

3-Nitrobenzenesulfonate, 3-Aminobenzenesulfonate, and 4-Aminobenzenesulfonate as Sole Carbon Sources for Bacteria

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Aerobic, carbon-limited, enrichment cultures containing 3-aminobenzenesulfonate or 3-nitrobenzenesulfonate as the sole source of carbon and energy yielded growth and complete substrate disappearance. Pure cultures of putative pseudomonads were isolated which utilized these compounds quantitatively. Degradation was compared with that of 2- and 4-aminobenzenesulfonate.

Aminobenzenesulfonates (ABSs) are widely used precursors in the manufacture of dyes and optical brighteners; 3-nitrobenzenesulfonate (3-NBS) is the precursor of metanilic acid (3-ABS) and is commonly used as a mild oxidant (10). Sulfonated aromatics are important water pollutants (11), which reflects a limited biodegradability (1), and amino- and nitro-substituted benzenesulfonates are expected to be more persistent than unsubstituted analogs (7). Oxygen uptake by bacterial cells in the presence of 3- and 4-ABS has been observed (15), but no proof of mineralization of any ABS was available until we isolated bacteria able to degrade 2- and 4-ABS completely (16). No utilization of 3-ABS as a carbon source was observed (16). Although bacterial metabolism of substituted nitrophenols has been reported (17), we know of no reports on the utilization of NBSs as carbon sources; however, 3-NBS (and 3-ABS) is subject to desulfonation (18). We now report isolation of bacteria and mass balances for the complete utilization of 3-NBS and 3-ABS as sole sources of carbon and energy in pure culture.

The NBSs used were purchased from Tokyo Chemical Industry (Tokyo, Japan), 3-ABS was obtained from Eastman Kodak, Co. (Rochester, N.Y.), 4-hydroxybenzenesulfonate was from BDH (Poole, England), and all other sulfonates were from Fluka (Buchs, Switzerland). All sulfonates were supplied as $\geq 98\%$ pure and were chromatographically pure. The sources of other chemicals are given elsewhere (16).

The mineral-salts medium (16) routinely contained an aromatic sulfonate (3 mM) as the sole source of carbon and energy. The unidentified bacterium strain S-1 (16) was used in some experiments. Organisms were stored at -80°C in 50% (vol/vol) glycerol. The enrichment procedure, isolation, and cultivation of organisms (16) were augmented by enrichment cultures at high ionic strength (with 2% [wt/vol] NaCl or Na_2SO_4 in the growth medium). The inocula were from an industrial sewage plant that treated sulfonated chemical waste (Ciba-Geigy AG, Basel, Switzerland). Enrichment cultures were evaluated after measurement of growth (optical density) and of substrate disappearance by reversed-phase high-pressure liquid chromatography (6, 16); sulfonates were eluted isocratically with 100 mM potassium phosphate buffer (pH 2.2) (for ABS) or buffer containing 20% (vol/vol) methanol (for NBS). Bacterial growth yields with limiting carbon sources and growth kinetics of the isolated organisms were examined as previously described (16). Protein, ammonium ion, sulfate ion, and sulfite ion were quantified by colorimetric methods cited elsewhere (16);

sulfate ion was also measured as suspended BaSO_4 precipitation (12). Nitrite was assayed colorimetrically (4), and its identity was confirmed by reversed-phase high-pressure liquid chromatography (8). Dissolved organic carbon was measured by standard methods (5). Cells for studies in nongrowing suspensions and cell-free extracts were grown in 2-liter Fernbach flasks, harvested in the late exponential phase ($9,000 \times g$, 20 min, 4°C), washed twice in cold 50 mM potassium phosphate buffer (pH 7.0), and used directly or stored at -20°C . Assay mixtures (initial volume, 10 ml) contained 500 μmol of potassium phosphate buffer (pH 7) and 150 mg of protein (whole cells or soluble protein from cells disrupted in a French pressure cell), and the reaction was started by adding 10 to 20 μmol of substrate. Reaction mixtures were shaken at 30°C in a water bath, and samples (1 ml) were taken at intervals to determine concentrations of substrate, sulfite ion, and sulfate ion after the removal of cells ($10,000 \times g$, 5 min, 4°C) or protein (16). Substrates were stable in control reactions, and biological material did not interfere in the determinations. Limited identification of the isolates was done (3), and Oxi/Ferm Tubes (Hoffmann-La Roche, Basel, Switzerland) and L-alanine-aminopeptidase (Bactident; Merck, Darmstadt, Federal Republic of Germany) were used.

Aerobic enrichment cultures to degrade 3-ABS or 3-NBS were done at 30°C in the presence and absence of 2% NaCl or Na_2SO_4 ; cultures were inoculated (10% [vol/vol]), incubated for 3 weeks, subcultured twice more into homologous medium, and evaluated. No degradation of sulfonate was observed under halophilic conditions. In contrast, partial degradation of 3-NBS was observed at low ionic strength, and full degradation was obtained by the addition of 0.1% (wt/vol) yeast extract. The yeast extract could not be replaced by a standard vitamin solution (13) and was not needed for growth on 3-NBS-minimal agar plates. The organism degrading 3-NBS, strain N-1, was isolated by alternate culturing in liquid and on solid selective medium. Purity was confirmed by plating on plate count agar; strain N-1 had a very narrow substrate spectrum, failing to grow with, e.g., acetate and succinate. 3-ABS was not degraded in the enrichment cultures, but a neglected sample of nonsterile 3-ABS-salts medium developed turbidity and the 3-ABS was totally degraded. The organism degrading 3-ABS, strain M-1, was isolated by alternately culturing in 3-ABS-salts medium and on plate count agar.

The isolates were rod-shaped bacteria that were gram-negative as judged by staining, by the L-alanine-aminopeptidase test, by growth on MacConkey agar no. 3, and by

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TABLE 1. Utilization of benzenesulfonates as sole sources of carbon and energy by pure cultures of bacteria

Sulfonate tested	Utilization ^a by strain:			
	N-1 ^b	M-1	S-1 ^c	O-1 ^c
2-ABS	—	—	—	+
3-ABS	—	+	—	—
4-ABS	—	—	+	—
2-NBS	—	—	—	—
3-NBS	+	—	—	—
4-NBS	—	—	—	—
4-Hydroxybenzenesulfonate	—	—	+	—
4-Methylbenzenesulfonate	—	+	—	+
Benzenesulfonate	—	+	—	+

^a Evaluated by measurement of growth (protein) and complete disappearance of the substrate.

^b Growth medium contained 0.1% yeast extract.

^c Data in part from Thurnheer et al. (16).

electron microscopy. Tests with Oxi/Ferm tubes led to the preliminary identification of strain M-1 as *Pseudomonas acidovorans* (Oxi/Ferm key 10007 and polar flagellae) and of strain N-1 as *Pseudomonas* sp. (Oxi/Ferm key 10005). Strain S-1 (16) showed characteristics of the *P. acidovorans* group (Oxi/Ferm key 10007) but was nonmotile.

Each strain had a narrow substrate range for benzenesulfonates (Table 1). No NBS or ABS, except that supplied in the enrichment, was degraded. Substrate disappearance was complete, and the negligible amounts of residual dissolved organic carbon (<5%) indicated complete substrate utilization. Growth of the strains M-1, N-1, and S-1 was proportional to the concentration of the added substrate (up to 6 mM for 3-ABS and 4-ABS and up to 3 mM for 3-NBS), and growth yields in the range of 3 to 4.6 g of protein per mol of carbon were observed, similar to control experiments (Table 2), thus confirming complete conversion of the substrate carbon to cell material and CO₂ (2). Growth with 3-NBS, 3-ABS, and 4-ABS was exponential (data not shown), and the specific degradation rates (Table 2) were similar to the rates for 2-ABS.

The sulfur from the sulfonate group was recovered quantitatively (96 to 100%) as sulfate at the end of growth. The amino group of 3- and 4-ABS was recovered in part (50 to 70%) as ammonium ion; the remainder was calculated to be in biomass. The nitro group of 3-NBS was recovered in 90% yield as nitrite; neither nitrate nor an organonitro compound was observed.

TABLE 2. Utilization of ABSs and NBS by four bacteria

Organism	Substrate	Growth rate μ (h ⁻¹)	Specific degradation rate (mkat/kg of protein)	Yield (g of protein/mol of carbon)
N-1 ^a	3-NBS	0.09	1.3	3.0
N-1	3-Hydroxybenzoate	0.23	2.0	4.5
O-1 ^b	2-ABS	0.10	1.5	5.6
M-1	3-ABS	0.06	0.8	3.3
M-1	Succinate	0.82	17.7	3.2
S-1	4-ABS	0.06	0.6	4.6
S-1	2-Aminobenzoate	0.17	1.4	4.6

^a Medium for N-1 contained 0.1% yeast extract.

^b Data from Thurnheer et al. (16).

4-ABS and 4-hydroxybenzenesulfonate were desulfonated by 4-ABS-grown cells of strain S-1 in suspension. Sulfate liberation was concomitant with substrate disappearance, and degradative rates of 20 μ kat/kg of protein for 4-ABS and 10 μ kat/kg of protein for 4-hydroxybenzenesulfonate were observed. No sulfite (<0.05 mM) was detected, but we presume it to be released and oxidized (9, 14). At least one unidentified transient intermediate per substrate was detected by high-pressure liquid chromatography during the degradation of 4-ABS (λ_{\min} , 219 nm; λ_{\max} , 251 nm) and 4-hydroxybenzenesulfonate (λ_{\min} , 218 nm; λ_{\max} , 233 nm; λ_{\min} , ca. 255 nm; λ_{\max} , 280 nm); their high polarity at pH 2.2 suggests charged species. No degradation of 4-ABS was observed in cell extracts of strain S-1, but 4-hydroxybenzenesulfonate was desulfonated (10 μ kat/kg of protein); the sulfonate group was recovered quantitatively as sulfate, and no sulfite was observed.

The isolation from industrial wastewater treatment plants of bacteria able to utilize 3-ABS and 3-NBS shows that the biological potential to degrade these compounds is present in that specific habitat. The low degradation rates observed, however, and the failure to obtain salt-tolerant cultures indicate that these compounds are at best poorly degraded in the halophilic environment of industrial wastewater treatment plants. The narrow substrate ranges (Table 1) (16) indicate that application of bacteria to treat sulfonated waste requires almost as many organisms as compounds. Bacteria with wide substrate spectra, rapid degradation rates, and salt tolerance could perhaps be developed (15a), but this would require a much better understanding of the degradative pathways and enzymes and their genes involved in sulfonate biodegradation.

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