GerN, an Endospore Germination Protein of *Bacillus cereus*,
Is an Na⁺/H⁺-K⁺ Antiporter

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GerN, a *Bacillus cereus* spore germination protein, exhibits homology to a widely distributed group of putative cation transporters or channel proteins. GerN complemented the Na⁺-sensitive phenotype of an *Escherichia coli* mutant that is deficient in Na⁺/H⁺ antiport activity (strain K14B). GerN also reduced the concentration of K⁺ required to support growth of an *E. coli* mutant deficient in K⁺ uptake (strain TK2420). In a fluorescence-based assay of everted *E. coli* K14B membrane vesicles, GerN exhibited robust Na⁺/H⁺ antiport activity, with a *Km* for Na⁺ estimated at 1.5 mM at pH 8.0 and 25 mM at pH 7.0. Li⁺, but not K⁺, served as a substrate. GerN-mediated Na⁺/H⁺ antiport was further demonstrated in everted vesicles as energy-dependent accumulation of 22Na⁺. GerN also used K⁺ as a coupling ion without completely replacing H⁺, as indicated by partial inhibition by K⁺ of H⁺ uptake into right-side-out vesicles loaded with Na⁺. K⁺ translocation as part of the antiport was supported by the stimulatory effect of intravesicular K⁺ on 22Na⁺ uptake by everted vesicles and the dependence of GerN-mediated 86Rb⁺ efflux on the presence of Na⁺ in *trans*. The inhibitory patterns of protonophore and thiocyanate were most consistent with an electrogenic Na⁺/H⁺-K⁺ antiport. GerN-mediated Na⁺/H⁺-K⁺ antiport was much more rapid than GerN-mediated Na⁺/H⁺ antiport.

Endospore germination completes the developmental program of *Bacillus* species, which supports the formation of a dormant, stress-resistant spore under conditions of nutrient deprivation and then allows the emergence of a vegetative cell upon appropriate signaling, e.g., by specific nutrients (10, 25, 26). Features of many of the ger genes that are required for optimal germination of *Bacillus* spores suggest that they are receptors for particular nutrient germinants and/or transport proteins (18, 19). Transporters are likely to be centrally involved in endospore germination, since there are significant outward fluxes of Na⁺, K⁺, and H⁺, as well as the subsequent reuptake of K⁺ in early stages of germination (27). Recently, a new spore germination gene that is required for inosine-dependent germination of *Bacillus cereus* spores has been identified and designated gerN (30). The deduced product of *gerN*, like a previously reported spore germination gene, *grmA*, from *Bacillus megaterium* (29), is a member of the CPA-2 monovalent cation-proton antiporter family of membrane transport proteins (23). This large family of cation transporters contains a putative iron transport protein, MagA, from *Magnetospirillum* sp. (20), Kef(C) and Kef(B) proteins, which are K⁺ efflux systems activated by glutathione adducts with electrophilic compounds (3, 7), and NapA, an Na⁺/H⁺ antiporter that has a role in Na⁺ resistance in *Enterococcus hirae* (33). Among these three types of proteins within the CPA-2 family, both GerN and GrmA most closely resemble NapA, to which they are, respectively, 43 and 48% identical and 67 and 71% similar in deduced amino acid sequence by BLAST (1) analysis. The possibility that NapA-like antiporters are involved in *Bacillus* spore germination and may be associated with receptors for specific nutrient germinants has been suggested (30). However, the actual catalytic activities of GerN and GrmA have not been documented. In the present study, two different mutants of *Escherichia coli* were first transformed with a plasmid that expressed *B. cereus* gerN and then used in complementation and membrane transport assays to clarify the activity of this putative transport protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains used in this study were DH5αMCR (Gibco-BRL), Na⁺/H⁺-antiporter-deficient K14B (strain CTC1004, with TrkJ gene deleted; 21), and potassium uptake-deficient TK2420 (Kdp−Kup−TrkJ−8). *E. coli* K14B has a normal complement of K⁺ uptake systems, but it has reduced levels of Na⁺/H⁺ antiport (mediating K⁺ efflux) relative to the wild type, presumably because of the cation substrate spectrum of one or more of the disrupted antiporter genes. *E. coli* TK2420 contains the normal complement of Na⁺ (K⁺)/H⁺ antiporters. *E. coli* strains were routinely grown at 37°C in LB medium (11). The effects of increasing concentrations of KCl on the growth of *E. coli* K14B were measured as described previously using LB medium supplemented with various concentrations of NaCl (11). The effects of increasing concentrations of KCl on the growth of *E. coli* K14B were measured as described previously in a defined medium (9) containing Na⁺ or in a medium in which the Na⁺-based buffer was replaced by morpholino propane sulfonic acid (MOPS) (12).

The plasmids used were pGEM3zf(+) (Promega) and pGerN, the same pGEM plasmid into which the open reading frame of the gerN gene from *B. cereus* was cloned behind the T7 promoter and a ribosome binding site. For construction of this recombinant plasmid, a promoterless copy of gerN was amplified from the *B. cereus* chromosome by PCR. The first primer, 5'-CCAG AGTCGTGAGTGGGAGGGATGATG-3', contains 3 bases at the 5’ end that provide aSacI restriction site. Bases 10 to 29 represent positions 458 to 477 in the GenBank AF246294 sequence except that A at 467 in the ribosome binding site was altered to G. The second primer, 5’-CCACTGCAGATGAT TATGGTATTAAGGTA-3’ contains anAccl restriction site at the 5’ end. Bases 10 to 30 represent the complement of the sequence of those bases that are 49 to 69 bases beyond the end of the GenBank gerN sequence. After digestion with SacI and Accl and purification of the product, the product was ligated with appropriately digested pGEM3zf(+) and used to transform *E. coli*. Recombinant
transporters were selected by conventional techniques, and the presence of the insert was confirmed. The recombinant plasmid pGerN was sequenced and found to contain only one base change, a silent change of C to T at position 1161.

Preparation of membrane vesicles and transport assays. Everted membrane vesicles were prepared by a method described by Rosen and colleagues (2, 22). Right-side-out membrane vesicles were prepared by the method of Kaback (14). A fluorescence assay of Na+/H+ antiport activity using acridine orange (AO) in everted membrane vesicles was performed as described by Goldberg et al. (11). The assay buffer contained 10 mM Tris-HEPES, 140 mM choline Cl, and 5 mM MgCl2. The pH of the assay buffer was varied as indicated for the individual experiments. For measurements of the fluorescence of ACMA (9-amino-6-chloro-methoxyacridine), right-side-out membrane vesicles were assayed in the same buffer as that for AO, at pH 8.0. ACMA was added to 500 nM, and a Perkin-Elmer LS50B luminescence spectrometer with an excitation wavelength of 410 nm and an emission wavelength of 490 nm was used. Right-side-out membrane vesicles were either loaded with 10 mM NaCl or not preloaded. A baseline of ACMA fluorescence was established, at which time 60 μg of vesicle protein was added, and the quenching of the fluorescence was monitored. All fluorescence assays were conducted at room temperature. The high concentration of chloride ion in both assays ensures that the transmembrane electrical component, Δψ, of the respiration-generated electrochemical proton gradient is dissipated by chloride ion movements, maximizing the pH gradient, ΔpH (more protons inside than out, in these assays), which is directly monitored by the probes. In experiments in which antiport is driven by substrate gradients and may, by its own electrogenic activity, generate a Δψ that would then constrain further antiport, the chloride ion fluxes would at least partially offset this constraint too, depending on the relative rates of antiport versus chloride ion equilibration.

Transport of radioactive 22Na+ was measured as described elsewhere (13). Everted membrane vesicles in 10 mM Tris-HEPES, pH 8.0, were either loaded with 10 mM potassium phosphate or not loaded. The assay, performed at 15°C, was initiated by diluting the vesicles 1:10 in 10 mM Tris-HEPES, pH 8.0, with or without the addition of 5 mM sodium phosphate. Samples were filtered, washed, and processed as for the 22Na+ uptake assays. Protein was measured by the method of Lowry et al. (16) using lysozyme as the standard.

FIG. 1. Effects of different concentrations of NaCl or KCl on the growth of E. coli strain KNabc or TK2420 transformed with control plasmid or pGerN. (A) Growth of E. coli KNabc transformants in the presence of increasing NaCl concentrations was measured after 15 h at 37°C. (B) Growth of E. coli TK2420 transformants in the presence of increasing KCl concentrations was measured after 15 h at 37°C. Open circles, control vector; solid circles, pGerN.

RESULTS

Complementation studies in E. coli mutant strains. As shown in Fig. 1, the recombinant plasmid that expresses gerN, pGerN, strongly complemented both the Na+-sensitive phenotype of E. coli KNabc and the phenotype of the K+ uptake-deficient E. coli strain TK2420. This pattern was consistent with the possibility that GerN is an antiporter that catalyzes both Na+ extrusion and K+ uptake, either with or without additional coupling ions. The possibility that the Na+ efflux was coupled to GerN-mediated K+ uptake was further suggested by the dependence of complementation of E. coli TK2420 by GerN on the presence of Na+; when the growth experiment with that strain was conducted in a Na+-free, MOPS-based buffer, there was no complementation (data not shown). Direct assays were necessary to test these inferences and explore other properties of the catalytic capacities of GerN.

Fluorescence-based assays of monovalent cation/H+ antiport in membrane vesicles. Everted membrane vesicles prepared from an E. coli KNabc transformant with a control plasmid exhibited no Na+, K+, or Li+/H+ antiport in fluorescence-based assays using AO. In this assay, addition of the electron donor D-lactate to the vesicles under the conditions described in Materials and Methods establishes a pH gradient (ΔpH), acid in, that results from respiration-dependent proton pumping into the vesicles. The fluorescence of the AO probe is quenched in response to that ΔpH. Antiport can then be assessed by the dequenching of AO fluorescence upon addition of a cation that is taken up into the vesicles in antiport with the intravesicular protons. The changes in probe quenching directly monitor the decrease in the ΔpH arising from the efflux of protons coupled to cation uptake. The transformant of E. coli KNabc with pGerN exhibited strong Na+ and Li+/H+ antiport, i.e., significant dequenching (Fig. 2), but no K+/H+ antiport. This pattern was consistent with the Na+/H+ antiporter activity in membrane vesicles prepared from E. coli KNabc transformed with pGEM3Zf(+) or pGerN. Formation of a ΔpH was monitored via AO quenching at pH 8.0 after the addition of 2 mM Tris-D-lactate to a mixture containing 70 μg of vesicle protein. The figure shows the effects of adding (at the arrows) various amounts of KCl, LiCl, or NaCl after the steady-state level of ΔpH (100% quenching) had been established.

FIG. 2. Na+ or Li+ or H+ antiport activity in everted membrane vesicles prepared from E. coli KNabc transformed with pGEM3Zf(+) or pGerN. Formation of a ΔpH was monitored via AO quenching at pH 8.0 after the addition of 2 mM Tris-D-lactate to a mixture containing 70 μg of vesicle protein. The figure shows the effects of adding (at the arrows) various amounts of KCl, LiCl, or NaCl after the steady-state level of ΔpH (100% quenching) had been established.
antiport. Consistently, addition of K\(^+\) before the electron donor did not alter the Na\(^+\)/H\(^+\) antiport, whereas Na\(^+\) and Li\(^+\) were cross-competitive (data not shown). The strong signal in the fluorescence assay made it possible to assess an apparent \(K_{\text{m}}\) for Na\(^+\) for GerN-dependent Na\(^+\)/H\(^+\) antiport. As shown in a reciprocal plot (Fig. 3), the \(K_{\text{m}}\) for Na\(^+\) was highly pH dependent, decreasing with increasing pH over the pH range of 7.0 to 8.0.

A fluorescence assay was next performed to address the question of whether GerN could use K\(^+\) as the coupling ion for Na\(^+\) antiport either instead of H\(^+\) or together with H\(^+\). Right-side-out vesicles were prepared from *E. coli* TK2420 and KNabc transformants and preloaded with 10 mM NaCl. At the start of the experiment, control vesicles (empty vector transformant) and GerN vesicles (pGerN transformant) were diluted into buffer either containing no added monovalent cation or containing 10 mM KCl, with no electron donor added. It was anticipated that there would be GerN-dependent uptake of H\(^+\) in exchange for the intravesicular Na\(^+\) in this right-side-out system, i.e., Na\(^+\)/H\(^+\) antiport driven by the outwardly directed Na\(^+\) gradient. If K\(^+\) could substitute for all or some of the H\(^+\) as the coupling ion, then fewer H\(^+\) ions would move inward. The development of a \(\Delta p\text{H}\) acid in, was monitored as described in Materials and Methods via ACMA fluorescence. As shown in Fig. 4, GerN-dependent H\(^+\) accumulation did indeed occur in both transformants. In addition, whereas the small H\(^+\) accumulation in the control vesicles was increased by addition of K\(^+\) to the extravesicular buffer, GerN-dependent H\(^+\) uptake was significantly reduced in the presence of K\(^+\).

The patterns were somewhat different in the two mutant strains, as expected from the absence of a different set of background transporters. In the *E. coli* KNabc transformants, in particular (Fig. 4, top), it was evident that there was still rapid initial movement of H\(^+\) dependent upon GerN, albeit reduced, when K\(^+\) was present. That is, H\(^+\) ions still move in antiport with Na\(^+\) when K\(^+\) is added, but fewer H\(^+\) ions move; some are likely to have been replaced by K\(^+\).

**Assays of \(^{22}\text{Na}^+\) accumulation and \(^{86}\text{Rb}^+\) efflux by everted vesicles.** In order to monitor the GerN-dependent fluxes of monovalent cations directly, assays of \(^{22}\text{Na}^+\) and \(^{86}\text{Rb}^+\) fluxes were next undertaken in everted membrane vesicle preparations. First, the accumulation of \(^{22}\text{Na}^+\) was assayed, with or without addition of an electron donor, and as a function of whether the vesicles had been preloaded with 10 mM K\(^+\). The buffers used in these assays did not contain high chloride concentrations, so both the \(\Delta p\text{H}\) and \(\Delta \phi\) components of the total electrochemical proton gradient, \(\Delta p\), were part of the available chemiosmotic driving force for the antiport as opposed to the completely \(\Delta p\text{H}\)-driven protocol used in the initial fluorescence assays (Fig. 2). The effect of the protonophore carbonyl cyanide-m-chlorophenylhydrazone (CCCP) was also examined. These experiments were conducted with control and pGerN transformants of *E. coli* KNabc. As shown in Fig. 5, GerN-dependent uptake of \(^{22}\text{Na}^+\) was significantly enhanced by the presence of intravesicular K\(^+\). In the absence of intravesicular K\(^+\) (Fig. 5, left panel), lactate-dependent uptake of \(^{22}\text{Na}^+\) was observed. Essentially no uptake was observed if lactate was omitted or CCCP was added. In the vesicles that contained K\(^+\) inside, more uptake of Na\(^+\) was observed even in the absence of lactate than in the vesicles lacking K\(^+\) inside; this uptake was transient (Fig. 5, right panel). The further addition of lactate to K\(^+\)-loaded vesicles resulted in rapid and sustained \(^{22}\text{Na}^+\) uptake without the subsequent loss of accumulated \(^{22}\text{Na}^+\) that was observed in the absence of the electron donor. CCCP inhibited both the lactate-dependent uptake of \(^{22}\text{Na}^+\) and the uptake that occurred in the absence of lactate, driven by the combined inward gradient of Na\(^+\) and outward gradient of K\(^+\). Plasmid controls conducted under the same sets of conditions exhibited no Na\(^+\) uptake (data not shown).

![FIG. 3. Double-reciprocal plot of NaCl concentration versus percent dequenching.](image-url)
It was of interest to ascertain whether the $K_m$ determined by the $^{22}Na^+$ uptake assay for Na$^+$ for the Na$^+$/H$^+$ antiport, i.e., in vesicles with no K$^+$ inside, was in the same range as that calculated from the fluorescence assay and whether intravesicular K$^+$ affected $K_m$ and/or $V_{max}$. As shown in Fig. 6, in assays conducted at pH 8.0, a $K_m$ of about 0.8 mM was calculated for both vesicles with and vesicles without intravesicular K$^+$. Thus, the $K_m$ was indeed in the same general range estimated by the fluorescence assay, and it was not changed by the addition of K$^+$ as a coupling ion. On the other hand, the $V_{max}$ was increased approximately twofold by the inclusion of intravesicular K$^+$. The $K_m$ and $V_{max}$ patterns at pH 7.0 were consistent both with the earlier finding of a higher $K_m$ at the lower pH than at pH 8.0 and with an increased $V_{max}$ in the presence of intravesicular K$^+$ (data not shown).

The partial, rather than complete, inhibition by K$^+$ of GerN- and Na$^+$/H$^+$-dependent H$^+$ uptake into right-side-out vesicles (Fig. 4) was most consistent with an Na$^+$/H$^+$-K$^+$ antiport, wherein a proton is a required counterion even when K$^+$ replaces at least 1 H$^+$ ion as part of the coupling ion complement and increases the antiport velocity. An Na$^+$/H$^+$-K$^+$ exchange could be electroneutral, e.g., if each turnover involved 2 Na$^+$ ions effluxing in exchange for 1 H$^+$ and 1 K$^+$ ion, or electrogenic, e.g., if a turnover involved efflux of 1 Na$^+$ ion in exchange for 1 H$^+$ and 1 K$^+$ ion. In order to assess this property, we examined the effect of thiocyanate on $^{22}Na^+$ uptake by K$^+$-loaded everted vesicles prepared from $E. coli$ KNabc cells expressing gerN. The protocol for thiocyanate treatment, described in Materials and Methods, included the permeant anion on both sides of the membrane at the start of the experiment. Transmembrane movements of the thiocyanate in response to a $\Delta \psi$ developed during lactate-dependent respiratory activity would be expected to reduce that $\Delta \psi$. As shown in Fig. 7, thiocyanate treatment completely abolished the stimulation of $^{22}Na^+$ uptake by lactate but did not affect lactate-independent, solute gradient-driven antiport.

Finally, GerN-dependent efflux of $^{86}Rb^+$ from everted membrane vesicles of $E. coli$ TK2420 was assessed both in the presence and in the absence of each of the following: Na$^+$ in the extravesicular buffer, added electron donor, and CCCP (Fig. 8). Significant $^{86}Rb^+$ efflux from control everted vesicles was observed even in this triple mutant, consistent with the likelihood that additional K$^+$ uptake pathways exist in $E. coli$. However, this background efflux exhibited no Na$^+$ dependence; in fact, extravesicular Na$^+$ modestly reduced the rate of $^{86}Rb^+$ efflux whether lactate or CCCP was added to the control vesicles. GerN-dependent $^{86}Rb^+$ efflux was much more rapid than in the control vesicles only when extravesicular Na$^+$ was

![FIG. 5. Uptake of $^{22}Na^+$ by everted membrane vesicles from $E. coli$ KNabc transformed with pGerN upon energization and/or loading with K$^+$. Membrane vesicles were either loaded (right) or not loaded (left) with K$^+$ as indicated in Materials and Methods. Uptake of $^{22}Na^+$ was measured either with no further additions (○) or in the presence of either 10 $\mu$M CCCP (●), 2.5 mM Tris-δ-lactate (△), or lactate plus CCCP (▲).](http://jb.asm.org/)

![FIG. 6. Effect of loading with K$^+$ on a double-reciprocal plot of $^{22}Na^+$ uptake by everted membrane vesicles from $E. coli$ KNabc transformed with pGerN. Initial uptake (10 s) of $^{22}Na^+$ was measured in Tris-δ-lactate-energized membrane vesicles loaded (●) or not loaded (○) with K$^+$.](http://jb.asm.org/)
FIG. 8. Effects of combinations of energization, Na⁺ in trans, and CCCP on GerN-dependent efflux of ⁸⁶Rb⁺ from everted membrane vesicles prepared from E. coli TK2420 transformants. Vesicles were loaded with K⁺-⁸⁶Rb⁺ as indicated in Materials and Methods. Efflux was initiated by 10-fold dilution into buffer containing no Na⁺ or 10 mM Na⁺ as indicated. Efflux was performed either with no further additions (○) or in the presence of either 2.5 mM Tris-δ-lactate (●), 10 μM CCCP (△), or both lactate and CCCP (▲).

The major result of this study is the demonstration that GerN catalyzes Na⁺/H⁺ antiport, as was anticipated by its sequence similarity to NapA, and also catalyzes Na⁺/H⁺-K⁺ antiport, which is significantly more rapid than Na⁺/H⁺ antiport. Li⁺, but not K⁺, can also serve as the cytoplasmic substrate. The Na⁺/H⁺ antiport observed was not just a reflection of contaminating K⁺. Rather, GerN must have a capacity for Na⁺/H⁺ antiport as one of its catalytic modes, since, in the fluorescence assays of antiport, Na⁺- and Li⁺-dependent movements of protons were observed. Evidence for coupling between Na⁺ efflux and K⁺ uptake included the dependence of the rate of Na⁺ uptake by everted vesicles on the presence of intravesicular K⁺ and the dependence of the rate of K⁺ (Rb⁺) efflux on extravesicular Na⁺. K⁺ apparently replaced some of the H⁺ ions that move in exchange for Na⁺ during Na⁺/H⁺ antiport but not all. There remained a significant Na⁺-dependent rapid H⁺ flux into right-side-out vesicles when abundant K⁺ was present on the outside (Fig. 4), consistent with an Na⁺/H⁺-K⁺ antiport. The two proposed GerN antiport modes are depicted in Fig. 9. The 2:1 stoichiometry of coupling ions entering to Na⁺ efflux was chosen arbitrarily as the simplest stoichiometry to use for diagrammatic purposes in representing the electrogenic antiports. The two H⁺ sites are distinguished because only part of the H⁺ ion complement can be replaced by K⁺ as the coupling ion. It will be worthwhile to confirm and extend deductions from these membrane vesicle assays in proteoliposomes in which purified GerN is the only protein. That system will be particularly useful, inasmuch...
GerN-mediated antiport is probably electrogenic as depicted in Fig. 9. That is, the ratio of H\(^+\) plus K\(^+\) moving into a right-side-out preparation to the Na\(^+\) moving out is greater than unity, so that a net positive charge moves inward. The geometric figures surrounding the two coupling ions suggest that these ions have distinct binding sites. The pH profile of Na\(^+\)/H\(^+\) antiport suggests that protons compete with Na\(^+\) on the cytoplasmic side of the membrane. (Right) Na\(^+\)/H\(^+\)-K\(^+\) antiport by GerN is supported by the finding of GerN-mediated and Na\(^+\)-dependent Rb\(^+\) (K\(^+\)) translocation with K\(^+\) replacing some, but not all, of the H\(^+\) ions that are transported in antiport with Na\(^+\). In the diagram, K\(^+\) is shown, hypothetically, as able to compete with H\(^+\) at only one of the H\(^+\) binding sites, and the antiport is shown as still requiring the full complement of coupling ions in this mode. GerN-mediated Na\(^+\)/H\(^+\)-K\(^+\) antiport is much more rapid than Na\(^+\)/H\(^+\) antiport; K\(^+\) increases the velocity of the antiport without affecting the K\(_{m}\) for Na\(^+\).

![Diagram showing proposed antiport activities of GerN.](image)

One of the notable properties of GerN-mediated antiport was the extremely high speed observed, especially when K\(^+\) was serving as one of the coupling ions. These high rates of antiport necessitated use of a low temperature for the radioactivity-based assays in order to observe a time course. They almost certainly reflect high turnover numbers given the expression conditions. Turnover numbers are best assessed in proteoliposomes and require determinations of the actual number of transporter molecules, neither of which has yet been accomplished for GerN. However, in the present experiments, gerN expression was under the control of the T7 promoter in E. coli strains that do not express a T7 polymerase. This is a device that we have found useful for producing very low levels of expression of membrane transport proteins that are toxic to particular E. coli strains when expressed at higher levels (13). Attempts to express gerN from stronger promoters in multicopy plasmids were unsuccessful with the strains used here (data not shown). No transformants that retained the correct recombinant plasmid were found. High levels of GerN may be toxic. It is not yet known whether gerN is expressed in vegetative cells of B. cereus or whether its expression is sporulation specific. High rates of GerN activity could be transiently important for some of the extensive early cation fluxes involved in germination (27). The rates are also of special note because some of the members of the CPA-2 family of transporters have been hypothesized to be channels (4); it may be that this structural group of transporters generally catalyzes rapid fluxes.

We hypothesize that it is the high speed of antiport catalyzed by GerN that accounts for certain features of the pattern of inhibition by thiocyanate. It was anticipated that electrogenic Na\(^+\)/H\(^+\)-K\(^+\) antiport would be stimulated by thiocyanate when driven entirely by inwardly directed Na\(^+\) and outwardly directed K\(^+\) gradients in everted vesicles. Under these conditions the antiport would be generating a \(\Delta \psi\) that would constrain the rate of antiport unless this back force was dissipated. The absence of a stimulatory effect by thiocyanate suggests that GerN-mediated antiport outpaces the rate at which thiocyanate can equilibrate across the membrane and dissipate the \(\Delta \psi\) that the antiport produces. Thiocyanate equilibration, like the high chloride ion concentration used in the fluorescence assays of antiport, is rapid enough to dissipate the \(\Delta \psi\) generated by respiration. It may also keep pace with \(\Delta \psi\) generation by GerN-mediated Na\(^+\)/H\(^+\) antiport, but not with the much more rapid GerN-mediated Na\(^+\)/H\(^+\)-K\(^+\) antiport. There is a precedent for the notion that the rate of a secondary antiport, but not respiration-dependent proton extrusion, might outpace the rate at which a permeant anion such as thiocyanate could equilibrate and dissipate the \(\Delta \psi\). The turnover number reported for E. coli NhaA is 89,000 min\(^{-1}\) (at pH 8.5) (28).
whereas turnover rates measured for cytochrome oxidase are cited in a range around 125 $\text{O}_2$ s$^{-1}$ (34).

CCCP also had an unanticipated effect on gradient-driven Na$^{+}$/H$^+$-K$^+$ antiport, as assayed by $^{22}\text{Na}^{+}$ uptake in everted vesicles containing K$^+$. CCCP had an inhibitory effect (Fig. 5, right graph). CCCP abolishes both components of the $\Delta \psi$ set up by respiration-dependent H$^+$ pumping into the everted vesicles. It was expected to inhibit respiration-driven antiport. By contrast, under conditions in which antiport was driven solely by opposing chemical gradients of two of the antiporter substrates, it might have stimulated or had no effect, as explained above for thioctanate. The inhibition observed could have its basis in a greater intrinsic velocity of the Na$^{+}$/H$^+$-K$^+$ antiport than the Na$^{+}$/H$^+$ antiport when the gradient-driven Na$^{+}$/H$^+$-K$^+$ antiport generated a $\Delta \psi$ in the presence of CCCP. Protons would have accumulated inside the vesicles in response to that potential. Perhaps the intravesicular H$^+$ concentration was sufficient to compete effectively with K$^+$. Any positive effect of dissipating the $\Delta \psi$ may have been more than offset because the velocity of the ensuing Na$^{+}$/H$^+$ antiport was much slower than the Na$^{+}$/H$^+$-K$^+$ antiport that occurred in the absence of CCCP. The reduction of Na$^{+}$-dependent efflux of $^{86}\text{Rb}^+$ by CCCP (Fig. 8) is consistent with this explanation.

Another notable property of GerN-mediated antiport is the decrease in the $K_m$ for Na$^{+}$ at a neutral versus an alkaline pH. Other Na$^{+}$/H$^+$ antiporters, especially NhaA of *E. coli*, are relatively inactive at neutral or low pHs. NhaA is tremendously activated at high pHs via increased specific activity and by regulation of expression (6, 28). The pattern of the pH effect on GerN activity in vesicles suggests that it is mediated by competition of H$^+$ with Na$^{+}$. Perhaps, during germination, this competition contributes to a temporal order of cation fluxes. Since the internal spore compartment is usually acidic (17, 24, 27), GerN-mediated activities might be suppressed until after the efflux of H$^+$ that occurs during germination has sufficiently alkalinized the internal spore compartment. Such speculations must be advanced with caution, since it is possible that additional features of GerN, relevant to its role in germination, have yet to be discovered. Moreover, the number of other ion transporters involved in germination, their identities, properties, and any ordering of their activities relative to GerN still need to be clarified. Also to be elucidated is the mechanistic basis for the specificity of GerN function with respect to the nature of the germination stimulus. That is, inosine-initiated germination appears to be much more dependent on GerN function than L-alanine-initiated germination (30). One of several possibilities is that the different germinant receptors sequester particular transporters in an inactive state and that when the receptor is stimulated by its germinant, its associated antiporter or group of transporters is activated either by their release or by some other mechanism.

The present findings on GerN raise the question of whether NapA, GrmA, and other homologues might also have the capacity for Na$^{+}$/H$^+$ + K$^+$ as well as Na$^{+}$/H$^+$ antiport. One or more of these proteins might then have a physiological role in K$^+$ acquisition and pH homeostasis in vegetative cells in addition to the role in Na$^{+}$ exclusion already proposed for NapA (33). Secondary Na$^{+}$/K$^+$ or Na$^{+}$/H$^+$-K$^+$ antiporter activities may be more widespread than has been appreciated. Verkhovskaya et al. (31) have suggested that *E. coli* has such an activity, but has yet to be identified with a specific gene. In *Bacillus* species and other gram-positive organisms, Tet(L) and Tet(K) proteins can mediate electrogenic exchange of a tetracycline-divalent-metal complex or Na$^{+}$ or K$^+$ for external H$^+$ or K$^+$ (12, 15). The possibility that an H$^+$ ion is involved in the Tet-mediated antiport even when K$^+$ is also a coupling ion, as suggested here for GerN, has not yet been tested. It has been shown, however, that in *Bacillus subtilis*, the chromosomally encoded Tet(L) protein plays a physiological role in pH homeostasis, Na$^{+}$ exclusion, and K$^+$ acquisition as well as in antibiotic resistance (5, 32). Thus, there is precedent for an antiporter that is involved in a particular stress response, i.e., antibiotic stress, also having roles in meeting other physiological challenges.

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