Active Potassium Extrusion Regulated by Intracellular pH in *Streptococcus faecalis*

Yoshimi Kakinuma and Kazuei Igarashi

From the Faculty of Pharmaceutical Sciences, Inohana Campus, Chiba University, 1-8-1 Inohana, Chiba 280, Japan

Potassium extrusion in bacteria is thought to play a role in the regulation of the cytoplasmic pH; in several organisms, it has been ascribed to secondary antiport of K⁺ for protons. *Streptococcus faecalis* exhibited a distinctive pattern: potassium extrusion occurred only when the cytoplasmic pH was alkaline and required the generation of ATP. The key observation is that glycolyzing cells suspended in an alkaline medium extruded K⁺, even against a K⁺ concentration gradient, provided the medium contained a weak permeant base (e.g., diethanolamine or methylamine). The amines render the cytoplasmic pH alkaline; when conditions were arranged to keep the cytoplasm neutral, no K⁺ extrusion was seen. Potassium extrusion required the presence of either glucose or arginine and was unaffected by protonophores and by inhibition of the F₁F₀ ATPase. When the medium contained [¹⁴C]methylamine, the cells accumulated the base to an extent stoichiometrically equivalent to the K⁺ lost. Concurrently, the cytoplasmic pH fell from 8.8 to 7.6, at which point K⁺ extrusion ceased. The results suggest that K⁺ extrusion is due to an ATP-driven transport system that expels K⁺ by exchange for H⁺ and is active only at alkaline cytoplasmic pH.

Potassium ion is the major cytoplasmic cation of growing bacterial cells and plays important roles in cell physiology. Cellular K⁺ activates various cytoplasmic enzymes, and K⁺ movements across the cell membrane are involved in the maintenance of turgor pressure and in the regulation of cytoplasmic pH. For these purposes, bacteria have evolved diverse potassium transport systems. Accumulation of K⁺ has been extensively studied, particularly in *Escherichia coli* and in *Streptococcus faecalis* (for reviews, see Refs. 1–3). Systems that extrude K⁺ are known to exist (4–8), but have not been fully characterized. Brey et al. (4) first discovered K⁺/H⁺ antiport in everted membrane vesicles of E. coli by examining the quenching of 9-aminoacridine. Kepes et al. (5) had noted earlier that the addition of N-ethylmaleimide to E. coli cells resulted in potassium efflux. Since N-ethylmaleimide-induced K⁺ efflux was found to be essentially electroneutral (6), this system may be a K⁺/H⁺ antiporter. In *Vibrio alginolyticus*, diethanolamine induced K⁺ efflux when added to cells growing at alkaline pH (7). The role of the membrane-permeant weak base is to alkalinize the cytoplasmic pH; alkalinization is a prerequisite for the operation of the transport system, which again appears to catalyze secondary antiport of K⁺ for H⁺. The K⁺/H⁺ antiporters are thought to function in the regulation of the K⁺ content, turgor pressure (9), and in pH homeostasis (10).

The present paper describes the extrusion of K⁺ by the fermentative bacterium *S. faecalis*. In this organism, cytoplasmic alkalinization is again a prerequisite for K⁺ extrusion, but the transport system differs from those described previously in that it requires the generation of ATP. We present evidence that *S. faecalis* contains an energy-dependent K⁺/H⁺ antiporter that is active at alkaline cytoplasmic pH and serves to acidify the cytoplasm (11). A preliminary report has appeared elsewhere (12).

**MATERIALS AND METHODS**

Organism and Growth Media—*S. faecalis* (faecium) ATCC 9790, supplied by F. M. Harold, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, was grown overnight on the complex medium (KTY) containing 10 g of Difco tryptone, 5 g of Difco yeast extract, 10 g of K₂HPO₄, and 10 g of glucose per liter (13). To prepare arginine-adapted cells, organisms were grown on the complex medium supplemented with 1% arginine and 0.1% galactose instead of glucose (14).

Transport Experiments—Overnight cultures were neutralized with KOH and washed twice with 2 mM MgSO₄. For uphill K⁺ extrusion, washed cells were suspended in 50 mM CHES-KOH (pH 9.0) containing 0.2 M KCl supplemented with glucose, arginine, or other reagents, at a cell density of 1 mg/ml, and incubated at 25 °C. For downhill K⁺ extrusion, cells were suspended in 50 mM CHES-NaOH (pH 9.0) containing 0.2 M NaCl. Cell samples (1.0 ml) were collected by filtration through Millipore filters (pore size 0.45 µm), washed twice with 2 mM MgSO₄, and extracted with hot 5% trichloroacetic acid. Aliquots were then analyzed for K⁺ and Na⁺ by flame photometry. Binding of the buffer K⁺ to the filters was subtracted from the data. To measure the uptake of [¹⁴C]methylamine (0.01 mCi/mmol), cell samples were collected by filtration and counted in a liquid scintillation counter. The cytoplasmic water space was taken to be 1.75 µl/mg of cells (15).

**RESULTS**

Energy-dependent Uphill Potassium Extrusion—Cells were suspended in the alkaline buffer (pH 9.0) containing at least 1 The abbreviations used are: CHES, 2-(cyclohexylamino)ethanesulfonic acid; TPF⁺, tetraphenylphosphonium ion; TCS, tetrachlorosalicylanilide; ΔpH, pH gradient; Δψ, membrane potential; DEAH⁺, protonated form of diethanolamine.

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† To whom correspondence and reprint requests should be addressed.
200 mM K', and K' extrusion from the cells was examined (Fig. 1). The intracellular concentration of K' at zero time was calculated to be about 300 mM, indicating a small chemical potential of K', directed outward. The parameters of the proton potential were determined under the same condition; there was no detectable Δψ and ΔpH was 1.3 units of interior acid (+80 mV; pHm, 7.7). The proton potential was thus +80 mV, opposite in polarity to that observed in neutral media. These cells did not extrude K'; even when 10 mM glucose was added, Δψ and ΔpH remained unchanged and no net K' movement occurred. However, when glucose was added together with 50 mM diethanolamine, there was a rapid extrusion of K' from the cells: within 50 min, the intracellular K' concentration had fallen to 100 mM. Note that potassium ion was actually extruded against a small concentration gradient, approximately 2-fold.

When glucose was absent, addition of 50 mM diethanolamine raised the cytoplasmic pH from 7.7 to 8.8 and reduced the proton potential almost to zero; however, no K' extrusion was induced. Uphill extrusion of K' from S. faecalis required the presence of both glucose and diethanolamine. K' extrusion was also examined in arginine-adapted cells, which can generate ATP via the arginine deiminase pathway (14). In those cells, addition of arginine together with 50 mM diethanolamine elicited K' extrusion, albeit less dramatically than glucose (data not shown). Since ATP is the only product common to these metabolic pathways, we infer that ATP, or a related metabolite, is required for potassium extrusion; for the present we shall designate it "ATP."

**ATP Is the Energy Source for Potassium Extrusion—** In principle, the finding that potassium extrusion requires ATP could have various explanations. For instance, ATP may be required in a regulatory capacity, as seems to be true for the major K' uptake system Ktr-I (16) and for Na'/H+ antiporter (2, 3, 17). Alternatively, ATP may serve as an energy donor, either directly or by generating electrochemical potentials of protons or of sodium ions.

Streptococci generate their proton potential by means of a proton-translocating ATPase (3, 18). Fig. 2 shows the effects of iodoacetic acid and ionophores on glucose-dependent K' extrusion. Uphill K' extrusion was completely inhibited by iodoacetic acid, an inhibitor of glycerolaldehyde-3-phosphate dehydrogenase in glycolysis, and also by arsenate (data not shown). However, it was not inhibited by the protonophores, TCS (Fig. 2) or carbonyl cyanide m-chlorophenylhydrazone (data not shown). Any K+ gradient established by the cells collapsed upon addition of gramicidin D, which transports all monovalent cations.

ATP-dependent K' extrusion was unaffected by treatment of the cells with dicyclohexylcarbodiimide, an inhibitor of the proton-translocating ATPase, and it was not impaired in mutant AS25, which lacks this enzyme (19) (data not shown). Finally, note that the activity of the F1F0-ATPase is optimal at pH 6.5 (3, 18); the enzyme should be virtually inactive at pH 8.5, established upon addition of diethanolamine. Taken together, these results virtually exclude the proton potential as a driving force for K+ extrusion.

S. faecalis at alkaline pH generates an electrochemical potential gradient for sodium ions, by means of a sodium-translocating ATPase (2, 13, 20). A role for the sodium potential in K+ extrusion is excluded by the finding that extrusion does not require sodium ions. Besides, our cells were grown on Na+-limited medium to avoid induction of the Na+-ATPase (13). We conclude that K' extrusion cannot be driven by either a proton or a sodium gradient. The system also does not require any special anion, for KCl could be replaced by potassium maleate or potassium phosphate (data not shown). It therefore appears that ATP serves as the energy donor for K+ expulsion.

**Potassium Extrusion Requires High Concentrations of a Membrane-permeable Amine—** Membrane-permeable amines traverse the cell membrane in their unprotonated forms by passive diffusion, bind intracellular protons, and accumulate in the protonated form due to the relative acidity of the cell interior (21). At low concentrations, amine accumulation is used as a measure of a pH gradient, interior acid (10). At high concentrations, however, amines collapse the pH gradient. As shown in Figs. 1 and 2, glucose-dependent K' extrusion requires the presence of diethanolamine, either to alkalize the cytoplasm or to provide a counterion for K'. The latter hypothesis implies that potassium extrusion is, in fact, ATP-dependent exchange of K' for the protonated species of the
base, DEAH⁺. As a test of this proposition, Fig. 3 shows ATP-dependent K⁺ extrusion at several concentrations of diethanolamine. Evidently, high concentrations of diethanolamine were required; even at 50 mM diethanolamine, the rate of K⁺ extrusion had not attained its maximum. It follows that the affinity of the hypothetical exchange system for DEAH⁺ would have to be very low, in the millimolar range. The existence of such a system is implausible, and we prefer to believe that the role of diethanolamine is to alkalinize the cytoplasmic pH.

Glucose-dependent uphill K⁺ extrusion was also observed in the presence of methylamine (Fig. 5) or of ethanolamine. However, triethanolamine and tris could not substitute for diethanolamine (data not shown).

Potassium Extrusion Requires an Alkaline Cytoplasmic pH—Fig. 4 shows ATP-dependent K⁺ extrusion as a function of the extracellular pH. Extrusion was observed only under alkaline conditions, pH 9.0 or 9.5. At pH 6.8 or 7.8, K⁺ was accumulated rather than excluded, possibly due to the operation of the uptake systems KtrI or KtrII (2, 3, 16). In agreement with this suggestion, when the experiment was repeated in sodium-free buffers and in the presence of TCS, K⁺ uptake at neutral pH was blocked (data not shown); by contrast, K⁺ extrusion at alkaline pH was still observed.

The ratio of protonated to unprotonated amine is a function of the medium pH. Since the pKₐ of diethanolamine at 25 °C is 8.8, the concentration of the free base decreases by 2 orders of magnitude between pH 9.0 and 7.0, and its availability may limit the rate of K⁺ extrusion at neutral pH. We infer that K⁺ extrusion does not depend on the protonation of the amine, but rather requires the cytoplasmic pH to be alkalinized to a pH above 8.

Accumulation of Permeant Amine during K⁺ Extrusion—To investigate the uptake of amines, we employed [¹⁴C]methylamine. Fig. 5 shows the time course of K⁺, Na⁺, and methylamine movements at pH 9.0. The cells were suspended in the alkaline buffer containing 50 mM NaCl to follow concurrent movements of Na⁺ ions. In the absence of amine, the cellular amounts of K⁺ and Na⁺ were unaffected by glucose. Upon addition of 50 mM methylamine, potassium ion was extruded, and an equivalent amount of methylamine accumulated in the cells. The sum total of the concentrations of K⁺, Na⁺, and methylamine remained constant at 340 mM (judging from the pKₐ of methylamine, 10.6 at 25 °C, we presume that most of the intracellular base was protonated). We infer that the cells exchange K⁺, not for Na⁺ but for an equivalent amount of the protonated amine.

Relationship of K⁺ Extrusion to Cytoplasmic pH—The results described above suggest that K⁺ extrusion operates only at alkaline cytoplasmic pH. This inference was confirmed by simultaneous measurements of K⁺ extrusion and of the pH, illustrated in Fig. 6. When cells were suspended in 50 mM CHES-KOH buffer (pH 9.0) containing 0.2 mM KC1 and 10 mM glucose, the cytoplasmic pH remained constant at 7.7 and ΔpH was almost 0; under these conditions, K⁺ extrusion did not occur. Addition of 20 mM diethanolamine at zero time induced cytoplasmic alkalinization to 8.9, and then K⁺ extrusion started. Concurrently with K⁺ extrusion, the cytoplasmic pH was acidified to 7.8, where K⁺ extrusion leveled off. A second addition of 30 mM diethanolamine also alkalinized the cytoplasmic pH and induced K⁺ extrusion, which ceased when the
intracellular pH reached about 8.0. During K⁺ extrusion, changes in the magnitude of ΔpH were negligible. Furthermore, addition of iodoacetic acid inhibited both K⁺ extrusion and acidification of cytoplasmic pH. Both the K⁺ gradient and the pH gradients established collapsed upon addition of gramicidin D (data not shown). In the absence of glucose, neither K⁺ extrusion nor cytoplasmic acidification were seen when the cytoplasmic pH was alkalinized to 8.8 by addition of diethanolamine. These results indicate that K⁺ extrusion requires both ATP and cytoplasmic alkalinization, and the K⁺ extrusion is tightly coupled to acidification of the cytoplasmic pH.

**DISCUSSION**

Potassium extrusion in a number of bacteria has been ascribed to a secondary antiporter that mediates exchange of K⁺ for H⁺ (4-8, 22), and we expected this mechanism to apply to *S. faecalis* as well. The results presented above are clearly incompatible with this notion and require us to postulate a primary ATP-linked potassium pump. The characteristics of the K⁺ extrusion system in *S. faecalis* can be summarized as follows. (i) Uphill K⁺ extrusion by glycolyzing cells is insensitive to dicyclohexylcarbodiimide and protonophores and is also Na⁺-independent. (ii) The system is sensitive to the cytoplasmic pH. Alkalization of the cytoplasmic pH to 8.8 is essential for K⁺ extrusion. (iii) The system stoichiometrically exchanges K⁺ for a permeant amine. (iv) K⁺ extrusion is accompanied by acidification of the cytoplasmic pH. On balance, we conclude that the system is not a porter but a primary transport system in which a chemical reaction supplies the driving force to exchange K⁺ for amine.

It is hard to distinguish K⁺/amine from K⁺/H⁺ antiport because we cannot directly measure proton movements. However, we favor the K⁺/H⁺ antiport mechanism for the following reasons. (i) K⁺/amine exchange occurred only at alkaline pH, as expected if the unprotonated form of amine is the species crossing the membrane (21). (ii) High concentrations of amine are required, suggesting that the amine serves to dissipate ΔpH (inside acidic) at alkaline pH (7). (iii) Most importantly, [¹⁴C]methylamine uptake coupled to K⁺ extrusion did not display saturation kinetics even at extracellular concentrations as high as 50 mM (Fig. 5; data not shown). In K⁺/amine antiporter (23) and the ammonium (amine) uptake system of *E. coli*, the *K₅* values for amine are at the micromolar level (24).

We conclude that, as shown in Fig. 7, unprotonated amine moves passively into the cells, alkalinizing the cytoplasmic pH. This activates the alkaline-sensitive ATP-driven K⁺/H⁺ antiporter, to expel internal K⁺ by exchange for external H⁺. The protons taken up are consumed by protonation of the internal amine, and the process continues until the internal pH has fallen to about 7.8.

The K⁺ gradient established by this system, about 2-fold, seems small for a primary transport system. We believe that the observed K⁺ gradient is not in equilibrium with ATP, but is regulated by the cytoplasmic pH. Besides, we should not ignore the leakiness of cell membrane to K⁺. When cells were suspended in K⁺-free buffer, downhill K⁺ release was observed in the absence of energy supply, accompanied by slow alkalinization of the cytoplasm. The process was stimulated by TTP⁺ and was more pronounced at alkaline than at neutral pH. Thus, in addition to ATP-driven K⁺ extrusion system, *S. faecalis* may contain yet another pathway for K⁺ release at alkaline pH. The nature of this pathway remains to be defined.

Bakker and Harold (16) observed energy-dependent downhill K⁺ efflux from *S. faecalis* at pH 7.0. K⁺ efflux was induced by TCS or dinitrophenol, only in the presence of glucose or arginine. It is clearly distinct from ATP-linked uphill K⁺ extrusion described here. Their observation could be explained by the hypothesis that the major K⁺ uptake system (*KtrI*) operates as an ATP-regulated K⁺/H⁺ symporter (2, 13, 16). Under conditions that depolarize the membrane but allow continued ATP generation, *KtrI* would catalyze K⁺ efflux. *S. faecalis* survives in the broad range of environmental pH, from 6 to 10 (11, 25). The cytoplasmic pH must be maintained
within 7.5 to 8.0 for optimal growth. Growth at alkaline pH requires acidification of the cytoplasmic pH (11). It is generally accepted that acidification of cytoplasmic pH in bacteria is due to secondary porters of Na⁺/H⁺ or K⁺/H⁺ (10, 26, 27). This cannot be true for S. faecalis. In this organism, the Na⁺/H⁺ antiporter (17) cannot acidify the cytoplasmic pH because the protonotive force is reversed at alkaline pH (11). Even in the medium containing 150–200 mM K⁺, this organism acidifies the cytoplasmic pH and grows well at alkaline pH. H⁺ influx via secondary K⁺/H⁺ antiporter, if it were present, cannot account for the magnitude of ΔpH at alkaline pH (11). We propose that ATP-linked K⁺/H⁺ antiport participates in pH homeostasis in this organism at alkaline pH. To regulate the circulation of important cations (K⁺, Na⁺, and H⁺) at alkaline pH, S. faecalis has evolved primary pumps to effect both Na⁺/K⁺ exchange (13) and K⁺/H⁺ exchange.

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