Amplification of the Na^+-ATPase of *Streptococcus faecalis* at alkaline pH

Yoshimi Kakinuma and Kazuei Igarashi

Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Chiba 260, Japan

Received 18 December 1989

The Na^+-ATPase activity of *Streptococcus faecalis* was influenced by the medium pH. Activities of the protonophore-resistant Na^+ extrusion and the KtrII (active K^+ uptake by the Na^+-ATPase) were maximal in the cells grown at pH 9.5, and were minimal in those grown at pH 6.0. In the cells grown at pH 7.5, they were moderately observed. The Na^+-stimulated ATPase activity of the cells grown at pH 9.5 was about 4-fold higher than that of the cells grown at pH 6.0. Thus, amplification of the Na^+-ATPase is remarkable at alkaline pH in this organism, possibly by an increase of the cytoplasmic Na^+ level as a signal.

ATPase, Na^+-; Induction; Alkaline pH; Proton potential; (*Streptococcus faecalis*)

1. INTRODUCTION

The fermentative bacterium *Streptococcus faecalis* contains two independent systems for sodium extrusion: the Na^+-translocating ATPase, which was discovered by Heefner and Harold [1], and a sodium/proton antiporter [2,3]. The Na^+-ATPase apparently exchanges Na^+ for K^+ ions [4]. According to our recent hypothesis of Na^+ circulation in growing *S. faecalis*, the Na^+-ATPase plays an important role especially at alkaline pH [5]. It is explained as follows. At acidic pH, the proton potential generated by the H^+-ATPase, whose activity is optimal around pH 6.5 [6], suffices to drive a Na^+/H^+ antiporter and other proton-linked transport systems (e.g. K^+ uptake via the KtrI system [7]). However, the proton potential is drastically decreased at pHs above 8, and, at pH 10, the proton potential turns to zero, even positive in some cases [8,9]. Under these conditions, the Na^+/H^+ antiporter cannot extrude Na^+ and the KtrI system does not operate. The only means by which *S. faecalis* can exchange Na^+ for K^+ is the Na^+-ATPase [5].

We examined here the effect of medium pH on amplification of the Na^+-ATPase in *S. faecalis*, and found that it is most remarkable in alkaline growth media.

Correspondence address: Y. Kakinuma, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Chiba 260, Japan

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DCCD, dicyclohexylcarbodiimide; TCS, tetrachlorosalicylanilide

2. MATERIALS AND METHODS

2.1. *Organisms and growth media*

All the experiments were conducted with *S. faecalis (faecium)* ATCC 9790, which was generously supplied by F.M. Harold (Colorado State University, Fort Collins, CO, USA) and the mutant derived from it. Organisms were grown on the complex medium KTY (10 g of Difco tryptone, 5 g of Difco yeast extract, 10 g of glucose and 10 g of K_2HPO_4) [4]. In some experiments, NaCl (0.2 M) was included into KTY medium, and concentrations of tryptone and yeast extract were limited to one-fourth of the original medium to decrease the Na^+ content [3]. The medium pH was adjusted with maleic acid and K_2CO_3 [8].

2.2. *Assay of sodium transport*

Sodium movements were followed with $^{22}Na^+$ as described previously [3]. 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 (pore size, 0.45 μm; Millipore Corp.), washed with the same buffer, and the radioactivity was measured with a liquid scintillation counter.

2.3. *Assay of ATPase activity of cell membrane*

Cell membrane preparation was obtained as described by Abrams [10] in the presence of 1 mM PMSF. ATPase activity was assayed as described elsewhere [4,11] in the presence of 0.5 mM DCCD.

2.4. *Assay of the KtrII activity*

To measure the KtrII activity [4], the cells were loaded with Na^+ by the monactin method [12], and resuspended in 50 mM Na^+-tricine buffer, pH 8.5, at a density of 1 mg/ml. After incubation with 10 mM glucose and 0.2 mM DCCD for 10 min, the reaction was initiated by the addition of 1 mM KCl. Cell samples were collected on membrane filters, washed with 2 mM $MgSO_4$, and the contents of Na^+ and K^+ of the cells were determined by flame photometry as described elsewhere [4].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

3. RESULTS

3.1. *Increase of the Na^+-ATPase activity at alkaline pH*

To find out the Na^+-ATPase activity of *S. faecalis* grown at different pHs, the ability to extrude sodium ions against a concentration gradient was first examined (fig.1). The wild-type strain 9790 was grown in KTY medium at pH 6.0, 7.2 or 9.5, respectively. The concentration of Na^+ ions in all media was 15 mM. Cells were suspended in a buffer, pH 8.5, containing 450 mM K^+ and 20 mM $^{22}Na^+$ since the Na^+-ATPase activity of the inverted membranes is optimal at pH 8.5 [4,11]. DCCD, TCS and valinomycin were included in cell suspension to dissipate the proton potential and to make the cytoplasmic pH equal to the buffer pH [13]. In the cells grown at pH 6.0, glucose-dependent $^{22}Na^+$ extrusion was little observed ($[Na^+]_{out}/[Na^+]_{in}$ of 1.3 at 20 min) (fig.1A). In the cells grown at pH 7.2, slight active sodium extrusion was observed ($[Na^+]_{out}/[Na^+]_{in}$ of 3.3 at 20 min) (fig.1B). In contrast, in the cells grown at pH 9.5, the activity of sodium extrusion was remarkable ($[Na^+]_{out}/[Na^+]_{in}$ of over 10 at 20 min) (fig.1C), suggesting that the Na^+-ATPase is highly amplified under these conditions. When the experiments were performed at pH 7.0 in the absence of the ionophores, active $^{22}Na^+$ extrusion against a concentration gradient of over 10-fold was observed in the cells grown at pH 6.0 and 7.2 (fig.1A,B), indicating that the sodium/proton antiporter operates in these cells [3].

Effect of medium pH on the Na^+-stimulated ATPase activity of the membranes was also examined (table 1).

Table 1

Increase of Na^+-stimulated ATPase activity in alkaline pH media

Medium (pH)	ATPase activity (μmol/min/mg protein)			
Addition:	None (*A*)	KCl[a]	NaCl[a] (*B*)	Na^+-stimulated (*B*−*A*)
KTY plus NaCl[b] (6.0)	0.20	0.21	0.24	0.04
KTY plus NaCl[b] (7.5)	0.23	0.20	0.31	0.08
KTY plus NaCl[b] (9.5)	0.27	0.21	0.42	0.15
KTY (6.0)	0.16	0.15	0.15	−[c]
KTY (7.5)	0.19	0.15	0.15	−[c]
KTY (9.5)	0.22	0.18	0.19	−[c]

[a] 25 mM
[b] 0.2 M
[c] not determined

Cells of strain 9790 grown on the media listed above were harvested at the middle logarithmic phase, and the cell membranes were prepared by the method of Abrams [10] in the presence of 1 mM PMSF. The Na^+-ATPase activity was assayed as described elsewhere [4,11] with and without 25 mM KCl and NaCl

First, cells were grown on KTY medium containing 0.2 M NaCl as the Na^+-ATPase is highly induced in media rich in Na^+ [4,11]. The ATPase activity, which is stimulated by Na^+ but not by K^+, was observed in all pH conditions. The specific activity of the Na^+-stimulated ATPase of the cells grown at pH 7.5 was twice as high as that of the cells grown at pH 6.0. Moreover, the activity of the cells grown at pH 9.5 was about 4-fold higher than that of the cells grown at pH 6.0. The ATPase activity was slightly inhibited by KCl,

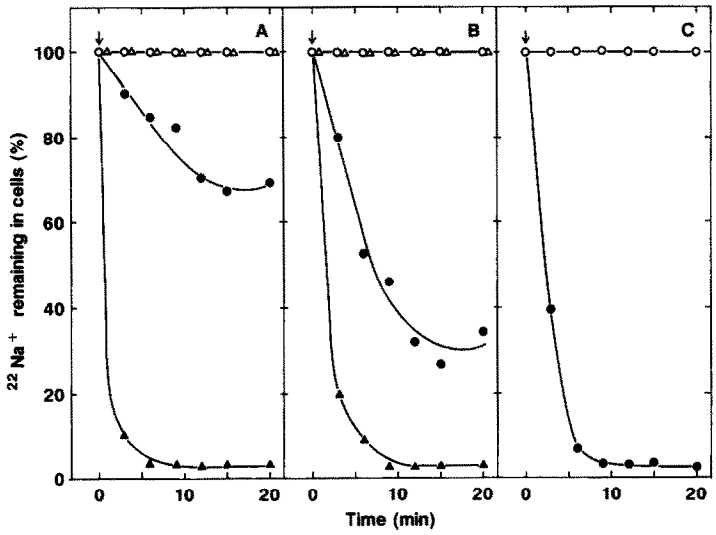

Fig.1. Sodium extrusion from *S. faecalis* grown at different pHs. Cells of strain 9790 were grown on KTY medium at pH 6.0 (A), at pH 7.2 (B) and at pH 9.5 (C), harvested at middle logarithmic phase, and resuspended at 4 mg/ml in 50 mM K^+-tricine buffer, pH 8.5 (circle) or pH 7.0 (triangle), containing 400 mM K^+-maleate. Then, 20 mM $^{22}NaCl$ (0.8 μCi/ml) was added and the cell suspensions were incubated at 25°C. After 60 min, the suspension was divided into aliquots, and glucose (10 mM) was added at 0 min. In the assays at pH 8.5, DCCD (0.4 mM), TCS (10 μM) and valinomycin (10 μM) were added at −10 min. 'Open' symbols represent no addition of glucose and 'closed' symbols represent the addition of glucose. The amounts represented as 100% were 45–50 nmol of Na^+ per mg of cells under all conditions.

but the reason for this is unknown [14]. As the high activity of sodium extrusion was observed in the cells grown on KTY medium (pH 9.5) (fig.1C), the Na^+-stimulated ATPase activity was expected to be found also in these cells. However, it was not detected (table 1). Fig.2 shows the double reciprocal plots of the Na^+-stimulated ATPase activities by the cells grown on KTY medium containing 0.2 M NaCl at different pHs.

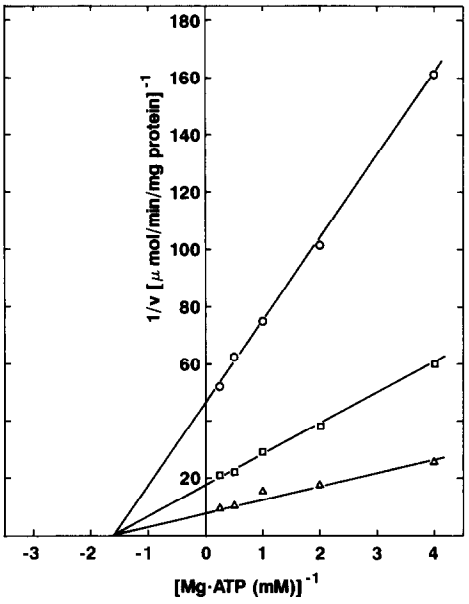

Fig.2. Double reciprocal plots of the Na^+-stimulated ATPase activity. Cells were grown on KTY medium containing 0.2 M NaCl at pH 6.0 (○), at pH 7.5 (□) and at pH 9.5 (△). Preparation of the cell membranes and the assay of ATPase activity were performed as described in section 2. The Na^+-stimulated ATPase activity was determined by $B-A$ as shown in table 1. The ratio of ATP:Mg^{2+} was kept constant at 1.

The K_m values for ATP of the individual activities were constant (about 0.6 mM), and V_{max} values were changed. These results indicate that the amount of Na^+-ATPase is increased by alkalinization of the medium pH.

3.2. *Increase of the KtrII activity at alkaline pH*

Two distinct potassium uptake systems, designated KtrI and KtrII, have been recognized in *S. faecalis* [4]. Accumulation of K^+ ions via KtrII was linked to the extrusion of Na^+ ions by the Na^+-ATPase. Kakinuma and Harold proposed that the KtrII is the Na^+-ATPase itself, which directly exchanges Na^+ for K^+ ions [2,4]. Fig.3 shows the KtrII activity by the cells grown on KTY medium containing 0.2 M NaCl at different pHs. The KtrII activity was slightly detected by the cells grown at pH 6.0 (fig.3A). The activity was increased by growing the cells at alkaline pH, and the maximum was observed by the cells grown at pH 9.5 (fig.3B,C). When cells were grown on KTY medium, the KtrII activity was much lower [4], but again, it tended to increase as the medium pH became higher (data not shown). Thus, the KtrII activity as well as the Na^+-ATPase is increased by alkalinization of the medium pH.

3.3. *Increase of the cellular Na^+ content at alkaline pH*

The cellular contents of Na^+ and K^+ ions of *S. faecalis* growing at different pHs were determined (fig.4). In 9790 growing on KTY medium, the intracellular concentration of Na^+ was low (fig.4A). The K^+ concentrations ranged from 420 to 500 mM. In KTY medium containing 0.2 M NaCl, the intracellular concentration of Na^+ of 9790 was also maintained at a low level (from 20 mM to 40 mM, assuming the cytoplasmic water space as 2 μl/mg of cells [7,8]),

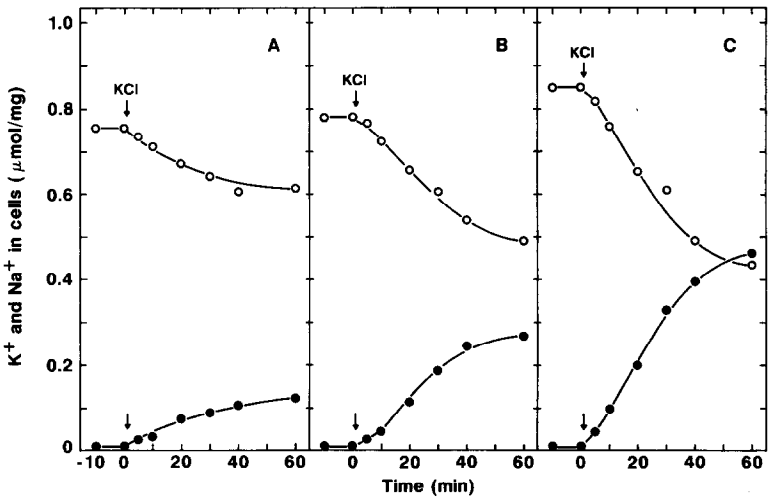

Fig.3. The KtrII activity of *S. faecalis* grown at different pHs. 9790 was grown on KTY medium containing 0.2 M NaCl at pH 6.0 (A), at pH 7.5 (B) and at pH 9.5 (C), loaded with Na^+ by the monactin method [12], and suspended in 50 mM Na^+-tricine buffer (pH 8.5) at 1 mg/ml. The suspension was supplemented with 10 mM glucose and 0.2 mM DCCD at −10 min; the uptake was initiated by the addition of 1 mM KCl at 0 min. The contents of Na^+ (○) and K^+ (●) were determined by flame photometry.

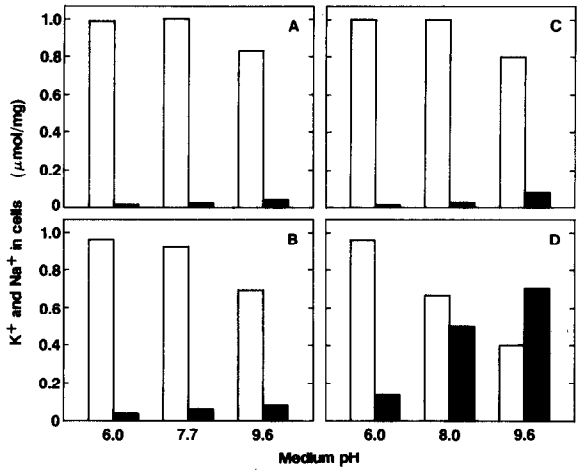

Fig.4. The cellular contents of Na^+ and K^+ of *S. faecalis* growing at different pHs. Cells of 9790 and Nak1 were grown on KTY medium or KTY medium containing 0.2 M NaCl. At the optical density of 0.2 at 600 nm, cells (10 ml of the culture) were collected on the filters, and washed twice with 2 mM $MgSO_4$. The contents of K^+ (open bar) and Na^+ (shaded bar) were determined by flame photometry. The concentrations of Na^+ and K^+ in media were 120 mM and 15 mM for KTY, and 120 mM and 220 mM for KTY plus NaCl, respectively. (A) 9790 in KTY; (B) 9790 in KTY plus NaCl; (C) Nak1 in KTY; (D) Nak1 in KTY plus NaCl.

although the values were slightly higher than those on KTY medium (fig.4A,B). Nak1 is a mutant lacking the Na^+-ATPase but not the Na^+/H^+ antiporter [14]. In Nak1 growing on KTY medium, the cytoplasmic Na^+ level was kept low at pH 6.0 and 8.0 (7–15 mM), but, at pH 9.5, the Na^+ concentration was increased to approximately 45 mM (fig.4C). In KTY medium containing 0.2 M NaCl at pH 6.0, the concentrations of Na^+ and K^+ of Nak1 were 70 and 480 mM, respectively. However, at pH 8.0 and 9.5, the K^+ contents had decreased (i.e. 200 mM at pH 9.6), and the Na^+ contents had increased remarkably (i.e. 320 mM at pH 9.6) (fig.4D). Thus, Na^+ ions are accumulated at alkaline pH when the Na^+-ATPase is blocked, indicating that the Na^+/H^+ antiporter cannot operate to exclude cytoplasmic Na^+ under these conditions [3,5,14]. The growth rate (h^{-1}) of Nak1 in KTY medium (pH 9.6) was decreased from 1.5 to 0.6 by addition of 0.2 M NaCl. Induction of the Na^+-ATPase is especially required at alkaline pH.

4. DISCUSSION

The Na^+-ATPase is amplified when cells are grown in media rich in sodium, particularly under conditions that limit the generation of a proton potential by the mutation, by the inclusion of the protonophore in media [4,11], and by alkalinization of medium pH as described here. In addition, when cells were grown on media containing monensin, the Na^+-ATPase activity was remarkably increased (Kakinuma, unpublished results). Taking into account the cellular Na^+ content under various conditions that highly induces the Na^+-ATPase, we speculate that an increase in $[Na^+]_{in}$ is the signal for amplification of Na^+-ATPase in *S. faecalis* [5].

Considering the physiological roles of a sodium potential in bacteria, Skulachev predicted an induction of Na^+-ATPase of *S. faecalis* by alkaline growth conditions [15]; to drive membrane-linked works of this organism under conditions where the proton potential is limited, the sodium potential should be generated by means of the Na^+-ATPase. Thus, a drop of the proton potential itself may have an effect on the amplification of the Na^+-ATPase at high pH. In *S. faecalis*, however, there is no evidence for any Na^+ potential-linked membrane processes [2,5], although it is mysterious in an energy economy. Only the KtrII system is Na^+-dependent [4]. In some culture conditions, we observed Na^+-dependent growths of *S. faecalis* at high pH. However, the Na^+ dependence was only seen in media poor in K^+ ions. Addition of K^+ ions replaced sodium ions (Kakinuma and Igarashi, in preparation), suggesting that Na^+ ions are required at alkaline pH only for K^+ circulation in this organism [5].

Acknowledgement: This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Heefner, D.L. and Harold, F.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2798–2802.
[2] Harold, F.M. and Kakinuma, Y. (1985) Ann. NY Acad. Sci. 456, 375–383.
[3] Kakinuma, Y. (1987) J. Bacteriol. 169, 3886–3890.
[4] Kakinuma, Y. and Harold, F.M. (1985) J. Biol. Chem. 260, 2086–2091.
[5] Kakinuma, Y. and Igarashi, K. (1989) J. Bioenerg. Biomemb. 21, 679–692.
[6] Kobayashi, H., Murakami, N. and Unemoto, T. (1982) J. Biol. Chem. 257, 13246–13252.
[7] Bakker, E.P. and Harold, F.M. (1980) J. Biol. Chem. 255, 433–440.
[8] Kakinuma, Y. (1987) J. Bacteriol. 169, 4403–4405.
[9] Kakinuma, Y. and Igarashi, K. (1988) J. Biol. Chem. 263, 14166–14170.
[10] Abrams, A. (1965) J. Biol. Chem. 240, 3675–3681.
[11] Kinoshita, N., Unemoto, T. and Kobayashi, H. (1984) J. Bacteriol. 158, 844–848.
[12] Harold, F.M. and Baarda, J.R. (1968) J. Bacteriol. 95, 816–823.
[13] Heefner, D.L. and Harold, F.M. (1980) J. Biol. Chem. 255, 11396–11402.
[14] Kakinuma, Y. and Igarashi, K. (1989) J. Bacteriol., submitted.
[15] Skulachev, V.P. (1985) Eur. J. Biochem. 151, 199–208.