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Overproduction of Transposon Tn*10*-Encoded Tetracycline Resistance Protein Results in Cell Death and Loss of Membrane Potential

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High-level expression of the Tn*10* tetracycline resistance protein TetA in *Escherichia coli* caused partial collapse of the membrane potential, arrest of growth, and killing of the cells. Since α-methylglucoside transport was not affected, the overproduced TetA protein may cause not destruction of membrane structure but rather unrestricted translocation of protons and/or ions across the membrane.

Transposon Tn*10* mediates high-level tetracycline resistance in *Escherichia coli* and other enteric bacteria (7, 12). Two genes of the transposon—*tetA*, encoding the resistance protein, and *tetR*, encoding a repressor—are involved in tetracycline resistance. Both genes are tightly regulated at the level of transcription by the *tetR* gene product (3, 4, 9, 11). Although the resistance mechanism is not fully understood, the data available indicate that resistance is based on TetA protein-mediated, energy-dependent export of the antibiotic from the cytoplasm (14, 15). Its localization within the bacterial cell inner membrane (13) is in agreement with such a function. A surprising finding was that strains with transposon Tn*10* located on a high-copy-number plasmid exhibited lower levels of resistance to tetracycline than did strains which carry the *tet* genes in a low-copy state (5, 6). Moreover, this negative gene dosage effect was correlated with overexpression of the *tetA* gene (16, 17).

Effects of TetA protein overproduction on growth and viability. To achieve controlled overproduction of TetA protein, the *tetA* gene of Tn*10* was cloned behind the *tac* promoter on multicopy plasmid pCB258. Expression from the *tac* promoter was controlled by the *lac* repressor encoded on the same plasmid. *E. coli* B strain CM12 (21) was transformed with plasmids pCB258 and pFDX127 (the parental plasmid of pCB258 lacking *tetA*, derived from pFDX104 [20]). Induction of *tetA* expression in CM12(pCB258) with isopropyl-β-D-thiogalactopyranoside (IPTG) resulted in a rapid decrease in growth rate and, upon longer incubation, complete stoppage of growth (Fig. 1a). Induction of *tetA* also stopped the increase of cell numbers followed by a decrease in the number of viable cells (Fig. 1b). In the control culture, CM12(pFDX127), no effect of induction of the *tac* promoter on growth rate or viability was detected. Since the only difference between plasmids pFDX127 and pCB258 was the presence of *tetA* on pCB258, the lethal effect observed could be correlated with the overproduction of TetA protein. Similar effects have previously been observed after overproduction of lactose permease (18) and the membrane-bound ATP synthase of *E. coli* (22).

Effect of TetA protein overproduction on membrane potential. The effect of induced overexpression of *tetA* on membrane potential ($\Delta\psi$) was tested by measuring uptake of the

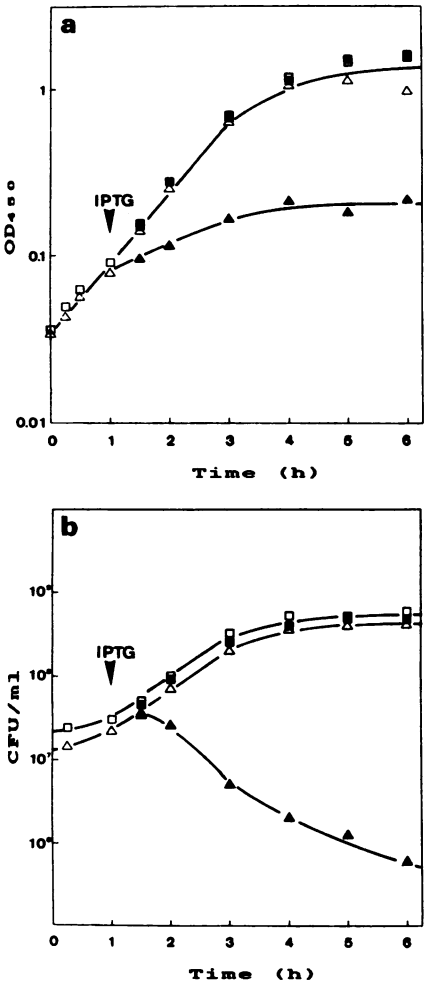

FIG. 1. Effects of overexpression of *tetA* on cell growth (a) and viability (b). Strains CM12(pFDX127) (□ and ■) and CM12 (pCB258) (△ and ▲) were grown in nutrient broth with 60 μg of ampicillin per ml. IPTG (5 mM) was added at the times indicated by arrowheads. OD_{450}, Optical density at 450 nm.

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TABLE 1. Effect of induced overproduction or TetA protein on transport of proline and α-methylglucoside

Substrate and plasmid carried by strain CM12	Relative rate of transport[a]	
	Without IPTG	With IPTG
[^{3}H]proline		
pFDX127	100	100
pCB258	100	12
[^{14}C]α-methylglucoside		
pFDX127	100	100
pCB258	100	85

[a] Cells were grown in nutrient broth with 60 μg of ampicillin per ml at 37°C. For uptake assays (37°C), they were suspended in 100 mM potassium phosphate buffer (pH 7.5) to an optical density of 0.5 at 450 nm. Three samples were taken within the first 60 s after addition of [^{3}H]proline (2 μM; specific activity, 0.27 Ci/mmol) or [α-^{14}C]methylglucoside (0.4 mM; specific activity, 0.4 mCi/mmol). Cells were collected on membrane filters (Sartorius SM111) and washed twice with potassium phosphate buffer, and the radioactivity was determined after drying. Initial uptake was calculated from three time points. The transport rates of CM12(pFDX127) were set as 100%. The actual rates were 26 pmol/2.5 × 10^8 cells and 2.4 nmol/2.5 × 10^8 cells for proline and α-methylglucoside, respectively.

radiolabeled lipophilic cation tetraphenylphosphonium (TPP^+). Exponentially growing cultures of CM12(pFDX127) and CM12(pCB258), either uninduced or induced with IPTG 15 min before the uptake assay, were used (Fig. 2). TPP^+ uptake by CM12(pFDX127) was not affected by addition of IPTG, and the membrane potential, estimated by the Nernst equation, was approximately −165 mV (assuming a cell volume of 2.5 μl/mg [dry weight] [21]). Addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone induced quick release of the TPP^+ accumulated by the cells. This shows that TPP^+ uptake is a direct reflection of the energized state of the cells. Strain CM12(pCB258) exhibited reduced TPP^+ uptake even in the absence of IPTG, which corresponded to a membrane potential of about −145 mV. In cultures of CM12(pCB258) induced with IPTG for 15 min, a drastic reduction in TPP^+ uptake was observed and the membrane potential was reduced to approximately −85 mV (Fig. 2a). A kinetic analysis revealed that 4 to 5 min after induction of *tetA*, TPP^+ uptake had decreased drastically (Fig. 2b). To analyze whether the decrease in the cell membrane potential caused by the overproduced TetA protein was a consequence of the destruction of the inner membrane, we measured the uptake of proline and α-methylglucoside. Active transport of proline depends on the proton motive force (10), of which Δψ is the main component at pHs of around 7.0. The rate of proline uptake was severely decreased upon induction of TetA protein synthesis (Table 1). α-Methylglucoside, which is taken up by the phosphoenolpyruvate sugar phosphotransferase system (19), was transported at similar rates by uninduced cells and cells overproducing TetA (Table 1). Since α-methylglucoside uptake was essentially unaffected, destruction of the cell inner membrane could be ruled out as an explanation for the decrease in Δψ observed. Rather, the effect of excess TetA protein on Δψ appears to be similar to that observed after addition of uncouplers, i.e., dissipation of Δψ by unrestricted translocation of protons and/or ions across the membrane.

How can the observed effect of the overproduced TetA protein be accounted for? According to current concepts, TetA protein functions as a carrier for tetracycline in the excretion of the antibiotic (which enters the cell presumably by passive diffusion [1, 2]). This transport of tetracycline was shown to be energy dependent (15) and may be driven by a countertransport of protons. Two models could account for the dissipating effect of TetA protein overproduction on membrane potential. (i) Rapid synthesis of a high number of

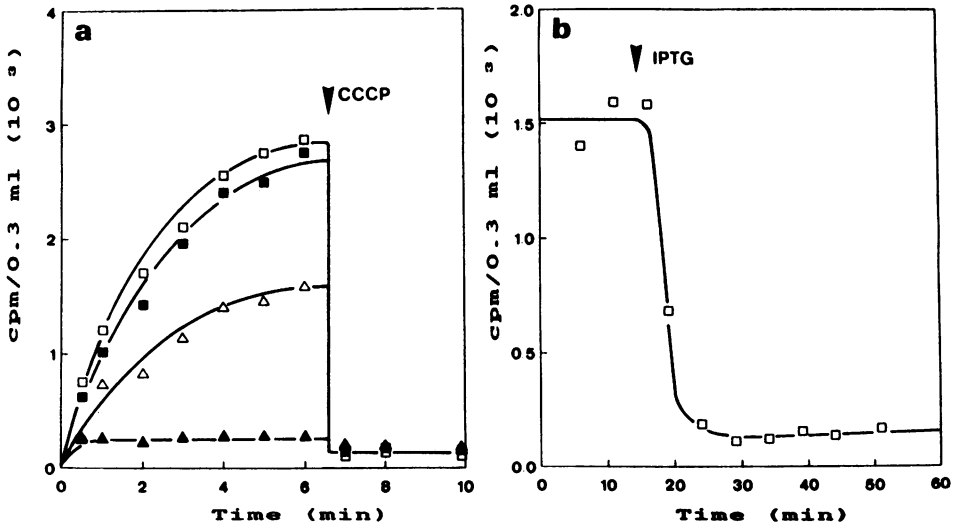

FIG. 2. (a) Effect of *tetA* overexpression on Δψ. *E. coli* B strain CM12 harboring several mutations which result in increased permeability (21) allowed TPP^+ uptake measurements in the absence of EDTA treatment. Strains CM12(pFDX127) (□ and ■) and CM12(pCB258) (△ and ▲) were grown in nutrient broth with 60 μg of ampicillin per ml. [^{3}H]TPP^+ (specific activity, 25 mCi/mmol) uptake was determined at an optical density of 0.3 at 450 nm. [^{3}H]TPP^+ was added to a final concentration of 5 μM, and culture samples were collected on GF/C membrane filters at 0.5 and 1 min and at 1-min intervals thereafter. Overexpression of *tetA* was induced (5 mM IPTG) at 15 min before addition of [^{3}H]TTP^+. At 6.5 min after TPP^+ addition, the uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 10 μM) was added. Open symbols represent uninduced cultures, and closed symbols represent induced cultures. (b) Change in membrane potential after induction of *tetA*. CM12(pCB258) was grown in nutrient broth supplemented with ampicillin (60 μg/ml). [^{3}H]TPP^+ uptake was measured at different times before and after addition of IPTG (5 mM) (arrowhead). The amount of [^{3}H]TPP^+ accumulated by the cells was determined at 6 min after addition of [^{3}H]TPP^+.

TetA protein molecules may result in insertion of the protein into the membrane in an abnormal conformation, which in turn may lead to an ion channel or to translocation of protons or ions in the absence of tetracycline. (ii) The observed effect of overproduced TetA protein on $\Delta\psi$ may be a consequence of the absence of sufficient quantities of other cellular components in the membrane. Such components, encoded by the host chromosome, may be involved in Tn*10*-mediated tetracycline resistance (8). Genetic analysis of mutants resistant to overproduced TetA protein may provide insight into the nature of the $\Delta\psi$-dissipating activity associated with overproduced TetA protein.

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