Electrogenic Antiport Activities of the Gram-positive Tet Proteins Include a Na⁺(K⁺)/K⁺ Mode That Mediates Net K⁺ Uptake*

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Two Gram-positive Tet proteins, TetA(L) from Bacillus subtilis and TetK from a Staphylococcus aureus plasmid, have previously been suggested to have multiple catalytic modes and roles. These include: tetracycline (Tc)-metal/H⁺ antiport for both proteins (Yamaguchi, A., Shinya, Y., Fujibira, E., Sawai, T., Noguchi, N., and Satusu, M. (1995) FEBS Lett. 365, 193–197; Cheng, J. Guffanti, A. A., Wang, W., Krulwich, T. A., and Bechhofer, D. H. (1996) J. Bacteriol. 178, 2853–2860); Na⁺(K⁺)/H⁺ antiport for both proteins (Cheng et al. (1996)); and an electrical potential-dependent K⁺ leak mode for TetK and highly truncated segments thereof that can facilitate net K⁺ uptake (Guay, G. G., Tuckman, M., McNicholas, P., and Rothstein, D. M. (1993) J. Bacteriol. 175, 4927–4929). Studies of membrane vesicles from Escherichia coli expressing low levels of complete and 3’-truncated versions of tetA(L) or tetK, now show that the full-length versions of both transporters catalyze electrogenic antiport and that demonstration of electrogenicity depends upon use of a low chloride buffer for the assay. The K⁺ uptake mode, assayed via 86Rb⁺ uptake, was also catalyzed by both full-length TetA(L) and TetK. This mode does not represent a potential-dependent leak. Such a leak was not demonstrable in energized membrane vesicles. Rather, Rb⁺ uptake occurred in right-side-out vesicles when the intravesicular space contained either Na⁺ or K⁺ but not choline. If an outwardly directed gradient of Na⁺ or K⁺ was present, Rb⁺ uptake occurred without energization in vesicles from cells transformed with a plasmid containing tetA(L) or tetK but not a control plasmid. Experiments in which a comparable exchange was carried out in low chloride buffers to which oxonol was added confirmed that the exchange was electrogenic. Thus, the K⁺ uptake mode is proposed to be a mode of the electrogenic monovalent cation/H⁺ antiport activity of TetA(L) and TetK in which K⁺ takes the place of the external protons. Truncated TetK and TetA(L) failed to catalyze either Tc-metal/H⁺ or Na⁺/H⁺ antiport in energized everted vesicles. Truncated TetK, but not TetA(L), did, however, exhibit modest, electrogenic Na⁺(K⁺)/Rb⁺ exchange as well as a small, potential-dependent leak of Rb⁺. The C-terminal halves of the TetA(L) and TetK proteins are thus required both for proton-coupled active transport activities of the multifunctional transporter and, perhaps, for minimizing cation leakiness.

Te³ enters bacterial cells in a non-carrier dependent fashion that is promoted by a transmembrane pH gradient, acid out (1). The antibiotic thus enters the cell best under neutral and acidic pH conditions and could inhibit cell protein synthesis strongly in sensitive cells in this pH range. Both Gram-positive and Gram-negative Tet efflux proteins catalyze similar exchange reactions which prevent cytoplasmic accumulation of the antibiotic. Te is actively extruded, as a complex with a divalent cation that bears a single positive charge, in exchange for external H⁺ (2, 3). The smaller (12-transmembrane segments) Gram-negative Tet proteins and the larger (14-transmembrane segments) Gram-positive Tet proteins share sequence similarity largely in the N-terminal six transmembrane segments regions (4, 5) but at least some motifs and/or residues in the C-terminal halves of each type of Te efflux protein cannot be modified without loss of activity (6, 7). Both the Gram-negative and Gram-positive Tet protein families contain examples that have further been shown to complement K⁺-uptake deficient mutants of Escherichia coli (8–11), but this net K⁺ uptake mode is not taken as a general property of Tet proteins. It has been attributed to an electrical potential-dependent K⁺ leak that could also be conferred by truncated forms of proteins that exhibit the property (10–12). Recently, studies in this laboratory have shown that the chromosomally encoded Bacillus subtilis TetA(L) protein and closely related TetK from a Staphylococcus aureus plasmid catalyze Na⁺(K⁺)/H⁺ antiport (13–17) in addition to Tc⁻Me²⁺/H⁺ antiport (2, 13, 14). These exchanges were evidently electrogenic, as assayed via energy-dependent Te-cobalt or Na⁺ uptake by everted vesicles of E. coli that expressed a cloned tetA(L) gene from a weak promoter (14). The exchanges were not inhibited by low nigericin concentrations that reduce the ΔpH but were significantly inhibited by valinomycin in the presence of K⁺, a combination that abolished the ΔΨ generated by respiration (14). Consistently, the antiports catalyzed by purified and reconstituted TetA(L) could be energized by an imposed potential (16). In addition, the important role of TetA(L) in acidifying the cytoplasm of B. subtilis relative to the external medium during growth at alkaline pH would require that the monovalent cation/H⁺ mode be electrogenic (15, 18). Since TetK could substitute for TetA(L) in a mutant of B. subtilis that had a disrupted tetA(L) gene, TetK is presumed to catalyze an electrogenic antiport similar to TetA(L). By contrast, the Te-metal/H⁺ antiport catalyzed by Gram-negative Tet proteins has been proposed to be electroneutral (19). Moreover, Yamaguchi and colleagues (3, 20) have experienced difficulty in demonstrating the Na⁺/H⁺ activity of TetK and indicate that the Te-metal/H⁺ antiport activity of TetK appeared to be electroneutral in preliminary work. One of

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The abbreviations used are: Tc, tetracycline; ΔpH, transmembrane pH gradient, acid out for right-side-out vesicles or cells; ΔΨ, transmembrane electrical potential, positive out for right-side-out vesicles or cells; MIC, minimal inhibitory concentration; MOPS, 4-morpholinepropanesulfonic acid.
the goals of the current study, therefore, was to examine the Tc-metal/H⁺ and Na⁺/H⁺ antiport activities of TetA(L) and TetK side-by-side in comparable preparations and to clarify their electrogenicity versus electroneutrality. The studies have strongly supported the multifunctional and electrogenic nature of both TetA(L) and TetK.

A second major goal of the studies was to test an alternate hypothesis to the putative K⁺ leak in explaining the ability of Tet proteins such as TetK to complement K⁺-uptake-deficient *E. coli*. The new hypothesis arises from the discovery that these Tet proteins are electrogenic monovalent cation/H⁺ antiporters, i.e., have a catalytic mode in which cytoplasmic Na⁺ or K⁺ is exchanged for a greater number of external H⁺. If K⁺ were able to occupy the external H⁺ sites, then an exchange of cytoplasmic monovalent cation for a greater number of K⁺ could account for the net uptake of K⁺ (Fig. 1). Thus, the net uptake of K⁺ catalyzed by a Tet protein could be one of its normal catalytic modes. Experiments were designed to test this hypothesis with TetK and to examine whether TetA(L), to which this kind of activity had never been attributed, might nonetheless possess a comparable capacity. If the capacity to catalyze net K⁺ uptake was in fact a function of the monovalent cation antiport mode, then TetA(L) might well demonstrate it. Or, a capacity to catalyze net K⁺ uptake might be restricted to those Tet proteins with both monovalent cation/H⁺ exchange activity and a particularly high affinity for K⁺. Both cloned TetA(L) and TetK were shown to restore Na⁺ exclusion capacity and resistance to a Δteta(L) strain of *B. subtilis*. In such experiments, the Na⁺ exclusion capacity of TetK, but not of TetA(L), was markedly reduced by the presence of K⁺. This suggested a higher K⁺ relative to Na⁺ affinity for TetK than for TetA(L) (15). The current studies support the hypothesis that net K⁺ uptake catalyzed by full-length forms of TetA(L) and TetK is a mode of the Na⁺/K⁺ antiport exchange of both proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** The *E. coli* strains used in this study are listed in Table I. The bacteria were routinely grown in Luria broth (LB) with KCl substituted for NaCl (LBK) (23) and then supplemented with NaCl as indicated. The potassium transport-deficient *E. coli* strain TRK2420 was grown on a defined medium (24) supplemented with various concentrations of KCl. The plasmid constructs containing full-length or truncated versions of tetA(L) or tetK were made by cloning polymerase chain reaction products into pGEM3Zf(+) behind the T7 promoter (Table I). Expression of such constructs in *E. coli* cells, without concomitant expression of a T7 polymerase, results in levels of expression that are sufficient for phenotypic effects of membrane transport proteins without the toxicity that most often results from greater overexpression of such genes (13). The primers for the full-length version of tetA(L) were tetF1S (GGAGGGGCCAGCATGTAATGCTCTTATTCACAGTC) and tetR1B (TCACTCATGGATCCAGGC-CAAGGT). The primers for the truncated version of tetA(L) were tetF2S and tetR2B (GGAGCTAAATTAGTCCATGATAATGACG). The primers for the truncated version of tetK were tetKF1S (CAGGTAACAGGATCCATGTTAGTTTAT) and tetKRB (AAGATA TATAAGGATCCAACTGTTCTTCAG). The primers for the truncated version of tetK were tetKB and tetKRB (GAAGTATAAGTGGTAGT- GATCCATGAAAT). The polymerase chain reaction products were blunt end ligated to pGEM3Zf(+) that had been cut with SmaI. The hexahistidine-tagged full-length version of tetA(L), pJQ2, was made as described previously (16).

**Complementation and Resistance Studies—** The various constructs were tested for their ability to enhance the growth of potassium transport-deficient *E. coli* TK2420 on various concentrations of KCl in a defined medium. Two-mL cultures were grown in 15-mL conical tubes with shaking at 37° C. They were inoculated with 10 μL of an 8-h culture (late logarithmic phase); the absorbance at 600 nm was recorded after 15 h. The concentration of KCl that allowed growth to an A₆₀₀ of at least 1.0 was defined as the minimum concentration that permitted growth. For complementation of *E. coli* strains NM81 or DH5α with various concentrations of NaCl, the bacteria containing various plasmid constructs were similarly tested on LB medium that was modified as indicated. The MIC was defined as that concentration of NaCl that stopped growth completely. The MIC of Tc was determined in a similar manner on cultures grown in LBK medium.

**Assays of Active Transport in Everted Vesicles—** Everted membrane vesicles were prepared in either 50 mM MOPS-KOH buffer or 50 mM Tris-HCl buffer, pH 7.5, as described previously (14). The transport of 50 μM [3H]Tc or 20 mM Na⁺ was performed as described (14). Controls without the energy source, NADH, were always performed to assess energy-dependent uptake. Binding controls were conducted in the presence of 5% butanol.

**Assays of Exchange in Right-side-out Vesicles—** Right-side-out membrane vesicles were prepared by the method of Kaback (25) by shocking spheroplasts in 10 mM Tris-HEPES, pH 7.5, plus 2 mM MgSO₄. Where indicated, the vesicles were passively loaded by incubation for 4 h at 20° C with various concentrations of NaCl, KCl, or choline-Cl. Exchange experiments were performed by diluting the vesicles 100-fold into buffer that contained a 100 μM final concentration of ³⁸Rb⁺/K⁺-Cl. To measure a ΔΨ, positive out, vesicles were incubated with 2 μM [³⁸H]tetraphenylphosphonium (26) and accumulation was measured by filtration onto OE67 filters (Schleicher & Schuell) that were then washed with buffer, dried, and counted by liquid scintillation. An internal vesicle volume of 2.2 μL/mg of protein (27) was used to calculate the ΔΨ from the Nernst equation.

**Fluorescence Based Assays of Transport-dependent Generation of an Electrical Potential—** The ΔΨ-dependent fluorescence of oxonol VI was used to measure the generation of a positive inside potential during exchange reactions. The assay mixture contained 10 mM Tris-HEPES, pH 7.5, plus 500 mM oxonol VI. The excitation wavelength was set at 580 nm and emission was at 631 nm (28). The change in fluorescence upon a 100-fold dilution of right-side-out membrane vesicles into the reaction tube was recorded. The final concentration of vesicles was usually 100 μg/mL unless otherwise stated. The fluorescence changes were quantitated by setting up various gradients of K⁺, outside greater than inside, adding 100 mM valinomycin, and recording the fluorescence change.

**RESULTS**

**Complementation or Resistance Properties Conferred upon *E. coli* Cells by Tet Constructs—** The salient properties conferred by the constructs were first examined in whole cells of suitable *E. coli* strains. *E. coli* TK2420 (K⁺-uptake-deficient) cells transformed with full-length and truncated tetA(L) or tetK genes were examined for complementation, i.e. a reduction in the
concentration of K⁺ required for growth relative to a vector control. The MIC for Tc was examined in wild type E. coli transformants and the MIC for NaCl was examined in both the wild type and in strain NM81 (Na⁺-sensitive). The studies of Na⁺ sensitivity were conducted both in the presence and absence of added K⁺. As shown in Table II, TetK strongly complemented the K⁺-requiring phenotype of E. coli inasmuch as the concentration of K⁺ required to reach an A₆₀₀ of 1.0 after 15 h of growth was 1.8 mM as opposed to 29 mM in the control plasmid transformant of E. coli TK2420. Tet(A(L)) also complemented significantly, albeit not as well as TetK, lowering the K⁺ concentration required to 6.5 mM. Truncated TetA(L) showed essentially no complementation while truncated TetK reduced the required K⁺ roughly by 50%. The full-length TetA(L) and TetK both strongly raised the MIC for Tc, whereas the truncated forms showed only modest positive impact upon resistance to the antibiotic. In both wild type and the NM81 strain of E. coli, only TetA(L) raised the MIC for Na⁺ significantly in LBK medium. In medium from which the added K⁺ was omitted, TetA(L), TetK, and the truncated TetK all showed some positive effect upon Na⁺ resistance.

It should be noted that in the pGEM vector used, and in the absence of the tet promoter region, the cloned genes were being expressed at low levels from the T7 promoter on the vector. It was not possible to use Western analyses to quantitate the amount of Tet protein in the different recombinant strains (or subsequent membranes therefrom) so that comparative effects may in part represent some difference in the ultimate amount of the particular protein that is actually found in the membrane. Under these conditions, none of the transformants showed a growth defect on LBK medium in the absence of high added Na⁺ concentrations as might be expected if they catalyzed a potential-dependent K⁺ leak. When the tet constructs were expressed more strongly from an inducible promoter in different plasmids, however, strong growth inhibition was observed, consistent with a generalized leakiness resulting from overexpression of a potentially toxic membrane protein (data not shown).

**Energy-dependent Tc-cobalt/H⁺ and Na⁺/H⁺ Antiport in Everted Vesicles of E. coli Expressing tet Genes**—Energized antiport activities that had previously been shown for the full-length TetA(L) and TetK proteins were assayed again for these proteins and their truncated forms. Tc-cobalt/H⁺ antiport was assayed in K-MOPS buffer. Na⁺/H⁺ antiport was assayed in this buffer as well as in Tris-Cl buffer to test the earlier suggestion that TetK might not transport Na⁺ as well as TetA(L) in the presence of elevated K⁺. Transformants of wild type E. coli were used as the starting material for vesicle preparations for these experiments. As shown in Fig. 2, TetA(L) and TetK both supported strong transport of Tc while neither truncated form did so. Similarly, as shown in Fig. 3, only the full-length forms catalyzed Na⁺/H⁺ antiport. Although not shown, a hexahistidine-tagged version of TetA(L) expressed in pJQ2, that had been used in earlier reconstitution work (16), also exhibited these activities. The efficacy of TetA(L) was not affected by the presence of K⁺, whereas the efficacy of TetK for Na⁺ transport was greatly diminished by added K⁺ consistent with the earlier indication that TetK has

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**Table I**

**Bacterial strains and plasmids used in this study**

| Bacterial strains or plasmids | Properties | Source or reference |
|------------------------------|------------|---------------------|
| E. coli DH5α MCR | F⁻ mcrAΔ(mrr-hsd, RMS-mcrBC)Δ80d lacZ ΔM15 ΔlacZYA-argF U169 deoR recA1 endA1 supE44 thi-1 gyrA96 relA1 | GI BRL |
| NM81 | metBLΔd, kanΔ41, nhaCΔ1, ΔnacZY, thrI | Padan et al. (21) |
| TK2420 | ΔkdpABC trdD1 ΔtrkA | Epstein et al. (22) |

**Table II**

**Growth characteristics of E. coli strains transformed with various constructs of tetA(L) and tet(K)**

All values are the average of at least six separate determinations ± S.D.

| Strain | Concentration of KCl Morris | MIC for tetracycline \( \mu g/ml \) |
|--------|-----------------------------|-------------------------------|
| TK2402/pGEM | 29.0 ± 3.2 | DH5α/pGEM |
| TK2420/pJG2 | 6.5 ± 1.0 | 1.0 ± 0.2 |
| TK2420/pJG3 | 1.8 ± 0.2 | 40.0 ± 3.0 |
| TK2420/pJG4 | 14.0 ± 1.7 | 27.0 ± 3.1 |
| TK2420/pJG5 | 21.8 ± 3.8 | 5.0 ± 0.5 |

| Strain | MIC for NaCl Morris | Medium LBK | Medium L |
|--------|---------------------|-----------|---------|
| NM81/pGEM | 0.54 ± 0.02 | 0.45 ± 0.03 |
| NM81/pJG2 | 0.70 ± 0.04 | 0.67 ± 0.02 |
| NM81/pJG3 | 0.68 ± 0.05 | 0.65 ± 0.04 |
| NM81/pJG4 | 0.60 ± 0.04 | 0.53 ± 0.03 |
| NM81/pJG5 | 0.53 ± 0.02 | 0.47 ± 0.02 |

**a** The minimal concentration of KCl in the growth medium permitting growth after 15 h to 1.0 or more at \( A_{600} \).

**b** MIC is the tetracycline concentration at which there is no growth after 15 h incubation.

**c** MIC for NaCl is the molar concentration of NaCl that prevents growth after 15 h in LBK or L medium.
TetK had not earlier been examined and was thus investigated (Fig. 2 are attributable to the Na
The higher background in these experiments as compared with those in
Values for uptake in the absence of the energy source were subtracted. The constructs assayed were transformants of
in the presence or absence of high K
lent cation/H
26450
a relatively higher preference for K
side-by-side with TetA(L). Everted vesicles, with K
on both sides of the membrane and energized as in the previous set of experiments, were treated either with low concentrations of nigericin (0.1 μM), valinomycin (1 μM), or both. The stimulation is consistent with nigericin minimizing Δψ-dependent loss of accumulated Tc from the energized, everted vesicles. If there is any inhibition of the antiport itself by the nigericin treatment it must be even smaller than the stimulatory relief afforded by the dampening of that Tc loss. By contrast, addition of valinomycin inhibited Tc uptake almost as much as the combination of nigericin and valinomycin which should together abolish the electrochemical proton gradient. Two different modifications of this protocol were also examined. Experiments had been conducted on the electrogenicity of the Gram-negative TetA(B) Tc-metal/H
in which nigericin was used at 2 μg/ml (19). This would be a concentration of 2.5 μM as compared with the much lower 0.1 μM used in our experiments. Since aberrant effects of nigericin, i.e., electrogenic exchange (29, 30), have been reported at unusually high ionophore concentration, such a concentration could be problematic. Although not shown, use of that concentration in experiments of the type depicted in Fig. 4, A and C, resulted in complete inhibition of transport. Also, high concentrations of chloride have been reported in assays using vesicle preparations for assessment of the capacity of an imposed diffusion potential to drive Tc-metal/H
antiport and no energization of antiport was observed (1). Since chloride is known to reduce and, in sufficient concentration, abolish a transmembrane potential across E. coli vesicle membranes (31, 32), it was possible that only an ineffectively small potential was actually generated in such experiments. As shown for the Tc-cobalt uptake mediated by TetA(L) and TetK (Fig. 4, B and D, respectively), use of buffers containing substantial added chloride for the ionophore experiments totally changed the inhibition pattern. Valinomycin no longer inhibited and nigericin (at the standard low concentration) inhibited completely, as expected if a Δψ did not exist and the ΔpH was now the sole driving force. The two ionophores were similarly examined, in both low and high chloride conditions, for their effects upon Na
H
antiport as monitored by Na
uptake (Fig. 5). In contrast with the experiments shown for Tc-cobalt uptake in Fig. 4 and for the Na
uptake experiments on TetA(L), assays of Na
uptake mediated by TetK were conducted in Tris buffers under both the low and high chloride condition. The chloride concentration used in the higher chloride condition was only sufficient to partially abolish the Δψ in the respiring vesicles because the attendant reduced K
concentrations minimized the inhibition of Na
translocation by TetK. The patterns of inhibition for both TetA(L) and TetK were the same as observed with the Tc-metal/H
antiport in the low chloride buffer, consistent with the electrogenicity of Na
H
antiport mediated by these proteins. Use of higher chloride concentrations reduced the total apparent dependence upon the Δψ, completely for TetA(L) and partially with TetK where lower chloride concentrations had been used.

86Rb
Uptake by Unenergized or Energized Right-side-out Vesicles—The monovalent cation/H
antiport mode of TetA(L) and TetK both appeared to be electrogenic and the complementation of TK2420 suggested that they could catalyze net K
uptake. Experiments were therefore developed to directly test the hypothesis that the net K
uptake was another reflection of the monovalent cation/H
exchange mode. Right-side-out membrane vesicles from E. coli TK2420 transformed with the same set of plasmids used in the experiments above were loaded with choline, KCl, or NaCl and were then diluted into Tris-HCl, MgCl2 buffer containing 100 μM 86Rb
-KCl, such that a 100-fold outwardly directed gradient of choline, KCl, or

FIG. 2. Uptake of Tc by everted vesicles of E. coli transformed by various Tet-encoding plasmids or control upon energization in the presence of cobalt. Everted membrane vesicles were prepared in 50 mM K-MOPS, pH 7.5. The vesicles were preincubated for 1 min with and without 2.5 mM K-NADH plus 100 μM CoCl2 after which 50 μM [3H]Tc was added. At various times the 50-μl reaction mixtures were filtered onto 0.22-μm GSWP (Millipore) filters with washing, twice, with 2 ml of K-MOPS. The filters were dried and the radioactivity was counted by liquid scintillation counting. In each case, for each time point, the amount of Tc taken up in the absence of an energy source was subtracted. The constructs assayed were transformants of E. coli DH5α as follows: pGEM (○); pJG2 (●); pJG3 (Δ); pJG4 (▲); or pJG5 (×).

FIG. 3. Uptake of Na
by everted vesicles of E. coli transformed by various Tet-encoding plasmids or control upon energization in the presence of high K
concentrations. The assay mixture contained 50 mM K-MOPS (open symbols) or 50 mM Tris-HCl (closed symbols), pH 7.5, plus 2.5 mM K-NADH and 20 mM 22Na
Values for uptake in the absence of the energy source were subtracted. The higher background in these experiments as compared with those in Fig. 2 are attributable to the Na
H
antiporter activity of the wild type (E. coli DH5α) strain used in these experiments, a choice made because of the stability of the tet-bearing plasmids. ○ and ●, pGEM vector; Δ and ▲, pJG2-Tet A(L); □ and ■, pJG3-Tet K; ○ and ◆, pJG4-Tet KT; ▲ and ▼, pJG5-Tet A(LT). Open, K
; closed, no K
.
NaCl was produced. The vesicles were not energized. Measurements of the transmembrane potential using tetraphenylphosphonium indicated that no potential developed at this concentration of chloride upon dilution without energization by an electron donor. Although not shown, none of the choline-loaded vesicle preparations exhibited Rb\(^{+}\) accumulation nor did the preparations from the transformant with truncated TetA(L), under any of the three conditions. As shown in Fig. 6, the preparations from the control transformant exhibited no Rb\(^{+}\) accumulation either. On the other hand, both the full-length TetA(L) and the TetK vesicles exhibited significant, transient Rb\(^{+}\) accumulation upon dilution of the NaCl or KCl-loaded vesicles, and the truncated TetK vesicles exhibited a smaller but reproducible level of Rb\(^{+}\) uptake in those conditions. Consistent with the absence of a potential, addition of SCN\(^{-}\) (to a final concentration of 500 \(\mu M\)) did not stimulate Rb\(^{+}\) uptake. Importantly, carbonyl cyanide \(p\)-chlorophenylhydrazone (to a final concentration of 10 \(\mu M\)) did not inhibit the uptake, consistent with the lack of involvement of protons in the exchange being measured.

It was important to assess whether the Rb\(^{+}\) uptake that was observed only upon dilution of K\(^{-}\) - or Na\(^{-}\) - loaded vesicles in the above experiments truly represented an electrogenic uptake. If so, a potential, positive-in, should be generated. Experiments with oxonol were conducted on K\(^{-}\) - versus choline-loaded vesicles to determine whether such a potential could be detected. The chloride content of the buffers was reduced by using 10 \(mM\) Tris-HEPES plus 2 \(mM\) KCl to load the vesicles so that any potential would not be immediately dissipated. Some chloride was retained because its complete elimination was found to impair the response of the probe. For each transformant type, K\(^{-}\) - or choline-loaded vesicles were diluted into buffer such that an outward gradient was generated (probe response designated \(F\)) or such that no gradient was generated (probe response designated \(F^*\)). Control experiments showed that although less \(^{86}\)Rb\(^{+}\) was accumulated under the gradient-producing conditions in this low chloride medium, its accumulation was easily demonstrable and remained dependent upon intravesicular K\(^{-}\) (data not shown). A lower level of accumulation was anticipated if a potential (positive-in) is allowed to develop more fully during an electrogenic exchange. The \(F/F^*\) ratios were calculated separately for the K\(^{-}\) - and choline-loaded vesicles. As shown in Table III, the response of the choline-loaded vesicles did not differ among the different transformant preparations. Moreover, every preparation elicited the same probe response whether or not a gradient was generated. By contrast, the K\(^{-}\) - loaded preparations from transformants with pJG2 and pJG3, encoding the two full-length Tet proteins, and pJG4, encoding the truncated TetK, elicited different probe responses upon generation of a gradient than its absence. That response, especially significant with the preparations in which full-length Tet proteins were expressed, was reflective of a significant potential, positive-inside, as calibrated by establishment of potentials of known magnitude. Preparations of plasmid control and pJG5 (truncated TetA(L)) preparations that were K\(^{-}\) - loaded both exhibited a \(F/F^*\) ratio slightly below

Fig. 4. The effects of nigericin and/or valinomycin on Tc uptake by everted vesicles from E. coli expressing either tetA(L) or tetK and assayed either in the presence of low or high chloride concentrations. Everted membrane vesicles were prepared in either 50 \(mM\) K-MOPS buffer, pH 7.5, or 50 \(mM\) Tris-HCl, 100 \(mM\) KCl, pH 7.5. Five-\(\mu L\) vesicles (30 mg of protein/ml) were added to reaction buffer containing 100 \(\mu M\) CoCl\(_2\) in the presence or absence of 2.5 \(mM\) K-NADH (for K-MOPS buffer) or 2.5 \(mM\) Tris-NADH (for Tris-KCl buffer) (●). To some reaction mixtures, 1.0 \(\mu M\) valinomycin (○) or 0.1 \(\mu M\) nigericin (△) were added. After 1 min of preincubation, uptake was initiated by the addition of 50 \(\mu M\) [\(^{3}H\)Tc]. The total reaction mixture of 50 \(\mu L\) was then handled as described in the legend to Fig. 2. Uptake in the absence of an energy source was subtracted for each time point. A, pJG2 in K-MOPS; B, pJG2 in Tris-KCl; C, pJG3 in K-MOPS; D, pJG3 in Tris-KCl.
unity, perhaps reflecting a small potential, negative inside upon dilution.

Choline-loaded and KCl-loaded vesicles were then examined in a different protocol to test whether energized vesicles from the same transformants, without an outwardly directed gradient of cation, exhibited a Tet-mediated, ΔΨ-dependent K⁺ leak. A leak was expected to be manifested as a potential-dependent (energization-dependent) but K⁺-independent accumulation of Rb⁺ found in any of the preparations containing Tet constructs, but absent in the plasmid control preparations. It was also of interest to examine whether energization would stimulate Rb⁺ uptake by K⁺-loaded vesicles as expected for an electrogenic antiport-dependent process. NaCl-loaded vesicles were not used because in preliminary experiments, the high level of

**FIG. 5.** The effects of nigericin and/or valinomycin on Na⁺ uptake by everted vesicles from *E. coli* expressing either tetA(L) or tetK and assayed either in the presence of low or high chloride concentrations. Everted membrane vesicles of *E. coli* DH5α expressing tetA(L) were prepared as described for the comparable preparation used in Fig. 4, in either K-MOPS or Tris-HCl buffer (○). To some reaction mixtures, 1.0 μM valinomycin (●) or 0.1 μM nigericin (▲) or both (△) were added. The vesicles were preincubated for 1 min in the presence or absence of 2.5 mM K-NADH (for K-MOPS buffer) or 2.5 mM Tris-NADH (for Tris-KCl buffer). Uptake was initiated by adding either 10 mM Na₂SO₄ (for no chloride conditions) or 20 mM NaCl (for high chloride conditions) plus 0.1 μCi of carrier-free ²²Na⁺. The vesicles expressing tetK were prepared in either 50 mM Tris-HEPES, pH 7.5, or 50 mM Tris-HCl, pH 7.5, both containing 5 mM K₂SO₄. As described above for the tetA(L) vesicles, radioactive Na⁺ was added in the presence or absence of NADH to reaction mixtures containing no additions or various additions of ionophores. Values for the uptake in the absence of the energy source were subtracted from the values obtained in its presence. A, pJG2 in K-MOPS; B, pJG2 in Tris-KCl; C, pJG3 in Tris-HEPES; C, pJG3 in Tris-HCl.

**FIG. 6.** ⁸⁶Rb⁺ uptake by unenergized right-side-out membrane vesicles from *E. coli* expressing various tet constructs in response to outwardly directed gradients of Na⁺ or K⁺. Membrane vesicles prepared in 10 mM Tris-HCl, 2 mM MgCl₂, pH 7.5, were passively loaded with 10 mM NaCl or 10 mM KCl (open symbols) or 100 μM of the same salt (closed symbols). Uptake was initiated by diluting 10 μl of the vesicles into 1 ml of Tris-MgCl₂ buffer containing a final concentration of 100 μM ⁸⁶Rb⁺-KCl. The dilution buffer for samples in which an outward cation gradient was generated (open symbols) contained no other added salt, but the dilution buffer for those samples in which no outward gradient was to be generated (closed symbols) contained NaCl or KCl at the same concentration (100 μM) as was present in the intravesicular space. At intervals, 200-μl samples were taken, filtered (on 0.45-μm HAWP filters (Millipore)), washed with 2 ml of Tris-MgCl₂ buffer, and dried for scintillation counting.
Electrogenic Exchange Modes of TetA(L) and Tet(K)

The studies conducted here confirm and extend earlier work indicating that both TetA(L) and TetK are multifunctional antiporters that catalyze electrogenic Tc-metal/H⁺ and Na⁺/H⁺ antiport. The successful demonstration of both of these activities and their electrogenericity clearly depends upon low expression levels of the proteins. Higher levels of expression make both cells and membrane leaky. For experiments in which ionophores are used to assess electrogenericity in an E. coli vesicle system, it is further important to use ionophore concentrations that avoid aberrant exchanges and to reduce the concentration of chloride sufficiently to avoid dissipation of the ΔΨ by the permeant anion alone. The totality of earlier experiments supports the conclusion by Kaneko and co-workers (19) that TetA(B) catalyzes a largely electroneutral Tc-metal/H⁺ antiport. However, these authors themselves indicate some discrepancies in their findings with the conclusion of complete electroneutrality, and the issue might merit re-examination. As discussed below, the specific catalytic properties and possible multifunctional features are important factors in the design of strategies to minimize the interference of antibiotic efflux systems with use of antimicrobial therapies.

The truncated versions of TetA(L) and TetK failed to exhibit any of the energy-dependent, proton-coupled activities of the full-length proteins, consistent with the evidence that residues in the C-terminal halves of TetK cannot be mutated without loss of active Tc efflux capacity (6). Nonetheless, there were some modest but reproducible protective effects of the truncated Tet proteins in the whole cell growth complementation experiments (Table I). Possibly the truncated forms retain the capacity to bind Tc, Tc-metal, and monovalent cations, and this accounts for those effects. Such a basis for modest complementation in similar experiments has previously been noted (33).

The current studies add a catalytic mode to the repertoire of the Gram-positive Tet proteins, i.e. a mode in which net K⁺ uptake is achieved via a full catalytic cycle in which more than one K⁺ is taken up in exchange for a single cytoplasmic Na⁺ or K⁺. Clearly, the full-length TetA(L) and TetK do not confer a leakiness upon E. coli membranes to K⁺ that allows electrogenic K⁺ entry (even down its chemical concentration gradient) in response to energization and establishment of a sizeable ΔΨ, inside-negative. The generation of a potential, inside-positive, during Na⁺/K⁺ exchange by unenergized vesicles is consistent with the operation of the whole catalytic antiport cycle but with the external Rb⁺ substituting for H⁺. Were only a partial cycle to be used for the exchange, the Rb⁺ accumulation

**DISCUSSION**

**TABLE III**

| Vessels from E. coli TK2420 | K⁺ gradient | Choline gradient |
|-----------------------------|-------------|------------------|
|                             | F⁺, ΔΨ (mV) | F⁺, ΔΨ (mV)      |
| pGEM                        | 0.91 ± 0.03 | 1.0 ± 0.02       |
| pJG2                        | 1.12 ± 0.02 | 0.91 ± 0.01      |
| pJG3                        | 1.20 ± 0.01 | 1.01 ± 0.01      |
| pJG4*                       | 1.05 ± 0.02 | 1.01 ± 0.02      |
| pJG5                        | 0.95 ± 0.03 | 1.00 ± 0.01      |

* Right-side membrane vesicles loaded with 10 mM Tris-HEPES plus 2 mM KCl or 2 mM choline Cl were diluted 100-fold into 10 mM Tris-HEPES, pH 7.5.

+ fluorescence intensity with a 100-fold K⁺ (or choline) gradient, in to out. F⁺ is fluorescence intensity in the absence of a K⁺ (or choline) gradient, K⁺ (or choline) equal in and out.

- The ΔΨ, positive in, was quantitated by measuring the fluorescence intensity increase upon addition of 100 nM valinomycin in the presence of various concentrations of K⁺ out > K⁺ in.

- 100 μg of membrane vesicle protein was used in most assays, but 200 μg of vesicle protein was used for pJG4 in order to better quantitate the signal.

**FIG. 7.** Rb⁺ uptake by energized and unenergized right-side-out membrane vesicles from E. coli expressing various tet constructs loaded with a concentration of choline or K⁺ that was the same as that in the external medium. Vesicles were loaded with either 200 μM choline-Cl (●, ○) or KCl (△, ▲) by incubating for 4 h at room temperature. To initiate uptake, 25 μl of the vesicles were diluted into 500 μl of 10 mM Tris-HCl, 2 mM MgCl₂, pH 7.5, containing a final concentration of 200 μM ³⁷Rb⁺-KCl. To half of the reaction mixtures, 10 mM Tris/λ-lactate (closed symbols) was added. 100 μl of samples were taken at various times and collected by filtration onto 0.45-μm HAWP (Millipore) filters. They were washed twice with Tris-MgCl₂ buffer, dried, and counted by liquid scintillation counting.
Another question of interest in connection with the net K⁺ porter activity and the extent to which this property occurs then even modest net Rb⁺ (35); this could reflect enhanced Na⁺ on the “fitness” of adapted genes of particular types, particularly with respect to minimizing positive selection for antibiotic efflux genes to pathogens. In assessments of those conditions in which the amount of transporter protein incorporated into the proteoliposome can be made comparable for different versions of the proteins. If Tet-mediated, electrogenic Rb⁺(K⁺) uptake depends upon the use of the H⁺-binding site and translocation pathway by these cations, and if the C-terminal part of the protein is required for proton binding and/or translocation, then even modest net Rb⁺ accumulation by truncated TetK is unanticipated under non-leaky conditions.

The finding that net K⁺ uptake by full-length Tet proteins is definitely a mode of the normal catalytic functions rather than a leak, is consistent with the robust growth of cells expressing low levels of these proteins. It is notable that TetA(L) behaved qualitatively similar to TetK although it had not earlier been implicated as having the capacity for net K⁺ uptake. As hypothesized at the start of the study, this capacity may be a correlate of possession by a Tet protein of Na⁺(K⁺)/H⁺ antiporter activity and the extent to which this property occurs broadly among Tet proteins has not been carefully examined. Another question of interest in connection with the net K⁺ uptake mode is whether it may have a physiological role, e.g. at particular pH values and/or K⁺ concentrations. It will be of importance to examine the possibility that the Gram-negative TetA(C) (e.g. from pBR322 or pACYC184) might catalyze a similar spectrum of activities to that shown here for the Gram-positive Tet proteins. TetA(C) is among the Tet proteins that can complement K⁺ uptake-deficient mutants of E. coli (8–10). Moreover, this gene has been shown to have a beneficial effect on the “fitness” of adapted E. coli in the absence of antibiotic (35); this could reflect enhanced Na⁺-resistance and K⁺ retrieval under some conditions. Whether TetK confers such a benefit on S. aureus will also be of interest to examine. Considerable current effort is directed toward reducing the prevalence and further spread of antibiotic-resistance genes among pathogenic bacteria or other organisms that might then transfer these genes to pathogens. In assessments of those conditions that will minimize positive selection for antibiotic efflux genes of particular types, e.g. tet genes, the full panoply of roles for the given efflux protein will be important information. For example, it might be important to consider the pH, Na⁺, and K⁺ concentration to which the organisms are exposed, rather than simply the exposure to Tc, when evaluating strategies for decreasing the prevalence of TetA(L) or TetK.

REFERENCES

1. Yamaguchi, A., Ohmori, H., Kaneko-Phadera, M., Nomura, T., and Sawai, T. (1991) Antimicrob. Agents Chemother. 35, 53–56
2. Yamaguchi, A., Udagawa, T., and Sawai, T. (1990) J. Biol. Chem. 265, 4809–4813
3. Yamaguchi, A., Shinya, Y., Fujihira, E., Sawai, T., Noguchi, N., and Sasatsu, M. (1995) FEBS Lett. 365, 183–187
4. Levy, S. B. (1992) Antimicrob. Agents Chemother. 21, 1–3
5. Paulsen, I. T., and Skurray, R. A. (1993) Gene (Amst.) 124, 1–11
6. Fujihira, E., Kimura, T., and Yamaguchi, A. (1997) FEBS Lett. 419, 211–214
7. Yamaguchi, A., Akasaka, T., Ono, N., Soneya, Y., Kakatani, M., and Sawai, T. (1992) J. Biol. Chem. 267, 7490–7498
8. Dusché, C. D., Salvacion, F. F., and Epstein, W. (1984) J. Bacteriol. 160, 1188–1190
9. Griffith, J. K., Cuellar, D. H., Fordyce, C. A., Hutchings, K. C., and Monodragons, A. A. (1994) Mol. Membr. Biol. 11, 271–277
10. Griffith, J. K., Kogoma, T., Corvo, D. L., Anderson, W. L., and Kazim, A. L. (1988) J. Bacteriol. 170, 598–604
11. Guay, G. G., Tuckman, M., McNicholas, P., and Rothstein, D. M. (1993) J. Bacteriol. 175, 4927–4929
12. Nakamura, T., Matsuura, Y., Ishihara, A., Kitagawa, T., Suzuki, F., and Unemoto, T. (1995) Biol. Pharm. Bull. 18, 1189–1193
13. Cheng, J., Guffanti, A. A., and Krulwich, T. A. (1994) J. Bacteriol. 269, 27365–27371
14. Guffanti, A. A., and Krulwich, T. A. (1995) J. Bacteriol. 177, 4557–4561
15. Cheng, J., Guffanti, A. A., Wang, W., Krulwich, T. A., and Bechhofer, D. H. (1996) J. Bacteriol. 178, 2853–2880
16. Cheng, J., Hicks, D. B., and Krulwich, T. A. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 14446–14451
17. Cheng, J., Baldwin, K., Guffanti, A. A., and Krulwich, T. A. (1996) Antimicrob. Agents Chemother. 40, 852–857
18. McNab, R. M., and Castle, A. M. (1987) Biophys. J. 52, 637–647
19. Kaneko, M., Yamaguchi, A., and Sawai, T. (1985) FEBS Lett. 193, 184–198
20. Hirata, T., Wakatabe, R., Matsuura, T., Nakamura, T., Soneya, Y., Fujihira, E., Kimura, T., and Yamaguchi, A. (1997) FEBS Lett. 412, 317–340
21. Padan, E., Maisler, N., Taglicht, D., Karpel, R., and Schuldiner, S. (1989) J. Biol. Chem. 264, 20287–20302
22. Epstein, W., Bourne, E., Leggatt, D., and Naprestek, J. (1993) Biochem. Soc. Trans. 21, 1006–1010
23. Goldberg, E. B., Arbel, T., Chen, J., Karpel, R., Mackie, G. A., Schuldiner, S., and Padan, E. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2615–2619
24. Epstein, W., and Kim, B. S. (1971) J. Bacteriol. 106, 639–644
25. Kabaš, H. R. (1971) Methods Enzymol. 22, 99–120
26. Schuldinier, S., and Kabaš, H. R. (1974) Biochemistry 13, 4541–4547
27. Kabaš, H. R., and Barnes, E. M., Jr. (1971) J. Biol. Chem. 246, 5523–5531
28. Waggner, A. S. (1979) Annu. Rev. Biophys. Bioeng. 8, 47–68
29. Johli, L. K., Doyle, R. J., and Streep, U. N. (1961) Cell 15, 753–763
30. Harrington, C. R., and Baddiley, J. (1964) J. Bacteriol. 89, 295–303
31. Nakamura, T., Hsu, C.-M., and Rosen, B. P. (1986) J. Bacteriol. 164, 193–197
32. McMurray, L. M., Stephan, M., and Levy, S. B. (1992) J. Bacteriol. 174, 6284–6297
33. Ivey, D. M., Guffanti, A. A., Shen, Z., Kudyan, N., and Krulwich, T. A. (1992) J. Bacteriol. 174, 4878–4884
34. Keyens, R. R., and Rupinov, G. (1982) J. Biol. Chem. 257, 1172–1176
35. Lenski, R. E., Simpson, S. C., and Nguyen, T. T. (1994) J. Bacteriol. 176, 3140–3147