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Alkalophilic *Bacillus firmus* RAB Generates Variants Which Can Grow at Lower Na^+ Concentrations Than the Parental Strain

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Obligately alkalophilic *Bacillus firmus* RAB cannot grow well on media containing less than 5 mM Na^+. However, variant strains can be isolated on plates containing 2 to 3 mM Na^+. These variants are observed only rarely in cultures that are plated before being subjected to repeated transfers in liquid medium. Cultures which have been transferred several times produce variants at an apparent frequency of 2×10^{-4}. Most of these variants are unstable, generating parental types at the high frequency of 10%; however, stable variants can be isolated. These strains grow better than the parental strain at very high pH values in the presence of 5 mM Na^+ and have enhanced activity of the Na^+-H^+ antiporter that has been implicated in pH homeostasis. By contrast, Na^+-coupled solute uptake is indistinguishable from that of the parental strain, and no obvious changes in the respiratory chain components are apparent in reduced versus oxidized difference spectra. The membranes of the variants show a marked enhancement, on sodium dodecyl sulfate-polyacrylamide gradient electrophoresis, in one polypeptide band with a molecular weight in the range of 90,000. The findings are discussed from the point of view of genetic mechanisms that might confer adaptability to even more extreme environments than usual and in view of earlier models relating the Na^+-translocating activities of the alkalophiles.

The alkalophilic bacilli are of special bioenergetic interest both because of their primary problem of maintaining a cytoplasmic pH that is much lower than that of the external milieu and because of secondary energetic problems that result from this necessity (11, 12). Clarification of the properties that relate to extreme alkalophily will increasingly require genetic approaches so that the number of genes and their products can be definitively assessed. Genes from alkalophilic bacilli have been successfully cloned and expressed in *Escherichia coli* by Horikoshi and his colleagues (14, 20), and there is a report, that awaits follow-up, of the transformation of *Bacillus subtilis* to a more alkalophilic phenotype with DNA from an alkalophile (24). As yet, however, no genetic transformations of the alkalophiles themselves have been reported.

Among our attempts to achieve such transformations, we have tried to use DNA from one alkalophilic species, *Bacillus alcalophilus*, to transform another alkalophilic species, *Bacillus firmus* RAB. Initial attempts were focused upon a difference in the Na^+ requirements of the two species. *B. alcalophilus* grows well without added Na^+ as long as no special precautions are taken to avoid normal contaminating Na^+ levels. *B. firmus* RAB, by contrast, is a more typical alkalophile in requiring substantial Na^+, in the range of 25 mM, for optimal growth (5). Both species apparently depend on the activity of a Na^+-H^+ antiporter to catalyze acidification of the cytoplasm relative to the exterior, but the antiporter from *B. alcalophilus* has a much higher affinity for Na^+ than that from *B. firmus* RAB (4, 13). Attempts were accordingly made to transform the latter species to a "higher-Na^+-affinity" phenotype with DNA from *B. alcalophilus*. Screening was carried out on plates containing medium that contained 2 to 3 mM Na^+, levels that would not permit the formation of discrete colonies of *B. firmus* RAB within 48 h. During the course of the experiments, however, it became clear that occasional cells of *B. firmus* RAB can grow at a lower-than-usual Na^+ concentration without any transformation. Some of these variants generate, with great frequency, strains with Na^+ requirements that resemble the original parent strain. Other variants are more stable in their increased ability to grow at lower Na^+ concentrations, although none of these strains exhibits as low a requirement for Na^+ as does *B. alcalophilus*.

We describe in this report the frequency of variant formation by *B. firmus* RAB and the properties of the variants with respect to growth, Na^+-coupled porter activities, and electrophoretic profiles of their membrane proteins. The data are thus far consistent with the existence of some genetic mechanism whereby variants express larger amounts of one specific protein or a small number of proteins; one of these proteins may be the Na^+-H^+ antiporter.

MATERIALS AND METHODS

Organisms and growth conditions. *B. firmus* RAB was grown at 30°C with aeration at pH 10.5 in L-malate-containing medium (8). The basal buffer contained 0.1% (wt/vol) $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$, and either 50 mM $NaHCO_3$-Na_2CO_3 or 50 mM $KHCO_3$-K_2CO_3 adjusted to pH values from 10.5 to 12.0 by varying the proportions of bicarbonate and carbonate salts. For experiments in which various concentrations of sodium carbonate were used, the total carbonate concentration was kept at 50 mM by compensatory amounts of potassium carbonate. The basal medium was supplemented with 0.02% (wt/vol) yeast extract, 1% (vol/vol) trace salts solution (9), and 50 mM potassium malate, added aseptically from separate sterile solutions. Growth experiments were performed in 500-ml sidearm flasks containing 50 ml of medium. Before the sidearm flasks were inoculated, late-logarithmic-phase cultures of cells were washed by centrifugation and suspension in 25 mM Tris hydrochloride plus 25 mM potassium phosphate (pH 9.0) (PT9). The sidearm flasks were inoculated to read between 10 and 20 Klett units on a Klett-Summerson colorimeter (no. 42 filter), and growth was monitored turbidimetrically.

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Petri plates containing either 25 mM $NaHCO_3$-Na_2CO_3 (pH 10.5) or 3 mM $NaHCO_3$-Na_2CO_3 plus 22 mM $KHCO_3$-K_2CO_3 (pH 10.5) were prepared from solutions made only in plasticware (Nalgene Labware Div., Nalge/Sybron Corp.) to avoid sodium leaching out of glassware. The petri plates contained 1% (wt/vol) purified agar (Difco Laboratories), trace salts solution (without sodium), and other supplements, all made in plasticware. Yeast extract was not included in the agar medium to minimize contaminating sodium. We had ascertained previously that *B. firmus* RAB grew well on agar medium without yeast extract as long as at least 5 mM sodium was present. Indeed, with plates prepared as described here and without added Na^+, it was even possible to demonstrate a marked requirement for sodium for growth of less stringent *B. alcalophilus*. Before being plated, logarithmically growing cells were washed with PT9, and all subsequent dilutions were made in PT9.

DNA preparations and DNA-DNA hybridization. Chromosomal DNA was isolated from logarithmic-phase cultures by a modification of Marmur's procedure (17) in which a phenol-chloroform-isoamyl alcohol (25:24:1) mixture is used for deproteinization (16).

^{3}H-labeled DNA was isolated from cells grown in the presence of 5 μCi of [^{3}H]thymidine per ml (56.4 Ci/mmol) by the method of Scher et al. (21) with the deoxyadenosine omitted. Labeled DNA was extracted by phenol and then suspended in 10 mM Tris (pH 7.5)–1 mM EDTA. The DNA was sheared by 10 to 20 passages through a 26-gauge needle and was then stored at −20°C.

The hybridization of ^{3}H-labeled DNA with nonradioactive DNA was assayed in liquid reaction mixtures by the approach of Wetmur and Davidson (25).

Assays of the Na^+-H^+ antiporter. Logarithmic-phase cells were starved in 100 mM potassium phosphate buffer (pH 7.0) for 15 h with shaking, as described previously (7). The starved cells were centrifuged for 10 min at 12,000 × *g*, washed, and suspended in 100 mM potassium carbonate (pH 9.0). After equilibration for 30 min at pH 9.0, the cells were centrifuged and concentrated to approximately 30 mg of cell protein per ml. $^{22}Na^+$ (as $^{22}NaCl$) was added to 5 mM, and cells were allowed to equilibrate for 60 min at room temperature (4). The efflux of radioactive sodium was initiated by diluting the cells 200-fold into 50 mM sodium carbonate–50 mM potassium carbonate (pH 11.0), with or without the addition of 10 mM potassium ascorbate and 1 mM phenazine methosulfate (PMS). Care was taken to add the ascorbate-PMS to the side of plastic tubes containing the dilution buffer so as to avoid auto-oxidation before the addition of the cells. On addition of the cells, the entire contents of the tube were rapidly mixed by vortexing. The efflux experiments were, unless otherwise specified, conducted at 15°C. At various intervals, suspensions were diluted by the addition of 3.5 ml of sodium-potassium carbonate (pH 11.0). The samples were rapidly filtered through glass fiber filters (25 mm; Whatman, Inc.). Zero time values were determined as described elsewhere (4). Radioactivity on the filters was measured by liquid scintillation counting. As indicated under Results, variations of this protocol were sometimes used in which the pH was lower, the concentration of Na^+ in the dilution buffer was lower, the temperature was higher, or energization by ascorbate-PMS was replaced by energization with a valinomycin-mediated potassium diffusion potential as described previously (4).

Assays of Na^+-solute symport. Cells starved as for the Na^+-H^+ antiporter assay were concentrated in 100 mM potassium carbonate (pH 9.0) and allowed to equilibrate. The uptake of α-aminoisobutyric acid (AIB) was assayed at 15°C in the presence or absence of ascorbate-PMS. Cells were diluted 200-fold into 95 mM potassium carbonate–5 mM sodium carbonate buffer (pH 11.0) containing 200 μM [^{14}C]AIB. Samples were taken at various times, washed with the dilution buffer, and counted as described above.

The uptake of AIB as well as malate was also assayed in cells that were not starved. Mid-logarithmic-phase cultures were harvested by centrifugation at 12,000 × *g* for 10 min. The cells were washed in one of several combinations of 50 mM potassium–sodium carbonate buffer (pH 10.5) containing sodium in concentrations of 1, 2, or 50 mM. The cells were suspended to 0.1 mg of cell protein per ml, aerated by rapid mixing, and assayed at room temperature for either AIB or malate uptake on addition of 200 μM [^{14}C]AIB or 100 μM [^{14}C]malate. Samples were taken and assayed for uptake as described previously (8).

Preparation of membrane vesicles and analyses of the membranes. Everted membrane vesicles were prepared by passing washed cells suspended in 20 mM TAPS (Tris hydroxymethyl-methylaminopropanesulfonic acid [pH 9.0]), 10 mM $MgCl_2$, and a small amount of DNAse through a precooled French pressure cell at 20,000 lb/in^2. Unbroken cells were removed by 10 min of centrifugation at 12,000 × *g*, and the membranes were recovered by a 60-min centrifugation at 250,000 × *g*. The membranes were washed once with 20 mM TAPS–10 mM $MgCl_2$ and suspended to 20 to 30 mg of protein per ml in 0.4 M sucrose–20 mM TAPS (pH 9.0)–10 mM NaCl, quick frozen in liquid N_2, and stored at −70°C. Dithionite-reduced minus ferricyanide-oxidized difference spectra were recorded at room temperature with a Perkin-Elmer 557 Dual Beam spectrophotometer, as described previously (15).

The polypeptide composition of everted membranes was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gradient electrophoresis by the procedure of Chua (2). Samples dissolved in 60 mM Na_2CO_3–60 mM dithiothreitol–2% SDS–10% glycerol were boiled for 2 min and loaded (100 μg per sample) onto 7.5 to 15% polyacrylamide gradient gels containing 0.1% SDS. Electrophoresis was carried out overnight at 50 V. Gels were stained with Coomassie brilliant blue R and destained as described by Chua (2).

Chemicals. [1-^{14}C]AIB (53 mCi/mmol) was from New England Nuclear Corp. L-[U-^{14}C]malic acid (40 mCi/mmol) was purchased from Amersham Corp. Acrylamide, bisacrylamide, and SDS were from Bio-Rad Laboratories. Ascorbic acid, PMS, and TAPS were products of Sigma Chemical Co. All other chemicals were obtained commercially at the highest purity available.

RESULTS

Growth characteristics of variant strains. Cultures of *B. firmus* RAB that are inoculated into the usual liquid medium directly from slants or from single colonies on plates containing 50 mM Na^+ fail to produce visible colonies within 48 h when plated on alkaline plates (pH 10.5) containing 2 to 3 mM Na^+; the rare observation of a colony on such plates within 48 h represents a frequency below 10^{-5}. After serial passage of *B. firmus* RAB cultures in Na^+-replete liquid medium, colonies are found with greater apparent frequency (2×10^{-4}) on plating on low-Na^+ plates. When the largest variant colonies are restreaked several times and then subcultured, they usually produce both large and small colonies on low-Na^+ plates, generating the wild-type characteristics (i.e., formation of very small colonies on the low-Na^+ plates

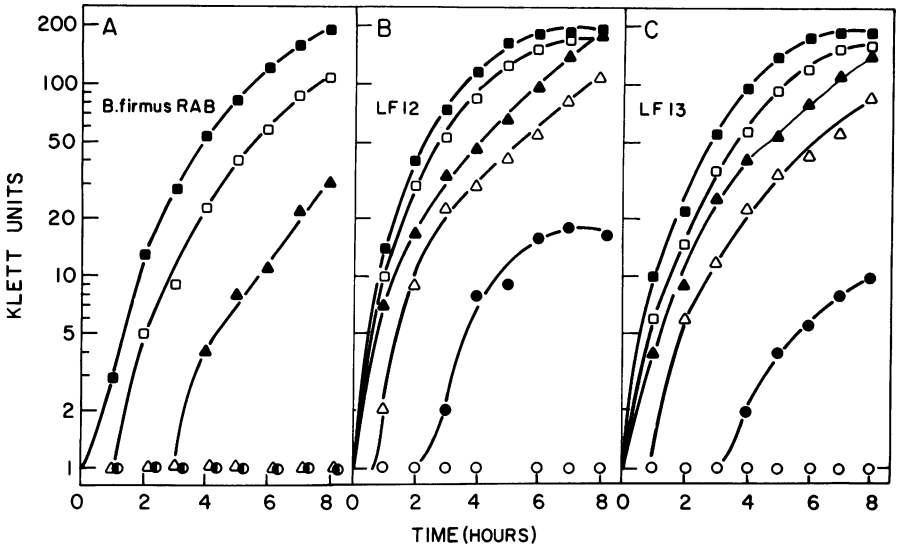

FIG. 1. Growth of *B. firmus* RAB and two stable variants at pH 10.5 in the presence of various concentrations of sodium. Media were prepared at pH 10.5, as described under Materials and Methods, and contained the following concentrations (millimolar) of added Na^+: 50 (■), 5 (□), 3 (▲), 2 (△), 1 (●), or zero (○). Growth was monitored turbidimetrically.

only after 48 h) with the high frequency of 10%. However, some of the variants with an enhanced ability to grow on low-Na^+ plates are stable. The generation of parental types can be assessed on plates with no more than a few hundred colonies; we estimate that the stable variants produce parental types with a frequency well below 10^{-3}. Two such stable variants, LF12 and LF13, were used in subsequent studies.

That these variants were not adventitious contaminants was determined in the following ways: the variants were morphologically indistinguishable from *B. firmus* RAB; they exhibited the same sensitivity to chloramphenicol and the same growth pattern—in which initial growth is followed by lysis—on lactose as does *B. firmus* RAB; and DNA-DNA hybridization data were consistent with identity to *B. firmus* RAB (greater than 90% homology), with *B. subtilis* and *B. alcalophilus* serving as negative controls.

Growth curves (Fig. 1) further indicate that the variants exhibit a subtle but reproducible enhancement in their ability to grow at low Na^+ concentrations relative to the parental strain. When cells of *B. firmus* RAB growing logarithmically in complete medium are washed and inoculated into liquid media containing no added Na^+ or concentrations of Na^+ up to 2 mM, no growth is observed. In the presence of 3 mM Na^+, modest growth occurs after a lag, whereas concentrations of 5 mM Na^+ and above support good growth of the parental strain. The two variants, on the other hand, grow modestly at 1 mM Na^+ and quite well at 2 mM Na^+ and above.

The variant strains also exhibited an enhanced ability to grow at extremely alkaline pH values in the presence of 5 mM Na^+; as the initial pH of the medium was increased from 11.0 to 12.0, the variants showed a modest inhibition of growth, whereas growth of the parental strain was severely compromised (Fig. 2).

Activity of Na^+-coupled porters. In view of the existing evidence for a crucial role for a Na^+-H^+ antiporter in the

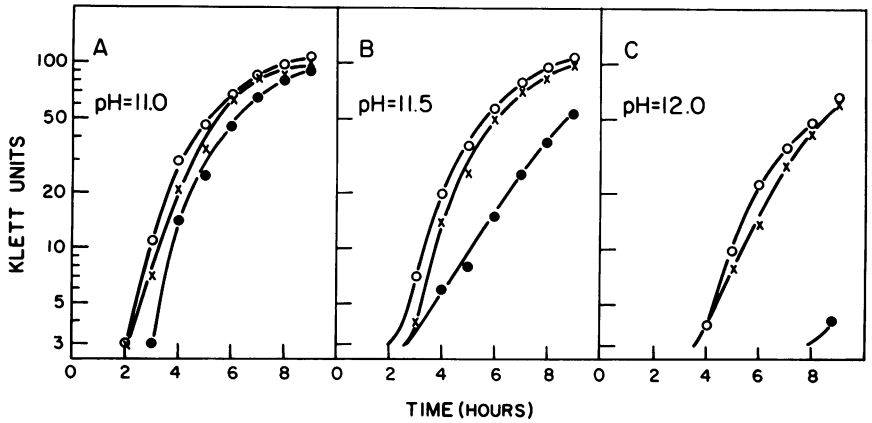

FIG. 2. Growth of *B. firmus* RAB and two stable variants in the presence of 5 mM sodium at pHs 11.0, 11.5, and 12.0. Media were prepared containing 5 mM sodium as described in Materials and Methods. The initial pH was adjusted to 11.0 (A), 11.5 (B), or 12.0 (C) by various combinations of potassium bicarbonate and potassium carbonate. Growth of *B. firmus* RAB (●), LF12 (○), and LF13 (X) was monitored turbidimetrically. During growth, the pH gradually fell (to approximately pH 10.5), but where growth was observed, it commenced at the initial pH of the medium.

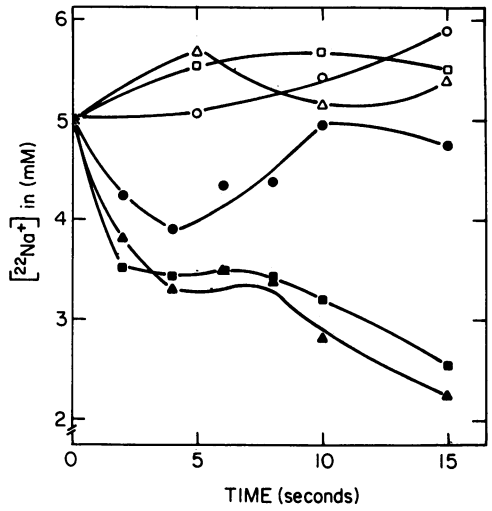

FIG. 3. Electron donor-dependent efflux of $^{22}Na^+$ from cells of *B. firmus* RAB and two stable variants. Starved whole cells of *B. firmus* RAB (●, ○), LF12 (▲, △), or LF13 (■, □) were equilibrated with 5 mM $^{22}Na^+$ at pH 9.0. Efflux of the $^{22}Na^+$ was monitored on dilution of the cells 200-fold into 50 mM potassium–50 mM sodium carbonate buffer (pH 11.0) at 15°C, to which either 10 mM potassium ascorbate–1 mM PMS (closed symbols) or no electron donor (open symbols) was added.

maintenance of a relatively acidified cytoplasm during growth at very high pH, this antiporter was examined first. Na^+-H^+ antiport activity was assessed by $\Delta\psi$-dependent $^{22}Na^+$ efflux from starved whole cells that were energized either by the addition of ascorbate-PMS, an electron donor, or by the establishment of a valinomycin-mediated potassium diffusion potential. Experiments were conducted at various external pH values, external Na^+ concentrations, and temperatures. A distinction between the variants and the parental *B. firmus* RAB with respect to antiporter activity could best be demonstrated under conditions in which the antiport was energized by the electron donor and was slowed by utilization of 15°C as the temperature and by the imposition of large chemical gradients against which the antiport had to work the exchange.

In the experiment shown in Fig. 3, cells equilibrated with 5 mM (radioactive) Na^+ inside at pH 9.0 were energized with ascorbate-PMS while they were diluted into buffer, at pH 11.0, containing 50 mM Na^+. In the absence of the electron donor, all three strains actually showed an increase in Na^+ inside, probably reflecting the uptake by the starved cells of bound radioactive Na^+ in response to a large Na^+ gradient, out > in. In the presence of the electron donor, the parental *B. firmus* RAB showed a modest efflux of $^{22}Na^+$, whereas the variants showed a more marked and sustained efflux of Na^+ from the interior against its electrochemical gradient. The data shown in Fig. 3 are the average values from four independent experiments. The difference between the parental and variant strains with respect to the higher initial rate of efflux and more complete efflux in the variants was highly reproducible. Other features of the average curves, e.g., the slow reentry of Na^+ into parental cells and the possibility of more than one phase of Na^+ efflux in the variants, were not always seen.

When antiport assays were conducted without low incubation temperatures and large, adverse chemical gradients of the substrates for the exchange, addition of the ascorbate-PMS resulted in $^{22}Na^+$ efflux that was extremely rapid in all three strains. By contrast, if the antiport was energized by a valinomycin-mediated potassium diffusion potential, there was almost no $^{22}Na^+$ efflux at pH 11; at pH 9.0, rapid efflux occurred from all three strains, with the variant strains often, but not always, showing more rapid efflux than the parental type (data not shown).

Assays of Na^+-coupled uptake of AIB under the same experimental conditions employed for the antiport assays shown in Fig. 3 failed to demonstrate any difference between Na^+-AIB symport activity in the parental and variant strains (Fig. 4). Moreover, no differences between the parental and variant strains were found for either AIB or malate uptake assayed in unstarved cells, at room temperature, or in the presence of 1, 2, or 50 mM Na^+ (data not shown).

Difference spectra. Reduced versus oxidized difference spectra were recorded for membrane vesicles from LF12 and LF13 at room temperature. The patterns of absorbance in the wavelengths expected for cytochromes were not different in any obvious qualitative or quantitative way from those of the parental *B. firmus* RAB (data not shown).

Membrane proteins. The membrane protein patterns of the two stable variants as well as of two less stable variant strains showed a dramatic and consistent difference from the parental pattern on gradient SDS-polyacrylamide electrophoresis. Both stable variants LF12 and LF13 and less stable variants RABTS and RABTL showed a marked enhancement in a polypeptide species with an apparent molecular weight in the range of 90,000 (Fig. 5). Careful scrutiny of several gels suggests that there may be a much more modest enhancement in LF12 and LF13 of at least one other

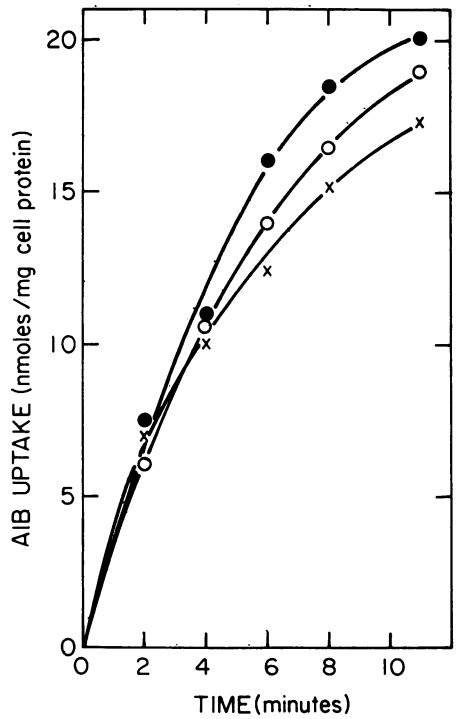

FIG. 4. Ascorbate-PMS-driven uptake of AIB by *B. firmus* RAB and two stable variants at pH 11.0. Starved cells of *B. firmus* RAB (●), LF12 (○), or LF13 (X) were assayed for AIB uptake, as described under Materials and Methods, at pH 11.0 and 15°C in the presence of 5 mM sodium. The background value for AIB taken up in the absence of the ascorbate-PMS was subtracted for each time point, but was always a small percentage of the experimental value.

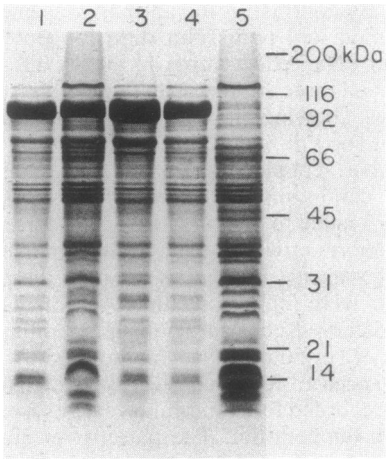

FIG. 5. SDS-polyacrylamide gradient gel electrophoresis of membrane polypeptides of *B. firmus* RAB and its variants. Everted membrane vesicles were prepared and subjected to SDS–7.5 to 15% polyacrylamide gradient gel electrophoresis as described under Materials and Methods. Lane 1, LF13; lane 2, LF12; lane 3, RABTS; lane 4, RABTL; and lane 5, *B. firmus* RAB. The molecular size markers (in kilodaltons [kDa]) are on the right side of the figure.

polypeptide, with a molecular weight near 45,000; the enhancement relative to the parental strain may be significant, especially when the percentage of the total protein aside from the major enhanced band is assessed.

DISCUSSION

Obligate alkalophiles such as *B. firmus* RAB, which grow optimally at about pH 10.5, are apparently well adapted to an extreme environment. The data presented here indicate, however, that this strain produces variants which are better adapted than the parent to even more extreme conditions, i.e., more extremely alkaline pH values and lower Na^+ concentrations. The findings are suggestive of some genetic mechanism which allows amplification of a certain gene or group of genes that are related to extreme alkalophily; in view of the existence of variant types with very different stabilities, the genetic mechanism may involve some sort of insertion or transposable element which results in either a stable or unstable change. It is notable, in view of these results, that another procaryotic inhabitant of a different extreme environment, *Halobacterium* sp., has been found to possess a high frequency of genetic variability, e.g., in expression of the bacterio-opsin gene, that is related to the activity of transposable genetic elements (3, 19, 22).

The biochemical data suggest that in the variants of *B. firmus* RAB which grow at lower Na^+ concentrations and higher pH values than the parental strain, the activity of the electrogenic Na^+-H^+ antiporter is enhanced, albeit difficult to demonstrate since the parental activity is already so high. Whether the enhancement in antiporter activity is the primary change, whether it is representative of increased expression of the structural gene for the antiporter, whether that enhancement is directly related to the large increase in the amount of an approximately 90,000-dalton membrane polypeptide, and whether there is one change or a whole group of variant properties are completely unknown at this time. The spectral data do not indicate that some respiratory chain component is amplified; had an increase in cytochromes been found, the increase in Na^+-H^+ antiporter activity could have been ascribed to the generation of a larger driving force. While subtle changes would not have been detected in the redox spectra, a larger driving force should have affected symport as well as antiport. Moreover, in at least some experiments in which the driving force was an artificial diffusion potential and respiration was inhibited by the presence of cyanide, there was still greater antiport activity in the stable variants than in the parental strain.

It is notable in connection with the antiport experiments that $^{22}Na^+$ efflux at the very high pH of 11.0 could be observed only when an electron donor was employed, whereas both electron donors and valinomycin-mediated potassium diffusion potentials are effective at more moderately alkaline pH values. These findings are similar to those reported previously for both Na^+-H^+ antiport activity (4) and ATP synthesis (7). We have suggested that at very alkaline pH values, bioenergetic processes that involve inward translocation of protons can no longer be energized by the diffusion potential because the chemical concentration of protons is limiting; energization by an electron donor may, under these circumstances, provide the requisite protons—via the proton-pumping activity of the respiratory chain—in some direct way.

In the variant strains, activity of the Na^+-translocating antiporter is altered without a parallel change in the activity of the Na^+-translocating symporters. In previous experiments with pleiotropic nonalkalophilic mutants of *B. alcalophilus* and *B. firmus* RAB, loss of Na^+-H^+ activity was accompanied by loss of Na^+ coupling to porters that catalyze uptake of AIB, malate, methionine, etc., even under conditions in which the absence of the antiporter would not itself cause a diminution in symport (1, 6). Because of these findings, we inferred a direct relationship between the Na^+-translocating antiporter and symporters and proposed, as one possible model, that the Na^+-translocating porters might share a common subunit. This model has become less inviting as evidence from other laboratories studying purified symporters consistently demonstrates that a single polypeptide can catalyze the cation-coupled symport (10, 18). Moreover, in a different alkalophile, Imae and colleagues (23) have shown that the antiporter can utilize Li^+ in place of Na^+, whereas at least some of the symporters cannot. The disjunction between antiport and symport activities reported here further leads us to favor explanations of the nonalkalophilic phenotype that do not involve a common subunit. It will be of interest to examine whether manipulation of the nature of the selective stress on the parental alkalophile strain makes it possible to isolate variants in which different activities, e.g., the symporters, are enhanced.

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