Biochimica et Biophysica Acta, 596 (1980) 118—128

BBA 78613

EFFECTS OF SALTS AND IONOPHORES ON PROLINE TRANSPORT IN A MODERATELY HALOPHILIC HALOTOLERANT BACTERIUM

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(Received May 22nd, 1979)

Key words: Halotolerance; Active transport; Proton motive force; Proline transport

Summary

The effect of salt on proline uptake in a moderately halophilic halotolerant bacterium was studied. Cells were grown either on low salt or high salt media. A correlation was found between the salt concentrations in the growth media and the optimal concentration for uptake. The uptake rate was stimulated 2—3-fold by NaCl, as compared to KCl.

The K_m, V and activation energies values for proline uptake, as well as the external pH effect, were similar in low-salt-grown cells and high-salt-grown cells. This suggests that the halotolerance of the transport system is not due to alterations of the system during growth at various conditions, but rather to its intrinsic ability to function under extreme environmental conditions.

The uptake was inhibited by cyanide and carbonyl cyanide *m*-chlorophenylhydrazone, but not by arsenate, indicating that the electrochemical proton gradient ($\Delta\overline{\mu}_{H^+}$), generated by respiration, is the main driving force for proline transport. In low-salt-grown cells, at pH 6.0, partial inhibition was exerted by nigericin or valinomycin, whereas at pH 8.0 the uptake was inhibited by valinomycin only. Similar, although less pronounced effects were found in high-salt-grown cells. The data suggest that at pH 6.0 proline transport is driven by $\Delta\overline{\mu}_{H^+}$ (composed of electrical potential ($\Delta\psi$) and pH gradient), whereas at pH 8.0 $\Delta\psi$ is the main driving force.

Procedures of pretreatment with EDTA were developed to enable the penetration of the ionophores into the cells.

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Moderately halophilic, halotolerant bacteria are unique in their ability to grow in the presence of an extremely wide range of NaCl concentrations [1]. The halotolerance of such organisms is explicable by two mechanisms, as suggested by Brown [2]: (a) their proteins are intrinsically better functional under extreme environmental conditions; (b) the intracellular milieu can be modified so that the inhibitory effect of the environment is diminished.

The halotolerance of peripheral systems might be expected, a priori, to be the product of the first mechanism. However, in the moderately halophilic, halotolerant bacterium Ba_1 [3], the halotolerance of the respiratory system, located at the cell membrane, has been ascribed to the second mechanism. At suboptimal salt concentrations, larger amounts of respiratory enzymes are produced which compensate for their low activity; at high salt concentrations, protective substances are involved in the resistance to salts [3—5]. It seemed, therefore, worthwhile to investigate the basis for the halotolerance of a membranal system in close contact with the external environment, such as a transport system.

The present study shows that the proline transport system in the halotolerant bacterium Ba_1 operates within a wide range of salt concentrations. The transport is driven by the proton motive force ($\Delta\overline{\mu}_{H^+}$) [6—10]. At pH 6.0 $\Delta\overline{\mu}_{H^+}$ is predominant, whereas at pH 8.0, the electrical potential ($\Delta\psi$), is the main driving force, irrespective of salt concentration.

Materials and Methods

Organism, culture media and buffers. A moderately halophilic halotolerant bacterium, Ba_1, isolated from the Dead Sea evaporation ponds by Rafaeli-Eshkol and Avidor [3] was used in this study. The bacterium is an obligatory aerobic, Gram-negative rod. Cultures were maintained on a solid medium consisting of 2.3% nutrient agar (Difco), 0.5% bacto agar (Difco), 100 mM $MgCl_2$, 4.0 M NaCl and 50 mM KCl. Growth media consisted of 0.8% nutrient broth (Difco), 100 mM $MgCl_2$ (sterilized separately), and either 50 mM of each NaCl and KCl for low-salt-grown cells or 1.5 M NaCl and 0.5 M KCl for high-salt-grown cells. The pH of the media was adjusted to pH 6.8 with NaOH. The bacteria were grown in Erlenmeyer flasks, with shaking at 37°C, until the cell suspensions had reached an absorbance of 0.5—0.6 as measured at 540 nm.

Buffered salt solutions contained 10 mM Hepes-10 mM maleate (pH 8.0 or pH 6.0, as indicated), 100 mM $MgCl_2$ and NaCl + KCl at designated molarities. The designated molarity of the latter two is the sum of their equal concentrations (e.g., 0.1 M salt specifies 50 mM each of NaCl and KCl), except for the 2.0 M concentration which consists of 1.5 M NaCl and 0.5 M KCl.

Proline uptake assay. Cells were harvested by centrifugation at 15 000 × *g* for 10 min at 4°C, washed twice in buffered salt solution containing NaCl and KCl at concentrations indicated and resuspended at a dilution of 1 : 50 (1 g. wet wt. per 50 ml of the same solution). Unless otherwise specified, the buffered salt solution for low-salt-grown cells was 0.1 M salt and for high-salt-grown cells 2.0 M salt. A standard assay mixture (1 ml) contained 10 mM

Hepes-10 mM maleate buffer (pH 8.0), 100 mM $MgCl_2$, 100 μg chloramphenicol, 20 mM glucose, 0.1 M salt for low-salt-grown cells or 2.0 M salt for high-salt-grown cells, and 200 μl of the 1 : 50 cell suspension (i.e., 0.3 mg protein). The reaction mixtures were incubated at 30°C in flat-bottomed flasks, to ensure adequate aeration. Proline uptake was initiated by the addition of [^{3}H]-proline (25 Ci/mol) at a final concentration of 50 μM. Samples of 200 μl were withdrawn at 15, 30, 60 and 120 s, filtered through a membrane filter of 0.45 μm pore size (Sartorius) and immediately washed with 5 ml of the corresponding buffered salt solution. The filters were dried, transferred into vials, scintillation fluid (Insta-gel, Packard) was added, and the radioactivity was counted in a liquid scintillation spectrometer. Radioactivity was plotted vs. time and the rate of uptake calculated from the slope, which was linear for at least during the first two min.

EDTA treatment. The EDTA treatment was performed according to Leive [11]. The low-salt-grown cells were washed twice with 100 mM Tris-HCl buffer (pH 8.0), containing 0.1 M salt, resuspended in the same solution and warmed to 37°C for 1 min. The EDTA was added to a final concentration of 0.5 mM (unless otherwise specified), and after 2 min shaking at 37°C, $MgCl_2$ was added to a final concentration of 100 mM. The cells were harvested by centrifugation, and resuspended at a 1 : 50 dilution in 10 mM Hepes-10 mM maleate buffer at pH indicated, containing 100 mM $MgCl_2$ and 0.1 M salt. The high-salt-grown cells were similarly treated with EDTA, but 0.3 M salt was used during the procedure.

Incubation with ionophores. Incubation with CCCP was performed directly without pretreatment of the cells. Before incubation with valinomycin or nigericin the cells were treated as follows: low-salt-grown cells were washed with buffered salt solution containing 10 mM Hepes-10 mM maleate (pH 8.0), 100 mM $MgCl_2$ and 2.0 M salt, and treated with EDTA as described above. The cells were then added to proline uptake mixtures, containing either nigericin or valinomycin at concentrations indicated. After 5 min preincubation at 30°C, [^{3}H]proline was added and sample were removed as before. The high-salt-grown cells were washed with buffered salt solution containing 10 mM Hepes-10 mM maleate (pH 8.0), 100 mM $MgCl_2$ and 0.3 M salt, and treated with EDTA as described above. The cells were then preincubated at 30°C for 5 min with either nigericin or valinomycin at 0.3 M salt and transferred to the proline assay mixtures containing 0.3 or 2.0 M salt.

Assay of nucleotides released from the cells. After incubation under the conditions described in the text, the cells were separated by centrifugation. Cold perchloric acid was added to the supernatant to a final concentration of 0.5 M, and the cell pellet resuspended in an equal volume of 0.5 M cold perchloric acid. After 20 min at 0°C and centrifugation, the absorbance of the extracts was measured at 260 nm.

Protein determination. Protein was determined according to Lowry [12].

Electron microscopy. Cells were washed and collected by centrifugation as before. The cell pellets were fixed with 5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) containing salt at concentrations indicated in the experiments. After 30 min at 4°C they were post-fixed for 1 h with 1% OsO_4 in the same buffer. The samples were then dehydrated by a series of graded alcohols

and embedded in Epon 812 [13]. Thin sections were cut with LKB ultramicrotome with the use of glass knives. The sections were stained on the grids with a saturated solution of uranyl acetate in 30% ethanol for 10 min at 55°C and post-stained with 0.03% lead citrate for 8 min at room temperature. The sections were examined with a Jeol 100-B electron microscope.

Materials. L-[3,4(*n*)-^{3}H]proline was purchased from the Radiochemical Centre, Amersham, England. Nigericin was the kind gift of C. Carmeli, Tel Aviv University. All materials used were of analytical grade.

Results

The effect of salt concentration on proline uptake

As may be seen in Fig. 1, proline uptake in low-salt-grown cells reached a maximal value at salt concentrations of 0.5 M. In high-salt-grown cells, uptake was maximal at salt concentrations of 1.0 M. In both types of cells, the maximal rate of uptake was dependent not only on the total concentration of salt, but also on the concentration of NaCl itself.

The reduction in proline uptake at low NaCl concentration was more pronounced in high-salt-grown cells at high salt concentration (Fig. 1). When NaCl was omitted from the external medium, the uptake was about 50% of that with NaCl, in both types of cells, at optimal salt concentrations.

The marked inhibition of proline uptake in low-salt-grown cells at 2.0 salt (Fig. 1) was most likely due to cell plasmolysis, as was revealed by electromicroscopy (Fig. 2). Reincubation of the plasmolyzed cells in 0.1 M salt for 30 min resulted in deplasmolysis and complete recovery of the uptake.

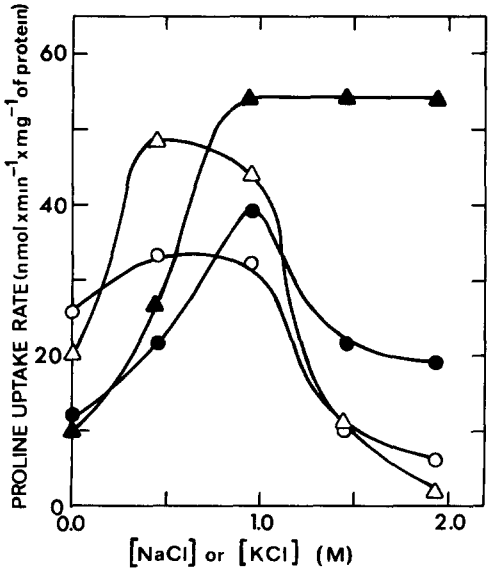

Fig. 1. The effect of NaCl and KCl concentration on proline uptake in low-salt-grown and high-salt-grown cells. The rate of proline uptake was measured in low-salt-grown (○, △) and high-salt-grown cells (●, ▲). The assay system (1 ml) contained either 50 mM NaCl and 0—1.95 M KCl (○, ●) or 50 mM KCl and 0—1.95 M NaCl (△, ▲). The procedure for uptake measurement was as described in Materials and Methods.

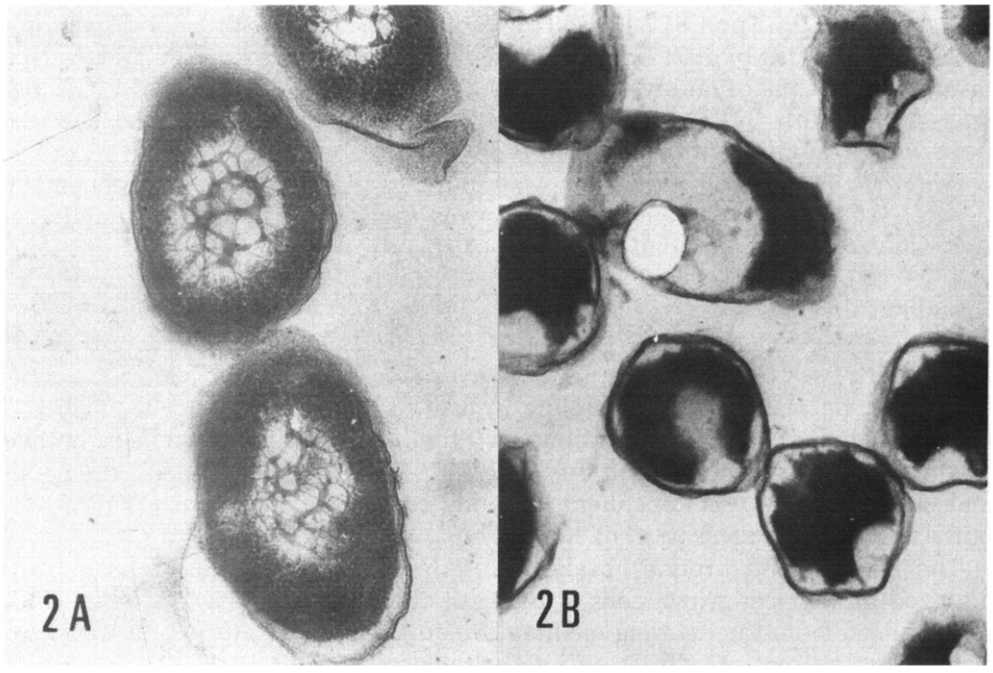

Fig. 2. Effect of salt concentration on the morphology of low-salt-grown cells. Cells were incubated for 30 min in 10 mM Hepes-10 mM maleate (pH 8.0) containing 100 mM $MgCl_2$ and the following concentrations of NaCl and KCl: (A) 50 mM each, (B) 1.5 M NaCl and 0.5 M KCl. Fixation with glutaraldehyde followed by OsO_4 was performed at the same salt concentrations. Preparations for electron microscopy were as described in Materials and Methods. Magnification: × 48 000.

The removal of Mg^{2+} had no effect on proline uptake at optimal salt concentrations; 0.8 M in the case of low-salt-grown cells, 2.0 M for high-salt-grown cells. However, at suboptimal salt concentrations (0.1 M for low-salt-grown cells and 0.3 M for high-salt-grown cells) Mg^{2+} depletion resulted in variable degrees of inhibition.

Kinetic parameters and characteristics of proline uptake

As shown in Table I, the K_m and V values for proline uptake for the low-salt-

TABLE I

THE EFFECT OF SALT CONCENTRATION ON KINETIC PARAMETERS OF PROLINE UPTAKE IN LOW-SALT-GROWN CELLS AND HIGH-SALT-GROWN CELLS

Procedure for proline uptake measurement was as described in Materials and Methods. Proline concentration was in the range of 1—500 μM. The K_m and V values were calculated according to Lineweaver and Burk.

Cells	NaCl (M)	KCl (M)	K_m (μM)	V (nmol · min^{-1} · mg^{-1} protein)
Low-salt-grown	0.05	0.05	5.3 ± 1.2 *	48 ± 6
	0.40	0.40	9.1 ± 1.9	63 ± 8
High-salt grown	0.40	0.40	8.8 ± 1.8	56 ± 7
	1.50	0.50	19.0 ± 3.2	91 ± 10

* Standard deviation of the mean.

grown cells and high-salt-grown cells were quite similar, when measured at the same salt concentration of 0.8 M. At salt concentrations identical to those of the growth media, 0.1 M for low-salt-grown cells and 2.0 M for high-salt-grown cells, the respective K_m and V values were different (Table I).

On the basis of the uptake rates at 10—45°C, activation energies were found to be 9.6 and 10.0 kcal per mol for low-salt-grown cells and high-salt-grown cells, respectively.

The effect of the external H^+ concentration on the rate of proline uptake was similar in low-salt-grown cells and high-salt-grown cells. An approximate 8-fold increase was found when the external pH was raised from 5.0 to 9.0.

The radioactivity taken up by the cells was virtually washed out by addition of excess of non-radioactive proline to the external medium, indicating that all the transported proline was not changed under the assay conditions.

Effect of EDTA treatment

Pretreatment with EDTA is essential for penetration of the ionophores, nigericin and valinomycin into Gram-negative bacterial cells [14].

When low-salt-grown cells were treated with EDTA at 0.1 M salt concentration, the rate of uptake decreased by 80%, even though Mg^{2+} was present in the reaction mixture. Washing of low-salt-grown cells with 2.0 M salt prior to EDTA treatment, lowered the latter's inhibitory effect to 10—20% (Table II). In high-salt-grown cells, EDTA treatment at 1.0—2.0 M salt concentrations stimulated the uptake by about 70%. At lower salt concentrations, 0.3—0.5 M, uptake decreased, and the stimulation exerted by EDTA was less pronounced (Table II). The decreased rate of uptake following EDTA treatment at low salt concentrations may be attributed to non-specific damage of the cell envelope. As shown in Table III, EDTA treatment of low-salt-grown cells and high-salt-

TABLE II

THE EFFECT OF EDTA TREATMENT ON PROLINE UPTAKE IN LOW-SALT-GROWN AND HIGH-SALT-GROWN CELLS

Procedures for EDTA treatment and proline uptake measurements were as described in Materials and Methods. The relative rate of 1.00 refers to 30 and 60 nmol · min^{-1} · mg^{-1} protein for low-salt-grown and high-salt-grown cells, respectively.

Cells	EDTA treatment	NaCl (M)	KCl (M)	Proline uptake (relative rate)
Low-salt-grown	—	0.05	0.05	1.00
	+			0.23
Low-salt-grown *	—	0.05	0.05	0.90
	+			0.86
High-salt-grown	—	1.50	0.50	1.00
	+			1.74
	—	0.50	0.50	1.00
	+			1.72
	—	0.25	0.25	0.84
	+			0.89
	—	0.15	0.15	0.57
	+			0.76

* LS-cells prewashed with 2.0 M salt.

TABLE III

THE EFFECT OF SALTS ON NUCLEOTIDE RELEASE DURING EDTA TREATMENT IN LOW-SALT-GROWN AND HIGH-SALT-GROWN CELLS

Procedures for EDTA treatment and nucleotide assay as described in Materials and Methods. 100% nucleotide refers to the sum of the pellet and supernatant optical densities.

Cells	NaCl (M)	KCl (M)	Treatment with EDTA (mM)	A_{260} (%)
Low-salt-grown			—	12
	0.05	0.05	0.05	69
			2.5	81
			—	11
	1.50	0.50	0.5	9
			2.5	15
High-salt-grown			—	19
	0.15	0.15	2.5	34
			10.0	42
			—	18
	1.50	0.50	2.5	20
			10.0	24

grown cells at 0.1 M and 0.3 M salt concentrations, respectively, resulted in the release of nucleotides from the cells. At 2.0 M salt concentration, only a very small amount of nucleotides was found in the medium.

A procedure of EDTA treatment was developed to enable penetration of nigericin and valimomycin into the cells, and at the same time cause minimal damage to the proline uptake system. The low-salt-grown cells were washed with 2.0 M salt, resuspended and treated with EDTA in 0.1 M salt, and then incubated with the ionophores. The high-salt-grown cells were washed with 0.3 M salt, treated with EDTA and incubated with the ionophores at the same salt concentration. The uptake was consequently measured either in 0.3 or 2.0 M salt.

The effects of the ionophores on proline uptake

In low-salt-grown cells titration studies with CCCP showed a marked decrease of proline uptake as the CCCP concentration increased (Fig. 3A). The effect was more pronounced at pH 6.0 than at pH 8.0. In the presence of 0.5 to 1.0 μM nigericin, at pH 6.0 the uptake rate decelerated to 25% of the control value. At pH 8.0 the uptake was not inhibited by nigericin, and at concentrations of 0.2 to 0.5 μM there was even slight stimulation (Fig. 3B). Valinomycin inhibited the uptake at both pH values, though its effect was more pronounced at pH 8.0 (Fig. 3C).

In high-salt-grown cells, at 2.0 M salt concentration, CCCP inhibition of proline uptake was similar to that in low-salt-grown cells (Fig. 4A). In the high-salt-grown cells at 0.3 M salt concentration, pH 6.0, nigericin at concentrations of 0.5 μM or greater produced only a partial inhibition of 70%. At pH 8.0, the inhibition was less pronounced (Fig. 4B). When high-salt-grown cells, preincubated with nigericin in 0.3 M salt concentration, were transferred to 2.0 M salt,

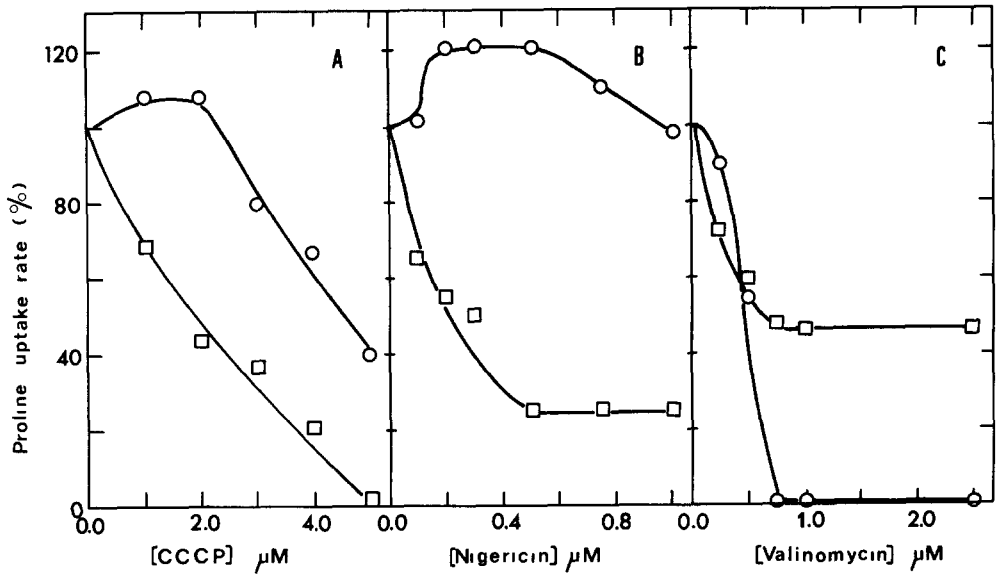

Fig. 3. Effects of ionophores on proline uptake in low-salt-grown cells. Reaction mixtures contained either CCCP (A), nigericin (B) or valinomycin (C). Preincubation with nigericin or valinomycin were carried out with EDTA-treated cells. The transport was measured either at pH 8.0 (○) or at pH 6.0 (□), as described in Materials and Methods. The relative rates of 100 refers to 7.5 and 33 nmol · min^{-1} · mg^{-1} protein at pH 6.0 and pH 8.0, respectively.

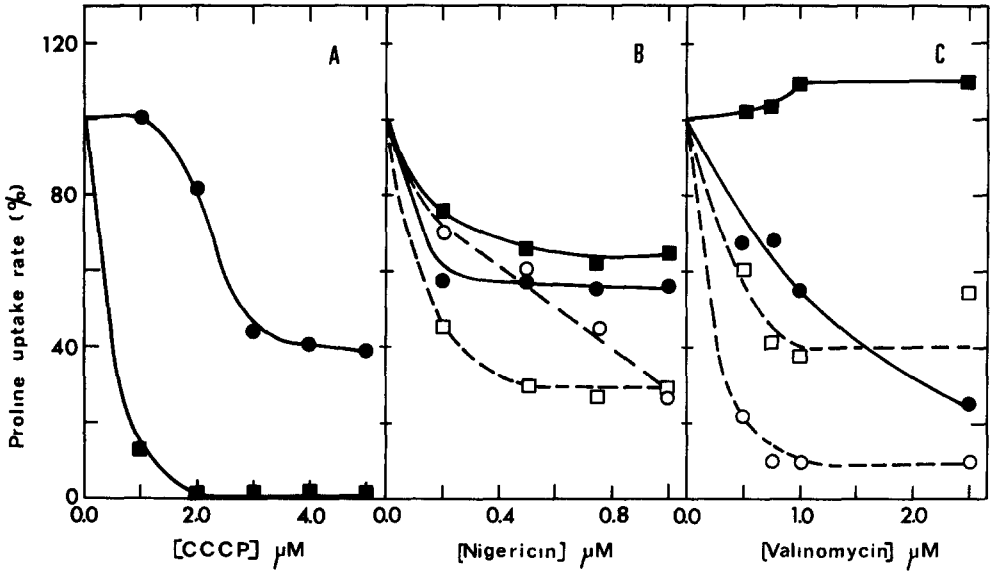

Fig. 4. Effects of ionophores on proline uptake in high-salt-grown cells. Cells were preincubated in reaction mixtures containing 2.0 M salt and CCCP at pH 8.0 (●) or pH 6.0 (■). Preincubation with nigericin or valinomycin was carried out with EDTA-treated cells in reaction mixtures containing 0.3 M salt. Proline uptake was measured in reaction mixtures at pH 8.0 (○, ●) or 6.0 (□, ■) containing NaCl and KCl, either 0.15 M each (○, □) or 1.5 M and 0.5 M (●, ■), respectively, and either CCCP (A), nigericin (B) or valinomycin (C). Procedures for EDTA treatment and uptake measurements were as described in Materials and Methods. The rate of 100 refers to 20 and 37 nmol · min^{-1} · mg^{-1} protein at pH 6.0 and 8.0, respectively, at 2.0 M salt.

partial inhibition of about 50% occurred at both pH values (Fig. 4B). Valinomycin at 0.3 M salt inhibited the uptake in high-salt-grown cells at both pH values, though a higher degree of inhibition was observed at pH 8.0 than at pH 6.0 (Fig. 4C). The effect of valinomycin was less pronounced when high-salt-grown cells, preincubated with the ionophore in 0.3 M salt, were transferred to 2.0 salt (Fig. 4C).

Effects of arsenate and cyanide on proline uptake

Arsenate, which inhibits reactions involving high energy phosphate bonds, at concentrations of up to 5 mM and after preincubation for 10 min had no effect on proline uptake in low-salt-grown cells and high-salt-grown cells. Cyanide, a respiratory inhibitor, at 10 mM concentration, caused over 90% inhibition in both cell types.

Discussion

The data presented in this article show that in the moderately halophilic, halotolerant bacterium Ba_1, proline uptake can take place within a wide range of salt concentrations.

The uptake in cells grown at both high and low salt concentrations shows: (1) identical K_m and V values when measured at the same salt concentration, (2) similar pH dependence and energy of activation, (3) almost no change in lipid composition and lipid to protein ratio (Tietz, A. and Peleg, E., unpublished). Therefore as pertains to proline transport, the halotolerance is not attributable to membrane or cell-component modification, engendered by growth at various salt concentrations [3—5]. Maintenance of a constant intracellular salt concentration as the underlying mechanism [15], is also not likely, since Ba_1 cells have an internal salt concentration similar to that of the growth medium [16]. Our findings suggest that it is the very nature of the transport proteins in this organism that enables them to function within a wide range of salt concentrations.

The relatively low rate of proline uptake observed at sub-optimal salt concentrations might be due to both the osmotic effect [17], which increases efflux, and to the low rate of respiration [3] accompanied by decreased influx. On the other hand, at high external salt concentrations low-salt-grown cells undergo plasmolysis and the uptake is markedly reduced. Decreased uptake cannot be ascribed to changes in K_m, since alterations in salt concentrations produce but slight discrepancies in K_m values (Table I). The stimulatory effect of NaCl on proline uptake might reflect its enhancement of respiration. The patterns of proline uptake (Fig. 1) and the rate of respiration [3] as a function of external NaCl concentration are very similar for both low-salt-grown and high-salt-grown cells. In addition, NaCl directly affects the activity of key enzymes of the respiratory chain in Ba_1 cells [16]. Unlike several transport systems in other halophiles and some non-halophiles [6,18—21], the NaCl stimulation of proline uptake in Ba_1 cells does not appear to be directly coupled to the sodium electrochemical gradient ($\Delta\bar{\mu}_{Na^+}$). Even in the absence of NaCl from the external medium a considerable proline uptake is observed in Ba_1 cells. In addition, the intracellular concentrations of NaCl in these cells

was shown to be the same as the extracellular one at 0.1 to 4.0 M NaCl concentrations [16].

The marked inhibition of proline uptake exerted by cyanide verifies that the transport is indeed linked to respiration. The inhibitory effects of the ionophores CCCP, nigericin and valinomycin, as well as the system's insensitivity to arsenate, demonstrates that the driving force in uptake is the electrochemical proton gradient generated by respiration and not a high energy phoshate bond [22].

In numerous bacterial transport systems, the main driving force for accumulation of solutes against their concentration gradients is the electrochemical proton gradient across the cell membrane ($\Delta\bar{\mu}_{H^+}$), i.e. the proton motive force [6—10]. This force consists of an electrical parameter, the membrane potential ($\Delta\psi$) and a chemical one, the proton gradient (Δ pH), according to the following equation:

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - \frac{2.3RT}{F} \cdot \Delta\text{pH}$$

The magnitude of the variables may be altered by changes in the external pH or by treatment of the cells with ionophores (Refs. 23—25 and Friedberg, I. and Kaback, H.R. (1979) in preparation). Ramos and Kaback have shown that at high external pH values, the main driving force for transport of various substrates into membrane vesicles of E. coli is $\Delta\psi$, whereas at low pH values some substrates are driven primarily by $\Delta\bar{\mu}_{H^+}$, others by Δ pH [26]. In intact cells of *Micrococcus lysodeikticus* it has been shown that at basic environment phosphate transport is driven mainly by $\Delta\psi$ whereas at an acidic one the main driving force is Δ pH [27].

In Ba_1 cells, the partial inhibition exerted by either nigericin or valinomycin at pH 6.0, on proline uptake in low-salt-grown and high-salt-grown cells at 0.1 and 0.3 M salt concentrations respectively, implies that in both cell types $\Delta\bar{\mu}_{H^+}$ is the driving force for the transport of proline from an acidic environment. At pH 8.0 and at these salt concentrations, valinomycin virtually inhibited the uptake in both cell types, thereby pointing to $\Delta\psi$ as the main driving force for uptake at the basic environment. Similar effects have been found for the isolated membrane vesicles of *E. coli* [26].

The inhibition of proline uptake by nigericin in high-salt-grown cells at pH 8.0 might result from electrogenic transfer of K^+, and dissipation of $\Delta\psi$, by nigericin at high salt concentration [28,29]. In low-salt-grown cells, on the contrary, low concentrations of nigericin stimulated proline uptake at pH 8.0 when the internal pH of the cell might be lower than the external one [23]. In this case ΔpH would be of a direction opposed to that prevailing at an external pH of 6.0 and might inhibit proline uptake. Nigericin, by dissipating ΔpH (without altering $\Delta\psi$) would be expected to lessen the inhibition evoked by the reversed ΔpH.

The effects of both ionophores were less pronounced at 2.0 M than at 0.3 M salt concentrations. This may be ascribed to possible alterations in the conformation of the ionophores or a change in their partition coefficients between aqueous and lipid phases as already suggested by Garty et al. [30] for extreme halophiles. Even so, one may infer from the partial inhibition exerted by nigeri-

cin at pH 6.0 and the inhibition caused by valinomycin at pH 8.0 that $\Delta\overline{\mu}_{H^+}$ and $\Delta\psi$ are the respective driving forces at these pH values.

In the course of searching for optimal conditions for ionophore penetration, the EDTA used in these experiments was found to variously affect the cells according to the salt concentrations. At low salt concentration, the EDTA treatment inhibited proline uptake while at high salt concentrations it was ineffective. The effect at low salt concentrations may be attributed to non-specific damage of the cell envelope as evidenced by the release of nucleotides from the cells. The EDTA stimulation of proline uptake in high-salt-grown cells at high salt concentration might be due to chelation of contaminating toxic cations.

Acknowledgement

We are indebted to Dr. Shimon Schuldiner for helpful discussions.

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