Enterococcus hirae vacuolar ATPase is expressed in response to pH as well as sodium

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Abstract The *Enterococcus hirae ntp* operon encodes both a vacuolar ATPase, which transports Na^+ as well as Li^+, and the KtrII K^+ transporter. A plasmid, in which the chloramphenicol acetyltransferase gene (CAT) was placed downstream of the *ntp* promoter, was introduced into a mutant totally defective in Na^+ extrusion. The CAT activity of this transformant was increased preferentially by addition of NaCl, but not by LiCl, in the media or by elevating the medium pH, correlating well with the increase in amounts of the ATPase subunits observed by Western blotting. The physiological significance of these responses of the *ntp* promoter is discussed.

Key words: Vacuolar ATPase; *ntp* operon; Promoter; Chloramphenicol acetyltransferase; *Enterococcus hirae*

1. Introduction

The Gram-positive bacterium *Enterococcus hirae* has two sodium extrusion systems: a secondary Na^+/H^+ antiporter [1,2] and an ATP-driven sodium pump [3], a vacuolar-type (V-) ATPase [4]. As this bacterium lacks the respiratory chain, the proton electrochemical gradient (proton potential) across the cell membrane is generated by proton expulsion via the F_0F_1,H^+-translocating ATPase. Since the activity of the H^+-ATPase is optimal around pH 6.5 [5], the proton potential is generated well at low pH, but it is minimal at high pH [6]. Therefore, the secondary Na^+/H^+ antiporter works well to extrude Na^+ at low pH, but not at high pH. V-ATPase is thus especially important for Na^+ extrusion at high pH [4,6].

E. hirae V-ATPase is encoded by the *ntp* gene cluster consisting of 11 *ntp* genes: *ntpFIKECGABDHJ* [7]; Northern blot experiments have revealed that this operon is transcribed as a single mRNA of more than 10 kb [8]. It is now clear that the gene products of *ntpF* to *ntpD* make up the subunits of the V-ATPase complex [9]. Furthermore we have shown that the *ntpJ* gene, a tailed cistron of this operon, encodes a component of the KtrII K^+ transport system independent of proton potential [10]. *E. hirae* V-ATPase is not constitutive. The amount of this ATPase increased in cells grown in high Na^+ medium [11,12] or grown at high pH [13]. A further increase in its level was observed when the proton potential was dissipated by mutation of the F_0F_1,H^+-ATPase or by the inclusion of a protonophore such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) in the medium [11,12]. Since the Na^+/H^+ antiporter does not lower the cytoplasmic Na^+ concentration ($[Na^+]_{in}$) under these conditions, these results suggest that an increase in the V-ATPase level occurs by an increase of $[Na^+]_{in}$ as the signal [6]. A recent Northern blot experiment has revealed that de novo synthesis of *E. hirae* V-ATPase is regulated by $[Na^+]_{in}$ at the transcriptional level [8]. Under the culture condition where proton potential was dissipated and medium K^+ concentration was very low, the growth of *E. hirae* depended upon the presence of Na^+ in the medium [14], suggesting that the sodium-responsive *ntp* operon is important for homeostasis of both K^+ and Na^+ in this bacterium at limited proton potential. However, the effect of other factors as the signal has not so far been studied.

In this paper we examined the effect of monovalent cation and medium pH on the *ntp* promoter activity, and found that expression of the *ntp* operon was preferentially stimulated by Na^+, but not by Li^+, or by elevating the medium pH.

2. Materials and methods

2.1. Strains and media

E. hirae ATCC 9790 and mutants WD4, in which the *napA* gene encoding the Na^+/H^+ antiporter was disrupted [2], and 7683, which is a mutant defective in both activities of NapA antiporter and V-ATPase [15], derived from 9790 were used. It is now suggested that the *ntpI* gene was point-mutated in 7683 [16]. Cells were grown in a standard complex medium, KTY or NaTY (pH 7.8)[12], or a completely defined medium TrisM (pH 7.5)[14] supplemented with 200 mM KCl (TrisKM). TrisKM medium contained 200 mM K^+ and was contaminated with 0.5 mM Na^+ [14]. Cell growth was monitored by measuring the optical density at 540 nm by a spectrophotometer.

2.2. Plasmid construction

An *Escherichia coli-Bacillus subtilis* shuttle vector pHE was constructed from pHY300PLK by replacing its tetracycline resistance gene with the erythromycin resistance gene of pC3 [17]. The 500 bp *Taq*I fragment of pKAZ151 [7], which has the *ntp* operon promoter region (P*ntp*), was first subcloned into the *Acc*I site of pUC119 (pKAZ101), and the 600 bp *Hin*dIII-*Eco*RI fragment of pKAZ101 was then subcloned into *Hin*dIII and *Eco*RI sites of pHE, giving pHEex. The 685 bp fragment containing the *Staphylococcus aureus* chloramphenicol acetyltransferase (CAT) gene was amplified by PCR with pKAZ111CAT01 [18] as a template. The primers used were PK1, 5′-GGCTCTAGAGCAGACAAGTAAGCC-3′, and PK2, 5′-GCCGGATCCTCTTCAACTAACGGG-3′. PK1 and PK2 contained new *Xba*I and *Bam*HI sites, respectively (underlined). The 685 bp *Xba*I-*Bam*HI fragment of the CAT gene, which does not have its own promoter, was then placed at *Xba*I-*Bam*HI sites downstream of the *ntp* promoter region, which extends from −300 to +50 region including the initiation codon for the initial *ntpF* cistron of this operon, of pHEex (pHECAT1) (Fig. 1). The initiation codon of the CAT gene was located 60 bp downstream of the initiation site of the *ntp* operon. The plasmid was transformed into 9790 or 7683 by electroporation as described previously [17].

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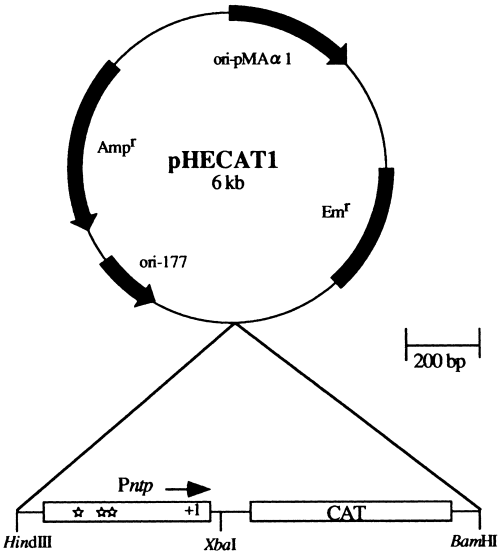

Fig. 1. Structure of pHECAT1. The *E. coli-B. subtilis* shuttle plasmid pHECAT1, originated from pHY300PLK, has the 500 bp fragment of the *E. hirae ntp* operon promoter (P*ntp*) and the 685 bp CAT gene fragment placed downstream of P*ntp*, as described in Section 2. The initiation codon of the CAT gene was located 60 bp downstream of the start site (+) of the *ntp* operon. Stars represent the location of palindromic sequences in the 5′ region of P*ntp*.

2.3. Transport experiment

For measurement of extrusion of Na^+ or Li^+, cells were suspended in 50 mM Tris-maleate (pH 7.5) containing 0.4 M KCl at a cell density of 1 mg protein/ml, and incubated at 25°C with 20 mM NaCl or LiCl for 60 min. After addition of 10 mM glucose, cell samples (0.2 ml) were collected at intervals by filtration through a Millipore filter (0.45 μm pore size), washed once with 2 mM $MgSO_4$, and extracted with hot 5% trichloroacetic acid. Aliquots were then analyzed for Na^+ or Li^+ by atomic absorption.

2.4. Measurement of the CAT activity

Cells were cultured in various media, harvested at middle logarithmic phase, and the cell lysates were prepared as described elsewhere [8]. The CAT activity was assayed in 250 μl of the reaction mixture (80 mM Tris-HCl (pH 8.0), 1 mM chloramphenicol, 0.1 mM [^{14}C]acetyl CoA (0.074 MBq/mmol), and 50 μg protein of the lysate). The reaction was initiated by addition of acetyl CoA, and the radioactivity of [^{14}C]acetylchloramphenicol produced in the reaction mixture was counted using a toluene-based scintillation cocktail. The activity was expressed as cpm/min/mg protein.

2.5. Others

Western blotting of the cell lysates was performed as described previously by use of antiserum against purified *E. hirae* V-ATPase, and visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase [8]. Determination of the contents of K^+ and Na^+ in cells growing in various media was done after separating the cells from medium by centrifugation through mineral oil [9]. Protein was determined by the method of Lowry et al. [19] with bovine serum albumin as standard.

3. Results

3.1. E. hirae V-ATPase transports Li^+ as well as Na^+

Recent kinetic analysis of the ATP hydrolysis of purified *E. hirae* V-ATPase has indicated that the activity is stimulated by Li^+ as well as Na^+ with similar K_m values [9], but Li^+ translocation by the V-ATPase has not been investigated. Lithium extrusion from whole cells was examined by strain WD4 which is deficient in the NapA antiporter (Fig. 2). Cells were cultured in NaTY medium containing about 120 mM Na^+ so as to induce V-ATPase [12]. Glucose-dependent lithium extrusion (Fig. 2A) as well as sodium extrusion (Fig. 2B) was clearly observed with this mutant. The extrusion was not inhibited by addition of valinomycin and CCCP which dissipate proton potential (data not shown). Since *E. hirae* has only two Na^+ extrusion systems, no sodium or lithium extrusion was observed with WD4 cells cultured in Na^+-depleted medium [20], which was confirmed in this study (data not shown). From these observations, we concluded that V-ATPase transports Li^+ equally well as Na^+.

3.2. The ntp promoter is preferentially stimulated by Na^+ but not by Li^+

The effect of monovalent cations on the *ntp* promoter activity was monitored by expression of the CAT gene (Fig. 1). The transformant 7683/pHECAT1 was cultured in defined medium TrisKM containing various concentrations of NaCl or LiCl, and the CAT activities of the cell lysates were measured (Fig. 3A). The growth of this transformant in this medium was little affected by the presence of up to 100 mM NaCl with the growth rate (h^{-1}) being 0.7–0.6. The CAT activity of the cells grown in this medium without NaCl was minimal, but it was remarkably increased by addition of NaCl; the activity of the cells in medium containing 50 mM NaCl was about 30-fold higher than the value in the absence of NaCl (Fig. 3A). The CAT activity slightly decreased at higher NaCl concentrations. Lithium was very toxic for the growth of *E. hirae*. The growth rate of the transformant in TrisKM medium containing 50 mM LiCl was 0.15 (h^{-1}), and the cell growth stopped at 100 mM LiCl. In contrast to the case of the addition of NaCl, CAT activity of the lysates was not detected even by addition of up to 50 mM LiCl (Fig. 3A). No active extrusion of Na^+ or Li^+ was observed with 7683 [15], meaning that the cytoplasmic Na^+ (Li^+) concentration is expected to be similar to that in medium. The effect of these

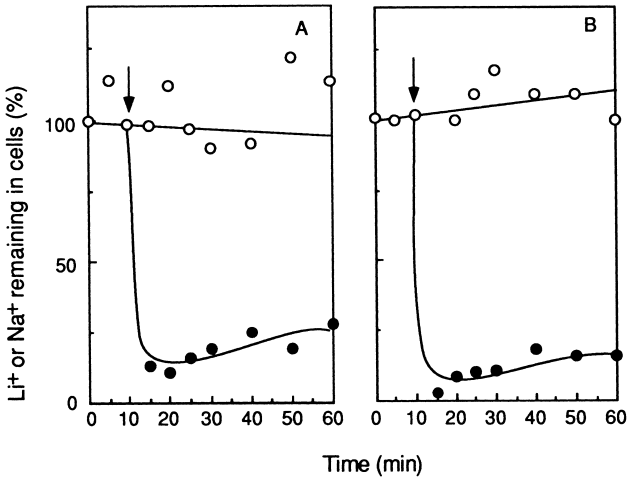

Fig. 2. Extrusion of lithium or sodium ions from *E. hirae* cells. Strain WD4 defective in NapA Na^+/H^+ antiporter was cultured in NaTY medium, washed twice with 2 mM $MgSO_4$ and suspended in 50 mM Tris-maleate (pH 7.5) containing 400 mM KCl. After loading with 20 mM LiCl (A) or 20 mM NaCl (B) for 60 min, cells were divided into aliquots, and the cellular contents of Li^+ and Na^+ were determined by atomic absorption as described in Section 2. Glucose (10 mM) was added at the time indicated by the arrow. Symbols: ○, control; ●, glucose.

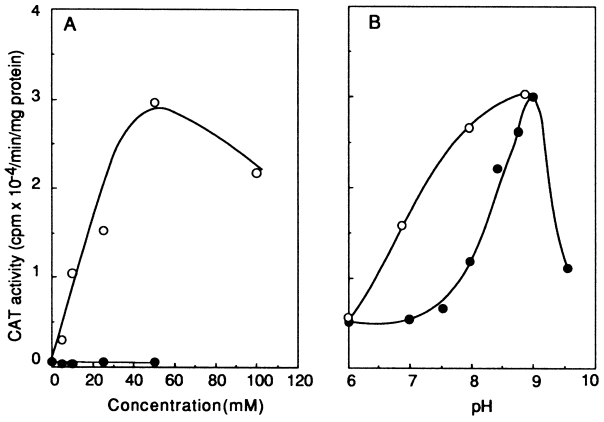

Fig. 3. CAT activities of cells cultured in various media. Strain 7683/pHECAT1 was cultured in TrisKM medium containing various concentrations of NaCl (○) or LiCl (●) (A) or in KTY medium at different pHs in the presence (●) or absence (○) of gramicidin D (5 μg/ml) (B). The cell lysates were prepared and the CAT activities of the lysates were determined as described in Section 2.

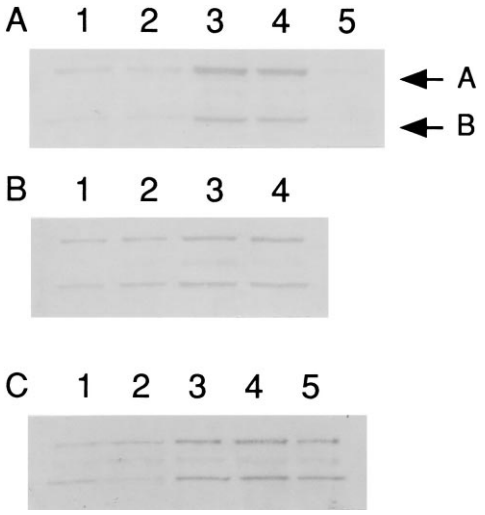

Fig. 4. Western blotting of the cell lysates with anti-*E. hirae* V-ATPase serum. Denatured gel electrophoresis (13.5% gel) of the cell lysates (3 μg), prepared from cells cultured in various media as described in the experiments of Fig. 3, was performed. Blotting and visualization was performed as described elsewhere [8]. The arrows indicate the A and B subunits of V-ATPase, respectively. A: Effect of LiCl or NaCl. Lane 1, 20 mM LiCl; lane 2, 10 mM LiCl; lane 3, 20 mM NaCl; lane 4, 10 mM NaCl; lane 5, control. B: Effect of medium pH in the absence of gramicidin D. Lane 1, pH 6; lane 2, pH 7; lane 3, pH 8; lane 4, pH 9. C: Effect of medium pH in the presence of gramicidin D. Lane 1, pH 6; lane 2, pH 6.5; lane 3, pH 7.7; lane 4, pH 9; lane 5, pH 9.5.

salts on the amounts of V-ATPase subunits in 7683/pHECAT1 cells was investigated by Western blotting with anti-V-ATPase serum (Fig. 4A). We have observed that expression of the *ntp* operon and assembly of the V-ATPase complex were normal in 7683 although it was functionally inactive (data not shown). An increase in the amount of V-ATPase in this strain was significant by culturing in media containing NaCl but not in those containing LiCl. Other salts such as KCl or CsCl at a concentration of 30 mM did not affect either CAT activity or the amount of ATPase subunits in 7683/pHECAT1.

We also examined *ntp* promoter activity in 9790/pHECAT1 cultured in the same defined medium in the presence of gramicidin D, making the cell membrane freely permeable to monovalent cations. The CAT activity was also induced by addition of NaCl but not by LiCl in media. An increase in the amount of V-ATPase in 9790 was significant by culturing in media containing gramicidin D and NaCl, but not LiCl (data not shown). From these results it is suggested that expression of the *ntp* operon is stimulated preferentially by Na^+ but not by other monovalent cations.

3.3. The ntp promoter is stimulated by elevating the medium pH

Since (i) the *ntpJ* gene product, one of the components of the KtrII K^+ transport system, is important for potassium accumulation under the culture condition where proton potential is not generated [9], and (ii) establishment of proton potential of *E. hirae* is negligible at alkaline external pH [6,21], it is likely that the *ntp* promoter is affected by a change in medium pH. Fig. 3B shows the effect of medium pH on the CAT activity of 7683/pHECAT1. Cells were cultured in KTY medium at different pHs in the presence or absence of gramicidin D; KTY medium contained 120 mM K^+ and 15 mM Na^+. In the absence of the ionophore, this strain grew well in this medium from pH 6 to pH 10. The CAT activity increased in response to an elevation of external pH (Fig. 3B); the activity at pH 9.0 was about 6-fold the value at pH 6.0. The cellular contents of Na^+ and K^+ in these 7683/pHECAT1 cells growing under the same condition were determined (Table 1). The Na^+ contents in cells grown at pHs 7.0–9.0 were relatively constant although the Na^+ content in cells grown at pH 6.0 was slightly lower than others. The internal K^+ contents in cells grown at different pHs were maintained at high levels. These results suggest that induction of the CAT activity at high pH did not result from an increase in $[Na^+]_{in}$.

The addition of gramicidin D in KTY medium did not inhibit the growth of 7683 around pH 7.5–8.0 but inhibited that at pH 6.0 and 9.0; the growth rates (h^{-1}) at pH 6.0 and 9.0 were 0.3 and 0.2, respectively. The CAT activities at pHs 7–8 in the presence of the ionophore were kept low in contrast to those in its absence (Fig. 3B). The CAT activity around pH 9 in the presence of the ionophore was high, equal to that in its absence (Fig. 3B). The internal Na^+ contents varied slightly from pH 6.0 to pH 9.0, which is insignificant. The K^+ contents in the presence of gramicidin D were particularly

Table 1
Effect of external pH on the cellular contents of Na^+ and K^+

Medium pH	Addition	Contents (μmol/mg protein) of	
		Na^+	K^+
A	None		
6.0		0.13 ± 0.03	2.72 ± 0.53
7.0		0.24 ± 0.03	2.31 ± 0.13
8.0		0.20 ± 0.08	2.16 ± 0.47
9.0		0.20 ± 0.03	2.19 ± 0.17
B	Gramicidin D[a]		
6.0		0.24 ± 0.05	1.34 ± 0.03
7.0		0.19 ± 0.43	1.54 ± 0.21
8.0		0.22 ± 0.08	2.16 ± 0.02
9.0		0.31 ± 0.03	2.64 ± 0.01

Cells were cultured in the same media as shown in Fig. 3, and the cation contents were determined as described in Section 2. The data from duplicate experiments ± S.E.M. are represented.
[a]5 μg/ml.

lower at acidic pHs although the reason is unclear and under investigation. Western blotting experiment revealed that the amounts of Na^+-ATPase increased, whether or not gramicidin D was present in the medium, predominantly at high pHs (Fig. 4B,C). These results suggest that expression of the *ntp* operon is also affected by pH, independent of the internal sodium level.

4. Discussion

In this paper we found that the response of the *ntp* promoter was specific to Na^+ ions, not to other monovalent cations, and, interestingly, expression of the operon was stimulated by elevating the medium pH. This notion was supported by the observation that the amount of V-ATPase subunits was increased by elevating medium Na^+ concentration or medium pH (Fig. 4). We did not examine the effect of high pH on *ntp* promoter activity in the absence of Na^+, since *E. hirae* grew in Na^+-depleted TrisKM medium within a narrow pH range from pH 6 to 7.5. Therefore, it is unknown if a high pH response of the promoter needs the presence of sodium ion.

Li^+ is toxic for cell physiology. The glycolytic activity was inhibited by Li^+ loading in *E. hirae* [22]; pyruvate kinase may be involved in Li^+ sensitivity as reported in *E. coli* [23]. It is reported that the NapA Na^+/H^+ antiporter recognizes Li^+ as well as Na^+ [24]. Transcription of the *napA* gene was induced at high concentrations of LiCl as well as NaCl in the media [25]. Therefore, it is likely that the NapA antiporter works for lithium detoxification. However, the NapA antiporter does not work for lithium extrusion at high pH where the proton potential was negligible [6]. Instead, V-ATPase works for Li^+ extrusion at alkaline pH. The *ntp* operon encoding Li^+-transporting ATPase, a powerful machinery for lithium detoxification, does not respond to the increase of $[Li^+]_{in}$ as shown in Section 3. We think that the alkaline pH, instead of $[Li^+]_{in}$, works as the signal for induction of the operon which detoxifies lithium.

The sodium energetics of *E. hirae* is still unclear, because an Na^+-linked transport system has not been found in this bacterium. The size of the Na^+ electrochemical gradient, more than –150 mV, generated by V-ATPase in this bacterium probably provides the driving force for uncovered Na^+-linked energy-consuming systems [13]. As strain WD4 grew well in high Na^+ media with a broad pH range from acidic to alkaline pHs, we concluded that V-ATPase is the main sodium extrusion system and the NapA antiporter is the supplementary one in *E. hirae* [20]. We think that sodium-dependent induction of V-ATPase is to generate the Na^+ gradient as the driving force. Even if external Na^+ is low, the response to high pH of the *ntp* operon is also physiologically important; the KtrII K^+ transport system, encoded by the *ntp* operon, works for K^+ accumulation under the condition where proton potential is limited [14]. Thus a dual response of the *ntp* operon to sodium and pH is physiologically important for sodium circulation, potassium accumulation and lithium detoxification of this bacterium.

The cytoplasmic pH of 7683 growing in KTY medium at pH 6–9 was maintained at about 7–8. Addition of gramicidin D induced the internal pH close to the value of the external pH [21]. The CAT activities at pHs 6.0 and 9.0 (Fig. 3B) were not changed by addition of gramicidin D, suggesting the possibility that the *ntp* promoter responded not to the internal pH but to the medium pH. It is interesting that the CAT activity decreased significantly by addition of gramicidin D at pH 7–8. Other factors such as the size of proton potential (or membrane potential) may affect the *ntp* promoter activity, but the details require further investigation.

There are three palindromic sequences in the 5′ untranslated region of the *ntp* promoter [7](Fig. 1), which are probably linked with transcriptional regulation of the operon. We are now working on identifying the specific DNA site for regulation and isolating mutants defective in regulation of expression of the *ntp* operon.

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