JOURNAL OF BACTERIOLOGY, Mar. 1983, p. 1163-1171
0021-9193/83/031163-09$02.00/0
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Vol. 153, No. 3

Development of Salt-Resistant Active Transport in a Moderately Halophilic Bacterium

D. J. KUSHNER,[1]* FRANCETTE HAMAIDE,[1] AND R. A. MACLEOD[2]

Department of Biology, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada,[1] and Department of Microbiology, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec H9X 1C0, Canada[2]

Received 16 August 1982/Accepted 29 November 1982

The moderately halophilic bacterium *Vibrio costicola* accumulates α-aminoisobutyric acid (AIB) by active transport. Substantial amounts of Na^+ ions are needed for this transport. This is not due to an ionic requirement for respiration; cells respire as well as KCl as in NaCl but do not transport AIB in KCl. In cells grown in the presence of 1.0 or 2.0 M NaCl, AIB transport took place in higher NaCl concentrations than in cells grown in the presence of 0.5 M NaCl. The latter cells developed salt-resistant transport when they were exposed to 1.0 M NaCl in the presence of chloramphenicol and other antibiotics that inhibit protein synthesis. Two levels of salt-resistant transport were observed. One level (resistance to 3.0 M NaCl) developed in 1.0 M NaCl without the addition of nutrients, did not seem to require an increase in internal solute concentration, and was not lost when cells grown in 1.0 M NaCl were suspended in 0.5 M NaCl. The second level (resistance to 4.0 M NaCl) developed in 1.0 M NaCl only when nutrients were added, may have required an increased internal solute concentration, and was lost when 1.0 M NaCl-grown cells were suspended in 0.5 M NaCl or KCl. Among the substances that stimulated the development of salt-resistant AIB transport, betaine was especially active. Furthermore, direct addition of betaine permitted cells to transport AIB at higher NaCl concentrations. High salt concentrations inhibited endogenous respiration to a lesser extent than AIB transport, especially in 0.5 M NaCl-grown cells. Thus, these concentrations of salt did not inhibit AIB transport by inhibiting respiration. However, oxidation of glucose and oxidation of succinate were at least as sensitive to high salt concentrations as AIB transport, suggesting that a salt-sensitive transport step(s) is involved in the oxidation of these substrates.

Moderately halophilic bacteria can grow over a wide range of NaCl concentrations, roughly from 0.5 to 3.0 M (9). For two species, *Vibrio costicola* and *Micrococcus halodenitrificans* (now *Paracoccus halodenitrificans*), this ability does not seem to be due to the selection of more or less salt-resistant populations (4). As has been pointed out previously (9), the ability to grow over a wide range of salt concentrations could mean that cells possess enzymes or structures that can function at these concentrations or that cells can form enzymes or structures with different salt responses, depending on the NaCl concentration at which they grow. In the few studies that have been carried out, however, growth at different salt concentrations did not lead to the formation of enzymes (9), ribosomes (19), or protein-synthesizing systems (20; M. Kogut and W. Madira, unpublished data) with different salt responses.

All of these studies were concerned with intracellular proteins. It is not certain what the intracellular environments of the organisms studied are, but they are certainly different from the external environment, and they may be much lower in ionic concentrations (9, 20).

Uncertainties about the internal environment may be partly avoided by transport studies. At least some of the transport system is in direct contact with the external medium. We studied some characteristics of the transport system for α-aminoisobutyric acid (AIB) in a moderate halophile, *V. costicola*. The ionic requirements of this system were characterized, and the response of this system to high concentrations of NaCl was studied. We found that the salt tolerance of transport changes after growth in the presence of different NaCl concentrations or even after exposure to different concentrations of NaCl or other salts under conditions in which

no growth occurs. Mechanisms that may account for these findings were considered and explored.

MATERIALS AND METHODS

V. costicola NRCC 37001 and the methods used for maintaining this bacterium have been described previously (6, 16). Growth and incubation were carried out at 25°C in shaken Erlenmeyer flasks (usually 125 ml) containing 0.2 volume of growth medium. This medium (proteose peptone tryptone medium [PPT]) contained 0.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 0.5% tryptone (Difco), and different concentrations of NaCl (usually 0.5 or 1.0 M). In some experiments the concentration of the nutrient material was doubled without any change in the results obtained.

Preliminary experiments showed that AIB transport (measured in 1.0 M salts, in which the rate was maximal for cells grown in 0.5 or 1.0 M NaCl [see below]) increased about 60% from the exponential phase of growth to just before the stationary phase and then remained fairly constant. Accordingly, for the experiments described here, cells were harvested just before the stationary phase, usually after 16 h of growth, at a cell density of about 1 mg (dry weight) per ml.

For transport experiments, cells were usually washed twice with a solution containing 0.5 or 1.0 M NaCl (according to the NaCl concentration in the growth medium), 8 mM KCl, 0.5 mM $MgSO_4$, 0.2 mM KH_2PO_4, and 50 mM Tris buffer (pH 7.2). (Such solutions are designated 0.5 or 1.0 M salts; if higher NaCl concentrations were used, solutions are designated according to the NaCl concentrations as 2.0 M salts, etc.) Cells were then suspended in the same solution to a density of about 3 mg (dry weight) per ml. To measure transport, 100-μl portions of a cell suspension were added to 50-ml Erlenmeyer flasks containing 4.9 ml of salts of the concentrations indicated below and 200 μM [1-^{14}C]AIB (0.5 μCi/μmol). The flasks were shaken at 25°C, and at intervals 0.5-ml samples of the suspension were filtered through Amicon microporous filters (25 mm; pore size, 0.45 μm) and washed with 5.0 ml of the corresponding salts solution. The filters were placed in scintillation vials containing 10 ml of Aquasol cocktail (New England Nuclear Corp.), and radiation was determined by scintillation counting.

In experiments in which the ionic requirements for transport were studied, the compositions of the washing and suspending media were changed as described below.

Respiration was measured with an O_2 electrode (Biological Oxygen Monitor; Yellow Springs Instruments Co., Yellow Springs, Ohio).

All experiments were repeated several times, and results were consistent. Typical experiments are described below. In several experiments, duplicate samples were removed to measure AIB uptake; results usually agreed within 5%.

RESULTS

Cationic requirements for AIB transport. *V. costicola*, which requires NaCl for growth, also requires this compound for AIB transport. In solutions in which NaCl was replaced by KCl no AIB transport took place. Transport increased with Na^+ concentration (Fig. 1). No transport took place in CsCl or NH_4Cl (data not shown).

Evidence for active transport of AIB. No transport occurred when cells were incubated under anaerobic conditions in tubes gassed with N_2. Addition of glucose (2.5 mM) caused only a 10% stimulation of AIB uptake. However, the cells showed measurable endogenous respiration (see below), which presumably provided sufficient energy. Most transport experiments were carried out without addition of glucose or other substrates. All of the cell-bound radioactive material could be extracted by exposing cells to water at 100°C for 10 min. Chromatographic studies, in which we used two different solvent systems (*n*-butyl alcohol-acetic acid-water and phenol-water) as described by Drapeau et al. (3) for *Alteromonas haloplanktis* (previously designated marine pseudomonad B16), showed that the cell-bound AIB moved to the same positions as pure AIB.

These experiments suggested that cell-bound AIB is free in the cytoplasm in an unchanged condition. Estimates based on a calculation of

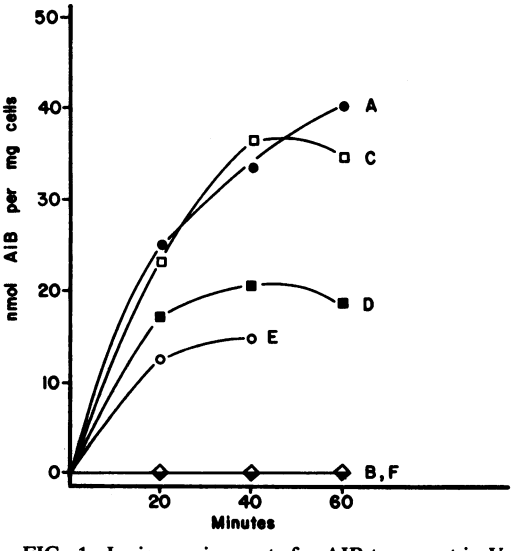

FIG. 1. Ionic requirements for AIB transport in *V. costicola*. Cells grown in 0.5% PPT containing 1.0 M NaCl were washed, and transport was measured, in different solutions. In addition to the major salts, solutions contained KCl, $MgSO_4$, Tris buffer, and NaH_2PO_4 (line A) or KH_2PO_4 (lines B through F), as described in the text. Line A, 1.0 M NaCl; line B, 1.0 M KCl; line C, 0.9 M NaCl + 0.1 M KCl; line D, 0.5 M NaCl + 0.5 M KCl; line E, 0.9 M KCl + 0.1 M NaCl; line F, 0.99 M KCl + 0.01 M NaCl. In other experiments, transport was as great in the absence of K^+ ions as in the presence of K^+ ions.

cell water as 5 μl/mg of protein showed that at least a 200-fold concentration of AIB in cell water took place. Together with the evidence that energy is needed for AIB uptake, these results confirm that active transport is involved in AIB uptake in *V. costicola*, as it is in a number of other bacteria.

Effect of NaCl concentration in the growth medium on the salt response of AIB transport. Figure 2 shows the effects of different NaCl concentrations on AIB transport by cells grown in media containing either 0.5 or 1.0 M NaCl. Cells grown at the lower salt concentration (Fig. 2A) transported AIB best at 0.5 to 1.0 M NaCl and still transported AIB at 2.0 M NaCl. Transport almost disappeared in 3.0 M NaCl and did disappear in 4.0 M NaCl. In other experiments, in which we used larger numbers of cells, we found that occasionally cells grown in 0.5 M NaCl had about 3% as much AIB uptake after 1 h in 4.0 M NaCl as in 1.0 M NaCl, but never more than this.

In contrast, cells grown in 1.0 M NaCl (Fig. 2B) took up considerable amounts of AIB in 3.0 M NaCl and even in 4.0 M NaCl, although maximum uptake still occurred between 0.5 and 1.0 M. The relative response to a lower NaCl concentration (0.25 M) was the same in both kinds of cells. At still lower concentrations, cells lysed, and transport could not be measured. AIB transport in cells grown in 2.0 M NaCl responded to different NaCl concentrations in a manner similar to AIB transport in 1.0 M-grown cells, with somewhat higher relative transport in 4.0 M NaCl (data not shown).

In the experiments described above, cells were subjected to sudden large osmotic changes. We were not certain whether the observed changes in the ability to transport AIB at the highest NaCl concentrations represented inhibition or destruction of the transport system. To distinguish between these possibilities, cells grown, washed, and suspended in 0.5 or 1.0 M NaCl were centrifuged and suspended in 4.0 M salts for 30 min. These cells were then centrifuged and suspended in 1.0 M salts, and their AIB transport was compared with transport of the original suspension in 1.0 M salts. We found (data not shown) that all of these rather drastic changes in the osmotic environments of the cells reduced the AIB transport in 1.0 M salts for cells grown in the lower or higher NaCl concentration by only about 10%. Thus, the transport system had not been destroyed, and the results shown in Fig. 2 represent true inhibition by the higher salt concentrations.

Development of salt-resistant transport. We thought that it was possible that the more salt-resistant transport in cells grown at the higher NaCl concentration might be due to the formation of a new protein(s) involved in transport. Accordingly, we carried out experiments on the development of salt resistance (as measured by the ability to transport AIB in 3.0 or 4.0 M salts) in cells grown in 0.5 M NaCl and then transferred to different media containing 1.0 M NaCl.

The results of several experiments (Table 1) showed that the appearance of resistance to the highest salt concentration occurred gradually, requiring 2 h or more. The most striking observation was that development of resistance took

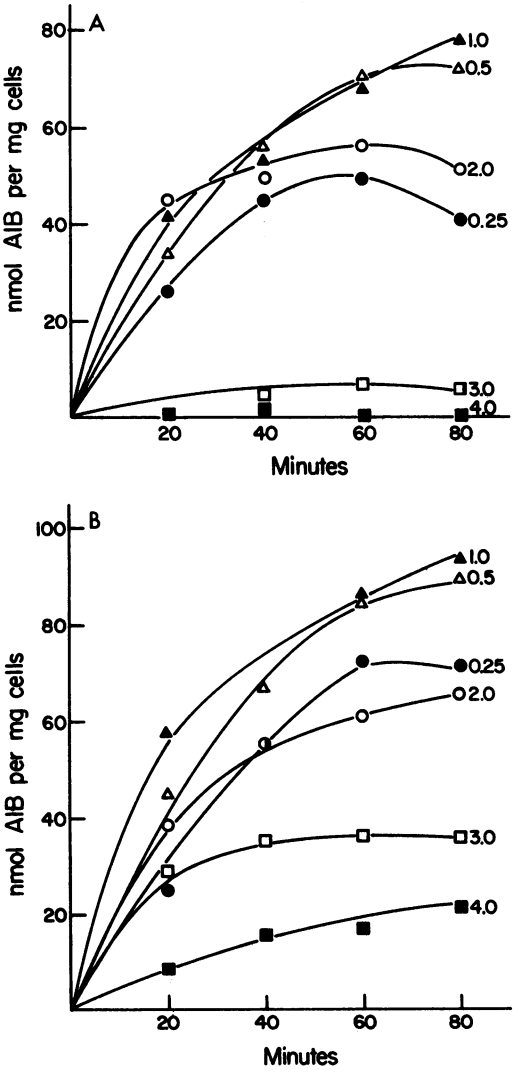

FIG. 2. Effect of NaCl concentration on AIB transport in *V. costicola*. (A) Cells grown in 0.5% PPT containing 0.5 M NaCl. (B) Cells grown in 0.5% PPT containing 1.0 M NaCl. Cells were washed in 0.5 M salts (A) or 1.0 M salts (B). The NaCl concentrations in which transport was measured are shown by the curves.

TABLE 1. Development of transport resistant to 4 M NaCl in cells grown in 0.5 M NaCl and incubated in other salts[a]

Expt	Incubation medium	Time (h)	AIB uptake[b]	
			1.0 M NaCl	4 M NaCl
1	0.5 M NaCl + PPT	0	30	0
		7.5	61	1
2	1.0 M NaCl + PPT	0	18	0
		2	19	10
		4	31	30
		6	33	52
	1.0 M NaCl + PPT + chloramphenicol	2	49	19
		4	52	16
		6	33	42
3	1.0 M salts	0	21	0
		4.5	16	8
	1.0 M salts + NH_4Cl	4.5	13	6
	1.0 M salts + glucose	4.5	38	7
	1.0 M salts + glucose + NH_4Cl	4.5	19	28
	1.0 M KCl + glucose + NH_4Cl	4.5	7	0

[a] Exponential cultures (0.2 to 0.4 mg [dry weight] per ml) in 0.5 M NaCl containing PPT were diluted with equal volumes of 0.5 M NaCl containing PPT and 1.5 M NaCl containing PPT in experiments 1 and 2, respectively. In experiment 3, washed cells were incubated in the media indicated. Final concentrations: PPT, 0.5%; glucose, 0.1 M; NH_4Cl, 0.2 M; chloramphenicol, 100 μg/ml. Medium containing 1.0 M KCl also contained 0.4 mM $MgSO_4$, 0.2 mM KH_2PO_4, and 50 mM Tris buffer (pH 7.2). All cell suspensions were shaken at 25°C. At the times shown, cells were washed and suspended in 1.0 M salts, and transport was measured as in the experiment shown in Fig. 2.

[b] Uptake in nanomoles per milligram (dry weight) at 60 min for 1.0 M NaCl and relative uptake at 4 M NaCl expressed as a percentage of uptake at 1 M NaCl.

place in the presence of chloramphenicol concentrations that quite effectively blocked growth and have been shown to inhibit protein synthesis (6). Therefore, formation of a salt-resistant transport system does not depend on protein synthesis. In a defined salts medium, neither glucose nor NH_4Cl alone stimulated development of resistance to 4.0 M salts, but the two compounds together did have a stimulatory effect. No resistance developed in cells incubated in 0.5 M NaCl or in 1.0 M KCl, even if nutrient medium was added.

We also found that transport resistant to 4.0 M salts developed in the presence of streptomycin and erythromycin. However, a good deal of cell lysis occurred during long-term incubation with these antibiotics; perhaps because of this, the final level of activity obtained was low, even in the optimal salt concentration (1.0 M).

Nutrient requirements for development of salt-resistant transport were studied further. These experiments showed that cells grown in 0.5 M NaCl could develop resistance regards AIB transport much more easily to 3.0 M salts than to 4.0 M salts (Table 2). Thus, exposure of cells grown in 0.5 M NaCl to 1.0 M salts alone caused transport to become resistant to 3.0 M salts, but not to 4.0 M salts. Adding different complex nutrients (PPT, Casamino Acids, or yeast extract) for short periods caused a striking increase in resistance of transport to 3.0 M salts. In fact, sometimes the level of resistance was greater than the level in cells grown in 1.0 M NaCl. The active amino acids in the Casamino Acid mixture have not been identified yet. Individual amino acids (proline, L-glutamate, L-me-

TABLE 2. Effect of medium constituents on development of transport resistant to 3 and 4 M salts[a]

Expt	Incubation medium	Time (h)	Relative AIB uptake[b]	
			3 M salts	4 M salts
1	0.5 M salts	2	5	3
	1.0 M salts	2	21	3
	1.0 M salts + 1% PPT	2	63	17
	1.0 M salts + 1% Casamino Acids	2	56	27
	1.0 M salts + 1% yeast extract	2	74	16
2	0.5 M salts	4	0.5	0
	1.0 M salts	4	14	0.8
	1.0 M salts + 0.2 M NH_4Cl	4	14	3
	1.0 M salts + 0.1 M glucose	4	32	3
	1.0 M salts + 0.1 M glucose + 0.2 M NH_4Cl	4	64	7
3	0.5 M salts	2	1	0
	0.5 M salts + 1 mM betaine	2	13	0.8
	1.0 M salts	2	15	1
	1.0 M salts + 1 mM betaine	2	40	52
4	1.0 M salts	2	15	0
	1.0 M salts + 50 mM betaine	2	82	32
	1.0 M salts + 50 mM choline	2	2	0.1
	1.0 M salts + 50 mM ethanolamine	2	4	0

[a] Experiments were carried out as described in Table 1, footnote *a*.

[b] AIB uptake was measured after 30 min in 1.0, 3.0, and 4.0 M salts. The treatments shown had no consistent large effects on uptake in 1.0 M salts. Relative uptake values in 3.0 and 4.0 M salts are expressed as percentages of uptake in 1.0 M salts.

TABLE 3. Effect of betaine on AIB transport at different NaCl concentrations[a]

NaCl conc (M)		AIB uptake (nmol/mg [dry wt] of cells)	
Growth	Experimental	Control	+Betaine (100 mM)
0.5	0.5	158	126
	1.0	119	119
	2.0	54	90
	3.0	8	30
	4.0	0.1	0.3
1.0	1.0	112	89
	2.0	56	72
	3.0	13	26
	4.0	6	8

[a] AIB uptake was measured after 60 min of incubation. Measurements at 20 and 40 min showed similar effects of betaine. The growth medium contained 1% PPT and 0.5 or 1.0 M NaCl.

thionine, and DL-alanine) added to 1.0 M salts together with 0.1 M glucose did not increase transport resistance to 3.0 M salts. Glycerol and sucrose had no stimulatory effect on the development of salt-resistant transport when they were added to 1.0 M NaCl (data not shown).

Betaine, a substance whose involvement with salt tolerance in moderate halophiles is becoming increasingly apparent (see below), greatly stimulated development of salt-resistant transport. The chemically related compounds choline and ethanolamine did not. On the contrary, less resistance developed, even to 3.0 M salts, in the presence of these compounds than in their absence (Table 2).

Next, we performed experiments to determine whether direct addition of some of the substances tested in the experiments shown in Table 2 had any effect on AIB transport in different salt concentrations. We found (Table 3) that betaine stimulated AIB transport in 2.0 and 3.0 M salts but not in 4.0 M salts. At lower salt concentrations betaine inhibited AIB transport. Choline, ethanolamine, and the amino acids shown in Table 2 did not affect AIB transport when they were added directly to cells exposed to different salt concentrations (data not shown).

Loss of salt-resistant transport. AIB transport resistant to the highest salt concentration developed slowly when cells grown in the presence of 0.5 M NaCl were incubated in solutions containing 1.0 M NaCl. In contrast, this resistance was quickly lost when cells grown in the presence of 1.0 M NaCl were put into lower salt concentrations. Table 4 shows that this loss occurred in 0.5 M NaCl or 0.5 M KCl, but not in 1.0 M NaCl. In 1.0 M KCl, some resistance was lost, but not all. In the experiments shown in Table 4, cells were washed twice, but in other experiments a single wash gave the same results. However, as Table 4 also shows, cells grown in 1.0 M NaCl and then exposed to 0.5 M NaCl lost little of their ability to transport AIB in 3.0 M salts.

Correlation of salt-resistant transport with internal solute concentrations. Although all of the internal solutes of *V. costicola* have not been accounted for, we thought that some index of the internal solute concentrations in *V. costicola* cells might be obtained by measuring the salt concentrations required to prevent lysis, as was done by Christian and Ingram (1). We measured lysis both by the loss of turbidity (optical density at 650 nm) and by the release of UV (260 nm)-absorbing substances from the cells; the latter method was found to be more precise for determining the salt concentration at which lysis occurred. Figure 3 shows the method used to calculate this lysis point and also shows that cells grown in the presence of 1.0 M NaCl had a higher lysis point than cells grown in the presence of 0.5 M NaCl, as expected (1).

When cells grown in the presence of 0.5 M NaCl were incubated in 1.0 M NaCl containing PPT for 2 h or more, the lysis point increased substantially, usually twofold or more. When cells grown in the presence of 0.5 M NaCl were incubated in 1.0 M salts with or without glucose and ammonium chloride (as in Table 1), the lysis point remained unchanged in some experiments; in others, it rose 50 to 70%. When cells grown in 1.0 M NaCl were diluted in 0.5 M salts, the lysis point decreased about 30%.

Even when there was no increase in the lysis point, cells incubated in 1.0 M salts developed

TABLE 4. Loss of salt-resistant transport by cells grown in 1.0 M NaCl[a]

Expt	Incubation medium	Relative AIB uptake[b]	
		3 M salts	4 M salts
1	1.0 M salts		23
	0.5 M salts		0.7
	1.0 M KCl		16
	0.5 M KCl		0.8
2	1.0 M salts	26	14
	0.5 M salts	33	1

[a] In experiment 1, cells were washed twice and suspended in salts solution before being assayed for AIB transport in 1.0, 3.0, and 4.0 M salts. The salts and KCl solutions used were the same as those used in the experiments shown in Table 1. AIB uptake was measured after 60 min. In experiment 2, cells were incubated for 4 h in the incubation medium, and AIB uptake was measured after 30 min.

[b] Relative uptake values in 3.0 and 4.0 M salts are expressed as percentages of uptake in 1.0 M salts.

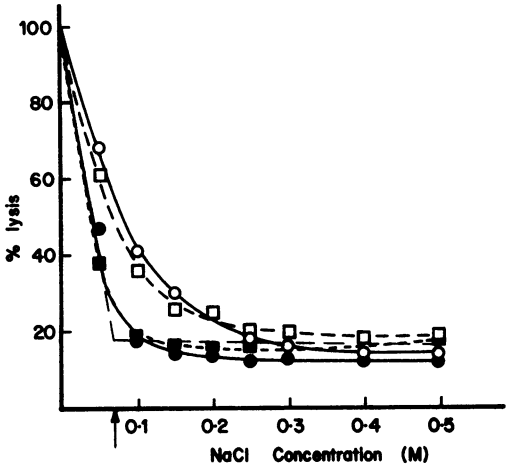

FIG. 3. Measurement of lysis point values. Cells grown in 1% PPT containing 0.5 or 1.0 M NaCl were washed in the same salt concentration as the growth medium, incubated for 2 h in 1% PPT containing 0.5 or 1.0 M NaCl, washed in the same salt concentration as the incubation medium, and suspended in different NaCl concentrations. Lysis was determined by the release of UV (260 nm)-absorbing material; 100% lysis was defined as the lysis occurring in distilled water. The lysis point was defined as the point of intersection of extensions of the descending and stationary curves; such an extension is shown on one curve of the graph. Symbols: ■, cells grown in 0.5 M NaCl and incubated in 0.5 M NaCl (lysis point, 0.065 M); □, cells grown in 0.5 M NaCl and incubated in 1.0 M NaCl (lysis point, 0.105 M); ●, cells grown in 1.0 M NaCl and incubated in 0.5 M NaCl (lysis point, 0.070 M); ○, cells grown in 1.0 M NaCl and incubated in 1.0 M NaCl (lysis point, 0.13 M).

transport resistant to 3.0 M salts, but not to 4.0 M salts. Our experiments, in which we used an admittedly crude method for estimating internal solute concentrations, suggested that higher internal solute concentrations are needed for resistance to 4.0 M salts than for resistance to 3.0 M salts.

Effects of salts on respiration of *V. costicola*. We thought that high salt concentrations or the lack of Na^+ ions might inhibit transport by interfering with the energy supply. Accordingly, the effects of different salts on respiration were measured.

Table 5 shows the effects of different salt concentrations on the respiration of cells grown in 0.5 or 1.0 M NaCl. Endogenous respiration was somewhat more sensitive in the former cells than in the latter. Thus, for cells grown in 0.5 M NaCl, the endogenous respiration in 4.0 M salts was 13% of the endogenous respiration in 1.0 M salts; for cells grown in 1.0 M NaCl, the corresponding value was 27%. This difference is less striking than the difference in AIB transport, which was virtually eliminated by 4.0 M salts in 0.5 M NaCl-grown cells. This suggests that the highest salt concentration does not act primarily by inhibiting respiration.

These experiments also showed (Table 5) that there was a large difference in the salt sensitivity of respiration of substrates by the two kinds of cells. The higher salt concentrations had much stronger inhibitory effects on respiration (as measured by the difference between respiration in the presence of the substrate and endogenous respiration) of glucose and succinate by cells that had been grown at the lower salt concentration than on respiration by cells that had been grown at the higher salt concentration. In 3.0 M salts and possibly in 4.0 M salts, some oxidation of glucose and succinate occurred in the 1.0 M NaCl-grown cells but not in the 0.5 M NaCl-grown cells. Glycerol oxidation was about equally salt sensitive in the two kinds of cells.

Table 5 also shows that endogenous respiration and respiration of substrates (with the exception of succinate) were approximately the same in 1.0 M KCl as in 1.0 M NaCl. Thus, the inability of cells to transport AIB in 1.0 M KCl

TABLE 5. Effect of salts on respiration of *V. costicola*

Concn of NaCl in growth medium (M)	Experimental conditions	Respiration (μg of O_2 used per min per mg [dry wt])[a]			
		Endogenous	Glucose	Succinate	Glycerol
0.5	0.5 M NaCl	1.14 ± 0.14	1.66 ± 0.03	4.44 ± 0.13	2.60 ± 0.22
	1.0 M NaCl	0.78 ± 0.09	1.14 ± 0.01	3.82 ± 0.07	2.24 ± 0.09
	3.0 M NaCl	0.17 ± 0.02	0.13 ± 0.01	0.17 ± 0.02	0.54 ± 0.11
	4.0 M NaCl	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.31 ± 0.05
1.0	1.0 M NaCl	0.74 ± 0.02	1.04 ± 0.04	3.74 ± 0.15	1.46 ± 0.07
	3.0 M NaCl	0.34 ± 0.02	0.40 ± 0.03	0.74 ± 0.08	0.60 ± 0.03
	4.0 M NaCl	0.20 ± 0.02	0.26 ± 0.03	0.26 ± 0.03	0.44 ± 0.05
	1.0 M KCl	0.86 ± 0.02	1.11 ± 0.09	2.33 ± 0.15	1.69 ± 0.20

[a] Mean results of two experiments are shown ± standard deviation of the mean. Endogenous values were not subtracted from oxidation rates in the presence of substrates. Cells (0.20 to 0.25 mg [dry weight] per ml) were assayed in the salt solution shown supplemented with 20 mM D-glucose, sodium succinate, or glycerol.

(Fig. 1) was not due to a failure of the cells to respire.

DISCUSSION

Transport studies with *V. costicola* and other halophilic and salt-tolerant microorganisms may prove to be valuable for studying mechanisms of salt tolerance, which have generally attracted less attention than mechanisms of salt requirement.

V. costicola has an active transport system for AIB, which requires substantial concentrations of Na^+ ions. Endogenous respiration in these bacteria, which provides energy for transport, takes place as well in 1.0 M KCl as in 1.0 M NaCl and may not require Na^+ ions. (The possibility that small amounts of NaCl present as contaminants in KCl are needed for respiration has not been excluded.) Drapeau and MacLeod (2) found that the Na^+ requirement for AIB transport in *A. haloplanktis* (*Pseudomonas* B16) was considerably higher than the Na^+ requirement for respiration. In this organism, as in *V. costicola*, the Na^+ requirement for transport is not primarily due to the effect of this ion on respiration. Niven and MacLeod (14) showed that there is a sodium ion-proton antiport system in *A. haloplanktis*, which is involved in the transport of K^+ ions and presumably other substances. We suspect that such a system also exists in *V. costicola*.

AIB transport in *V. costicola* is very salt tolerant. It is little affected by 2.0 M NaCl and may still continue in 3.0 and 4.0 M NaCl. One of our major findings is that the response to higher salt concentrations changes according to the salt concentration at which the cells are grown. This was one of the first examples within one microorganism of adaptation of a physiological process to growth at different salt concentrations (10).

Differences in salt tolerance of transport seem to be correlated with differences in salt tolerance of growth in other microorganisms. Lindman (11, 12) recently showed that more salt-tolerant yeast species could transport glucosamine at higher NaCl concentrations than less salt-tolerant species.

While our work was in progress, Peleg et al. (15) reported that cells of the moderately halophilic, halotolerant bacterium Ba_1 grown in 2.0 M salt could transport proline at considerably higher salt concentrations than cells grown in 0.2 M salt. These authors suggested that transport inhibition by high salt concentrations in the latter cells was due to plasmolysis and supported this with electron micrographs.

Preliminary electron microscope studies with *V. costicola* have shown that considerable plasmolysis occurs when cells grown in 0.5 or 1.0 M NaCl are placed in 3.0 or 4.0 M salts. However, the degree of plasmolysis seemed to be the same for the cells grown in low and high salt concentrations. We considered the possibility that plasmolysis damaged the transport system, but found that this did not occur.

An adaptation of AIB transport to growth of *Halomonas elongata* in different NaCl concentrations has been found very recently (E. Martin, T. Duryea, R. Vreeland, L. Hilsabeck, and C. Davis, unpublished data). This type of microbial adaptation may be widespread.

Probably our own most interesting finding is that salt-resistant transport develops in nongrowing cells by a process that apparently does not involve protein synthesis; this development occurs in a few hours and is not inhibited by chloramphenicol or other antibiotics. We distinguished two levels of resistance, resistance to 3.0 M NaCl and resistance to 4.0 M NaCl. The first level developed in 0.5 M NaCl-grown cells when these cells were placed in 1.0 M NaCl alone, was not always accompanied by increased levels of intracellular solutes, as measured by lysis point experiments, and was not lost when 1.0 M NaCl-grown cells were placed in 0.5 M NaCl. The second, higher level of resistance developed more slowly, required nutrients in addition to 1.0 M NaCl, was quickly lost when 1.0 M NaCl-grown cells were placed in 0.5 M NaCl or KCl, and seemed to require an increased level of intracellular solutes.

We are considering the possibility that resistance to the highest NaCl concentration (4.0 M) is due to an overall increased concentration of intracellular solutes. We still do not know what these solutes are. Shindler et al. (16) found that the levels of Na^+ and K^+ ions increased with the external Na^+ concentration of the growth medium, to give values roughly equal to this concentration; however, there is some doubt that these ions exist as free ions in the cytoplasm (9, 20). In certain microorganisms the levels of internal polyols and amino acids increase with increasing external salt concentration (9, 13, 18). We have found no polyols in *V. costicola* and no consistent changes in concentrations of free amino acids at different external salt concentrations (K. Hanna and D. J. Kushner, unpublished data).

Could one or a few solutes be especially important in the development of salt-resistant transport? Betaine is of special interest here; we found that this substance was very effective in the development of salt-resistant AIB transport (Table 2). Furthermore, simply adding betaine stimulated transport of AIB at high salt concentrations, although betaine inhibited such transport at lower salt concentrations (Table 3).

Betaine seems to be involved in salt tolerance

in other bacteria. Shkedy-Vinkler and Avi-Dor (17) have shown that betaine can stimulate respiration of succinate and other substances by the halophilic bacterium Ba_1 in high salt concentrations. (However, we are not certain whether this is an effect on respiration or an effect on transport [see below].) Very recently Galinski and Trüper (5) found that intracellular betaine concentrations varied directly with the NaCl concentration of the growth medium for the halophilic phototrophic bacterium *Ectothiorhodospira halochloris*.

We still cannot explain the easy acquisition of resistance to 3.0 M NaCl. Our results could suggest that a gradual increase in salt concentration (i.e., from 0.5 to 1.0 to 3.0 M) is less inhibitory than a direct increase from 0.5 to 3.0 M. If this were so, however, we would expect that resistance to 3.0 M NaCl would be lost when 1.0 M NaCl-grown cells were placed in 0.5 M NaCl. This did not occur.

In the experiments on AIB transport described above, cells were incubated without added substrate and obtained the necessary energy from endogenous respiration. The highest NaCl concentrations permitted cells grown in the presence of 0.5 M NaCl to respire. Of course, this does not prove that this respiration generated energy in a form that could be used for transport; in fact, the cells no longer transported AIB.

Oxidation of glucose and oxidation of succinate were at least as sensitive to high salt concentrations as AIB transport was. This was especially evident in the 0.5 M NaCl-grown cells, in which high salt concentrations completely inhibited oxidation of these substrates, although they did not completely inhibit endogenous respiration. This suggests that transport may be involved in respiration of these substrates. In *A. haloplanktis*, it has been shown that oxidation of a number of substrates is Na^+ dependent, primarily because this ion is needed for the transport of these substrates (R. Droniuk and R. A. MacLeod, manuscript in preparation). Further experiments on the energetics of transport by *V. costicola* at different salt concentrations are now being carried out.

We have also considered the possibility that salt-resistant transport is associated with changes in membrane composition. Lipids do change in a number of halophilic bacteria when they are grown in different salt concentrations (7, 8). However, Peleg et al. (15) found no changes in lipid composition or in lipid-to-protein ratios in membranes of strain Ba_1 grown in high or low concentrations. We have found that growing *V. costicola* in higher salt concentrations leads to a decrease in the proportion of phosphatidylethanolamine and an increase in the proportions of phosphatidylglycerol and cardiolipin (M. Kogut, M. Kates, K. Hanna, and D. J. Kushner, unpublished data). The system described above should permit us to investigate the relationship, if any, between lipid composition and salt-resistant transport.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to D.J.K. and R.A.M. Part of this work was carried out at the Macdonald Campus of McGill University while D.J.K. was on sabbatical leave from the University of Ottawa.

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