Replacement of Amino Acid Sequence Features of α- and c-Subunits of ATP Synthases of Alkaliphilic Bacillus with the Bacillus Consensus Sequence Results in Defective Oxidative Phosphorylation and Non-fermentative Growth at pH 10.5*

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Mitchell’s (Mitchell, P. (1961) Nature 191, 144–148) chemiosmotic model of energy coupling posits a bulk electrochemical proton gradient (Δp) as the sole driving force for proton-coupled ATP synthesis via oxidative phosphorylation (OXPHOS) and for other bioenergetic work. Two properties of proton-coupled OXPHOS by alkaliphilic Bacillus species pose a challenge to this tenet: robust ATP synthesis at pH 10.5 that does not correlate with the magnitude of the Δp and the failure of artificially imposed potentials to substitute for respiration-generated potentials in energizing ATP synthesis at high pH (Krulwich, T. (1995) Mol. Microbiol. 15, 403–410). Here we show that these properties, in alkaliphilic Bacillus pseudofirmus OF4, depend upon alkaliphile-specific features in the proton pathway through the α- and c-subunits of ATP synthase. Site-directed changes were made in six such features to the corresponding sequence in Bacillus megaterium, which reflects the consensus sequence for non-alkaliphilic Bacillus. Five of the six single mutants assembled an active ATPase/ATP synthase, and four of these mutants exhibited a specific defect in non-fermentative growth at high pH. Most of these mutants lost the ability to generate the high phosphorylation potentials at low bulk Δp that are characteristic of alkaliphiles. The αLys180 and αGly212 residues that are predicted to be in the proton uptake pathway of the α-subunit were specifically implicated in pH-dependent restriction of proton flux through the ATP synthase to and from the bulk phase. The evidence included greatly enhanced ATP synthesis in response to an artificially imposed potential at high pH. The findings demonstrate that the ATP synthase of extreme alkaliphiles has special features that are required for non-fermentative growth and OXPHOS at high pH.

Aerobic organisms maximize catabolic energy conservation by carrying out OXPHOS.1 Energy stored in NADH or FADH2 during catabolism is used to produce a bulk ∆p, acid and positive out, across the mitochondrial or bacterial cell membrane by respiration-dependent proton extrusion. Inward proton flux through the proton-coupled ATP synthase, energized by the Δp, then leads to ATP production (1–4). Among the important unresolved issues is whether protons are always captured from the bulk medium as in Mitchell’s (1) chemiosmotic model or whether they can be sequestered as they emerge from the respiratory chain (2, 5–9). Robust H+‐coupled OXPHOS by extremely alkaliphilic Bacillus strains growing on non-fermentable carbon sources at external pH values ≥10.5 poses one of the most striking challenges to the strictly bulk energization model (10–13). At such pH values, maintenance of a cytoplasmic pH that is much lower than the external pH, i.e. a ΔpH that is acid in, lowers the total chemiosmotic driving force, and yet OXPHOS proceeds optimally (10, 13).

A variety of solutions to the energetic conundrum of alkaliphile OXPHOS have been proposed (for reviews, see Refs. 10 and 14). We have hypothesized that special properties of the alkaliphile ATP synthase are needed for OXPHOS at high pH that depend upon the presence of specific amino acid residues or stretches of amino acids in functionally important regions of the membrane-embedded α- and c-subunits of the enzyme (10, 15). Other hypotheses have suggested global features of the alkaliphile membrane, membrane surface, or cell wall-associated polymers (see Refs 10 and 14) that contribute to the resolution of the alkaliphile energetic problem. It has recently been proposed that a single global feature of this kind, i.e. a sufficiently low pH near the membrane surface, can completely account for alkaliphile OXPHOS (14), eliminating any need for special features of the enzymes that directly participate in OXPHOS. The goal of the current studies was to test whether in fact apparent alkaliphile-specific features of two membrane-embedded ATP synthase subunits are specifically important for non-fermentative growth of alkaliphilic Bacillus pseudofirmus OF4 by changing them to the consensus sequence for non-alkaliphilic Bacillus species. If the alkaliphile F0 sequence features are required for OXPHOS at high pH, mutants expressing normal levels of an altered ATP synthase would be expected to be specifically defective in non-fermentative growth and OXPHOS at pH 10.5. If, on the other hand, there are

ΔGp, phosphorylation potential; Δp, transmembrane electrochemical proton gradient; ΔpH, transmembrane pH gradient; ΔV, transmembrane electrical potential; DCCD, dicucylohexylcarbodiimide; F0-ATP synthase, membrane-embedded sector of the F0F1-ATP synthase; TMH, transmembrane helix; MOPS, 4-morpholinepropanesulfonic acid; Tricine, N-2-hydroxy-1,1-bis(hydroxymethyl)ethyglycine.

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1 The abbreviations used are: OXPHOS, oxidative phosphorylation;
features of the surface layers that obviate the need for special adaptations of the alkaliphile OXPHOS machinery itself, the mutants would be without a specific OXPHOS phenotype at high pH.

Two signatures of alkaliphile OXPHOS are exhibited by whole cells and ADP + P_i-loaded membrane vesicles of alkaliphile *B. pseudofirmus* OF4 that lack cell wall polymers (10–13). Along with non-fermentative growth at high pH, these signature properties were used in this study to evaluate the OXPHOS phenotypes of the mutants and develop information about potential roles of particular ATP synthase features. First, the “quantitative signature” of alkaliphile OXPHOS is the generation of a higher ΔGp (reflecting the [ATP]/[ADP][P_i] ratio expressed in mV) at pH 10.5 than at pH 7.5 even though the Δp is about 3 times higher at the lower pH. In a strictly bulk chemiosmotic model, the ΔGp should exhibit a direct relationship to the bulk Δp. The lower Δp at high pH results from a large “chemiosmotically reversed” bulk pH gradient, acid in, that is required at pH 10.5. Although the other component of the Δp, the Δψ, increases in the productive direction (positive out) at the higher pH, this increase does not come close to offsetting the chemiosmotically adverse pH gradient (10, 13).

The second property of alkaliphile OXPHOS that is at odds with strict coupling to a bulk Δp is a “qualitative signature” that is also assessed in one group of mutants studied here. This “signature” is the inability of a valinomycin-mediated potassium diffusion potential to energize ATP synthesis comparably to a respiration-generated Δψ of the same magnitude. At elevated pH values at which the Δψ is the sole Δp component energizing ATP synthesis, an artificially imposed potential of the same magnitude would be predicted by the chemiosmotic model to work just as well as the respiration-generated potential. Rather, overall energization of ATP synthesis by the imposed potential is much lower than energization by respiration in alkaliphile cells and ADP + P_i-loaded membrane vesicles (11, 12). Most notably, although the imposed potentials can still energize transport up to external pH values of 10.5, they lose their ability to energize ATP synthesis totally as the external pH rises above 9.2 where alkaliphile growth on OXPHOS-requiring substrates is still optimal (10–13). We hypothesized that failure of imposed potentials to energize ATP synthesis above pH 9.2 reflects a pH-dependent gating of proton entry into the alkaliphile ATP synthase. Presumably such gating would also block proton loss to the bulk through the synthase when the alkalinity is particularly high and the bulk Δp is particularly low (Ref. 10 and Fig. 1A). An element of the ATP synthase responsible for such gating would be expected to reside in the proton uptake pathway of the a-subunit through which protons entering from the bulk pass *en route* to the critical carboxylate of the rotary c-subunit assembly (4, 16–20). At pH values above the hypothesized gating pH, protons presumably could gain entry without first equilibrating with the bulk phase as proposed by others, e.g., by a proton-gathering element (21, 22), from anionic surface lipids (23), from protons trapped in the surface water layer (14), or from an interacting proton-extruding respiratory chain complex (24).

In this study, we first built on the initial observations of alkaliphile-specific sequence features in a- and c-subunits of the F_0-ATP synthase (15) using the expanded data base and greatly increased information about the *Escherichia coli* enzyme to identify the a- and c-subunit features to be mutated. Two single amino acids and one hydrophilic loop segment in the alkaliphile a-subunit and two single amino acids and one membrane-embedded segment in the alkaliphile c-subunit differed significantly from the homologous neutrophile sequences. These sequences of *B. pseudofirmus* OF4 were changed to that of non-alkaliphilic *Bacillus megaterium*, which represents the consensus sequence for *Bacillus*. Five of the six mutants were found to assemble an active ATP synthase, but they still exhibited a defect in non-fermentative growth at high pH. Assays of the two signatures of alkaliphile OXPHOS at pH 10.5 further indicated that the sequence features in these mutants are likely to have different mechanistic roles.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Genetic Manipulations, and Growth Conditions—** Alkaliphilic *B. pseudofirmus* OF4 strain 811M, a methionine auxotroph of *B. pseudofirmus* OF4 (25), was the parent strain for the F_0 mutant studies. Homologous recombination using the temperature-sensitive plasmid pG + host 4 (Appligene, Pleasanton, CA) was used to construct a Δ*atpB*-P_c (ΔP_c) strain by methods described previously (26). Mutations were made in a cassette that was reintroduced into the deletion strain (26) so that a new EcoRI restriction site was created in codons 162–163 of ATPB. The primers used for this and other constructs made in this study are shown in Table 1. The wild type used for all the experiments was prepared by reintroducing the wild type sequence, with the added EcoRI site, into the deletion strain. This strain exhibited no difference from the parent wild type strain in its capacity for non-fermentative growth. All the other mutations changed *B. pseudofirmus* OF4 sequences for single amino acids or small stretches of amino acids to the sequence(s) found in the equivalent position(s) of the *B. megaterium* ATP synthase. Single amino acid mutations were made by the method of Kunkel et al. (27) with a bacteriophage M13m19 F_c template that was identical to the cassette cloned into pG + host 4. The multiple amino acid replacements (α-loop and cTMH1) were made by the PCR overlap extension method described by Ho et al. (28). The mutations that were constructed included the following in the a-subunit: α-loop, residues 97–106 (equivalent to 128–137 in *E. coli*), from FELYNPTTKE to FAIVIDHN; α180 (218 in *E. coli*), from Lys to Gly, α212 (245 in *E. coli*), from Gly to Ser; double mutant α180 and α212, Lys to Gly and Gly to Ser, respectively. The mutants in the c-subunit were the followings: cTMH1, residues 15–23 (22–30 in *E. coli*), from VAGAIHAV to LGAGIHNGL; c33 (40 in *E. coli*), from Thr to Ala; and c51 (58 in *E. coli*), from Pro to Ala. The F_c segment of each mutant was entirely sequenced and verified to have only the desired mutation(s). Initial characterization of growth properties were nonetheless conducted on four to six independent mutants of each type to be sure that each strain used for subsequent detailed studies represented a phenotype that was reproducibly found as a result of the particular mutagenic change. Cloning was performed in *E. coli* XL-1 Blue (Promega, Madison, WI) grown in Luria broth using 250 μg/ml erythromycin as selection for pG + host 4. *B. pseudofirmus* OF4 strain 811M and mutagenic derivatives were routinely grown at 30 °C in a semisedimented medium containing 0.1% yeast extract with mineral salts and buffered with 0.1 M MOPS-NaOH at pH 7.5 or 0.1 α Na_2CO_3/H_2CO_3 at pH 10.5 (26). Growth experiments were conducted in which the sole carbon source was the yeast extract or with added 50 mM glucose or 50 mM sodium i-malate. Growth on glucose is reported as the A_600 after subtracting the amount of growth on yeast extract alone. The ΔF_p strain exhibited very little growth on malate in excess of the growth on yeast extract alone, but this small fermentative component was subtracted from the raw A_600 data for malate growth so that the values presented represent the growth yield from non-fermentative growth.

**Preparation of Everted Membrane Vesicles and ATPase Assays—** Everted membrane vesicles were prepared from overnight cultures grown at pH 7.5 with malate as the energy source as described earlier (29) except that a battery of protease inhibitors was included in the French press buffer (1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.06 μM bestatin, 1 μg/ml pepstatin A, 1 μM E64 (N-trans-epoxy succinyl)-leucine 4-guanidinobutylamide), 1 μM phosphoramidon, and 1 mM phenylmethylsulfonyl fluoride, final concentrations) (29). Oligomycin-stimulated ATPase assays, which were carried out for 3 min at 37 °C, contained, in a 0.5-ml volume, 20 mM Tricine-NaOH, pH 8.0, 5 mM ATP (sodium salt, Sigma), 2.5 mM MgCl_2, 30 mM oligomycin, 50 mM Na_2SO_4, and 50 μg of membrane protein (29). Unstimulated ATPase activity was determined using 0.4 mg of membrane protein in a 0.5-ml volume containing 50 mM Tricine-NaOH, pH 8.0, 5 mM MgCl_2, and 5 mM ATP. The reactions were incubated for 15 min at 37 °C and terminated by trichloroacetic acid addition (5% final concentration). The precipitated protein was removed by centrifugation, and the supernatants were analyzed for liberated P_i by the method of LeBel et al. (30). Blank
and P, standard tubes received an equivalent volume of membranes after trichloroacetic acid addition. For DCCD inhibition, everted membrane vesicles were preincubated with 4.8 µl of methanol (no DCCD) or 25 mM DCCD (to 100 µM final concentration) in 1.2 ml at a concentration of 2.6 mg of protein/ml for 30 min at room temperature in 20 mM MOPS-NaOH, pH 7.0, 5 mM MgCl₂. They were then diluted 4-fold into assay buffer (67 mM Tricine-NaOH, pH 8.0, 5 mM MgCl₂) and equilibrated at 37 °C, and the reaction was carried out for 30 min after addition of 5 mM ATP. Reactions were terminated by addition of trichloroacetic acid and analyzed as described above. Control experiments were carried out with purified B. pseudofermentum OP4 F₁ ATPase prepared as described earlier (31). Instead of membranes, 50 µg of purified F₁ was included in the 1.2-ml preincubation mixture, and P, was determined by the malachite green assay (32). The experiments showed no DCCD inhibition of F₁ under these conditions.

**Western Analyses**—Everted membrane vesicles equivalent to 5 µg of protein were loaded in each lane (except for the ΔF₁ mutant for which 50 µg of protein was used), resolved on 12% SDS-polyacrylamide minigels (33), and transferred electrophoretically to nitrocellulose membranes, which were then blocked with 5% skim milk in Tris-buffered saline (1×) containing 0.05% Tween-20, to 500 µg/ml after which valinomycin was added (34). The same mixtures were used to load the vesicles for generation of a diffusion potential at pH 8.3, for the vesicles to be energized by a diffusion potential at pH 9.3, the final pH of the loaded solution was 9.3. For assays of respiration-dependent ATP synthesis, the ADP + P, loaded vesicles were diluted 1:20, to 500 µg of protein/ml, in either pH 7.5 buffer containing 25 mM sodium phosphate, 0.25 mM succinate, 5 mM MgCl₂, and 200 mM K₂SO₄ or into pH 10.5 buffer containing 25 mM Na₂CO₃, 0.25 mM succinate, 5 mM MgCl₂, and 200 mM K₂SO₄, and immediately thereafter 10 mM ascorbate plus 0.1 mM phenazine methosulfate was added to start the reaction. For energization of ATP synthesis by a valinomycin-mediated potassium diffusion potential, the right-side-out vesicles were loaded at pH 8.3 with the same concentrations of ADP + P, as in the experiments with ascorbate-phenazine methosulfate energization. The vesicles were concentrated to ~20 mg of protein/ml after which valinomycin was added to a final concentration of 1 µM. For DCCD treatment, 200 µM DCCD was added to the concentrated cell mixture 5 min before the valinomycin addition. It was also included in the dilution buffer for those samples. To initiate synthesis, the vesicles were diluted 500-fold into a potassium-free buffer of the same pH as the internal pH, either 8.3 or 9.3, consisting of 25 mM sodium phosphate, 0.25 mM succinate, 5 mM MgCl₂, 200 mM K₂SO₄, and 1 µM valinomycin. This was calculated, by the Nernst equation, to generate a potential of ~160 mV. A set of controls in which the external buffer contained the equivalent potassium ion concentration as the internal concentration was conducted routinely.

### Table I

| Primer name | Sequence (5‘–3’)** | Corresponding sequence| Purpose |
|-------------|-------------------|----------------------|---------|
| F₅-BamF    | GCGGATCCTCTATATATATGGAAGCT | 2743–2765 | Cloning of F₅ |
| F₅-EcoR    | GCGGATCCTCTATTGATTGGAAGCT | 5895–5878 | Same |
| F₅-markedF | GGGGATCCTCTATATATATGGAAGCT | 3892–3819 | Insertion of EcoRI site into codons 162–163 of α-subunit |
| ΔF₅-BamF1  | GCGGATCCTCTATATATATGGAAGCT | 3797–3776 | Same |
| ΔF₅-SacR1  | AATGCAGTCATGGAAGCT | 2420–2439 | Deletion of F₅ |
| ΔF₅-SacF1  | AATGCAGTCATGGAAGCT | 3339–3330 | Same |
| ΔF₅-KpnR1  | AATGCAGTCATGGAAGCT | 4947–4968 | Same |
| Bme α-loopF | CTCTAGATCTGCAATGGAAGCT | 5687–5666 | Same |
| Bme α-loopR | CTCTAGATCTGCAATGGAAGCT | 3631–3650 | α-Loop mutation |
| Bme TMH1F  | CTCTAGATCTGCAATGGAAGCT | 1199–1200 | cTMH1 mutation |
| Bme TMH1R  | CTCTAGATCTGCAATGGAAGCT | 4171–4174 | Same |
| αK180G     | CTCTAGATCTGCAATGGAAGCT | 3835–3876 | αK180G mutation |
| aG212S     | CTCTAGATCTGCAATGGAAGCT | 3934–3966 | aG212S mutation |
| cT33A      | CTCTAGATCTGCAATGGAAGCT | 4211–4241 | cT33A mutation |
| cP51A      | CTCTAGATCTGCAATGGAAGCT | 4299–4292 | cP51A mutation |

**Underlined sequences are restriction sites; mutagenized nucleotides are in bold.**

**According to GenBank accession number AF330160.
RESULTS

Identification of Alkaliphile-specific Sequence Features in the a- and c-Subunits of the ATP Synthase—In Fig. 1B, the alkaliphile-specific sequence features included in the study after analyses of new sequence alignments are indicated on topological models of the a- and c-subunits by numbered boxes. The alignments used to identify these features included the two alkaliphiles that were in the earlier alignments, Bacillus alcalophilus and Bacillus halodurans C-125, and the extensively studied thermophilic Bacillus subtilis. The model (courtesy of Mark E. Girvin) of the a-subunit region containing features 1–3, a-loop, aLys190, and aGly212. The model had the lowest energy resulting from 100 independent torsion angle dynamic simulations using helical backbone H-bond and dihedral angle constraints along with long range constraints identified in the E. coli subunit (49). F, an alignment illustrating the features in the c-subunit displayed only by extreme alkaliphiles. Shown are the TMH1 feature in which the glycines of the conserved XGXXGXXG region are largely or completely replaced by alanines, the cThr21 residue instead of a conserved alanine, and cPro15 in place of a glycine or alanine in other bacteria. The essential carbonylate, cGlu45, is shaded. Numbering refers to B. pseudofirmus OF4 at the top and to E. coli at the bottom. The National Center for Biotechnology Information gene identifier numbers for the data shown are: B. pseudofirmus OF4, 12061041 (A) and 142546 (C); B. halodurans C-125, 15616322 (A) and 15616321; B. alcalophilus, 142566 (A) and 142567 (C); O. iheyensis, 23100436 (A) and 23100435 (C); B. megaterium, 142555 (A) and 142556 (C); G. stearothermophilus, 534857 (A) and 534858 (C); E. coli, 15804336 (A) and 15804337 (C).

with an upper pH limit for growth of about 9.5 and, importantly, little apparent ability to grow on non-fermentable carbon sources that would necessitate a capacity for OXPHOS (43). The other new alkaliphile sequence data in the alignments is for the H+-coupled ATP synthase of the alkaliphilic, thermophilic Bacillus strain TA2.1A that grows only up to pH 10 but still exhibits the quantitative signature found for B. pseudofirmus OF4 (44–46). Sequence data from three different neutralophile Bacillus species and the extensively studied E. coli are also included in the alignments.

The first feature of the alkaliphile a-subunit, the a-loop feature (numbered 1 in Fig. 1B), is part of the periplasmic loop between TMH2 and TMH3. As illustrated by the boxed area of hydrophathy plots in Fig. 1C, the region nearest aTMH2 displays a more polar, hydroxylamino acid-rich character in alkaliphiles than in Bacillus neutralophiles and E. coli. Modeled
and Fillingame (47), the alkaliphiles have 6–8 hydroxy or charged amino acids of 9–10 total residues (FELYNPTTHE in B. pseudofirmus OF4) compared with the 2–4 found in the 8–10-amino acid stretch of various neutrophiles (FAIVIDHIN in B. megaterium). If modeled as a longer loop proposed at this position (48), the difference is preserved. The a-loop extends from a region of aTMH2 proposed to be part of the proton uptake pathway through the ATP synthase and to initiate the Δp-dependent conformational changes during proton uptake (17, 19). In the current study, the a-loop mutant has the FAIVIDHIN sequence of B. megaterium in place of its slightly longer and more polar native sequence. The two other a-subunit features included in the study were the single amino acid deviations noted earlier in aTMH4 and aTMH5 (15), numbered 2 and 3, respectively, in Fig. 1B. The alkaliphiles have a lysine at aTMH4 position 180 (Gly in neutrophiles) and glycine in position 212 (Ser in Bacillus neutrophiles and His in E. coli). The E. coli residues corresponding to αLys480 and αGly212 are among the “second site suppressor pairs” found to functionally interact and considered likely to be in physical proximity within the proton uptake pathway (17) as modeled for the alkaliphile subunit by methods used in studies of E. coli (49) (Fig. 1E). Hartzog and Cain (50) replaced the E. coli equivalents of αLys480 and αGly212 with the alkaliphile residues in those positions. Single mutants lost the capacity for growth on succinate, whereas this capacity was restored in the double mutant. Therefore, both the single mutations of the alkaliphile residues and a double mutant with changes to the Bacillus consensus sequence (αK180G, αG212S, and αK180GαG212S) were constructed in B. pseudofirmus OF4 for the current study.

The three c-subunit features chosen for study so far have been found only in extreme alkaliphiles (Fig. 1, B and F), a correlation re-enforced by analyses of newly isolated alkaliphile strains (51). One of these features is a proline residue that is 3 residues away, on the N-terminal side, from the essential c-subunit carboxylate (cGlu54 in B. pseudofirmus OF4 and cAsp91 in E. coli, see residue numbered 6 in Fig. 1B). It is 6 residues away from a conserved proline (cPro57 in B. pseudofirmus OF4) that flanks the carboxylate on the C-terminal side. Arechaga and Jones (52) called PXXXEXP “the alkaliphile motif” and suggested that it might influence the ion binding site. On the opposite helix of the c-subunit hairpin, cTMH1, a consensus sequence of XGXGXGXXG in the middle of the helix has been reported from alignments of 38 c-subunits, and the glycines were suggested to allow for the tight packing of adjacent helices in the ring (53). The presence of small, uncharged residues in cTMH1 was also suggested to accommodate movements of cTMH2 that would be essential during the protonation-deprotonation cycle of the carboxylate (49, 54). Thus the substitution of at least 3 of the glycine residues of this motif with alanines in extreme alkaliphiles represents another feature of interest (numbered 4 in Fig. 1B). The mutant in which the B. pseudofirmus OF4 sequence VAGAIAVAI was changed to the B. megaterium sequence LGAGIGNGL is designated as the cTMH1 mutant. The third feature of the c-subunit included in the study is threonine, cThr23 of the alkaliphiles, substituting for a conserved alanine in the loop region involved in binding of F1, (55). This feature is numbered 5 in Fig. 1B.

**Growth Phenotypes of the ATP Synthase Mutant Panel**—None of the aτ mutations exhibited a reduced growth yield on glucose at either pH 7.5 or 10.5 (Fig. 2) even though one of the mutants, the cTMH1 mutant, exhibited a large growth defect on malate at both pH 7.5 and 10.5. Although not shown, the ΔF0 strain of alkaliphilc B. pseudofirmus OF4 similarly did not exhibit a defect in growth on glucose, although such a deficit has been observed in a Δaτ strain of Bacillus subtilis (56). This lack of a significant contribution of the hydrolytic activity of the alkaliphile F0F1-ATP synthase to fermentative growth is consistent with observations that the alkaliphile enzymes are substantially “locked” in a synthetic conformation (29, 31, 46). This in turn has been hypothesized by Keis et al. (46) to result from the especially basic character of the C-terminal domain of alkaliphile c-subunits, resulting in interactions strongly favoring the synthetic over the hydrolytic conformation of the enzyme (57, 58). In contrast to their normal growth yield on glucose, the mutants exhibited diverse deficits in non-fermentative growth. The αGly212 mutant grew to a normal growth yield on malate at pH 10.5 in semidefined medium (Fig. 2), but this mutant as well as the αK180GαG212S double mutant and CP51A mutant failed to grow in a defined medium that had a high amine-nitrogen concentration and more strongly stresses the pH homeostatic mechanism at high pH (Refs. 59 and 60 and data not shown). The remaining mutants exhibited a substantial defect in growth yield on the semidefined malate-containing medium that was specific for pH 10.5. The defect was particularly severe in the a-loop, αK180G (single or double), and CP51A mutants (Fig. 2), although the ATPase activity of their membranes was at 80% or more of wild type levels (see below). Growth rates on malate at pH 10.5 examined side-by-side with the wild type and ΔF0 strains were zero or negligible for the cTMH1, CP51A, and both the single and double αK180G mutants. The a-loop mutant exhibited modest growth after a prolonged lag. The αG212S mutant grew at the same rate as wild type, and the cT33A mutant grew at a rate that was about 15% greater than that of the wild type (data not shown).

**Assays of ATPase Content and Activity of Membrane Vesicles**—The cTMH1 mutant, with its alanine-rich region replaced...
by a consensus glycine-rich region, had greatly reduced levels of ATPase protein in the membrane and was not included in further experiments. All the other mutants assembled an ATPase at protein levels comparable to the wild type (Table II). The ATPase (hydrolytic) activity of the enzymes from alkali-

| Strain          | β-Subunit content (Western) | Octylglucoside-stimulated ATPase activity | Unstimulated ATPase activity | DCCD inhibition, % of unstimulated ATPase activity^c |
|-----------------|-----------------------------|------------------------------------------|------------------------------|---------------------------------------------------|
| Wild type       | %                          | %                                       | %                           | %                                                 |
| 𝛼-Loop          | 98 ± 28                    | 113 ± 19                                 | 123 ± 8                     | 50 ± 6                                            |
| aK180G          | 116 ± 27                   | 113 ± 19                                 | 127 ± 49                    | 64 ± 9                                            |
| aG212S          | 106 ± 29                   | 111 ± 10                                 | 133 ± 36                    | 63 ± 5                                            |
| aK180G/aG212S   | 109 ± 28                   | 115 ± 10                                 | 135 ± 37                    | 49 ± 6                                            |
| cTMH1           | 5 ± 4                      | 11 ± 6                                   | ND                          | ND                                                |
| cT33A           | 101 ± 14                   | 82 ± 7                                   | 88 ± 22                     | 45 ± 8                                            |
| cP51A           | 99 ± 15                    | 82 ± 13                                  | 161 ± 34                    | 58 ± 16                                           |

^a DCCD inhibition of the unstimulated ATPase activity was determined by preincubation of the vesicles with or without 100 μM DCCD for 30 min as described under “Experimental Procedures.” Under these conditions, purified B. pseudofirmus OF1 F1 was not inhibited by DCCD (see “Experimental Procedures”).

^b μmol of P_i released min^{-1} mg of protein^{-1}.

^c ND, not determined.

ATP Synthesis and Δp Generation Upon Re-energization of ATP-depleted Cells—ATP resynthesis was assayed in wild type and mutant cells that were grown at pH 8.5 and then washed with nutrient-free pH 8.5 buffer under conditions that resulted in depletion of cytoplasmic ATP (see “Experimental Procedures”). The cells were then re-energized in buffered malate at pH 7.5 or 10.5. Growth of the wild type at pH 10.5 enhances the ability of re-energized cells to synthesize ATP at pH 10.5 (11), but pH 8.5 had to be used for growth here since most of the mutants were incapable of substantial growth at the higher pH. The lower growth pH led to generation of significantly lower ATP/ADP ratios than observed in re-energization experiments using pH 10.5-grown cells and than those found in growing cells (11, 13). This has been observed before with pH 7.5-grown cells (11) and probably results from the presence of a suboptimal complement of membrane lipids and respiratory chain components (10). However, upon energization in the current protocol, the wild type and all the mutants generated normal Δψ values in a range of ~137 to ~163 mV at pH 7.5 and ~168 to ~193 mV at pH 10.5, showing that each population of cells was energetically functional. The wild type ATP/ADP ratio increased over 3-fold during the 3-h incubation period at pH 10.5 under these conditions. Bioenergetic parameters (i.e. [ATP], [ADP], [P_i], Δψ, and Δψ') were determined at zero time, 1 h, and 3 h, and the ΔGp/Δp ratio was calculated. The ΔGp/Δp ratio would reflect the protons traversing the ATP synthase per ATP synthesized if the synthase functions in equilibrium with the bulk force but would reflect the discrepancy between the actual driving force and measured bulk Δp if protons are captured without equilibration. The typical ΔGp/Δp ratio during wild type OXPHOS at pH 10.5 is 13 (10, 13), a value that was reached within the 3-h resynthesis experiment (Fig. 3, top panel). By contrast, the typical ΔGp/Δp ratio during wild type OXPHOS at pH 7.5 is 2.5–3, and this ratio was observed throughout the resynthesis experiment at pH 7.5 for all the mutants as well as the wild type strain (Fig. 3, bottom panel). At pH 10.5, the mutants showed diverse deviations from the wild type capacity to resynthesize ATP. ATP resynthesis was compromised severely in the a-loop, aLys^{180}, and cPro^{51} mutants, which was much less affected in the aGly^{212} mutant, and was significantly enhanced in the cThr^{23} mutant (Fig. 3, top panel). A correlation was observed between the capacity to synthesize ATP at a high ΔGp/Δp ratio at pH 10.5 and a capacity to acidify the cytoplasm. This interesting relationship suggests that proton recapture may be a physiologically important adjunct to other mechanisms of alkaliphile pH homeostasis (10). Notably, the double aK180G and aG212S mutant exhibited a significant increase in cytoplasmic pH relative to all the other strains right after the upward pH shift to 10.5. This supported a role for this pair of residues in preventing proton loss through the ATP synthase. The individual aK180G, aG212S, and cP51A mutants exhibited somewhat less cytoplasmic alkalization. The a-loop mutant exhibited no difference in initial cytoplasmic pH change from the wild type, and the cT33A mutant reproducibly exhibited a slightly lower cytoplasmic pH than wild type, paralleling its larger ΔGp generation.

ATP Synthesis by ADP + P_i-loaded Right-side-out Membrane Vesicles—Greatly reduced respiration-dependent ATP synthesis was observed, relative to wild type preparations, in ADP + P_i-loaded vesicles from single or double aK180G mutants, the a-loop mutant, and the cP51A mutant at pH 10.5, while the aG212S mutant was only modestly affected (Fig. 4). Interestingly, vesicles of the cT33A mutant synthesized ATP at pH 10.5 after a lag and then quickly showed a decline in intravesicular ATP. At pH 7.5, ATP was synthesized by all the preparations, although there were small discernible deficits, relative to wild type preparations, in ADP and P_i-loaded vesicles with dATP and dTTP, respectively. The ATP synthase of B. pseudofirmus OF1 is known to synthesize ATP from ADP and P_i, but not from dATP and dTTP (28, 29). The ATP synthase of B. pseudofirmus OF1 is known to synthesize ATP from ADP and P_i, but not from dATP and dTTP (28, 29). The ATP synthase of B. pseudofirmus OF1 is known to synthesize ATP from ADP and P_i, but not from dATP and dTTP (28, 29). The ATP synthase of B. pseudofirmus OF1 is known to synthesize ATP from ADP and P_i, but not from dATP and dTTP (28, 29).
Fig. 3. Resynthesis of ATP by washed wild type and mutant cells upon energization by malate at either pH 7.5 or 10.5. Cells were grown at pH 8.5 for 3 h with malate as energy source and with the yeast extract lowered to 0.02% prior to washing the cells free of nutrients. Cytoplasmic ATP was depleted during the cell preparation (see "Experimental Procedures"). Re-energization was achieved by diluting the washed cells into buffer at either pH 7.5 or 10.5 that contained 50 mM sodium DL-malate (zero time). The cells were incubated for 3 h during which samples were assayed for [ATP], [ADP], [Pi], pH, and ΔΨ from which the ΔGp and Δp values were calculated. The S.D. of the values used to calculate the parameters shown was within 10% of the mean of duplicate determinations in at least three independent experiments. Top, pH 10.5; bottom, pH 7.5.

Fig. 4. Respiration-dependent synthesis of ATP by ADP + P↓-loaded right-side-out membrane vesicles of wild type and mutants. Vesicles were prepared from cells that were grown at pH 8.5. Assays were initiated by the addition of electron donor, 10 mM ascorbate, plus 0.1 mM phenazine methosulfate and were conducted as described under "Experimental Procedures." Growth at pH 8.5 rather than 10.5 accounts for reduced ATP synthesis by wild type relative to synthesis at pH 7.5 in these experiments as compared with the earlier experiments where OXPHOS at pH 10.5 was higher (12). The S.D. of the values shown was within 10%, and the means were derived from duplicate assays on at least three independent vesicle preparations.

Table III

| Strain            | pH 8.3 | pH 9.3 (−DCCD only) |
|-------------------|--------|---------------------|
|                   | −DCCD | +DCCD†              |
| Wild type         | 2.05 ± 0.18  | 0.15 ± 0.05         | 0.06 ± 0.02 |
| a-Loop            | 2.53 ± 0.21  | 0.13 ± 0.04         | 0.15 ± 0.07 |
| aK180G            | 3.86 ± 0.39  | 0.14 ± 0.04         | 1.08 ± 0.20 |
| aG212S            | 3.88 ± 0.48  | 0.16 ± 0.04         | 0.91 ± 0.07 |
| aK180G/aG212S     | 3.98 ± 0.34  | 0.15 ± 0.06         | 1.78 ± 0.33 |

* Pretreatment for 5 min with 200 μM DCCD.
† Experiments at pH 9.3 were conducted only on samples that were not heated with DCCD because DCCD inhibition is not optimal at that pH level.

Discussion

This study shows that specific features of the membrane-embedded subunits of proton-coupled alkaliphile ATP synthase are required for non-fermentative growth and for the robust OXPHOS that wild type B. pseudofirmus OF4 carries out at very high pH. Four of the five single a- and c-subunit mutants as well as the double mutant, with consensus Bacillus sequences replacing alkaliphile sequence features, assembled an active ATP synthase at wild type levels (Table II). All but the aG212S mutant exhibited deficits in non-fermentative growth at high pH in standard semidefined medium that were profound except in the cT33A mutant (Fig. 2). The details of their underlying deficits in OXPHOS were quite distinct from one another, indicating that a number of properties are involved in function of wild type ATP synthase at pH 10.5. The results are consistent with Williams’ (2, 6) early expectation that specific properties of proton-consuming as well as proton-pumping OXPHOS complexes would be important for proton transfers that are faster than proton equilibration with the bulk. Various global factors that can increase proton retention near the membrane surface are also likely to contribute to alkaliphile OXPHOS as suggested by others (for reviews, see Refs. 10 and 14). The current results, however, show that the aggregate global adaptations are not sufficient to support the OXPHOS observed in B. pseudofirmus OF4.

The effects of single aK180G and aG212S mutations versus the effects of their combined mutation are consistent with a functional interaction between the residues at these positions.
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The data include the enhanced DCCD inhibition of ATPase activity (in the absence of octylglucoside) of the single mutants but not the double mutant (Table II), the greater stimulation of ATP synthesis by a diffusion potential in the double mutant than in the single mutants (Table III), and the greater cytoplasmic alkalization of the double mutant than of the single mutants immediately after an alkaline shift (Fig. 3). However, the aGly212 and aLys180 residues do not exhibit complete functional equivalence. Whereas the aK180G-containing single and double mutants exhibited severe deficits in growth on malate in semidifed medium, the aG212S exhibited no such deficit (Fig. 2), showing a non-fermentative growth deficit only on the more stringent high amine nitrogen-malate medium. The absence of a growth phenotype for the aG212S mutant on malate in semidifed medium at pH 10.5 is consistent with earlier observations that small defects in pH homeostasis, as might be caused by compromised proton loss through the ATP synthase, are more evident in the more challenging pH shift protocol (or growth phenotypes at pH values of 11 and above) than in growth experiments on malate at pH 10.5 (61). The growth deficits of the mutants in the current panel on semidifed malate-containing medium at pH 10.5 appear to correlate best with loss of the quantitative signature of alkaliphile OXPHOS, i.e. a robust capacity of ATP synthesis per se and a high ΔGp/Δp ratio at high pH. We hypothesize that the growth deficit of the aLys180-containing mutants on this medium at pH 10.5 reflects an important role for the positively charged lysine residue in ATP synthesis itself at high pH, e.g. proton flow to the c-subunit carboxylate, in addition to its role in proton retention. The aGly212 residue does not appear to have an equivalent role in the ATP synthesis function. Two pieces of evidence support this lack of functional equivalence between aLys180 and aGly212 in ATP synthesis. Both the ΔGp/Δp profile of cells of the aG212S mutant at pH 10.5 (Fig. 3) and the capacity of vesicles from the aG212S mutant to carry out respiration-dependent ATP synthesis at pH 10.5 (Fig. 4) were much closer to wild type patterns than to those of the aK180G mutant. It will be of particular interest to probe the role of aLys180 in ATP synthase synthesis further by examining different substitutions at the aLys180 position alone and in combination with changes in aGly212 or the conserved aArg172.

Mutation of the a-loop did not alter any of the indices of a gating element, but this mutation was nonetheless accompanied by a severe deficit in non-fermentative growth at pH 10.5 and loss of the quantitative signature of alkaliphile OXPHOS, i.e. the ability to carry out OXPHOS at pH 10.5 with a high ΔGp/Δp ratio. The a-loop is in a position near a region of aTMIH2 that is proposed to have an important role in the proton uptake pathway (17, 19) and might monitor the status of the aLys180-aGly212 pair. It is a candidate for the hypothesized element that facilitates sequestered proton capture by the gated ATP synthase at high pH (Fig. 1A).

While removed from the proton uptake pathway of the a-subunit, the two c-subunit features studied in detail are also of great interest. The cP51A mutation severely compromised non-fermentative growth and OXPHOS at high pH. It might modify the structure of the region surrounding the critical cGlu4 carboxylate so that proton loss from this part of the ATP synthase complex is specifically minimized. This is consistent with the presence of a proline at the cPro51 position only in extreme alkaliphiles (Fig. 1F). Alternatively this feature might have a role in assembly of a high number of c-subunit monomers in the rotor at elevated pH values. The different c-subunit stoichiometry found in different ATPases is a topic of intense recent interest especially because this stoichiometry is expected to have bioenergetic implications (4, 62–65).

Finally the phenotype of the cT33A mutant was distinct from those of the other mutants in the panel. The growth rate and bioenergetic profile of the cT33A mutant in whole cells suggested that it should be a "superalkaliphile". Of particular note was its rapid development of an extraordinarily high ΔGp/Δp relative to the wild type strain in the ATP resynthesis experiments (Fig. 3, top panel). Yet the cT33A mutant exhibited a deficit in growth yield on non-fermentative substrates at high pH. This deficit indicates that there is functional value at high pH of the unusual threonine in the interface region between the c-subunit and the stalk that connects to the catalytic subunits. This role for cThr33 is reflected in the kinetics of respiration-dependent ATP synthesis by right-side-out vesicles from the cT33A mutant at pH 10.5, i.e. the pattern of initial synthesis followed by a decline in ATP (Fig. 4). We hypothesize that the cThr33 residue prevents rapid torque generation at high pH that, in the cT33A mutant, is followed by slippage. This might result in an ATP synthesis-hydrolysis oscillation that would account for the reduced growth yield on malate at pH 10.5 even though the ATP synthase is highly capable of synthesizing ATP over the full pH range of the alkaliphile.

The goal of the current study was to test the hypothesis that the alkaliphile-specific features of the a- and c-subunits of ATP synthase are critically involved in OXPHOS and non-fermentative growth. The experimental design therefore involved making a specific mutagenic change to the consensus sequence for non-alkaliphiles that was likely to allow most of the mutants to assemble an active ATP synthase. The current observation of diverse pH 10.5-specific deficits in fermentative growth and OXPHOS features found in these mutants demonstrates that the alkaliphile-specific features of the synthase are important for OXPHOS at pH 10.5. It will now be of interest to focus on these different features and their particular mechanistic roles by replacing them with multiple substitutions that will further probe their function. Mechanistic insights into the adaptations of ATP synthesis by the alkaliphile may be expected to be of general interest with respect to proton coupling mechanisms in OXPHOS.

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REFERENCES
1. Mitchell, P. (1961) Nature 191, 144–148
2. Williams, R. J. P. (1961) J. Mol. Biol. 1, 1–13
3. Boyer, P. D. (1995) Annu. Rev. Biochem. 66, 717–749
4. Stock, D., Gibbons, C., Arechaga, I., Leslie, A. G. W. & Walker, J. E. (2000) Curr. Opin. Struct. Biol. 10, 672–679
5. Boyer, P. D. (1998) Biochim. Biophys. Acta 1365, 3–9
6. Williams, R. J. P. (1978) Biochim. Biophys. Acta 505, 1–44
7. Slater, E. C., Berden, J. A. & Herweijer, M. A. (1985) Biochim. Biophys. Acta 811, 217–231
8. Rottenberg, H. (1985) Mod. Cell Biol. 4, 47–83
9. Kell, D. B. (1979) Biochim. Biophys. Acta 594, 55–99
10. Llano, E., M. & Englichreth, E. (1997) Trends Biochem. Sci. 22, 420–423
11. Fillingame, R. H., Angervine, C. M. & Dimitriev, O. Y. (2003) FEBS Lett. 555, 29–34
12. Angervine, C. M. & Fillingame, R. H. (2003) J. Biol. Chem. 278, 6066–6074
13. Angervine, C. M., Herold, K. A. & Fillingame, R. H. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 13179–13183
14. Cai, B. D. (2000) Bioenerg. Biomembr. 32, 365–371
15. Marantz, Y., Nachiei, E., Aagaard, A., Brzezinski, P. & Gutman, M. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 8590–8595
16. Nachiei, E., Gutman, M., Ttior, J. & Oesterhelt, D. (2002) Biochim. Biophys. Acta 1507, 416–426
17. Hanes, T. H. & Dencher, N. A. (2002) FEBS Lett. 528, 35–39
18. Qu, Z.-H., Yu, L. & Yu, C.-A. (1992) Biochemistry 31, 3297–3302
19. Clejan, S., Angervine, C. A., Cohen, M. A. & Kruelwich, T. A. (1989) J. Bacteriol. 171, 1744–1746
Alkaliphile OXPHOS and Special Properties of ATP Synthase

26. Ito, M., Guffanti, A. A., Zeng, Y. J., Ivey, D. M. & Krulwich, T. A. (1997) J. Bacteriol. 179, 3851–3857
27. Kunkel, T. A., Bebenek, K. & McClary, J. (1991) Methods Enzymol. 204, 125–129
28. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) Gene (Amst.) 77, 51–59
29. Hicks, D. B. & Krulwich, T. A. (1990) J. Biol. Chem. 265, 20547–20554
30. LeBel, D., Poirier, G. G. & Beaudoin, A. R. (1978) Anal. Biochem. 86, 86–89
31. Hicks, D. B. & Krulwich, T. A. (1986) J. Biol. Chem. 261, 12896–12902
32. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. (1979) Anal. Chem. 51, 95–97
33. Schagger, H. & von Jagow, G. (1987) Anal. Biochem. 166, 368–379
34. Krulwich, T. A. & Guffanti, A. A. (1979) J. Bioenerg. Biomembr. 21, 663–667
35. Cole, H., Wimpenney, J. W. T. & Hughes, D. E. (1967) Biochim. Biophys. Acta 143, 445–453
36. Stanley, P. E. & Williams, S. G. (1969) Anal. Biochem. 29, 381–392
37. Chapman, A. G., Fall, L. & Atkinson, D. E. (1971) J. Bacteriol. 106, 1072–1086
38. Rose, J. & Slater, E. C. (1972) Biochim. Biophys. Acta 297, 275–296
39. Chen, K. Y. & Cheng, S. (1988) Biochem. Biophys. Res. Commun. 156, 185–191
40. Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogawa, N., Kuhara, S. & Horikoshi, K. (2000) Nucleic Acids Res. 28, 4317–4331
41. Takami, H., Takaki, Y. & Uchiyama, I. (2002) Nucleic Acids Res. 30, 9027–9035
42. Arne, R. & Ohtani, M. (1990) Biochem. J. 266, 933–936
43. Lu, J., Nogi, Y. & Takami, H. (2001) FEMS Microbiol. Lett. 205, 291–297
44. Oslos, K., Kris, S., Morgan, H. W., Dimroth, P. & Cook, G. M. (2003) J. Bacteriol. 185, 461–465
45. Cook, G. M., Kris, S., Morgan, H. W., von Ballmoos, C., Matthey, U., Kaim, G. & Dimroth, P. (2003) J. Bacteriol. 185, 4442–4449
46. Kris, S., Kaim, G., Dimroth, P. & Cook, G. M. (2004) Biochim. Biophys. Acta 1676, 112–117
47. Vallyavitel, I. F. & Fillingame, R. H. (1998) J. Biol. Chem. 273, 16241–16247
48. Zhang, D. & Vik, S. B. (2003) Biochemistry 42, 331–337
49. Rastogi, V. K. & Girvin, M. E. (1998) Nature 402, 263–268
50. Hartzog, P. E. & Cain, B. D. (1994) J. Biol. Chem. 269, 32313–32317
51. Ivey, D. M. & Donelson, J. L. (2002) American Society for Microbiology 102nd General Meeting, Salt Lake City, Utah, May 19–23, 2002, p. 274, Abstr. K-36, American Society for Microbiology Press, Washington, D. C.
52. Arechaga, I. & Jones, P. C. (2001) FEBS Lett. 494, 1–5
53. Vonck, J., van Nidda, T. K., Meier, T., Matthey, U., Mills, D. J., Kuhlbrandt, W. & Dimroth, P. (2002) J. Mol. Biol. 321, 367–376
54. Hong, S., Ko, Y. H. & Pedersen, P. L. (2001) Arch. Biochem. Biophys. 394, 275–279
55. Hermolin, J., Dimitriev, O. Y., Zhang, Y. & Fillingame, R. H. (1999) J. Biol. Chem. 274, 17011–17016
56. Santana, M., Ionescu, M. S., Vertes, A., Longin, R., Kunst, F., Danchin, A. & Glaser, P. (1994) J. Bacteriol. 176, 6802–6811
57. Tsumada, S. P., Rodgers, A. J., Aggeler, R., Wiles, M. C., Yoshida, M. & Capaldi, R. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6560–6564
58. Suzuki, T., Murakami, T., Iino, R., Suzuki, J., Ono, S., Hirakihara, Y. & Yoshida, M. (2003) J. Biol. Chem. 278, 46040–46046
59. Hicks, D. B., Wang, Z., Wei, Y., Kent, R., Guffanti, A. A., Bencs, H., Bechhofer, D. H. & Krulwich, T. A. (2003) Proc. Natl. Acad. Sci. U. S. A., 100, 10213–10218
60. Wei, Y., Southworth, T. W., Kloster, H., Ito, M., Guffanti, A. A., Moir, A. & Krulwich, T. A. (2003) J. Bacteriol. 185, 5133–5147
61. Gilmour, J., Messner, P., Guffanti, A. A., Kent, R., Scheberl, A., Kendrick, N. & Krulwich, T. A. (2000) J. Bacteriol. 182, 5969–5981
62. Muller, D. J., Dencher, N. A., Meier, T., Dimroth, P., Suda, K., Stahlberg, H., Engel, A., Seelert, H. & Matthey, W. (2001) FEBS Lett. 504, 219–222
63. Schmedt, R. A., Cu, J., Williams, J. R. & Bruslow, W. S. (1998) J. Bacteriol. 180, 3205–3208
64. Stahlberg, H., Muller, D. J., Suda, K., Fothiadis, Engel, A., Meier, T., Matthey, U. & Dimroth, P. (2001) EMBO Rep. 2, 229–233
65. Murata, T., Arechaga, I., Fearnley, I. M., Kakinuma, Y., Yamato, I. & Walker, J. E. (2003) J. Biol. Chem. 278, 21162–21167