Reduction of Nitroaromatic Compounds by Anaerobic Bacteria Isolated from the Human Gastrointestinal Tract

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Human intestinal microbial flora were screened for their abilities to reduce nitroaromatic compounds by growing them on brain heart infusion agar plates containing 1-nitropyrene. Bacteria metabolizing 1-nitropyrene, detected by the appearance of clear zones around the colonies, were identified as Clostridium leuhtum, Clostridium paraparatif picum, Clostridium clostridiforme, another Clostridium sp., and an Eubacterium sp. These bacteria produced aromatic amines from nitroaromatic compounds, as shown by thin-layer chromatography, high-pressure liquid chromatography, and biochemical tests. Incubation of three of these bacteria with 1-nitropyrene, 1,3-dinitropyrene, and 1,6-dinitropyrene inactivated the direct-acting mutagenicity associated with these compounds. Menadione and o-iodosobenzoic acid inhibited nitroreductase activity in all of the isolates, indicating the involvement of sulfhydryl groups in the active site of the enzyme. The optimum pH for nitroreductase activity was 8.0. Only the Clostridium sp. required added flavin adenine dinucleotide for nitroreductase activity. The nitroreductases were constitutive and extracellular. An activity stain for the detection of nitroreductase on anaerobic native polyacrylamide gels was developed. This activity stain revealed only one isozyme in each bacterium but showed that the nitroreductases from different bacteria had distinct electrophoretic mobilities.

Nitroaromatic compounds (nitro-PAHs) are ubiquitous environmental contaminants that are formed by the nitration of PAHs from various combustion sources. These compounds have been detected in carbon black photocopy toners, urban-air particulates, diesel fuel emissions, used motor oils, barbecued food, and tea leaves. Nitro-PAHs, especially nitropyrenes, are potent mutagens in mammalian cells. They have carcinogenic activity in laboratory animals and may pose a human health risk.

Nitro-PAHs are metabolized by both reductive and oxidative pathways (nitroreduction, ring oxidation, or a combination of the two). Reduction of nitro groups is catalyzed by several mammalian enzymes and also by intestinal bacteria. Exposure of humans to nitro-PAHs usually occurs via inhalation of diesel engine exhaust particulates. These compounds can also reach the intestinal tract through ingestion of food. In a study, Ohnishi et al. measured the amounts of 1-nitropyrene and 1,6-dinitropyrene in several grilled foods and estimated the exposure of individuals to nitroaromatic compounds through inhalation and ingestion of food during a 20-year period.

Intestinal microflora, which are dominated by obligate anaerobes, can modify a wide range of environmental chemicals either directly or through the enterohepatic circulation. Various anaerobic human, monkey, and rat intestinal microflora are capable of rapidly metabolizing 1-nitropyrene, dinitropyrenes, and 6-nitrochrysene to aromatic amines. In addition, anaerobic bacteria commonly associated with the gastrointestinal tract reduce nitro-PAHs to aromatic amines, indicating nitroreductase activity in these bacteria. Kinouchi and Ohnishi have studied the enzymes that convert 1-nitropyrene to 1-aminoypyrene in Bacteroides fragilis and have purified one of these enzymes.

In this study, we have developed a screening method to detect anaerobic bacteria capable of producing nitroreductases and have shown that the isolated bacteria decrease the mutagenicity of nitro-PAHs. We also have partially characterized the nitroreductase activities associated with these bacteria.

MATERIALS AND METHODS

Chemicals. 4-Nitrobenzoic acid and 4-amino benzoic acid were obtained from Chem Service, Media, Pa. 1-Nitropyrene, 1,3- and 1,6-dinitropyrene, 1-amino pyrene, and pyrene were obtained from Chemsyn Science Laboratories, Leno x, Kans., and Aldrich Chemical Co., Milwaukee, Wis. 1-Nitro[4,5,9,10-14C]pyrene (specific activity, 57.4 mCi/mmol; radiochemical purity, >99%) was obtained from Chemsyn. Tetracycline hydrochloride, menadione, o-iodosobenzoic acid, flavin adenine dinucleotide (FAD), and NADH were purchased from Sigma Chemical Co., St. Louis, Mo. N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDD) and high-pressure liquid chromatography(HPLC)-grade solvents were purchased from Fisher Chemical Co., Pittsburgh, Pa. All other chemicals were of reagent grade and of the highest purity available.

Media and cultural conditions. Brain heart infusion (BHI) agar from Difco Laboratories, Detroit, Mich., was prepared as previously reported (31) and used for the isolation and detection of nitroreductase-producing bacteria from intestinal microbial flora. BHI broth, prereduced and anaerobically sterilized (PRAS), from Carr-Scarborough Microbiologists, Stone Mountain, Ga., was used for the growth and maintenance of nitroreductase-producing bacteria.

Isolation of nitroreductase-producing bacteria. Bacteria from human intestinal microflora were isolated on BHI agar
plates containing 16 μg of 1-nitropyrene per ml, which produced a yellow background in the plates. Fecal material from one individual was collected in a degassed container and used as the source of the isolates. One gram of feces was added to 9 ml of BHI (PRAS) broth. Serial dilutions of human feces in BHI (PRAS) broth were made, and 0.1 ml of each dilution was spread on each of the plates. The plates were incubated at 37°C under both aerobic and anaerobic conditions. They were observed after 2 days for clearance of 1-nitropyrene around bacterial colonies. *Clostridium perfringens* ATCC 3626, from the American Type Culture Collection, Rockville, Md., was used as a positive control for nitroreductase activity (4). Colonies surrounded by clear zones were transferred to BHI plates containing 1-nitropyrene for single-colony isolation. The isolated colonies were transferred to BHI (PRAS) broth for growth, maintenance, and identification.

Identification of nitroreductase-producing anaerobic bacteria. The bacteria capable of reducing 1-nitropyrene were identified by colony morphology, Gram stain, biochemical tests, volatile and nonvolatile fatty acids, and protein analysis according to the methods described elsewhere (13, 21, 26, 36).

Assay of enzyme activity. Nitroreductase activity was assayed by adding 4-nitrobenzoic acid to an enzyme-containing solution and measuring the amount of 4-aminobenzoic acid produced (40). 4-Nitrobenzoic acid was added to tubes containing combined supernatants and cell extracts from overnight cultures of each bacterial isolate. Noninoculated BHI (PRAS) broth was used as a control. The tubes were incubated at 37°C for 1 h and then acidified with 0.21% (final concentration) trichloroacetic acid. Sodium nitrite (final concentration, 0.007%) was added to the mixtures to form a diazonium salt with 4-aminobenzoic acid. The mixtures were incubated at room temperature for 20 min. Next, ammonium sulfate (final concentration, 0.04%) was added to neutralize the sodium nitrite. Following incubation for 3 min at room temperature, NEDD (final concentration, 0.35%) was added. The diazonium salt formed from 4-aminobenzoic acid couples with NEDD in acidic solutions and produces a red-purple azo dye. The A_{540} was measured with a Beckman DU-7 spectrophotometer. The amount of 4-aminobenzoic acid produced was calculated from a standard curve.

One unit of enzyme was defined as the amount of enzyme necessary to produce 1 μg of 4-aminobenzoic acid at 37°C under anaerobic conditions in 1 h. The specific activity of the enzyme was measured by calculating the units of enzymatic activity per milligram of total soluble protein in supernatants and cell extracts.

Detection of arylamines in bacterial colonies following growth with nitroaromatic compounds. 1-Nitropyrene or 6-nitrochrysene, dissolved in dimethyl sulfoxide, was added to the BHI agar at a concentration of 16 to 32 μg/ml of the medium before incubation with bacteria. The cultures were incubated in the dark at 37°C for 48 h and were tested for the presence of arylamine. Thirteen milliliters of a solution containing 0.21% trichloroacetic acid and 0.007% sodium nitrite was added to each 9-cm-diameter culture plate. The plates were incubated at room temperature for 20 min. One milliliter of 0.5% ammonium sulfate was added to the plates, which were incubated for another 0.29 mg/ml) was added to the plate. The plates were incubated at room temperature and observed for the appearance of a red-purple color in the colonies.

Analyses of 1-nitropyrene metabolites by TLC and HPLC.

1-Nitropyrene (final concentration, 16 μg/ml) was added to BHI (PRAS) broth and inoculated with 0.2 ml of an overnight bacterial culture. After incubation, the cells were broken by sonic oscillation and the cultures were extracted three times with equal volumes of ethyl acetate. The combined extracts were dried by rotary evaporation under reduced pressure and then redissolved in methanol for thin-layer chromatography (TLC) and HPLC.

The ethyl acetate extracts from the culture media were spotted onto a TLC plate (silica gel, 0.2-mm thickness; E. Merck AG, Darmstadt, Germany). 1-Aminopyrene was spotted on the plate as a standard, and the plates were developed in ethyl acetate-benzene (1:9 [vol/vol]). The plates were then observed for 1-aminopyrene spots with UV light.

For study of metabolites by HPLC, 3 μCi of 14C-labeled 1-nitropyrene and 8 μg of nonlabeled 1-nitropyrene per ml were added to BHI (PRAS) broth and the medium was inoculated with the bacterial isolates. The cultures were incubated at 37°C for 3 days, sonicated for 12 min (four sonifications of 3 min each), and extracted with ethyl acetate. The extracts were dried under anhydrous sodium sulfate, and the residues were dissolved in methanol. The samples were then analyzed by HPLC, using a Beckman model 100A liquid chromatograph with a diode array detector as previously described (14). One-minute fractions were collected, and the radioactivity present in each fraction was measured (14, 24, 32). Authentic 1-aminopyrene and 1-nitropyrene were used as chromatographic markers.

Mutagenicity assay. 1-Nitropyrene (final concentration, 8 μg/ml) and 1,3-dinitropyrene and 1,6-dinitropyrene (final concentration, 1 μg/ml) were added to separate tubes containing BHI (PRAS) medium. The media were inoculated and incubated for 0 to 5 days at 37°C. The metabolites were extracted with ethyl acetate and concentrated as described above. Tubes of un inoculated BHI (PRAS) medium containing nitro-PAHs were used as controls. Nitro-PAHs were also added to ethyl acetate extracts of bacterial cultures and were tested in the mutagenicity assay as controls. Ethyl acetate-extracted metabolites were dried, dissolved in dimethyl sulfoxide, and tested for mutagenicity by using *S. typhimurium* TA98 (5).

Effect of pH on nitroreductase activity. Tubes containing 10 ml of BHI broth were adjusted to pHs from 4.5 to 9.5 with HCl and NaOH and were inoculated with 0.2 ml of an overnight culture of each isolate. 4-Nitrobenzoic acid (0.29 mg/ml) was added to all of the cultures. Nitroreductase activity was assayed by testing for the formation of 4-aminobenzoic acid with NEDD as described elsewhere (40).

Protein determination and cell count. After sonication and centrifugation, the soluble protein in the combined supernatant and cell extract from each isolate was measured (22). The amount of protein detected in noninoculated BHI (PRAS) broth was subtracted from the total amount of protein. Bacterial cells were enumerated with a Petroff-Hauser bacteria counter.

Cofactor requirement. Two tubes of M10 medium prepared by the method of Caldwell and Bryant (3) were inoculated with each isolate, and FAD (final concentration, 50 μg/ml) was added to one tube. 4-Nitrobenzoic acid (final concentration, 0.29 mg/ml) was added to the other tube. The cultures were incubated for 2 days and assayed for nitroreductase activity by testing for the formation of 4-aminobenzoic acid from 4-nitrobenzoic acid.

Effects of inhibitors on nitroreductase activity. Overnight cultures of bacteria in BHI (PRAS) broth were sonicated...
with an ultrasonic cleaner for a total of 12 min and centrifuged at 5,000 × g for 10 min. Tetracycline hydrochloride (final concentration, 15 μg/ml) and menadione or o,o-diosbenzoic acid (final concentration, 0.32 mg/ml) were added to the combined supernatant and cell extract. Cultures without inhibitors were used as controls. Replicate culture tubes were incubated for 15 min at 37°C, and then 4-nitrobenzoic acid was added to the tubes with or without inhibitors (final concentration, 0.29 mg/ml). The cultures were incubated at 37°C for 1 h and assayed for the formation of 4-aminobenzoic acid.

Nitroreductase activity assay on native polyacrylamide gels. A non-denaturing anaerobic gel was prepared and run as previously described (30). The gel was then incubated, with occasional shaking, anaerobically for 1.5 h at 37°C in a Ziploc bag containing 30 ml of degassed M10 medium supplemented with 0.24 mg of 4-nitrobenzoic acid per ml and with FAD and NADH (final concentration of each, 50 μg/ml). The solution was decanted, and the gel was incubated for another hour. The gel then was incubated for 20 min at room temperature in a solution containing 0.21% trichloroacetic acid and 0.007% sodium nitrite. Ammonium sulfamate (final concentration, 0.004%) was then added to the gel. After 3 min, NEDD (final concentration, 0.027%) was added to the gel to develop the nitroreductase bands. The gel was gently shaken at room temperature in the bag until the bands of red-purple color, indicating 4-aminobenzoic acid, had developed on the gel.

RESULTS

Isolation and identification of nitroreductase-producing bacteria. Following incubation of bacteria from human intestinal microflora on the medium containing 1-nitropyrene, many different bacterial colonies developed on the plates under both anaerobic and aerobic conditions. There were no clear zones on the plates that were incubated aerobically, but on plates incubated under anaerobic conditions, some colonies were surrounded by clear zones on the yellow background of the plates. These colonies were transferred anaerobically to plates containing 1-nitropyrene for single-colony isolation. The 1-nitropyrene was utilized, and clear zones surrounding the bacteria developed on the plates (Fig. 1).

The bacterial isolates were identified as a Clostridium sp., Clostridium leptum, Clostridium paraputrificum, Clostridium clostridiforme, and a Eubacterium sp. One of the isolates, designated NP4, was not identified. Table 1 lists the anaerobic bacteria and the amount of 4-nitrobenzoic acid utilized per milligram of protein, as calculated from duplicates of the combined supernatants and cell extracts from each bacterium.

The bacteria listed in Table 1 also metabolized 6-nitrochrysene, 1-amino-7-nitrofluorene, 4-nitrobenzoic acid, and 1,3- and 1,6-dinitrobenzene, as was shown by the development of clear zones, amine production, and mutagenicity tests (data not shown).

Identification of metabolites. HPLC, TLC, and biochemical tests were used for identification of the metabolites produced by the isolates in BH1 (PRAS) broth containing 1-nitropyrene. HPLC analysis of the ethyl acetate extracts from C. perfringens and five of the isolates revealed several UV-absorbing peaks. The compounds eluting from 2 to 16 min were derived from the culture medium and not from 1-nitropyrene (Fig. 2). This was determined by experiments with 14C-labeled 1-nitropyrene that revealed only two peaks of radioactivity (data not shown). The retention time and UV spectrum of the peak eluting at 17 min were identical to those of authentic 1-aminothrene (Fig. 2). The peak eluting at 31 min corresponded to 1-nitropyrene (Fig. 2).

On TLC plates, the major metabolite from each of the isolates migrated with the authentic 1-aminothrene and was fluorescent, as was the authentic 1-aminothrene standard. We concluded that 1-nitropyrene was reduced by nitroreductases from all of the different bacteria to 1-aminothrene.

The ability of the isolates to convert other nitroaromatic compounds to amines was also tested. After the addition of 4-nitrobenzoic acid to broth cultures or after growth of bacterial isolates on agar plates containing 1-nitropyrene or 6-nitrochrysene, the amines in the bacterial cultures was detected by the addition of NEDD. The purple azo dye was produced after the reduction of 4-nitrobenzoic acid in broth cultures or after the reduction of 1-nitropyrene or 6-nitrochrysene in bacterial colonies. There was no color development in bacterial isolates grown without nitroaromatic compounds or in bacteria that did not reduce these nitroaromatic compounds.

Mutagenicity tests. The S. typhimurium reversion assay using strain TA89 was used to determine the direct-acting mutagenic activity of the ethyl acetate-extracted metabolites of bacteria incubated with 1-nitropyrene, 1,3-dinitrobenzene, and 6-nitrochrysene. The results are presented in Table 1. The data indicate that the number of revertants was significantly higher in the samples from the isolates grown on 1-nitropyrene than in the samples from the isolates grown on 6-nitrochrysene.
grew at neutral to slightly alkaline pHs. The enzyme was active at all the pHs at which the bacteria grew, but the optimum pH for activity was 8.0.

**Nitroreductase characteristics in bacterial isolates.** The supernatants of overnight cultures of bacteria incubated in the absence of 4-nitrobenzoic acid were tested for nitroreductase activity. The conversion of 4-nitrobenzoic acid to 4-aminobenzoic acid under anaerobic conditions occurred in all of the cultures tested. The exclusion of oxygen was required in this procedure. These results indicate that the enzyme was constitutive and extracellular and required anaerobiosis for activity. Only the enzyme from one of the isolates (Clostridium sp.) required added FAD for activity.

**Comparison of enzyme activity in different isolates.** The amounts of enzyme produced by different bacterial isolates were compared by measuring the units of enzyme produced per milligram of soluble protein and the numbers of cells that produced 1 U of enzyme in two replicate cultures (Table 3). There were differences in the amount of enzyme produced by each bacterium and in the enzymatic activity per milligram of protein produced by each bacterium. The *Eubacterium* sp. produced the highest amount of enzyme and *C. clostridiforme* produced the least.

**Effect of inhibitors on enzymatic activity of nitroreductase.** The effects of sulphydryl-modifying chemicals on nitroreductase activity are shown in Table 4. Menadione and o-iodosobenzoic acid inhibited the enzymatic activity of nitroreductases from all of the isolates. The percentage of inhibition with menadione varied from 38.4% for the *Eubacterium* sp. to 77.4% for isolate NP4. With o-iodosobenzoic acid, inhibition varied from 58.4% for the *Eubacterium* sp. to 77.0% for *Clostridium* sp.

**Comparison of nitroreductases from different isolates on native gels.** Ammonium sulfate-fractionated crude extracts

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**TABLE 3. Nitroreductase activity of proteins from anaerobic bacteria isolated from human gastrointestinal tract**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>U° of enzyme</th>
<th>Per mg of protein</th>
<th>Per 10^6 bacteria</th>
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<tbody>
<tr>
<td><em>C. leptum</em></td>
<td>16.7 ± 6.6</td>
<td>86.4 ± 12.4</td>
<td></td>
</tr>
<tr>
<td><em>Eubacterium</em> sp.</td>
<td>14.6 ± 1.9</td>
<td>188.7 ± 17.6</td>
<td></td>
</tr>
<tr>
<td><em>C. clostridiforme</em></td>
<td>16.8 ± 2.7</td>
<td>11.0 ± 4.7</td>
<td></td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>11.5 ± 1.0</td>
<td>147.3 ± 79.0</td>
<td></td>
</tr>
<tr>
<td><em>C. paraputrificum</em></td>
<td>12.0 ± 3.2</td>
<td>85.6 ± 11.6</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium</em> sp.</td>
<td>25.0 ± 7.1</td>
<td>80.0 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

*a* One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µg of 4-aminobenzoic acid at 37°C in 1 h under anaerobic conditions.
from cultures grown in BHI (PRAS) broth were used for the detection of enzymes from three strains on the gels. The enzymes from different isolates were detected by using native gels under anaerobic conditions. Enzyme bands were red-purple on the gels where 4-nitrobenzoic acid had been reduced to 4-aminobenzoic acid and converted to azo dye by NEDD. Five units of enzyme was sufficient to produce a red-purple band on the gels. There was only one enzyme band in each lane, indicating that there was only one nitroreductase isozyme in each bacterium. The electrophoretic mobilities of the enzyme bands were slightly different for each strain, indicating that different forms of nitroreductase were produced by these strains (Fig. 3).

**DISCUSSION**

The abilities of pure cultures of bacteria and of intestinal microflora from humans and several animal species to reduce nitro-PAHs to amino-PAHs has been shown previously (4, 5, 14, 17, 23, 24). However, the specific bacteria in the human gastrointestinal tract that reduce these environmental pollutants have not been previously identified. In this study, a simple method for rapid detection and isolation of bacteria capable of reduction of nitro-PAHs has been described.

Although we used feces from only one individual for isolation of these microorganisms, other bacteria probably would be detected by using this method with other sources of isolates. Four of the bacterial strains isolated belonged to the genus Clostridium. Some of these bacteria were not previously known to produce nitroreductases. HPLC, TLC, and biochemical tests revealed that aromatic amines were the major metabolites produced from nitro compounds by these bacteria. The presence of 1-aminopyrene, 6-aminochrysene, and 4-aminobenzoic acid as major metabolites from the corresponding nitroaromatic compounds indicates that the first reaction in the biotransformation of nitroaromatic compounds by intestinal bacteria is accomplished by a nitroreductase.

The Ames (S. typhimurium) assay was used to monitor the effect of biotransformation by three of the isolates on the mutagenicity of 1-nitropyrene and of 1,3- and 1,6-dinitropyrene. The three isolates were capable of metabolizing all three nitro-PAHs to products that were considerably less mutagenic. Kinouchi and Ohnishi (19) attributed the decrease in the mutagenicity of 1-nitropyrene after incubation with B. fragilis to the rapid metabolism of 1-nitropyrene to 1-aminopyrene, which has little direct-acting mutagenicity.

The isolates used in the present study virtually eliminated the mutagenicity of 1-nitropyrene and of 1,3- and 1,6-dinitropyrene. No traces of active N-hydroxylamines were detected in the metabolites of these bacteria after exposure to nitro-PAHs (HPLC results showed only two peaks of radioactivity). Thus these organisms could be useful for anaerobic detoxification of nitro-PAHs under appropriate conditions. Previously it was shown that intestinal microflora of different animal species produce compounds from 1,8-dinitropyrene that are less mutagenic than the parent compound (5). Moller et al. (25) administered 2-nitrofluorene to germfree and conventional rats and found that a smaller amount of mutagenic metabolites of this environmental contaminant was excreted in the urine and feces of conventional rats than of germfree rats. Their study clearly demonstrates the importance of intestinal microflora in the metabolism of nitro-PAHs in vivo. In our study, the specific organisms contributing to detoxification have been identified.

Nitroreductases from these organisms seemed to have a wide spectrum of substrates, as shown by the reduction of several nitroaromatic compounds, but the efficiency of reduction varied with different substrates (data not shown). The bacterial isolates grew in a narrow pH range under anaerobic conditions and produced active enzymes with a pH optimum of 8.0. Arora et al. (1) noted similarly that the optimum pH for nitroreductase activity of Vibrio cholerae was 8.0 in phosphate buffer.

Our isolates grew and produced the enzyme in a low-nutrient broth medium (M10 medium). The activity was lower in M10 medium than in the rich BHI (PRAS) medium (data not shown), probably because of differences in growth rates. FAD was necessary for nitroreductase activity only in the Clostridium sp. Similarly, nitroreductase activity of B. fragilis is markedly enhanced by the addition of flavin (19). A small amount of riboflavin (0.01 μg/ml), present in the yeast extract in M10 medium, conceivably may have satisfied a flavin requirement for the nitroreductases from the other bacterial isolates.

The enzymes from these isolates were inhibited by both menadione and o-iodosobenzoic acid, which bind to sulphydryl groups. This indicates that sulphydryl groups are essential for the catalytic function of the enzyme and suggests that there may be cysteine residues in the active site. Kinouchi and Ohnishi (19) also noted the inhibition of

**TABLE 4. Effects of menadione and o-iodosobenzoic acid on nitroreductase activity**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>% Inhibition by:</th>
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<tr>
<td></td>
<td>Menadione</td>
</tr>
<tr>
<td>Clostridium sp.</td>
<td>69.8</td>
</tr>
<tr>
<td>C. leptum</td>
<td>71.8</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>92.3</td>
</tr>
<tr>
<td>Eubacterium sp.</td>
<td>38.4</td>
</tr>
<tr>
<td>Isolate NP4</td>
<td>77.2</td>
</tr>
</tbody>
</table>

**FIG. 3. Activity staining of nitroreductase on native polyacrylamide gel.** Crude extracts were used for the detection of enzymes. Lane 1, C. paraputreficum; lane 2, a Eubacterium sp.; lane 3, C. perfringens. Arrows show the nitroreductase bands.
nitroreductases from *B. fragilis* by menadione and o-hydrobenzoic acid.

A simplified activity assay for direct detection of nitroreductase activity on native polyacrylamide gels after electrophoresis was developed by using 4-nitrobenzoic acid. This compound is a model substrate for nitroreductase (29). Previously, the detection of nitroreductase in *Escherichia coli* on gels was accomplished by slicing the gel and assaying for reductase activity (2). The method described here detects structural differences among nitroreductases from different bacteria by using crude extracts and can be used for purification of enzymes in preparative gels. The need for purification of highly oxygen-sensitive nitroreductase enzymes by conventional methods for this purpose has thus been eliminated.

The presence of different nitroreductases in different isolates was indicated by the distinct electrophoretic mobilities of the enzyme in an activity assay on native polyacrylamide gels. We detected only one nitroreductase isozyme in each bacterium. Kinouchi and Ohnishi (19) detected four different nitroreductase isozymes (nitroreductases I to IV) from *B. fragilis*. If more than one isozyme of nitroreductase was present in our isolates, they either migrated together on the native gels or could not be detected by this method.

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