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Mechanisms of tetracycline and rifampicin resistance in *Bacteroides fragilis* mutants obtained by ionizing radiation

Ali O. Azghani[a] and Robert Fuerst[b]

[a]*Department of Biochemistry, University of Texas Health Center, Tyler, TX, and* [b]*Department of Biology, Texas Woman's University, Denton, TX, U.S.A.*

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SUMMARY

Based on a dose-survival curve, a radiation dose of 3.99 C/kg was used to induce antibiotic-resistant mutants in *Bacteroides fragilis*. *Escherichia coli* B/r membrane fragments were employed as a reducing agent. Antibiotic-resistant mutants of *B. fragilis* were utilized to study the mechanism by which these organisms become resistant to selected chemotherapeutic agents. Decreased accumulation of tetracycline by resistant mutants of *B. fragilis* suggests that the resistance to this antibiotic is associated with the outer membrane permeability. There is a marked difference in the inhibitory action of rifampicin on RNA polymerase activity in rifampicin-sensitive and -resistant strains of *B. fragilis*. This enzyme is, therefore, the likely target for inhibition of bacterial growth in this anaerobe by rifampicin.

INTRODUCTION

The importance of Gram-negative obligate anaerobic bacteria, especially *Bacteroides*, has been recognized during the past few years. The growing interest in genetic studies of *Bacteroides* is due partly to the involvement of this organism in disease and the emergence of antibiotic-resistant strains [17,18,20]. This creates great concern, because these Gram-negative anaerobes are the major bacterial species in the human intestine and may constitute a significant reservoir of antibiotic resistance genes in the gut.

Correspondence: A.O. Azghani, Department of Biochemistry, University of Texas Health Center, P.O. Box 2003, Tyler, TX 75710, U.S.A.

The mutation to rifampicin resistance (rif^r) in aerobic bacteria, including *E. coli*, is localized in the *rpoB* gene encoding the beta subunit of RNA polymerase [14]. Studies on the *rpoB* gene cloned from rif^r mutants on the *E. coli* chromosome revealed that the mutant genes contain an ATTA transversion, in which a valine residue is substituted in the mutant RNA polymerase for the aspartic acid residue of the normal beta subunit [16].

The genetic bases of the resistance of *Bacteroides* species to tetracycline have been studied by a number of investigators [8,19], and different species of plasmids were found to be responsible for carrying the resistance genes. A conjugative plasmid was identified in *B. orchraceus* which specifies resistance to chloramphenicol, tetracycline, kanamycin, and

streptomycin. The plasmid was transferred by conjugation to *E. coli* and was maintained in this new host. Studies on low-level tetracycline-resistant *E. coli* cells demonstrated a change in major outer membrane proteins or porins [15]. The lipopolysaccharide component also affects tetracycline activity [12].

In this communication, we report the mechanism of action of two antibiotics, rifampicin and tetracycline, on plasmid-free *B. fragilis*. Antibiotic-resistant mutants of the organism were obtained after exposure to Cs-137 gamma radiation.

MATERIAL AND METHODS

Bacteroides fragilis VPI 531 was obtained from the Anaerobic Laboratory, Virginia Polytechnic Institute (Blacksburg, VA), and maintained in pre-reduced chopped meat broth. The identity of the strain was confirmed by the methods outlined in the Anaerobe Laboratory Manual [10].

Cultures for daily experiments were grown in Schaedler broth (Difco), or brain heart infusion (Gibco) complemented with hemin (Sigma) 1%, cysteine-HCl (Nutritional Biochemical Corp.) 0.05%, and resazurin (Sigma) 0.4%. Antibiotic medium No. 3 (Gibco) supplemented with hemin was used for the antibiotic sensitivity tests.

The cultures were kept oxygen-free during the manipulation by using the VPI anaerobic culture inoculation system (Bellco), and the plate cultures were placed in an anaerobic GasPack jar (BBL) during the incubation period.

A Mark I Cs-137 gamma irradiator (Shepherd and Associates) was employed to induce mutations in *B. fragilis* cells. Bacterial cells were suspended in 10 ml of pre-reduced phosphate buffer (pH 6.8) with 0.2 μl of *E. coli* B/r cytoplasmic membrane fragments [1] as a reducing agent. The cells were then exposed in a modified 100 ml Turner bulb to 1.29×10^3 roentgen/min (R/min) of Cs-137 for a range of time intervals. Prior to irradiation, the air in the Turner bulb was replaced with a mixture of 10% H_2, 10% CO_2 and 80% N_2.

The selection of antibiotic-resistant mutants was carried out on anaerobic brain heart infusion (ABHI) agar containing 10 μg/ml of the test antibiotic. Aliquots of the irradiated cell suspension were inoculated into ABHI broth and incubated anaerobically at 37°C for 8 h. Pour plates were made with ABHI agar containing 10 μg/ml of test antibiotic. The plates were then incubated for 24 h at 37°C in a GasPack jar. Mutant colonies obtained on agar plates were picked and inoculated into chopped meat broth with 10 μg/ml of test antibiotic, and maintained for later studies.

The isolation of RNA polymerase was performed as described [7]. RNA polymerase was coprecipitated with DNA from cell lysates. The enzyme was then eluted from the precipitate by adding 0.5 ml of 0.01 M Tris-HCl (pH 7.4) containing 2.0 M NaCl and freshly prepared 0.01 M dithiothreitol. The extracts were stable at −70°C (Forma Scientific) for many months.

The RNA polymerase activity was assayed using [^{3}H]ATP according to the method of Clark et al. [6]. To determine the effect of rifampicin on the activity of RNA polymerase, desired concentrations of the antibiotic and the enzyme were mixed and incubated at 37°C for 10 min. This was then added into the reaction mixture and the incorporation of [^{3}H]ATP was measured.

The uptake of tetracycline (7(n)-^{3}H, 697 Ci/mmol, NEN) by bacterial cells was determined using two techniques. In the first procedure [13], the cells were grown anaerobically in mid to late log phase at 37°C, centrifuged, and resuspended in fresh Schaedler broth. After 15 min incubation at 37°C, [^{3}H]tetracycline was added to the culture to a final concentration of 1 μg/ml. After incubation at 37°C for 1 h, 100 μl samples were removed and diluted 1:17 in cold saline buffer and centrifuged at 4°C for 10 min to remove the free tetracycline in the media. The cell pellets were resuspended in 0.5 ml of saline buffer and dissolved in 10 ml of Aquasol scintillation cocktail. The activity of the accumulated tetracycline was then counted in a Beckman 9,000 liquid scintillation counter. In a slight modification of the above procedure [11], cells were resuspended in Schaedler broth containing 0.5 μg/ml of tetracycline prior to the addition of the labeled

antibiotic. After 3 h incubation at 30°C, the preceding procedure for tetracycline accumulation was followed.

Attempts were made to use 'plasmid-free strains' of *B. fragilis* for this project. The synthesis of possible plasmid DNA in *B. fragilis* was impaired by elevating the incubation temperature as described for other organisms [4]. The bacteria were then tested for the presence of plasmids by agarose gel electrophoresis [21].

The production of beta-lactamase was determined according to the procedure of Bourgault and Rosenblatt [3]. The nitrocefin was a gift from Dr. Donald Nash, University of Texas Health Center at Tyler.

RESULTS

In order to exclude the involvement of plasmid DNA in this study, extrachromosomal DNA was eliminated from *B. fragilis* by elevating the incubation temperature. The test organism was proven to be plasmid-free on 0.7% agarose gel electrophoresis.

Effects of ionizing radiation on the survival of *B. fragilis* were determined (Fig. 1). Exposure to 1.54×10^4 R (3.99 coulomb/kg) induced an $LD_{99.9}$ in the *Bacteroides* population samples. This dose was obtained after 12 min irradiation and used to induce mutations in the test organisms.

Mutants of *B. fragilis* resistant to the antibiotics tetracycline and rifampicin were isolated. The results of cellular morphology, colony characteristics and biochemical activities, including gas-liquid chromatography analysis of volatile fatty acid metabolism, indicated that these mutants and their parent strain share similar characteristics.

The sensitivity to various kinds of antibiotics of *B. fragilis* rif^r strains was compared to that of the parent strain. As shown in Table 1, higher concentrations of rifampicin and rifamycin were required to inhibit the growth of the mutants. However, the mutant strains exhibited increased sensitivities to penicillin.

B. fragilis rifampicin-resistant (rif^r) mutants

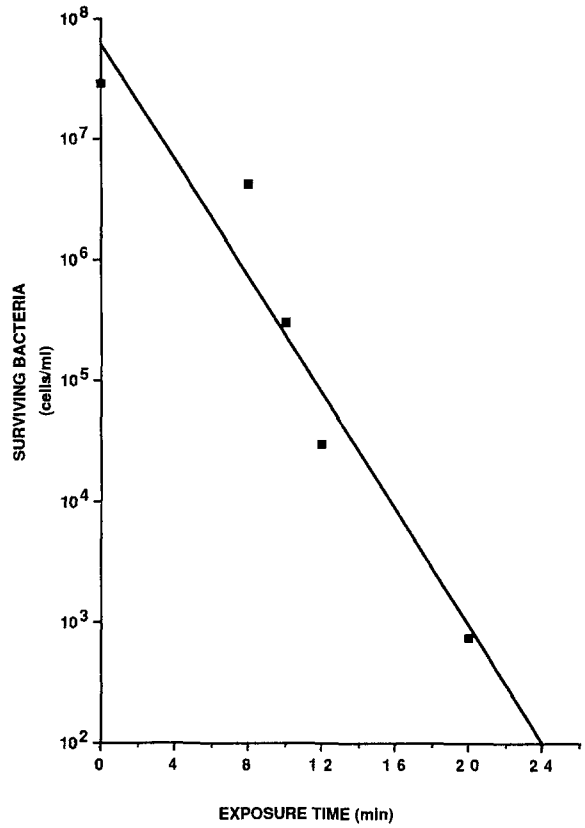

Fig. 1. Effects of 1.29×10^3 R/min of Cs-137 on the survival of *B. fragilis* at various time intervals.

Table 1

Minimal inhibitory concentrations (MICs) of different antibiotics tested against *B. fragilis* rifampicin-resistant and wild-type strains

Antibiotics	MICs (μg/ml)	
	B. fragilis wild type	*B. fragilis* rif^r mutants
Rifampicin	0.39	100
Rifamycin	0.39	>100
Penicillin	>250[a]	50[a]
Tetracycline	1.56	3.12
Chloramphenicol	0.39	9.39

[a] U/ml.

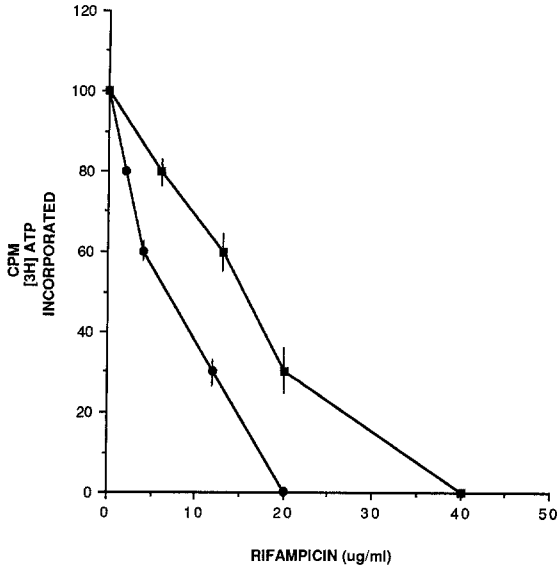

Fig. 2. Inhibitory action of rifampicin on the incorporation of [^{3}H]ATP by RNA polymerase, extracted from *B. fragilis* strains. The bars indicate standard deviation. ●, wild type; ■, rif^r.

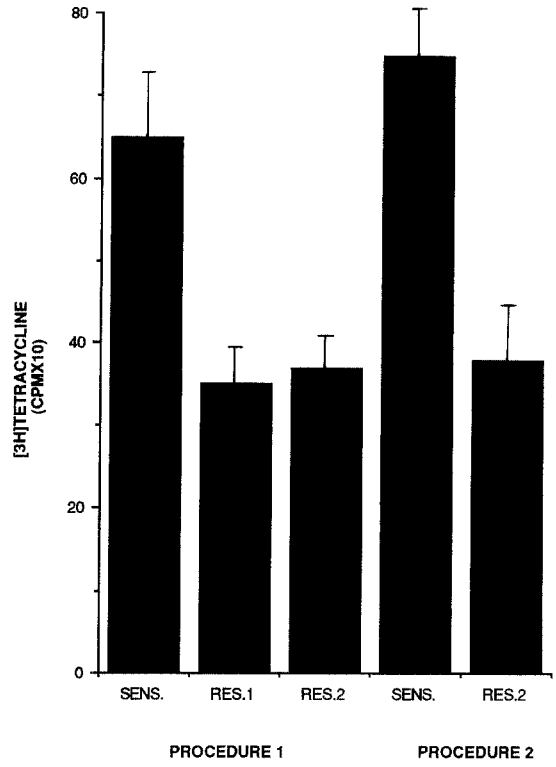

Fig. 3. Uptake of [^{3}H]tetracycline by sensitive and resistant mutants of *B. fragilis* after 60 min incubation at 37°C with 1 μg/ml of labeled tetracycline. The second procedure was done after preincubation of the strains with 0.5 μg/ml of unlabeled tetracycline for 3 h. The bars indicate standard deviation.

were used to determine whether RNA polymerase was a possible target site for rifampicin. Partially purified enzymes obtained from the parent strain and rifampicin mutants were examined for in nitro RNA synthesis activities. RNA polymerase of *E. coli* (Sigma) was used as the control. It was found that a 10 min incubation of the enzymes with the assay mixture was sufficient to synthesize a measurable amount of RNA molecules in vitro.

Fig. 2 indicates the in vitro inhibitory action of rifampicin on the incorporation of [^{3}H]ATP by the RNA polymerase isolated from the *B. fragilis* parent type and rifampicin-resistant mutants. The amount of radioactivity incorporated by the enzymes extracted from rif^r mutants was reduced 50% by the inclusion of 16 μg of rifampicin per ml of reaction mixture. However, only 6 μg/ml of the antibiotic inhibited the incorporation of [^{3}H]ATP by 50% when the enzyme from wild-type *B. fragilis* was utilized. Therefore, the mutant strains showed a greater than 2.5-fold decrease in sensitivity to rifampicin.

Accumulation of labeled tetracycline was measured in both wild-type and the tetracycline-resist-

Table 2

Minimal inhibitory concentrations (MICs) of different antibiotics tested against the parent strain and tetracycline-resistant variants of *B. fragilis* VPI 531

Antibiotics	MICs (μg/ml)	
	B. fragilis wild type	*B. fragilis* tet^r mutants
Tetracycline	1.56	25
Doxycycline	0.78	1.56
Minocycline	0.39	1.56
Penicillin	250[a]	50[a]
Rifampicin	0.39	3.12
Chloramphenicol	0.39	1.56

[a] U/ml.

ant (tet^r) mutants of *B. fragilis*. Uptake of the drug by either strain was time-dependent, and the difference in drug accumulation activity by sensitive and resistant strains was significant after 30 and 60 min of incubation.

Fig. 3 shows the accumulation of [^{3}H]-tetracycline by normal and tetracycline-resistant *B. fragilis* strains after 1 h incubation. Statistical analysis of the data indicated that the tet^r mutants accumulated significantly less tetracycline than the parent strains. The presence of subinhibitory concentrations of the antibiotic prior to the uptake assay did not affect the ability of the cells to accumulate tetracycline (Fig. 3, procedure 2).

B. fragilis tet^r mutants were sensitive to the more lipophilic tetracycline derivatives, minocycline and doxycycline. These mutants also became more sensitive to penicillin than the parent strain (Table 2).

DISCUSSION

Effects of ionizing radiation on the survival of *B. fragilis* were determined. Graphic illustrations of the results obtained (Fig. 1) indicate that the survival of *B. fragilis* cells following ionizing radiation follows the exponential kinetics of the single target theory similar to that of *Pseudomonas aeruginosa* [2] and *E. coli* [5].

As a result of insult with radiation and antibiotic treatment, *B. fragilis* mutants resistant to rifampicin were isolated. In the presence of the antibiotic, the in vitro activity of RNA polymerase was determined for rif^r *B. fragilis* and compared with that of the wild type. The enzyme from rif^r strains exhibited a greater than 2.5-fold decrease in sensitivity to rifampicin. This finding is in agreement with the results observed with aerobic bacteria, *E. coli* and *Staphylococcus aureus* [9], and indicates that rifampicins inhibit bacterial growth via inactivation of RNA polymerase. The difference between the minimal inhibitory concentrations of the sensitive and resistant strains to other antibiotics such as penicillin and tetracycline, however, suggested that other factor(s), such as alterations in the permeability of the bacterial membrane, also contributed to resistance in the responses toward the antibiotics.

Using two different methods, it was possible to show that the resistance to tetracycline in *B. fragilis* was related to a decreased uptake of the antibiotic. This suggests that the radiation-induced changes in membrane permeability could produce mutant strains that are resistant to tetracycline.

Studies on low-level tetracycline-resistant *E. coli* demonstrated a change in the major outer membrane proteins, called porins [15], some of which are involved in the formation of water-filled channels, whereas mutations in the lipopolysaccharide of this organism were shown to alter the sensitivity of these mutants to the more lipophilic tetracycline [3]. The fact that *B. fragilis* tet^r mutants remained sensitive to doxycycline and minocycline (lipophilic derivatives of tetracycline) indicated that the alterations in outer membrane proteins rather than lipopolysaccharide decreased the rate of entry of tetracycline into the mutants cells.

The antibiotic-resistant cells showed increased sensitivity to penicillin (Tables 1 and 2). Assays in the presence of nitrocefin indicated that these mutants produced beta-lactamase, and thus the increased sensitivity of mutants to penicillin may be due to cell surface membrane alterations.

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