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Mineralization of the Sulfonated Azo Dye Mordant Yellow 3 by a 6-Aminonaphthalene-2-Sulfonate-Degrading Bacterial Consortium

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Under anaerobic conditions the sulfonated azo dye Mordant Yellow 3 was reduced by the biomass of a bacterial consortium grown aerobically with 6-aminonaphthalene-2-sulfonic acid. Stoichiometric amounts of the aromatic amines 6-aminonaphthalene-2-sulfonate and 5-aminosalicylate were generated and excreted into the medium. After re-aeration of the culture, these amines were mineralized by different members of the bacterial culture. Thus, total degradation of a sulfonated azo dye was achieved by using an alternating anaerobic-aerobic treatment. The ability of the mixed bacterial culture to reduce the azo dye was correlated with the presence of strain BN6, which possessed the ability to oxidize various naphthalenesulfonic acids. It is suggested that strain BN6 has a transport system for naphthalenesulfonic acids which also catalyzes uptake of sulfonated azo dyes. These dyes are then gratuitously reduced in the cytoplasm by unspecific reductases.

Sulfonated azo compounds are widely used as dyes for textiles, food, and cosmetics. Both aromatic sulfonic acid and azo groups are rare among natural products and thus confer a xenobiotic character to sulfonated azo dyes. In conventional sewage plants, certain aromatic compounds carrying SO_3H groups as substituents, such as naphthol- and naphthylaminesulfonic acids, resist biodegradation or are incompletely degraded. As a consequence, 5 to 15% of the organic carbon content of Rhine River water is caused by sulfonated compounds and its secondary products (6).

The isolation of a mixed bacterial community growing aerobically with 6-aminonaphthalene-2-sulfonic acid (6A2NS) or various other substituted naphthalenesulfonic acids as the sole source of carbon and energy has been reported from this laboratory (12). The complete degradation of 6A2NS was basically described by a mutualistic interaction of two strains. Strain BN6 effected the initial conversion of 6A2NS to 5-aminosalicylate (5AS) in quantitative amounts, whereas the partner strain, BN9, could grow with 5AS but not with 6A2NS.

Under anaerobic conditions, many bacteria gratuitously reduce azo compounds to the corresponding amines (18). In this report, this metabolic trait is used for total degradation of the sulfonated azo dye Mordant Yellow 3 (MY3) by an anaerobic-aerobic process, using a 6A2NS-degrading bacterial culture.

MATERIALS AND METHODS

Bacterial strains and media. The 6A2NS-utilizing mixed bacterial community and the isolation of strains BN6 and BN9 from this multispecies bacterial culture have been described by Nörtemann et al. (12). The 6A2NS-degrading, mixed bacterial community was grown aerobically as already described by these authors. For anaerobic transformations, the aerobically grown mixed culture was transferred to Hungate tubes (17 ml). The azo dyes and, in some cases, additional carbon sources were added, and the tubes were completely filled with the bacterial culture, sealed with screw caps, and incubated at 30°C. To prevent possible contamination with oxygen during sampling, tubes were opened only once, and only as many Hungate tubes were incubated as measurements were planned.

Analytical methods. Azo dyes and metabolites were analyzed by high-pressure liquid chromatography (HPLC; HPLC data and chromatography control station 840, equipped with a programmable multiwavelength detector, model 490; Waters Associates Inc., Milford, Mass.). A reverse-phase column (125 by 4.6 mm [internal diameter]; Bischoff, Leonberg, Germany) packed with 5-μm particles of Lichrosorb RP8 (Merck, Darmstadt, Germany) was used as the stationary phase. The separated compounds were detected by simultaneous observation at 210 nm and at the wavelength of maximal absorbance of the azo dyes.

For the simultaneous analysis of MY3, 6A2NS, and 5AS by HPLC, the following solvent gradient was used (flow rate, 1 ml/min). From 0 to 10 min the mobile phase consisted of 80% (vol/vol) water and 20% (vol/vol) methanol. From 10 to 20 min a linear solvent gradient was applied with a final composition of 20% (vol/vol) water and 80% (vol/vol) methanol. From 20 to 25 min the solvent system consisted of 20% (vol/vol) water and 80% (vol/vol) methanol. Tetrabutylammonium phosphate (low-UV PIC A; Waters) as ion-pair reagent was present in the solvent systems in accordance with the instructions of the manufacturer. Under these conditions, the respective retention times (R_t) were as follows: 5AS R_t = 3.6 min; 6A2NS R_t = 6.0 min; and MY3 R_t = 22.5 min. In some experiments, the concentration of MY3 was determined with a spectrophotometer (Uvikon 810; Kontron, Zurich, Switzerland) based on an extinction coefficient, ε_{364nm}, of 27,700 M^{-1} cm^{-1} (pH 7.2).

Dissolved organic carbon was assayed with a total organic carbon analyzer, 915-B (Beckman Instruments, Fullerton, Calif.). Samples (50 μl) of culture fluid were injected after removal of cells and particles by centrifugation. Dissolved oxygen was determined with an oxygen electrode (YSI 5300; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The protein content of cell extracts was determined with bovine serum albumin as a standard (2). For the mixed bacterial community, an optical density at 546 nm (OD_{546}) of 1 correlated to a protein content of 0.115 mg/ml. Glucose

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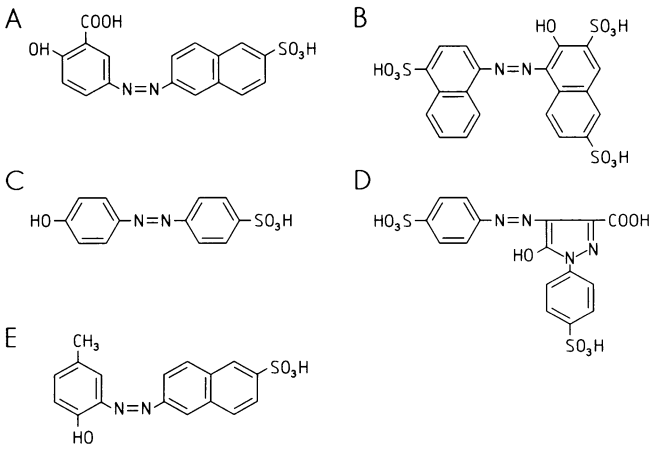

FIG. 1. Azo compounds used in this study. (A) MY 3 (CI 14095); (B) amaranth (CI 16185 = Acid Red 27 = FD&C Red 2); (C) 4-hydroxyazobenzene-4'-sulfonic acid; (D) tartrazine (CI 19140 = Acid Yellow 23 = FD&C Yellow 5); (E) Acid Yellow 21 (CI 14230). CI, color index (from reference 16).

was determined in cell culture supernatants by an enzymatic test (10).

Chemicals. 6A2NS, MY3, and Acid Yellow were obtained from Bayer AG, Leverkusen, Germany. 5AS, tartrazine, and amaranth were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals used for mineral salts media and buffer solutions were from E. Merck AG. Biochemicals were from Boehringer, Mannheim, Germany.

RESULTS

The ability of the 6A2NS-degrading mixed bacterial culture to reduce sulfonated azo dyes was tested with the yellow azo dye MY3 (Fig. 1). This compound was selected as a model because reductive cleavage of the azo bond of MY3 would yield 6A2NS and 5AS, which are both growth substrates for the 6A2NS-degrading mixed culture under aerobic conditions (12).

No decoloration of the azo dye was observed when the 6A2NS-degrading mixed bacterial community was incubated aerobically. When the coculture, however, was grown aerobically with 6A2NS and then incubated anaerobically in stoppered tubes, the medium was decolorized. Within 5 days, the culture (OD_{546} = 2.0) converted 50% of the MY3 initially present. No reaction was observed in a control experiment without cells.

The stoichiometric reduction of an azo bond yielding aromatic amines requires four reduction equivalents. We found that the rate of MY3 turnover by the mixed bacterial community under anaerobic conditions was considerably enhanced in the presence of glucose. After 3 days of incubation under the same conditions as described above, MY3 was completely metabolized.

For HPLC analysis of the products formed, the mixed culture was grown aerobically with 6A2NS (OD_{546} = 2.1) and incubated anaerobically with the azo dye. In the presence or absence of glucose, the expected reduction products of MY3, 6A2NS and 5AS, were formed at nearly stoichiometric amounts. The average recoveries ranged from 90 to 100% for 6A2NS and from 70 to 90% for 5AS. The formation of dark polymers under the aerobic conditions of HPLC analysis indicated that part of the 5AS was lost by autoxidation. The reaction rates without and with (10 mM) the addition of glucose were 0.26 and 0.95 μmol h^{-1} mg of $protein^{-1}$, respectively.

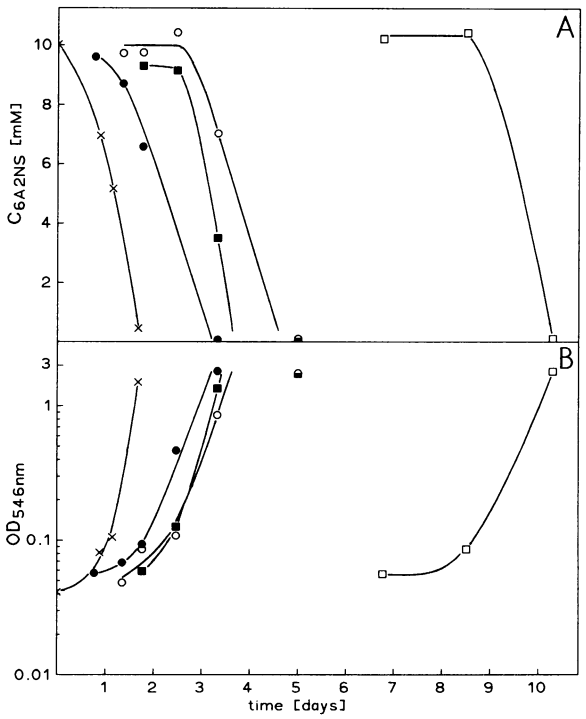

FIG. 2. Influence of different anaerobic incubation periods on ability of the 6A2NS-degrading mixed bacterial community to restore the potential to degrade 6A2NS aerobically. The coculture was grown aerobically with 6A2NS to a cell density (OD_{546}) of 1.7. The culture was then transferred to Hungate tubes and incubated anaerobically at 30°C. After the indicated time intervals, the respective tubes were opened and 0.5 ml from each was transferred into flasks containing 20 ml of mineral medium with 6A2NS (10 mM). The flasks were incubated aerobically on a shaker, and growth and 6A2NS turnover were determined. Disappearance of 6A2NS (A) and increase in OD (B) after different intervals of anaerobic incubation: (×) 0 h; (●) 19 h; (■) 33 h; (○) 42 h; (□) 162 h.

Glucose could not be replaced by a component of industrial sewage such as methanol, ethanol, acetone, or isopropanol. The addition of 1-butanol (3 mM), however, caused a threefold enhancement of the turnoverrate of MY3.

The ability of the 6A2NS-degrading mixed bacterial community to cleave reductively the azo bond under anaerobic conditions, generating equal amounts of 6A2NS and 5AS, makes it possible to mineralize sulfonated azo compounds such as MY3 by an alternating anaerobic-aerobic treatment process. Therefore, we tested the ability of the 6A2NS-degrading culture to maintain its viability under anaerobic conditions. Thus, the culture first was grown aerobically with 6A2NS and then was exposed to anaerobic conditions in Hungate tubes for different time intervals. After this treatment, the cultures were transferred to fresh medium with 6A2NS and incubated aerobically (Fig. 2). Oxygen starvation for a period of ≤19 h did not affect the ability of the cells to restore the catabolic potential towards 6A2NS. When the culture was incubated in Hungate tubes for a longer time period, we observed a considerable increase in the length of the lag phase after the culture was aerated again. Even after 7 days in closed Hungate tubes, the culture

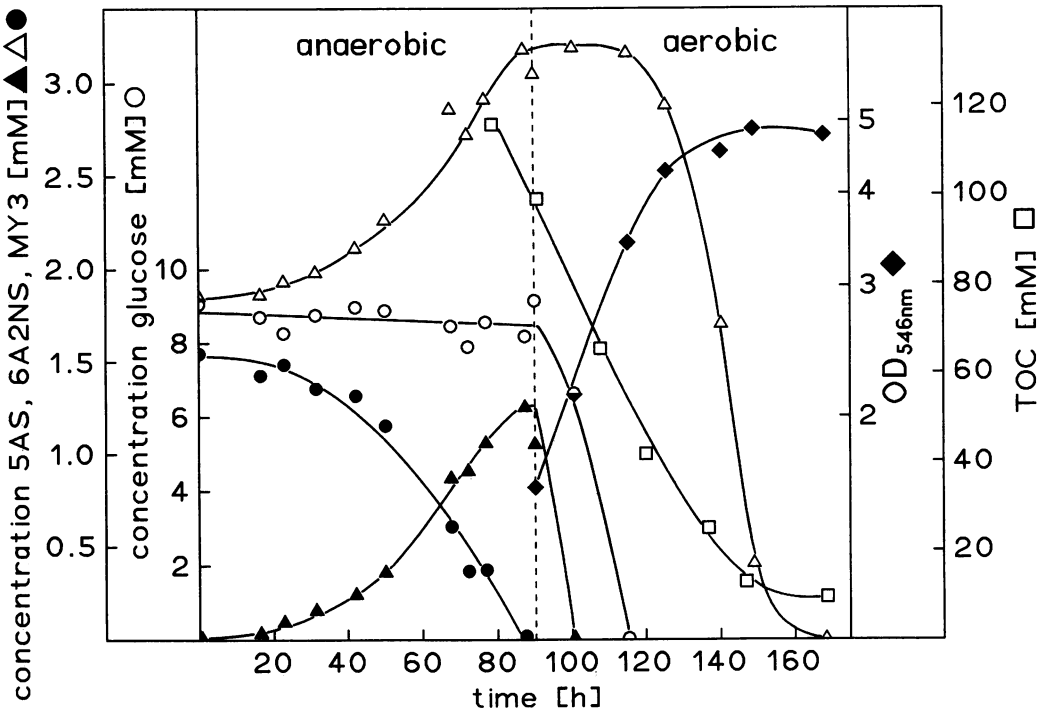

FIG. 3. Total degradation of the azo dye MY3 by a 6A2NS-degrading mixed bacterial community. The mixed bacterial community was grown aerobically with 6A2NS to an OD_{546} of 1.8. The culture was then incubated anaerobically in Hungate tubes containing MY3 (1.5 mM) and glucose (10 mM). After complete decoloration of MY3, the culture was transferred to Erlenmeyer flasks, which were shaken aerobically. The initial concentration of 6A2NS during anaerobic incubation was due to the residual amount of substrate from the preceding aerobic cultivation. TOC, total organic carbon.

still restored the ability to grow with 6A2NS, albeit after a prolonged lag period (Fig. 2).

To demonstrate total mineralization of MY3, the dye was first incubated anaerobically with the 6A2NS-degrading mixed culture. Glucose was added to enhance the reduction rate of the azo dye. Analysis by HPLC revealed the disappearance of MY3 (1.5 mM) and formation of stoichiometric amounts of 6A2NS and 5AS (Fig. 3). After complete reduction of MY3, air was reintroduced into the culture and aerobic degradation of 6A2NS and 5AS by the bacterial community was monitored. The degradation of 5AS started immediately after reintroduction of air, while catabolic activity of 6A2NS was restored only after a prolonged lag period. Nevertheless, at the end of the experiment complete degradation of 5AS and 6A2NS was shown by HPLC and total organic carbon analysis of the culture fluid (Fig. 3). Thus, the ability of the bacterial community to completely mineralize the sulfonated azo dye was demonstrated. Under

TABLE 1. Relative turnover rates of MY3 by cells of the 6A2NS-degrading mixed bacterial culture and of strain BN6 grown aerobically under different conditions[a]

Bacterial culture	Growth with:	Specific turnover rate (μmol of MY3 h^{-1} mg of protein^{-1})
Mixed	6A2NS	94
Mixed	Nutrient broth	4
Mixed	Acetate	5
BN6	Glucose-2NS	130
BN6	Glucose	29

[a] The mixed bacterial community was grown aerobically with 6A2NS (10 mM), nutrient broth, or acetate (20 mM). Strain BN6 was grown with glucose, and in one experiment it was induced for the metabolism of naphthalenesulfonic acids by the addition of naphthalene-2-sulfonic acid (2NS; 0.5 mM) 6 h before the start of anaerobic incubation.

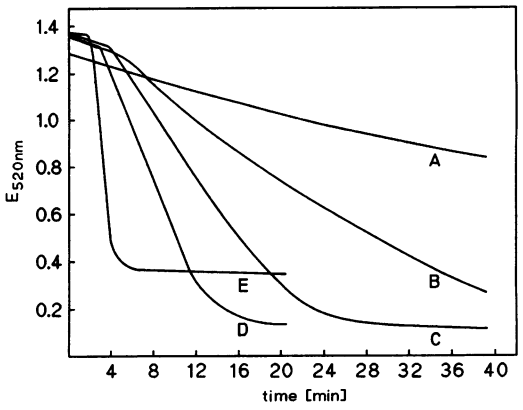

FIG. 4. Spectrophotometric demonstration of an azo reductase in cell extracts from strain BN6. Cell extract was prepared from cells of strain BN6 grown aerobically with glucose. The decrease in the concentration of amaranth was determined spectrophotometrically in gas-tight cuvettes. The anaerobic reaction mixture contained, in 1.6 ml, Tris HCl (50 mM; pH 7.5), NADH (0.6 mM), and amaranth (0.06 mM). The buffer was made anaerobic by boiling and gassing with N_2. The concentration of FAD was varied from 0 to 1 mM. The test was started by the addition of cell extract (12.5 mg of protein). A, Spontaneous reaction with 0.2 mM FAD (without protein); B, 0 mM FAD; C, 0.05 mM FAD; D, 0.3 mM FAD; E, 1.0 mM FAD.

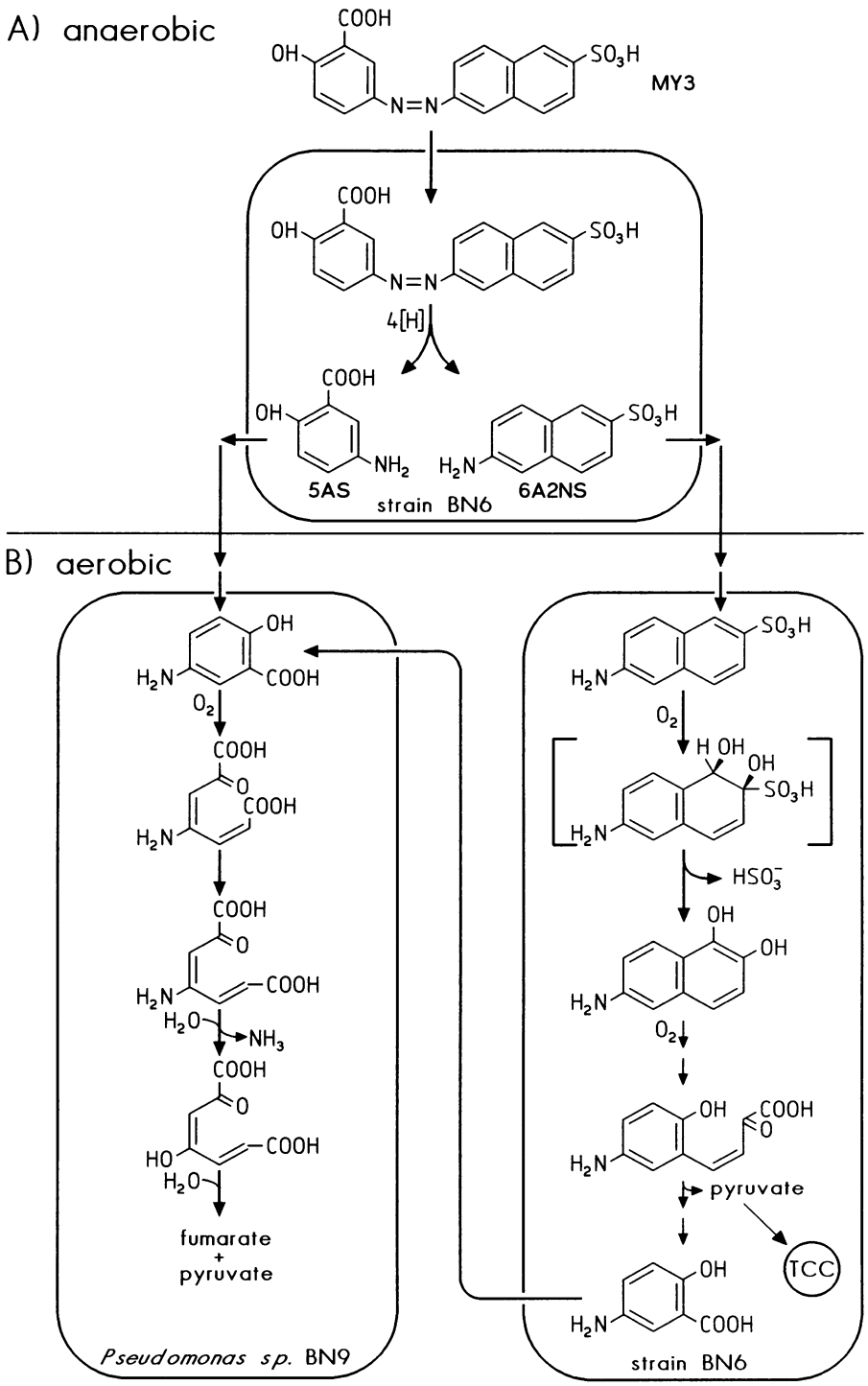

FIG. 5. Proposed pathway for degradation of the azo dye MY3 by a mixed bacterial community. The catabolic pathway of 6A2NS has been described by Nörtemann et al. (12), and that of 5AS has been described by Stolz (17).

anaerobic conditions only a very small amount of glucose (0.38 mM) was consumed. Residual glucose was immediately metabolized upon aeration of the culture.

Under aerobic conditions, the coculture used in this study harbors the ability to convert and desulfonate a wide range of sulfonated naphthalenes (12). To determine the possible use of the culture to mineralize different sulfonated azo dyes, the ability of the culture to reduce various azo compounds was studied. After aerobic growth with 6A2NS, the mixed bacterial community (OD_{546} = 1.9) was incubated anaerobically with the azo dyes (0.5 mM each) amaranth (Fig. 1B), 4-hydroxyazobenzene-4'-sulfonic acid (Fig. 1C), tartrazine

(Fig. 1D), and Acid Yellow 21 (Fig. 1E) together with MY3 as a control. After 3 days of anaerobic incubation, 37% of amaranth, 43% of 4-hydroxyazobenzene-4'-sulfonic acid, 98% of Acid Yellow 21, and 51% of MY3 were reduced, while 94% of the initial amount of tartrazine was still present. When glucose (10 mM) was added as a source of reduction equivalents, the azo dyes, except tartrazine (16% turnover), were completely metabolized.

To determine which strain of the mixed bacterial community was responsible for the reduction of the azo dye, cells of the mixed culture, strain BN6, strain BN9, and, as a control, *Escherichia coli* were incubated anaerobically with MY3. Only the mixed culture and strain BN6 showed significant activities with MY3. The reduction rate of MY3 by the mixed culture and strain BN6 was significantly higher in cultures which had been induced with naphthalenesulfonic acids (Table 1). Pure cultures of strain BN6 showed higher specific activities with MY3 than did the 6A2NS-degrading mixed culture (Table 1). Obviously, strain BN6 is the active member in the reduction of MY3 by the mixed bacterial community.

There were at least two possible ways for glucose to enhance reduction of MY3. It could act as a donor of reduction equivalents [e.g., via NAD(P)H or reduced flavin adenine dinucleotide ($FADH_2$)], or its addition could result in more actively respiring cells, thus rapidly depleting the medium of oxygen and enabling azo reductase to transfer reduction equivalents to the azo dye. Therefore, the rates of oxygen depletion in the mixed culture (OD_{546} = 2.1) in the presence of MY3 with or without added glucose were compared. Under both conditions, a complete (<3% of the original oxygen tension) removal of the dissolved oxygen was observed within 6 min. Obviously, the endogenous respiration of the cells is high enough for complete and rapid removal of oxygen within the Hungate tubes. In another experiment, the course of the redox potential was monitored in closed culture vessels containing a redox indicator (resazurin). With or without glucose, the cultures reached the half-redox potential of resazurin (pH 7.0; −51 mV) in less than 10 min and a minimal potential of about −100 mV.

Several bacterial azo reductase activities have been described in cell extracts (13, 15, 19, 20). When the red azo dye amaranth (Fig. 1B) was incubated in oxygen-free buffer with NADH as a source of reduction equivalents, a slow decoloration of the azo dye was observed. Addition of cell extract from strain BN6 did not cause an immediate increase in the reaction rate. However, the reaction rate increased drastically after a few minutes (Fig. 4). The addition of flavin adenine dinucleotide (FAD) to the reaction mixture clearly enhanced this reaction and shortened the lag phase. A possible explanation for this pronounced effect is that FAD is reduced enzymatically by NADH and $FADH_2$ can then spontaneously reduce the azo dye (13).

DISCUSSION

Up to now the ability to mineralize azo compounds aerobically has been restricted to a few bacterial strains which utilize certain carboxyl-substituted structures (21, 22). Adaptation experiments with these cultures to growth with the industrially important sulfonated analogs were unsuccessful (9). This corresponds to observations in this laboratory with MY3, which did not serve as a carbon or energy source in aerobic enrichment cultures.

On the other hand, it has been known for a long time that various biological systems possess the ability to cleave the azo bond reductively under anaerobic conditions (1, 18). In mammalian tissues, azo reductase activity is mainly found in liver. A NAD(P)H-cytochrome *c* reductase and the cytochrome P_{450} system have been implicated in this reaction (7, 8). Under physiological conditions, the intestinal flora is mainly responsible for the reduction of azo dyes ingested as food additives (14). Correspondingly, the intestinal bacteria *Proteus vulgaris* and *Streptococcus faecalis* were found to reduce azo compounds (4, 13, 15).

Most investigators studying the reduction of azo dyes by bacteria noticed the extreme lack of specificity of the azo reductase system and showed that cell extract or aged (damaged) cells reduced azo dyes more efficiently and metabolized a wider range of azo dyes than intact cells (3, 11, 20). It was therefore suggested, especially in the case of sulfonated azo dyes, that permeation through the cell membrane is the rate-limiting step during bacterial reduction of azo dyes (11, 13). The actual reduction in the cytoplasm of the cells has been suggested to be an unspecific process involving either soluble or enzyme-bound FADHs (5, 13, 15).

Obviously, strain BN6 is actively involved in the reduction of MY3. This organism alone or as a member of the mixed culture can efficiently reduce MY3. This suggests that in these cultures only cells of strain BN6 have the ability to take up the azo dyes. Because strain BN6 must have an uptake system for the anions of sulfonated naphthalenes and, particularly, naphthylamine sulfonates, it seems reasonable that this inducible transport system may also take up sulfonated azo compounds gratuitously.

This is the first demonstration of complete biological mineralization of a sulfonated azo dye. The pathway for the mineralization of MY3 can be depicted schematically as shown in Fig. 5. When deprived of oxygen, the biomass of the mixed bacterial culture, which has been grown aerobically with 6A2NS, can reduce MY3 to 6A2NS and 5AS. These metabolites can be totally degraded and utilized by the culture when aerobic conditions are restored.

This system could be useful for the treatment of azo dye-containing waste-waters because, under anaerobic conditions, it reduces a wide range of azo dyes and aerobically oxidizes many different aminonaphthalenesulfonic acids.

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