β -Galactoside Transport in an Alkaline-tolerant Strain of *Bacillus circulans*

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An alkaline-tolerant bacterium, which grew on various carbohydrates between pH 6 and 9.5, was isolated from soil and identified as *Bacillus circulans*. Lactose-grown organisms exhibited a transmembrane pH gradient (ΔpH) of -47 mV at pH 6.6, but no ΔpH at pH 9.0. The transmembrane electrical potential ($\Delta\psi$) was -66 mV at pH 6.6 and -115 mV at pH 9.0. Thus the total protonmotive forces at the two pH values were essentially the same. Lactose-grown organisms transported thiomethyl β -D-galactopyranoside (TMG) at pH 6.6 and at pH 9.0, but transport at the alkaline pH was dependent upon addition of ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) or preincubation with lactose. In the presence of ascorbate/TMPD, the TMG transport system exhibited similar kinetics and substrate specificities at pH 6.6 and pH 9.0, and resulted in accumulation of chemically unmodified TMG to a concentration approximately 180 times greater than the external concentration. Experiments in which a diffusion potential was generated in starved organisms or in which organisms were treated with nigericin indicated a lack of correlation between the rate of TMG uptake and the magnitude of $\Delta\psi$. By contrast, the rate of TMG uptake correlated with cellular ATP levels in organisms incubated at different pH values and in organisms treated with *N,N'*-dicyclohexylcarbodiimide, arsenate or nigericin.

INTRODUCTION

Bacterial processes such as motility (Larsen *et al.*, 1974; Thipoyathasana & Valentine, 1974), active transport (Kashket & Wilson, 1974; Pavlasova & Harold, 1968; West & Mitchell, 1972) and ATP synthesis (Maloney *et al.*, 1974; Tsuchiya & Rosen, 1976; Tsuchiya, 1977) depend on the presence of an activated membrane state. It is now generally believed that the activated state of the membrane consists of a protonmotive force (p.m.f.; Mitchell, 1961, 1963). According to Mitchell's chemiosmotic hypothesis, the p.m.f. is generated by the extrusion of protons during the oxidation of substrates via the respiratory chain and during ATP hydrolysis. This results in the acidification of the external *milieu*, generating a pH gradient across the cell membrane (ΔpH , interior alkaline). Moreover, the unequal distribution of protons results in the establishment of a transmembrane electrical potential ($\Delta\psi$, interior negative). Indeed, the extrusion of protons has been shown to occur when substrates are oxidized by the respiratory chain (Hertzberg & Hinkle, 1974; Scholes & Mitchell, 1970) or on hydrolysis of ATP by bacterial (Ca^{2+} , Mg^{2+})-ATPase (Hertzberg & Hinkle, 1974; West & Mitchell, 1974). Synthesis of ATP occurs in response to either a ΔpH or a $\Delta\psi$ of sufficient magnitude (Wilson *et al.*, 1976), and various respiration-coupled transport systems have been shown to respond directly to either the ΔpH or the total p.m.f. (Flagg & Wilson, 1977; Ramos & Kaback, 1977*a, b*). Another category of active transport systems, usually lost on osmotic shock, are believed to be coupled in some direct way to the phosphate bond energy of ATP (Berger, 1973; Berger & Heppel, 1974; Klein & Boyer, 1972).

The ATP can be produced from glycolysis, but in aerobes would primarily arise from the vectorial action of a p.m.f. on the membrane-bound ATPase.

Can an organism which grows in an alkaline environment utilize a p.m.f. generating a large enough electrical potential to compensate for the lack of a proton gradient or even a 'reversed' ΔpH ? Or will such an organism subsist on the ATP produced fermentatively, and utilize a phosphotransferase system and/or ATP-dependent transport mechanisms for solute transport? In an attempt to answer these questions, work was initiated on alkalophilic and alkaline-tolerant *Bacillus* species. In a truly alkalophilic species, *Bacillus alcalophilus*, growth on L-malate and transport of α -aminoisobutyric acid (AIB) occurred at pH values up to 11.5 and were optimal at pH 10.5. In that highly alkaline pH range, *B. alcalophilus* maintained a large ΔpH , interior acid. While the $\Delta\psi$ was also large, and increased with increasing pH, the total p.m.f. was very low. Transport of AIB occurred by electrogenic symport with sodium ions in response to the $\Delta\psi$ (Guffanti *et al.*, 1978). The studies reported here were conducted on a newly isolated, alkaline-tolerant strain of *Bacillus circulans*. This obligate aerobe grew in media ranging from pH 6 to 9.5. At the lower end of its pH range, both a ΔpH and $\Delta\psi$ were demonstrable. At the higher pH values, ΔpH was zero (but not 'reversed') and $\Delta\psi$ was significantly higher than at lower pH values. Studies of β -galactoside transport indicated that ATP is directly involved in the energization of this transport system.

METHODS

Organism and growth conditions. A bacterium which was able to tolerate alkaline pH was isolated from soil by enrichment culture techniques, using sequential transfers in liquid medium and final single colony isolation from solid medium. The medium employed for enrichment as well as for subsequent growth and maintenance contained 25 mM-Tris, 25 mM-potassium phosphate, 7.6 mM- $(\text{NH}_4)_2\text{SO}_4$ and 0.1 mM- MgSO_4 , adjusted to pH 9.0. This basal medium, referred to as PT9, was completed by the aseptic addition of sterile solutions of trace salts (Hegeman, 1966) to a final concentration of 1% (v/v), 0.1% (w/v) yeast extract and carbon source (25 mM for disaccharides, 50 mM for monosaccharides). An identical medium, adjusted to pH 6.6 is referred to as PT6.6. Lactose was the carbon source used for enrichment and for most of the experiments described here. Organisms were grown at 30 °C on a New Brunswick G25 rotatory shaker with continuous shaking at 200 rev. min⁻¹. Growth was monitored turbidimetrically with a Klett-Summers colorimeter (no. 42 filter). The ability of the organism to grow was determined as a function of pH. Since PT9 became somewhat more acid even when the buffer concentration was doubled, only the initial growth rate was used. The organism grew well between pH 6.5 and 9.5 with either lactose or glucose as the carbon source.

Identification of the alkaline-tolerant organism. The bacterium isolated at high pH was an aerobic, rod-shaped, Gram-positive, peritrichously flagellated, spore-forming organism identified as *Bacillus circulans* on the basis of its morphological and physiological characteristics (Buchanan & Gibbons, 1974; Gordon *et al.*, 1973). It appeared to have amounts of loose capsular material adhering to its surface. The G+C content, as estimated by the temperature melt method of Marmur & Doty (1959), was 50.5% when determined on DNA extracted by the method of Marmur (1961). In media with NH_4^+ as nitrogen source and adjusted to pH 9 or pH 6.6, the organism grew on carbohydrates such as arabinose, fructose, galactose, gluconate, glucose, glycerol, lactose, maltose, mannitol, mannose, rhamnose, sucrose or xylose. Organic acids and amino acids were not utilized as carbon sources.

Chemicals. Lysozyme (EC 3.2.1.17; egg white), *N,N*-dicyclohexylcarbodiimide (DCCD), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), isopropyl β -D-thiogalactoside (IPTG), *o*-nitrophenyl β -D-galactoside (ONPG), firefly tails, ATP (disodium salt) and chloramphenicol were obtained from Sigma. Valinomycin was purchased from Calbiochem. Amersham-Searle provided 4-*O*- β -D-galactopyranosyl-D-[1-¹⁴C]-glucopyranose ([¹⁴C]lactose) and New England Nuclear provided [G-³H]inulin, [¹⁴C]methylamine hydrochloride, 5,5-dimethyl-[2-¹⁴C]oxazolidine-2,4-dione (DMO) and [¹⁴C]methyl β -D-thiogalactoside ([¹⁴C]TMG). Nigericin and [³H]triphenylmethylphosphonium bromide (TPMP⁺) were generously provided by Dr H. R. Kaback. All other chemicals were obtained commercially at the highest purity available.

Measurement of lactose and TMG uptake. Organisms growing exponentially on PT9 with lactose as carbon source were washed twice in PT basal medium of the appropriate pH and suspended in the same medium. Cell suspensions (about 0.1 mg protein ml⁻¹) were treated with chloramphenicol (40 $\mu\text{g ml}^{-1}$) for 10 min at 25 °C prior to uptake experiments. Either 20 μM -[¹⁴C]lactose (10 $\mu\text{Ci } \mu\text{mol}^{-1}$) or 200 μM -[¹⁴C]TMG (0.33 $\mu\text{Ci } \mu\text{mol}^{-1}$) was added to aerated cells at 25 °C to start the reaction. Suspensions were aerated by

rapid stirring during the incubations with radioactive substrate. Samples (1 ml) were taken at appropriate intervals, quickly filtered through Matheson-Higgins filters (pore size 0.45 μm) and washed with 10 ml buffer. The filters were dried and placed in glass scintillation vials. Scintillation cocktail (10 ml Econofluor; New England Nuclear) was added to each vial, and radioactivity was measured with a Beckman LS-230 spectrometer.

Extraction and identification of accumulated TMG. Organisms were allowed to accumulate [^{14}C]TMG for 30 min, after which they were centrifuged, washed and resuspended in 0.2 ml 5% (v/v) butanol, as described by Egan & Morse (1966). The radioactive extract was spotted on Whatman no.1 filter paper and descending chromatography was performed with 1-butanol/acetic acid/water (2:1:1, by vol.; Winkler & Wilson, 1966).

Determination of ΔpH and $\Delta\psi$. Intracellular water volume was determined by the method of Maloney *et al.* (1975), using [^3H]inulin (270 Ci g^{-1}). The internal water volume was calculated to be 3.4 μl (mg protein) $^{-1}$. Protein was determined by the method of Lowry *et al.* (1951), using lysozyme as a standard.

The ΔpH was measured by uptake of a weak acid or weak base using flow dialysis in an apparatus modified according to Ramos *et al.* (1976). Organisms were washed twice in the appropriate basal medium (PT9 or PT6-6) and pretreated with 1 mM-EDTA for 10 min in a variation of the method of Padan *et al.* (1976). The cells were then washed free of EDTA and resuspended (final concentration 10 to 15 mg protein ml^{-1}) in the upper chamber of the flow dialysis apparatus. The intracellular cell volume occupied 4 to 5% of the total volume of 800 μl in the upper chamber. Either the weak acid [^{14}C]DMO (8.8 mCi mmol^{-1}) or the weak base [^{14}C]methylamine (52.2 mCi mmol^{-1}) was added to a final concentration of 141 μM or 53 μM , respectively. Flow dialysis was performed exactly as described by Ramos *et al.* (1976). Control experiments were carried out with heat-killed organisms to obtain values for non-specific binding of DMO or methylamine. In those experiments in which a ΔpH was demonstrated using DMO, addition of 0.2 μM -nigericin completely abolished DMO uptake. The internal pH was calculated according to the method of Waddell & Butler (1959) and ΔpH was expressed as the difference between internal and external pH.

The electrical potential across the membrane was determined by measuring the accumulation of the lipophilic cation TPMP $^{+}$, using a filtration assay (Schuldiner & Kaback, 1975). [^3H]TPMP $^{+}$ (45 mCi mmol^{-1}) was added to a washed cell suspension (0.1 mg protein ml^{-1}) to a final concentration of 25 μM . Suspensions were gassed with water-saturated oxygen and steady-state concentration values from TPMP $^{+}$ uptake experiments were used to calculate $\Delta\psi$ from the Nernst equation:

$$\Delta\psi = 58.8 \log \frac{[\text{TPMP}^{+}]_{\text{in}}}{[\text{TPMP}^{+}]_{\text{out}}}$$

Values for non-specific binding were determined from zero-time points. The total p.m.f. was calculated by inserting the values for ΔpH and $\Delta\psi$ into the equation: $\text{p.m.f.} = \Delta\psi - Z\Delta\text{pH}$.

Measurement of ATP. Exponentially growing organisms were washed with PT9 or PT6-6 and ATP was extracted with 30% (v/v) perchloric acid according to the method of Cole *et al.* (1967). The ATP was measured by the firefly assay in a Beckman LS-230 spectrometer with the coincidence switch off, as described by Stanley & Williams (1969). On each day a new ATP standard was determined and a fresh firefly lantern extract was prepared.

RESULTS

The p.m.f. was determined in organisms of the alkaline-tolerant *B. circulans* strain which had been grown on lactose at pH 9.0, then washed and resuspended at pH 6.6 or pH 9.0. At pH 6.6, these organisms exhibited only a small ΔpH which was increased by the addition of ascorbate/TMPD. No ΔpH was observed at an external pH of 9.0 (Table 1). At more alkaline pH values, up to pH 10.0, the ΔpH remained zero; no 'reversed' ΔpH (i.e. interior acid) was found (data not shown). The $\Delta\psi$ was significantly higher at pH 9.0 than at pH 6.6, and was slightly elevated by the addition of ascorbate/TMPD at both pH values. Because of the higher $\Delta\psi$ at pH 9.0, the total p.m.f.s at the two pH values were not significantly different even though a ΔpH was generated only at pH 6.6.

A study of β -galactoside transport by lactose-grown cells was then undertaken. Since the *B. circulans* strain used only carbohydrates for growth, we first investigated whether a phosphotransferase system might be employed for transport, thus circumventing the need for respiration-derived energy. All attempts to detect a phosphoenolpyruvate:hexose phosphotransferase system by the method of Tanaka *et al.* (1967) were negative.

In preliminary experiments, *B. circulans* took up TMG at pH 6.6, but showed no TMG transport at alkaline pH. Lactose accumulation was linear with time at pH 6.6, but at pH 9.0

Table 1. *pH gradient and electrical potential across the membrane of lactose-grown B. circulans at pH 6.6 and 9.0*

Organisms grown on PT9/lactose were washed twice and resuspended in PT9 or PT6.6. ΔpH and $\Delta\psi$ were measured in the absence or presence of 20 mM-ascorbate plus 2 mM-TMPD (designated AT). The ΔpH was determined by the distribution in flow dialysis of [^{14}C]DMO at pH 6.6 and 9.0. [^{14}C]Methylamine uptake was also assayed at pH 9.0. The membrane potential was measured by the accumulation of [^3H]TPMP $^+$.

External pH	Internal pH		ΔpH (mV)		$\Delta\psi$ (mV)		P.m.f. (mV)	
	Without AT	With AT	Without AT	With AT	Without AT	With AT	Without AT	With AT
6.6	7.4	7.7	-47	-71	-66	-83	-113	-154
9.0	9.0	9.0	0	0	-115	-138	-115	-138

the rate of accumulation increased dramatically with time. Preincubation (30 min, 30 °C) in PT9 containing 25 mM-lactose abolished the accelerating kinetics at alkaline pH. Addition of ascorbate/TMPD mimicked the effects of preincubation with lactose. In the presence of the artificial electron donor, the initial rate of lactose accumulation at alkaline pH was stimulated and the accelerating kinetics were abolished (Fig. 1). Moreover, in the presence of ascorbate/TMPD, TMG uptake was observed at pH 9.0. The initial rate of TMG uptake exhibited at pH 9.0 in the presence of ascorbate/TMPD was the same as that found at pH 6.6 either with or without ascorbate/TMPD (Fig. 2). In most of the subsequent transport experiments, ascorbate/TMPD was included in the reaction mixtures.

Uptake of TMG was examined over a range of TMG concentrations from 1 μM to 1 mM at both pH values, but only one saturable system was observed. The apparent K_m at pH 9.0 or 6.6 was 100 to 150 μM . When an external concentration of 200 μM -TMG was used and the steady-state level of TMG accumulation was measured in the presence of ascorbate/TMPD, cells were found to concentrate TMG 185-fold at pH 6.6 and 175-fold at pH 9.0 over the concentration in the medium.

In initial determinations of the inhibition of TMG uptake by lactose, a stimulatory effect (25%) was found at low lactose concentrations (1 μM , 2 μM), possibly due to inhibition of efflux. Inhibition of TMG uptake by higher concentrations of lactose (10 or 20 μM) was competitive, with an apparent K_i of 7.7 μM (data not shown). Both IPTG and ONPG inhibited TMG uptake at pH 9.0 and 6.6 to approximately the same extent (Table 2). Galactose and melibiose also inhibited considerably when added at concentrations 10 times that of TMG. Other sugars tested showed less than 30% inhibition.

To eliminate the possibility that TMG was trapped inside the organisms by an enzymic conversion, organisms were allowed to accumulate the radioactively labelled analogue, and then excess non-radioactive TMG was added to half the suspension. The accumulated TMG was rapidly displaced (data not shown). Intracellular radioactivity accumulated during uptake of [^{14}C]TMG (extracted from the organisms by butanol) co-chromatographed with a reference sample of authentic [^{14}C]TMG.

The mechanism of energization of the TMG transport system was investigated. It was expected that in the absence of a phosphotransferase system, the p.m.f. and/or the presence of ATP *per se* would be required for TMG transport. First the role of the $\Delta\psi$ was examined. Lactose-grown organisms were starved by incubation for 30 min with 10 mM- NaN_3 plus 40 μg chloramphenicol ml^{-1} and then incubated in 50 mM-Tris/HCl, pH 6.6 or pH 9.0, in the presence of 100 mM-KCl and 2 μM -valinomycin. After incubation for 1 h, each suspension was diluted into KCl-free or KCl-containing Tris/HCl buffer at pH 6.6 or pH 9.0 containing 10 mM- NaN_3 . A diffusion potential, resulting from valinomycin-mediated K^+ efflux, was expected to be generated in the K^+ -free but not in the K^+ -containing suspensions. A

Fig. 1

Fig. 2

Fig. 1. Lactose accumulation at pH 9.0 in the presence or absence of an artificial electron donor. Organisms grown on PT9/lactose were assayed for lactose uptake (20 μ M final concentration) in PT9 in the absence (●) or presence (○) of 20 mM-ascorbate plus 2 mM-TMPD.

Fig. 2. Transport of TMG at pH 9.0 and pH 6.6 in the presence or absence of an artificial electron donor. Organisms grown on PT9/lactose were assayed for TMG uptake (200 μ M final concentration) in PT9 (●, ○) or PT6.6 (▲, △) in the absence (●, ▲) or presence (○, △) of 20 mM-ascorbate plus 2 mM-TMPD.

Table 2. *Specificity of TMG transport at pH 6.6 and 9.0*

Washed organisms were preincubated for 2 min with the indicated additions (final concentration 2 mM) before uptake of [14 C]TMG (final concentration 200 μ M) was measured in the presence of ascorbate/TMPD. Transport of TMG by control suspensions (no additions) was 15.0 nmol min $^{-1}$ (mg protein) $^{-1}$ at pH 6.6 and 16.5 nmol min $^{-1}$ (mg protein) $^{-1}$ at pH 9.0.

Addition	Inhibition (%)	
	pH 6.6	pH 9.0
Lactose	96	91
ONPG	70	77
IPTG	67	58
Melibiose	74	82
Galactose	46	60
Glucose	26	28
Cellobiose	24	20
Sucrose	26	12
Maltose	20	7
Methyl α -glucoside	14	10
Mannose	0	0
Fructose	0	0

significant diffusion potential was observed at both pH values, as indicated by TPMP $^{+}$ uptake (Fig. 3). However, the rate of TMG uptake, which was markedly lowered by the azide treatment, was not stimulated by the increase in the $\Delta\psi$.

Abolition of a transmembrane Δ pH by nigericin causes an increase in the $\Delta\psi$ in isolated membrane vesicles (Ramos *et al.*, 1976). No evidence for a proton symport mechanism for TMG uptake was found in the *B. circulans* strain; addition of TMG did not diminish the Δ pH at pH 6.6 and alkalization of the medium did not occur concomitantly with TMG uptake (data not shown). Treatment of the organisms with nigericin abolished the Δ pH at pH 6.6, as monitored by DMO uptake. There was a slight concomitant increase in the $\Delta\psi$, from -116 to -128 mV, while cellular ATP levels declined from 4.8 to 1.6 nmol (mg protein) $^{-1}$.

Fig. 3. Effect of valinomycin-induced K^+ diffusion potential on the uptake $200 \mu M$ - $[^{14}C]$ TMG and $25 \mu M$ - $[^3H]$ TPMP $^+$. Organisms grown on PT9/lactose to the late-exponential phase were washed and resuspended in 50 mM-Tris/HCl, pH 9.0 or 6.6. The suspensions were starved for 30 min at $30^\circ C$ in the presence of 10 mM- NaN_3 and $40 \mu g$ chloramphenicol ml^{-1} . The suspensions were then concentrated 100-fold by centrifugation and resuspension in 50 mM-Tris/HCl, pH 9.0 or 6.6, plus 100 mM-KCl, 2 μM -valinomycin and 10 mM- NaN_3 . After incubation at $30^\circ C$ for 1 h, the uptake experiments were initiated by a 100-fold dilution of the suspensions into 50 mM-Tris/HCl at pH 9.0 (a) or pH 6.6 (b), both containing 2 μM -valinomycin and either 100 mM-KCl (●, ▲) or 100 mM-NaCl (○, △), and TMG (●, ○) or TPMP $^+$ (▲, △) uptake was assayed.

Table 3. Effect of DCCD on $\Delta\psi$, cellular ATP content and TMG uptake

Organisms grown on PT9/lactose to the late-exponential phase were washed and resuspended in either PT6.6 or PT9. Suspensions were assayed for TMG uptake ($200 \mu M$ final concentration), $\Delta\psi$ and ATP in the presence of ascorbate (20 mM) and TMPD (2 mM) and either with or without 100 μM -DCCD. $\Delta\psi$ was determined by the distribution of $[^3H]$ TPMP $^+$.

External pH	ATP content [nmol (mg protein) $^{-1}$]		TMG uptake [nmol min $^{-1}$ (mg protein) $^{-1}$]		$\Delta\psi$ (mV)	
	With DCCD	Without DCCD	With DCCD	Without DCCD	With DCCD	Without DCCD
6.6	1.04	4.51	1.9	12.7	-82	-84
9.0	0.83	4.42	2.3	12.8	-99	-125

The rate of TMG uptake declined from 15.8 to 6.0 nmol min $^{-1}$ (mg protein) $^{-1}$, i.e. it correlated with the cellular ATP levels and not with the $\Delta\psi$.

Several other observations suggested that TMG uptake was directly dependent on ATP. Organisms incubated at pH 6.6 contained 1.17 nmol ATP (mg protein) $^{-1}$. Those treatments which facilitated TMG uptake at pH 9.0 also raised the cellular ATP levels. Thus, pre-incubation with lactose, as described earlier, caused a four- to five-fold increase in the ATP levels at pH 6.6 and an eight- to nine-fold increase in the ATP levels at pH 9.0. Incubation with ascorbate/TMPD also raised the cellular ATP levels at both pH 6.6 and 9.0 to 4.55 and 4.48 nmol ATP (mg protein) $^{-1}$, respectively. In the presence of ascorbate/TMPD, DCCD markedly inhibited TMG uptake; the cellular ATP levels, but not the $\Delta\psi$, were correspondingly lowered (Table 3). An effect of arsenate on cellular ATP levels could also be demonstrated; incubation with arsenate was effective only when carried out in the presence of growth substrate. Treatment of cells with arsenate in the presence of lactose did not affect the $\Delta\psi$, but prevented most or all of the increase in ATP levels that had been found previously on preincubation with lactose (Table 4). Under these conditions, the rate

Table 4. *Effect of arsenate on $\Delta\psi$, cellular ATP content and TMG uptake*

Organisms grown on PT9/lactose to the late-exponential phase were washed and resuspended in 50 mM-Tris/HCl, pH 9.0. The organisms were incubated with or without 10 mM-sodium arsenate for 10 min at 30 °C in the presence of 25 mM-lactose, trace salts, 0.1 % (w/v) yeast extract and chloramphenicol (40 μ g ml⁻¹). After washing and resuspending at pH 6.6 or 9.0, with or without arsenate, the suspensions were assayed for TMG uptake, $\Delta\psi$ and ATP. $\Delta\psi$ was determined by the distribution of [³H]TPMP⁺.

External pH	ATP content [nmol (mg protein) ⁻¹]		TMG uptake [nmol min ⁻¹ (mg protein) ⁻¹]		$\Delta\psi$ (mV)	
	With arsenate	Without arsenate	With arsenate	Without arsenate	With arsenate	Without arsenate
6.6	1.07	4.76	4.7	13.3	-98	-91
9.0	1.01	4.85	4.2	11.8	-141	-148

of TMG uptake was inhibited by over 50%. Prolonged incubation with arsenate caused more marked reductions in both cellular ATP and TMG uptake, but the longer treatments also caused some decrease in the $\Delta\psi$.

DISCUSSION

Little information beyond general growth characteristics is available concerning species of *Bacillus* that can grow at alkaline pH (Chislett & Kushner, 1961; Kushner & Lisson, 1959; Ohta *et al.*, 1975). Extensive work has been done on the uptake of organic acids (Bisschop *et al.*, 1975b) and amino acids (Bisschop *et al.*, 1975a; Clark & Young, 1974; Konings *et al.*, 1971; Konings & Freese, 1972) by *B. subtilis* in both whole organisms and membrane vesicles at near neutral pH. From the present study and our previous work on *B. alcalophilus* (Guffanti *et al.*, 1978), some possible generalizations begin to emerge. In both the true alkalophile and in the alkaline-tolerant *B. circulans* the cytoplasmic pH never rose above 9.0 to 9.5. It is not surprising that an upper cytoplasmic pH limit, compatible with life, should be in this range; both RNA stability and the pK values of protein amino groups would be relevant factors. The alkalophile could grow at even higher pH values, thus withstanding the bioenergetic constraints imposed by a reversed Δ pH. The alkaline-tolerant species, by contrast, did not grow above pH 9.0 to 9.5, and therefore was not subject to a reversed Δ pH. We are currently studying two additional alkaline-tolerant *Bacillus* species and one newly isolated alkalophilic *Bacillus* species; so far the same pattern of pH limits *vis à vis* external and cytoplasmic pH has been found. The basis for the difference between alkalophilic and alkaline-tolerant strains, such that only the former grow at pH values that necessitate maintenance of a reversed Δ pH, may include such factors as anti-porters which can establish a high $\Delta\psi$ at very alkaline pH values and enzymes for ATP synthesis, which, in the alkalophile, would have to function under conditions in which there was a negligible p.m.f. (Guffanti *et al.*, 1978). It is notable that both the alkaline-tolerant *B. circulans* and *B. alcalophilus* (Guffanti *et al.*, 1978) exhibited a rise in $\Delta\psi$ as the external pH was raised. By contrast, the $\Delta\psi$ of *Escherichia coli* (Padan *et al.*, 1976) and *Arthrobacter pyridinolis* (K. G. Mandel & T. A. Krulwich, unpublished results), which grow at neutral pH, remain constant over a range of pH.

The transport system for TMG in the alkaline-tolerant strain of *B. circulans* was clearly an active transport system which involved accumulation of the substrate, against a concentration gradient, by a saturable carrier dependent upon metabolic energy. The approximately 180-fold concentration of TMG above the external concentration compared favourably with TMG accumulation by *E. coli* (Winkler & Wilson, 1966). The transport system for TMG in *B. circulans* displayed an affinity for lactose, other β -galactosides and the α -galactoside melibiose, a specificity similar to that of the lactose permease system in *E. coli* (Kennedy, 1970).

Since *B. circulans* is an aerobe, it was particularly difficult to separate the p.m.f. and ATP as necessary sources of energy for transport. Several lines of evidence, however, indicated a direct requirement for ATP: (i) generation of a $\Delta\psi$ in starved organisms was not accompanied by a stimulation of TMG transport; (ii) treatment of cells with nigericin at pH 6.6 abolished the ΔpH with a concomitant increase in the $\Delta\psi$, while cellular ATP levels and the rate of TMG uptake were lowered; (iii) those conditions which facilitated transport at pH 9.0, i.e. preincubation with lactose or addition of ascorbate/TMPD, raised cellular ATP levels correspondingly; (iv) the stimulation of TMG transport by ascorbate/TMPD was abolished by DCCD, an ATPase inhibitor; and (v) treatment of cells with arsenate under conditions in which the $\Delta\psi$ was unaffected, lowered both the levels of cellular ATP and the rate of TMG transport. However, none of these experiments ruled out the possibility that a p.m.f. might be required for TMG transport in addition to ATP.

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