Mutants of *Streptococcus faecalis* Sensitive to Alkaline pH Lack Na\(^+\)-ATPase

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Alkali-sensitive mutants which grow at pH 7.5 but not at pH 9.5 in Na\(^+\)-rich media were isolated from *Streptococcus faecalis* ATCC 9790. One of the mutants, designated Nak1, lacked activities of both Na\(^+\)-stimulated ATPase and KtrII (active K\(^+\) uptake by sodium ATPase). These activities were restored in a spontaneous revertant designated Nak1R. Active sodium extrusion from Nak1 was observed at pH 7.0, which allows the cells to generate a proton potential, but not at pH 9.5, which reverses the proton potential, making it positive. Sodium extrusion at pH 7.0 was inhibited by addition of dicyclohexylcarbodiimide and protonophores. Even at pH 9.5, Nak1 did grow well in Na\(^+\)-poor media. In Na\(^+\)-rich media at pH 7.5, growth of Nak1 but not that of 9790 was severely inhibited by a protonophore. These results indicate that mutant Nak1 lacks sodium ATPase but contains a sodium/proton antiporter and that sodium ATPase is essential for the growth of this organism at high pH in Na\(^+\)-rich conditions.

Bacteria have evolved diverse mechanisms for active extrusion of sodium ions. Secondary sodium/proton antiporters are widely distributed (16), and some bacteria have been found to produce a primary sodium pump. In *Klebsiella aerogenes*, sodium extrusion is linked to the decarboxylation of oxaloacetate (2). In *Vibrio alginolyticus*, sodium extrusion is coupled directly to the respiratory chain (17). Heefner and Harold found that in the fermentative bacterium *Streptococcus faecalis*, sodium is expelled by an ATP-driven primary pump, i.e., sodium ATPase (8). Those researchers also noted Na\(^+\)/H\(^+\) antiporter activity in some of their preparations (7-9). They attributed this to proteolytic damage to the sodium ATPase molecules and proposed that *S. faecalis* possesses a single system for sodium extrusion which exchanges sodium ions for protons (6, 8). Sodium ATPase is induced when the cells are grown in medium rich in Na\(^+\) (12, 15).

In a study of potassium accumulation via the KtrII system of *S. faecalis*, Kakinuma and Harold revealed that the system responsible for K\(^+\) accumulation is sodium ATPase (4, 12). That is, sodium ATPase apparently exchanges Na\(^+\) for K\(^+\) rather than for H\(^+\). In addition, the activity of a sodium/proton antiporter was found in intact cells grown under conditions that do not induce production of sodium ATPase (10). On the basis of these studies, it is now proposed that *S. faecalis* contains two independent systems for sodium extrusion: (i) an inducible sodium ATPase which exchanges Na\(^+\) for K\(^+\) and (ii) a constitutive sodium/proton antiporter (4, 10, 12, 14).

To find out the significance of the existence of two extrusion systems in Na\(^+\) circulation, mutants defective in the individual systems are invaluable. By selection for sensitivity to the concentration of medium Na\(^+\), a mutant defective in sodium extrusion activity has been isolated in *S. faecalis* (3). However, this mutant lacked the activities of both sodium ATPase and the sodium/proton antiporter (3, 7). We report here the isolation of a mutant whose growth in Na\(^+\)-rich medium is inhibited at high pH; this mutant lacks Na\(^+\)-ATPase activity but retains the Na\(^+\)/H\(^+\) antiporter.

MATERIALS AND METHODS

Organisms and growth media. All of the experiments were conducted with *S. faecalis* (faecium) ATCC 9790, which was generously supplied by F. M. Harold (Colorado State University, Fort Collins), and mutants derived from it. Organisms were grown on complex medium NaTY (10 g of Difco tryptone, 5 g of Difco yeast extract, 10 g of glucose, 8.5 g of Na\(_2\)HPO\(_4\)) or KTY (tryptone, yeast extract, and glucose as for NaTY plus 10 g of K\(_2\)HPO\(_4\)) (12). Cell growth was monitored by measuring the optical density at 600 nm.

In some experiments, the overnight culture was neutralized with KOH and incubated with glucose for 30 min to allow the cells to take up potassium (K\(^+\)-loaded cells); in others, the cells were loaded with Na\(^+\) by the monactin method (12). In either case, the cells were collected by centrifugation, washed twice with 2 mM MgSO\(_4\), and suspended as described for the individual experiments.

Isolation of a mutant sensitive to high pH. Mutants unable to grow at pH 9.5 on NaTY medium were induced by N-methyl-N'-nitro-N-nitrosoguanidine (200 \(\mu\)g/ml) at 37°C for 30 min. The mutants were enriched after two cycles of penicillin (200 U/ml) treatment in NaTY medium at pH 9.5. Three colonies which grew at pH 7.5 but not at pH 9.5 were picked up by replica plating. Glucose was omitted from the selective plates to minimize the pH drop during growth. All mutants grew on SF medium (the selective medium for *S. faecalis* and *S. faecium*; Difco Laboratories). The spontaneous reversion frequency of Nak1, one of the mutants, was \(10^{-8}\), indicating a single mutation.

Transport experiments. To measure the KtrII activity (4, 12), the cells were loaded with Na\(^+\) and suspended in 50 mM Na\(^-\)-tricine buffer, pH 8.5, at a density of 1 mg (dry weight) per ml. After incubation with 10 mM glucose and 0.2 mM dicyclohexylcarbodiimide (DCCD) for 10 min, the reaction was initiated by addition of 1 mM KCl. Cell samples were collected by filtration on membrane filters (pore size, 0.45 \(\mu\)m; ADVANTEC, Toyo Corp.) and washed with 2 mM MgSO\(_4\). Sodium and potassium contents were determined by flame photometry after extraction of the cells with hot 5% trichloroacetic acid.

Sodium extrusion from intact cells was monitored with \(^{22}\)Na\(^+\) as described previously (7, 10). Cells harvested in the

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late-logarithmic phase of growth were used directly as K$^+$-loaded cells. Washed cells were suspended at 4 mg (dry weight) per ml in a buffer with 20 mM $^{22}$NaCl (0.8 µCi/ml) and incubated at 25°C for 60 min. At intervals, samples (0.2 ml) were filtered through membrane filters and washed with the same buffer, and the radioactivity was measured with a liquid scintillation counter.

**Reagents.** Radioactive materials were purchased from DuPont, NEN Research Products. Other reagents used were analytical grade.

**Other methods.** Cell membrane preparations were obtained as described by Abrams (1), except for the presence of 1 mM phenylmethylsulfonyl fluoride, from cells harvested in the late-logarithmic phase. ATPase activity was assayed in 0.5 mM DCCD by a procedure described elsewhere (12). ATP-dependent $^{22}$Na$^+$ uptake by membrane vesicles was measured as described by Heefner and Harold (8). The membrane potential and pH gradient (interior acid) of intact cells were calculated on the basis of the distribution of $[^3]$H[tetraphenylphosphonium ion and $[^3]$H]benzylamine, respectively (11), as described previously.

**RESULTS**

**Isolation of mutants sensitive to high pH.** The rationale for isolation of the mutant defective in sodium ATPase was based on our hypothesis that sodium ATPase is induced by an increase of cytoplasmic Na$^+$ (10, 14). *S. faecalis* tolerates a wide range of environmental pHs, from 5 to 10 (11). At low pH, the proton potential generated via H$^+$/Na$^+$-ATPase is large enough to drive a Na$^+/H^+$ antiporter. The proton potential is drastically decreased at pHs above 8. At pH 10, the proton potential turns to zero; it even becomes positive in some cases (11, 13). The Na$^+/H^+$ antiporter cannot operate to exclude cytoplasmic Na$^+$ ions under these conditions, and then induction of sodium ATPase is especially required. Therefore, mutants lacking sodium ATPase should be unable to grow at high pH.

Mutants whose growth is inhibited at high pH were isolated as described in Materials and Methods. Figure 1 shows the effect of medium pH on the growth of 9790 (the parent strain), Nak1, one of the pH-sensitive mutants, and Nak1R, the revertant obtained from Nak1, in complex medium NaTY. When the medium pH was shifted from 7.5 to 6.0 by addition of maleic acid or to 9.5 by addition of 50 mM Na$_2$CO$_3$ in the early logarithmic phase of cell growth, 9790 continued to grow with no lag phase (Fig. 1A). Nak1 also grew well at pH 6.0, but in contrast, its growth was considerably slowed at pH 9.5 (Fig. 1B). Nak1R recovered the ability to grow at high pH (Fig. 1C). Importantly, Nak1 grew well at pH 9.5 when the medium pH was raised by 50 mM K$_2$CO$_3$ (Fig. 1B) or by addition of 100 mM KCl to NaTY medium containing carbonate (data not shown). Nak1 grew on KTY medium (pH 9.5) at a rate of 1.4/h. Thus, Nak1 is not sensitive to high pH as such, only to the combination of a high Na$^+$ concentration and a high pH. The contents of K$^+$ and Na$^+$ in growing 9790 cells at pH 9.5 were 0.77 and 0.10 µmol/mg of cells, respectively. However, the K$^+$ and Na$^+$ contents in Nak1 cells on NaTY medium containing Na$_2$CO$_3$ at pH 9.5 (Fig. 1B) were 0.17 and 0.72 µmol/mg, respectively. Thus, the inability of Nak1 to grow at high pH resulted from defective exchange of Na$^+$ for K$^+$.

One possible interpretation is that Nak1 is leaky to Na$^+$ and/or K$^+$. Passive effluxes of K$^+$ and Na$^+$ from 9790 and Nak1 cells were examined. Potassium-loaded 9790 and Nak1 cells were suspended in K$^+$-free buffer containing 50 mM Na$^+$ at pHs 7.5 and 9.5. K$^+$ efflux from these strains was very slow, although K$^+$ efflux was slightly faster at pH 9.5 than at pH 7.5. At 30 min after suspension of Nak1 cells in a buffer at pH 7.5, more than 95% of the cellular K$^+$ was retained. In mutant 7683, which is defective in sodium extrusion and was isolated by Harold et al. (3), about 80% of the cellular K$^+$ leaked out within 30 min under the same conditions (3). When Na$^+$-loaded cells were suspended in Na$^+$-free buffer at pH 7.5 or 9.5, no remarkable differences in Na$^+$ efflux rates were observed between 9790 and Nak1. These results suggest that the mutation does not affect membrane permeability for K$^+$ and Na$^+$.

**Defect of Na$^+$-ATPase in the mutant.** The sodium ATPase in *S. faecalis* exchanges Na$^+$ for K$^+$ (12). In media poor in K$^+$, growth of this organism at high pH depends on Na$^+$ ions (Y.K., unpublished data). Thus, on the basis of our hypothesis (14), it is likely that active exchange of Na$^+$ for K$^+$ at high pH is mediated by sodium ATPase. To determine whether Nak1 is defective in sodium ATPase, the activity of KtrII, active exchange of Na$^+$ for K$^+$ by sodium ATPase (12), was examined (Fig. 2). Cells were grown on NaTY medium (pH 7.5). KtrII activity, which 9790 showed (Fig. 2A), was not detected in Nak1 (Fig. 2B) but was restored in the revertant Nak1R (Fig. 2C). Na$^+$-stimulated ATPase activity of the cell membranes was also examined in these strains (Table 1). In the membranes of 9790 cells grown on NaTY medium, stimulation of ATPase activity by Na$^+$ was readily observed. It was increased about twofold by growing the cells on NaTY medium containing 0.5 M NaCl (12). The KtrII activity of 9790 grown on this medium was also increased (data not shown). In Nak1 cells grown on these media, however, ATPase activity was not stimulated but rather slightly inhibited by NaCl. The increase of Na$^+$-stimulated ATPase activity in media rich in Na$^+$ was restored in Nak1R. These results indicate that the Nak1 mutant is defective in sodium ATPase.

Although the ATPase activity of 9790 was not affected by KCl, those of Nak1 and Nak1R were slightly inhibited by KCl (Table 1). Thus, it is likely that the ATPase activity of the mutant is sensitive to Cl$^-$, although the reason for this is unknown.

**Sodium/proton antiporter of the mutant.** As described...
above, Nak1 can grow on NaTY medium, even in 0.5 M NaCl at a slightly high pH (Fig. 1B). Under these conditions, Na⁺ ions must be expelled from cells via a system other than sodium ATPase. Figure 3 shows the effects of protonophores on the growth of cells in NaTY medium at pH 7.5. Harold and Van Brunt (5) demonstrated that circulation of H⁺ and K⁺ is not obligatory for *S. faecalis* to grow, provided that the cells are grown on a rich medium with a high concentration of K⁺ and a slightly high pH (7.5 to 7.7). The parent strain continued to grow, even in the presence of 40 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 3A). Under this condition, sodium ATPase is highly induced (12, 15). We understand this by the notion that inducible Na⁺-ATPase functions for K⁺-Na⁺ exchange whenever the proton potential is short-circuited (14). By contrast, growth of Nak1 lacking sodium ATPase was strongly inhibited by CCCP (Fig. 3B). Growth was restored by addition of 100 mM KCl. Growth of Nak1 on KTY medium at pH 7.5 was not inhibited by CCCP (data not shown). Nak1R recovered the resistance of its growth to CCCP (Fig. 3C). These results suggest that the proton potential-dependent system for sodium extrusion remains functional in the mutant.

The ability of mutant Nak1 to extrude sodium against a concentration gradient was examined at pHs 7.0 and 9.5 (Fig. 4). K⁺-loaded cells were suspended in a buffer containing 450 mM K⁺ and 20 mM 22Na⁺. Upon addition of glucose, 22Na⁺ was quickly expelled at pH 7.0, against a concentration gradient of over 10-fold (Fig. 4A). However, when DCCD, tetrachlorosalicylanilide, and valinomycin were included in the cell suspension to dissipate the proton potential, 22Na⁺ was not expelled but rather accumulated slightly. On the other hand, at pH 9.5, active 22Na⁺ extrusion was not observed, even in the absence of protonophores (Fig. 4B). In

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**TABLE 1.** Na⁺-stimulated ATPase activities of membranes of 9790, Nak1, and Nak1R

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>ATPase activity (nmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No addition (A)</td>
</tr>
<tr>
<td>9790</td>
<td>NaTY</td>
<td>105.7</td>
</tr>
<tr>
<td></td>
<td>NaTY + NaCl</td>
<td>148.3</td>
</tr>
<tr>
<td>Nak1</td>
<td>NaTY</td>
<td>192.7</td>
</tr>
<tr>
<td></td>
<td>NaTY + NaCl</td>
<td>198.6</td>
</tr>
<tr>
<td>Nak1R</td>
<td>NaTY</td>
<td>144.2</td>
</tr>
<tr>
<td></td>
<td>NaTY + NaCl</td>
<td>216.5</td>
</tr>
</tbody>
</table>

⁴ Cells were grown on the media listed, and membrane preparations were obtained by the method reported by Abrams (1), with 1 mM phenylmethylsulfonyl fluoride. ATPase activity was assayed as described previously (12), with or without KCl or NaCl. The values are averages of three independent experiments, and the standard deviations were below 5%.

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**FIG. 2.** KtrII activities of 9790, Nak1, and Nak1R. Cells were grown on NaTY medium, loaded with Na⁺, and suspended in 50 mM Na⁺-tricine buffer (pH 8.5) at 1 mg/mL. The suspension was supplemented with 10 mM glucose and 0.2 mM DCCD at -10 min; uptake was initiated by addition of 1 mM KCl at 0 min. Na⁺ (-O) and K⁺ (●) contents were determined by flame photometry. A, 9790; B, Nak1; C, Nak1R.

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**FIG. 3.** Effect of CCCP on the growth of 9790, Nak1, and Nak1R. Cells were grown on NaTY medium at pH 7.5 (O). Arrow a indicates addition of 40 μM CCCP (●), and arrow b indicates addition of 100 mM KCl (▲). A, 9790; B, Nak1; C, Nak1R.

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**FIG. 4.** Active Na⁺ extrusion activity of Nak1. Nak1 cells were grown on NaTY medium, harvested in the late-logarithmic phase, and suspended at 4 mg/mL in 50 mM K⁺-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.0) containing 400 mM K⁺-maleate (A), or 50 mM K⁺-CHES (2-(cyclohexylamino)ethanesulfonic acid) buffer (pH 9.5) containing 400 mM K⁺-maleate (B). A 20 mM concentration of 22NaCl (0.8 μCi/mL) was then added, and the cell suspensions were incubated at 25°C. After 60 min, the suspension was divided into aliquots, and at 3 min, 10 mM glucose (Glc) was added. Symbols: O, no glucose; ●, 10 mM glucose at 3 min; ▲, 0.4 mM DCCD, 10 μM tetrachlorosalicylanilide, and 10 μM valinomycin at -10 min and glucose at 3 min.
the parent strain containing sodium ATPase, active $^{22}\text{Na}^+$ extrusion, which is resistant to the protonophores, was observed at pHs 7.0 and 9.5 (data not shown; 7-10). In addition, inverted membrane vesicles of Nak1 exhibited ATP-driven uptake of $^{22}\text{Na}^+$, which was completely inhibited by protonophores (data not shown). We infer that the Na$^+$/H$^+$ antiporter (10) operates in Nak1 and that the mutant lacks only Na$^+$-ATPase.

**DISCUSSION**

The first mutant of *S. faecalis* defective in Na$^+$-ATPase, designated 7683, was selected by inability to grow in media rich in Na$^+$ (3). This Na$^+$-sensitive mutant showed no active Na$^+$ extrusion activity (3, 7). The hypothesis of Heefner and Harold (6, 8), that *S. faecalis* contains a single system for sodium extrusion, was based on the properties of this mutant. That is, two types of revertants were obtained from 7683; one, R-I, has antiporter activity, and the other, R-II, has sodium pump activity. Heefner and Harold proposed a modular model for the sodium pump composed of an antiporter subunit and a catalytic subunit (6, 8).

We believe that 7683 is pleiotropic, or at least a double mutant, for three reasons. (i) The cells are also very leaky to K$^+$ ions (3); (ii) several membrane proteins were shown to be missing in 7683 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Y.K., unpublished data); and (iii) most importantly, 7683 was isolated by mutagenesis with 25 $\mu$g of N-methyl-N' -nitro-N-nitosoguanidine per ml for 4 h (3), probably too harsh a treatment to induce a single-gene mutation. Our attempts to isolate a mutant with the same phenotype as 7683 by the same selection following treatment with N-methyl-N'-nitro-N-nitosoguanidine (200 $\mu$g/ml, 30 min) were unsuccessful.

In this study, we isolated a new mutant defective in sodium extrusion at high pH. Two further alkali-sensitive mutants isolated here also lacked Na$^+$-ATPase. In light of the reversion frequency of the mutation, Nak1 appears to be a single-gene mutant lacking only sodium ATPase but not the Na$^+$/H$^+$ antiporter. These results support our hypothesis that *S. faecalis* contains two independent systems for Na$^+$ extrusion (10, 14).

In view of the Na$^+$ circulation of *S. faecalis* at high pH, we expected the antiporter to be the pathway by which sodium ions enter the cells (4, 14). At high pH, the proton potential (mostly pH gradient) is reversed (11, 13). If the Na$^+$/H$^+$ antiporter were active at high pH, Na$^+$ uptake via the antiporter energized by a reversed proton potential would be expected in Nak1 at high pH. The proton potential under the conditions shown in Fig. 4B was actually positive (+55 mV of $\Delta$H and -20 mV of $\Delta$\phi), but Na$^+$ uptake was not observed. In general, Na$^+$/H$^+$ antiporters in bacteria remain active at high pH (16). Is the antiporter of *S. faecalis* exceptional in being inactive at high pH? The pathway of sodium influx at high pH remains unknown.

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**LITERATURE CITED**