APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1990, p. 2146–2151
0099-2240/90/072146-06$02.00/0

Vol. 56, No. 7

Azoreductase Activity of Anaerobic Bacteria Isolated from Human Intestinal Microflora

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Received 29 December 1989/Accepted 4 May 1990

A plate assay was developed for the detection of anaerobic bacteria that produce azoreductases. With this plate assay, 10 strains of anaerobic bacteria capable of reducing azo dyes were isolated from human feces and identified as *Eubacterium hadrum* (2 strains), *Eubacterium* spp. (2 species), *Clostridium clostridiiforme*, a *Butyrivibrio* sp., a *Bacteroides* sp., *Clostridium paraputrificum*, *Clostridium nexile*, and a *Clostridium* sp. The average rate of reduction of Direct Blue 15 dye (a dimethoxybenzidine-based dye) in these strains ranged from 16 to 135 nmol of dye per min per mg of protein. The enzymes were inactivated by oxygen. In seven isolates, a flavin compound (riboflavin, flavin adenine dinucleotide, or flavin mononucleotide) was required for azoreductase activity. In the other three isolates and in *Clostridium perfringens*, no added flavin was required for activity. Nondenaturing polyacrylamide gel electrophoresis showed that each bacterium expressed only one azoreductase isozyme. At least three types of azoreductase enzyme were produced by the different isolates. All of the azoreductases were produced constitutively and released extracellularly.

Azo dye compounds represent a large group of chemicals which are extensively used in the textile, pharmaceutical, food, and cosmetic industries. Although the commonly used azo dyes are not mutagenic in the standard Ames plate assay (26), they are reduced by azoreductases from intestinal bacteria and, to a lesser extent, by enzymes of the cytosolic and microsomal fractions of the liver (3–5, 16, 18, 19). The first catabolic step in the reduction of azo dyes, which is accompanied by a decrease in the visible light absorbance of the dye and then decolorization of the dye, is the reduction of the azo bridge (Fig. 1) to produce aromatic amines (3–5, 15). Aromatic amines, which are known human carcinogens, have been found in the urine of dyestuff workers and test animals following the administration of azo dyes (3–5, 18).

Azo dye compounds are linked to bladder cancer in humans and to hepatocarcinoma and nuclear anomalies in intestinal epithelial cells in mice (18, 21, 24). Thus, a number of azo dyes have been classified as carcinogenic (10). The ability of the intestinal microflora of human and other animal species to reduce the azo groups of xenobiotic compounds has been known for many years (1, 3–6, 18). However, the specific organisms of the intestinal microflora participating in azo dye reduction are poorly understood (1).

In this study, we developed a plate assay capable of detecting the specific anaerobic bacteria from human intestinal flora that participate in the reduction of azo dyes. The production of enzymes by these organisms was compared, and the cofactor requirements of the azoreductases were studied.

MATERIALS AND METHODS

Media and culture conditions. The medium used for the isolation of azoreductase-producing bacteria from intestinal microbial flora was brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) prepared in accordance with manufacturer instructions and supplemented with the following ingredients per liter: Bacto-Agar (Difco), 2 g; yeast extract, 0.5 g; vitamin K_1, 1 mg; hemin, 5 mg; cysteine, 0.5 g; and either Direct Blue 15 (3,3′-dimethoxybenzidine-based dye) or Nitro Red [4-amino-5-hydroxy-3-(4-nitrophenylazo)-2,7-naphthalenedisulfonic acid disodium salt], 80 mg (Fig. 1). The medium used for the growth and maintenance of the azoreductase-producing bacteria was BHI broth (prereduced and anaerobically sterilized; Carr-Scarborough Microbiologicals, Stone Mountain, Ga.). M10 medium was prepared as described by Caldwell and Bryant (2), except that the volatile fatty acids were replaced by acetic acid and propionic acid.

Isolation of azoreductase-producing bacteria. Azoreductase-producing bacteria were isolated from human intestinal microflora as follows. One gram of freshly voided human feces was diluted in 9 ml of BHI broth under anaerobic gas (5% CO_2, 10% hydrogen, 85% nitrogen). Tenfold serial dilutions of the sample were made in BHI broth, and 0.1-ml dilutions of 10^{-5} to 10^{-9} were plated on BHI agar plates containing Direct Blue 15 or Nitro Red (final concentration, 80 mg/ml). The plates were incubated under both aerobic and anaerobic conditions at 37°C and observed for clearance of the dye surrounding the colonies. Approximately 20% of the colonies developed clear zones after plating of the dilutions of fecal samples from the same source at different times. The colonies which cleared the dye were transferred to BHI agar plates containing Direct Blue 15 for single-colony isolation. The isolated colonies were transferred to BHI broth for growth and maintenance.

Identification of azoreductase-producing anaerobic bacteria. The anaerobic bacteria capable of reducing Direct Blue 15 were identified by the methods described by Holdeman et al. (11) and in *Bergey's Manual of Systematic Bacteriology* (14, 27) with media from Carr-Scarborough Microbiologicals. The identification of the isolates was verified by comparing the morphology, staining characteristics, results of biochemical tests, and cellular proteins with those of known cultures of bacteria from the American Type Culture Collection, Rockville, Md. Proteins were compared by gel electrophoresis in polyacrylamide gels by the methods described by Moore et al. (22).

Kinetics of degradation of azo dyes by different isolates. The

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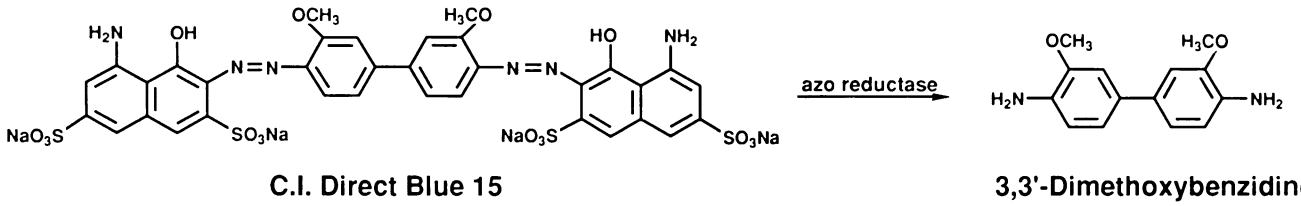

FIG. 1. Structures of Direct Blue 15 and its reduction product. C.I., Color index.

bacterial isolates which could reduce Direct Blue 15 under anaerobic conditions were used for this experiment. To overnight cultures grown in BHI broth, 50 μmol of sterile Direct Blue 15 was added per ml. The cultures were incubated at 37°C. Two milliliters from each of five replicate cultures was removed aseptically under anaerobic conditions at 2-h intervals. The samples were centrifuged at 15,000 × *g* for 5 min in tightly capped tubes to ensure anaerobiosis. The supernatant was used to measure the disappearance of the dye over time with a DU-7 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at a wavelength of 615 nm, the lambda max for Direct Blue 15 (3), and with water for a blank.

Enzymatic activity in the supernatants of the isolates. Overnight cultures of the isolates grown in BHI broth were centrifuged at 15,000 × *g* for 15 min in tightly capped tubes to ensure anaerobiosis. The supernatants were transferred to tubes containing 2 ml of heavy mineral oil under anaerobic conditions for the assay of enzymatic activity. Direct Blue 15 and tetracycline were added to the supernatants to final concentrations of 50 to 100 μM and 15 μg/ml, respectively, and the tubes were incubated at 37°C. For a control, BHI broth with Direct Blue 15 was used. The cultures were observed and monitored for a decrease in the A_{615} until the dye was completely cleared and reduced, at which point the absorbance reached a constant level. The time required for total reduction of the dye was noted. The amount of soluble protein from each strain was measured in the supernatants prior to incubation with the dye. The pellets from these cultures were used for bacterial counting.

Protein determination and bacterial counting. The amount of soluble protein produced by each bacterium in the spent medium after incubation (which was used for the determination of enzymatic activity) was determined by the method of Lowry et al. (17) with bovine serum albumin as a standard. The amount of protein in the uninoculated medium was small and was subtracted from the value obtained for soluble protein from each strain in the spent medium. Bacterial counting (in the pellets from the cultures used for the determination of enzymatic activity) was performed by plating serial dilutions of bacteria on BHI agar plates.

Effects of cofactors on the azoreductase activity of the isolates. M10 medium was used for this experiment and was prepared as either broth or agar medium. Flavin adenine dinucleotide (FAD), flavin mononucleotide, or riboflavin (final concentration, 50 μg/ml) and Direct Blue 15 (final concentration, 25 μg/ml) were added to the medium. Only the dye was added to the control medium. The bacteria from a single colony of either the new isolates or *Clostridium perfringens* were used as inocula. Cultures were inoculated under anaerobic conditions and incubated anaerobically at 37°C. Other compounds tested at a concentration of 50 μg/ml were NAD, NADH, NADPH, glutathione, and cytochrome *c*. M10 medium plates containing, in addition to 50 μg of FAD per ml, mersalyl acid (final concentration, 50 μg/ml) were also prepared. They were inoculated with *C. perfringens* or one of the new isolates.

Native gel electrophoresis of protein for the detection of azoreductase. The enzyme in culture supernatants was precipitated by the addition of 60% ammonium sulfate, and the precipitate was dissolved in BHI broth. Bromophenol blue was added to the protein as a marker before the sample was loaded on the gel. In some experiments, pelleted cells were broken by sonic oscillation and loaded directly on the gel after the addition of bromophenol blue. The separating gel for nondenaturing polyacrylamide gels contained 10% acrylamide and 0.26% bis and was prepared in accordance with the instructions provided by Sigma Chemical Co., St. Louis, Mo. (technical bulletin no. MKR-137). The stacking gel contained 2.5% acrylamide and 0.5 μg of riboflavin per ml. The electrode buffer contained 5 mM Tris and 37.3 mM glycine (pH 8.3). Thirty to 50 μg of each protein sample was used for loading the gel. A model V16 vertical slab gel electrophoresis apparatus (Bethesda Research Laboratories, Gaithersburg, Md.) was used for electrophoresis. A constant current of 6 mA was applied to the gel until the bromophenol blue was close to the anodic end of the gel. All steps for the preparation and running of the gel had to be performed under anaerobic conditions to ensure the activity of the enzyme. At the conclusion of the run, the gel was stained with Nitro Red or Direct Blue 15 and incubated at 37°C under anaerobic conditions.

RESULTS

Detection of azoreductase-producing anaerobic bacteria. Following the anaerobic incubation of *C. perfringens* and the fecal dilutions for 24 h on BHI agar containing either Direct Blue 15 or Nitro Red, colonies of *C. perfringens* and many human intestinal bacteria were observed. Clear zones, which surrounded the colonies of *C. perfringens* and some of the colonies of intestinal bacteria (Fig. 2), indicated that these

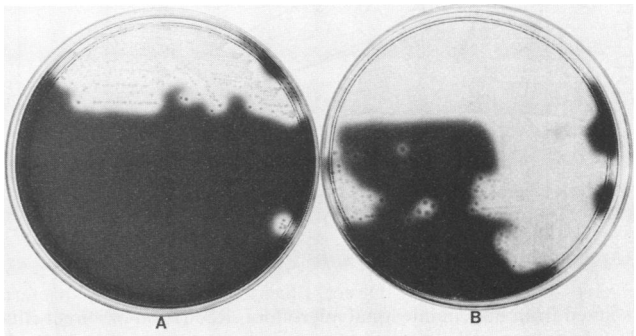

FIG. 2. Appearance of clear zones around bacterial colonies due to the reduction of Direct Blue 15 on BHI agar plates. (A) *C. perfringens*. (B) *C. paraputrificum*.

TABLE 1. Azoreductase production by anaerobic bacteria from human intestinal microflora isolated from human feces and the average rate of reduction of Direct Blue 15 by each bacterium

Bacterial isolate	Avg rate of reduction of Direct Blue 15 (nmol/min per mg of protein[a])
Bacteroides sp.	16
Butyrivibrio sp.	70
E. hadrum 1	22
E. hadrum 2	21
Eubacterium sp.	22
Eubacterium sp.	24
C. nexile	ND[b]
Clostridium sp.	135
C. clostridiiforme	131
C. paraputrificum	126
C. perfringens (control)	249

[a] Soluble protein from spent growth medium.
[b] ND, Not done.

bacteria had reduced the Direct Blue 15 and Nitro Red. Morphologically distinct colonies from human intestinal bacteria that reduced the dyes were transferred anaerobically to fresh plates with Direct Blue 15. First partial and then complete clearing of the dye occurred, indicating that the production of the enzyme increased with time.

The isolated bacteria were all strict anaerobes. The list of azoreductase-producing anaerobes identified is shown in Table 1. *Eubacterium hadrum* 1 and 2 were slightly different in growth rate and azoreductase production. The protein profiles of the two isolates differed slightly from each other and from that of *E. hadrum* ATCC 29173, so the strains were considered to be different.

Comparison of the kinetics of reduction of azo dyes by different isolates. The reduction of Direct Blue 15 by different isolates was compared in BHI broth. A decrease in the A_{615} of Direct Blue 15 occurred at different rates in the supernatants from different bacteria. Figure 3 shows the reduction of Direct Blue 15 for six of our isolates and *C. perfringens*. The rate of reduction of dye was highest for *C. perfringens*. Among our new isolates, *Clostridium paraputrificum* had the highest rate of reduction and the *Butyrivibrio* sp. had the lowest.

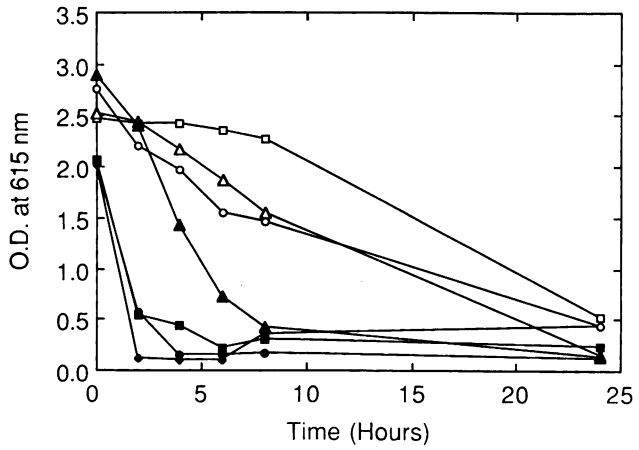

FIG. 3. Reduction of Direct Blue 15 over time by bacteria isolated from human intestinal microflora. Reduction of Direct Blue 15 was determined spectrophotometrically at 615 nm. Symbols: ◆, *C. perfringens*; ●, *C. paraputrificum*; ■, *C. clostridiiforme*; ▲, *Eubacterium* sp.; △, *E. hadrum*; ○, *Bacteroides* sp.; □, *Butyrivibrio* sp. O.D., Optical density.

TABLE 2. Comparison of the azoreductase activity of proteins from different bacteria and the units of enzyme produced by 10^{10} cells of each bacterium

Bacterium	U of activity[a]/mg of soluble protein	U of extracellular enzyme/10^{10} bacteria
C. perfringens	0.249	29.40
C. clostridiiforme	0.130	17.54
E. hadrum 2	0.021	2.56
Butyrivibrio sp.	0.070	1.36
Bacteroides sp.	0.016	1.38

[a] Amount of enzyme needed to reduce 1 μmol of Direct Blue 15 per min.

The average rate of reduction of Direct Blue 15 in the supernatant of each bacterium was measured to compare the azoreductase activity of the different isolates in five replicate cultures. For determination of the average rate, the time necessary for complete reduction of a given amount of Direct Blue 15 by 1 mg of soluble bacterial protein from each bacterium was measured, regardless of the kinetics of degradation. The average rate of reduction ranged from 16 to 135 nmol/min per mg of soluble protein for our isolates (Table 1).

Comparison of azoreductase production in different isolates. To evaluate whether differences in azoreductase activity were due to differences in enzyme production or in the specific activity of extracellular proteins (units of enzyme per milligram of soluble protein), we compared the production of enzymes in three bacteria with low enzymatic activity with that in two bacteria with high enzymatic activity. The procedure involved measuring the number of bacteria needed to produce 1 U of extracellular enzyme. One unit of enzymatic activity in the supernatant of a bacterial culture was defined as the amount of enzyme necessary to reduce 1 μmol of Direct Blue 15 in 1 min under anaerobic conditions at 37°C. The same bacterial culture was used for the determination of enzymatic activity, measurement of protein, and bacterial counting.

C. perfringens produced the highest enzyme activity per number of bacteria. *Clostridium clostridiiforme* enzyme production per cell was higher than that of the other species, except for *C. perfringens*. The amount of enzyme produced in *C. clostridiiforme* was 6.85 times higher than that produced in *E. hadrum* 2 and 12.7 times higher than that produced in the *Bacteroides* sp. and the *Butyrivibrio* sp. (Table 2). However, *E. hadrum* 2 had a faster growth rate than did the *Bacteroides* sp. or the *Butyrivibrio* sp., as was evident from the turbidity of the cultures.

In addition, the specific activities of supernatant proteins from different bacteria differed. The specific activity was highest for *C. perfringens* and *C. clostridiiforme* (Table 2). The activity per milligram of protein from *C. clostridiiforme* was 1.8 times higher than that from the *Butyrivibrio* sp., 4.6 times higher than that from *E. hadrum* 2, and 8.18 times higher than that from the *Bacteroides* sp. The *Bacteroides* sp. had the lowest growth rate and produced the lowest amount of enzyme. Its supernatant had a low activity per milligram of protein.

Effects of cofactors on azoreductase. *C. perfringens* and some of our isolates reduced the dye in cultures, with or without FAD (Table 3). For other isolates, reduction required FAD, although the bacteria grew in both the presence and the absence of FAD.

TABLE 3. Effects of FAD on azoreductase production and activity in anaerobic bacteria

Bacterium	Reaction[a] of the following with Direct Blue 15:			
	Bacterial culture + FAD	Bacterial culture + no FAD	Bacterial culture + FAD + tetracycline	Boiled bacteria + FAD + tetracycline
C. perfringens	+	+	NA	NA
Clostridium sp.	+	+	NA	NA
C. nexile	+	−	+	−
C. clostridiiforme	+	−	+	−
C. paraputrificum	+	+	NA	NA
Butyrivibrio sp.	+	−	+	−
Bacteroides sp.	+	−	+	−
Eubacterium sp.	+	−	+	−
Eubacterium sp.	+	+	NA	NA
E. hadrum 2	+	−	+	−

[a] +, Reduction of Direct Blue 15; NA, not applicable (species were not tested with added FAD, since they were active without it); −, no reduction of Direct Blue 15.

To find out whether flavin-containing compounds were necessary for the production or activity of the enzyme, we divided the cultures from isolates requiring flavin for dye reduction into four parts, with the following treatments: (i) FAD addition, (ii) tetracycline and FAD addition (to prevent further growth of the bacteria and to test for the activity of enzyme already produced at the time of FAD addition), (iii) FAD and tetracycline addition after boiling of the culture for 2 min (to inactivate the enzyme already produced), and (iv) no treatment (enzyme and dye were present, but no FAD was present). All of these isolates were tetracycline sensitive. The dye was reduced in all bacterial cultures after the addition of either FAD alone or FAD and tetracycline. This result shows that the enzyme was present but did not function in the absence of flavin-containing compounds (Table 3) and that flavin-containing compounds were necessary for the activity of the enzyme but not for enzyme biosynthesis.

Mersalyl acid, an inhibitor of azoreductase in rat liver microsomes, did not inhibit the azoreductase activity of either *C. perfringens* or our isolates (Table 4).

TABLE 4. Effects of the addition of different cofactors on the azoreductase activity of intestinal anaerobic bacteria

Cofactor[a]	Reaction[b] with Direct Blue 15 in:					
	C. perfringens	*E. hadrum* 1	*E. hadrum* 2	*C. clostridiiforme*	*Butyrivibrio* sp.	*Bacteroides* sp.
FAD	+	+	+	+	+	+
Flavin mononucleotide	+	+	+	+	+	+
Riboflavin	+	+	+	+	+	+
Glutathione	+	−	−	−	−	−
Cytochrome *c*	+	−	−	−	−	−
NAD	+	−	−	−	−	−
NADH	+	−	−	−	−	−
NADPH	+	−	−	−	−	−
FAD + mersalyl acid	+	+	+	+	+	+
None	+	−	−	−	−	−

[a] 50 μg of each of the listed cofactors per ml was added to medium M10 containing Direct Blue 15.

[b] +, Reduction of Direct Blue 15; −, no reduction of Direct Blue 15.

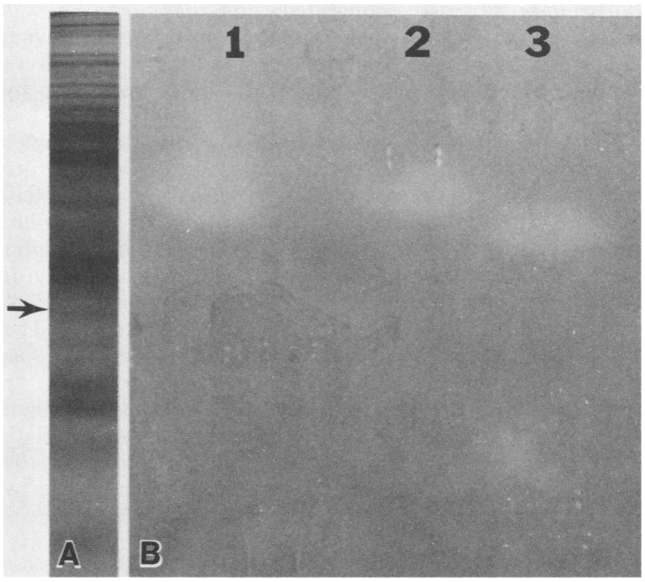

FIG. 4. Nondenaturing polyacrylamide gel electrophoresis of proteins from different isolates and *C. perfringens*. (A) *C. perfringens* soluble protein stained with Coomassie brilliant blue. The arrow indicates the location of the azoreductase band (the dimensions of the gel change after staining). (B) Soluble proteins from *C. perfringens* and isolates stained with Nitro Red. The dye was reduced at the location of the enzyme band. Lanes: 1, *Clostridium* sp.; 2, *C. paraputrificum*; 3, *C. perfringens*.

The effects of other cofactors on the enzymatic activity of *C. perfringens* and five isolates were tested with Direct Blue 15 (Table 4). All of the isolates grew on all of the minimal medium plates, regardless of the addition or type of cofactor. Direct Blue 15 was reduced on all of the plates with *C. perfringens*, even in the absence of cofactor. The selected isolates, however, showed enzymatic activity and clearance of Direct Blue 15 only on plates with flavin-containing compounds (FAD, flavin mononucleotide, or riboflavin).

Comparison of the azoreductase structures in different isolates. The enzymes from these bacteria were compared on nondenaturing polyacrylamide gels under anaerobic conditions. An amount of ammonium sulfate (60%) over the amount necessary to precipitate the enzyme from *Streptococcus faecalis* (29) was used to ensure complete precipitation of the enzyme. After electrophoresis, the azoreductase from each bacterium was located by the clear band which developed on the gel, which was stained with either Direct Blue 15 or Nitro Red (Fig.4).

As in the plate assay, the development of clear bands on the gels was faster with Nitro Red staining than with Direct Blue 15 staining. Both dyes, however, were cleared at the same location on the gel. Only a single clear band was present for each strain, indicating that only one isozyme was produced by each of the bacteria tested. The azoreductase band was detected in both the supernatant and the broken cell pellet.

The locations of the enzyme bands differed for some of the bacteria. Since the nondenaturing gel did not contain sodium dodecyl sulfate, the location of the band depended on both the molecular weight and the charge of the enzyme. The azoreductase from *E. hadrum* 1 migrated the same distance as the enzyme from *E. hadrum* 2. It differed from the enzymes from *C. clostridiiforme* and *C. perfringens* (data not shown). The enzymes from *C. paraputrificum* and the

Clostridium sp. also comigrated, and the migration was slightly different from that of the *C. perfringens* enzyme (Fig. 4). At least three different forms of the enzyme were expressed by these species and could be distinguished by differences in molecular weight and/or charge.

Some characteristics of azoreductase. When the samples containing enzymes were briefly exposed to oxygen before being loaded on the gel, the enzymes were completely inactivated. Even when the subsequent steps of electrophoresis and staining with azo dye all were performed under anaerobic conditions, the enzymes did not regain activity. It appears that the enzymes from all of these isolates are irreversibly inactivated in the presence of oxygen. The absolute exclusion of oxygen during all of the steps was necessary for the detection of enzyme bands on the gel.

When the 15,000 × *g* cell supernatants from overnight cultures grown in the absence of dye were incubated with Direct Blue 15, dye reduction occurred, indicating that the enzyme was extracellular and did not require induction by azo dye for production.

DISCUSSION

Previously we showed that anaerobic bacteria from human intestinal microflora have azoreductase activity and identified the aromatic amines produced by the breakdown of benzidine-based azo dyes (3–5, 18). Subsequently we developed an assay that detects the specific anaerobic bacteria participating in the reduction of azo dyes in human intestinal microflora. This assay will be useful for screening for azoreductase-producing anaerobic bacteria. We isolated, from the feces of a healthy individual, several species of bacteria representative of the organisms capable of producing azoreductase. Other bacteria with azoreductase activity would probably have been identified by our assay if we had used other sources of intestinal microflora.

Gas chromatography-mass spectrometry analysis of a product of the reduction of Direct Blue 15 by five of the isolates showed that the Direct Blue dye was converted to dimethoxybenzidine (Fig. 1; unpublished results), as we previously showed for human intestinal flora (3–5, 18).

Except for *C. paraputrificum*, the organisms found to have azoreductase were species not previously reported to reduce azo dye compounds (1). These were differences in the rates of reduction of azo dyes by different bacteria, as noted in other studies (1, 4). These differences were due to variations in growth rates and differences in the production and/or activity of the enzyme.

The differences in the rates of reduction of Nitro Red and Direct Blue 15 dye observed were consistent with previous reports for other azo dyes (23, 29). The results of activity staining after nondenaturing gel electrophoresis indicated that the same azoreductase reduced both dyes.

Some of our isolates required the addition of a flavin cofactor for the initiation of their enzymatic activity. We hypothesize that soluble flavin could be reduced by an azoreductase and then reduce the azo dye to the corresponding amine (8, 9, 28, 29). Perhaps the protein responsible for azoreduction in these isolates is a flavoprotein similar to the azoreductase of *S. faecalis* (9).

In our experiments, no other cofactors that were tested could substitute for flavin to restore enzymatic activity. Some of these cofactors have been shown to be essential for the activity of azoreductases from other sources (25). *C. perfringens* and three of the isolates which did not need flavin-containing compounds for azoreductase activity also had higher rates of reduction of azo dye in BHI broth. *C. clostridiiforme* and a *Eubacterium* sp. reduced azo dye in the absence of flavin-containing compounds but at a slower rate than in the presence of flavin-containing compounds.

The enzymes in our isolates were different from the azoreductases in liver microsomes and in *Pseudomonas cepacia* and *Pseudomonas* spp. (12, 13, 15). Mersalyl acid inhibits liver microsomal azoreductase but not the microbial azoreductase in our isolates (25). The azoreductase in our isolates, like the liver microsomal azoreductase, became irreversibly inactivated in the presence of oxygen, unlike the azoreductases in *Pseudomonas* spp. and *P. cepacia* (12, 13, 15, 20). The azoreductase in our isolates also differed from that of the nematode *Ascaris lumbricoides* var. *suum* (7), which retained only 30% of its activity in the presence of flavin (7).

Our gel electrophoresis system suggested the presence of only one azoreductase isozyme in each bacterium. In contrast, liver microsomes contain several different types of azoreductase (25). Size and/or charge differences existed in the azoreductase isozymes produced by the different bacteria, as shown by electrophoresis. At least three different forms of this enzyme were detected in different strains.

Since azoreductase was produced constitutively, it may have other functions vital to the cell. The enzyme was secreted extracellularly in all of these isolates. The supernatants had more enzyme than did the cell lysate pellets. Previous reports on azoreductase from intestinal bacteria have used only cell extracts (1, 9).

ACKNOWLEDGMENTS

Special thanks are due to Amy Boast for technical assistance, to Tom Heinze for mass spectrometry analysis, and to John B. Sutherland, William Melchior, and K. Barry Delclos for helpful comments.

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