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Isolation and Properties of Metronidazole-Resistant Mutants of *Bacteroides fragilis*

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Metronidazole-resistant mutants of *Bacteroides fragilis*, isolated after mutagenesis, had diminished ability to take up and metabolize the drug. All the metronidazole-resistant strains had depressed levels of pyruvate dehydrogenase compared with parent cultures. Their end products of glucose metabolism also differed from normal *B. fragilis* products and were consistent with deficiencies in pyruvate dehydrogenase activity.

Metronidazole is active in vitro and in vivo against strictly anaerobic bacteria. It has been used effectively in the treatment of anaerobic infections (16) and prophylactically in gynecological and colonic surgery (5, 18). Reports of strictly anaerobic bacteria showing resistance to metronidazole are rare, and its rapid bactericidal action on these bacteria (14, 15) has been attributed to reduction of the nitro group to an active intermediate (7). Interaction of the reduced intermediate with bacterial deoxyribonucleic acid has been proposed (7) as a basis for the toxicity of the drug.

It has recently been reported (15) that facultatively anaerobic bacteria take up metronidazole slowly, although extracts of their cells had no affect on the biological activity. This implies that facultative anaerobes do not activate intracellular metronidazole. In the present work, we report the isolation of metronidazole-resistant mutants of the strictly anaerobic bacterium *Bacteroides fragilis*. Direct comparisons of the properties of mutant and parent strains are made, especially with respect to uptake and metabolism of metronidazole. The finding that metronidazole-resistant mutants of *B. fragilis* have decreased pyruvate dehydrogenase activity is discussed in terms of the mode of action of this drug.

MATERIALS AND METHODS

Bacterial strains. Strains of *B. fragilis* used in this work were isolated from clinical specimens and identified, as previously described (3). *B. fragilis* strain AM24 was isolated from a urinary tract infection and was found in preliminary screening to be metronidazole resistant. *B fragilis* strains AM17 and AM78 were originally supplied by S. M. Finegold.

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Chemicals. All chemicals used for buffers and assays were analytical grade reagents. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was obtained from K & K Laboratories, Inc., Plainview, N.Y., and ethyl methane sulfonate (EMS) was from Eastman Kodak Co., Rochester, N.Y.

Media. Anaerobic brain heart infusion (ABHI) broth and a minimal medium in which growth of *B. fragilis* was glucose-dependent (AB broth) were prepared as described previously (1). ABHIA plates were prepared by adding 2% agar to brain heart infusion broth, sterilizing, and then supplementing immediately before use. ABHI-G broth was ABHI broth supplemented with 1% glucose. Sterile aqueous stock solutions of metronidazole (May & Baker, Melbourne, Australia) and rifampin (Ciba-Geigy, Melbourne, Australia) were used for the addition of these agents to ABHIA, just before pouring plates and where appropriate, to liquid media and cultures.

Mutagenic treatments and selection of metronidazole-resistant mutants. Cells of *B. fragilis* were cultured, treated with MNNG (80 to 100 μg/ml) and incubated in ABHI broth as previously described (1). Samples (0.1 to 0.2 ml) of the broth cultures were spread on ABHIA plates containing either rifampin (100 μg/ml) or metronidazole (10 or 25 μg/ml), and the plates were incubated anaerobically for 24 to 48 h at 37°C. Single colonies were subcultured to plates containing the same level of antimicrobial agent used in the original selection. For treatment with EMS, cells grown as for MNNG treatment were resuspended in reduced phosphate buffer (0.05 M, pH 7.4, supplemented as above) and diluted fourfold in similar buffer containing 75 μl of EMS. After 15 min at room temperature, suspensions were subcultured to ABHI broths; mutants were expressed and isolated as described above.

Concentrations of MNNG and EMS were chosen to give a 90 to 99% reduction in viable count under the conditions of testing for each strain.

Susceptibility tests. A disk diffusion method was used with the following antimicrobial agents: ampicillin, 10 μg; penicillin G, 2 U; cephaloridine, 25 μg; cephalothin, 30 μg; chloramphenicol, 10 μg; cloxacillin, 10 μg; doxycycline, 30 μg; erythromycin, 10 μg; tetra-

cycline, 10 μg; and lincomycin, 2 μg (all from Oxoid, London, England); lincomycin, 10 μg; clindamycin, 10 μg (Upjohn, Kalamazoo, Mich.); metronidazole, 5 μg (May and Baker, Melbourne, Australia). Disks were applied to freshly prepared ABHIA plates that had been inoculated by flooding with overnight broth cultures. Sensitivity patterns were recorded after 24 h at 37°C or 48 h at 37°C for slower growing strains. Minimal inhibitory concentrations (MICs) of antibiotics were determined by an agar dilution technique as previously described (2).

Growth curves and inhibition of growth by antimicrobial agents. Growth was initiated by the addition of overnight broth cultures to 20 ml of reduced medium in 100-ml side-arm flasks or 10 ml in Klett tubes giving initial turbidities of 30 to 60 Klett units. Flasks and tubes were sealed with butyl rubber stoppers, removed from the anaerobic chamber and incubated at 37°C. Growth was followed turbidimetrically in a Klett-Summerson colorimeter. Where appropriate, antimicrobial agents were added anaerobically by injection through the rubber stopper.

Detection of acids and alcohols by gas-liquid chromatography. A Packard model 427 chromatograph fitted with a flame ionization detector, coupled to a Hewlett-Packard 3380A integrator, was used for gas-liquid chromatography analyses. Samples for acid analyses were prepared as described by Holdeman and Moore (6), and 1 μl was injected onto a glass column (2 m by 4 mm I.D.) containing 15% SP1220–1% H_3PO_4 on 80/100 mesh Chromosorb W AW (Supelco, Inc., Bellefonte, Pa.) and operated under isothermal conditions (injection, 190°C; oven, 135°C; detector, 150°C) with N_2 carrier gas (40 ml/min). To achieve greater separation of methylated pyruvic and lactic acids, a temperature program (initial, 105°C; then 5°C/min to 150°C) was used. Alcohols were analyzed on a column (2 m by 2 mm I. D.) packed with Chromosorb 103, 80/100 mesh (Johns Manville Products Corp., Manville, N.J.), coated with 5% (wt/wt) KOH. Acidic products were removed from samples by adding the carbonate form of Dowex-1 (1 by 8-400) (Sigma Chemical Co., St. Louis, Mo.). The resin was removed by centrifugation, and 1 μl of the supernatant fluid was injected. A temperature program (2 min at 145°C; then 5°C/min to 190°C with a 5-min hold at the final temperature) gave good resolution of C_1 to C_6 alcohols of *n* and *iso* configurations.

Detection of gases by gas-liquid chromatography. Samples (1 ml) of culture headspace were analyzed on a 16-foot (ca. 488-cm) column containing Porapak Q and R (Waters Associates, Inc., Milford, Mass.) with 8 feet (ca. 244 cm) of each resin in series (Porapak Q at injector end) at 40°C with helium carrier (15 ml/min). Detector and injection temperatures were 100°C, and filament temperature of the thermal conductivity detector was 190°C.

Extraction and assay of metronidazole. Metronidazole was assayed colorimetrically by a method based on that of Populaire et al. (13). Samples (2 ml) were made alkaline (0.5 ml of 1 N KOH) and extracted with 5 ml of chloroform by inverting 20 times and then centrifuging to break any emulsion. The extraction procedure was repeated three times. Pooled organic phases were evaporated to dryness at 45°C under air and dissolved in 2 ml of ethanol. To assay metronidazole, 0.1 ml of a 7.5% aqueous solution of titanium chloride was added. After 15 min at room temperature, 1 ml of 1% *p*-dimethylaminobenzaldehyde was added, and the mixture was incubated at 65°C for 15 min. Ethanol was then added to give a final volume of 20 ml. Absorbance at 494 nm was proportional to metronidazole concentration over the range 5 to 50 μg/ml.

Assay of pyruvate dehydrogenase. Extracts of cells for assay of pyruvate dehydrogenase were prepared as follows: 100 ml of AB or ABHI broth was inoculated (1%) and grown for 18 h at 37°C before cells were pelleted (7,000 × *g*, 4°C, 10 min), washed once, and then resuspended in 5 ml of phosphate buffer (0.1 M, pH 7.0) with 250 μM 2-mercaptoethanol (PME buffer). Cells were disrupted by sonication and transferred to N_2-flushed tubes on ice and then into the anaerobic chamber for assay. Based on a previously described assay (8), reaction mixtures (10 ml) contained 2 mM sodium pyruvate, 0.075 mM HS–coenzyme A and 1.25 mM methyl viologen in PME buffer. Reactions were started in the anaerobic chamber by addition of extract (10 to 500 μl), tubes were sealed immediately and removed from the chamber, and changes in absorbance at 37°C were monitored at 1-min intervals in a Klett-Summerson colorimeter. Units of activity were arbitrarily defined as rate of change of Klett units per minute, and specific activity was expressed as units per milligram of protein from the average of two to five separate determinations. All assays were performed anaerobically due to oxygen sensitivity of the enzyme activity (data not shown). In some experiments, metronidazole, 1-nitro-3-naphthalene, or Furadantin was added to reaction mixtures before addition of extract to test the ability of these compounds to compete with methyl viologen as electron acceptors.

Metabolism of metronidazole. The amount of metronidazole metabolized by broken cell preparations of susceptible and resistant strains was measured by the pyruvate dehydrogenase assay. When metronidazole was present in this assay, it caused a delay in the reduction of methyl viologen proportional to the amount of metronidazole. Rates of metabolism of metronidazole were calculated from the length of the delay, which was linear with respect to metronidazole concentration up to 20 μM, the highest concentration tested.

RESULTS

Isolation of metronidazole-resistant mutants. Mutants able to grow in the presence of relatively high concentrations of metronidazole were isolated from four strains of *B. fragilis* after treatment with MNNG. The mutants were isolated at low frequencies from broth cultures incubated for 18 to 24 h after mutagenesis. In most cases, less than 12 colonies grew on a metronidazole agar plate inoculated with approximately 5×10^8 viable cells from a broth culture. It was assumed that mutants appearing at such a low frequency were clonally derived and would have similar properties. Several

strains of *B. fragilis* failed to yield any mutants after treatment with MNNG, and no EMS-induced mutants were isolated from any of the strains. However, after treatment with either of these mutagens, all strains yielded rifampin-resistant mutants.

The origins of metronidazole-resistant strains used in this work are given in Table 1. Most mutants were selected on agar containing 25 μg of metronidazole per ml, and these mutants had relatively high levels of resistance (MIC, 100 to 150 μg/ml). Although selection at 10 μg/ml yielded mutants on two occasions from strain AM78, no mutants were isolated from this strain when 25 μg of metronidazole per ml was used in the same experiments. The mutants from strain AM78 which were only moderately resistant (MIC, 25 μg/ml) had different properties from the high-level resistance mutants (see below).

Properties of metronidazole-resistant strains. Cultural and biochemical characteristics. By Gram stain and microscopic appearance, the resistant strains did not differ greatly from the parental cultures; cells were small, pleomorphic, and gram-negative with bipolar staining typical of this species (6). Occasional filamentous forms were seen. The capsules of resistant strains AM24, AM72, and AM75, with high metronidazole MICs, were larger than those seen for parent strain AM78 and for the moderately resistant mutants isolated from it. All the parent strains showed biochemical reactions expected for *B. fragilis* (6), and mutants of strain AM78 were identical to the parent in this respect. Mutant strains AM70, AM72, and AM75 and isolate AM24 had atypical biochemical reactions in that they all hydrolyzed trehalose and mannitol. Strains AM24 and AM72 gave weakly positive reactions with indole reagent and were strongly catalase positive on ABHIA plates.

Antibiotic susceptibilities. Metronidazole-resistant mutants of strain AM78 had patterns of susceptibility to antibiotic disks similar to the parent culture and produced high levels of the *B. fragilis*-type β-lactamase typical of the parent strain (1). The MICs of penicillin G were 800 to 1,100 μg/ml for the mutants, compared to 1,400 μg/ml for the parent strain.

Metronidazole-resistant mutant strains AM70, AM72, and AM75 and the clinical isolate, strain AM24, all with high metronidazole MICs, were resistant to clindamycin, lincomycin, and erythromycin; strain AM70 was also resistant to tetracycline. All of these mutant strains gave increased zone diameters with β-lactam antibiotic disks, especially ampicillin, compared with those of the parent strains.

Susceptibility to related chemicals. Parent and mutant strains were tested for susceptibility to a range of compounds similar to metronidazole. There was no inhibition of growth by the following compounds, up to 1 mM: aminobenzimidazole; pyrrole; 2,5-dimethylpyrrole; pyrazole methylindole; imidazole; and thioproline. However, all the metronidazole-resistant mutants showed increased resistance to 1-nitronaphthalene (MICs four- to sixfold those of parent strains, which were 100 to 200 μM), and the high-level resistant mutants were also more resistant to Furadantin (MICs at least twofold those of parent strains, which were 400 to 500 μM).

Growth rates and utilization of carbon sources. Addition of metronidazole to logarithmically growing ABHI broth cultures of mutants AM75 and AM78.5, at concentrations inhibitory to the parent cultures (strains AM17 and AM78, respectively), had no effect on the growth of the mutants (Fig. 1). At higher concentrations of metronidazole (25 and 50 μg/ml), parent strain AM17 lysed and there was some inhibition of growth of mutant AM78.5 (data not shown). However, all metronidazole-resistant mutants grew more slowly than their parent strains in ABHI without metronidazole and produced comparatively less cell mass when grown under conditions of limiting glucose concentrations (0.05, 0.1, and 0.2%) in a minimal medium where growth was glucose dependent.

The ability of the parent strains and metronidazole-resistant mutants to utilize other car-

TABLE 1. *Metronidazole-resistant strains of B. fragilis used in this work*

Strain	Parent	Mutagenic treatment (μg of MNNG per ml)	Selection[a]	Metronidazole MIC (μg/ml)
AM70	AM39	80	25	100
AM72	AM21	100	25	100
AM75	AM17	60	25	100
AM78.1	AM78	100	10	25
AM78.5	AM78	100	10	25
AM24[b]		None	None	150

[a] Micrograms of metronidazole per milliliter in ABHIA plates.
[b] Isolated from urinary tract infection.

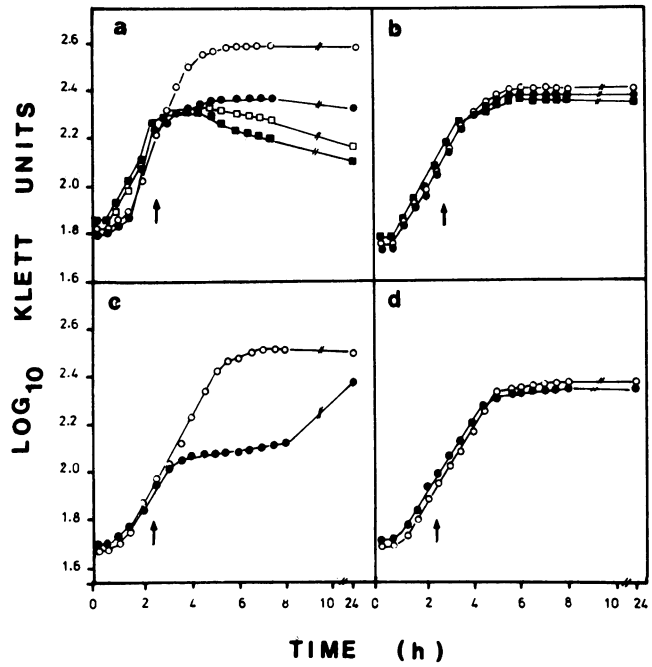

FIG. 1. *Growth of B. fragilis parent strains (a, AM17; c, AM78) and metronidazole-resistant mutants (b, AM75; d, AM78.5). Metronidazole was added anaerobically during logarithmic growth (times arrowed): no metronidazole* (○); *10 μg/ml* (●); *25 μg/ml* (□); *50 μg/ml* (■). *Experimental details are given in the text.*

bon sources was tested with washed, glucose-grown cells. There was no growth on lactate, citrate, glutamate, propionate, acetate, histidine, or tryptophan with parent strains AM17 and AM78 and mutants AM72, AM78.1, and AM78.5. Parent strain AM78 and high-level resistant mutant AM72 could grow on pyruvate as a sole carbon source, but mutants of strain AM78 failed to grow on pyruvate. All of these strains grew well on xylose and usually reached turbidities comparable to those achieved in glucose broths. Metabolic end products in xylose broth were similar to those detected in glucose-grown cultures (see below).

Production of acids and alcohols. The short-chain organic acids produced from fermentation of glucose by parent strains were typical of *B. fragilis* (6). In contrast, all the metronidazole-resistant strains produced elevated levels of lactic acid, and the mutants from AM78 also showed increased propionic and pyruvic acids (Fig. 2). Pyruvic acid, which is not normally detected in cultures of *B. fragilis* (6), was identified by co-injection of standard and culture (methylated separately) under isothermal and temperature programmed operating conditions. Analyses of alcohols produced in the same cultures showed that, apart from the AM78 mutants, resistant strains all produced increased amounts of ethanol, compared with the parent cultures (Fig. 3).

Some of these differences between metronidazole-resistant and parent strains might have been related to differences in growth rates. Accordingly, cultures were sampled at various stages of growth and analyzed for volatile and nonvolatile acids. Although proportions of products did change slightly, samples taken at any stage during growth contained representative products, and the distinctions between parent and mutant strains were still apparent. No pyruvic acid was detected in parent culture at any stage. Significant levels of lactic and propionic acids were detected in samples from late-stationary-phase cultures of some parent strains.

Production of gases. Elevated levels of CO_2 were detected in gas analyses of cultures of resistant strains AM72, AM75, and AM24 compared with the parent strains and mutant AM78.5 (Fig. 4). Production of H_2S was similar in all strains tested; the detection of H_2 by this system used was poor, and no reliable results were obtained.

Uptake of metronidazole by resistant strains. Addition of metronidazole to logarithmically growing cultures of susceptible strains AM17 and AM78 caused cessation of growth (Fig. 1). The uptake of metronidazole was stud-

ied by measuring the concentration of drug remaining in the medium. No metronidazole was detectable in the medium after 2 to 3 h; the calculated rate of uptake was 15 μg/ml per h. This is in contrast to the gradual disappearance (2.6 μg/ml per h) from supernatant fluids of cultures of resistant strains. No metronidazole was detected in association with whole cells of any of the strains after 24 h of incubation; broken cell preparations were not tested.

Pyruvate dehydrogenase activity in susceptible and resistant strains. Metronidazole susceptibility of strictly anaerobic bacteria has been attributed to the reduction of the drug by

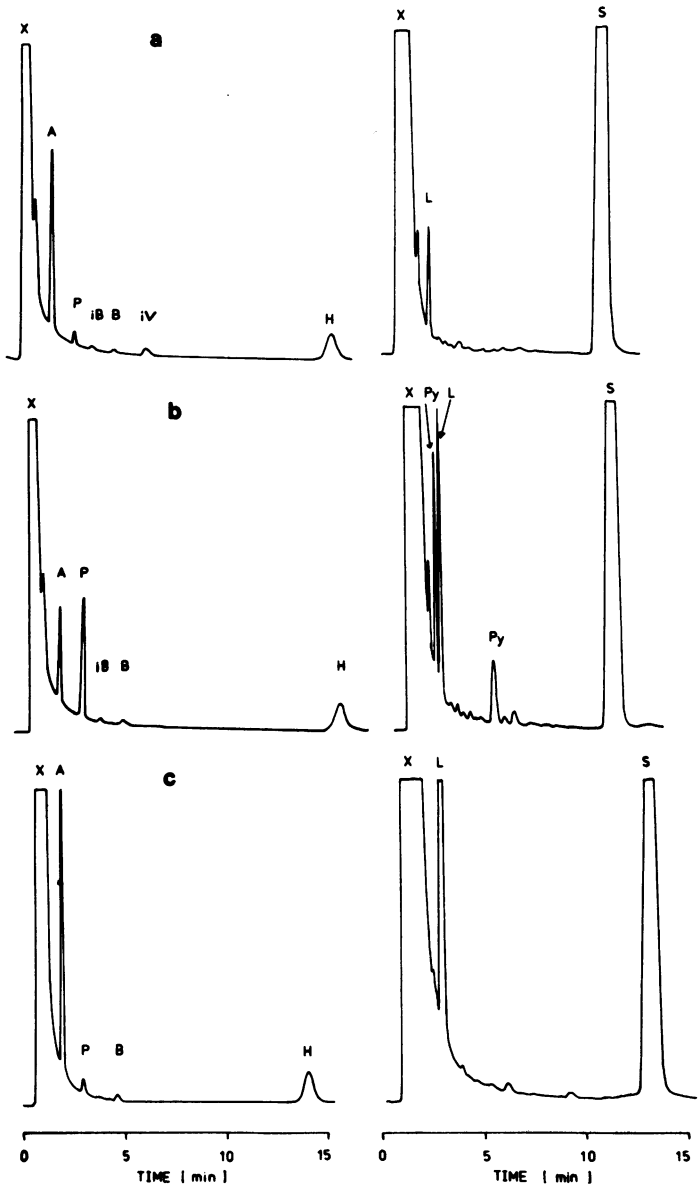

FIG. 2. *Short-chain organic acids produced from glucose fermentation by B. fragilis strains: (a) parent strain AM17 (parent strain AM78 gave a similar profile); (b) metronidazole-resistant strain AM78.5; (c) metronidazole-resistant strain AM72 (strains AM75 and AM24 gave similar profiles). Volatile acids: A, acetic; P, propionic; iB, isobutyric; B, butyric; iV, isovaleric; H, hexanoic (internal standard). Methylated acids: L, lactic; Py, pyruvic; S, succinic; X, solvent peak. Experimental details are given in the text.*

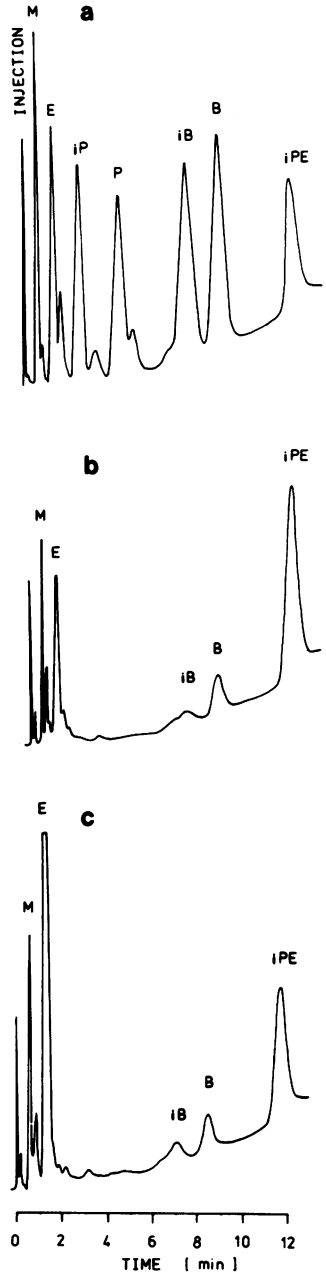

FIG. 3. *Alcohols produced by B. fragilis strains. (a) Standard alcohol mixture (5 mM methanol [M], ethanol [E], isopropanol [iP], propanol [P], isobutanol [iB], butanol [B], and iso-pentanol [iPE]); iPE was the internal standard included in all tests; (b) parent strain AM78; other parent strains and metronidazole-resistant mutants of strain AM78 gave this profile; (c) metronidazole-resistant strain AM75; strains AM72 and AM24 gave similar profiles. Experimental details are given in the text.*

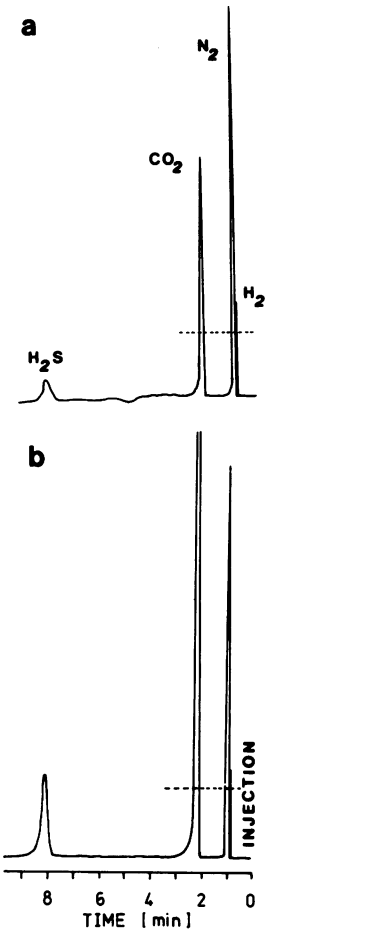

FIG. 4. *Gases produced from glucose fermentation by B. fragilis strains. (a) Parent strain AM78 (parent strain AM17 and metronidazole-resistant strain AM78.5 gave similar profiles); (b) metronidazole-resistant strain AM24 (metronidazole-resistant strains AM72 and AM75 gave similar profiles). The dashed line indicates contribution of relevant chamber gases (H_2 and CO_2). Experimental details are given in the text.*

its substitution for ferredoxin as terminal electron acceptor (12) in the phosphoroclastic reaction. This reaction involves decarboxylation of pyruvic acid, then substrate level phosphorylation. This reaction involves decarboxylation of pyruvic acid, then substrate-level phosphorylation to yield acetyl-phosphate and hydrogen. The kinetics of reduction of metronidazole by cell-free extracts were studied with the redox dye methyl viologen, which remains oxidized cause the metronidazole-resistant mutants of AM78 accumulated pyruvate, and pyruvate dehydrogenase has been implicated in metronida-

zole reduction (11), pyruvate was chosen as electron donor in these studies.

Pyruvate dehydrogenase activities were assayed in fresh extracts prepared anaerobically from cells grown to stationary phase (when activity was maximal) in AB and ABHI broth (Table 2). Specific activities were greater for ABHI-grown cells compared to AB-grown cells for all strains. All metronidazole-resistant strains had less enzyme activity compared with the parental strains, and the activities in highly resistant strains AM72, AM75, and AM24 were barely detectable. No activity was detected in extracts from *E. coli* cells grown anaerobically and tested at comparable protein levels. The presence of either metronidazole or 1-nitronaphthalene in reaction mixtures containing extracts of *Bacteroides* cells delayed the reduction of methyl viologen, and the length of the delay depended on the initial activity of pyruvate dehydrogenase (Fig. 5). Furadantin had no effect on the rate of methyl viologen reduction when tested with extracts either of parent strains or of mutant strains AM78.1 and AM72.

The mutants metabolized metronidazole very slowly compared with wild-type *B. fragilis*. Rates of metabolism by all extracts were based on a linear relationship between delay in reduction of methyl viologen and the amount of metronidazole initially present. Strain AM72 (high-level resistance) metabolized metronidazole at a rate of 0.2 nmol/mg of protein per min compared with 25.9 nmol for strain AM78.1 (moderately resistant) and 113.6 nmol for strain AM78 (wild type). A similar situation was found with respect to metabolism of 1-nitronaphthalene for these strains. These differences could not be attributed to a decreased affinity of the enzyme from mutant strains for pyruvate. The K_m's for wild-type and mutant enzyme (from a moderately resistant strain) were 0.21 and 0.28 mM, respectively.

DISCUSSION

The metronidazole-resistant strains described in this study were moderately (MIC, 25 μg/ml) or highly (MIC, 100 μg/ml) resistant to the drug compared with normal *B. fragilis* strains. Concentrations of metronidazole attainable in tissues and body fluids after moderate doses (18) are probably adequate to prevent emergence of strains with moderate resistance. Strains with metronidazole MICs of 100 μg/ml or higher, however, may provide a more serious challenge to successful therapy of anaerobic infections with the drug, since only massive doses regularly achieve blood levels of this magnitude (4, 17). It is of interest to note that the clinical isolate, strain AM24, was resistant to high levels of metronidazole. Mutants with high-level resistance had altered biochemical properties, drug susceptibility patterns, and metabolic end products, producing a phenotype quite disparate from the normal *B. fragilis*.

We found that normal *B. fragilis* strains produced pyruvate dehydrogenase with properties similar to those described for the pyruvate dehydrogenase from *Trichomonas foetus* (8) and other dehydrogenases in *B. fragilis* (10). These enzymes are oxygen sensitive and unstable during storage. In *Trichomonas* and *Clostridium* species, they are involved in the phosphoroclastic reaction, yielding acetate, CO_2, and H_2 and generating high-energy compounds. Presumably they serve a similar function in *Bacteroides*. We further demonstrated that metronidazole was reduced preferentially to methyl viologen in extracts with pyruvate dehydrogenase activity. A similar observation was reported for *Trichomonas* by Lindmark and Müller (8). The finding

TABLE 2. *Pyruvate dehydrogenase activities in extracts from metronidazole-susceptible and -resistant strains*

Strain	Metronidazole, MIC (μg/ml)	Pyruvate dehydrogenase sp act[a]	
		AB broth	ABHI broth
AM17	1.0	91.3	110.6
AM24	100	NT[b]	0.49
AM72	100	0[c] (<0.1)[d]	0.48 (0.4)
AM75	100	0 (<0.1)	0.13 (0.1)
AM78	2.5	119.8	173.0
AM78.1	25	6.8 (5.7)	46.6 (26.9)
AM78.5	25	5.1 (4.3)	30.2 (17.4)
E. coli C600	NT[b]	0[c]	0[c]

[a] See text for details of assay.
[b] NT, Not tested.
[c] No activity (changes in absorbance) detected despite large amounts of extract (0.5 ml) used in assay.
[d] Figures in parentheses are percentages of the parent strain activity.

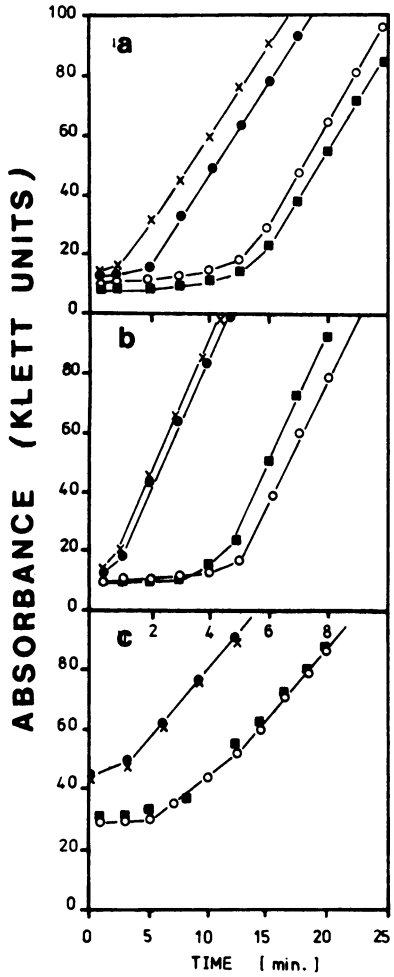

FIG. 5. *Effects of metronidazole, 1-nitronaphthalene, and Furadantin on pyruvate dehydrogenase activity in extracts of metronidazole-susceptible (strain AM78) and -resistant strains (AM78.1 and AM 72). Test compounds (8 μM for AM78, AM78.1; 5 μM for AM72) were added to incubation mixtures before addition of extracts: (a) 10 μl of AM 78 extract (2,200 U of pyruvate dehydrogenase per ml, 20.7 mg of protein per ml); (b) 50 μl of AM78.1 extract (660 U/ml, 24.7 mg/ml); (c) 500 μl of AM72 extract (14.7 U/ml, 16.0 mg/ml). Time was measured from the addition of extract. The first absorbance reading was recorded at 30 s and subsequently at 1-min intervals. Normal reaction, no addition (●); metronidazole added (○); 1-nitronaphthalene added (■); Furadantin added (×).*

that metronidazole-resistant strains were also resistant to 1-nitronaphthalene was consistent with the observation that this compound also delayed reduction of methyl viologen by pyruvate dehydrogenase. Although our high-level resistant mutants were also resistant to Furadantin, this compound had no effect on reduction of methyl viologen. If Furadantin is normally activated by reduction, the site of this reduction is presumably different from that for metronidazole. All of the metronidazole-resistant strains produced depressed levels of pyruvate dehydrogenase, and there was an inverse relationship between the level of resistance and the specific activity of this enzyme. These results suggest that the major site of metronidazole reduction is the phosphoroclastic reaction, with metronidazole as preferential electron acceptor during electron transfers. The nature of the electron transfer protein associated with this reaction is not known. Attempts to demonstrate ferredoxin in *B. fragilis* have been unsuccessful (11).

The changes in metabolic end products in metronidazole-resistant *B. fragilis* are consistent with their comparatively low levels of pyruvate dehydrogenase activity. Strains moderately resistant to metronidazole had dehydrogenase levels about 25% of the normal. They produced elevated levels of lactate, and accumulated pyruvate. On the other hand, high-level resistant strains were almost lacking dehydrogenase activity, producing more lactate, ethanol, and CO_2 than normal, and did not accumulate pyruvate. All the metronidazole-resistant strains studied had slower growth rates compared with normal *B. fragilis*, and our results suggest that the energy yield from glucose is less in these strains. In some metronidazole-resistant strains, the metabolic disadvantages of diminished dehydrogenase activity (manifested as slower growth rates and lower biomass yields) were compensated for by metabolism of pyruvate via other, presumably minor, pathways. Thus, high-level resistant mutants, like wild-type *B. fragilis*, were able to utilize pyruvate as a sole source of carbon, whereas moderately resistant strains failed to grow on pyruvate. The accumulation of pyruvate by the latter strains, when grown on glucose, indicates an inability to utilize pyruvate by alternative pathways.

Although as yet we have no evidence to link the phenotypic properties of metronidazole-resistant mutants to a single mutation, this is suggested by the similarity in phenotypes of the mutants isolated in the laboratory and the strain isolated from a clinical specimen.

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