ISOLATION AND PROPERTIES OF
METRONIDAZOLE-RESISTANT MUTANTS OF CLOSTRIDIUM
PERFRINGENS

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SUMMARY. Clostridium perfringens strains resistant to metronidazole and tinidazole were isolated from the sensitive parent strain CM288 after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine. Strain CM288 was already resistant to rifampicin and nalidixic acid; these genetic markers helped to confirm the identity of mutants. All mutants showed similar characteristics: they grew more slowly than the parent strain and failed to reach the same maximum turbidity; uptake of metronidazole and tinidazole from culture fluids was slow and end products of glucose metabolism were different from those of the parent. Pyruvate dehydrogenase activity was not detected in broken cell preparations of the mutant strains although this enzyme was readily detected in the parent strain. Changes in end products of glucose metabolism were consistent with the absence of pyruvate dehydrogenase activity because pyruvate was accumulated during growth and lactate levels were higher whereas acetate, CO₂ and ethanol levels were diminished.

INTRODUCTION

Metronidazole and some other 5-nitroimidazole derivatives have been used therapeutically in infections caused by anaerobic protozoa and bacteria (Grunberg and Titsworth, 1973) and prophylactically in gynaecological and colonic surgery (Willis et al., 1975; Hunt et al., 1979). The exclusive activity of these compounds against anaerobes (Edwards, Dye and Carne, 1973; Tanowitz et al., 1975) is thought to be related to the unique ability of anaerobes to take up the drugs and rapidly metabolise them to reduced intermediate substances (Ings, McFadzean and Ormerod, 1974; Tally et al., 1978), which kill bacteria by interacting with DNA (LaRusso et al., 1977). The proposed site of metronidazole reduction is the pyruvate phosphoroclastic reaction (O'Brien and Morris, 1972; Edwards et al., 1973) in which ferredoxin serves as the terminal electron acceptor in the generation of acetate, H₂, CO₂ and energy. Bactericidal 5-nitroimidazoles have less negative redox potentials (e.g., metronidazole -0.415 V) than ferredoxin (-0.460 V) and probably compete for electrons otherwise.
available to ferredoxin (Dornbusch and Nord, 1974; Chien and Mizuba, 1978). In a previous report (Britz and Wilkinson, 1979) we showed that metronidazole-resistant mutants of \textit{Bacteroides fragilis} had depressed levels of pyruvate dehydrogenase activity and that there was an inverse relationship between the degree of resistance and the specific activity of the enzyme. Mutant strains were biochemically distinct from parent strains and changes in end products of glucose metabolism were consistent with deficiencies in pyruvate dehydrogenase activity. In the present communication we report the isolation and characterisation of \textit{Clostridium perfringens} strains resistant to metronidazole and tinidazole after mutagenesis with \textit{N}-methyl-\textit{N'}-nitro-\textit{N}-nitroso-guanidine.

\textbf{Materials and methods}

\textit{Bacterial strains.} \textit{C. perfringens} strain CW504, a nalidixic-acid resistant mutant of the wild-type strain CW362 (Rood, Scott and Duncan, 1978) was obtained from Dr J.I. Rood and given our strain number CM283. Strain CM288 was a rifampicin-resistant mutant of CM283 that arose spontaneously.

\textit{Chemicals and radioisotopes.} All chemicals were of analytical grade and were obtained from standard commercial sources. Uniformly labelled [\textit{14}C] glucose (specific activity 345 mCi/mmol) was from New England Nuclear, Boston, Massachusetts. \textit{N}-methyl-\textit{N'}-nitro-\textit{N}-nitroso-guanidine (MNNG) was from K \& K Laboratories, Plainview, New York and phenethylamine from Eastman Kodak, Rochester, NY. Nalidixic acid was from Sterling Pharmaceuticals Pty, Sydney, and rifampicin from Ciba-Geigy, Sydney. Metronidazole and tinidazole were generous gifts from their manufacturers (May and Baker Ltd, Melbourne, and Pfizer Ltd, Sydney, respectively).

\textit{Culture media.} Anaerobic brain heart infusion (ABHI) broth was prepared as described by Britz and Wilkinson (1978a). Plates for the isolation of mutants were prepared from ABHI broth solidified with 2\% agar and supplemented with metronidazole (25 mg/L) or rifampicin and nalidixic acid (50 mg of each/L) immediately before pouring. Proteose peptone broth was prepared as described by Jayko and Lichstein (1959) from Difco Proteose Peptone No. 3 and usually contained 0.4\% glucose (PPG broth). Fluid thioglycollate medium (FTM) (Difco) was prepared according to the maker's instructions.

\textit{Growth conditions and growth curves.} Plates were inoculated in air and incubated in anaerobic jars in an atmosphere of 10\% CO\textsubscript{2}, 5\% H\textsubscript{2} and 85\% N\textsubscript{2}. Inoculated broths were equilibrated in this atmosphere before bottles or tubes were sealed and incubated at 37°C. Bacterial growth was followed turbidimetrically, with a Klett-Summerson colorimeter, in 15-ml screw-capped tubes sealed with butyl rubber stoppers. Ten-ml volumes of reduced medium were inoculated with bacteria from overnight broth cultures to achieve initial turbidities of 10-20 Klett units (about 10\textsuperscript{7} organisms/ml). When appropriate, antimicrobial agents or MNNG were added by injection through the rubber stoppers.

\textit{Mutagenic treatment and selection of metronidazole-resistant mutants.} A stock solution of MNNG 10 g/L was prepared in ABHI broth. Sufficient stock solution was added to mid-exponential cultures of strain CM288 in ABHI broth to give a final concentration of 150 mg/L, which caused a rapid decrease in the rate of growth. Four hours after the addition of MNNG, 1 ml of culture was removed into each of five sterile centrifuge tubes. Cells were centrifuged at 1500 g for 15 min; the resultant pellet from each sample was resuspended in 9 ml of thioglycollate medium and incubated at 37°C. After overnight incubation 0.3-ml volumes were spread onto ABHI agar containing metronidazole and incubated for 48 h at 37°C. Metronidazole-resistant colonies were purified on ABHI agar containing the drug; mutants arising from each tube of thioglycollate medium were assumed to be clonally derived. Identity of mutants was confirmed by testing for resistance to rifampicin and nalidixic acid and for production of a lecithinase which was neutralised by \textit{C. perfringens} antitoxin. Other biochemical characteristics were checked by methods described by Holdeman and Mgore (1975).

\textit{Susceptibility testing.} The disk diffusion and agar-dilution methods used have been
described (Britz and Wilkinson, 1978a and b). For agar-dilution tests, approximately $10^3$ organisms were spot-inoculated onto the surface of antibiotic-containing agar from cultures appropriately diluted in ABHI broth.

Detection of acids and alcohols by gas-liquid chromatography (GLC) was as described previously (Britz and Wilkinson, 1979). Samples for acid analyses were prepared as described by Holdeman and Moore (1975), and 1 µl was injected onto a glass column (2 m × 4 mm internal diameter) containing 15% SP 1220-1% H₃PO₄ on 80/100 mesh Chromosorb W AW (Supelco Inc., Bellefonte, PA.) and operated isothermally at 145°C with N₂ carrier gas (40 ml/min). Greater separation of methylated pyruvic and lactic acids was achieved by use of a temperature-programmed procedure (see Britz and Wilkinson, 1979). Alcohols were analysed on a column (2 m × 2 mm internal diameter) packed with Chromosorb 103, 80/100 mesh (Johns Manville Products, Manville, NJ.); the resin was treated with 5% KOH before packing. 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. The column was operated isothermally at 145°C.

Assay procedures. Metronidazole and tinidazole were assayed colorimetrically as described by Britz and Wilkinson (1979) by a method based on that of Populaire et al. (1968). Pyruvate dehydrogenase activity was assayed by measuring the rate of reduction of methyl viologen in an atmosphere of N₂ (Lindmark and Müller, 1973) as described in detail by Britz and Wilkinson (1979). Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Glucose was estimated in cultures by the Somogyi-Nelson procedure (Nelson, 1944) after trichloroacetic acid precipitation of proteins and subsequent neutralisation.

Volatile fatty acid (VFA) and CO₂ production from [U-¹⁴C] glucose. ABHI broth cultures (10 ml) were set up in 100-ml reagent bottles sealed with rubber stoppers through which had been inserted plastic centrewells containing fluted filter paper. During mid-exponential growth, 1 µCi of [U-¹⁴C] glucose was added and growth at 37°C was allowed to continue overnight. Growth was stopped by addition of 1 ml of 50% H₂SO₄ by syringe, after which 0.15 ml of phenethylamine was injected onto the filter paper in the centrewell. The culture was shaken at room temperature for 1 h during which CO₂ was collected onto the filter paper. The centrewell was then removed and the radioactivity measured by scintillation counting (Britz and Lowther, 1981). Portions of the culture, 0.6 ml and 50 µl respectively, were removed for analysis of volatile fatty acids and for estimation of residual radioactivity. The remaining culture was methylated (Holdeman and Moore, 1975) and the derivatives converted to their corresponding hydroxamates after extraction into 15 ml of chloroform. The hydroxamates were separated by paper chromatography on Whatman No. 1 paper by the method of Thompson (1951). The solvent used was the organic phase resulting from the separation of an emulsion of octanol, formic acid and water (3:1:3), with the chromatography tank humidified with the aqueous phase.

RESULTS AND DISCUSSION

Spontaneously occurring mutants of C. perfringens strain CM288 resistant to metronidazole were not isolated and resistance to metronidazole induced by the mutagen MNNG was achieved only once. On this occasion, mutants were isolated from four of the five tubes of thioglycollate medium used to resuscitate cells after exposure to MNNG; 3–5 colonies were isolated from each selective plate, indicating that mutation to metronidazole resistance was a rare event. Mutants arising from each resuscitation broth were assumed to be clonally derived, although it is possible that all mutants arose from a single mutational event during the period of exposure to the mutagen.

All mutants were similar to the parent strain in gram-staining properties and colonial morphology, although colonies of the mutants were smaller than those formed by the parent strain on plates incubated for the same period. Mutants were
resistant to rifampicin and nalidixic acid but sensitive to other drugs tested by the disk-diffusion method with 10 µg disks, viz., ampicillin, chloramphenicol, clindamycin, lincomycin, erythromycin and tetracycline. Mutants showed fermentation properties expected of *C. perfringens* and their lecithinase was neutralised by *C. perfringens* antitoxin; mutants produced weak reactions in the lecithinase test probably because of their impaired growth (see below). All mutants showed similar resistance to metronidazole and tinidazole, being inhibited by metronidazole 25–35 mg/L and tinidazole 20 mg/L. The parent strain was inhibited by both drugs at a concentration of 0.1 mg/L.

Fig. 1 shows the growth characteristics of mutant and parent strains in ABHI broth. All mutants grew more slowly than the parent strain and failed to reach the same maximum turbidity; dry weights of cells from cultures of mutant strains were
20–40% lower than those of the parent strain. Notably, mutants had a longer lag period before initiation of exponential growth, although cultures were inoculated with similar numbers of cells. All mutants removed metronidazole and tinidazole from culture fluids more slowly than the parent strain CM288. The results for metronidazole are shown in fig. 2. Strain CM288 removed 80–90% of the metronidazole or tinidazole within 40 min of addition of drug, during which time the growth rate rapidly decreased.

The major metabolic end products of parent strain CM288 grown in ABHI or PPG broths were acetic, butyric and lactic acids and ethanol, with propionic and pyruvic acids as minor products; typical concentrations of products found in PPG broth are listed in table I. All mutant strains consistently produced lower concentrations of the volatile acids and ethanol while producing higher concentrations of pyruvic and lactic acids. Strain CM288 converted 12.5% of [14C] glucose supplied into CO2, whereas three of the four mutant strains converted < 1% of the sugar into CO2. The amount of radioactivity extracted into ether or converted to hydroxamates indicated that proportionately less labelled glucose was converted into volatile fatty acids by the mutants (7–16%, mean 11%) than by the parent strain (22%). The distribution of label from [14C] glucose into volatile fatty acids was different for the parent and mutant strains. The mutants produced proportionately less acetic acid and proportionately more propionic acid, although the proportion of butyric acid was similar for mutant and parent strains (data not shown). These observations agree with the GLC analyses of culture fluids because total fatty acid production from glucose was reduced and acetic acid production was grossly impaired. In general, marginally less glucose was utilised by the mutant strains than by the parent (table II): an average of 3% more residual glucose was detected in cultures of mutants compared with the parent strain after growth for 24 h providing that the concentration of glucose supplied was at least 20 g/L. The observation that residual glucose concentration was similar in cultures of parent and mutant strains suggests that all the strains used similar amounts of glucose but that the mutants metabolised glucose less efficiently.

Because diminished levels of pyruvate dehydrogenase were implicated in metronidazole resistance in B. fragilis (Britz and Wilkinson, 1979) broken-cell preparations of

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MIC of metronidazole (mg/L)</th>
<th>Concentration (mmol/L)* of pyruvic acid</th>
<th>lactic acid</th>
<th>acetic acid</th>
<th>propionic acid</th>
<th>butyric acid</th>
<th>Conc (mm) of CO2†</th>
<th>Production of CO2†</th>
<th>Dry weight (g/L)</th>
</tr>
</thead>
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<tr>
<td>CM288</td>
<td>0.1</td>
<td>0.2</td>
<td>5.2</td>
<td>50.0</td>
<td>0.20</td>
<td>1.23</td>
<td>19.2</td>
<td>12.5</td>
<td>0.84</td>
</tr>
<tr>
<td>CM289</td>
<td>25</td>
<td>4.3</td>
<td>8.1</td>
<td>4.4</td>
<td>0.01</td>
<td>0.04</td>
<td>2.4</td>
<td>2.0</td>
<td>0.68</td>
</tr>
<tr>
<td>CM292</td>
<td>35</td>
<td>1.4</td>
<td>21.5</td>
<td>9.7</td>
<td>0.05</td>
<td>0.06</td>
<td>4.4</td>
<td>0.1</td>
<td>0.74</td>
</tr>
<tr>
<td>CM293</td>
<td>35</td>
<td>1.9</td>
<td>18.4</td>
<td>32.4</td>
<td>0.12</td>
<td>0.12</td>
<td>8.3</td>
<td>2.4</td>
<td>0.51</td>
</tr>
<tr>
<td>CM294</td>
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<td>0.6</td>
<td>13.9</td>
<td>8.4</td>
<td>0.07</td>
<td>0.09</td>
<td>3.4</td>
<td>0.7</td>
<td>0.56</td>
</tr>
</tbody>
</table>

* Corrected for endogenous acids in PPG broth.
† Percentage radioactivity recovered relative to supplied [U-14C] glucose.
‡ Estimated from 10-ml cultures.
the metronidazole-resistant strains of *C. perfringens* were assayed for pyruvate dehydrogenase activity. Parent strain CM288 produced pyruvate dehydrogenase with a typical specific activity of 340 units/mg protein which could be detected with 50–100 μg protein in the assay. Pyruvate dehydrogenase activity was not detected in any of the mutants despite the use of up to 11 mg of protein in the assay.

Many properties of the metronidazole-resistant *C. perfringens* mutants can be interpreted in terms of loss of pyruvate dehydrogenase activity. Impairment of the phosphoroclastic reaction would decrease acetate, CO₂, H₂ and energy production and the resultant metabolic disadvantage would explain the slower rates of glucose utilisation as well as the decrease in biomass. Pyruvate would become available to other reactions, e.g., lactate synthesis, or would accumulate. An alternative interpretation of slow growth and slow uptake of metronidazole by mutants is a generalised transport deficiency resulting from cell-surface changes. In a previous study, metronidazole-resistant mutants of *B. fragilis* did show some traits consistent with cell-surface changes, such as resistance to antimicrobial agents other than metronidazole, e.g., erythromycin, clindamycin and lincomycin, and changes in the amount of capsular material produced (Britz and Wilkinson, 1979). Such changes were not seen, however, in metronidazole-resistant mutants of *C. perfringens*. The trait common to metronidazole-resistant mutants of both species was an alteration in pyruvate dehydrogenase activity. Although this enzyme appears to be central in determining metronidazole sensitivity in these species, it is apparent that even total loss of pyruvate dehydrogenase does not lead to complete resistance to the drug. Many reactions feed electrons to ferredoxin (Valentine, 1964), so that metronidazole and tinidazole can still substitute for ferredoxin despite virtual “closedown” of the phosphoroclastic reaction. Because low redox potential electron transfer is essential for anaerobic metabolism, complete loss of such processes, presumably essential for greater resistance to metronidazole, would be lethal.

### REFERENCES


