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Macrolide Accumulation by *Bacteroides fragilis* ATCC 25285

YOSHINORI MUTO,* KAORI BANDOH, KUNITOMO WATANABE, NAOKI KATOH, AND KAZUE UENO

Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Tsukasamachi 40, Gifu, Japan

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The accumulation of macrolide antibiotics in *Bacteroides fragilis* ATCC 25285 was increased in the order erythromycin, josamycin, and rokitamycin, depending on hydrophobicity. The half-times of efflux were also prolonged in the same order. Furthermore, MICs of the antibiotics were correlated with the extent of hydrophobicity. These findings suggest that the macrolide antibiotics are accumulated in *B. fragilis* by means of their hydrophobic properties, and the efficient accumulation of the drugs may explain the susceptibility of this gram-negative bacterium to macrolides.

There are several lines of evidence that macrolides are intrinsically inactive against gram-negative bacteria (1, 7). Since the site of action of macrolides has been thought to be the ribosomal subunits located in cytoplasm (16), efficient permeation across the surface envelope is required for the drugs to be effective. It has thus been proposed that the resistance in gram-negative organisms is due to the permeability barrier of the outer membrane (7, 15, 18, 19). However, some anaerobic gram-negative rods such as *Bacteroides* spp. which carry complete outer membranes (11) are usually susceptible to lower concentrations of macrolide antibiotics (21, 23), suggesting differences in the properties of the outer membrane between gram-negative anaerobes and aerobes. Although some information on the transport of macrolide antibiotics in aerobic bacteria (7, 15) is available, little is known about antibiotic transport in anaerobic gram-negative bacteria.

In view of the considerable clinical significance of the anaerobic bacteria (6), particularly the *Bacteroides* group, this study was undertaken to examine macrolide transport in *Bacteroides fragilis*. Since we were particularly interested in relating the permeability of macrolide antibiotics to their physical properties such as hydrophobicity, the transport of three macrolides which differ greatly in their hydrophobicity was systematically assessed.

B. fragilis ATCC 25285 was grown at 35°C in an anaerobic glove box containing an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. The medium was GAM (Gifu Anaerobic Medium) broth (Nissui Seiyaku Co.) containing (per liter) 10 g of Proteose Peptone, 13.5 g of serum digest, 5 g of yeast extract, 2.4 g of beef extract, 1.2 g of liver extract, 3 g of glucose, 2.5 g of potassium dihydrogen phosphate, 3 g of sodium chloride, 5 g of soluble starch, 0.3 g of L-cysteine hydrochloride, 0.3 g of sodium thioglycolate, 3 g of soy peptone, and 10 g of peptone. The medium was supplemented with 0.0005% hemin and menadione. Bacteria were harvested by centrifugation, washed, and suspended in anaerobic salt solution (ABS) containing 20 mM MOPS (morpholinepropanesulfonic acid)-Tris (pH 7.0), 155 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 1.7 mM $CaCl_2$, and 3 mM L-cysteine, all under anaerobic conditions. Antibacterial activity (expressed as MIC) was determined by the agar dilution technique, as described previously (12). Octanol-water partition coefficients for macrolide antibiotics were obtained by the method of Ohno et al. (20), which measured the drug concentration in octanol and water phases at 25°C spectrophotometrically.

Uptake from the extracellular medium was measured in the anaerobic glove box for [^{14}C]erythromycin (specific activity, 3.68 mCi/mmol), [^{14}C]josamycin (specific activity, 4.55 mCi/mmol), and [^{14}C]rokitamycin (specific activity, 4.32 mCi/mmol). Bacteria were suspended in 1 ml of ABS to produce a final optical density at 420 nm of 6.0 to 7.0. After preincubation for 5 min at 37°C, the reaction was started by adding each ^{14}C-macrolide to a final concentration of 100 μM. At time intervals after addition of the drug, portions (0.1 ml) were removed and filtered through 0.45 μm HAWP membrane filters (Millipore Corp.) prewashed with ABS containing 100 μg of nonradioactive macrolide per ml. The filters were washed with 10 ml of the same solution at 4°C and put into scintillation vials for measurement of radioactivity. The amount of each drug bound to membrane filters in the absence of bacteria was subtracted from the experimental values.

The efflux of antibiotics was measured by the decrease in ^{14}C-macrolides from preloaded cells. To load the ^{14}C-macrolides, bacteria were suspended in 1 ml of ABS and incubated with 100 μM ^{14}C-labeled drug for 10 min at 37°C. This period of incubation allowed the drug in the cell to reach maximal levels (Fig. 1). Immediately after the 10-min incubation, 50 ml of ABS was added to dilute the extracellular drug, and the efflux rate of the preloaded ^{14}C-drug was measured by filtering 2.5 ml of cell suspension through Millipore filters at different times. The washed filters were counted for radioactivity. Data were fitted to a first-order exponential function: $C_t/C_0 = e^{-kt}$ ($k = 0.693/t_{1/2}$), where C_0 and C_t are the cellular ^{14}C-macrolide at zero and t times, respectively; $t_{1/2}$ is the time at which C_t reaches one-half of C_0; and k is the first-order rate constant. C_0 was determined by directly filtering the preloaded cells without any intervening efflux interval. The $t_{1/2}$ values were determined from the slopes of the plots of ln C_t/C_0 versus t.

Erythromycin, josamycin, and rokitamycin as well as the corresponding ^{14}C-labeled drugs were kindly supplied by Toyo Jozo Co., Ltd., Shizuoka, Japan.

Figure 1 shows the uptake of erythromycin, josamycin, and rokitamycin by *B. fragilis* ATCC 25285. The profiles of time course of the drug uptake were similar for three macrolides, showing an initial rapid-uptake phase within 10 min, followed by a slower release phase. The initial velocity was calculated by measuring the amount accumulated during the first 5 min. The preliminary studies indicated the linearity of the drug uptake during this time period. Of the drugs

* Corresponding author.

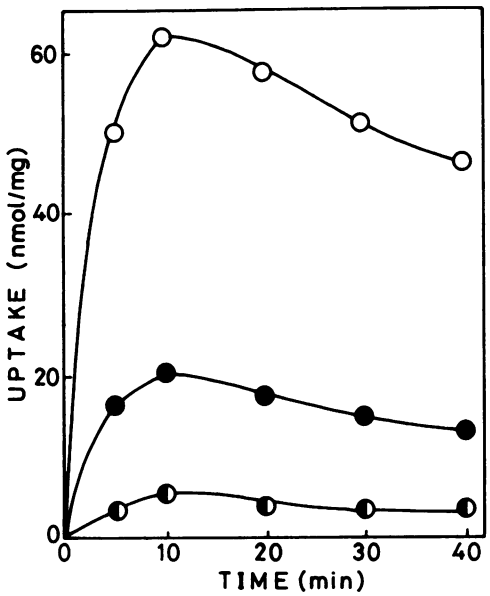

FIG. 1. Uptake of macrolides by *B. fragilis* ATCC 25285. Accumulation of macrolide antibiotics was measured in an anaerobic glove box using ^{14}C-labeled drugs (○, rokitamycin; ●, josamycin; ◐, erythromycin).

studied, rokitamycin had the highest initial velocity of accumulation. The velocities (in nanomoles per minute per milligram of dry weight) were as follows: rokitamycin, 10.1; josamycin, 3.32; and erythromycin, 0.68. The maximal levels of accumulation (nanomoles per milligram of dry weight) determined at 10 min were as follows: erythromycin, 5.4; josamycin, 20.5; and rokitamycin, 63.2. If the cellular volume of *B. fragilis* was assumed to be similar to that of *Escherichia coli* (2.7 μl/mg of dry weight [14]), the maximal concentration would be 2 mM for erythromycin and 23 mM for rokitamycin. These concentrations of antibiotics in the cell are 20- to 230-fold greater than that of the extracellular medium (100 μM). At present, the molecular mechanism by which *B. fragilis* can accumulate macrolide antibiotics against a concentration gradient remains to be explored. Many reports have demonstrated that bacterial cells can actively accumulate antibiotics such as aminoglycosides (2) and tetracyclines (13) in an energy-dependent manner. Although such energy-dependent uptake of aminoglycosides (3) and tetracycline (5) has also been reported for *B. fragilis*, addition of glucose or carbonyl cyanide *m*-chlorophenylhydrazone to the cell suspension did not affect the macrolide accumulation in *B. fragilis* ATCC 25285 (data not shown). These results suggest that the accumulation of macrolides in *B. fragilis* is not mediated by the energy-dependent mechanism, but further work is required to elucidate the precise mechanism of the macrolide uptake.

Efflux from cells is also important in the accumulation of antibiotics in the cell. We therefore examined efflux of macrolide antibiotics from *B. fragilis* cells. When the cells preloaded with ^{14}C-labeled macrolide were diluted by adding ABS, the concentration of the drug in the cells decreased exponentially with time. The time courses of efflux were fitted to an exponential function described and plotted using a semilog scale (Fig. 2). The linear relationship between ln C_t/C_0 versus time indicated that the efflux process followed a single exponential curve. From the slopes of semilog plots, $t_{1/2}$ (half-time for drug efflux) was calculated. The $t_{1/2}$ values

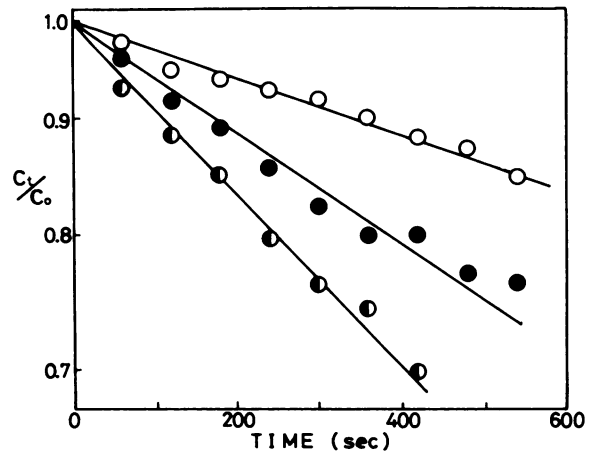

FIG. 2. Efflux of macrolides from *B. fragilis*. To determine $t_{1/2}$ values, the data were plotted using a semilog scale. C_t is the cellular ^{14}C-macrolide present at time t, and C_0 is the zero-time cellular ^{14}C-macrolide (○, rokitamycin; ●, josamycin; ◐, erythromycin).

of rokitamycin, josamycin, and erythromycin for efflux were 2,310, 1,205, and 792 s, respectively. Since $t_{1/2}$ is inversely proportional to velocity for drug efflux, the present results demonstrate that erythromycin possessed the highest efflux rate, followed in decreasing order by josamycin and rokitamycin.

The octanol-water partition coefficient is an index of hydrophobicity (4). The partition coefficients were reported to be 42 for erythromycin, 244 for josamycin, and 1,202 for rokitamycin (20). The partition coefficients of three macrolide antibiotics correlated well with the values of initial velocity for uptake (Fig. 3A) and $t_{1/2}$ for efflux (Fig. 3B). This indicates that the velocity for uptake and $t_{1/2}$ for efflux of the antibiotics may be dependent on the hydrophobicity of the drugs. It can therefore be postulated that the increase of drug hydrophobicity may result in the enhancement of the drug accumulation in the cell. To assess this possibility, we determined the MICs of three macrolide antibiotics for *B. fragilis* ATCC 25285. The values were 0.78, 0.10, and 0.05 μg/ml for erythromycin, josamycin, and rokitamycin, respectively. These values were found to correlate well with the octanol-water partition coefficients (Fig. 3C).

The results obtained in the present investigation indicate that macrolide antibiotics are accumulated by a process

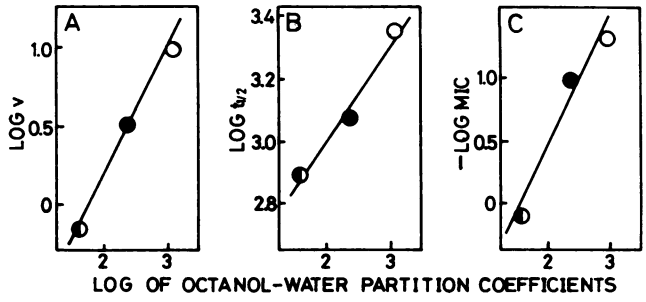

FIG. 3. Correlation of octanol-water partition coefficient with initial velocity of uptake, $t_{1/2}$ for efflux, and MICs of macrolide antibiotics. Ordinate: logarithm of the initial velocities (v) for macrolide uptake (A), $t_{1/2}$ values for macrolide efflux (B), or MICs of macrolide antibiotics (C). Abscissa: logarithm of the octanol-water partition coefficients (○, rokitamycin; ●, josamycin; ◐, erythromycin). Correlation coefficients calculated by the least-squares method for three macrolide antibiotics ranged between 0.97 and 0.99.

dependent on their hydrophobicity. Moreover, the antibacterial activity expressed in terms of the MICs increased with the drug hydrophobicity. These observations suggest that the antimicrobial activity of macrolides against *B. fragilis* correlates with the extent of drug accumulation in the bacteria. In the present study, however, the concentration of antibiotics used for the transport studies (100 μM) was in excess of the MICs. It is likely that antibiotics at this relatively high concentration may have some effects on the properties of macrolide transport itself. Therefore, the relationship between antibacterial activity and drug accumulation have to be interpreted with caution. The extent of drug accumulation in a cell is determined by the dynamic equilibrium between the influx and efflux processes (22). Efflux of macrolide antibiotics was seen in *B. fragilis* cells (Fig. 1). Furthermore, kinetic data (Fig. 2) demonstrated that the efflux rates of three macrolides were inversely correlated with the maximal amounts of accumulation of the drugs (Fig. 1). Thus, a decrease in efflux and an increase in uptake contribute to the efficient accumulation of the macrolides in *B. fragilis* cells.

Substantial evidence indicates that gram-negative bacteria are deficient in a pathway for penetration of hydrophobic substances, like macrolides, crystal violet, and detergents (8, 15, 19). However, results with *B. fragilis* presented above indicate that the bacteria were susceptible to macrolide antibiotics and that the drugs were efficiently accumulated in these cells, depending on the hydrophobicity of the drugs. Similar observations have also been reported for some *Neisseria* strains (24) and several mutant strains such as the "deep rough" *Salmonella* mutant (17). These and other investigations (19) have shown that the barrier for hydrophobic solutes was lipopolysaccharide located in the outer membrane and that alteration of the chemical composition or the amount of lipopolysaccharide in the outer membrane was responsible for elevated permeability of hydrophobic solutes (19). In this context, it is interesting to note that lipopolysaccharide isolated from the outer membrane of *B. fragilis* was chemically different from endotoxins of aerobic gram-negative bacteria and exhibited reduced biological potency (9, 10). Thus, relatively high permeability of macrolide antibiotics in *B. fragilis* cells may be related to the specificity of chemical composition of lipopolysaccharide. Further studies on the transport of macrolide and the physicochemical nature of surface envelopes in *B. fragilis* will provide valuable information concerning the transport mechanism of hydrophobic drugs.

LITERATURE CITED

1. **Atkinson, B. A.** 1980. Species incidence, trends of susceptibility to antibiotics in the U.S., and minimum inhibitory concentrations, p. 607–722. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
2. **Bryan, L. E.** 1984. Aminoglycoside resistance, p. 241–277. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., New York.
3. **Bryan, L. E., S. K. Kowand, and H. M. Van Den Elzen.** 1979. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: *Clostridium perfringens* and *Bacteroides fragilis*. Antimicrob. Agents Chemother. **15**:7–13.
4. **Dunn, W. J., III, ad C. Hansch.** 1974. Chemicobiological interactions and the use of partition coefficients in their correlation. Chem.-Biol. Interactions **9**:75–95.
5. **Fayolle, F., G. Privitera, and M. Sebald.** 1980. Tetracycline transport in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **18**:502–505.
6. **Finegold, S. M.** 1977. General aspects of anaerobic infection, p. 68–77. *In* S. M. Finegold (ed.), Anaerobic bacteria in human disease. Academic Press, Inc., New York.
7. **Godfrey, A. J., and L. E. Bryan.** 1984. Intrinsic resistance and whole cell factors contributing to antibiotic resistance, p. 113–145. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., New York.
8. **Hancock, R. E. W.** 1984. Alterations in outer membrane permeability. Annu. Rev. Microbiol. **38**:237–264.
9. **Hofstad, T., K. Sveen, and G. Dahlen.** 1977. Chemical composition, serological reactivity and endotoxicity of lipopolysaccharides extracted in different ways from *Bacteroides fragilis*, *Bacteroides melaninogenicus* and *Bacteroides oralis*. Acta Pathol. Microbiol. Scand. Sect. B **85**:262–270.
10. **Kasper, D. L.** 1976. Chemical and biological characterization of the lipopolysaccharide of *Bacteroides fragilis* subspecies *fragilis*. J. Infect. Dis. **134**:59–66.
11. **Kasper, D. L., and M. W. Seiler.** 1975. Immunochemical characterization of the outer membrane complex of *Bacteroides fragilis* subspecies *fragilis*. J. Infect. Dis. **132**:440–450.
12. **Kesado, T., K. Watanabe, Y. Asahi, M. Isono, and K. Ueno.** 1984. Comparative antibacterial activities of 7α-methoxy cephalosporins and 7β-methoxyiminoacetamido cephalosporins against *Bacteroides fragilis*. Antimicrob. Agents Chemother. **25**:131–133.
13. **Levy, S. B.** 1984. Resistance to the tetracyclines, p. 191–240. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., New York.
14. **Maloney, P. C., E. R. Kashket, and T. H. Wilson.** 1975. Methods for studying transport in bacteria, p. 1–49. *In* E. D. Korn, (ed.), Methods in membrane biology. Plenum Publishing Corp., New York.
15. **Mao, J. C.-H., and M. Putterman.** 1968. Accumulation in gram-positive and gram-negative bacteria as a mechanism of resistance to erythromycin. J. Bacteriol. **95**:1111–1117.
16. **Mao, J. C.-H., and R. G. Wiegand.** 1968. Mode of action of macrolides. Biochim. Biophys. Acta **157**:404–413.
17. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta **433**:118–132.
18. **Nikaido, H., and T. Nakae.** 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. **20**:163–250.
19. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
20. **Ohno, M., K. Ohta, and M. Morishita.** 1987. Physicochemical properties of rokitamycin. Iyakuhin Kenkyu **18**:634–639. (In Japanese.)
21. **Quentin, C., B. de Barbeyrac, N. Dubosc-Marchenary, and C. Bebear.** 1985. Susceptibility of anaerobic bacteria to eight macrolides and related drugs. Recent Adv. Chemother. Antimicrob. Sect. **2**:1511–1512.
22. **Stein, W. D.** 1986. Physical basis of movement across cell membrane, p. 1–68. *In* W. D. Stein, (ed.), Transport and diffusion across cell membranes. Academic Press, Inc., New York.
23. **Watanabe, K. N. Kato, K. Sawa, M. Aoki, and K. Ueno.** 1984. Antimicrobial activity of TMS-19-Q(3″-propionylleucomycin A_5) against anaerobic bacteria and *Ureaplasma*. Chemotherapy (Tokyo). **32**:54–59. (In Japanese.)
24. **Wolf-Watz, H., T. Elmros, S. Normark, and G. D. Bloom.** 1975. Cell envelope of *Neisseria gonorrhoeae*: outer membrane and peptidoglycan composition of penicillin-sensitive and -resistant strains. Infect. Immun. **11**:1332–1341.