Tobramycin Uptake in *Escherichia coli* Is Driven by Either Electrical Potential or ATP

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Aminoglycoside antibiotics such as streptomycin and tobramycin must traverse the bacterial cytoplasmic membrane prior to initiating lethal effects. Previous data on *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* have demonstrated that transport of aminoglycosides is regulated by ΔΨ, the electrical component of the proton motive force. However, several laboratories have observed that growth of bacterial cells can occur in the apparent absence of ΔΨ, and we wished to confirm that these studies with *E. coli* and further investigate whether transport of aminoglycosides could occur in the absence of a membrane potential. Treatment of acrA strain CL2 with the protonophore carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) dissipated ΔΨ, decreased intracellular ATP levels, and resulted in cessation of growth; after a variable period of time (3 to 7 h), growth resumed, ultimately achieving growth rates comparable to those of untreated cells. Absence of ΔΨ in these cells was confirmed by absence of [3H]tobramycin uptake as measured by membrane filtration, lack of flagellar motion, and inability of these cells to transport proline (but not methionine). Regrowth was associated with restoration of normal intracellular ATP as measured by luciferase-bioluminescence assay. Unlike unacclimatized CL2 cells treated with CCCP, these cells transported [3H]tobramycin similarly to untreated cells; aminoglycoside-induced killing was seen in association with transport. These studies suggest that under certain circumstances aminoglycoside transport can be driven by ATP (or other high-energy activated phosphate donors) alone, in the absence of a measurable ΔΨ. *uncBC* mutants of CL2 incapable of interconverting ΔΨ and ATP were treated with CCCP, resulting in dissipation of ΔΨ but no alteration in ATP content. Despite maintenance of normal ATP, there was no transport of [3H]tobramycin, confirming that under normal growth conditions ATP has no role in the transport of aminoglycosides.

Facultative bacteria can actively transport low-molecular-weight substrates across the bacterial cytoplasmic membrane. The proximate energy for these transport processes is most often either ATP or the electrochemical proton gradient. The electrochemical proton gradient (proton motive force, ΔμH+) is expressed by the equation ΔμH+ = ΔΨZ + ΔpH, where ΔΨ and ΔpH are the electrical and chemical components of ΔμH+ (Z = 61.5 at 37°C). The active transport of some substrates, such as the amino acids proline and lysine, is under certain circumstances directly proportional to the magnitude of ΔμH+ or one of its components (ΔΨ alone or ΔpH alone) (1, 5, 20). The transport of other substrates, such as the amino acids methionine and leucine, requires only ATP and is independent of ΔμH+ (22).

The members of the aminoglycoside class of antibiotics are small cationic molecules that must traverse the bacterial cytoplasmic membrane prior to interacting with the ribosome and initiating lethal events. Data from our laboratory on *Staphylococcus aureus* have demonstrated that the transport of aminoglycosides, as well as aminoglycoside-induced killing, is regulated by the proton motive force. More specifically, uptake is dependent on ΔΨ, the electrical component of the proton motive force, and is also gated; i.e., a minimum threshold of energy is required for the induction of transport (12, 33). These observations, which have been confirmed by other investigators with a variety of bacterial strains, including *Escherichia coli*, *Bacillus subtilis*, and *S. aureus*, are consistent with Mitchell’s chemiosmotic hypothesis (4, 9, 16). This hypothesis predicts that the transport of cations is driven by ΔΨ alone, which is interior negative (36, 37). However, there are little data on whether or not ΔΨ has a thermodynamic or regulatory role in aminoglycoside transport. In addition, intracellular ATP levels and ΔμH+ are directly coupled and interconvertible via the bidirectional proton-translocating ATPase enzyme complex (H+-ATPase complex) so that perturbations of one energy mode will frequently affect the other. For example, protonophores, chemicals which dissipate ΔμH+, will also decrease ATP levels because of reverse proton flow through the H+-ATPase complex. Although many experimental manipulations used in previous studies of aminoglycoside transport would not be expected to alter intracellular ATP content, ATP levels were not directly measured, and the importance of ATP as an independent factor in the energy-dependent transport of aminoglycosides is unknown.

Several laboratories have observed that growth of bacterial cells can occur in the apparent absence of ΔΨ (27, 19). In these studies, chemicals known to dissipate ΔΨ were added to the media of *E. coli* or *Enterococcus faecalis* cells during logarithmic growth; initially there was cessation of growth,
but after a static period growth resumed. We have confirmed these observations with *E. coli* and noted that cells growing in the presence of the protrophore carbonyl cyanide-m-chlorophenylhydrazone (CCCP) were killed when exposed to aminoglycoside antibiotics. This suggested that aminoglycoside transport may not require membrane potential and that, under different environmental conditions, transport could be driven either by ΔΨ or an alternate energy mode, most likely intracellular ATP. Although the process by which aminoglycosides are transported into cells is not known to be a component of normal cell growth, we nevertheless thought that this process might serve as a model for other vital cellular transport processes presumed to be dependent on ΔΨ alone. These processes must be able to function efficiently via an alternate bioenergetic mode for growth to continue when the membrane potential is perturbed. To investigate this phenomenon further, we examined aminoglycoside transport in a whole-cell system, utilizing *acrA* mutants of *E. coli* in which energetic parameters could be readily manipulated and quantified (8, 20).

### MATERIALS AND METHODS

**Growth and preparation of cells.** See Table 1 for characteristics and derivation of strains used in this study. Cells were grown overnight to early logarithmic phase in modified LBM medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 5 g of glucose per liter of water) or M9 minimal medium containing 0.5% glucose or 0.5% succinate as a carbon source at 37°C in a gyratory shaker bath (New Brunswick Scientific model G76). For most experiments, medium pH was maintained at 7.5 by additional buffers, either 50 mM potassium phosphate (pH 7.5) or 100 mM Tris hydrochloride (pH 7.5).

**Viability assays.** Growth was monitored by optical density determinations on a Coleman spectrophotometer (model 6-20A) at 600 nm. Growth rates or cell viability or both were also determined with and without antibiotics by standard pour plate techniques, using LBM agar (LBM medium plus 1.5% agar).

**Determination of ΔΨ.** The determination of ΔΨ (interior negative) was performed by measuring the distribution of the lipophilic cation [3H]tetraphenylphosphonium (TPP+) as described previously (12). In these studies we used TPP+ (specific activity, 26 Ci/mmol; 962 GBq/mmol) at a final concentration of 20 μM in 1-ml aliquots of log-phase cells in M9 or LBM medium, pH 7.5, at 37°C. When noted, cells were washed and resuspended in 50 mM potassium phosphate buffer, pH 7.5, prior to determination of TPP+.

### Table 1. *E. coli* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Genetic marker(s)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL2</td>
<td>HfrH</td>
<td>galE28 thi-1 relAI rpsL acrA</td>
<td>W. Coleman (8)</td>
</tr>
<tr>
<td>PL2</td>
<td>HfrH</td>
<td>galE28 thi-1 relAI rpsL</td>
<td>W. Coleman (8)</td>
</tr>
<tr>
<td>ASI</td>
<td>F-</td>
<td>acrA</td>
<td>Y. Imae (20)</td>
</tr>
<tr>
<td>CK1801</td>
<td>F-</td>
<td>ΔlacY169 araD139 thi-1 rpsL relAI Δ(uuncB-uncC)</td>
<td>C. Kuromato (7)</td>
</tr>
<tr>
<td>KD119a</td>
<td>F-</td>
<td>gyrB inu:Tn10</td>
<td>K. Drlica</td>
</tr>
<tr>
<td>HF1</td>
<td>F-</td>
<td>As CK1801 but tua:Tn10</td>
<td>This studya</td>
</tr>
<tr>
<td>HF2</td>
<td>HfrH</td>
<td>As CL2 but also ΔuncBC tua:Tn10</td>
<td>This studyb</td>
</tr>
</tbody>
</table>

a P1 · KD119a × CK1801, selection for Tc, gyrB+, inability to grow on minimal medium with succinate as sole carbon source.

b P1 · HFI × CL2, selection for Tc, inability to grow in minimal medium with succinate as sole carbon source.

Samples were filtered through glass microfiber filters (Whatman GF/C) and washed twice with 4 volumes of 0.1 M lithium chloride. Concentration gradients were calculated by using a value of 5.85 × 10^-6 M of intracellular fluid potassium of cell protein (42). Protein content was measured as described by Lowry et al., with crystalline bovine serum albumin as the standard (32). ΔΨ was calculated from the Nerst equation (ΔΨ = 61.5 log [TPP+]m/[TPP+]om) from steady-state distribution values obtained during [3H]TPP+ uptake experiments. All experiments were done at pH 7.5, where ΔΨ = 0 and ΔμT = ΔΨ, as derived from the equation given above.

**ATP determinations.** ATP levels were determined by a modification of the luciferin-luciferase bioluminescence assay (26) on cells grown in M9 medium, using prepared standard reagents on a Moonlight 500 luminometer (Analytical Luminescence Laboratory, Inc., San Diego, Calif.). Samples were left at 4°C prior to determination of ATP levels. For experiments done in LBM, cells were washed and immediately resuspended in 50 mM potassium phosphate buffer (with or without CCCP), pH 7.5. Measurements were done on 25-μl aliquots of whole cells (diluted in M9 medium or buffer when necessary) after standard ATP curves were generated with standards containing known amounts of ATP in M9 or potassium phosphate buffer. Standards were prepared with or without addition of CCCP to ensure that presence of CCCP in the reaction mixture had no effect on measurements of ATP levels. All samples were measured at least in triplicate.

**Flagellar motion assay.** The presence or absence of flagellar motility was inferred from the presence or absence of cell movement observed by phase-contrast microscopy of viable bacterial cells, using a Nikon Fluophot phase-contrast microscope (Microphot V series, Japan).

**Transport assays.** For [3H]tobramycin uptake, filtration assays were carried out in logarithmic-phase cells as described previously (35), using [3H]tobramycin (specific activity, 4.9 Ci/mmol; 181 GBq/mmol), except that glass microfiber filters (Whatman GF/C) were used and 2-ml filtered samples were washed twice with 1 volume of 3% sodium chloride.

For proline and methionine uptake, filtration assays were performed on logarithmic-phase cells which were washed and resuspended in 50 mM potassium phosphate buffer, pH 7.5 (with or without CCCP). Cells were then chlormycetin (100 μg/ml final concentration) 3 min prior to addition of either [3H]proline (specific activity, 31 Ci/mmol; 1.15 TBq/mmol; final concentration, 1 μM) or [3H]methionine (specific activity, 85 Ci/mmol; 3.15 TBq/mmol; final concentration, 1 μM). One-milliliter samples were removed at the indicated times, filtered through Whatman GF/F filters, and washed with 2 volumes of 0.1 M lithium chloride.

**Materials.** [3H]TPP+, [3H]tobramycin, [3H]proline, and [3H]methionine were purchased from Amer sham Searle. ATP and CCCP were purchased from Sigma Chemical Co. Unlabeled tobramycin (potency, 975 μg/mg) was purchased from Eli Lilly. Unlabeled tetraphenylphosphonium chloride was purchased from ICN Pharmaceuticals. Reagents for the luciferin-luciferase bioluminescence assay were obtained through Analytical Luminescence Laboratory, Inc.

**RESULTS**

We used *acrA* mutants of *E. coli* K-12 (Table 1) to study the bioenergetics of aminoglycoside transport since these strains are defective in their outer membrane barrier function and demonstrate increased susceptibility to many sub-
strain CL2; addition of EDTA did not further stimulate uptake of TPP⁺ in strain CL2 (data not shown). Treatment of strain CL2 with the protonophore CCCP resulted in dissipation of ΔΨ in a dose-dependent manner (Fig. 1, inset). Concentrations of CCCP of >8 μM eliminated ΔΨ, as calculated from [³H]TPP⁺ uptake, in these cells.

Next, we examined growth as monitored turbidimetrically in exponential-phase CL2 cultures treated with concentrations of CCCP shown previously to dissipate ΔΨ (final concentration, 16 or 32 μM). After an initial period of approximately 15 to 50 min, there was no further increase in cell mass in continuously aerated broth cultures, as demonstrated by the optical density plateau. After a variable period of time, generally 3 to 7 h, cell growth resumed, as evidenced by both increase in culture turbidity and increase in CFU as measured by standard pour plate techniques. On microscopic examination, these cells demonstrated normal bacterial morphology. Ultimately, after prolonged exposure to CCCP, growth rates of CCCP-treated cells became comparable to that of untreated cells.

Figure 2A shows a representative experiment with acrA strain CL2 which was exposed to 16 μM CCCP. For discussion purposes, we have defined five phases of growth, I through V, in these cells. Phase I is the phase of exponential growth prior to the addition of CCCP; phase II is the brief initial period after CCCP addition, during which there is a transition and decline in the rate of increase in culture turbidity; phase III is the phase of growth; phase IV is the phase of early resumption of growth; and phase V occurs when rates of growth for CCCP-treated cells are equivalent to those of untreated (phase I) cells. Figure 2A (inset) demonstrates the equivalent growth rates of phase I and phase V cells (passaged for >5,000 cell doublings in the presence of 16 μM CCCP). These studies were also repeated in strain AS1, another acrA mutant (20), and similar growth curves were obtained at CCCP concentrations of 30 to 50 μM (data not shown).

The observation that growth resumed after a plateau phase in concentrations of CCCP known to dissipate ΔΨ suggested that the energy requirements for all growth-related transport processes did not absolutely include an electrochemical proton gradient. Importantly, ΔΨ and ΔpH are interconvertible through the proton-translocating ATPase complex, and treatment of cells with CCCP might also be expected to indirectly affect (i.e., decrease) intracellular ATP levels. We therefore measured both ΔΨ (since ΔpH = ΔΨ at pH 7.5) and ATP levels during the various phases of growth to characterize better the total cellular bioenergetics of our system. Figure 2B shows relative levels of ΔΨ (as determined by TPP⁺ uptake) and intracellular ATP (as measured by bioluminiscence assay) in strain CL2 during growth phases I, II, and V in enriched medium (LBM plus 0.5% glucose, pH 7.5). Treatment with CCCP (phase II) is associated with dissipation of ΔΨ as well as a decrease in ATP content to 50% of normal cell base-line values in these experiments. Data were similar for cells grown in minimal medium containing glucose (M9 plus 0.5% glucose, pH 7.5), but when succinate was used as the sole carbon source (M9 plus 0.5% succinate, pH 7.5), ATP levels fell to <10% of normal cell base-line values (data not shown). ΔΨ and ATP levels throughout phase III were similar to those measured in phase II (data not shown). During regrowth (phases IV and V), ΔΨ remains unmeasurable, but intracellular ATP levels have returned to base line, suggesting that phase V growth is associated with restoration of intracellular ATP.

We next undertook a series of experiments to exclude...
ATP can drive tobramycin uptake in E. coli.

All samples were measured at least in triplicate.

ATP concentrations were determined by a modification of the luminescence method (16). ATP standards were prepared in 5 mM Tris-HCl (pH 7.5) and analyzed as described in the text.

FIG. 2. X (a) Representative experimental data demonstrating the effects of 16 mM CCP (which disperses A') on cell growth as measured by optical density in modified LB medium. A, phase II: cells were harvested at the growth condition described above (b) Measurements of A'. ATP levels during phases I, II, and stationary phase. (c) Cells under the growth condition described above (d) Measurements of A'. ATP levels during phases I, II, and stationary phase. (e) Cells under the growth condition described above (f) Measurements of A'. ATP levels during phases I, II, and stationary phase. (g) Measurements of A'. ATP levels during phases I, II, and stationary phase.
rigorously the possibility that the apparent absence of $\Delta \Psi$ in phase II through V cells was simply due to experimental artifact or inability to measure true values of $\Delta \Psi$ in these cells with the TPP$^+$ probe. First, we directly examined whether or not CCCP-treated cells were motile at pH 7.5 (where $\Delta \mu_{H^+} = \Delta \Psi$), using phase-contrast microscopy. The flagellar motor is known to be $\Delta \mu_{H^+}$-dependent and is gated at $\sim$-30 mV (25). Swimming motion was observed in phase I but not phase V cells, implying that $\Delta \Psi$ in phase V cells was below the levels necessary for flagellar motion (i.e., $> -30$ mV). In addition, we measured uptake of the amino acid proline in phase I and phase V cells. In E. coli, there are at least two mechanisms of proline uptake, one driven by the sodium electrochemical gradient ($\Delta \mu_{Na^+}$) and the other driven by the proton electrochemical gradient (5, 21). However, in E. coli $\Delta \mu_{Na^+}$ is generated by Na$^+$/$H^+$ antiport, and as a result both proline transport systems require $\Delta \mu_{H^+}$. Phase V cells, unlike phase I cells, did not take up proline (Fig. 3A). However, when phase V cells were then resuspended in CCCP-free potassium phosphate buffer, there was uptake of proline comparable to that demonstrated in phase I cells. For comparison, the uptake of methionine in phase I and V cells is also shown (Fig. 3B). Methionine is known to be transported via an ATP-dependent, osmotically shockable carrier system (22). Equivalent uptake of methionine was seen in phase I and V cells. Ability to transport methionine but not proline during phase V supports the conclusion that these cells lack a membrane potential but maintain normal levels of ATP. The reestablishment of proline transport in phase V cells resuspended in CCCP-free buffer suggests that, in the absence of protonophores, phase V cells regenerate $\Delta \mu_{H^+}$ from ATP stores via an intact H$^+$-ATPase complex.

To ensure that CCCP was not "lost" from our experimental system by breakdown or adherence to glass, a bioassay for CCCP was performed and confirmed that CCCP was still present in the medium of phase V cells. When phase V cells were separated from their medium by filtration and resuspended in fresh CCCP-containing medium, they continued to grow without an alteration in growth rate, while the filtrate completely inhibited growth when added to fresh phase I cultures. Finally, to ensure that phase V cells do not develop resistance to CCCP or the TPP$^+$ probe by restoration of an outer membrane barrier analogous to that seen in wild-type (acrA$^+$) cells or in recently described "uncoupler resistant" mutants (30, 41), we performed additional control experiments. In these studies, phase I and V cells were washed and resuspended in either 50 mM potassium phosphate buffer, pH 7.5, or buffer containing 16 mM CCCP, and $[^3H]TPP^+$ uptake was then measured (Fig. 3C). As anticipated from the proline uptake experiments (Fig. 3A), phase I cells resuspended in buffer exhibited uptake of TPP$^+$ comparable to that of cells in LBM. However, phase I, II, or V cells resuspended in buffer containing CCCP exhibited no uptake of TPP$^+$, even in the presence of 5 mM EDTA. When phase V cells were resuspended in CCCP-free buffer, TPP$^+$ uptake occurred almost immediately and was comparable to that of cells never exposed to CCCP, presumably by restoration of $\Delta \Psi$ at the expense of ATP via an intact H$^+$-ATPase. Phase II cells also exhibited TPP$^+$ uptake when resuspended in
CCCP-free buffer, but this was significantly less than the uptake exhibited by the phase V cells, perhaps because of the decreased ATP levels of phase II cells relative to phase V cells (Fig. 2B). In all cases, subsequent addition of CCCP to the buffer immediately reabolished TPP$^+$ uptake. The instantaneous ability to affect TPP$^+$ uptake by addition or removal of CCCP excludes the possibility that phase V growth is due to a phenotypic alteration of outer membrane function.

Having sufficiently characterized and validated the bioenergetic characteristics of our model system, we then examined the relationship between cellular energy and aminoglycoside uptake. Figure 4 demonstrates the comparison of uptake of $[^3$H]tobramycin as determined by membrane filtration in phase I, II, and V CL2 cells. Uptake occurred in both phases I and V, but not in phase II cells. Aminoglycoside-induced killing was seen in association with uptake during phases I and V, but was abolished during phase II, confirming that measured counts represent functional aminoglycoside uptake and not merely binding to the bacterial cell envelope. These studies also suggest that energy-dependent aminoglycoside uptake in phase V is driven by ATP (or another high-energy activated phosphate donor), since $\Delta\Psi$ is zero.

The unique role of ATP in phase V aminoglycoside transport was further demonstrated by treatment of phase V cells with increasing concentrations of arsenate. When treated with concentrations of arsenate which decrease intracellular ATP (range, 15 to 100% of controls) without altering $\Delta\Psi$ (28), aminoglycoside-induced killing was reduced in a dose-dependent manner and was proportional ($r = 0.98$) to the levels of intracellular ATP (data not shown). Arsenate treatment had no effect on aminoglycoside-induced killing in phase I cells, suggesting that phase I uptake is ATP independent.

To characterize further the energy requirements of uptake in normally growing (phase I) cells, we used a strain carrying the $\Delta$uncBC mutation (7). This class of mutants is deficient in all proteins of the proton-translocating ATPase complex and is unable to hydrolyze ATP in response to treatment with the protonophore CCCP (3). In these cells, $\Delta\Psi$ can be selectively dissipated without decreasing intracellular ATP levels. Figure 5A demonstrates the comparison of $[^3$H]tobramycin uptake in HF2 (a $\Delta$uncBC derivative of CL2) before and immediately after the addition of 8 $\mu$M CCCP (phases I and II). Similar data were also obtained at higher (16 $\mu$M) concentrations of CCCP (data not shown). Figure 5B shows the relative levels of ATP and $\Delta\Psi$ in strain HF2 and its parent strain CL2 during phase I (no CCCP) or II (plus 8 $\mu$M CCCP). Despite maintenance of normal intracellular ATP levels in strain HF2 during phase II, there was no uptake of $[^3$H]tobramycin. Killing was also abolished in these cells (data not shown), further confirming previous studies with arsenate and suggesting that ATP has no role in phase I aminoglycoside uptake or killing. Like the parent strain CL2, HF2 cells will ultimately regrow in the presence of CCCP (phase V) and will then transport aminoglycosides in an ATP-dependent fashion (data not shown). These data also suggest that regrowth is not associated with an alteration in the $H^+-$ATPase complex described in one class of uncoupler-resistant mutants (30).

**DISCUSSION**

This work enlarges on previous studies from our laboratory and others demonstrating a regulatory role for $\Delta\Psi$ in the transport of aminoglycosides across the bacterial cytoplasmic membrane (4, 9, 12, 16, 33). Furthermore, we have confirmed previous studies by others suggesting that continued cell growth can occur in the absence of an electrochemical proton gradient (19, 27). By careful quantification of cellular energy metabolism, including both $\Delta\Psi$ and ATP, we have confirmed that, in normally growing cells, treatment with CCCP (which dissipates $\Delta\Psi$) blocks aminoglycoside transport, even in the presence of normal cellular ATP levels. However, we have also demonstrated that, after a period of adaptation, cells are able to grow in the presence of CCCP and in the apparent absence of a membrane potential (phase V growth). In a rigorously characterized system, these cells can be grown at rates equivalent to that of untreated (phase I) cells in glucose- but not succinate-containing medium and maintain normal cellular ATP levels despite the absence of $\Delta\Psi$. In addition, these cells transport aminoglycosides in proportion to their ATP content and not in response to $\Delta\Psi$.

Many previous studies have corroborated our data suggesting a role for $\Delta\Psi$ as one of several independent factors which regulate the energy-dependent transport of aminoglycosides in normally growing bacteria (4, 9, 12, 33). In most of these studies the role of ATP was not specifically addressed, but more recent data from Goss et al., as well as the data here, confirm that transport is generally not influenced by the levels of intracellular ATP (17). Although the bioenergetics of aminoglycoside transport have been fairly well characterized, neither the function of energy (thermodynamic, regulatory) nor the mechanism by which aminoglycosides penetrate the cytoplasmic membrane has been elucidated (10, 43). The existence of an ATP-dependent transport system (phase V transport) that transports aminoglycosides in a manner similar to transport observed during normal growth conditions suggests that the role of energy,
either ATP or $\Delta \Psi$, is similar in phase I or phase V cells. Other factors, such as growth rate, have been shown to affect aminoglycoside transport independently of $\Delta \Psi$ (29, 38, 39), but there were no apparent differences in growth rate between phase I and V cells during logarithmic growth.

That cells can grow in the absence of a measurable membrane potential suggests that many critical cellular endergonic processes thought previously to be completely or partially dependent on $\Delta \mu_{\text{H}^+}$ ($\Delta \Psi$ and/or $\Delta \text{pH}$) can also be efficiently driven by ATP alone after a period of adaptation. While transport of aminoglycosides is not a crucial cellular function, it may serve as a useful model for other physiologic events that under normal growth conditions are independent of ATP. We have also used our model system to study protein export, another cellular transport process about which there are conflicting in vivo and in vitro data regarding the roles of ATP and membrane potential (3, 6). Preliminary results indicate that efficient protein transport does occur in the absence of a membrane potential (13a). Although treatment of cells with CCCP is not a physiologic event, a decrease in the magnitude of $\Delta \mu_{\text{H}^+}$ or $\Delta \text{pH}$ does occur in association with natural environmental conditions which bacteria may encounter, including stationary-phase growth (23, 24), anaerobic growth (23, 24, 34), or low pH (3, 33, 34, 40). We believe that the ability to switch energy modes may represent a general adaptive response to such hostile environments.

One potential explanation for the phenomenology of phase V growth would be development of resistance to the uncoupling agent CCCP. Uncoupler-resistant mutants have been described in both E. coli and Bacillus species (11, 18, 30, 41). At least some of the E. coli mutants described are thought to result in exclusion of uncoupling agents from the energized cytoplasmic membrane via alterations in outer membrane barrier function (30, 41); in Bacillus spp., there are also mutations that remain sensitive to uncoupling agents but whose membranes are somehow altered to function at reduced (but still measurable) levels of membrane potential (11, 18). The relatively short (i.e., 3- to 7-h) period required for resumption of growth in CL2 cells treated with CCCP suggests that mutation is not the mechanism of phase V growth. Phenotypically, phase V cells show no evidence of enhanced outer membrane barrier function when compared with untreated CL2 cells (Fig. 3B); also, the ability to transport proline should be maintained because of the persistence of an intact membrane potential. In addition, measurable $\Delta \mu_{\text{H}^+}$ in our cells was much less (no flagellar motion, no measurable TPP$^+$ or proline uptake) than even the minimum levels required by the membrane-altered Bacillus mutants that have been reported (30). Even were the membrane potential in phase V cells to derive from an electrochemical gradient other than a gradient of protons (for example, Na$^+$), the effective gradient should still be measurable by either the TPP$^+$ probe or ability to transport proline (2, 5). Thus, it is quite unlikely that any type of electrical or chemical membrane potential can account for the transport of aminoglycosides in phase V cells.

While the mechanism of aminoglycoside transport in phase V cells has not been thoroughly investigated, it is clearly associated with the restoration of intracellular ATP levels. When UncBC mutants are treated with CCCP, ATP levels do not fall. Nevertheless, they undergo an identical phase of growth cessation prior to entering phases IV and V. The nature of the adaptive response associated with a fall in $\Delta \mu_{\text{H}^+}$ has not been explored. However, preliminary experiments in which phase III cells were pulsed with chloram-
phenicol and subsequently washed and resuspended in CCP-containing medium show that the delay in onset of growth seen in phases IV and V is proportional to the duration of the chloramphenicol pulse. This suggests that induction of protein synthesis is required for adaptation and regrowth in the absence of ΔψH⁺. Since many events required for rapid growth were thought to be ΔψH⁺-dependent, it is highly unlikely that the induction process represents a novel aminoglycoside transport system. More likely, the proteins involved in both phase V growth and phase V aminoglycoside transport would be part of a global energy regulatory process that is induced by a fall in membrane potential. This could be a new, as yet uncharacterized global response system or a component of one of the other previously described stress response systems known to be operative in bacterial cells (15).

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