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Sodium-Coupled Energy Transduction in the Newly Isolated Thermoalkaliphilic Strain LBS3

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Strain LBS3 is a novel anaerobic thermoalkaliphilic bacterium that grows optimally at pH 9.5 and 50°C. Since a high concentration of Na^+ ions is required for growth, we have analyzed the primary bioenergetic mechanism of energy transduction in this organism. For this purpose, a method was devised for the isolation of right-side-out membrane vesicles that are functional for the energy-dependent uptake of solutes. A strict requirement for Na^+ was observed for the uptake of several amino acids, and in the case of L-leucine, it was concluded that amino acid uptake occurs in symport with Na^+ ions. Further characterization of the leucine transport system revealed that its pH and temperature optima closely match the conditions that support the growth of strain LBS3. The ATPase activity associated with inside-out membrane vesicles was found to be stimulated by both Na^+ and Li^+ ions. These data suggest that the primary mechanism of energy transduction in the anaerobic thermoalkaliphilic strain LBS3 is dependent on sodium cycling. The implications of this finding for the mechanism of intracellular pH regulation are discussed.

The newly isolated strain LBS3 is a gram-positive, spore-forming, anaerobic thermoalkaliphile that grows optimally at 50°C and an alkaline pH of 9.5. The strain was isolated from a hot-lake inlet at Lake Bogoria in Kenya and represents a new group within the gram-positive *Clostridium-Bacillus* subphyllum (24). Only a few thermoalkaliphiles are known to date, like the anaerobes *Clostridium paradoxum* (19) and *Clostridium thermoalkalophilum* (18), the hyperthermophile *Thermococcus alcaliphilus* (11), and a few moderately thermophilic alkaliphilic methanogens (2).

Strain LBS3 is an anaerobe, a thermophile, and an alkaliphile. Its physiological characteristics therefore must fit this complex habitat. As an anaerobic bacterium, it is faced with the problem that metabolic energy is mainly obtained by substrate level phosphorylation (13). The electrochemical ion gradient across the cytoplasmic membrane is usually generated by a cytoplasmic-membrane-bound H^+- or Na^+-ATPase (5), although other mechanisms have been reported recently (see reference 12). Most anaerobes studied to date use H^+ as a coupling ion in energy-transducing processes. In recent years, a number of anaerobes have been found to also use Na^+ as a coupling ion (1, 4), and one anaerobic thermophile, *Clostridium fervidus*, has actually been found to rely completely on Na^+ in energy transduction (26).

Aerobic alkaliphiles rely mainly on primary, respiration-dependent H^+ pumping for the generation of an electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$). To counteract the H^+ expulsion and to maintain an intracellular pH of around 8.2, these bacteria possess Na^+/H^+ antiporters (9, 23). K^+/H^+ antiporters have also been implicated in the regulation of the intracellular pH (29) in such organisms as the moderately halophilic and alkaliphilic methanogen *Methanolobus taylorii* GS-16 (22). In this respect, K^+ ions play a role in pH homeostasis in *Bacillus alcalophilus* (14) and in the ureolytic bacterium *Bacillus pasteurii* (10). In the latter case, K^+ ions can be replaced by ammonium ions under certain conditions (10). The low magnitude of the $\Delta\tilde{\mu}_{H^+}$ in alkaliphiles has led to the suggestion that these bacteria may use the electrochemical gradient of sodium ions ($\Delta\tilde{\mu}_{Na^+}$) for the generation of ATP. However, to date, the activity of the membrane-bound ATPase in all aerobic alkaliphiles appears to be exclusively H^+ coupled (15).

As a thermophilic bacterium, strain LBS3 is, under normal conditions, confronted with the problem that the passive permeation of H^+ and Na^+ across the cytoplasmic membrane increases with temperature (32). The permeability of membranes to H^+, both in neutrophilic thermophiles and in mesophiles, is about 10^2- to 10^3-fold higher than their permeability to Na^+ ions. To account for the increased membrane permeability to H^+ at higher temperatures, aerobic thermophiles dramatically increase their respiration-linked H^+ pumping activity to assure the maintenance of both a viable $\Delta\tilde{\mu}_{H^+}$ and the intracellular pH. The anaerobic thermophile *C. fervidus* does not possess an effective H^+ pumping system. This organism has solved the problem by using Na^+ as the only coupling ion in energy transduction (26). As a consequence, *C. fervidus* cannot control its internal pH and grows only at around pH 7.

Like most, if not all, alkaliphiles, anaerobic alkaliphilic thermophiles obviously have to control their internal pH. In view of the considerations presented above, the bioenergetics of these organisms becomes an intriguing problem. It is not known if anaerobic thermoalkaliphiles depend on H^+ or Na^+ ions, or both, for energy transduction. In anaerobic thermoalkaliphiles, as in aerobic alkaliphiles (15), the membrane would have to be highly impermeable to H^+ for H^+-linked energy transduction to occur. With Na^+-linked energy transduction, on the other hand, there is the problem of maintenance of the intracellular pH of these organisms. The following question arises: is Na^+ the only viable option as an ion for energy-conserving processes in anaerobic thermoalkaliphiles? Are these organisms predominantly dependent on Na^+ ions for energy-requiring

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processes, such as the uptake of solutes and flagellar action (3, 30)? In this report, we demonstrate that the primary mechanism of energy transduction in the anaerobic thermoalkaliphilic strain LBS3 is dependent on sodium cycling.

MATERIALS AND METHODS

Bacterial strains and cell growth. The newly isolated strain LBS3 (24) was grown anaerobically by the Hungate technique (8, 21) on a medium containing $(NH_4)_2SO_4$ (1.0 g/liter), NH_4Cl (0.4 g/liter), $Na_2S_2O_4$ (0.1 g/liter), K_2HPO_4 (0.5 g/liter), $MgSO_4$ (0.1 g/liter), $CaCl_2$ (0.05 g/liter), tryptone (0.25 g/liter), yeast extract (0.25 g/liter), $FeCl_3$ (0.01 g/liter), resazurine (0.001 g/liter), trace element solution 141 (Deutsche Sammlung von Mikroorganismen; 10 ml), vitamin solution 141 (Deutsche Sammlung von Mikroorganismen; 10 ml), $NaHCO_3$ (2.0 g/liter), Na_2CO_3 (2.0 g/liter), and cysteine (0.5 g/liter). As a carbon source, Bacto Peptone (Difco) was added at 2.0% (wt/vol). After distribution into 15-ml Hungate tubes (9 ml of medium per tube) or 100-ml serum bottles (20 or 50 ml per bottle), the medium was sterilized. The vitamin solution was injected from filter-sterilized solutions into autoclaved tubes containing the above-described medium. The carbonates were concentrated 10-fold and sterilized separately. For each 9 ml of medium, 1 ml of the sterile carbonate solution was added aseptically prior to addition of the sample or culture material; the final pH was 9.5. Cells were incubated at 50°C and pH 9.5. Large amounts of cells were grown in a pH-controlled 19-liter bioengineering fermentor. Cells were harvested in the exponential phase of growth at a density of 3×10^8 to 4×10^8/ml, washed twice with 100 mM potassium phosphate (pH 9.0), resuspended in a very small volume of the same buffer, centrifuged, and frozen as pellets in liquid nitrogen.

Membrane vesicle isolation. For the preparation of right-side-out membrane vesicles, 1 g of cells was resuspended in a final volume of 20 ml of 100 mM potassium phosphate, pH 8.0, containing 10 mM $MgSO_4$. Lysozyme was added at 1 mg/ml, and the suspension was incubated at 45°C with gentle shaking for 30 min. Cells were concentrated by centrifugation at 13,300 × *g* for 10 min, and the pellet was resuspended in 2 to 3 ml of 100 mM potassium phosphate, pH 8.0, supplemented with 2% (wt/vol) glucose. Incubation was continued at 45°C for 10 min, and lysis was induced by dilution of the suspension into 300 ml of 50 mM potassium phosphate, pH 8.0, supplemented with 10 mM $MgSO_4$ and 35 μg each of DNase and RNase per ml. Incubation was continued for 30 min at 45°C. Subsequently, Na-EDTA was added to a final concentration of 15 mM, and after 5 min, $MgSO_4$ was added at 4 mM. To remove cells and larger cell debris, the suspension was centrifuged at 3,330 × *g* for 15 min, followed by centrifugation of the supernatant at 13,300 × *g* for 30 min to collect the membrane vesicles. The pellet was resuspended in 100 mM potassium phosphate (pH 8.0), washed once, resuspended, and stored in 0.1-ml aliquots under liquid nitrogen.

For the preparation of inside-out membrane vesicles, washed cells (1 g) were resuspended in 5 ml of 100 mM Tricine, pH 7.0, containing DNase and RNase (each at 10 μg/ml) and then disrupted in a French pressure cell by two passes at 8,000 lb/in^2. After a centrifugation step at 13,300 × *g* to remove cell debris, membranes were collected from the supernatant after an ultracentrifugation step at 267,000 × *g*. Membranes were resuspended in a very small volume and rapidly frozen in liquid nitrogen.

Uptake of amino acids in membrane vesicles. Uptake of ^{14}C-labelled amino acids by membrane vesicles of strain LBS3, driven by an artificial gradient, was measured at 40°C. Membrane vesicles were concentrated by centrifugation and resuspended in 100 mM potassium phosphate, pH 8.0, at a protein concentration of 16 to 24 mg/ml in the absence or presence of 2 nmol of valinomycin per mg of protein. After incubation for 60 min on ice, 2 μl of the membrane vesicle suspension was diluted 100-fold in buffers of the following compositions under the described conditions: (i) 100 mM sodium phosphate, pH 8.0, without prior incubation with valinomycin, to impose a chemical gradient of sodium ions ($\Delta\bar{\mu}_{Na^+}$); (ii) 100 mM sodium phosphate, pH 8.0, in the presence of valinomycin, to impose a transmembrane electrical potential ($\Delta\Psi$) and a $\Delta\bar{\mu}_{Na^+}$ (i.e., a $\Delta\bar{\mu}_{Na^+}$); and (iii) 100 mM potassium phosphate, pH 8.0, to avoid imposing a gradient. In all cases, the dilution buffer (200 μl) contained the following U-^{14}C-labelled L-amino acids at the noted final concentrations: L-leucine, 0.81 μM (311 mCi/mmol); L-serine, 0.47 μM (540 mCi/mmol); L-glutamate, 0.11 μM (46 mCi/mmol); L-glutamine, 0.93 μM (270 mCi/mmol); L-arginine, 0.79 μM (318 mCi/mmol); L-threonine, 0.44 μM (226 mCi/mmol); L-phenylalanine, 4.8 μM (5.22 μCi/mmol); L-lysine, 0.89 μM (282 mCi/mmol); L-asparagine, 0.89 μM (228 mCi/mmol); and L-alanine, 1.44 μM (174 mCi/mmol).

To determine the pH dependency of $\Delta\bar{\mu}_{Na^+}$-driven uptake of leucine, membrane vesicles were diluted 100-fold in 100 mM sodium phosphate (pH 5 to 9) or 100 mM sodium carbonate (pH 9 to 11). The sodium dependency of leucine uptake was measured in a similar manner except that sodium was replaced by potassium.

ATPase activity measurements. ATPase activity measurements were performed with inside-out membrane vesicles that were obtained by French press treatment of cells. Membrane vesicles stored under liquid nitrogen were quickly thawed and diluted 40-fold into 100 mM Tricine (pH 7.0) at 4°C. The ATPase activity was estimated from the amount of released P_i measured by a modification (6) of the method of Lanzetta et al. (16). The assay buffer contained 100 mM Tricine, pH 7.0, supplemented with 3 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 0.05% (vol/vol) Triton X-100. NaCl, KCl, or LiCl was present at 50 mM unless otherwise noted. Assays were performed in a 96-well microtiter plate with a well volume of 300 μl. Each well contained 5 μg of membrane protein (in 10 μl) and 10 μl of assay buffer. After a 1-min incubation at 46°C, the reaction was initiated by the addition of 10 μl of Tris-ATP (2 mM final concentration). Reactions were stopped after 10 min by the addition of 200 μl of malachite green solution (6). Color development was terminated after 5 min by the addition of 10 μl of a 34% (wt/vol) citric acid solution. The assay was measured immediately at 620 nm in a multititer counter. For the determination of pH dependency, membrane vesicles were diluted 40-fold in 100 mM Tricine buffer prepared at various pH values, preincubated for 60 min on ice, and assayed for ATPase activity. The effects of inhibitors on the ATPase activity were measured in assay buffer in the presence of 50 mM NaCl (final concentration). Membrane vesicles were preincubated for 60 min on ice in the presence of *N,N′*-dicyclohexylcarbodiimide (200 or 50 μM), carbonyl cyanide *m*-chlorophenylhydrazone (20 or 10 μM), vanadate (200 or 50 μM), or nitrate (25 or 10 mM KNO_3). Controls in which no membrane vesicles were used in the assay were included. Calibration was achieved by using a series of P_i standards.

Protein determination. Protein was determined according to the method of Lowry et al. (20) with bovine serum albumin as a standard.

Materials. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, United Kingdom. All other chemicals were of reagent grade and were obtained from commercial sources.

TABLE 1. Uptake of ^{14}C-labelled amino acids by membrane vesicles of strain LBS3 upon imposition of a $\Delta\bar{\mu}_{Na^+}$ at 40°C

Amino acid	Initial uptake rate[a] (nmol/min × mg of protein)
L-Arginine	15.5
L-Phenylalanine	13.5
L-Threonine	5.2
L-Alanine	4.5
L-Lysine	2.6
L-Asparagine	2.2
L-Leucine	1.6
L-Serine	1.2
L-Glutamine	1.1
L-Glutamate	0.03

[a] Membrane vesicles of strain LBS3 were loaded with 100 mM potassium phosphate, pH 8.0, and diluted 100-fold into 100 mM sodium or potassium phosphate, pH 8.0, supplemented with the indicated ^{14}C-labelled amino acid. The initial rates of amino acid uptake were determined during the first 7.5 s. The rates of amino acid uptake in the absence of a sodium gradient ranged from 0.01 to 0.05 nmol/min × mg of protein.

RESULTS

Uptake of amino acids by strain LBS3 membrane vesicles. From the newly isolated strain LBS3, membrane vesicles were isolated by lysozyme treatment and osmotic lysis. To determine whether the membrane vesicles had retained transport activity, the uptake of ^{14}C-labelled amino acids was analyzed in response to an imposed $\Delta\bar{\mu}_{Na^+}$. In the initial experiments, a $\Delta\bar{\mu}_{Na^+}$ was chosen because strain LBS3 requires a high sodium concentration for growth, i.e., 170 mM (24). Membrane vesicles were loaded with potassium ions and diluted into a buffer (pH 8.0, 40°C) containing sodium or potassium ions to generate a $\Delta\bar{\mu}_{Na^+}$ or no gradient, respectively. Except for L-glutamate, all tested amino acids were accumulated by the membrane vesicles in a sodium-dependent manner (Table 1). Especially high uptake rates were observed for L-arginine and L-phenylalanine. These data suggest that the uptake of most amino acids by strain LBS3 is dependent on Na^+ ions.

The uptake of L-leucine was studied in greater detail. Membrane vesicles were loaded with potassium ions and diluted into a buffer containing sodium ions either in the absence or in the presence of the K^+ ionophore valinomycin in order to generate a $\Delta\bar{\mu}_{Na^+}$ or $\Delta\bar{\mu}_{Na^+}$ (i.e., $\Delta\Psi - \Delta\bar{\mu}_{Na^+}$), respectively. As shown in Fig. 1, rapid uptake of leucine occurred in the

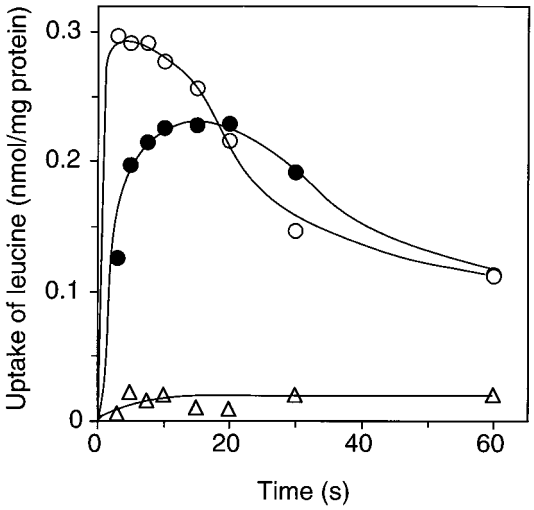

FIG. 1. Transport of leucine by membrane vesicles of strain LBS3. Transport of [^{14}C-]L-leucine was measured at pH 8.0 and 40°C in the presence of a $\Delta\bar{\mu}_{Na^+}$ (●), a $\Delta\Psi$ and a $\Delta\bar{\mu}_{Na^+}$ (i.e., a $\Delta\bar{\mu}_{Na^+}$) (○), or no gradient (△). Further experimental details are described in Materials and Methods.

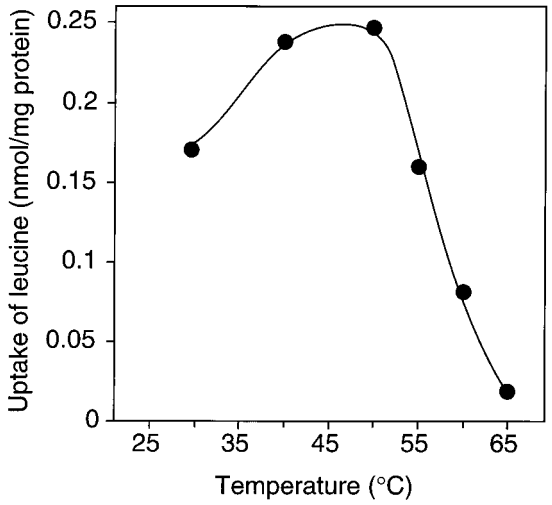

FIG. 3. Effect of temperature on initial rates of $\Delta\bar{\mu}_{Na^+}$-driven leucine uptake by membrane vesicles of strain LBS3. The $\Delta\bar{\mu}_{Na^+}$ was imposed at pH 8.0 and the temperature indicated as described in Materials and Methods. The initial rate of uptake were determined in the first 7.5 s.

presence of both a $\Delta\bar{\mu}_{Na^+}$ and a $\Delta\bar{\mu}_{Na^+}$. However, uptake was accelerated by the imposition of a $\Delta\Psi$ on top of the chemical gradient of sodium ions. These data demonstrate that L-leucine uptake by strain LBS3 occurs in symport with Na^+ ions.

Effect of pH and temperature on L-leucine uptake. Since strain LBS3 is an anaerobic thermoalkaliphile that grows optimally at 50°C and an alkaline pH of 9.5, the pH and temperature dependencies of L-leucine uptake were studied. An imposed $\Delta\Psi$ is usually rapidly dissipated at higher temperatures, while a chemical gradient of Na^+ is more stable. Therefore, experiments were performed with a $\Delta\bar{\mu}_{Na^+}$ of −120 mV only. The initial L-leucine uptake rate was maximal at pH 8.0 when assayed at 40°C (Fig. 2), although high rates of uptake were observed over a broader range of pH values, i.e., 7.0 to 10.0. When assayed at pH 8.0, uptake was optimal at 50°C (Fig. 3) but decreased rapidly at higher temperatures. Both the pH range and the temperature range that support uptake of L-leucine correspond to the physiological borders that allow the growth of strain LBS3 (24).

Ion dependency of L-leucine uptake. To establish whether L-leucine uptake is strictly coupled to Na^+ ion transport, the influence of other cations was tested. Many Na^+-dependent transport systems are able to utilize Li^+ ions instead of Na^+. As shown in Fig. 4, no uptake was observed when K^+-loaded vesicles were diluted into a buffer containing Li^+ as the counterion. Even when the Li^+ concentration was gradually lowered from 100 to 10 mM, no uptake of leucine was observed (data not shown). It is, therefore, concluded that L-leucine uptake is strictly coupled to Na^+ symport. Next, the Na^+ de-

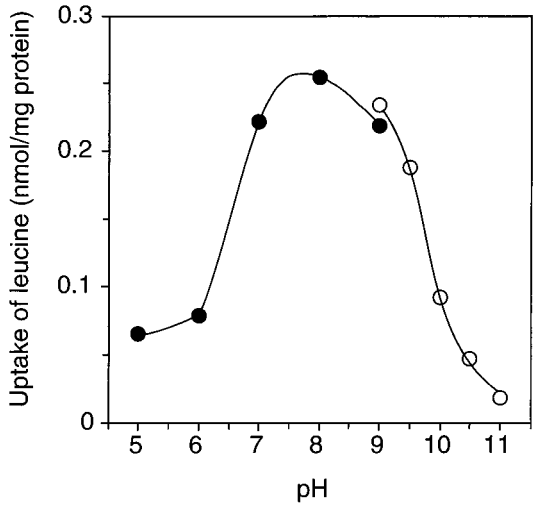

FIG. 2. Initial rates of $\Delta\bar{\mu}_{Na^+}$-driven leucine uptake by membrane vesicles of strain LBS3 as a function of the external pH. The $\Delta\bar{\mu}_{Na^+}$ was imposed at 40°C and the pH indicated as described in Materials and Methods. A $\Delta\bar{\mu}_{Na^+}$ of −120 mV was generated by dilution of the K^+-loaded membrane vesicles in 100 mM sodium phosphate (●) or 100 mM sodium carbonate (○). The initial rate of uptake were determined in the first 7.5 s.

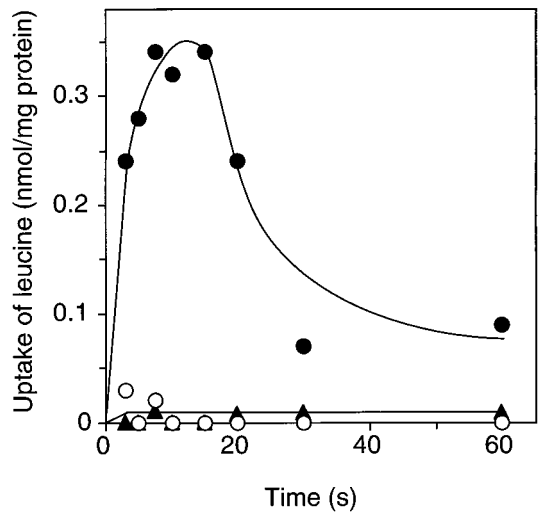

FIG. 4. Transport of leucine by membrane vesicles via a chemical gradient of Na^+ or Li^+ ions. Uptake experiments were performed at pH 8.0 and 40°C as described in Materials and Methods. Potassium-loaded membrane vesicles were diluted into buffer containing sodium (●), potassium (○), or lithium (▲).

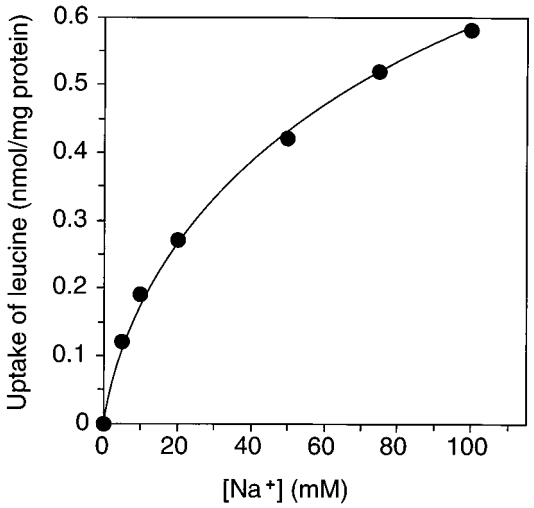

FIG. 5. Effect of sodium ion concentration on initial rate of $\Delta\bar{\mu}_{Na^+}$-driven leucine uptake by membrane vesicles of strain LBS3. Uptake experiments were performed at pH 8.0 and 40°C as described in Materials and Methods. The internal and external ionic strengths were kept equal, and externally the potassium was replaced by sodium. The initial rate of uptake was determined in the first 7.5 s.

pendency of the initial rate of L-leucine uptake was determined. As shown in Fig. 5, L-leucine uptake exhibits a rather low affinity for Na^+ ions, with an apparent K_m of about 45 mM. Uptake was saturated at an Na^+ concentration of around 130 mM (data not shown). It is concluded that L-leucine uptake specifically requires a high concentration of sodium ions.

Characterization of the ion dependency of the membrane-bound ATPase. The data presented above suggest that the secondary transport of amino acids by strain LBS3 is coupled to Na^+ transport and thus depends on the sodium motive force. Since strain LBS3 is a strict anaerobe that relies on substrate level phosphorylation for the production of metabolic energy, the generation of a sodium motive force depends on either a proton-translocating ATPase that acts in concert with a Na^+/H^+ antiporter or a primary sodium pump, such as a sodium-translocating ATPase. To test the latter possibility, inside-out membrane vesicles were prepared and the ion dependency of the membrane-bound ATPase activity was measured. ATPase activity was measured by following the release of P_i upon addition of Mg-ATP. The rate of autohydrolysis of Tris-ATP in the buffer system used was subtracted from the observed activity. The maximum ATP autohydrolysis rate in the chosen buffer system was lower than 124 nmol of P_i released per min per mg of protein and varied with the pH. Maximum activity was observed when the membranes were solubilized with the detergent Triton X-100 (Table 2). The optimal concentration of Triton X-100 was between 0.05 and 0.1% (vol/vol) (data not shown). In the remaining assays, Triton X-100 was employed at a concentration of 0.05% (vol/vol). The ATPase activity showed a broad pH dependency and was maximal at pH 7.0 to 7.5 when assayed in the presence of Na^+ or Li^+. In the absence of these ions, a low ATPase activity was detected (Fig. 6). The remaining activity is presumably due to the presence of contaminating Na^+ ions in the buffer solution. Next, we determined the Na^+ ion dependency of the ATPase activity. For this purpose, special precautions were taken to prevent the presence of contaminating amounts of Na^+ by using a low-Na^+ buffer and plastic ware. Saturation of the stimulation of the ATPase activity occurred with only 1 mM Na^+ (Fig. 7). At 100 and 200 mM Na^+, inhibition of the ATPase activity was observed. These data demonstrate that the membrane-bound ATPase of strain LBS3 is Na^+ stimulated, suggesting that it may be an Na^+-translocating ATPase.

TABLE 2. Effect of inhibitors on the sodium-stimulated ATPase activity of inside-out membrane vesicles of strain LBS3

Compound	Concentration	Relative rate of ATP hydrolysis[a] (% of control)
Control	NA[b]	100
Control + Triton X-100	NA	67
DCCD[c]		64
	200 μM	59
CCCP[d]	10 μM	123
	20 μM	127
Vanadate	50 μM	118
	200 μM	123
KNO_3	10 mM	110
	25 mM	103

[a] 100% activity corresponds to the release of 97 nmol of P_i per min per mg of protein. ATPase activity was measured at pH 7.0 and 46°C as described in Materials and Methods.
[b] NA, not applicable.
[c] DCCD, *N,N'*-dicyclohexylcarbodiimide.
[d] CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Effect of inhibitors and activators on ATPase activity. To characterize the type of ATPase, the effects of a number of inhibitors on the Na^+-stimulated ATPase activity in the absence of Triton X-100 were tested. In the presence of the F-type inhibitor *N,N'*-dicyclohexylcarbodiimide, moderate inhibition of the ATP activity was observed (Table 2). On the other hand, the ATPase activity was slightly stimulated by the P-type ATPase inhibitor vanadate, indicating that the enzyme does not belong to this group of ATPases. The V-type inhibitor NO_3^- had no effect on the ATPase activity. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone caused a slight stimulation of the ATPase activity, but this effect was not as

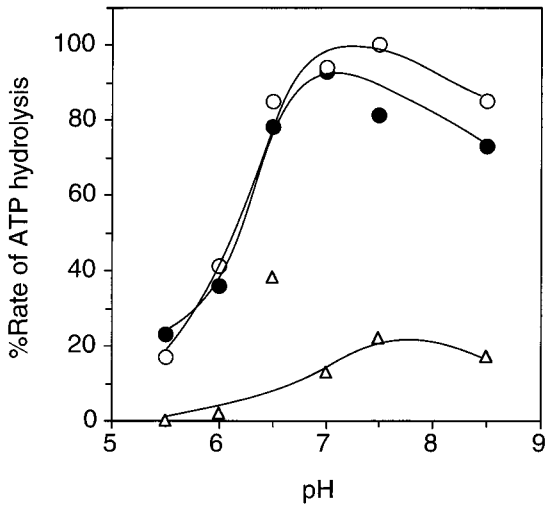

FIG. 6. Effect of pH on the rate of ATP hydrolysis by inside-out membrane vesicles of strain LBS3. P_i release was measured upon addition of 2 mM Mg-ATP in the presence of sodium (●), lithium (○), or potassium (△) ions (each at 50 mM) as described in Materials and Methods; 100% activity corresponds to the release of 100 nmol of P_i per min per mg of protein, and 0% activity corresponds to the rate of ATP autohydrolysis measured in this buffer system at the specified pH value.

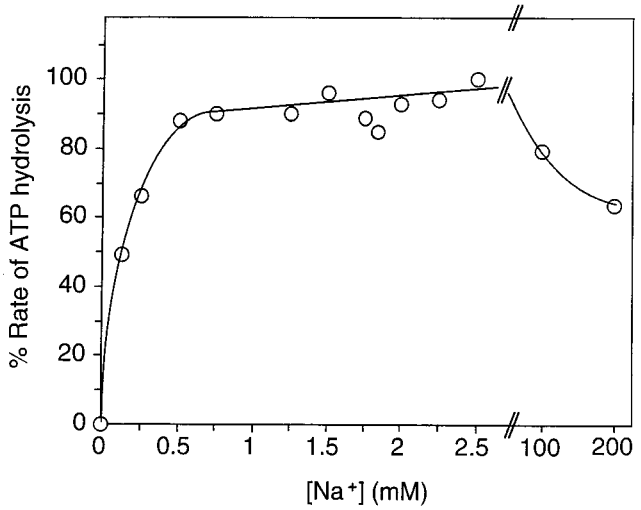

FIG. 7. Effect of increasing Na^+ concentration on the ATP-hydrolyzing activity in inside-out membrane vesicles of strain LBS3. Release of P_i was measured at pH 7.0 and 46°C upon addition of 2 mM Mg-ATP as described in Materials and Methods; 100% activity corresponds to the release of 98 nmol of P_i per min per mg of protein, and 0% activity corresponds to the rate of ATP autohydrolysis measured in this buffer system at the specified salt concentration.

strong as that observed by Hicks and Krulwich (7) for H^+-translocating ATPases.

DISCUSSION

Strain LBS3 belongs to a newly recognized group of anaerobic thermoalkaliphilic bacteria. The bioenergetics of this novel group of organisms has not been studied before. The present work demonstrates a strict Na^+requirement for the uptake of several amino acids by membrane vesicles derived from strain LBS3, while the membrane-bound ATPase was found to be Na^+ stimulated. These data suggest that energy transduction in strain LBS3 depends on sodium cycling.

Experiments with membrane vesicles were initiated to prevent problems associated with the oxygen sensitivity of metabolic energy generation in intact cells (1). The exclusion of oxygen from the transport assays proved to be unnecessary. Anaerobic conditions (i.e., nitrogen gas) had no effect on the transport and ATPase activity (25) (data not shown). Right-side-out membrane vesicles of strain LBS3 proved to be a convenient and reproducible system for transport studies. Several amino acids are accumulated by these vesicles in an Na^+-dependent fashion. In the case of L-leucine, it was shown that uptake occurs in symport with Na^+ and in an electrogenic manner. Further characterization of the leucine transport system revealed that its pH and temperature optima closely match the conditions that support the growth of this organism. In contrast to the Na^+-dependent uptake systems in the neutrophilic anaerobe *C. fervidus* (25), Na^+ could not be replaced by Li^+. Similar findings were obtained with regard to the uptake of solutes by alkaliphiles (3, 14). On the other hand, despite its high specificity, the L-leucine transport system shows only a poor affinity for Na^+ ions, and more than 100 mM Na^+ is needed to saturate the system. The optimum sodium concentration of the growth medium is 170 mM (24), and it thus appears that there is a significant requirement for sodium ions for both growth and energy transduction. In addition, strain LBS3 does not grow in medium without Na^+, and in its absence the cells become immobile (24).

Strain LBS3 exhibits an ATPase activity associated with inside-out membrane vesicles that is stimulated by both Na^+ and Li^+ ions. Saturation occurred bacteria at only 1 mM Na^+. Na^+-stimulated ATPases have also been found in the anaerobic bacteria *Propionigenium modestum* (17), *C. fervidus* (27), and *Enterococcus hirae* (31) and in the methanogenic archaeon *Methanosarcina mazei* Gö1 (1). It seems likely that this ATPase functions as a primary Na^+ pump, although definitive proof lies in the demonstration of this activity. The inhibition spectrum suggests that the ATPase from strain LBS3 is an F-type enzyme, although care should be taken with indirect classification on the basis of inhibitors alone.

Since energy transduction in strain LBS3 appears to be dependent on Na^+ cycling, the following question remains: does this organism maintain its intracellular pH, and if so, what mechanism is used for pH homeostasis? At pH 10 to 11, alkaliphiles usually maintain an intracellular pH that is about 2 pH units lower than the pH of the suspending medium. Since the lack of pH homeostasis in alkaliphiles is unprecedented to this date (9, 15), we assume that strain LBS3 is not an exception in this respect. In aerobic alkaliphiles, the activity of an Na^+/H^+ antiporter has been found to play an important role in internal pH regulation (15, 23, 28). Since strain LBS3 primarily relies on an Na^+-translocating ATPase for the generation of a sodium gradient across the membrane, it is difficult to image how an Na^+/H^+ antiporter, would contribute to pH homeostasis. The possibility exists that the internal pH is regulated by a K^+/H^+ antiporter, which allows the influx of H^+ at the expense of the release of K^+. K^+/H^+ antiporters have been implicated in pH regulation in other bacteria before (22, 29). In addition, the intracellular formation of acids (e.g. acetate, which is produced when the cells are grown on starch [24]) may contribute to pH homeostasis.

Another important factor for energy transduction (32) and growth (33) is the temperature. The permeability of the membrane to H^+ and Na^+ ions increases as the temperature rises, and a recent study on the permeability characteristics of liposomes derived from the membrane lipids of various neutrophilic bacteria suggests that there is a relation between the maximum growth temperature and the permeability of the membrane to H^+ (32). Since Na^+ ions are 10^2- to 10^3-fold less permeative than H^+, extreme thermophiles may gain an advantage by switching to Na^+-coupled energy transduction under conditions in which the permeability to H^+ becomes too high. A high permeability of the membrane to H^+ would make it even more difficult for anaerobic thermoalkaliphiles to regulate their intracellular pH. It is therefore not unlikely that the lipid bilayer of these organisms is adapted to cope with the phenomenon. On the other hand, a low permeability of the membrane to H^+ does not alone suffice for pH homeostasis. With both primary and secondary energy transduction being exclusively coupled to Na^+ another mechanism of pH regulation, such as the K^+/H^+ antiporter, must be operational in these organisms. Acidification of the intracellular pH relative to the pH of external medium could also directly result from $\Delta\Psi$-driven expulsion of OH^- (or uptake of H^+ which is phenomenologically similar to expulsion of OH^- but is less likely to occur because of the infinitely low concentration of H^+ at pH 10). This could be passive or could involve a pH-regulated channel. Obviously, studies are needed to determine if and how these bacteria regulate their intracellular pH, an important aspect of the bioenergetics of anaerobic thermoalkaliphiles.

In conclusion, energy transduction in the anaerobic thermoalkaliphilic strain LBS3 is coupled to sodium ions. This contrasts with studies on the bioenergetics of aerobic alkali-

philes, which rely on H^+-linked energy transduction. Since this is the first report on the bioenergetics of an anaerobic thermoalkaliphile, many questions remain and it will be important to elucidate how these organisms maintain their intracellular pH under alkaline conditions.

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