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Tetracycline Transport in *Bacteroides fragilis*

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In a susceptible strain of *Bacteroides fragilis*, tetracycline uptake is biphasic. The initial phase is independent of adenosine 5'-triphosphate synthesis, which is coupled to fumarate reduction; this phase is not altered by expression of tetracycline resistance genes in a resistant strain. The second phase appears to occur by active transport, since it is largely reduced by rotenone, an inhibitor of electron transport to fumarate; moreover, this phase is under negative control of the tetracycline resistance gene.

Tetracycline (Tc) resistance is at present wide-spread among clinical isolates of the *Bacteroides fragilis* group (3; manuscript in preparation). In this laboratory we have particularly studied *B. fragilis* 92. Its resistance to tetracycline is transferable to susceptible strains and is very likely plasmid mediated (11). In gram-negative facultative anaerobes, plasmid-mediated tetracycline resistance is generally an inducible property. Levy and McMurry (5, 10) have identified in *Escherichia coli* a tetracycline-inducible protein, TET, involved in the transport system for tetracycline, and they have shown that the synthesis of this protein is negatively controlled. The presence of a Tc plasmid led to changes in the cell envelope to resistant strains.

In *B. fragilis* 92, tetracycline resistance is also an inducible property (Tc^r_i), and mutants were obtained which are constitutive for this property (Tc^r_c mutants) (11). It was therefore decided to study in *B. fragilis* whether the acquisition of tetracycline resistance by a susceptible strain is associated with a modification of the tetracycline transport system. We shall present results on tetracycline transport: first, in a susceptible, plasmid-free strain; second, in a transcipient of this strain with an inducible tetracycline resistance; and finally, in a transcipient with a constitutive tetracycline resistance. Experiments were also carried out in the presence of rotenone, an inhibitor of fumarate reductase, which is a key enzyme for the energetic metabolism of *B. fragilis* (4, 8, 9).

It is shown in this paper that in susceptible cells or in uninduced Tc^r_i cells, tetracycline uptake is biphasic, characterized by an initial rapid uptake which is independent of the fumarate pathway, followed by a slower uptake which appears to occur by an active fumarate-dependent transport. This latter phase is lacking in induced Tc^r_i cells and in Tc^r_c cells, showing that this phase is under negative control of the tetracycline resistance genes.

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MATERIALS AND METHODS

Strains. The following strains were used: 638, a plasmid-free strain of *B. fragilis* (11), susceptible to tetracycline (minimum inhibitory concentration, 0.064 μg/ml); strain 638-4, an inducible, tetracycline-resistant strain (minimum inhibitory concentration, 16 μg/ml) obtained by mating strain 638 and a tetracycline-induced culture of strain 92; and 638-6, a constitutive tetracycline-resistant strain (minimum inhibitory concentration, 32 μg/ml) obtained by mating strain 638 and a Tc constitutive derivative of strain 92 (11). These strains are rifampin resistant and hemin dependent.

Media. Media used included the following: SSC solution (NaCl, 0.15 M; sodium citrate, 0.015 M); SSC/10 solution, a one-tenth dilution of SSC; TY broth, containing 30 g of Trypticase (BBL Microbiology Systems), 20 g of yeast extract (Difco), 1 g of sodium thioglycolate, and 5×10^3 g of hemin per liter at pH 7.4 and autoclaved for 30 min at 112°C; and TGY broth, which was the previous medium supplemented with 5 g of glucose per liter.

Growth conditions. Cultures were generally incubated in anaerobic jars (Baird and Taitloch) at 34°C in an atmosphere of H_2-CO_2 (85:15, vol/vol). For kinetics experiments with sampling at short time intervals, the previous media were reduced and distributed anaerobically (anaerobic chamber, Celster Isotechnie) in serum bottles (Wheaton, glass, no. 223743) closed with butyl rubber stoppers (Wheaton, no. 224154) and aluminum vial seals (Wheaton, no. 224183), using a vial seal crimper (Wheaton, no. 224303). The bottle, like the anaerobic chamber, contained an oxygen-free gas mixture (N_2-H_2-CO_2, 85:5:10, vol/vol/vol). Media were autoclaved for 30 min at 105°C. Inoculation and sampling of these reduced media were made through the stoppers by using a sterile 1-ml syringe and a needle of 0.45 by 13 mm. All manipulations were made without air bubbling. Induction by tetracycline was carried out by growing the strains overnight in the presence of tetracycline at 0.5 μg/ml.

Tetracycline transport studies. Studies of tetracycline transport were done with [7-^{3}H]tetracycline (New England Nuclear Corp.) at a specific activity of

0.5 to 1 Ci/mmol. Solutions of tetracycline were made in methanol, and fresh solutions were used.

An overnight culture in TGY broth was centrifuged for 10 min at 5,000 rpm; cells were suspended in 1 ml of TGY broth and added through a syringe into a TGY bottle to produce a final optical density at 530 nm (A_{530}) of 6 to 12. At this A_{530}, no growth occurred, and a constant A_{530} value could be obtained during the experiment. The bottles were maintained at 34°C for 10 min, and [7-^{3}H]tetracycline was added by syringe to a final concentration of 0.5 μg/ml. The time of tetracycline addition was the zero time (t_0) of the experiment. At various intervals of time, samples of 0.2 ml were taken, immediately diluted 10 times in cold SSC/10 solution, and spun quickly at 5,000 rpm. The bacteria were suspended in 1.5 ml of cold SSC/10 solution. A portion of this supension was used for radioactivity counting: the samples were counted by liquid scintillation counting in Aquasol (New England Nuclear Corp.) at a 74% counting efficiency. Another sample of the suspension was used for optical density evaluation (A_{530}) with a spectrophotometer (Monospac 103, Jobin et Yvon). Results are given as counts per minute per A_{530} unit of cells.

Chemicals. Tetracycline hydrochloride was from Sigma; rotenone was from Sigma and was used at 3×10^{-4} M.

RESULTS

Two-phase accumulation of tetracycline by the tetracycline-susceptible strain. When tetracycline accumulation was studied in the susceptible strain, the results (Fig. 1) showed a biphasic transport, with an initial and rapid phase terminating at 5 min, followed by a slower phase which lasted for 30 min at least. The tetracycline uptake calculated during the first 6 min was 0.4 to 0.5 ng/A_{530} unit per min. During this phase, tetracycline accumulation was proportional to cell concentration (data not shown). Taking into account the degree of purity of the radioactive tetracycline used (97%) and the fact that the cellular radioactivity accumulation was proportional to cell concentration in our experiments, it can be assumed that tetracycline accumulation is responsible for the radioactivity measured.

Induction of resistance to tetracycline in the inducible Tc^r strain leads to a modification in the kinetics of accumulation. When identical experimental conditions were used with the Tc^r strain 638-4, which was not induced for tetracycline resistance, the results obtained were identical to those obtained with the susceptible strain. A two-phase uptake was observed, with a rapid phase terminating at 5 min, followed by a slower phase (Fig. 2). When this strain was previously induced by overnight incubation in the presence of nonradioactive tetracycline (0.5 μg/ml), however, the second phase of tetracycline uptake did not occur (Fig. 2).

An identical monophasic curve was obtained with the constitutive strain 638-6 in the absence of tetracycline induction (Fig. 2).

Effect of an electron transport inhibitor on tetracycline accumulation by susceptible cells. These experiments were carried out to determine whether one of the tetracycline uptake phases was an active process. In *B. fragilis*, fumarate reductase activity is hemin dependent, and it is an essential step in the energetic metabolism of this species because the reaction is coupled with a concomitant phosphorylation of adenosine phosphates to adenosine 5'-triphosphate (4, 8, 9). Rotenone significantly inhibits electron transport between H_2 and fumarate (4) and was used in our system as an energy inhibitor.

In a control experiment, an overnight culture of strain 638 in TGY broth was centrifuged and suspended in a bottle of TY broth containing 0.2% sodium fumarate. After 20 min of incubation at 34°C to adapt the cell to this substrate, radioactive tetracycline was added (t_0) and tetracycline transport was studied as described pre-

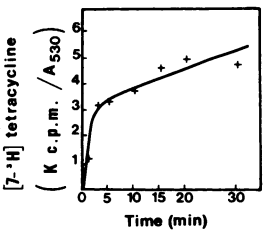

FIG. 1. *Uptake of tetracycline by B. fragilis 638 susceptible to tetracycline. An overnight culture in TGY broth was centrifuged and suspended at an A_{530} value of 6 in reduced TGY broth. At t_0 [7-^{3}H]tetracycline (specific activity, 0.57 Ci/mmol) was added at 0.5 μg/ml. At various intervals of time samples were taken for radioactivity counting and for A_{530} evaluation, as described in the text.*

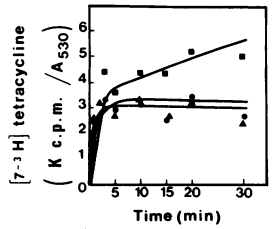

FIG. 2. *Uptake of tetracycline by an inducible (638-4) and a constitutive (638-6) tetracycline-resistant strain of B. fragilis. With the inducible strain 638-4, tetracycline uptake was studied in uninduced cells (■) and in induced cells (▲). No tetracycline induction was done with the constitutive strain (●). Experimental conditions were the same as for Fig. 1, except for the induced strain 638-4 (▲), for which the overnight incubation was made in TGY broth supplemented with nonradioactive tetracycline at 0.5 μg/ml.*

viously. Under these conditions, a biphasic uptake was again observed, similar to results on glucose-containing medium (Fig. 3).

When rotenone was added at 3×10^{-4} M 20 min after the addition of fumarate and radioactive tetracycline was added 20 min later, the second phase of tetracycline uptake was strongly reduced (Fig. 3), showing that only the initial rapid phase of tetracycline uptake is independent of the fumarate pathway. However, the second phase seems to be an active energy-dependent process. In unreported experiments using rotenone and glucose instead of fumarate as the source of energy, no inhibition of the second phase of tetracycline penetration was observed. This clearly shows that the second phase of tetracycline penetration can be energized by adenosine 5'-triphosphate synthesized by pathways other than electron transport to fumarate.

DISCUSSION

These results show clearly that tetracycline accumulation in *B. fragilis* is a biphasic process as it is in *E. coli* (2, 6, 7).

The initial phase of tetracycline uptake, which lasts 5 min, is rotenone insensitive and does not require energy produced by fumarate reduction. During this phase, tetracycline accumulation is either a passive phenomenon, i.e., through pores of the outer membrane (1), or an active process using energy obtained by an alternative pathway. In *B. fragilis*, most adenosine 5'-triphosphate is synthesized by a reaction coupled with fumarate reduction (8), but adenosine 5'-triphosphate synthesis by substrate level phosphorylation is likely. We were unable to differentiate both possibilities; the other energy inhibitors available were active on the fumarate pathway at a later stage than rotenone.

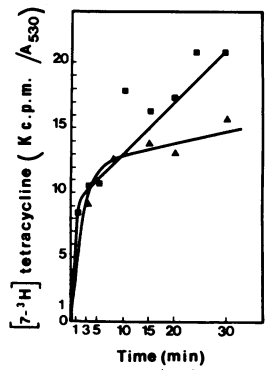

FIG. 3. *Uptake of tetracycline by the tetracycline-susceptible B. fragilis strain 638 in TY medium containing sodium fumarate at 0.2% in the absence (■) and in the presence (▲) of rotenone at 3×10^{-4} M. An overnight culture in TGY broth was suspended at an A_{530} of 6 in TY broth containing sodium fumarate at 0.2%. After 20 min of incubation of 34°C, rotenone was added at 0.3 mM to one of the flasks. After 20 min of incubation of 34°C, [7-³H]tetracycline (specific activity, 0.94 Ci/mmol) was added (t_0) at 0.5 μg/ml to both flasks. Experimental conditions were the same as for Fig. 1.*

The fact that this first uptake phase could be present both in susceptible and resistant cells whose minimum inhibitory concentrations differ by 8 $\log_2$ shows that this phase does not play a critical role in the tetracycline resistance level in *B. fragilis*. The initial phase is not modified by induction of the cells by tetracycline. However, in *E. coli*, the primary effect of induction is to induce a new transport system leading to a reduced initial uptake (5). Since the proportion of tetracycline incorporated by this mechanism is relatively high in *B. fragilis*, it is possible that after the 5 min of initial uptake, the cell localization of tetracycline is such that the antibiotic is inactive (i.e., tetracycline does not really get into contact with ribosomes). The complete insensitivity of the first phase to rotenone, which inhibits adenosine 5'-triphosphate synthesis via fumarate reduction, is in contrast with what was observed in a tetracycline-resistant *E. coli* harboring plasmid R100. In this strain, certain energetic inhibitors partially inhibit the initial phase of tetracycline entry (5).

The second, slower phase, which is energy dependent, corresponds to an active transport process. This phase does not occur in induced resistant cells or in constitutive strains. In uninduced resistant cells its lack is related to suboptimal expression of tetracycline resistance, which is negatively controlled as in staphylococci and *E. coli* (5, 6). These results suggest a very common mechanism of tetracycline transport in bacteria, and we are trying to demonstrate in *B. fragilis* the presence of a membrane protein playing a role in this negative control.

In a susceptible *B. fragilis* strain, the second uptake phase is relatively small in extent as compared to the initial uptake phase. Tetracycline resistance is supposed to be the result of at least two mechanisms, a diminution of antibiotic active transport and a loss of specific action on the ribosomes; our results suggest that the inhibition of antibiotic active transport has a less important role in tetracycline resistance expression in *B. fragilis* than in *E. coli*.

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