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Reduction of Nitrosubstituted Aromatic Compounds by the Halophilic Anaerobic Eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*

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The moderately halophilic, obligately anaerobic eubacteria *Haloanaerobium praevalens* DSM 2228 and *Sporohalobacter marismortui* ATCC 35420 are able to reduce a variety of nitrosubstituted aromatic compounds at a high rate to the corresponding amines. Compounds degraded included nitrobenzene, *o*-nitrophenol, *m*-nitrophenol, *p*-nitrophenol, nitroanilines, 2,4-dinitrophenol, and 2,4-dinitroaniline. Most of these compounds, when added at concentrations of 50 to 100 mg/liter, were completely transformed within 24 h, but at the highest concentrations growth rates were somewhat lowered. Growth of *H. praevalens* in the presence of ^{14}C-labeled *p*-nitrophenol showed that the compound was not incorporated by the cells or degraded to acid-volatile compounds.

Nitrosubstituted aromatic compounds such as nitrobenzene and nitrophenols are widely used in the manufacture of herbicides, fungicides, insecticides, azo dyes, explosives, and pharmaceuticals. Nitroaromatic compounds are released into the environment during the hydrolysis of several organophosphorus pesticides, such as parathion. Nitrophenols and other nitrosubstituted aromatic compounds are generally considered to be highly resistant to microbial degradation. Slow degradation of nitroaromatic compounds in fresh water and in sewage sludge, under aerobic as well as under anaerobic conditions, has been reported (5, 8, 9, 17). A few aerobic bacteria (most of them belonging to the genus *Pseudomonas*) have been shown to degrade nitrophenols in pure culture; in this process the nitro group is released as nitrite, and then the aromatic ring is oxidized (6, 21, 22). Even less is known about anaerobic bacteria that degrade nitrophenols and other nitrosubstituted aromatic compounds. To our knowledge no anaerobic bacteria have been reported to completely break down nitroaromatic compounds in pure culture; a few reports of bacterial consortia degrading nitrophenols to methane exist (5, 19), and it has been suggested that the initial step in the process is a reduction of the nitro group to an amino group (19). A number of bacteria have been shown to reduce nitrosubstituted aromatic compounds. These include obligately anaerobic *Clostridium* and *Eubacterium* species (2, 15) and also aerobic *Pseudomonas* species. In the last case, the nitro group may be a substitute for oxygen in anaerobic respiration (16).

In the course of our studies on obligately anaerobic halophilic eubacteria belonging to the family *Haloanaerobiaceae* (13, 14), it was found that *Haloanaerobium praevalens* and *Sporohalobacter marismortui* rapidly reduced nitrobenzene, *p*-nitrophenol, and other nitroaromatic compounds. In this article, we describe the range of compounds reduced and provide information on the nature of the process.

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MATERIALS AND METHODS

Chemicals. Nitrobenzene and *o*-nitrophenol were obtained from BDH, Poole, England; *o*-aminophenol, *m*-aminophenol, *p*-aminophenol, *o*-nitroaniline, *m*-nitroaniline, *p*-nitroaniline, and 2,4-dinitroaniline were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. *p*-Nitrophenol and *p*-nitroaniline were from Riedel-De Haen, Hannover, Germany. *m*-Nitrophenol, 2,4-dinitrophenol and [U-^{14}C]*p*-nitrophenol were obtained from Sigma Chemical Co., St. Louis, Mo. Tryptone and yeast extract were purchased from Difco Laboratories, Detroit, Mich.

Bacterial strains and culture conditions. *H. praevalens* DSM 2228 (20) was obtained from J. G. Zeikus (Michigan Biotechnology Institute, Lansing) and was grown in medium (pH 7.0) containing the following ingredients (in grams per liter): NaCl, 130; KCl, 1.0; $MgSO_4 \cdot 7H_2O$, 8.8; tryptone, 10; yeast extract, 10; L-cysteine hydrochloride, 0.5; and glucose, 2.5. *S. marismortui* ATCC 35420 (14) was grown in anaerobic medium (pH 6.5) with the following composition (in grams per liter): NaCl, 140; $MgCl_2 \cdot 6H_2O$, 20.3; $CaCl_2 \cdot 2H_2O$, 7.35; KCl, 3.7; glucose, 5.0; yeast extract, 5.0; L-cysteine hydrochloride, 0.5; and PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer to a final concentration of 25 mM. All anaerobic media were prepared by boiling under nitrogen, and anaerobic culture techniques described by Balch et al. (3) were used throughout. The glucose and PIPES buffer were added from concentrated autoclaved anoxic solutions to the autoclaved medium. The cells were grown at 37 or 40°C in 25-ml stoppered serum tubes (3) containing 10 ml of growth medium under a gas phase of nitrogen. *Escherichia coli* K-12 was grown anaerobically in tubes completely filled with medium (pH 7.3) containing 4.0 g of tryptone per liter, 2.0 g of yeast extract per liter, and 5 g of glucose (autoclaved separately) per liter.

Degradation of nitrosubstituted aromatic compounds. Nitrosubstituted aromatic compounds were added to the growth medium by injecting concentrated anoxic solutions through the stopper. Some of the substances tested were dissolved in ethanol; the final ethanol concentration added to the cultures never exceeded 0.5% (vol/vol) and did not

inhibit bacterial growth. In some experiments the concentrations of yeast extract and tryptone in the growth medium were lowered to 2.5 g/liter, as indicated below. Growth was monitored by removing samples by means of a syringe needle through the stopper and measuring the cultures' turbidities at 600 nm. For *H. praevalens*, an A_{600} of 1.0 corresponded to 0.44 mg (dry weight) of cells per ml.

To monitor the extent of breakdown of the aromatic nitro compounds added and the appearance of specific degradation products, we used a spectrophotometric assay (1). Culture samples were centrifuged, and the supernatant fluids were diluted fourfold with 1 M Tris hydrochloride (pH 9); a pH adjustment was necessary before spectrophotometric assessment, since for some of the compounds tested the absorption spectrum is pH dependent. Absorption spectra in the range of 200 to 500 nm were recorded with a Hewlett Packard Co. (Palo Alto, Calif.) model 8452A diode array spectrophotometer, against Tris buffer, against the supernatant of a culture without added aromatic compounds, and/or against uninoculated growth medium with or without addition of the compounds tested. For calculations of *p*-nitrophenol concentrations, a specific A_{400} of 1.89×10^4 M^{-1} cm^{-1} was used. To test for aromatic degradation products, cultures were also extracted with chloroform (50% by volume), and the absorption spectra of the chloroform extracts were recorded as described above and compared with those of known aromatic compounds in chloroform.

To determine the fate of added *p*-nitrophenol during degradation by *H. praevalens*, the incorporation of radiolabeled *p*-nitrophenol was tested. Growth medium containing 1% yeast extract, 1% tryptone, and 50 mg of *p*-nitrophenol per liter was supplemented with [U-^{14}C]*p*-nitrophenol (0.02 μCi/ml) and inoculated with *H. praevalens*. After different incubation times, samples were withdrawn and filtered through Millipore filters (pore size, 0.45 μm). The filters were washed with 10 ml of cold 10% trichloroacetic acid and dried. Next, 5 ml of Instagel (Packard Instrument Co., Inc., Rockville, Md.) scintillation cocktail was added, and the radioactivity in the filters was measured in a scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.; model LS 2800). In addition, the radioactivity of the culture supernatant was measured. To test whether CO_2 or other acid-volatile products are formed during the degradation of *p*-nitrophenol, culture supernatant samples were acidified with HCl and incubated overnight in a desiccator above KOH, and the radioactivity remaining was determined.

Other analytical methods. *p*-Aminophenol and aniline were assayed colorimetrically by reaction with *p*-dimethylaminobenzaldehyde. To 0.2 ml of culture supernatant, 0.8 ml of water and 0.05 ml of 1 M HCl were added, and then 3 ml of ethanol, 0.5 ml of 5% *p*-dimethylaminobenzaldehyde in ethanol, and 0.5 ml of 15.7% citric acid in 6% NaOH were added. After 10 min, 2.5 ml of water was added to the tubes, and the A_{440} was measured. *o*-Aminophenol, *m*-aminophenol, and aniline could also be detected in this test, albeit at a lower sensitivity. Nitrite in culture supernatants was assayed colorimetrically (21).

RESULTS

The halophilic, anaerobic eubacteria *H. praevalens* and *S. marismortui* were found to transform a wide variety of nitrosubstituted aromatic compounds, as demonstrated by the decrease in light absorption at the specific wavelengths absorbed by the compounds tested. Compounds transformed by both strains include nitrobenzene, *o*-nitrophenol, *m*-nitrophenol, *p*-nitrophenol, *o*-nitroaniline, *m*-nitroaniline, *p*-nitroaniline, 2,4-dinitrophenol, and 2,4-dinitroaniline. The compounds were generally added at a concentration of 50 mg/liter, which did not inhibit growth. 2,4-Dinitrophenol was found to be transformed when added at a concentration of 10 mg/liter; higher concentrations inhibited growth. None of these compounds were transformed by *E. coli*.

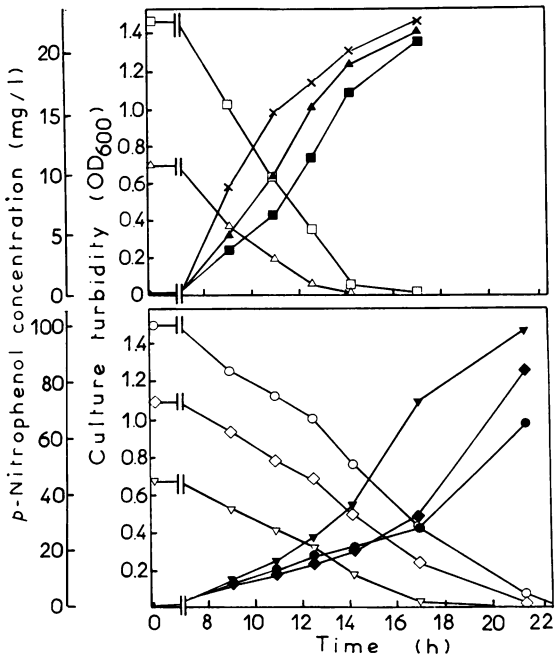

FIG. 1. Transformation of *p*-nitrophenol during growth of *H. praevalens*. *H. praevalens* was grown at 40°C in standard growth medium containing 10 g of tryptone per liter, 10 g of yeast extract per liter, and 2.5 g of glucose per liter and supplemented with 12.5 (△), 25 (□), 50 (▽), 75 (◇), or 100 (○) mg of *p*-nitrophenol per liter. Cultures were inoculated with a 1% bacterial inoculum grown in the absence of *p*-nitrophenol. At different times, samples were withdrawn, the culture turbidity (optical density at 600 nm [OD_{600}]) (closed symbols) was compared with that of a control without added *p*-nitrophenol (×), and the remaining *p*-nitrophenol concentration (open symbols) was assessed according to the A_{400} of dilutions of culture supernatant with 1 M Tris hydrochloride (pH 9.0). In control experiments without inoculum, the *p*-nitrophenol concentration remained constant throughout the incubation period (not shown).

The transformation of *p*-nitrophenol by *H. praevalens* was investigated in more detail. Concentrations of up to 100 to 125 mg of *p*-nitrophenol per liter were tolerated, but at concentrations above 25 mg/liter growth was slowed down significantly. However, at all concentrations tested, the *p*-nitrophenol added disappeared completely within 24 h (Fig. 1). Chemical determinations of aromatic amines showed a quantitative conversion of *p*-nitrophenol to *p*-aminophenol (Fig. 2). Similarly, it was shown that nitrobenzene was reduced to aniline. Aniline was also detected by its absorption spectrum in chloroform extracts of cultures grown in the presence of nitrobenzene. As aromatic amines were not degraded by the organisms tested, it can be concluded that the only transformation that took place was the reduction of the nitro groups to amino groups. Nitrite, which is a known product of nitrophenol degradation by aerobic bacteria (21, 22), was not detected in *o*-nitrophenol- or *p*-nitrophenol-degrading cultures.

To obtain additional information on the fate of *p*-nitrophe-

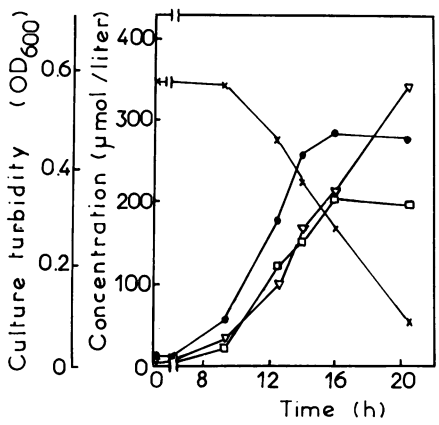

FIG. 2. Formation of *p*-aminophenol from *p*-nitrophenol by *H. praevalens*. *H. praevalens* was grown in medium containing 2.5 g of tryptone per liter, 2.5 g of yeast extract per liter, 2.5 g of glucose per liter, and 50 mg of *p*-nitrophenol per liter. At different times, samples were withdrawn, the culture turbidity (optical density at 600 nm [OD_{600}]) (□) was compared with that of a control without added *p*-nitrophenol (●), the remaining *p*-nitrophenol concentration (×) was assessed as described in the legend to Fig. 1, and the concentration of *p*-aminophenol was determined colorimetrically (▽). In control experiments in which *p*-aminophenol (50 mg/liter) was added to *H. praevalens* cultures, its concentration remained constant throughout the incubation period (not shown).

nol during degradation by *H. praevalens*, a culture containing 50 mg of *p*-nitrophenol per liter was supplemented with ^{14}C-labeled *p*-nitrophenol. No significant amounts of labeled material were incorporated into the cells, the radioactivity in the culture supernant remained constant, and no label appeared in acid-volatile products (Table 1).

DISCUSSION

Though considerable knowledge about the anaerobic degradation of aromatic compounds has accumulated (4, 7, 17, 19), little is known about the anaerobic breakdown of nitrosubstituted aromatic rings. Anaerobic sludge was reported to slowly degrade nitrophenols to methane and carbon dioxide (5), but the nitrophenols were found to inhibit the methanogenic step, and the bacteria responsible for the process were not characterized. A few reports of bacterial consortia degrading nitrophenols to methane have been published (4, 5). During methanogenic fermentation of nitrophenols in sewage sludge, the nitro group was presumed to undergo reduction to form a substituent amino group. The resulting intermediate aminophenol subsequently underwent mineralization (5). Aromatic amines are highly reactive, and in the presence of oxygen, polymerization products are formed. In soil systems these amines react with humic acids; these reactions lead to immobilization of the products and make further degradation extremely difficult (16).

TABLE 1. Incubation of *H. praevalens* in the presence of ^{14}C-labeled *p*-nitrophenol[a]

Incubation time (h)	Concn (mM) of:		% of total radioactivity in[b]:		
	p-Nitrophenol	*p*-Aminophenol	Cells	Supernatant	Acidified supernatant
0	0.50	0	<1	98	ND[c]
18	0.19	0.27	<1	99	ND
36	0	0.50	<1	96	ND
60	0	0.48	<1	95	104

[a] *H. praevalens* was inoculated into medium containing 14% NaCl, 1% yeast extract, and 1% tryptone, in addition to the other medium components described in the text and 0.5 mM *p*-nitrophenol and 7×10^4 cpm of [U-^{14}C]*p*-nitrophenol per ml, and the culture was incubated at 35°C. After different periods, samples were withdrawn, the concentrations of *p*-nitrophenol and *p*-aminophenol were determined spectrophotometrically, and the radioactivity in the different fractions was measured.

[b] At any sampling time, the total radioactivity of the sample was considered to be 100%. Total radioactivity values did not change significantly during the experiment.

[c] ND, not determined.

The obligately anaerobic bacteria of the family *Haloanaerobiaceae* were not previously known for their metabolic versatility, and the range of substrates utilized was reported to be restricted to simple sugars and amino acids (13, 14, 20). *H. praevalens* was known to degrade carbohydrates (including pectin and *N*-acetylglucosamine), amino acids, and peptides (20). The findings described in this article show that the variety of organic compounds that can be transformed in hypersaline environments may be much greater than previously assumed (10, 11).

The use of anaerobic halophilic bacteria in the industrial fermentation of complex organic matter has been proposed (18), and preliminary tests of breakdown of pretreated lignitic material have been performed (12). The results showed a certain promise in the use of anaerobic halophiles in biotechnology. The reduction of nitrosubstituted aromatic compounds by members of the *Haloanaerobiaceae*, as described in this article, shows that anaerobic halophilic bacteria may have a considerable practical use.

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