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Topical Review

Bioenergetics of Alkalophilic Bacteria

Terry Ann Krulwich
Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York, 10029

Introduction

Alkalophilic bacteria pose a variety of interesting bioenergetic questions that all arise from the necessity of maintaining a cytoplasmic pH that is much lower than optimal external pH values for growth. Alkalophiles will be defined here as organisms which grow optimally at external pH values of 10 to 11. Such organisms, usually species of *Bacillus*, can readily be isolated from natural or industrial enrichments (Horikoshi & Akiba, 1982; Krulwich & Guffanti, 1983). The alkalophiles that have been studied thus far are all aerobes which require low concentrations of Na^+, but are not halophilic or even marine; however, halophilic alkalophiles clearly can be isolated (Tindall, Mills & Grant, 1980). There are obligately alkalophilic strains which exhibit little or no growth below pH 8.5 as well as strains that are facultative with respect to pH (Gee, Lund, Metcalf & Peel, 1980; Koyama, Takinishi & Nosoh 1983). The alkalophilic bacteria as defined here are bioenergetically distinct from the more abundant alkaline-tolerant bacteria, which include many cyanobacteria, marine vibrios, and bacilli, Alkaline-tolerant bacteria are generally capable of growth in the neutral range of pH, but grow well or even best at external pH values of 9–9.5. Since this is probably just about the upper threshold of cytoplasmic pH that is compatible with viability, the alkaline-tolerant species live right on—but not above—the edge. The cytoplasm would not have to be acidified nearly as much relative to the outside in an alkaline-tolerant species, and a momentary failure of pH homeostasis would have far less disastrous consequences for such organisms than for the true alkalophiles. For halophilic or marine species growing at pH 9, the constraints imposed by the specific ionic components of the external milieu may well play a more important role in the bioenergetic adaptations than pH.

It could be foreseen (Garland, 1977) that the alkalophile's central problem of pH homeostasis, of interest *per se*, might lead to secondary bioenergetic problems of equal importance and interest. As proposed by Mitchell (1961, 1963), aerobic organisms, growing on nonfermentable carbon sources, transduce energy via an intermediary involvement of an electrochemical proton gradient between the cytoplasm and the bulk phase of the medium, outside acid and positive. The two bioenergetically equivalent components of this electrochemical proton gradient or protonmotive force (pmf) are a pH gradient (ΔpH, acid out) and a gradient of electrical potential ($\Delta\psi$, positive out). The pmf generated during respiration or other primary proton pumping events would be made available for ATP synthesis, motility, solute transport and other bioenergetic work (Harold, 1977). Since alkalophiles clearly must maintain a substantial pH gradient, *acid in*, the chemical gradient of protons would be adverse with respect to development of an adequate pmf. Alkalophiles would either possess mechanisms of offsetting that "reversed" pH gradient with an extremely high $\Delta\psi$ or would have found ways, using a modest pmf, of carrying out the bioenergetic work that usually depends upon a substantial pmf.

The Total Protonmotive Force of Alkalophilic Bacilli is Low

Alkalophilic *Bacillus firmus* RAB and *Bacillus alcalophilus* have been used for most of the studies conducted in my laboratory. These species are obligately alkalophilic, with an optimal pH of 10.5 for growth on malate-containing media (Guffanti, Susman, Blanco & Krulwich, 1978; Guffanti, Blanco, Benenson & Krulwich, 1980). The pmf was first de-

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Table. Protonmotive force pattern of cells of alkalophilic *Bacillus firmus* RAB[a]

pH_{out}	Cation in buffer	Malate (10 mM) present	pH_{in}	58.8 ΔpH	Δψ (mV)	pmf (mV)
10.5	Na^+	Yes	8.3	+127	−179	−52
	Na^+	No	9.4	+65	−162	−97
	K^+	No	10.5	0	−152	−152
9.0	Na^+	Yes	7.8	+74	−174	−103
	Na^+	No	8.0	+57	−144	−87
	K^+	No	9.4	−24	−142	−166
7.0	Na^+	Yes	7.7	−42	−50	−92
	Na^+	No	7.7	−40	−41	−81
	K^+	No	7.7	−41	0	−41

[a] Cells growing logarithmically at pH 10.5 were washed rapidly and resuspended in buffers, as indicated. Data are taken from Kitada et al., 1982.

termined as a function of pH_{out} in experiments using washed cell suspensions incubated in non-nutrient buffers. Under these experimental conditions, pH homeostasis is evident in both *B. alcalophilus* and *B. firmus* RAB, with the cytoplasmic pH maintained at pH 9.5 or lower at values of pH_{out} up to 11 or 11.5 (Guffanti et al., 1978, 1980). In the same series of experiments, Δψ values were found to increase over a range of pH from 8.0 to 11.5, from approximately −80 to about −150 mV. Total pmf values for cells of *B. alcalophilus* and *B. firmus* RAB at pH 10.5 were calculated to be about −75 and −60 mV, respectively (Guffanti et al., 1978, 1980). In fact, those estimates were probably somewhat high relative to the pmf of actively growing cells. As shown in the Table for *B. firmus* RAB, cells washed rapidly and resuspended at pH 10.5 in the presence of both Na^+ and malate maintain a pH_{in} lower than 8.5, and exhibit Δψ values near −180 mV, i.e., exhibit a total pmf just slightly above −50 mV. The pmf is higher at pH 9.0 because of the smaller "reversal" of the pH gradient; nonetheless, growth is better at pH 10.5 than at 9.0. Washed cells of *B. firmus* RAB that are suspended in buffer at pH 7.0 remain viable for a few hours but do not grow. Such cells generate a very small pmf relative to that formed at alkaline pH values, and the pmf is composed about equally of a Δψ and a ΔpH of conventional orientations (Table; Kitada, Guffanti & Krulwich, 1982).

Importantly, the Δψ values found for *B. firmus* RAB and *B. alcalophilus* at high pH are completely comparable to those reported for another alkalophilic strain, *Bacillus* YN-1, by Hirota and Imae (1983). They are also comparable to those reported by Hoddinott, Reid and Ingledew (1978) for *Bacillus pasteurii*. *B. pasteurii*, however, generated substantial total pmfs at pH 9.0 and 10.0 (higher pH values were not examined), because the acidification of the interior was modest (Hoddinott et al., 1978), thus resembling the pattern of an alkaline-tolerant rather than a truly alkalophilic organism. Indeed, the strain employed by Hoddinott et al. (1978) grew better at pH 9.0 than at higher pH values, and exploratory studies conducted in my laboratory with strains of *B. pasteurii* obtained from the American Type Culture Collection failed to turn up any strains that grew optimally at very alkaline pH.

It continues to be of importance to ask whether the low pmf values calculated for the extreme obligate alkalophiles might somehow result from determinations of the Δψ that are falsely low. Most of the measurements have been made with lipophilic cations, especially $TPMP^+$ and TPP^+, using either flow dialysis or filtration assays of the type developed by other investigators (Schuldiner & Kaback, 1975; Ramos, Schuldiner & Kaback, 1976). These probes might yield falsely low values if, at higher Δψ values, they fail to measure the full Δψ, as may be indicated in calibration experiments using theoretical Nernst potentials (Kashket, Blanchard & Metzger, 1980; Shioi, Matsuura & Imae, 1980; Guffanti, Blumenfeld & Krulwich, 1981*a*). On the other hand, because of their binding properties and the need for adequate correction for binding (e.g. Rottenberg, 1984), problems of overestimation also exist. In our own studies, there is a fairly close correlation between Δψ values obtained using $^{86}Rb^+$ in the presence of valinomycin and those obtained using TPP^+ (Guffanti, Bornstein & Krulwich, 1981*b*). In addition, results from studies of the relationship between $\Delta\bar{\mu}_{solute}$ and Δψ for solutes whose uptake is Δψ-dependent are most supportive of the view that the Δψ values we calculate are slightly overestimated rather than the converse (Guffanti et al., 1984). Thus, the quantitative dilemma presented by an inadequate pmf, and resulting from the need to maintain a relatively acidified cytoplasm, is likely a real and cogent one.

By contrast to the obligate alkalophiles, the al-

kaline-tolerant strain of *B. firmus* did not, apparently, possess the ability to acidify the interior substantially relative to the external milieu at very alkaline pH (Guffanti et al., 1980). Since growth was limited to external pH values of 9.0 to 9.5, it is likely that cytoplasmic pH values in that range are the approximate limit that is compatible with viability. This conclusion is supported by studies in which the loss of viability of alkalophilic *B. firmus* RAB upon failure of active pH homeostasis is determined as a function of external pH (Kitada et al., 1982).

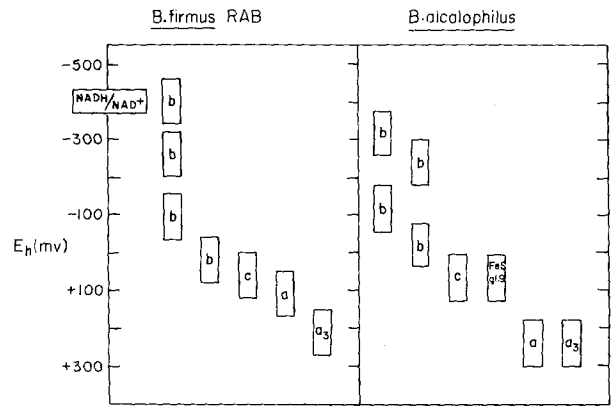

Fig. 1. Summary of the respiratory chain components identified on the basis of redox potentiometry in membranes from *Bacillus alcalophilus* and *Bacillus firmus* RAB at pH 9.0. The data are taken from Lewis et al., 1981 and Kitada et al., 1983. The blocks representing each component indicate the potential range over which the component becomes 9–91% oxidized or reduced. The lateral positioning is arbitrary

The Respiratory Chain Pumps Protons Optimally at Alkaline pH

It was clear, early on in studies of the extremely alkalophilic bacilli, that the respiratory chain catalyzes primary proton extrusion. In the absence of Na^+ and K^+, both whole cells and right-side-out-membrane vesicles generate a conventional ΔpH, i.e., acid out, during respiration (Mandel, Guffanti & Krulwich, 1980; Krulwich, Guffanti, Bornstein & Hoffstein, 1982; Kitada et al., 1982; Table).

Membranes of *B. alcalophilus* and *B. firmus* RAB are intensely red and contain more than 5 nmol of cytochrome heme per mg of membrane protein (Lewis, Belkina & Krulwich, 1980). The concentrations of *b*- and *c*-type cytochromes are especially high. Studies of the redox species in alkalophile membranes indicate the presence of a multiplicity of components as distinguished by mid-point potentials (Lewis et al., 1981; Kitada, Lewis & Krulwich, 1983). A summary of the species that have been titrated at pH 9.0 in membranes of *B. alcalophilus* and *B. firmus* RAB are shown in Fig. 1. Strekas (1984), in resonance Raman spectroscopic studies of the membrane preparations from the same two alkalophilic species, resolved *b*, *c*, and *a*-type cytochromes and found preliminary evidence in support of the apparent heterogeneity of the *b*-type cytochromes. More recently, both soluble and membrane-associated *c*-cytochromes have been distinguished in *B. firmus* RAB. The latter cytochrome *c* copurifies with the cytochrome oxidase, which has been characterized as possessing two major polypeptide subunits, 2 moles of copper, and 2 moles of heme *a* (Kitada & Krulwich, 1984). It is not yet clear whether the *b*-type cytochromes with different mid-point potentials actually represent distinct proteins; evidence to date is consistent with the possibility that only one protein is involved. Titrations of membranes of *B. alcalophilus* using EPR indicate the presence of numerous iron-sulfur clusters and, specifically, the presence of a Rieske FeS protein which is essentially isopotential with the cytochrome *c* (Lewis et al., 1981). Both alkalophilic bacilli also contain appreciable amounts of membrane quinone, originally thought to be ubiquinone, but more recently confirmed to be menaquinone.

The large amounts of respiratory chain components appear to be an adaptation to energy costs of life at high pH rather than the amplification of a chain to compensate for its compromised function. The growth yields of the organisms on malate are comparable to those of other aerobes, as are their respiratory rates (Lewis et al., 1980). The Y_{mal} for *B. firmus* RAB, for example, is 38 mg/dry wt/mmol of L-malate (Lewis, Krulwich, Reynafarje & Lehninger, 1983). More direct assessments of respiratory chain function, respiration-dependent proton translocation, are even more to the point. H^+/O stoichiometries were determined in oxygen pulse experiments with *B. firmus* RAB, using endogenous substrates in the presence of K^+ and valinomycin. Lewis et al. (1983) found H^+/O values as high as 13, with a frequently observed value of 9, when the determinations were made at pH 9.0. At pH 7.0, the H^+/O ratios were much lower, i.e., near 4; the low pmf values observed at pH 7.0 (leading to an inability to grow?) are probably secondary to poor function of the respiratory chain at this pH. It will be of interest to examine respiratory chain function in facultative alkalophiles as a function of pH.

Nonalkalophilic mutant derivatives have been isolated from some alkalophilic bacilli (Krulwich, Mandel, Bornstein & Guffanti, 1979; Guffanti et al., 1980). These mutants are identified on plates containing pH 7.0 media and have lost the ability to

grow above pH 9.0. Among a constellation of pleiotropic properties, strains selected in this way are found to have greatly reduced levels of membrane cytochromes, especially lower concentrations of *b*- and *c*-type cytochromes (Lewis et al., 1980). Their respiratory rates, in terms of oxygen consumption, are comparable to those of the wild type parent and other aerobes, but their growth yields and their H^+/O ratios are low (Lewis et al., 1983).

There is no current evidence that the extreme, nonmarine alkalophiles possess a respiration-driven, primary Na^+ pump of the kind described in the alkaline-tolerant marine vibrios (Tokuda & Unemoto, 1981, 1982). Na^+ does not stimulate respiration in *B. firmus* RAB or *B. alcalophilus*, and extensive observations of Na^+ movements are best explained by a secondary porter catalyzing Na^+/H^+ exchange that is $\Delta\psi$-dependent rather than a primary pump that produces a $\Delta\psi$. It will be of interest to assay for such activities as Na^+-dependent NADH oxidase in both conventional and marine alkalophiles.

Regulation of Cytoplasmic pH Involves an Electrogenic, Secondary Na^+/H^+ Antiporter

The first indication for the importance of a Na^+/H^+ antiporter came from the observation that the typical pmf pattern of whole cells is only reproduced in energized membrane vesicles from alkalophiles if Na^+ is present (Mandel et al., 1980; Krulwich et al., 1982); the Na^+-dependent acidification of the vesicle interior is also dependent upon a $\Delta\psi$, positive out. The Na^+ concentration required for net proton uptake in vitro correlates with that required for growth of the given alkalophile species (Krulwich et al., 1982). Nonalkalophilic mutant derivatives of both alkalophilic species fail to exhibit Na^+-dependent acidification of either the cytoplasmic or intravesicular space (Mandel et al., 1980; Krulwich et al., 1982). As already noted, vesicles of both wild type and mutant species exhibit respiration-dependent generation of a ΔpH, acid out, if Na^+ and K^+ are omitted. Vesicles from *B. alcalophilus* may have an electroneutral K^+/H^+ exchange activity, since the presence of K^+ allows the $\Delta\psi$-independent dissipation of the ΔpH established by primary proton pumping but not net proton uptake (Mandel et al., 1980). No such activity was observed in *B. firmus* RAB vesicles; as with whole cells (Table), net proton extrusion was observed in K^+-containing buffers as long as Na^+ was omitted (Krulwich et al., 1982).

Everted membrane vesicles from *B. alcalophilus*, upon energization with NADH, exhibit $\Delta\psi$- and Na^+ (or Li^+)-dependent proton extrusion and accumulation of $^{22}Na^+$ (Mandel et al., 1980); the nonalkalophic mutant derivative of *B. alcalophilus* does not exhibit these activities. ATP can substitute for NADH in energizing the antiport in the everted vesicle preparations from the wild type, but the ATP clearly acts via an obligatory, intermediary $\Delta\psi$ (Guffanti, 1983). Thus, as assayed in vesicle preparations, net proton accumulation on the cytoplasmic side of the membrane occurs secondary to respiration-dependent proton translocation and is catalyzed by an apparently electrogenic Na^+/H^+ antiporter. The antiporter is active in the very alkaline range of pH, and is inactive at pH 7.0 (Kitada et al., 1982). Sensitivity of the electrogenic Na^+/H^+ antiporter from *Escherichia coli* (Bassilana, Damiano & Leblanc, 1984) and of the electroneutral antiporter in renal brush border vesicles (Aronson, Nee & Suhm, 1982) to regulation by protons has been reported, and these porters have both been hypothesized by at least some investigators to have a role in pH homeostasis (Krulwich, 1983).

Assays of the antiporter in whole cells have been conducted by starving the cells so that they can be passively loaded with $^{22}Na^+$, and then energizing efflux of the radioactive cation by generating a valinomycin-mediated K^+ diffusion potential (Garcia, Guffanti & Krulwich, 1983). In vivo assays of this type confirm the estimates of the K_m for Na^+ for *B. alcalophilus* and *B. firmus* RAB that are calculated from the experiments with vesicles, and again demonstrate the absence of electrogenic antiporter activity in nonalkalophilic mutant strains. The mutants did exhibit a *$\Delta\psi$-independent* loss of $^{22}Na^+$, but there is no evidence that this flux is an antiport, and it might well be a leak. Using the cell assay, it has also been shown that: Li^+ is competitive with Na^+ (expected, since Li^+ can serve as a substrate for the antiport); the rate of Na^+ efflux increases linearly with the magnitude of the $\Delta\psi$ over an appreciable range, as expected for an exchange with a H^+/Na^+ ratio greater than one, but with no indication of gating; and the proton inhibitory site is on the cytoplasmic side of the membrane (Garcia et al., 1983).

Under the experimental conditions used in the cell assay, the Na^+/H^+ antiport actually functioned less well at very highly alkaline external pH values than at pH 7.0. This is perplexing in view of the normal conditions under which the antiport operates. Our working hypothesis, which will be elaborated further in connection with ATP synthesis, is that the normal conditions under which the antiporter operates also involve energization by respiration rather than a diffusion potential of potassium ions, and that the protons pumped by respiration may be made available as a substrate for the anti-

porter without complete equilibration with the bulk medium. When the antiport is energized by the diffusion potential, the extremely low concentration of protons in the medium at very alkaline pH may limit the rate of the exchange.

Recently, the assay approach employed with whole cells has been successfully applied to a reconstituted system. Octyl glucoside extracts of alkalophile membranes have been reconstituted into proteoliposomes made with alkalophile lipids (Seto-Young, Garcia & Krulwich, 1985). The proteoliposomes are prepared with potassium inside and are loaded with $^{22}Na^+$. Li^+-sensitive efflux of $^{22}Na^+$ against its electrochemical gradient is observed upon generation of a diffusion potential at pH 9.0 but not at pH 7.0. The protein that catalyzes the antiport has not yet been purified and identified. Koyama et al. (1983) examined SDS-polyacrylamide gels of membrane proteins from facultatively alkalophilic *Bacillus* YN-2 grown at either pH 10.2 or 7.5, and noted three membrane protein bands in the M_r range between 40,000 to 60,000 that were associated with high pH. On the other hand, our attention has been focused on a membrane protein of about 90,000 M_r as a possible candidate for the antiporter. SDS-polyacrylamide gels of the membranes of variants of *B. firmus* RAB with enhanced antiporter activity show one consistent change, i.e., the intensification of a band of around 90,000 M_r.

The crucial role of Na^+ in pH homeostasis in the very alkaline range of pH values has been supported by a variety of investigations of both moderate (Hamaide, Kushner & Sprott, 1983; Miller, Turpin & Canvin, 1984) and extreme alkalophiles. The requirement for Na^+ is demonstrated most dramatically in experiments in which alkalophiles are subjected to changes in Na^+ concentration and/or dramatic shifts in pH_{out}. Kitada et al. (1982) showed that resuspension of *B. firmus* RAB at pH 10.5 in buffers without Na^+ resulted in an immediate rise in pH_{in} to 10.5 (shown in the Table) and a rapid loss of viability. Subsequently, McLaggan, Selwyn and Dawson (1984) reported the results of pH-jump experiments on a facultative alkalophile, *Exiguobacterium aurantiacum,* that had been isolated from potato plant effluent and requires Na^+ for growth at pH 10.0 (Gee et al., 1980). Cells of *E. aurantiacum* were incubated in glucose-containing buffer at pH 8.85 and then subjected to a sudden change in the pH_{out} to pH 9.4 or 9.48. In the absence of Na^+ (K^+ present instead), the pH_{in} rises as much as the external pH during the shift. On the other hand, when Na^+ is present, the cells maintain their pH_{in} below that of the external medium throughout, and rapidly achieve a new steady-state cytoplasmic pH. Thus the alkalophile exhibits a remarkable ability to maintain its cytoplasmic pH in the face of a sudden change in the external pH. In similar experiments in *E. coli,* the bacteria initially respond to a pH jump with a total collapse of the ΔpH, followed by a relatively slow recovery that presumably utilizes the active pH homeostasis machinery (Zilberstein, Agmon, Schuldiner & Padan, 1982*a*, 1984; Kroll & Booth, 1983). Since Na^+ added to the alkalophile at the time of the pH jump was quite efficacious in pH homeostasis, McLaggan et al. (1984) suggested that there must be a route that allows very rapid entry of Na^+, perhaps a pH-controlled Na^+ entry pathway of the type proposed *a priori* by Booth and Kroll (1983).

Experiments on pH homeostasis in obligately alkalophilic *B. firmus* RAB confirm the finding of McLaggan et al. (1984) with respect to the capacity of alkalophiles to maintain pH_{in} during a large pH jump and also provide an indication that Na^+-coupled solute uptake systems can provide the Na^+ entry pathway that is requisite for pH homeostasis (Krulwich, Federbush & Guffanti, 1985*b*). Maintenance of both viability and a relatively acidified cytoplasm by *B. firmus* RAB upon shift from Na^+-containing medium to Na^+-containing buffer at pH 10.5 was enhanced by the presence of malate (Kitada et al., 1982; Table). It seemed unlikely that the malate was acting as a crucial energy source since cells of *B. firmus* RAB require extensive starvation in order to become depleted of endogenous energy reserves. If the malate enhanced pH homeostasis by providing an entry route for Na^+, it might be possible to show that even a nonmetabolizable substrate whose Na^+-coupled uptake would consume energy could still have a net beneficial effect with respect to regulation of intracellular pH. Indeed, α-aminoisobutyric acid (AIB), a nonmetabolizable amino acid analogue whose uptake by *B. firmus* RAB is coupled to Na^+, enabled the cells to maintain a somewhat lower pH_{in} during the shift from the growth medium to buffer and also had a positive effect upon retention of viability.

Most impressive were pH-jump experiments in which cells were first equilibrated at pH 8.5 and then subjected to a rapid jump in the external pH to 10.5. In the absence of Na^+ cells exhibited an immediate rise in pH_{in} to 10.5, whereas in the presence of a high concentration of Na^+ (50 mM), the pH_{in} remained below 8.5 for the first 30 sec and then slowly drifted upward to pH 9.0 over the next 2.5 min. Inclusion of both 50 mM Na^+ and AIB (500 μM) resulted in an initial *decrease* in pH_{in} and then the establishment of a new steady-state right near the starting pH_{in}. Moreover, a low concentration of Na^+ (2.5 mM) which did not allow *B. firmus* RAB to maintain an acidified cytoplasm during the shift in

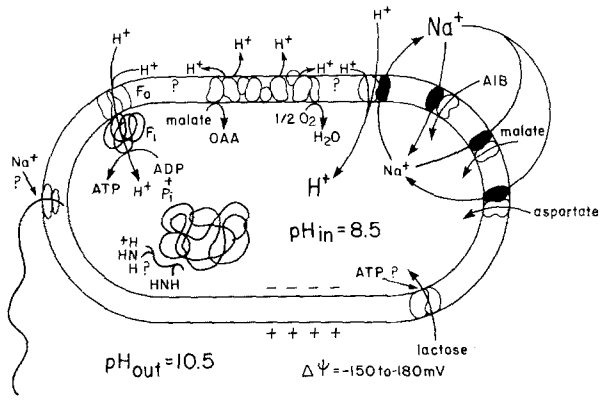

Fig. 2. Schematic representation of an alkalophile cell. Features of the model include: typical cytoplasmic pH and $\Delta\psi$ values during growth on malate at pH 10.5; a proton-pumping respiratory chain; a Na^+/H^+ antiporter, catalyzing an electrogenic exchange of H^+_{out} (perhaps utilizing some protons that are transported along a localized pathway?) for Na^+_{in}; Na^+ solute symporters that complete the Na^+ cycle; an F_1F_0 = ATPase involved in ATP synthesis and, perhaps, utilizing some localized proton pathway; some basic molecules shown as protecting the chromosome, representative of unidentified, basic buffering components of the cytoplasm; an involvement of Na^+ in flagellar rotation; and an ATP-dependent lactose transport system. The Na^+-translocating porters are shown with a defined Na^+ site; that site is probably not, according to the most recent data, a distinct subunit. Some regulatory or common synthetic step may link the Na^+-translocating porters, or a pleiotropic nonalkalophilic phenotype may be produced by mutational generation of a Na^+ leak

the absence of AIB, was rendered quite efficacious in its presence (Krulwich et al., 1985*b*). While these studies do not preclude the existence of an independent pH-regulated pathway for Na^+ entry, there is no specific evidence for such a pathway and were one to exist, it would be surprising if a solute that facilitates Na^+ entry would have the dramatic effects observed. Thus while there remains the possibility that a special channel for Na^+ entry exists in these organisms, an argument can be made for the hypothesis that the Na^+-translocating sites of the numerous symporters are the dominant or even sole routes of Na^+ entry. There may be regulatory effects of pH on the symporters which appropriately link their activity with respect to Na^+ entry to the need for pH homeostasis. For example, it may be that a sufficient rise in pH_{in} uncouples the cation uptake from uptake of the solute so that substantial Na^+ uptake occurs using the symporter proteins even in a solute-poor milieu. A model of an alkalophile cell is presented in Fig. 2, and includes the Na^+ cycle composed of the Na^+/H^+ antiporter and Na^+/solute symporters.

In *B. firmus* RAB there is no current evidence for antiport activities other than the Na^+/H^+ antiporter that have a role in pH homeostasis; indeed, as noted earlier, if there is a secondary K^+/H^+ antiport in this species at all, it is not a very active one. Thus, in at least one extreme alkalophile, the Na^+ cycle presented may well be both necessary and sufficient for pH homeostasis in the alkaline range of pH. With at least one alkaline-tolerant marine bacterium, however, it has been proposed that a K^+/H^+ antiporter plays an important auxiliary role at alkaline pH (Nakamura, Tokuda & Unemoto, 1984). By contrast, studies using vesicles from the facultative alkalophile, *Bacillus* YN-2000 (formerly YN-2), Koyama and Nosoh (1985) have found that K^+ (proposed to be acting via a K^+/H^+ antiporter) functioned in *alkalization* of the cytoplasm in the near neutral range of pH_{out} whereas Na^+ was the crucial cation for intravesicular pH maintenance in the highly alkaline range.

While it is clear that pH homeostasis in the alkalophilic bacteria is completely dependent upon active mechanisms—primarily, if not entirely, a secondary Na^+/H^+ antiporter—it is of interest to determine whether the cytoplasm and the barrier membrane possess any unusual properties that may relate to the extreme environment inhabited by the organisms. In this connection, it is of interest that *B. alcalophilus* and *B. firmus* RAB both have extraordinarily high cytoplasmic buffering capacities in the alkaline range of pH (Krulwich, Agus, Schneier, & Guffanti, 1985*a*). Since removal of Na^+ results in immediate alkalinization of the interior, the buffering (whose chemical nature is unknown) is no protection against global failure of active mechanisms; perhaps there are basic proteins or amines of some type which protect specific molecules within the cell (this possibility is indicated with an appropriate question mark in Fig. 2). With respect to the membrane lipids, Koga, Nishihara and Morii (1982) studied the lipids of alkalophilic *Bacillus* A-007 and found neutral lipids containing diacylglycerols and appreciable quantities of squalene and dehydrosqualene, and polar lipids composed of seven species of phospholipids including 7% cardiolipin and 4% bis(monoacylglycerol) phosphate. The latter was subsequently found to be 1-acyl-*sn*-glycero-3-phosphoryl-1′(3′-acyl)-*sn*-glycerol (Nishihara, Morii & Koga, 1982). Glycolipids and phosphoglycolipids were not found in *Bacillus* A-007 or in two other alkalophilic strains (Koga et al., 1982). The details of the membrane chemistry of the alkalophiles, and the structure of the membrane as a function of pH, will clearly be of interest. One factor that may compromise growth of certain alkalophiles at low pH is some constraint imposed by the chemistry of the membrane. During studies of the buffering capacity of whole alkalophile cells at various external pH

values (Krulwich et al., 1985*a*), loss of cell integrity was apparent at pH values not far below 7.0.

Solute Transport is Mediated by Na^+-Coupled or ATP-Dependent Mechanisms that Bypass the Problem of the Low Protonmotive Force

Studies of ion transport in alkalophiles have been dominated by investigations of cation antiports. In addition to the important and ubiquitous Na^+/H^+ antiporter and the demonstrable but less well-understood K^+/H^+ antiporter, a Na^+/Ca^{++} antiporter has been found in at least one alkalophile, *Bacillus* A-007, by Ando, Yabuki and Kusaka (1981). It is not yet known how potassium is accumulated or what transport systems exist for anions. One intriguing report by Ando et al. (1983) indicates the existence of H^+- and K^+-coupled uptake of phosphate in vesicles from alkalophilic *Bacillus* A-007. This porter(s) is an apparent exception to the general finding of Na^+-coupling in the alkalophilic bacteria. Further and more detailed studies of anion transport will be of considerable interest.

The alkalophiles solve the problem of accumulating solutes in the presence of a low pmf largely by using Na^+ as a coupling ion for solute uptake instead of coupling solute uptake to that of protons as do most bacteria (Harold, 1977). Na^+/solute symports are not unusual; they are the dominant mode of transport in eukaryotic cells (Crane, 1977) and in halophilic (Lanyi, 1979) and other marine bacteria (Skulachev, 1984). In these latter bacteria, the use of Na^+ relates primarily to the high Na^+ content of the environment. Extrusion of Na^+ from the cell interior by antiporters and primary pumps generates high electrochemical gradients of Na^+ in marine and high-salt media. Na^+-coupled transport systems take advantage of these gradients. By contrast, the best-studied extreme alkalophiles live in environments and media that have conventional salt concentrations. The use of Na^+-coupled transport as a major mode reflects an adaptation by the alkalophiles to a specific bioenergetic problem rather than to life in a salty milieu. Energization of solute uptake by an electrochemical gradient of Na^+, produced secondary to respiration-dependent proton translocation, allows the alkalophile to use the substantial $\Delta\psi$ and to be "blind" to the reversed pH gradient.

Na^+-dependent uptake of AIB and several other amino acids was first demonstrated by Koyama, Kiyamiya and Nosoh (1976) and then Kitada and Horikoshi (1977, 1979) in alkalophilic *Bacillus* 8-1. Na^+ decreases the K_m for substrate, whereas the V_{max} is unchanged. Li^+, K^+ and NH_4^+ could not substitute for Na^+ in this strain. Subsequently, Kitada and Horikoshi (1980*a*,*b*) showed that membrane vesicles from *Bacillus* 8-1 exhibit electron donor- and Na^+-dependent uptake of amino acids and that imposition of a sodium ion gradient causes transient uptake of AIB. Uptake of AIB and several other amino acids by *B. alcalophilus* (Guffanti et al., 1978; Guffanti, Cohn, Koback & Krulwich, 1981*c*) and *B. firmus* RAB (Guffanti et al., 1980) is similarly energized by an electrochemical gradient of Na^+ with the cation lowering the K_m for solute (Bonner, Mann, Guffanti & Krulwich, 1982). Studies of passive efflux and exchange of AIB in unenergized vesicles, by methods largely developed by Kaczorowski and Kaback (1979), showed that: both efflux and exchange are highly dependent upon Na^+; the two processes occur at comparable rates; and both processes are inhibited by generation of a pmf, outside positive. These findings suggest that the ternary complex between cation, solute, and carrier is positively charged and that "return of the unloaded carrier" or its mechanistic equivalent for transmembrane carriers is not rate limiting for efflux (Bonner et al., 1982). The rates of passive efflux and exchange of AIB in vesicles from *B. alcalophilus* occur at comparable rates at pH 7.0 and 9.0 but are much slower at pH 5.5 (Bonner et al., 1982); it is possible that changes in the membrane lipids affect porter function at pH values below neutral.

Interestingly, the nonalkalophilic mutant derivatives, which have altered cytochrome patterns and lack activity of the Na^+/H^+ antiporter, also have pleiotropically lost Na^+-coupled solute transport. Relevant observations include: the mutant strains do not exhibit Na^+-dependent uptake of AIB, malate, aspartate, methionine or other normally Na^+-coupled solutes, even at very initial time points or if monensin is added in low concentrations to replace the antiporter (Krulwich et al, 1979; Guffanti et al., 1981*c*); the nonalkalophilic strains similarly do not exhibit Na^+-dependent efflux or exchange of those solutes from unenergized vesicles—processes which would not depend upon a functional antiporter (Guffanti et al., 1981*c*; Bonner et al., 1982); the nonalkalophilic mutant strains do actively transport solutes, apparently utilizing the same porters that exist in the wild type parents, but now coupling uptake via those porters to protons rather than Na^+ (Guffanti et al., 1981*c*; Bonner et al., 1982); the frequency of mutation to nonalkalophily, near one in 10^9 cells, is consistent with one mutation—perhaps with some restriction in site—or possibly two mutations (Lewis, Kaback & Krulwich, 1982); the pleiotropic phenotype is found in all of the many such strains isolated, be they spontaneous mutants or mutants selected after mutagenesis, and all of the

dozen or more revertants examined regain the entire wild type phenotype (Lewis et al., 1982).

The observation of the concomitant mutational loss of all Na^+-coupled functions led to the interference of a relationship between the Na^+-translocating antiporter and symporters. A model was presented in which these porters share a common Na^+-translocating subunit which confers Na^+-coupling upon a second solute-specific subunit (Guffanti et al., 1981*c*). The porter could be envisioned to possess the latent capacity for coupling to protons, which is only expressed upon mutational loss of the "Na^+ subunit." The changes in cellular cytochromes might, in this sort of model, be secondary effects resulting from the loss of Na^+-dependent functions and the consequent alterations in pH_{in} (Lewis et al., 1982). During the years since the working hypothesis was presented, no definitive evidence has emerged, although Li^+ has been shown to be a substrate for the antiporter in at least some alkalophiles which cannot use Li^+ to replace Na^+ for symport (Sugiyama, Matsukura & Imae, 1985). Moreover, the finding that the purified symporters from other bacteria are single polypeptides that catalyze ion-coupled solute translocation (Newman, Foster, Wilson & Kaback, 1981; Hirata, Kambe & Kagawa, 1984) has made the "subunit" model less inviting. Recent data have finally presented a situation (short of the ultimate structural studies that would confirm or disprove a common subunit model) in which a change in the Na^+/H^+ antiporter is disjoined from any apparent change in the Na^+/solute symporters. Variants of *B. firmus* RAB, which appear to possess enhanced Na^+/H^+ antiporter activity, do not have parallel changes in Na^+/AIB symport. These findings would seem to contradict the model invoking a common Na^+-translocating subunit.

Other plausible explanations for the data on the nonalkalophilic mutants include a mutational change in some cellular effector that regulates several membrane functions, loss of some processing or insertion step common to some class of membrane proteins, or some change in the biosynthetic machinery affecting a particular class of membrane proteins (Krulwich, 1983). A mutation has been described in *E. coli* that is at least partially analogous to the mutations to nonalkalophily. The *phs* mutation leads to sensitivity to alkaline growth pH, loss of the ability to grow on substrates whose uptake is Na^+-coupled, and at least partial loss of activities attributed to the Na^+/H^+ antiporter in *E. coli* (Zilberstein et al., 1982*a*,*b*). Recent mapping studies by Booth and his colleagues (Rowland, Giffard & Booth, 1984; I.R. Booth, *personal communication*) indicate that the *phs* mutation is a mutation in the RNA polymerase such that transcription of some group of proteins is affected. It will be of great interest to correlate this finding with the specific pleiotropic effects of the mutation, especially the sensitivity to alkaline pH in *E. coli*. It will also make it of interest to look for a similar basis for the pleiotropic mutations of alkalophilic bacteria to nonalkalophily. At least one other possible interpretation of the phenotype of the nonalkalophilic strains merits consideration at this time. The nonalkalophilic mutant derivative of *B. firmus* RAB, strain RABN, exhibits only very poor growth at pH 7.0, and its growth is further inhibited by Na^+ (A.A. Guffanti, *unpublished*). This strain may be relatively leaky to Na^+ (Garcia et al., 1983; Seto-Young et al., 1985). Perhaps the mutational event results in a membrane leak for Na^+ which precludes Na^+-coupling to bioenergetic work, allows expression of the latent capacity for proton coupling, and has secondary effects of the pmf and respiratory chain function.

Both the ability of nonalkalophilic mutants to grow obligate alkalophiles at all at pH 7.0 and the observation that the growth is consistently poor are of interest. Perhaps the proton-coupled mutants can grow because they are able to use the full, albeit small, pmf that is divided equally into $\Delta\psi$ and ΔpH at pH 7.0 (Table; Kitada et al., 1982). On the other hand, the Na^+-coupled wild type cell would only be able to utilize the $\Delta\psi$ component, and the rate of solute uptake—which is very low at pH 7.0 in whole respiring cells—might be too low to support growth (Kitada et al., 1982). Interestingly, Kallas and Castenholz (1982) suggested that the growth-arresting event in exposure of an alkaline-tolerant cyanobacterium to relatively low pH might be on solute transport.

Finally, with respect to solute transport in the alkalophiles, there are at least some sugar transport systems that are not symport systems. Transport of β-galactosides, for example, by *B. alcalophilus* seemed to depend upon ATP or some derivative thereof rather than on the pmf and/or Na^+ (Guffanti, Blanco & Krulwich, 1979). The presence of this type of transporter in the alkalophiles is indicated in Fig. 2.

Motility is also Energized by an Electrochemical Gradient of Na^+

Alkalophilic, as well as some of the alkaline-tolerant marine bacteria, appear to require an electrochemical gradient of Na^+ to energize motility, thus utilizing the same means of bypassing the low pmf as is used for most solute transport. Hirota, Kitada and Imae (1981) showed that the swimming speed of alkalophilic *Bacillus* YN-1 was a function of the

Na^+ concentration and that monensin strongly inhibited motility. Hirota and Imae (1983) subsequently measured the relevant gradients, finding that at 100 mM Na^+ in the medium the cellular concentration of Na^+ was 30 mM while the $\Delta\psi$ was -170 mV. They presented evidence for a dependence of motility both on the $\Delta\psi$ and Na^+, with a linear dependence upon the $\Delta\psi$, and a total threshold for motility of about -100 mV. Although data are presented which support the energetic equivalence of the Na^+ gradient and the $\Delta\psi$, no determinations were done in which a gradient of Na^+ was shown to energize flagellar motion in the absence of a $\Delta\psi$. Importantly, Li^+ could substitute for Na^+ for the purposes of pH homeostasis in *Bacillus* YN-1, but could not be utilized for motility (Sugiyama et al., 1985). Kitada et al. (1982) similarly found that Na^+ is required for motility of *B. firmus* RAB and that motility also seems to require a $\Delta\psi$ of sufficient magnitude. The flagella from *B. firmus* RAB have been purified (Guffanti & Eisenstein, 1983); the single flagellin subunit has an M_r of 40,000 and has far fewer basic amino acids than flagella from neutralophiles. An electrochemical gradient of Na^+ also energizes motility of marine *Vibrio alginolyticus*, whereas a mutant strain of that species defective in Na^+-coupled functions can apparently use a pmf (Chernyak et al., 1983).

The use of Na^+ "in place of a proton" for motility raises interesting questions and, perhaps, opportunities. Is the normal site of cation interaction for motility flexible enough so that a single mutational change, to a loss of Na^+-coupled functions, will allow accommodation of either a proton or Na^+ as suggested by the reports on *Vibrio?* Are Na^+-type and H^+-type flagellar motors structurally distinct? Does Na^+ really replace the proton or does it initiate some microscopic cycle in which protons are still the entity that interacts with the flagellar apparatus? Clearly, there are analogies between these questions and those raised by various mutational events which alter the cation specificity of symporters, both in alkalophiles and in other bacteria (e.g. the melibiose carrier of *E. coli* which can now be probed both genetically and using reconstituted systems; Niiya, Yamasaki, Wilson & Tsuchiya, 1982; Shiota; Yazyu & Tsuchiya, 1984).

ATP Synthesis is Catalyzed by an F_1F_0-ATPase

Alkalophiles grow optimally at pH values at which their total pmf is near -50 mV (Table). Their phosphorylation potentials (ΔG_p) are in the same range as those of other aerobes, with conventional concentrations of cytoplasmic P_i and nucleotides (e.g. 4.0 mM ATP, 0.5 mM ADP, and 10 mM P_i in typical measurements after energization of *B. firmus* RAB). Moreover, in ADP + P_i-loaded, right-side-out vesicles from *B. alcalophilus* in which the internal ADP and P_i concentrations are experimentally fixed, a similarly large quantitative discrepancy is found between the pmf generated upon addition of an electron donor and the amount of ATP formed (Guffanti et al., 1981*b*).

Thus were ATP synthesis to be energized by chemiosmotic coupling to the bulk pmf, working upon a proton-translocating (F_1F_0-) ATPase, either large and variable H^+/ATP stoichiometries would be required or one would need to uncover a large error in the pmf measurements. As already noted, the cytoplasmic pH measurements are not serious candidates for large underestimations, and corroborative measurements make large underestimations of the $\Delta\psi$ unlikely as well. Therefore, our initial expectation was that the solution to the pmf problem with respect to ATP synthesis by the alkalophiles would be the same as for other bioenergetic processes, i.e., the use of an electrochemical gradient of Na^+. Indeed, Skulachev and his colleagues (Chernyak, et al., 1983; Skulachev, 1984) have presented a model Na^+ cycle for the marine vibrios in which a Na^+-dependent (vanadate-sensitive) ATP synthase is assigned the role of ATP generation. Perhaps in some marine aerobes there is a Na^+-translocating ATP synthase, but in the extreme alkalophiles, such a Na^+-dependent solution to the bioenergetic problem does not appear to obtain. In both whole cell experiments (Guffanti, Chiu & Krulwich, 1985) and experiments with ADP + P_i-loaded, right-side-out vesicles of *B. alcalophilus* (Guffanti et al., 1981*b*) and *B. firmus* RAB (Krulwich & Guffanti, 1983), no requirement for or stimulation by added Na^+ can be demonstrated for ATP synthesis. By contrast, ATP synthesis in these experimental systems is DCCD-sensitive and—in weakly buffered vesicles of *B. alcalophilus*—appears to involve influx of protons concomitant with ATP synthesis. Similarly, no significant stimulation of ATP hydrolysis by Na^+ (or inhibition by vanadate) is demonstrable in everted vesicle preparations of *B. alcalophilus* or *B. firmus* RAB, whereas ATP hydrolysis is clearly accompanied by inward proton pumping (Krulwich & Guffanti, 1983; Guffanti, 1983).

On the contrary, alkalophile membranes do contain an F_1F_0-ATPase. Although the F_0 from an alkalophile is yet to be characterized, soluble F_1-ATPases have been purified from two different alkalophilic bacilli. Koyama, Koshiya and Nosoh (1980) first reported the purification of an F_1-ATPase, to a specific activity of 9.2 units/mg, from

an alkalophilic *Bacillus*. Recently David Hicks in my laboratory has achieved the purification of the F_1-ATPase from *B. firmus* RAB by extraction from everted membrane vesicles by low ionic strength treatment followed by hydrophobic chromatography and sucrose gradient centrifugation. The enzyme exhibits a typical 5-subunit structure on SDS gels. The ATPase activity, like that of Koyama et al. (1980), is labile at 4°C, and has a high ratio of Ca^{++}/Mg^{++} ATPase activity. The purified F_1-ATPase from *B. firmus* RAB has a specific activity of 70–80 units/mg with Ca^{++} and 0.5 units/mg with Mg^{++}. The activity with Mg^{++} is stimulated by sulfite anion, methanol, and octylglucoside, 10-, 30-, and 100-fold, respectively. The purified enzyme is not markedly affected by cations.

The F_1F_0-ATPase of the alkalophile might generate large ΔG_p values at low pmf by functioning in subcellular organelles, which provide a mocroenvironment with a much larger driving force. However, fine structural studies (Krulwich, 1982) fail to show the presence of intracyptoplasmic membranes that would accommodate such a solution. In recent preparations, we have improved the resolution of the analysis by adjusting the fixation conditions to better approximate the cytoplasmic pH; still, only occasional spore membranes and mesosomal membranes typical of the septal region in bacilli are observed.

Compounding the quantitative problem is a striking qualitative phenomenon. It was noted in the discussion of the Na^+/H^+ antiporter that use of a valinomycin-mediated K^+ diffusion potential resulted in more substantial rates of $^{22}Na^+$ efflux at neutral pH_{out} than at the very alkaline pH values at which the antiporter actually functions. Similarly, and strikingly, ATP synthesis by starved whole cells of *B. firmus* RAB occurred in response to such a diffusion potential or in response to the addition of either malate or ascorbate/PMS at pH 7.0; at the neutral pH value malate was somewhat more efficacious than the diffusion potential, as assessed by $\Delta G_p/\Delta\psi$ ratios, but the difference was only 3.4 *vs.* 2.3 (Guffanti et al., 1984). By contrast, at pH 9.0, diffusion potentials of −170 mV were totally ineffective in energizing ATP synthesis whereas respiration-generated potentials of the same magnitude (initiated by addition of either malate or ascorbate/PMS and measured with TPP^+) produced typically high ΔG_p values with a $\Delta G_p/\Delta\psi$ ratio of 2.5. Nor was the valinomycin-mediated K^+ diffusion potential simply incompetent at pH 9.0: TPP^+ measured a $\Delta\psi$ close to that predicted from the Nernst relationship; and AIB uptake in the presence of Na^+ was energized as effectively by the diffusion potential at pH 9.0—$\Delta\bar{\mu}_{AIB}/\Delta\psi$ = 0.55—as by respiration at the same pH—$\Delta\bar{\mu}_{AIB}/\Delta\psi$ = 0.65.

Several other methodological or essentially trivial explanations for the lack of equivalence between a diffusion potential and a respiration-derived potential at alkaline pH have been eliminated. First, the phenomenon can be reproduced in membrane vesicles and is thus not dependent upon some cytoplasmic activity in the respiring cells (Guffanti et al., 1985). Second, an examination and evaluation has been made of the proton influx that accompanies the generation of large diffusion potentials. Experiments in which Tris-free and Na^+-free buffers were compared with the buffers used in the original experiments show that in any given buffer the proton influx accompanying the diffusion potential is, if anything, greater at pH 7.0 than at 9.0 and the TPP^+ uptake, if different at all, is slightly greater at pH 9.0 (Guffanti et al., 1985). Thus whatever the effect of proton influx on the pmf, it is more adverse at the pH at which a diffusion potential does energize ATP synthesis than at the alkaline pH at which it does not. Perhaps, then, the failure of the diffusion potential to energize ATP synthesis at pH 9.0 relates to an insufficiently low pH_{in}. This possibility is eliminated by numerous observations of malate-initiated ATP synthesis under conditions in which the pH_{in} was anywhere in a range from pH 7.0 to 9.5.

Nor is the inefficacy of the diffusion potential at pH 9.0, but not at pH 7.0, a result of extremely rapid decay of the diffusion potential at high pH and/or an extremely fast rate of hydrolysis of ATP made at pH 9.0. Time course of TPP^+ and AIB uptake in response to a diffusion potential at pH 9.0 are superimposable, eliminating the possibility that the TPP^+ response occurs long after the potential is dissipated. Experiments in which respiration-initiated ATP synthesis is arrested by the addition of cyanide (either with or without gramicidin) show, moreover, that the hydrolysis of ATP at pH 9.0 follows a monitorable time course over about 30 to 90 sec. Therefore, even if the diffusion potential had been short lived, the short burst of ATP synthesis would have been detected.

A number of experiments were also conducted in which a short burst of respiration (terminated by the addition of cyanide) was allowed just before or during the generation of a diffusion potential at pH 9.0. The results were negative with respect to facilitating energization by the diffusion potential at pH 9.0. Currently, our inclination is towards a working model which focuses on the observation that for the two alkalophile porters which must translocate protons inward from a very alkaline medium, respira-

tion energizes much more effectively than a diffusion potential. That greater effectiveness, though, is highly pH dependent, pointing again to the proton as the relevant factor. The model shown in Fig. 3 shows respiration as pumping protons along some localized pathway that makes at least some of the protons directly available to the proton-utilizing antiporter and ATPase while others are released into the bulk. Whereas at pH 7.0 a diffusion potential energizes those processes, drawing upon the protons in the medium, the proton concentration may start to limit the reaction at pH 9.0, let alone the much more alkaline growth pH values. Energy coupling hypotheses involving some sort of localized pathway for proton flow have been developed by many other investigators (Skulachev, 1982; Westerhoff et al., 1984; Ferguson, 1985). The potential advantage of the alkalophiles for exploration of such hypotheses is that the constraints of the milieu are so extreme that the mechanism(s) for localized proton flow could represent a gene product or set of gene products that are essential for growth at very high pH and could be identified as such.

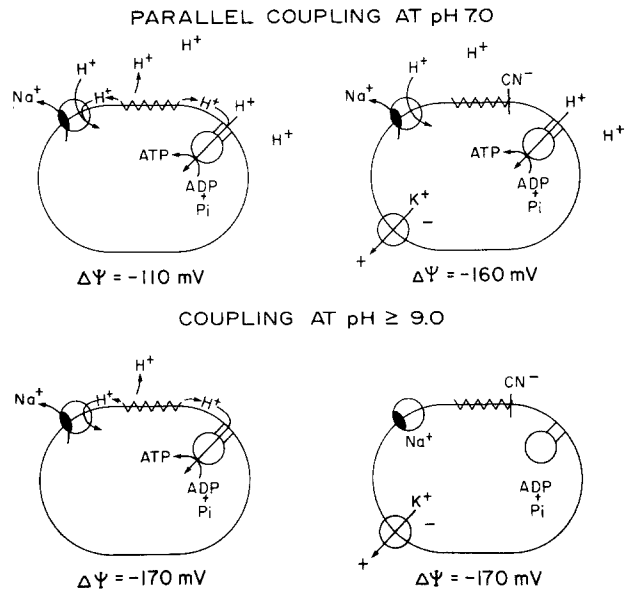

Fig. 3. A model of energy coupling to the Na^+/H^+ antiporter and ATPase of the alkalophile explaining pH-dependent differences between diffusion potentials and respiration-derived potentials on the basis of a localized pathway for protons pumped by respiration

Genetic Studies of the Alkalophiles are in Initial Stages

Ultimately, successful resolution of some of the more elusive bioenergetic issues will be greatly facilitated by the availability of genetic tools. There is one report, not subsequently extended or confirmed, of the use of DNA from alkalophilic *Bacillus* YN-1 and YN-2 to transform a restriction- and modification-deficient strain of *Bacillus subtilis* into an alkalophilic phenotype as assessed by growth on plates at pH 10.0 (Takinishi et al., 1983). The transformants were identified as transformants to prototrophy for one of two markers in the recipient strain as well as the ability to grow at pH 10.0. The isolates retain other markers of the recipient strain and are sensitive to a phage that is lytic for *B. subtilis*. These results, if truly reflective of transformation to an alkaline phenotype might suggest that no more than one or two genes are required for at least a partial alkalophilic phenotype. DNA-DNA hybridization experiments carried out in my laboratory indicate no relatedness between the alkalophiles and *B. subtilis* and very little relatedness between the two alkalophilic species. Transformation has been achieved of alkalophile strains by plasmids isolated from *B. subtilis*, but the reproducibility and efficiency are still problematic.

As already indicated, at least one alkalophile, *B. firmus* RAB, has recently been found to generate genetic variants with enhanced antiporter activity; these variants are detected on plates with Na^+ concentrations that barely support growth of the normal wild type. The variants are produced at high frequencies and, usually, will yield both small and big colony types when subsequently isolated and replated on low Na^+ plates. Occasionally a variant can be isolated which retains the apparently enhanced antiporter activity. As these observations are subjected to further, and intensive, investigation, it will be of interest to keep in mind the complex genetic findings with *Halobacterium*, another organism that inhabits an extreme environment (DasSarma, Rajbhandary & Khorana, 1983; Pfeifer et al., 1983).

Horikoshi and his colleagues (Kudo, Kato & Horikoshi, 1983; Honda, Kudo & Horikoshi, 1985; Kudo et al., 1985) have successfully cloned the penicillinase and xylanase genes from an alkalophilic *Bacillus* species in *E. coli*. The penicillinase produced by the *E. coli* was released into the culture medium, suggesting that the cloned DNA fragment elicited some change in the outer membrane of the host (Kudo et al., 1983). Kudo et al. (1985) have also cloned an alkalophilic promoter sequence in *B. subtilis* in which its expression was dependent upon the phase of the host growth cycle.

Summary

The central problem for organisms which grow optimally, and in some cases obligately, at pH values of 10 to 11, is the maintenance of a relatively acidified cytoplasm. A key component of the pH homeostatic mechanism is an electrogenic Na^+/H^+ antiporter which—by virtue of kinetic properties and/or its concentration in the membrane—catalyzes net proton uptake while the organisms extrude protons during respiration. The antiporter is also capable of maintaining a constant pH_{in} during profound elevations in pH_{out} as long as Na^+ entry is facilitated by the presence of solutes which are taken up with Na^+. Secondary to the problem of acidifying the interior is the adverse effect of the large pH gradient, acid in, on the total pmf of alkalophile cells. For the purposes of solute uptake and motility, the organisms appear to largely bypass the problem of a low pmf by utilizing a sodium motive force for energization. However, ATP synthesis appears not to resolve the energetics problem by using Na^+ or by incorporating the proton-translocating ATPase into intracellular organelles. The current data suggest that effective proton pumping carried out by the alkalophile respiratory chain at high pH may deliver at least some portion of the protons to the proton-utilizing catalysts, i.e., the F_1F_0-ATPase and the Na^+/H^+ antiporter, by some localized pathway.

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