However, the doubling time was slightly longer with 1,6NDS (11 h) and the maximum sulfite concentration in the late exponential growth phase was ≤25 μM. This culture could still grow with 2,6NDS; however, without further readaptation to this substrate a slightly increased doubling time (9.5 h) was observed. The 1,6NDS-grown culture of *Moraxella* strain ASL4 could grow with an equimolar mixture of 2,6NDS and 1,6NDS, with the former being the preferred substrate (Fig. 2). That both substrates compete for the same initial enzyme is indicated by an experiment shown in Fig. 3. 2,6NDS-grown cells of strain ASL4 were washed and incubated with an equimolar mixture of 2,6NDS and 1,6NDS. Obviously, 1,6NDS did not disappear from the culture fluid before the major part of 2,6NDS had been taken up (≤0.2 mM). Similar competition and mutual inhibitory effects had been observed between 2NS and 1NS as substrates for *Pseudomonas testosteroni* C22 (4) or between 6-aminonaphthalene-2-sulfonic acid and 2NS as substrates for *Pseudomonas* strain BN6 (8).

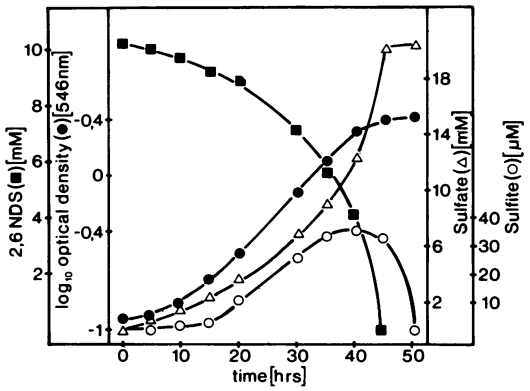

FIG. 1. Growth of *Moraxella* strain ASL4 with 2,6NDS. A mineral salts medium with 2,6NDS as the sole carbon source was inoculated with an exponentially growing culture of strain ASL4. Growth was followed photometrically at 546 nm. The concentration of 2,6NDS was measured by HPLC. Sulfate was determined by the method of Bertolacini and Barney (1); sulfite was quantified by the method of Johnston et al. (7).

Relative rates of oxidation of substituted naphthalenes. 2,6NDS-grown cells of strain ASL4 exhibited low activities for other naphthalenesulfonic acids with the exception of 1,6NDS, 6-hydroxynaphthalene-2-sulfonic acid, 6-aminonaphthalene-2-sulfonic acid, and 2,6NDC. 2,6NDC is the only substrate which is transformed into a product (dead end product) that can be detected in the culture fluid. Specific activities of oxidation of different substrates were assayed polarographically (Table 1). It is noteworthy that strain ASL4 exhibited very low activities with 2NS and with naphthalene. Those activities were completely lost after the strain had been cultivated with 2,6NDS as the sole carbon source over a period of 20 months.

Mechanism of desulfonation. Catabolism of naphthalene (6), of 2NS and 1NS (3, 4) and of amino- and hydroxynaph-

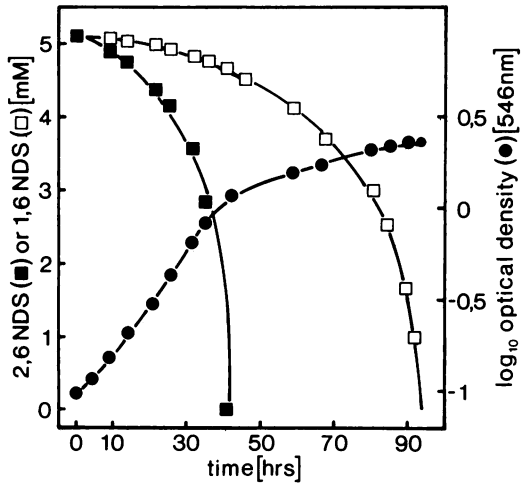

FIG. 2. Utilization of a mixture of 2,6NDS and 1,6NDS by a 1,6NDS-pregrown culture of strain ASL4. The mineral salts medium containing equimolar concentrations of 2,6NDS and 1,6NDS was inoculated with a culture growing exponentially with 1,6NDS as the sole carbon source. The increase in cell density was determined photometrically at 546 nm. Concentrations of disulfonic acids were monitored by HPLC.

thalene-2-sulfonates (8) is initiated by 1,2-dioxygenation of the aromatic nucleus. With naphthalenesulfonates as growth substrates, the naphthalene skeleton is dioxygenated in such a way that a 1,2-dihydroxy-1,2-dihydronaphthalene-2-sulfonate is generated. This hypothetical intermediate could eliminate sulfite spontaneously. According to published observations of monosulfonated naphthalenes (3, 4, 8), a corresponding initial 1,2-dioxygenation mechanism could be demonstrated by cooxidation of 2,6NDC as a structural analog of 2,6NDS. 2,6NDS-grown cells of strain ASL4 readily cooxidized 2,6NDC.

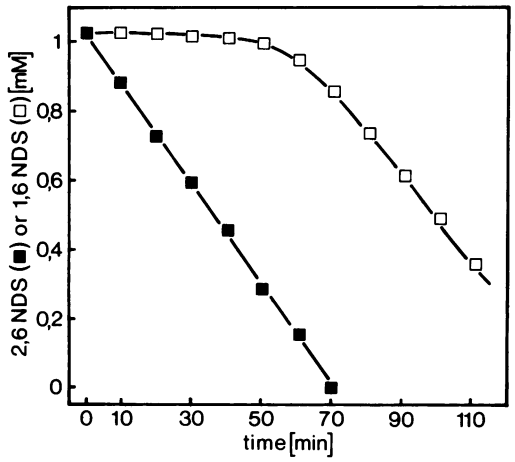

FIG. 3. Turnover of 2,6NDS by *Moraxella* strain ASL4 in the presence of 1,6NDS. Cells were grown in mineral salts medium with 2,6NDS (10 mM) as the sole carbon source. The cells were harvested during late exponential growth and resuspended in phosphate buffer (pH 7.3, 50 mM) containing 2,6NDS and 1,6NDS. Substrate concentrations were determined by HPLC. Cell density corresponded to 1.4 mg of protein per ml.

TABLE 1. Oxidation of naphthalene and substituted naphthalenes by resting cells of *Moraxella* strain ASL4 grown with 2,6NDS[a]

Substrate	Relative activity (%)[b]
2,6NDS	100
1,6NDS	92
Naphthalene-1,5-disulfonic acid	7
Naphthalene-1,4-disulfonic acid	≤5
Naphthalene-2,7-disulfonic acid	≤5
1NS	≤5
2NS	6
6-Hydroxynaphthalene-2-sulfonic acid	34
6-Aminonaphthalene-2-sulfonic acid	21
Naphthalene-1,3,5-trisulfonic acid	≤5
Naphthalene-1,3,6-trisulfonic acid	≤5
2,6NDC	10
Naphthalene	≤5

[a] Cells were grown in mineral salts medium containing 10 mM 2,6NDS as the sole carbon source and harvested during the late exponential growth phase. Cells were suspended in phosphate buffer (50 mM, pH 7.3), corresponding to an optical density at 546 nm of 1.5.

[b] Activity in relation to that of 2,6NDS. The specific oxygen uptake rate of this substrate was 173 μmol of O_2 per min per g of protein. Relative activities described as ≤5% were too low to be determined more accurately by this method.

Obviously, these two substrates competed for the same initial catabolic enzyme because 2,6NDC turnover was reversibly inhibited by 2,6NDS and the catabolic activity was fully restored after consumption of 2,6NDS (Fig. 4). To identify the oxidation product of 2,6NDC, *Moraxella* strain ASL4 was grown in a 10-liter fermentor with 2,6NDS (10 mM), harvested at the end of the exponential growth phase, and suspended in phosphate buffer (pH 7.3; 50 mM). The cell density was adjusted to an optical density at 546 nm of 10. 2,6NDC (3 mmol) was cooxidized within 6 h at 28°C. Cells were removed by centrifugation. The reaction product was

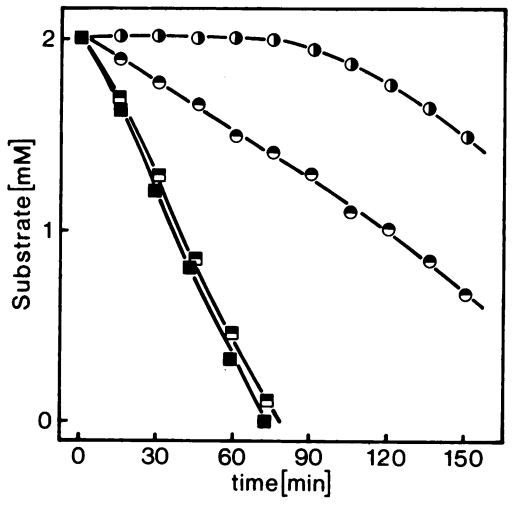

FIG. 4. Turnover of 2,6NDC by resting cells of *Moraxella* strain ASL4 in the presence of 2,6NDS. Cells were grown with 2,6NDS as the sole carbon source and used as described for Fig. 3. The cell suspension (1.4 mg of protein per ml) was incubated at 30°C with a mixture of 2,6NDS and 2,6NDC (initial concentrations were 2.0 mM each). The disappearance of 2,6NDC (◑) and 2,6NDS (■) from the culture fluid was monitored by HPLC. The uptake of 2,6NDC (◓) and 2,6NDS (■) alone was used as a control.

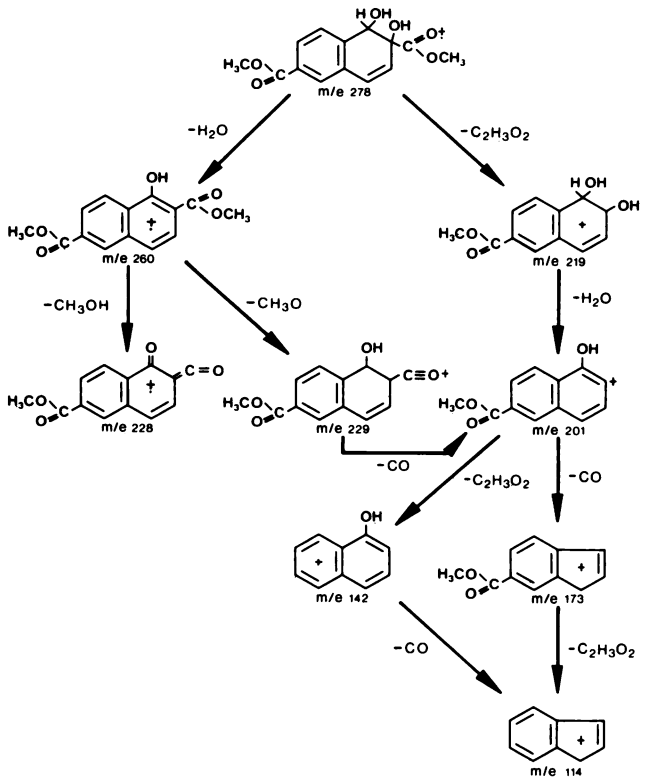

FIG. 5. Suggested pattern of fragmentation of 1,2-dihydroxy-1,2-dihydronaphthalene-2,6-dicarboxylic acid dimethyl ester derived from characteristic ions of the mass spectrum.

extracted from the acidified (pH 2) supernatant with ethyl acetate. After being methylated by the addition of a small excess of ether diazomethane (5) and purified by preparative thin-layer chromatography (silica gel 60PF 254; solvent system, acetone-chloroform, 80:20), the methyl ester crystallized from light petroleum ether (60 to 80°C). The methylated product was identified as 1,2-dihydroxy-1,2-dihydronaphthalene-2,6-dicarboxylic acid dimethylester (mp 141°C, uncorrected). Physical evidence for the identity of the product was provided by high-resolution mass spectrometry (determined mass, 174.3195, corresponding to the brutto formula $C_{16}H_{18}O_4$). The main fragmentation pattern, in accordance with the structure, is given in Fig. 5. Further identification was obtained by proton magnetic resonance spectrometry (Table 2) and infrared spectral data (νOH, 3520 cm^{-1}; νCO, 1,760 and 1,720 cm^{-1}). UV absorption maxima in methanol were observed at 258 nm (ε 4,540) and 229 nm (ε 27,700). The structure of the cooxidation product clearly indicates that 2,6NDS catabolism is initiated by a dioxygenase which is rather unspecific but highly regioselective. The initial dioxygenation product of 2,6NDS must be 1,2-dihydroxy-1,2-dihydronaphthalene-2,6-disulfonic acid, which, in contrast to the analogous dicarboxylic acid, eliminates 1 mol of sulfite spontaneously with rearomatization (see Fig. 7).

TABLE 2. Proton magnetic resonance data of 1,2-dihydroxy-1,2-dihydronaphthalene-2,6-dicarboxylic acid dimethyl ester[a]

Proton	Chemical shift (ppm)	Description	Formula
1	5.18	1 H (s, hydroxymethine)	
2	7.66	1 H (d, OH, $J_{1,2}$ = 8 Hz)	
3	7.96	1 H (d, OH, $J_{1,2}$ = 8 Hz)	
4	3.91	6 H (2 s, CH_3)	
5	5.92	1 H (d, olefinic, $J_{5,6}$ = 10 Hz)	
6	6.69	1 H (d, olefinic, $J_{6,5}$ = 10 Hz)	
7	7.79	1 H (s, aromatic)	
8	7.67–8.00	2 H (m, aromatic)	

[a] The compound was dissolved in deuterochloroform, and the spectra were recorded at 100 MHz.

5SS from 2,6NDS. Metabolites were not detected in the culture fluid if growing or resting cells of strain ASL4 oxidized 2,6NDS. However, cultures which had passed the exponential growth phase developed an unusual brownish color if an excess of 2,6NDS was available. Under these conditions, a single metabolite was detected in the culture fluid. This was identified with authentic 5SS by HPLC (solvent system 20% solvent B: retention time 130 s or 50% solvent B: retention time 100 s, flow rate, 40 ml/h). The UV spectrum (λ_{max} 238 and 300 nm) was measured in situ under stopped flow conditions.

Although 5SS is an extremely polar metabolite, strain ASL4 could take up this substrate instantaneously and grow with it on solid media at a concentration ≤5 mM. Growth in batch culture proved extremely difficult because of the toxicity of 5SS. A pronounced growth inhibitory effect of 5SS on 2,6NDS-utilizing cells of strain ASL4 was noticed already at a concentration of ≤0.1 mM. At ≤1.0 mM, 5SS growth was totally inhibited. Nevertheless, *Moraxella* strain ASL4 could utilize 5SS in batch culture if the substrate was introduced in portions at intervals so that the actual concentration in the culture fluid never exceeded 0.1 mM. Thus optical densities ≤0.5 could be attained after ≤5 mM 5SS had been degraded.

Alternatively, the organism was readily grown with 5SS in continuous culture. A chemostat was run at a dilution rate of D = 0.04 (h^{-1}). The concentration of 5SS in the reservoir

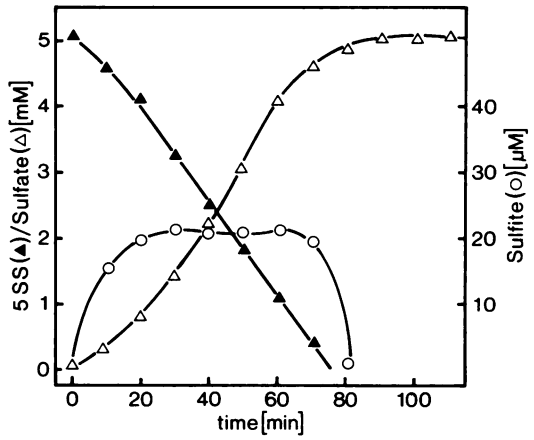

FIG. 6. Turnover of 5SS by 2,6NDS-grown cells of *Moraxella* strain ASL4. Cells were grown in mineral salts medium with 2,6NDS (10 mM) as the sole carbon source and harvested during late exponential growth. They were washed twice in phosphate buffer (pH 7.3, 50 mM) to remove residual 2,6NDS and resuspended (optical density at 546 nm, 2; corresponding to 1.45 mg of protein per ml). 5SS was added (initial concentration, 5 mM) and its uptake was monitored by HPLC. Sulfite and sulfate were determined by the methods given in the legend to Fig. 1.

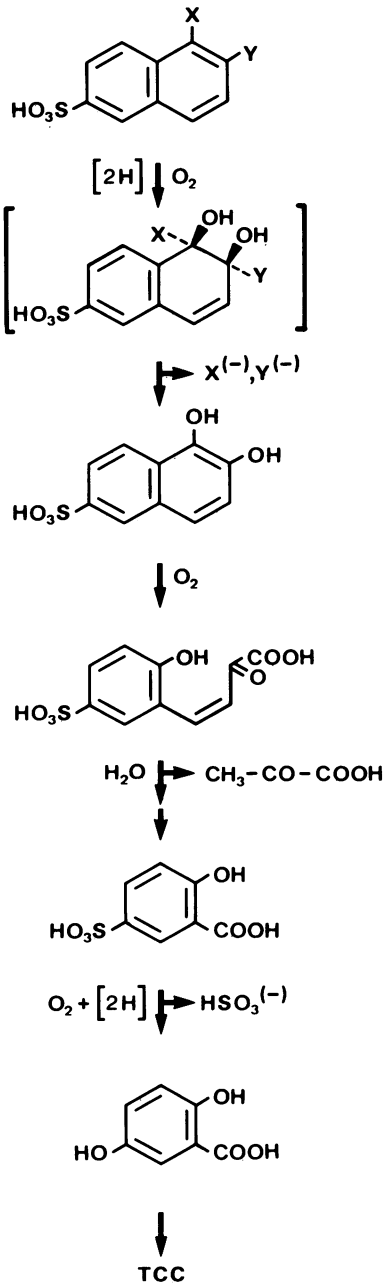

FIG. 7. Proposed pathway for the degradation of 1NS and 2NS (X or Y = SO_3H) by *Moraxella* strain ASL4.

was 0.1 mM, and after acclimation of the system over a period of 1 week, it was increased in a stepwise fashion up to 10 mM. Under steady-state conditions, the maximum optical density at 546 nm was 1.4. The critical dilution rate (D_c) value was attained at a dilution rate of 0.35 h^{-1}, which corresponded to a doubling time of 5 h.

Desulfonation of 5SS. To investigate the mechanism of desulfonation of 5SS, 2,6NDS-grown cells of *Moraxella* strain ASL4 were incubated with excess amounts of 5SS at a concentration (5 mM) which would not allow growth (Fig. 6). Sulfate was excreted into the medium in stoichiometric amounts with transient accumulation of small amounts of sulfite. During turnover of 5SS, small amounts of gentisate (≤0.4 mM) were detected in the culture fluid by HPLC. This metabolite was identified by comparison with an authentic compound by its retention time and UV spectrum measured in situ.

Although 2,6NDS-grown cells of *Moraxella* strain ASL4 exhibited high activities for 5SS, neither cell extracts nor the particulate fraction from disrupted cells (French press) was able to oxidize 5SS alone or in the presence of NADPH. That gentisate may be generated from 5SS by an oxygenolytic elimination of sulfite is indicated by the ability of 2,6NDS- or 5SS-grown cells to eliminate nitrite from 5-nitrosalicylate. Cooxidation of this structural analog corresponds to a mechanism described by Spain et al. (11) for the enzymatic oxidation of 4-nitrophenol by a *Moraxella* species capable of growth with this xenobiotic compound.

2,6NDS- and 5SS-grown cells of *Moraxella* strain ASL4 took up oxygen at the expense of gentisate. Correspondingly high gentisate 1,2-dioxygenase activities were measured (1,900 μmol/min per g of protein) in cell extracts of this organism. In contrast, no activity was found for catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, salicylate 1- or 5-hydroxylase, or any protocatechuate dioxygenase.

Induction of catabolic activities. The 2,6NDS-degrading activity of strain ASL4 was not constitutive and could be induced only with 2,6NDS. Neither 5SS nor gentisate served as an inducer, in contrast with organisms degrading naphthalene monosulfonic acids. The proposed catabolic pathway of 2,6NDS or 1,6NDS (Fig. 7) corresponds to the pathway discussed for the catabolism of 1NS or 2NS. The major part of these substrates was degraded via gentisate rather than salicylate. The latter metabolites proved to be potent inducers of the enzymes of the entire pathway (4). Hydroxy- and aminosalicylates are also indispensable as inducers for enzymes of hydroxy- and aminonaphthalene sulfonate catabolism (8). Catabolism of naphthalene disulfonic acid is unique because one of the sulfonic acid groups was eliminated at a late catabolic step, probably by oxygenation of sulfosalicylic acid. Furthermore, the catabolic enzymes of the disulfonic acid pathway were induced by the disulfonic acid itself or an early metabolite but not by 5SS or gentisate.

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