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REVIEW

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pH homeostasis and ATP synthesis: studies of two processes that necessitate inward proton translocation in extremely alkaliphilic *Bacillus* species

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Abstract Alkaliphilic *Bacillus* species that are isolated from nonmarine, moderate salt, and moderate temperature environments offer the opportunity to explore strategies that have developed for solving the energetic challenges of aerobic growth at pH values between 10 and 11. Such bacteria share many structural, metabolic, genomic, and regulatory features with nonextremophilic species such as *Bacillus subtilis*. Comparative studies can therefore illuminate the specific features of gene organization and special features of gene products that are homologs of those found in nonextremophiles, and potentially identify novel gene products of importance in alkaliphily. We have focused our studies on the facultative alkaliphile *Bacillus firmus* OF4, which is routinely grown on malate-containing medium at either pH 7.5 or 10.5. Current work is directed toward clarification of the characteristics and energetics of membrane-associated proteins that must catalyze inward proton movements. One group of such proteins are the Na^+/H^+ antiporters that enable cells to adapt to a sudden upward shift in pH and to maintain a cytoplasmic pH that is 2–2.3 units below the external pH in the most alkaline range of pH for growth. Another is the proton-translocating ATP synthase that catalyzes robust production of ATP under conditions in which the external proton concentration and the bulk chemiosmotic driving force are low. Three gene loci that are candidates for Na^+/H^+ antiporter encoding genes with roles in Na^+- dependent pH homeostasis have been identified. All of them have homologs in *B. subtilis*, in which pH homeostasis can be carried out with either K^+ or Na^+. The physiological importance of one of the *B. firmus* OF4 loci, *nhaC*, has been studied by targeted gene disruption, and the same approach is being extended to the others. The *atp* genes that encode the alkaliphile's F_1F_O-ATP synthase are found to have interesting motifs in areas of putative importance for proton translocation. As an initial step in studies that will probe the importance and possible roles of these motifs, the entire *atp* operon from *B. firmus* OF4 has been cloned and functionally expressed in an *Escherichia coli* mutant that has a full deletion of its *atp* genes. The transformant does not exhibit growth on succinate, but shows reproducible, modest increases in the aerobic growth yields on glucose as well as membrane ATPase activity that exhibits characteristics of the alkaliphile enzyme.

Key words Alkaliphile · Antiporters · ATP synthase · Oxidative phosphorylation

Introduction

From the point of view of membrane bioenergetics, the high external pH values at which extreme, aerobic alkaliphilic bacilli thrive present a special challenge vis a vis processes that depend on inward proton translocation. This is true both because the absolute concentration of protons in the bulk, e.g., at pH 10–11, presents a problem with respect to proton capture by a membrane-associated translocator and because the alkaliphile maintains a ΔpH such that the cytoplasmic proton concentration is higher than that in the medium. On an upward shift in outward pH, the more successful an alkaliphile is in maintaining an optimal cytoplasmic pH, the more problematic is further proton entry for continued adjustment to increasing pH or for any other process that requires proton uptake. That is, the outwardly directed proton gradient (ΔpH, acid in) that reflects successful pH homeostasis at pH 9, for example, is not fully offset energetically by a compensatory rise in the transmembrane electrical potential, positive out (Krulwich 1995). The ΔpH, acid in, makes it energetically more difficult therefore to acidify the cytoplasm further or to bring

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protons through the ATP synthase as the external pH is raised even further to optimal values for growth in the 10–11 range. Were alkaliphiles to grow only under fermentative conditions, then the generation of metabolic acids might sufficiently acidify the cytoplasm relative to the bulk and substrate-level phosphorylation might supply sufficient ATP to obviate the need for inward proton translocation. However, the most intensely studied group of nonmarine, extreme alkaliphiles are the alkaliphilic *Bacillus* species that can grow aerobically on nonfermentative substrates at pH values above 10.5 (Sturr et al. 1994; Ivey et al. 1998; Krulwich et al. [in press]).

The respiratory chains of aerobic alkaliphilic bacilli such as *Bacillus firmus* OF4, which has been the focus of work in our laboratories, are highly active over a broad pH range and translocate protons outward during electron transport. Electron transport is mediated by a series of respiratory chain complexes that are apparently of conventional types and are present in the membrane at high levels. No primary, respiration-dependent Na^+ translocation has been demonstrated for these bacteria. On the other hand, outward proton translocation has been directly shown to occur (Hicks and Krulwich 1995). Thus the large ΔpH, acid in, that is maintained by *B. firmus* OF4 and other aerobic alkaliphilic *Bacillus* species at high values of the growth pH must involve net proton accumulation concomitant with primary, outward proton extrusion. Moreover, a net driving force must be retained to support oxidative phosphorylation, which is yet another process of these alkaliphiles that is coupled to inward proton movement.

Inward proton translocation is a necessary part of active pH homeostasis

A recent minireview of the mechanisms whereby alkaliphiles achieve cytoplasmic pH regulation has appeared in this journal (Krulwich et al. 1997). This and other reviews (Krulwich 1995; Ivey et al. 1998; Krulwich et al. [in press]) present detailed discussions of evidence that pH homeostasis in respiring cells of extreme alkaliphiles depends on active secondary proton uptake that is mediated by membrane-associated antiporters. The salient features are reviewed only briefly here, and a summary is presented of the current state of characterization of the key transporters. It should also be noted that, at least in cells growing on fermentative substrates, there is a role for passive mechanisms in proton retention in or near the cell surface, e.g., a dependence on negatively charged cell wall polymers (Aono and Ohtani 1990; Aono et al. 1995). However, even in strains of alkaliphiles that have such mechanisms, they are not sufficient to support pH homeostasis on failure of active, antiporter-dependent mechanisms (Kudo et al. 1990; Hamamoto et al. 1994).

The antiporters whose mutational compromise leads to a nonalkaliphilic, pH homeostasis-minus phenotype catalyze electrogenic uptake of protons in exchange for cytoplasmic Na^+ during respiration. Because such coupling of inward proton translocation for pH homeostasis requires an adequate cytoplasmic concentration of Na^+, reentry mechanisms for Na^+ may be considered part of the pH homeostatic cycle under growth conditions in which Na^+ is not abundant. The Na^+ dependence of alkaliphile pH homeostasis is not observed in neutralophilic *Bacillus subtilis* in which exchange of either cytoplasmic Na^+ or K^+ for external protons can support pH homeostasis (Cheng et al. 1996). pH homeostasis in *Escherichia coli* may similarly utilize either K^+ or Na^+ in antiporter-mediated proton accumulation, because multiple mutants with undetectable Na^+/H^+ antiporter activity exhibit Na^+ sensitivity but still appear to grow at elevated pH in the absence of added Na^+ (Padan and Schuldiner 1996). Quite likely, the alkaliphile antiporters have developed Na^+ specificity to minimize the chance that cytoplasmic K^+ concentrations, which must be adequate for optimal cytoplasmic protein stability and activity, will be adversely depleted (Krulwich et al. 1997).

The first Na^+/H^+ antiporter-encoding gene from an extreme alkaliphile, the *nhaC* gene from *B. firmus* OF4, was cloned in a truncated form on the basis of its ability to functionally complement the Na^+-sensitive phenotype of antiporter-deficient *E. coli* mutant strains (Ivey et al. 1991). More recently, a targeted disruption of this gene in *B. firmus* OF4 facilitated a clarification of the role of this locus and also led to observations indicating that this alkaliphile must possess at least three distinct antiporters with roles in pH homeostasis (Ito et al. 1997). The *nhaC* gene clearly does not encode the dominant antiporter in this crucial cell process, because the strain with a deletion in the gene was still able to grow well at pH 10.5 in media with abundant Na^+. However, as the concentration of Na^+ was reduced to 10 mM and below at pH 10.5, or only to 25 mM at pH 7.5, poorer growth of the deletion strain than the wild type was observed; the phenotype was complemented by the cloned *nhaC* gene. Interestingly, the most pronounced phenotype was observed at pH 7.5 in pH 7.5-grown cells. Similarly, pH 7.5-grown cells in particular, and to a lesser extent cells grown at pH 10.5, were deficient in their ability to acidify the cytoplasm on a shift from pH 8.5 to 10.6 at low Na^+ concentrations. Taken together, the data support the conclusion that NhaC in *B. firmus* OF4 is a high-affinity Na^+/H^+ antiporter that is the major such high-affinity antiporter in pH 7.5-grown cells. Cells grown at high pH must have at least one additional high-affinity Na^+/H^+ antiporter, and cells grown at both pH values possess substantial constitutive activity of one or more lower affinity antiporters that is (are) likely to be the dominant antiporter(s) in pH homeostasis (Ito et al. 1997). Evidence from other alkaliphiles also suggests a multiplicity of distinct antiporters (Kitada et al. 1994).

A good candidate for a dominant antiporter is a recently identified *B. firmus* OF4 homolog (Krulwich et al. [in press]) of an operon that has been found in *Rhizobium meliloti*, where it has been named *pha* (for pH adaptation) (Putnoky et al. 1996). The importance of this locus in alkaliphile pH homeostasis is indicated by earlier identification of an apparently incomplete version of this seven-gene operon in alkaliphilic *Bacillus* C-125 (Kudo et al. 1990;

Hamamoto et al. 1994). A mutation in this operon that was corrected by a cloned fragment of the operon was associated with loss of the ability to grow at high pH concomitant with a pH homeostasis-negative phenotype and markedly reduced Na^+/H^+ antiport activity (Hamamoto et al. 1994). Interestingly, mutations in the *R. meliloti* operon have a K^+-related phenotype that is suggestive of K^+/H^+ loss (Putnoky et al. 1996), consistent with the expectation that homologous antiporters with roles in pH homeostasis may be Na^+ specific in alkaliphiles only. A homologous operon in *B. subtilis* (Oudega et al. 1997) that we have named *mrp* (for multiple resistance and pH adaptation) has been shown to have a role in both K^+- and Na^+-dependent pH homeostasis in a range of cation concentration below the optimum for the important TetA(L) antiporter of this species (unpublished data). A distinct locus has been identified in both *B. firmus* OF4 and in *B. subtilis* as resembling the *napA* gene that is proposed to encode a Na^+/H^+ antiporter in *Enterococcus hirae* (Wasser et al. 1992). The role of this locus has not yet been established in any *Bacillus* species, although another homolog is associated with germination in *Bacillus megaterium* (Tani et al. 1996). Currently, each of the candidate loci, in both *B. firmus* OF4 and *B. subtilis*, for antiporters with a role in pH homeostasis is being studied in our laboratories through the complementation capacity of cloned versions in antiporter-deficient bacterial strains and through targeted disruptions—singly and in combination. These studies should clarify the roles of these different loci in the alkaliphilic and neutralophilic *Bacillus* species and may point to generalizations about distinctions between the porters in the extremophile vs. nonextremophile. Thus far, no alkaliphile homolog of the *tet*-like genes, one of which has been shown to have an important role as a monovalent cation/proton antiporter in *B. subtilis* (Cheng et al. 1996), has been identified in an alkaliphilic *Bacillus*. However, the ongoing genome sequencing project of such an extremophile should soon lead to information about whether, for some reason, this structural type is absent from the alkaliphile, plays some other role(s) only, or has simply been missed as a participant in alkaliphile pH homeostasis.

Oxidative phosphorylation utilizing a proton-coupled ATP synthase is expected to necessitate inward proton translocation

Unlike pH homeostasis, which must involve proton accumulation in the cytoplasm relative to the outside, coupling of oxidative phosphorylation by alkaliphiles does not a priori need to be mediated via protons. Rather, it was anticipated that like some anaerobic and other fermentative bacteria, extreme alkaliphiles would possess a Na^+-coupled F_1F_O-ATPase that could, in the alkaliphile, be used in the synthetic direction and catalyze ATP synthesis. Even if the respiratory chain extrudes only protons, the bulk Δp would still be much lower than the bulk electrochemical Na^+ gradient across the alkaliphile-coupling membrane because only the magnitude of the Δp would be reduced by the ΔpH, acid in. However, studies in whole cells, isolated membrane vesicles (Ivey et al. 1998), and with purified, reconstituted preparations of F_1F_O-ATP synthases from two different alkaliphiles indicate that these synthases are exclusively proton-coupled (Hicks and Krulwich 1990; Hoffmann and Dimroth 1991a) and are not assembled from different gene products or in different gene product stoichiometries for use at different values of growth pH (Ivey et al. 1994). Is it possible, then, that the ostensibly low bulk Δp is not a sufficient challenge to merit any special adaptation for oxidative phosphorylation, as has at least been suggested (Hoffmann and Dimroth 1991b)? Three cogent and different pieces of data suggest that the challenge is far from trivial. First, an enormous quantitative discordance between the chemiosmotic driving force and the ATP synthesized is indicated from the most rigorously controlled studies, i.e., studies of *B. firmus* OF4 cells grown in carefully pH-controlled continuous cultures so that measurements of the Δp components and growth yields were taken on logarithmic phase cells growing at different constant pH values (Sturr et al. 1994). Second, in highly buffered batch cultures of *B. firmus* OF4 (Guffanti and Hicks 1991) as well as in lightly buffered batch cultures of *B. alcalophilus* (Hoffmann and Dimroth 1991b), the best ATP synthesis was observed at the highly alkaline pH values at which the Δp was lower than at near-neutral pH. That the Δp experienced by the alkaliphile is low is further supported by the observation that these soil organisms, including strains that grow at low Na^+ concentrations, utilize Na^+-coupled transporters for their ion-coupled symporters rather than H^+-coupled symporters (Krulwich 1995). In light of this adaptation and the fact that a Na^+-coupled ATPase is capable of supporting nonfermentative growth of *E. coli* (Kaim and Dimroth 1995), it is very striking that a proton-translocating ATP synthase is utilized by the alkaliphile. The third kind of evidence that some special adaptation exists to accomplish ATP synthesis at low Δp is that there is a lack of equivalence in the efficacy of artificially generated potentials across the alkaliphile membrane and respiration-derived $\Delta\Psi$ values of the same magnitude in energizing ATP synthesis (Guffanti et al. 1984; Guffanti and Krulwich 1992, 1994). Although an artificially imposed diffusion potential energizes Na^+/H^+ antiport and Na^+-coupled solute symport at both near-neutral and high pH, it fails to energize ATP synthesis above about pH 9.2. Respiration, by contrast, supports maximal ATP synthesis in the more alkaline range. There is a crucial importance of the respiratory chain itself in oxidative phosphorylation, beyond the bulk electrochemical gradient it generates (although that has been consistently found to be necessary; i.e., no ATP synthesis is seen if the $\Delta p = 0$). In particular, the caa_3-type terminal oxidase, which is one of two terminal oxidases in *B. firmus* OF4, appears to be of special importance. The level of this oxidase and the mRNA from which it is translated is upregulated by growth at high pH (Quirk et al. 1993), and mutations resulting in partial deficiencies in this enzyme complex lead to a non-alkaliphilic phenotype (Krulwich et al. 1996). In fact, disruption of the caa_3-type oxidase-

encoding *cta* operon in *B. firmus* OF4 leads to an inability to grow in malate even at near-neutral pH in spite of the presence of a *bd*-type oxidase that has quinol oxidase activity (Gilmour and Krulwich 1997).

However the alkaliphile resolves its problem of using a proton-translocating ATP synthase at high pH and low Δp, it is apparently not simply by utilizing far more substrate to generate that ATP because the molar growth yields on malate are at least as high at the alkaline end of the pH range for growth as at near-neutral pH (Guffanti and Hicks 1991; Sturr et al. 1994). Thus, a number of different kinds of models are being considered in which there is sequestration of some of the protons translocated by respiration so that they are captured by the ATP synthase without first having fully equilibrated with the alkaline bulk phase (Krulwich et al. [in press]). Sequestration in actual subcellular organelles or intramembrane organelles as suggested by Skulachev (1991) has not been supported by a number of different electron microscopic studies (Rhode et al. 1989; Sturr et al. 1994). Nor is some sort of sequestration of protons by acidic cell wall layers (e.g., as suggested by Kemper et al. 1993) necessary, let alone sufficient, for alkaliphile oxidative phosphorylation, because at least in strains such as *B. firmus* OF4, ATP synthesis shows the same energization pattern in wall-less membrane vesicle preparations as in cells (Guffanti and Krulwich 1994). Perhaps, then, protons move rapidly along the phospholipid or protein surfaces of the coupling membrane, and thereby reach the synthase on emerging from a respiratory chain complex before moving out into the bulk. Rapid proton movements along the surface have been suggested in bacteriorhodopsin-containing membranes (Heberle et al. 1994; Alexiev et al. 1995; Gutman and Nachliel 1995; Gabriel and Teissie 1996). There is some recent evidence that membrane lipid "remodeling" may be a feature of alkaliphile growth at its high end of the pH range, although earlier studies failed to indicate major differences in the lipids at high pH. Alternatively, protons might move directly from a respiratory chain complex, e.g., the caa_3-type oxidase, to the ATP synthase via protein–protein interactions. These interactions could occur between regions of the two putatively interacting complexes that are close to the membrane surface but not in direct contact with the bulk.

In connection with the sort of sequestration model involving protein–protein interactions, it is notable that there are "alkaliphile-specific motifs" in the deduced amino acid sequence of the *a* and *c* subunits of the F_O-ATPase of several alkaliphilic *Bacillus* species (Ivey and Krulwich 1991, 1992). These "motifs" are in regions of particular interest with respect to the proton translocation pathway and energy-coupling mechanism in the synthase. They include a "second proline" on the other side of the important *N′*,*N′*-dicyclohexylcarbodiimide- (DCCD-) binding carboxylate from a conserved proline in the second membrane-spanning region of the *c*-subunit; several G → A changes and one A → G change in a usually conserved set of glycines and alanines in the region of the first membrane-spanning region of the *c*-subunit that is opposite that carboxylate; and the presence of G218 (*E. coli* numbering) →K and H245 → G changes in regions of the *a*-subunit. Interestingly, if the latter changes are made in *E. coli* by site-directed mutagenesis, they must be made together rather than separately if synthase activity is to be preserved. This requirement suggests an interaction of these regions (Hartzog and Cain 1994), which is also indicated by other studies (Vik and Antonio 1994). The E219 neighboring the unusual K218 in the alkaliphile has recently been shown not to be essential for the proton translocation pathway (Valiyaveetil and Fillingame 1997). The R210 of the *a*-subunit is also nonessential for proton translocation per se (Valiyaveetil and Fillingame 1997) but is a strong candidate for involvement in the coupling mechanism and may be interactive with the carboxylate of the *c*-subunit (Fillingame 1990; Vik and Antonio 1994). In connection with emerging energy-coupling models from groups studying the *E. coli* and mitochondrial enzymes, there is the intriguing possibility that the K218 of the alkaliphile modify the pK of R210 in the *a*-subunit and that the "second proline" of the *c*-subunit might influence the structure of that crucial region in some functionally important way. If so, these motifs might contribute to some mechanistic adaptation with respect to the mode of proton delivery to the F_O-ATPase that facilitates oxidative phosphorylation at pH > 9.

As the first steps toward direct evaluation of these unusual sequence features of the alkaliphile F_O, the full *atp* operon has been cloned from a lambda library of *B. firmus* OF4 DNA into a pGEM vector and transformed into a strain of *E. coli* LE392 from which the *atp* operon is entirely deleted. *E. coli* LE392Δ*atp* (Angov and Brusilow 1988) was obtained from William Brusilow. As shown in Table 1, the

Table 1. Growth properties of *E. coli* LE392/pGEM, LE392Δ*atp*/pGEM, and LE392Δ*atp*/p16BS1 (carrying the *Bacillus firmus OF4* atp operon) in minimal media with different carbon sources

E. coli transformant	Maximal growth, A_{600}, on		
	5 mM glucose	50 mM glucose	50 mM succinate
LE392/pGEM	1.11	2.15	1.87
LE392Δ*atp*/pGEM	0.57	1.99	0.01
LE392Δ*atp*/p16BS1	0.64	2.01	0.01

The transformants were grown aerobically at 37°C, and turbidity at 600 nm was recorded when growth had ceased. The medium was minimal medium A (Miller 1992) supplemented with thiamine, methionine, and the indicated carbon source.

Table 2. ATPase activities of everted vesicles isolated from *E. coli* LE392/pGEM, LE392Δ*atp*/pGEM, and LE392Δ*atp*/p16BS1 (carrying *B. firmus* OF4 *atp* operon)

E. coli transformant	Specific ATPase activity (μmoles Pi $min^{-1}mg^{-1}$)	DCCD inhibition	Fold stimulation by octylglucoside + sulfite
LE392/pGEM	0.390	82%	1.5
LE392Δ*atp*/pGEM	0.011	0	0[a]
LE392Δ*atp*/p16BS1	0.067	29%	8.7

Everted vesicles were isolated by differential ultracentrifugation after cell breakage by French pressure treatment at 14000 p.s.i. Reactions to measure the stimulation of enzyme activity by the inclusion of octylglucoside and sodium sulfite in the assay contained 20 mM Tricine-NaOH, pH 8.0, 5 mM Na_2ATP, and 2.5 mM $MgCl_2$ with 30 mM octylglucoside and 50 mM sodium sulfite, and were compared to reactions lacking octylglucoside and sodium sulfite. These were carried out for 0, 1, 2, and 3 min at 37°C and were stopped by transfer to ice-cold tubes containing TCA. *N′*,*N′*-dicyclohexylcarbdiimide (DCCD) inhibition was determined by preincubation of samples for 30 min at 37°C with or without 100 μM DCCD; reactions contained 50 mM Tricine-NaOH, pH 8.0, 5 mM Na_2ATP, and 5 mM $MgCl_2$ and were carried out for 0, 2.5, 5, and 10 min. Pi content was analyzed by the method of LeBel et al. (1978).
[a] Octylglucoside and sulfite inhibited activity.

recombinant plasmid carrying the alkaliphile *atp* operon did not restore the capacity for growth of the *atp*-minus *E. coli* strain on succinate although the growth yield on glucose was reproducibly increased to a modest extent. Current experiments are under way to determine whether expression of the alkaliphile *atp* operon from a lower copy number plasmid or from a site of chromosomal integration in an *atp* deletion strain of *B. subtilis* might provide a heterologous system in which the alkaliphile operon could support nonfermentative growth. Although not shown, Western analyses of membrane vesicles from the *E. coli* transformant with the recombinant plasmid p16BS1, and control transformants of the wild-type and mutant *E. coli* strains, indicated that the LE392Δ*atp*/p16BS1 indeed contained alkaliphile ATP synthase polypeptides at levels comparable to the synthase content of wild-type *E. coli*, although some apparent cross-reactive breakdown products were also in evidence. The ATPase activity of the p16BS1 transformant was about sixfold higher than the control transformant of the *atp*-minus *E. coli* strain (Table 2), although still significantly lower than that of the wild type. The lower DCCD sensitivity and higher octylglucoside + sulfite stimulation of the p16BS1 transformant, relative to the *E. coli* wild type (Table 2), was consistent with properties of the alkaliphile ATP synthase found in earlier studies. Importantly, the stimulated activity of the transformant expressing the alkaliphile ATPase, 0.55 μmol $min^{-1}mg^{-1}$ membrane protein, is comparable to that of the wild-type *E. coli* preparations. As assays of actual proton movements indicated no increase in global proton leakiness in the preparations containing the alkaliphile ATPase, there may be some nontrivial reason that will be of biological interest (i.e., other than expression levels) for the failure of the alkaliphile ATPase genes to support growth on succinate in the mutant *E. coli* strain. Moreover, a functional full-length clone of the alkaliphile *atp* operon is now available for further optimization in this and other heterologous systems and for development for analysis of site-directed changes in such systems. Ultimately, the evaluation of specific mutations in the F_O-ATPase genes will have to be conducted in the alkaliphile itself where the relevance of the alkaliphile-specific motifs to alkaliphily can be assessed.

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