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Isolation and Characterization of New Facultatively Alkalophilic Strains of *Bacillus* Species

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Four facultatively alkalophilic isolates were purified from enrichment cultures initiated with lime-treated garden soil. Four isolates, OF1, OF3, OF4, and OF6, were obligately aerobic, spore-forming, gram-positive, motile rods which were capable of growth at both pH 7.5 and pH 10.5. Strains OF1 and OF6 grew best at the lower pH value; and whereas growth of these strains at pH 10.5 was completely dependent on added Na^+, growth at pH 7.5 was only partially dependent on added Na^+. Strains OF3 and OF4 grew better at pH 10.5 than at pH 7.5, with strain OF3 growing modestly over its entire pH range, while OF4 grew well. Growth of OF3 and OF4 was completely dependent on added Na^+ at both pH 7.5 and pH 10.5. DNA-DNA hybridization studies indicated that OF1 and OF6 are closely related strains but are not related to the other isolates, *Bacillus subtilis*, or two previously studied obligately alkalophilic bacilli. OF3 was unrelated to any of the other organisms examined in the study, whereas OF4 showed complete homology with obligately alkalophilic *Bacillus firmus* RAB. All four isolates maintained a cytoplasmic pH that was considerably lower than the external pH when the latter was 10.5. Although substantial transmembrane electrical potentials were observed, the total electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) was low at pH 10.5 in all the strains. By contrast, $\Delta\bar{\mu}_{H^+}$ was substantial at pH 7.5 and at that pH was composed entirely of an electrical potential. These results are in contrast to previous findings that obligately alkalophilic bacilli generate only small electrical potentials at near neutral pH. All the isolates exhibited substantial rates of respiration as measured by oxygen consumption. Neither respiration nor NADH oxidation by everted membrane vesicles was significantly stimulated by Na^+. Analyses of reduced versus oxidized difference spectra of membranes from OF4 showed that the total membrane cytochrome content was considerably higher in cells grown at pH 10.5 than at pH 7.5, with the levels of *c*- and *a*-type cytochromes exhibiting the largest pH-dependent differences. Initial examination of membrane protein profiles on gel electrophoresis also indicated a number of changes in pattern in each isolate, depending on the growth pH.

Alkalophilic bacteria, which grow well in a pH range from 10 to 11, are of ecological, industrial, and basic bioenergetic interest (8, 13). Studies of alkalophiles conducted thus far in our laboratory have focused on bacilli that are obligately alkalophilic. These species, especially *Bacillus alcalophilus* and *Bacillus firmus* RAB, cannot grow in the neutral range of pH and grow well only at pH 9.0 and above (4, 5). Other investigators have studied different species of alkalophiles, mostly bacilli, that can grow both in the neutral and highly alkaline pH ranges (3, 10, 21). It is not yet clear what precludes growth of the obligate alkalophiles at neutral pH, although possible factors include compromise of membrane integrity at neutral pH (12) and poor function of the respiratory chain at neutral but not alkaline pH (17). It is also unclear whether facultative alkalophiles express specific gene products or regulate the amounts of certain gene products in response to growth at high pH. Results of one study by Koyama et al. (10) indicate that there is a difference in the patterns of membrane proteins, as visualized after gel electrophoresis, in a facultative alkalophile grown at near neutral versus alkaline pH, but this question has not been intensively examined.

We recently undertook the task of isolating new alkalophilic strains, hoping to find among them facultative organisms which grow well at very alkaline as well as neutral pHs. It was anticipated that comparisons of the physiological properties of such strains and the well-characterized obligate alkalophiles might provide data relevant to the basis of obligate alkalophily. Moreover, it was expected that detailed studies of facultative alkalophiles at near neutral and very alkaline pHs would provide a productive experimental approach for identifying the gene product(s) required for alkalophily. We report here the characteristics of four newly isolated, facultatively alkalophilic *Bacillus* strains. One of them is closely related to obligately alkalophilic *Bacillus firmus* RAB which was isolated previously from soil from the same area (4) and which has been extensively studied in our laboratory.

MATERIALS AND METHODS

Isolation of alkalophilic bacteria. Bacteria that are able to grow at pH 10.5 were isolated from limed garden soil by enrichment culture in carbonate-buffered broth cultures with malate as the carbon source (5). The area from which the soil was taken was the same source from which alkalophilic *B. firmus* RAB had been isolated in 1980 (4). Single colonies were isolated on solid medium at pH 10.5 and restreaked several times. Four isolates, each from separate enrichments, were purified and were designated OF1, OF3, OF4, and OF6. All four isolates were spore-forming, gram-positive, rod-shaped bacteria which were obligately aerobic

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TABLE 1. Characteristics of alkalophilic isolates

Strain	Range of pH for growth on D, L-malate	Cell and spore morphology[a]	Colony color and morphology[b]	Growth on:			
				Glucose	Fructose	Lactose	Acetate
B. alcalophilus	9–11.5	Rod-shaped cells with small, round terminal spores that do not swell the cell	Circular, undulating, raised, cream-colored, 3.5-mm diam	+	+	+	+
B. firmus RAB	8.5–11	Rod-shaped cells with oval terminal spores that do not swell the cell	Circular, entire, raised, cream-colored, 1.5-mm diam	+	+	−	+
OF1	7–12	Rod-shaped cells with oval subterminal spores that do not swell the cell	Circular, entire, convex, deep yellow, 3-mm diam	+	+	−	−
OF3	7.5–12	Rod-shaped cells with terminal oval spores that swell the cell	Circular, undulating, raised, cream-colored, 1-mm diam	+	+	−	−
OF4	7.5–11.5	Rod-shaped cells with oval terminal spores that do not swell the cell	Circular, entire, raised, off-white, 1-mm diam	+	+	−	+
OF6	7–10.5	Rod-shaped cells with terminal elliptical spores that do not swell the cells	Circular, entire, raised, yellow, 5-mm diam	+	+	−	+

[a] These characteristics were the same at pH 7.5 and pH 10.5.
[b] On malate-carbonate plates (pH 10.5).

and motile. Additional characteristics of the isolates are listed in Table 1, together with those properties of obligately alkalophilic *B. firmus* RAB and *B. alcalophilus*.

Growth of the organisms. The four new isolates, as well as *B. firmus* RAB and *B. alcalophilus* ATCC 27647, were routinely grown with shaking at 30°C in L-malate containing medium at pH 10.5 (5). Two nonalkalophilic mutant derivatives of the two obligate alkalophiles *B. firmus* RABN and *B. alcalophilus* KM^{er} were grown in L-malate-containing medium at pH 6.8 (4). *Bacillus subtilis* BD99 was grown at pH 7.0 in Spizizen salts (24) with 50 mM L-malate as the carbon source, and supplemented with 50 μg/ml each of L-threonine, L-histidine, and L-tryptophan.

Growth experiments were performed in 500-ml sidearm flasks containing 50 ml of medium. For growth over a pH range of 7.0 to 12.0, the basal buffer contained 0.1% (wt/vol) ammonium sulfate, 0.1 mM magnesium sulfate, and either 50 mM $NaHCO_3$-Na_2CO_3 or 50 mM Na_2HPO_4-NaH_2PO_4; it was adjusted at intervals of 0.5 pH units from pH 9.0 to 12.0 for carbonate buffer and pH 7.0 to 9.0 for phosphate buffer. For experiments in which cells were grown with or without added sodium, the basal buffer consisted of 0.1% ammonium sulfate, 0.1 mM magnesium sulfate and 50 mM concentrations of one of the following: (i) K_2HPO_4-KH_2PO_4 (pH 7.5); (ii) Na_2HPO_4-NaH_2PO_4 (pH 7.5); (iii) K_2CO_3-$KHCO_3$ (pH 10.5); (iv) Na_2CO_3-$NaHCO_3$ (pH 10.5). The basal media were always supplemented with 0.1% (wt/vol) yeast extract–1% (vol/vol) trace salts–50 mM potassium malate, which was added aseptically from separate sterile solutions. The sidearm flasks were inoculated with late-logarithmic-phase cultures so that they read between 10 and 20 Klett units on a Klett-Summerson colorimeter (no. 42 filter). Growth at 30°C, with shaking, was followed turbidimetrically. The growth on carbon sources other than malate was followed at pH 10.5 in Na_2CO_3-$NaHCO_3$ medium to which 50 mM glucose, 50 mM acetate, 50 mM fructose, or 25 mM lactose was added aseptically.

DNA-DNA hybridization experiments. Cells were grown to the late logarithmic phase and DNA was extracted by a modified Marmur procedure (20) in which phenol-chloroform-isoamyl alcohol (25:24:1) was used for deproteinization (19). DNA labeled with tritium was prepared from cells that were grown in the presence of 5 μCi of [^{3}H]thymidine (56.4 Ci/mmol) per ml by the method of Scher et al. (22), with the deoxyadenosine omitted. Labeled DNA, extracted by the phenol method, was suspended in 10 mM Tris–1 mM EDTA (pH 7.5). The DNA was sheared by 10 to 20 passages through a 26-gauge needle and stored at −20°C. Hybridization of nonradioactive chromosomal DNA with ^{3}H-labeled DNA was performed in a liquid reaction mixture as described by Wetmur and Davidson (26).

Measurements of the protonmotive force. Both the $\Delta\psi$ (transmembrane electrical potential) and ΔpH (transmembrane pH gradient) components of the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) were measured in mid-logarithmic-phase cultures grown at either pH 7.5 or 10.5 on media containing sodium salts. Before the radioactive probes were added, the pH of the cultures was adjusted to exactly 7.5 or 10.5 by adding NaOH; this was not generally a major adjustment. $\Delta\psi$ was measured by a filtration assay after the addition of 2 μM [^{3}H]tetraphenylphosphonium (TPP^+) to vigorously aerated cells (23). The ΔpH was determined at pH 7.5 or 10.5 by assaying the distribution of the ^{14}C-labeled weak acids dimethyloxazolidine-2,4-dione (DMO) and benzoic acid at 40 and 34 μM, respectively, or the ^{14}C-labeled weak base methylamine at 4.3 μM; the filtration assay described by Zilberstein et al. (28) was employed. For all assays of $\Delta\psi$ or ΔpH, controls for probe binding were performed in the presence of 10 μM gramicidin or 5% butanol. Both gave similar values that were a small percentage of the experimental values and were subtracted from the experimental values. In calculating the magnitude of the $\Delta\psi$ and ΔpH, the value used for the internal cell water volume was 10 μl/mg of cell protein, the value previously determined for *B. firmus*

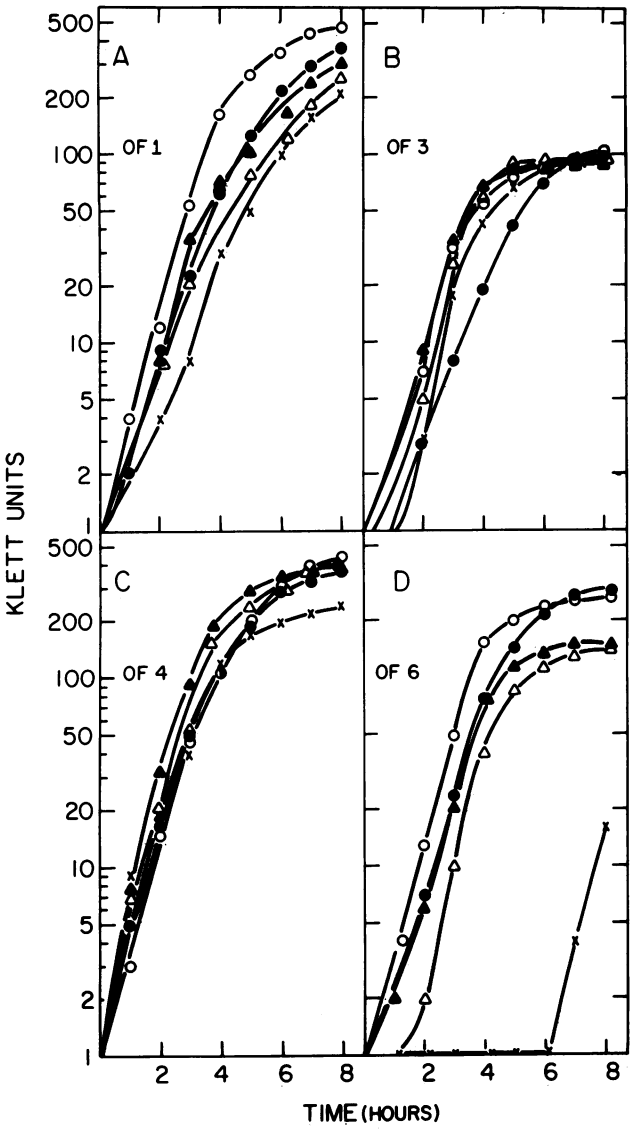

FIG. 1. Growth of the four new isolates at different pHs. The four isolates OF1 (A), OF3 (B), OF4 (C), and OF6 (D) were grown as described in the text, at the following initial pHs: pH 7.5 (●); pH 8.5 (○); pH 9.5 (▲); pH 10.5 (△); or pH 11.5 (×). The pH of the cultures was recorded during the course of the experiment; in all cases, growth, where it occurred, began while the culture was still at the initial pH. By the middle of the logarithmic phase of growth some changes in the culture pH had occurred, generally moving the pH of the culture gradually toward pH 9 to pH 10.

RAB. Protein was determined by the method of Lowry et al. (18), using bovine serum albumin as the standard.

Respiratory rates of whole cells. Logarithmically growing cells of the four isolates were harvested by centrifugation at 12,000 × *g* for 10 min. Cells grown at pH 7.5 were washed and suspended in 25 mM potassium phosphate (pH 7.0). Cells grown at pH 10.5 were washed and suspended in either 25 mM potassium carbonate (pH 9.0) or 25 mM sodium carbonate (pH 10.5). The cells that were suspended either at pH 7.5 or 9.0 in potassium buffers were assayed for oxygen consumption in the presence or absence of 10 mM NaCl; cells suspended at pH 10.5 were not exposed to Na^+-free conditions, because the absence of Na^+ leads to immediate

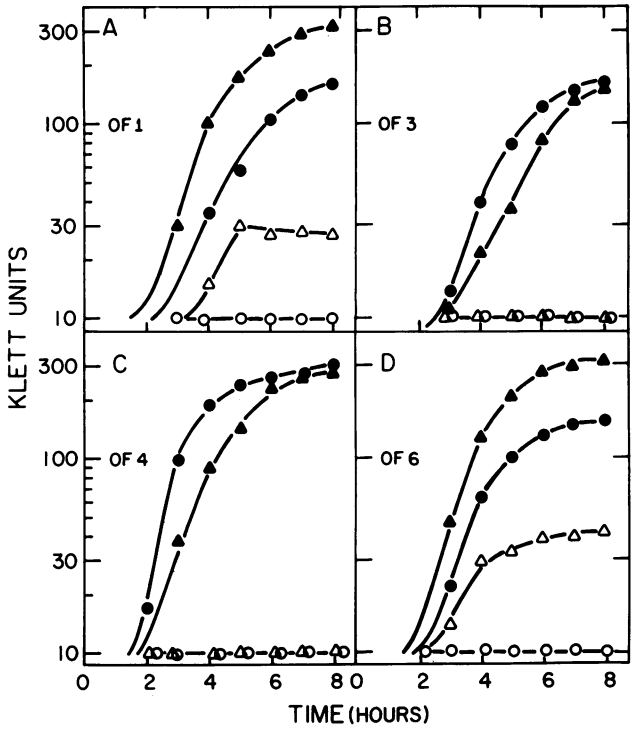

FIG. 2. Growth of the four isolates at pH 7.5 and pH 10.5 in the presence or absence of added sodium. The isolates OF1 (A), OF3 (B), OF4 (C), and OF6 (D) were grown as described in the text at pH 7.5 (△, ▲) or pH 10.5 (○, ●) with (closed symbols) and without (open symbols) 50 mM added sodium.

and deleterious alkalinization of the interior of most alkalophiles at that pH (9). The rate of oxygen consumption by the whole cells, using endogenous substrates, was measured with a Clark-type oxygen monitor (model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C, as described elsewhere (15).

Preparation of everted vesicles. Late-logarithmic-phase cultures were harvested by centrifugation at 10,000 × *g* for 10 min. Cells grown at pH 10.5 were washed by resuspension and centrifugation in 20 mM Tris hydrochloride–10 mM $MgCl_2$ (pH 9.0), while the cells grown at pH 7.5 were washed in 2 mM Tris hydrochloride–10 mM $MgCl_2$ (pH 7.5). The washed cells were suspended in either the pH 9.0 or pH 7.5 buffer used for the wash, a small amount of DNase was added, and the cells were passed through a prechilled French pressure cell at 20,000 lb/in^2. Intact cells were removed by centrifugation at 12,000 × *g* for 10 min. The everted vesicles were then pelleted by centrifugation at 250,000 × *g* for 60 min. The membranes were washed once in the same buffers described above, at either pH 9.0 or 7.5, by resuspension and centrifugation at 250,000 × *g* for 60 min. The final pellet used for NADH oxidase assays and gel electrophoresis was suspended to 20 mg of protein per ml by homogenization in 20 mM Tris hydrochloride (pH 7.5 or 9.0) containing 10% glycerol. The vesicle suspensions were then frozen in liquid nitrogen and stored at −70°C. Everted vesicles for cytochrome determinations were prepared similarly, except that the suspension buffer contained 0.4 M sucrose, 20 mM Tris hydroxymethyl-methylamine propane sulfonic acid (pH 9.0), and 10 mM NaCl.

Assays of NADH oxidase. NADH oxidase activity was assayed spectrophotometrically by a variation of the method

described by Tokuda and Unemoto (25). The assay mixture contained 0.2 mM Tris NADH, 20 mM Tris hydrochloride (pH 9.0) and about 20 μg of membrane protein in 1 ml to which, as indicated in specific experiments, either 10 mM NaCl or 10 mM KCl was sometimes added.

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

Determinations of membrane cytochrome contents of strain OF4. Dithionite-reduced versus thiocyanate-oxidized difference spectra were conducted at room temperature on everted membrane vesicles prepared from strain OF4 grown at pH 10.5 and pH 7.5. The procedures for recording the spectra and the calculation of the concentration of cytochromes *a*, *b*, and *c* were those used in previous studies (15).

Chemicals. Acrylamide, bisacrylamide, and SDS were purchased from Bio-Rad Laboratories, Richmond, Calif.). [*methyl*-^{3}H]thymidine (85 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. [^{14}C]methylamine (51.8 mCi/mmol), [*phenyl*-^{3}H]TPP$^+$ (35.5 Ci/mmol), [^{14}C]benzoic acid (25 mCi/mmol), and [2-^{14}C]DMO (950 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were obtained commercially at the highest purity available.

RESULTS

Growth characteristics. Three of the four new isolates, OF1, OF4, and OF6, grew well over a broad range of pH (Fig. 1). Of these three isolates, OF4 showed somewhat better growth at the very alkaline pHs, whereas OF1 and OF6 exhibited better growth at near neutral pHs than at very high pHs (Fig. 1 and 2). While the fourth isolate, OF3, did not grow very well at any of the pHs studied, it did show better growth at the very alkaline pHs than at near neutral pHs. Importantly, all of these strains, including the more alkalophilic ones, grew well at pH 7.5; the obligately alkalophilic species studied by us heretofore showed some growth at pH 8.5 to 9.0 (Table 1), but growth at those pHs was very poor relative to that at pH 10.0 to 11.0. All four of the isolates were completely dependent on added Na^+ for growth at pH 10.5, even without any special precautions to eliminate contaminating Na^+ (Fig. 2). By contrast, only the two strains which grew best at very alkaline pH, OF3 and OF4, were completely dependent on added Na^+ for growth at pH 7.5. OF1 and OF6 grew appreciably in the absence of added Na^+ at pH 7.5, although growth was greatly enhanced by the addition of Na^+ to the medium.

DNA homology among the isolates, obligate alkalophiles, and *B. subtilis*. The relatedness between the new isolates and *B. subtilis*, obligately alkalophilic *B. alcalophilus* and *B. firmus* RAB, and each other was assessed by DNA-DNA hybridization. Two of the new isolates, OF1 and OF6, which grew better at pH 7.5 than at pH 10.5 and were intensely yellow in color, appeared to be closely related to one another, probably because they represent strains of the same species (Table 2). Neither these isolates nor OF3, however, was related closely to *B. subtilis* or to the two obligate alkalophiles included in the study. OF4, on the other hand, exhibited complete homology with *B. firmus* RAB and presumably represents a strain thereof. Nonalkalophilic mutants that had previously been isolated on mutagenesis of *B. alcalophilus* or *B. firmus* RAB exhibited the expected homology with their parental strains, but the two obligately alkalophilic species showed little relatedness either to each other or to *B. subtilis*.

The $\Delta\bar{\mu}_{H^+}$ and respiratory rates of the isolates. The $\Delta\psi$ and ΔpH values for cells growing at pH 7.5 and pH 10.5 were determined for each of the isolates. All four isolates exhibited substantial $\Delta\bar{\mu}_{H^+}$ values, in a range from −164 to −190 mV, at pH 7.5, and this $\Delta\bar{\mu}_{H^+}$ was entirely composed of the $\Delta\psi$ component (Table 3). That is, at pH 7.5 there was no detectable chemical gradient of protons across the membrane, but a substantial $\Delta\psi$ was observed. At pH 10.5 all four isolates exhibited a cytoplasmic pH that was considerably below the pH outside of the cell. The apparent differences between the cytoplasmic pHs of the different strains may be real, but they might also reflect small errors introduced by assuming one standard internal water volume for all the calculations. The $\Delta\psi$ values observed at pH 10.5 were as high or even higher than those found at pH 7.5. Because, however, of the reversed ΔpH, the total $\Delta\bar{\mu}_{H^+}$ values at pH 10.5 were low in all of the isolates.

The respiratory rates of washed whole cells as a function of pH and the presence or the absence of added Na^+ (for pH 7.0 and pH 9.0 only) are shown in Table 4. Because a major aim of these determinations was to ascertain whether Na^+ stimulates respiration, endogenous rather than added substrates were used. Added substrates were likely to be taken up by Na^+ symport mechanisms, thus skewing the outcome. The respiratory rates with endogenous substrates were substantial for all the strains. OF4, the isolate which exhibited the best growth at pH 10.5, also exhibited the highest respiratory rate. Both OF4 and OF1 exhibited maximal respiratory rates at pH 10.5, and the presence of Na^+ had either no effect or only a modest stimulatory effect which could well be related to residual contaminating substrate which enters in a Na^+-dependent fashion. OF3 and OF6 respired most rapidly at pH 7.0 and pH 9.0, respectively, and Na^+ again had either no effect or only a small stimulatory effect on the respiratory rates.

TABLE 2. DNA homologies among the new isolates, obligate alkalophiles, and *B. subtilis*[a]

Strain	Percent DNA homology with ^{3}H-labeled DNA from:				
	B. alcalophilus	*B. firmus* RAB	OF1	OF4	OF6
B. alcalophilus	100	18	28	22	27
B. alcalophilus KM^{er}	84	—[b]	—	—	—
B. firmus RAB	36	100	33	95	33
B. firmus RABN	—	92	—	—	—
B. subtilis BD99	16	20	5	24	8
OF1	54	16	100	12	92
OF3	39	10	7	19	12
OF4	39	92	41	100	25
OF6	51	38	74	12	100

[a] The results are the averages of at least three experiments with at least two independent preparations in which the average hybridization between the probe and unlabeled DNA from the same strain was 50 ± 5%. The values shown were normalized to 100%. The standard deviations of the values were between 4 and 18%.

[b] —, Not determined.

TABLE 3. The protonmotive force of cells growing at pH 7.5 or pH 10.5

Strain	pH_{out}[a]	pH_{in}[b]	ΔpH		Δψ (mV)[d]	$\Delta\bar{\mu}_{H^+}$ (mV)
			Units	mV[c]		
OF1	7.5	7.5	0	0	−170 ± 25	−170
	10.5	8.7	1.8	103 ± 12	−168 ± 18	−65
OF3	7.5	7.5	0	0	−164 ± 9	−164
	10.5	7.9	2.6	153 ± 8	−207 ± 16	−54
OF4	7.5	7.5	0	0	−185 ± 10	−185
	10.5	8.0	2.5	147 ± 9	−204 ± 15	−57
OF6	7.5	7.5	0	0	−190 ± 17	−190
	10.5	8.2	2.3	135 ± 14	−214 ± 21	−79

[a] pH_{out}, pH outside of the cell.
[b] pH_{in}, pH in the cytoplasm.
[c] The ΔpH was determined by measuring the distribution of either [^{14}C]benzoic acid (pH outside of the cell, 7.5) or [^{14}C]methylamine (pH outside of the cell, 10.5) as described in the text. The results with benzoic acid were in complete agreement with determinations of ΔpH made with DMO. pH_{in}, pH in the cytoplasm.
[d] Δψ was determined by the distribution of [^{3}H]TPP^+, as described in the text.

NADH oxidase activity of everted vesicles. Although the stimulation of respiration by Na^+ at alkaline pH was, at best, modest in all of the isolates, it was important to probe more directly the possible existence of a respiration-driven primary Na^+ pump of the type described in certain alkaline-tolerant marine bacteria (25). Thus the stimulation of NADH oxidase by Na^+ that is one hallmark of the latter system was examined in the facultatively alkalophilic isolates. Everted vesicles of all four isolates exhibited low activities of NADH oxidase, with activity in vesicles from OF1 and OF4 being somewhat higher than that in the other two preparations (Table 5). Whereas the addition of either K^+ or Na^+ caused some stimulation of NADH oxidase in some of the preparations, there was no specific stimulatory effect of Na^+.

Electrophoretic profiles of membrane polypeptides. The electrophoretic patterns of the membrane polypeptides from the isolates (Fig. 3) support the finding that OF4 and *B. firmus* RAB are homologous strains of the same species; the patterns of the polypeptides from cells grown at pH 10.5 were similar. Interestingly, the polypeptide pattern of membranes from cells of OF4 grown at pH 7.5 showed, in this initial qualitative characterization, several marked differences in polypeptides from the membranes from cells of the same strain growth at pH 10.5. For example, two distinct polypeptides in the 95,000- to 115,000-molecular-weight range change inversely between the two membrane preparations, as do a number of polypeptides in the 24,000- to 35,000-molecular-weight range. The patterns of membrane polypeptides from the other three isolates were distinct in significant ways from OF4 and from each other. In each isolate there were also apparent differences in pattern between membrane polypeptides from cells grown at pH 7.5 versus cells grown at pH 10.5.

Cytochrome content of OF4 membranes. The total membrane cytochrome content of vesicles from strain OF4 (Fig. 4) was significantly higher when the cells were grown at pH 10.5 than when they were grown at pH 7.5. The differences were especially pronounced for cytochromes *a* and *c*.

TABLE 4. Respiratory rates of whole cells of the facultatively alkalophilic isolates, using endogenous substrates, as a function of pH and the presence of Na^+

Strain	Assay pH	Na^+ present[a]	Respiratory rate (natoms of O/min per mg of protein)
OF1	7.0	−	124
		+	124
	9.0	−	100
		+	125
	10.5	+	172
OF3	7.0	−	78
		+	110
	9.0	−	85
		+	105
	10.5	+	69
OF4	7.0	−	185
		+	218
	9.0	−	329
		+	411
	10.5	+	411
OF6	7.0	−	74
		+	74
	9.0	−	98
		+	126
	10.5	+	83

[a] Cells at pH 9.0 or 7.0 in 25 mM potassium phosphate buffer were assayed for oxygen consumption with either 10 mM KCl or 10 mM NaCl added. Cells at pH 10.5 were in 25 mM sodium carbonate buffer. Standard deviations were all within 10%.

DISCUSSION

The finding of new facultatively alkalophilic isolates reinforces prior findings with respect to bacteria that grow at extremely high pHs and also offers a number of new experimental opportunities. All of the isolates exhibited a pronounced ability to maintain a cytoplasmic pH that was considerably lower than the pH of the exterior at pH 10.5. This ability is apparently critical for growth of bacteria at

TABLE 5. NADH oxidase activity of everted vesicles from the isolates, assayed at pH 9.0 in the presence or absence of added Na^+

Strain	Cation added, 10 mM	NADH oxidase activity (μmol/min per mg of protein)
OF1	None	0.11
	K^+	0.15
	Na^+	0.14
OF3	None	0.05
	K^+	0.06
	Na^+	0.05
OF4	None	0.17
	K^+	0.19
	Na^+	0.19
OF6	None	0.06
	K^+	0.05
	Na^+	0.04

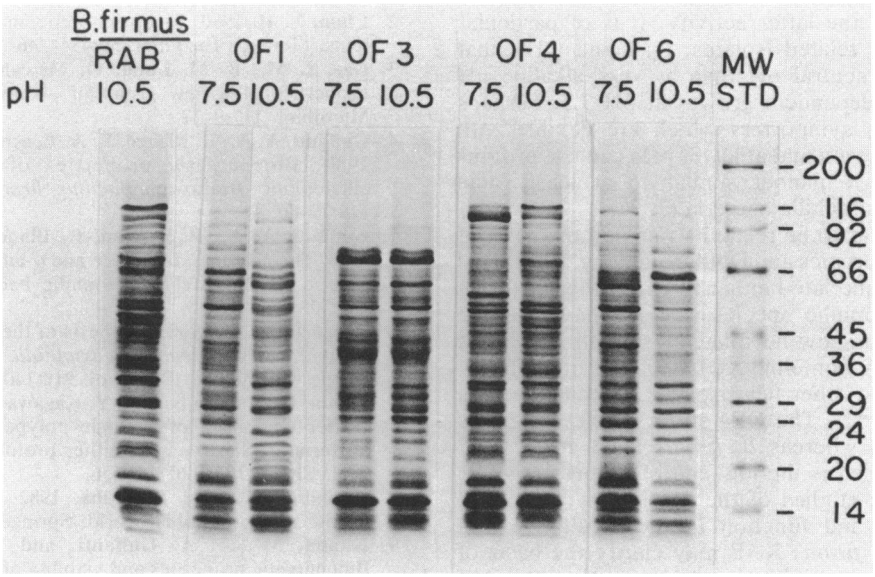

FIG. 3. SDS-polyacrylamide gradient gel electrophoresis of membrane polypeptides of *B. firmus* RAB grown at pH 10.5 and of the four isolates grown at either pH 7.5 or pH 10.5. Everted membrane vesicles were prepared and were subjected to SDS-7.5 to 15% polyacrylamide gradient gel electrophoresis as described in the text. The samples (100 μg of protein per lane) were applied to the lanes as indicated, with molecular weight standards (MW STD; in thousands) shown to the right of the figure.

pHs much above pH 9.0 and is a special property that distinguishes alkalophilic species—be they obligate or facultative—from alkaline-tolerant bacteria which grow well at pH 9.0 but not at extremely alkaline pHs (13). The new facultative strains generated substantial $\Delta\psi$ values that were positive outside of the cells at very alkaline pHs. However, as with the obligate alkalophiles, the large adverse ΔpH results in very low bulk $\Delta\bar{\mu}_{H^+}$ values at pH 10.5. Our laboratory has an ongoing interest in how aerobic bacilli carry out $\Delta\bar{\mu}_{H^+}$-dependent bioenergetic work, such as ATP synthesis, under conditions in which the putative driving force is so low.

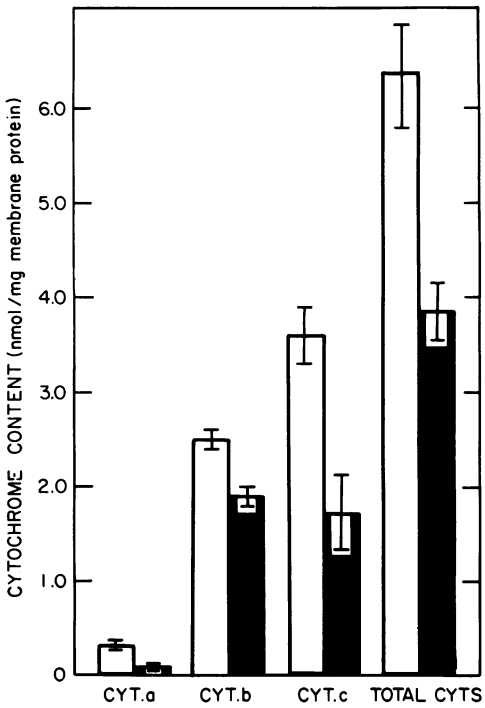

FIG. 4. The membrane cytochrome (CYT) content of OF4 grown at pH 10.5 or pH 7.5. Everted membrane vesicles of OF4 were prepared from cells grown at pH 7.5 (■) or pH 10.5 (□) and analyzed spectrally for cytochromes as described in the text. The range bars indicate the standard deviation.

Importantly, all four of the new facultative isolates generated very appreciable $\Delta\psi$ values at a near neutral pH as well as at an alkaline pH. At pH 7.5 the cytoplasmic pH was the same as the external pH, so that the $\Delta\bar{\mu}_{H^+}$ was equal to the substantial $\Delta\psi$. These findings are in contrast to previous determinations made on the obligate alkalophiles. *B. firmus* RAB, for example, generates only very low $\Delta\psi$ values when suspended at neutral pH (9), and results of studies of respiratory chain function indicate that the proton pumping capacity of the respiratory chain of that obligately alkalophilic strain is much lower at pH 7.0 than at pH 9.0 (17). Perhaps an important difference between obligate and facultative alkalophiles is the greater retention of respiratory chain function by the facultative species at lower pHs. Because in prior studies it has also been suggested that the membranes of the obligately alkalophilic bacteria lose integrity at moderately acidic pH, e.g., pH 6.5 (12), we are interested in the additional possibility that the membranes of the facultatively alkalophilic strains may be constituted to retain their barrier properties over a broader range of pHs than those of the obligately alkalophilic strains. Studies of the membrane lipids have been initiated.

The absolute dependence of growth at very alkaline pH on the presence of Na^+ is consistent with the possibility that, as in previously studied alkalophiles (13), pH homeostasis as well as solute symport are Na^+-dependent processes. Moreover, it is likely that pH homeostasis will involve a secondary Na^+/H^+ antiport of the type found in obligately alkalophilic bacilli (11) rather than a primary, respiration-dependent Na^+ pump of the kind reported in some alkaline-tolerant marine bacteria (1, 25). The lack of pronounced stimulation of respiration by Na^+ and the absence of any stimulation of NADH oxidation by Na^+ are inconsistent

with the presence of the latter activity. It is of particular interest that the two related isolates, OF1 and OF6, that grow better at near neutral pH than at very alkaline pH exhibit some Na^+-independent growth at pH 7.5. Perhaps these strains possess symporters which are flexible with respect to the coupling ion and at lower pHs can use protons in place of Na^+ in a manner similar to at least some symporters in nonalkalophilic bacteria (7, 27).

OF1 and OF6 appear to be related strains on the basis of physiological characteristics and DNA homology. These two strains and OF3 are unrelated either to each other or to the two obligately alkalophilic species that we have studied previously. They also show no relatedness with *B. subtilis* on the basis of DNA homology. OF4 on the other hand, while unrelated to the other new isolates, appears to be a strain of *B. firmus* RAB. The OF4 strain, however, grows very well at pH 7.5, whereas *B. firmus* RAB grows only poorly at pH 8.5, which is the low end of its pH range for growth. Comparative studies of the respiratory chain and membrane structure and function in the facultative and obligate strains of *B. firmus* RAB may clarify the basis of obligate alkalophily, i.e., the constraint(s) that prevent growth of such strains at near neutral or neutral pH. Moreover, OF4 and the other facultative strains may be useful for studies of those gene products that are required for alkalophily. The electrophoretic profiles of the membrane polypeptides indicate in a preliminary way that there are discrete changes associated with the growth pH. Of special interest in OF4 are changes in relatively high-molecular-weight species of membrane polypeptides that are in the same range as polypeptides that are altered in variants with an enhanced ability to grow at very alkaline pHs (14). Results of studies of such variants suggested the possibility that amplification of a membrane protein in the 95-kilodalton range is among a few possible changes associated with even more extreme alkalophily than that in the parental strain (14); thus, it is notable that in OF4, the membrane protein profile of which is quite similar to that of *B. firmus* RAB and its variants, a membrane protein in the same region is present in an apparently greater concentration during growth at the lower of the two pHs examined. Some of the membrane proteins that fluctuate quantitatively with growth pH will probably prove to be cytochromes, because the spectral data indicate an increase in all the cytochrome species during growth of OF4 at pH 10.5 as opposed to pH 7.5, with especially marked increases in cytochromes *a* and *c*.

It is anticipated that the facultative strain of OF4 and mutants thereof will be of greater experimental utility than the pleiotropic, nonalkalophilic mutant strains such as *B. firmus* RABN that were isolated by their ability to grow at pH 7.0 and had lost the ability to grow at pHs above 9.0 (16). Those mutant strains, in addition to their pleiotropy, generate very low $\Delta\tilde{\mu}_{H^+}$ values and grow only very poorly. They are thus difficult to maintain in pure culture and to grow in sufficient quantity for biochemical studies.

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