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Aminoglycoside Uptake Increased by *tet* Gene Expression

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The expression of extrachromosomal *tet* genes not only confers tetracycline resistance but also increases the susceptibilities of gram-negative bacteria to commonly used aminoglycoside antibiotics. We investigated the possibility that *tet* expression increases aminoglycoside susceptibility by increasing bacterial uptake of aminoglycoside. Studies of [^{3}H]gentamicin uptake in paired sets of *Escherichia coli* HB101 and *Salmonella typhimurium* LT2 expressing and not expressing *tet* showed that *tet* expression accelerates energy-dependent [^{3}H]gentamicin uptake. Increased [^{3}H]gentamicin uptake was accompanied by decreased bacterial protein synthesis and bacterial growth. Increased aminoglycoside uptake occurred whether *tet* expression was constitutive or induced, whether the *tet* gene was class B or C, and whether the *tet* gene was plasmid borne or integrated into the bacterial chromosome. *tet* expression produced no measurable change in membrane potential, suggesting that *tet* expression increases aminoglycoside uptake either by increasing the availability of specific carriers or by lowering the minimum membrane potential that is necessary for uptake.

Tetracycline resistance is common among gram-negative bacteria and is almost always due to the expression of extrachromosomal *tet* genes. These genes encode proteins which transport tetracycline out through the bacterial inner membrane (11, 13). In gram-negative bacteria, *tet* expression has additional effects: decrease of growth and viability (15), mediation of the uptake of potassium (7), increased susceptibilities to cadmium (8) and fusaric acid (1, 12), and increased susceptibilities to commonly used aminoglycoside antibiotics (9). The mechanisms of these pleiotropic effects of *tet* expression, including increased aminoglycoside susceptibility, are not understood.

Increased aminoglycoside susceptibility is particularly intriguing because of its potential clinical utility. We have shown previously that tetracycline increases the gentamicin susceptibility of tetracycline-resistant clinical isolates of gram-negative bacilli by inducing *tet* (14). This suggests that tetracycline might be used to induce *tet* expression and increase aminoglycoside activity in therapy for infections due to gram-negative bacilli.

The bactericidal action of aminoglycoside antibiotics depends on bacterial aminoglycoside uptake followed by interaction with the ribosomal target and inhibition of protein synthesis (16). We have demonstrated that *tet* expression increases the early bactericidal action of aminoglycosides (14), but the mechanism of this increased killing is unknown. Because *tet* expression produces an inner membrane protein, it seems more likely that *tet* expression alters aminoglycoside uptake across the inner membrane rather than increasing the susceptibility of ribosomes to inhibition by aminoglycosides. To test this possibility, we investigated the effect of *tet* expression on [^{3}H]gentamicin uptake while monitoring bacterial protein synthesis and growth.

Aminoglycoside uptake is usually proportional to the bacterial membrane potential (5, 16). We also investigated the effect of *tet* expression on bacterial membrane potential to determine if an effect on uptake was due to alteration of the transmembrane potential. These studies indicate that *tet* expression increases aminoglycoside uptake and that the increased uptake is not due to a measurable increase in the bacterial membrane potential.

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MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used in this study (Table 1) have been described previously (9).

[^{3}H]gentamicin. Gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) was radiolabeled by exposure to tritium gas (Dupont, NEN Research Products, Boston, Mass.). [^{3}H]gentamicin isomers were then purified from the crude radiolabeled mixture by chromatography on silica gel (Sigma) with a solvent of chloroform–methanol–30% ammonium hydroxide (2:1:1, vol/vol/vol). The purity and composition of each column fraction were assessed by comparison with gentamicin standards by thin-layer chromatography on silica gel by using the solvent described above. Column fractions

TABLE 1. Bacterial strains and plasmids[a]

Strain	Phenotype	LD_{90} (μg/ml)		Reference(s)
		Tc	Gm	
E. coli				
HB101	Tc^s	2	1	3, 9
HB101(pBR322)	Tc^{cr}	65	0.5	2, 9
HB101(pCC100)	Tc^{cr}	115	0.3	9
HB101(pCC42)	Tc^{ir}	65	0.5[b]	9
HB101(pSC101)	Tc^{ir}	40	0.6[b]	4, 9
HB101::Tn*10*	Tc^{ir}	200	1.4[b]	6, 9
S. typhimurium				
LT2	Tc^s	2	3	6, 9
LT2(pBR322)	Tc^{cr}	80	1	2, 9

[a] Only phenotypes that are relevant to this study are indicated. Abbreviations: Tc, tetracycline; Gm, gentamicin; s, susceptible; ir, inducible resistance; cr, constitutive resistance; LD_{90}, concentration of antibiotic that reduces plating efficiency by 90% (9).

[b] Gentamicin 90% lethal dose obtained on induction of the *tet* gene by tetracycline exposure.

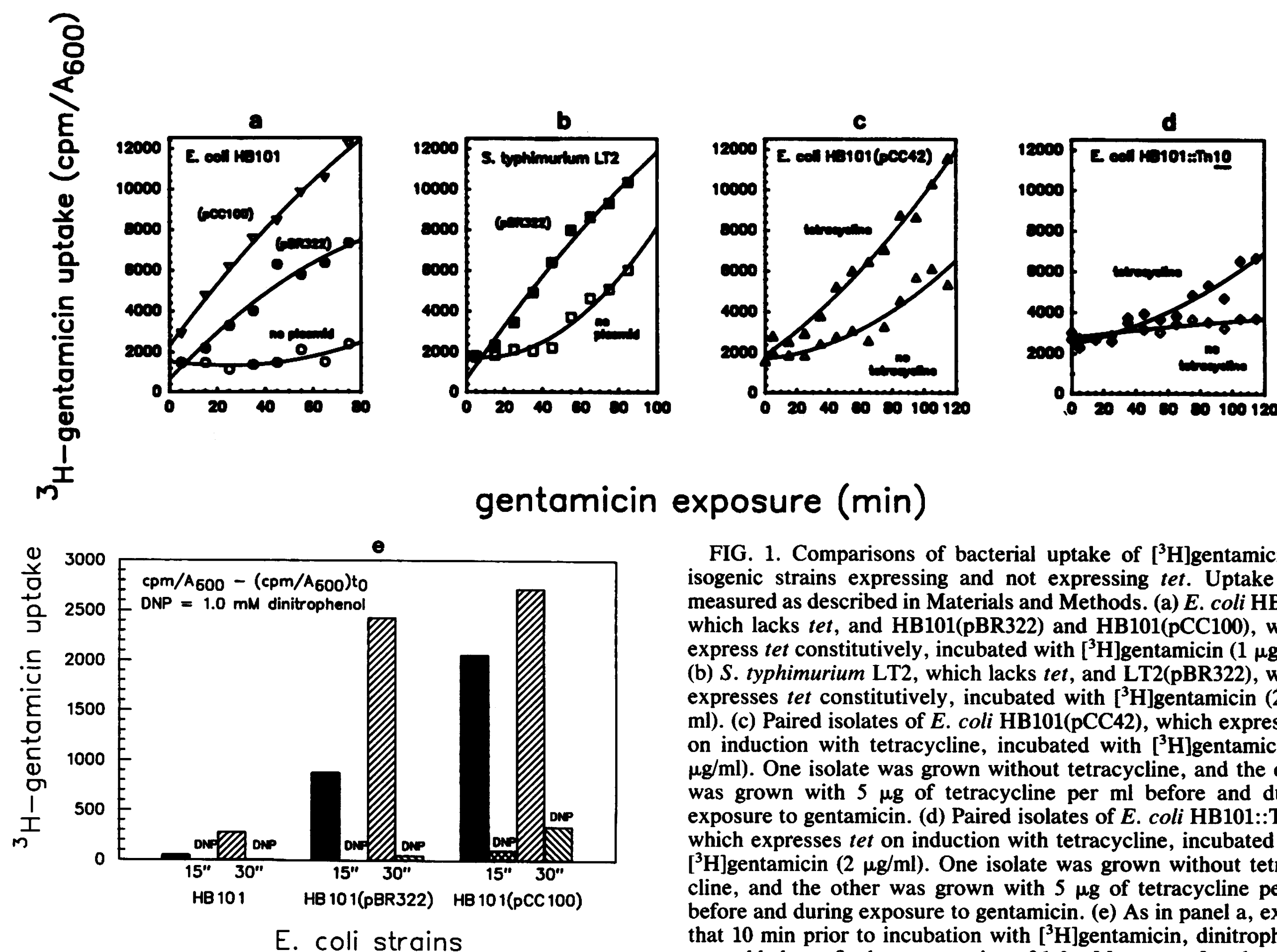

FIG. 1. Comparisons of bacterial uptake of [^{3}H]gentamicin in isogenic strains expressing and not expressing *tet*. Uptake was measured as described in Materials and Methods. (a) *E. coli* HB101, which lacks *tet*, and HB101(pBR322) and HB101(pCC100), which express *tet* constitutively, incubated with [^{3}H]gentamicin (1 μg/ml). (b) *S. typhimurium* LT2, which lacks *tet*, and LT2(pBR322), which expresses *tet* constitutively, incubated with [^{3}H]gentamicin (2 μg/ml). (c) Paired isolates of *E. coli* HB101(pCC42), which express *tet* on induction with tetracycline, incubated with [^{3}H]gentamicin (1 μg/ml). One isolate was grown without tetracycline, and the other was grown with 5 μg of tetracycline per ml before and during exposure to gentamicin. (d) Paired isolates of *E. coli* HB101::Tn*10*, which expresses *tet* on induction with tetracycline, incubated with [^{3}H]gentamicin (2 μg/ml). One isolate was grown without tetracycline, and the other was grown with 5 μg of tetracycline per ml before and during exposure to gentamicin. (e) As in panel a, except that 10 min prior to incubation with [^{3}H]gentamicin, dinitrophenol was added to a final concentration of 1.0 mM to one of each pair of cultures. Gentamicin uptake is reported as the change in uptake from zero time, i.e., $(cpm/A_{600}) - (cpm/A_{600})_{t_0}$, where cpm is counts per minute.

were pooled, evaporated to dryness, and suspended in distilled water, and the antibiotic activity of each fraction was normalized to the activity of nonradiolabeled gentamicin sulfate.

Preparation of cultures and measurement of growth. Log-phase cultures were diluted with L broth to approximately 2.5×10^7 bacteria per ml. The L broth contained (per liter of water) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 5 ml of 1 N NaOH, and 1 ml of 1 M $MgSO_4$. The final pH of the broth was 7.7 to 7.8. Either gentamicin sulfate (Sigma) or [^{3}H]gentamicin (described above) was added to the final specified concentrations. The cultures were then incubated by shaking at 37°C, and the growth of the cultures was determined at specified intervals from the optical density at 600 nm. *tet* expression was induced in *Escherichia coli* HB101(pCC42) and HB101::Tn*10* by exposure to a subinhibitory concentration of tetracycline (5 μg/ml) for 2 h prior to the addition of gentamicin.

Measurement of [^{3}H]gentamicin uptake. At the specified intervals, 1.0-ml samples were withdrawn from the cultures containing [^{3}H]gentamicin. The bacteria were separated from the radioactive supernatant by centrifugation for 30 s in a microcentrifuge, suspended in 1.0 ml of ice-cold 0.14 M NaCl, and collected on a 0.45-μm-pore-size HAWP membrane filter (Millipore Corp., Bedford, Mass.). The filters were then washed with 10 ml of ice-cold 0.14 M NaCl and dried, and radioactivity was determined by liquid scintillation spectrometry. The uptake at each point was normalized to the corresponding optical density at 600 nm.

Measurement of protein synthesis. At specified intervals, 1.0-ml samples of bacteria were withdrawn from the cultures containing nonradiolabeled gentamicin. The bacteria were collected by centrifugation, suspended in 0.5 ml of minimal medium containing [^{3}H]arginine (2 μCi/ml) (Dupont), and incubated by shaking at room temperature for 15 min. Equal volumes of ice-cold 20% trichloroacetic acid were then added. After 30 min at 4°C, the samples were collected on cellulose acetate membranes. The filters were then washed consecutively at 90 and 4°C with 5% trichloroacetic acid and washed with ethanol at 4°C, and radioactivity was determined by liquid scintillation spectrometry.

Measurement of membrane potential. The membrane potential was determined by the method of Kashket (10) by using the Nernst equation and the transmembrane distribution of the lipophilic cation [^{3}H]tetraphenylphosphonium$^+$ (Dupont) in the presence and absence of the protonophore carbonylcyanide *m*-chlorophenylhydrazone. [^{14}C]polyethylene glycol (M_r, 4,000; Dupont) was added to the incubation medium and used to quantitate the amount of extracellular tetraphenylphosphonium$^+$ trapped in the bacterial pellet (10). Five independent determinations of tetraphenylphosphonium$^+$ distribution were made for each strain. Bacteria were separated from the incubation medium by centrifugation, and the radioactivity was counted by liquid scintillation

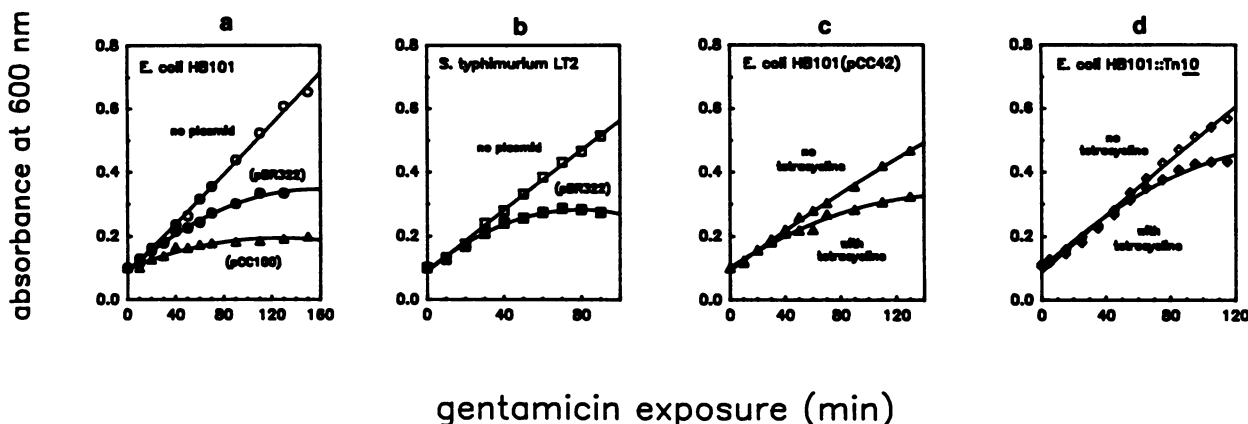

FIG. 2. Comparisons of inhibition of bacterial growth by gentamicin in isogenic strains expressing and not expressing *tet*. The effects of 1 μg (a and c) and 2 μg (b and d) of [^{3}H]gentamicin per ml on the growth of the specified strains were determined as described in Materials and Methods. (a) *E. coli* HB101, which lacks *tet*, and HB101(pBR322) and HB101(pCC100), which express *tet* constitutively. (b) *S. typhimurium* LT2, which lacks *tet*, and LT2(pBR322), which expresses *tet* constitutively. (c) Paired isolates of *E. coli* HB101(pCC42), which express *tet* on induction with tetracycline. One isolate was grown without tetracycline, and the other was grown with 5 μg of tetracycline per ml before and during exposure to gentamicin. (d) Paired isolates of *E. coli* HB101::Tn*10*, which express *tet* on induction with tetracycline. One isolate was grown without tetracycline, and the other was grown with 5 μg of tetracycline per ml before and during exposure to gentamicin.

spectrometry. The intracellular volume was calculated to be 0.95 μl by assuming a 2.5-μl intracellular volume per mg of bacteria (5) and 0.38 mg/ml of bacteria at an optical density of 600 nm (10). The cellular volumes of tetracycline-susceptible and tetracycline-resistant *E. coli* HB101 were identical when measured by electrical resistance with a cell counter (Particle Data Inc., Chicago, Ill.).

RESULTS

Gentamicin uptake. Figure 1 compares the [^{3}H]gentamicin uptake in *E. coli* HB101 and *Salmonella typhimurium* LT2 strains variously expressing *tet*. In all instances (Fig. 1a to d), *tet* expression accelerated the uptake of gentamicin by hastening the onset of uptake or increasing its rate or both. Dinitrophenol, a protonophore which dissipates the transmembrane proton gradient, prevented the increased uptake conferred by *tet* expression (Fig. 1e). Plasmids pBR322 and pCC100, which express *tet* constitutively, accelerated gentamicin uptake in *E. coli* HB101 (Fig. 1a). Plasmid pCC100, which confers a higher level of resistance to tetracycline than pBR322 does, effected a greater increase in gentamicin uptake than pBR322 did (Fig. 1a). Similarly, plasmid pBR322 accelerated gentamicin uptake in *S. typhimurium* LT2 (Fig. 1b).

Plasmid pCC42, which contains a class C *tet* gene derived from pBR322, and transposon Tn*10*, which contains an unrelated class B *tet* gene, both express *tet* upon exposure to tetracycline (9). In both HB101(pCC42) and HB101::Tn*10*, the induction of *tet* expression by tetracycline increased gentamicin uptake (Fig. 1c and d).

tet expression altered gentamicin uptake in the same way that increased gentamicin concentration did, by shortening the lag period prior to uptake or increasing the rate of uptake or both (data not shown) (16).

Inhibition of growth. In all cases, *tet* expression caused an earlier, more rapid inhibition of bacterial growth by gentamicin (Fig. 2). Plasmids pBR322 and pCC100, which express a class C *tet* gene constitutively, hastened growth inhibition by gentamicin in *E. coli* HB101 (Fig. 2a). Among the plasmids containing class C *tet* genes, there was a direct relationship between the level of tetracycline resistance conferred by the *tet* gene (Table 1) and the level of increase in the gentamicin growth inhibition. Thus, pCC100 caused a greater increase in gentamicin growth inhibition than did pBR322 (Fig. 2a), which caused a greater increase than did plasmid pSC101 (data not shown). Plasmid pBR322 accelerated the inhibition of *S. typhimurium* LT2 by gentamicin (Fig. 2b).

Plasmid pCC42 and transposon Tn*10*, which express their *tet* genes on induction, hastened gentamicin growth inhibition of *E. coli* HB101 (Fig. 2c and d) induced with tetracycline. In contrast, exposure to the same concentration of tetracycline had no effect on the growth rate of HB101 (pBR322) (data not shown), which contains a noninducible class C *tet* gene.

Inhibition of protein synthesis. Plasmids pBR322 and pCC100, which express *tet* constitutively, each promoted gentamicin inhibition of protein synthesis in *E. coli* HB101 (Fig. 3). Plasmid pCC100, which confers a higher level of tetracycline resistance than pBR322 does, produced an earlier inhibition of protein synthesis than pBR322 did.

Measurements of membrane potential. There was no measurable relationship between membrane potential and *tet* expression. The membrane potential in *E. coli* HB101, which lacks *tet*, was 97 mV. Membrane potentials in *E. coli* HB101 (pBR322) and HB101(pCC100), which express *tet* constitutively, were 99 and 93 mV, respectively. Standard deviations of membrane potential measurements were 8 to 9 mV.

DISCUSSION

In these studies, the mechanism by which *tet* expression increases the activity of aminoglycoside antibiotics against gram-negative bacteria was investigated. Aminoglycoside antibiotics must be taken up by gram-negative bacteria through an energy-requiring process before they exert their antimicrobial activities (16). Once inside the bacteria, the aminoglycosides interfere with ribosomal function and cause decreased protein synthesis. Because *tet* expression produces an inner membrane protein, we hypothesized that *tet* expression increases aminoglycoside activity by increasing aminoglycoside uptake across the bacterial inner membrane.

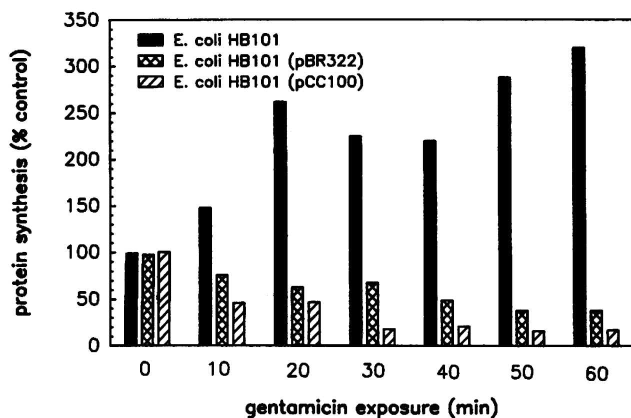

FIG. 3. Comparison of inhibition of protein synthesis by gentamicin in isogenic isolates expressing and not expressing *tet*. The effect of gentamicin sulfate (1 μg/ml) on the rate of protein synthesis was determined as described in Materials and Methods for *E. coli* HB101, which lacks *tet*, and HB101(pBR322) and HB101(pCC100), which express *tet* constitutively. The values reported are the percentages of results with paired zero time controls in the absence of gentamicin.

We investigated this possibility by comparing [^{3}H]gentamicin uptake in isogenic pairs of organisms expressing and not expressing *tet* and demonstrated that *tet* expression increases [^{3}H]gentamicin uptake.

In order to ensure that the increased [^{3}H]gentamicin uptake was due to *tet* expression and not to some other aspect of the plasmid or of the host bacterial strain, we studied the effect of *tet* expression on uptake in diverse conditions. *tet* expression increased [^{3}H]gentamicin uptake in all circumstances: whether the host bacteria was *E. coli* or *S. typhimurium*, whether the *tet* gene was plasmid borne (pBR322, pCC100, pCC42, and pSC101) or integrated into the bacterial chromosome (Tn*10*), whether *tet* expression was constitutive (pBR322 and pCC100) or induced (pCC42, pSC101, and Tn*10*), and whether the *tet* gene was class B (Tn*10*) or class C (pBR322, pCC100, pCC42, and pSC101).

Aminoglycoside uptake is usually dependent on the energy of the proton motive force and can be blocked by the protonophore dinitrophenol (5, 16). *tet* expression causes the production of a membrane protein, which might passively render the bacteria more leaky to aminoglycoside or might act as an energy-dependent pump or gated channel for aminoglycoside uptake. We investigated whether the increased aminoglycoside uptake conferred by *tet* expression was a passive phenomenon or an energy-dependent process. The increased uptake was blocked by dinitrophenol (Fig. 1e), demonstrating that it is dependent on the energy of the proton motive force.

Energy-dependent aminoglycoside uptake is usually proportional to the bacterial membrane potential, which serves as the driving force for aminoglycoside entry above a definable threshold (5). Damper and Epstein have defined a quantitative relationship between aminoglycoside susceptibility and the membrane potential (5). This equation allowed us to predict that the membrane potentials of *E. coli* HB101(pCC100) and HB101(pBR322) would be, respectively, 18 to 20 and 9 to 10 mV higher than that of HB101 if the alteration in aminoglycoside susceptibility produced by *tet* expression was due to an increase in the membrane potential. We found no measurable difference in the membrane potentials of these strains, however. The 18- to 20-mV difference in membrane potential predicted between HB101(pCC100) and HB101 would have been detected within the precision of membrane potential measurement by tetraphenylphosphonium$^+$ equilibration and was not detected. Thus, there is no evidence that the increased aminoglycoside uptake is driven by an increase in membrane potential. The possibilities should now be considered that *tet* expression either increases the availability of specific carrier molecules or reduces the minimum membrane potential that is necessary for uptake.

ACKNOWLEDGMENTS

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