Sequence analysis
Characterization of the N-ATPase, a distinct, laterally transferred Na\(^+\)-translocating form of the bacterial F-type membrane ATPase

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ABSTRACT
An analysis of the distribution of the Na\(^+\)-translocating ATPases among microbial genomes identified an atypical form of the F\(_{1}\)F\(_\text{Fo}\)-type ATPase that is present in the archaea Methanosarcina Barkeri and M. acetivorans, in a number of phylogenetically diverse marine and halotolerant bacteria and in pathogens Burkholderia ssp.

In complete genomes, representatives of this form (referred to here as N-ATPase) are always present as second copies, in addition to the typical proton-translocating ATP synthases. The N-ATPase is encoded by a highly conserved atpDCQRBEFAG operon and its subunits cluster separately from the equivalent subunits of the typical F\(_{1}\)F\(_\text{Fo}\)-type ATPases. Na\(^+\)-ATPase \(c\) subunits carry a full set of sodium-binding residues, indicating that most of these enzymes are Na\(^+\)-translocating ATPases that likely confer on their hosts the ability to extrude Na\(^+\) ions. Other distinctive properties of the N-ATPase operons include the absence of the delta subunit from its cytoplasmic sector and the presence of two additional membrane subunits, AtpQ (formerly gene 1) and AtpR (formerly gene X). We argue that N-ATPases are an early-diverging branch of membrane ATPases that, similarly to the eukaryotic V-type ATPases, do not synthesize ATP.

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1 INTRODUCTION
F\(_{1}\)F\(_\text{Fo}\)-type (F-type) and V\(_A\)/V\(_\text{O}\)-type ATPases are membrane-anchored rotary enzymes that couple translocation of H\(^+\) or Na\(^+\) ions across the membrane to the synthesis or hydrolysis of ATP. Although most of their subunits are evolutionarily related, the two classes of ATPases have clear differences in structure and phylogenetic distribution: F-type ATPases are found in bacteria, mitochondria and chloroplasts, whereas V\(_A\)/V\(_\text{O}\)-type ATPases are found in eukaryotic cell membranes (in particular, vacuoles), as well as in all archaea and in some bacteria (Hilario and Gogarten, 1998; Forgac, 2007; von Ballmoos et al., 2008; Mulikidjianian et al., 2009). V\(_A\)/V\(_\text{O}\)-type ATPases are often subdivided into two classes based (i) on the ability of the prokaryotic (A-type) enzyme to function in the direction of ATP synthesis and (ii) presence of additional subunits in the eukaryotic (V-type) enzyme that normally functions only as an ion-translocating ATPase, using the energy of ATP hydrolysis to acidify cellular compartments (Hilario and Gogarten, 1998; Forgac, 2007).

Structural characterization of the Na\(^+\)-binding sites in the \(c\)-subunits of F- and V-type ATPases (Meier et al., 2005; Murata et al., 2005) allowed unequivocal assignment of the cation specificity for the membrane ATPases encoded in numerous sequenced genomes: only those \(c\)-subunits that contain full sets of Na\(^+\) ligands appeared to transport Na\(^+\), the loss of at least one of those ligands correlated with the loss of Na\(^+\) specificity (Mulikidjianian et al., 2008a; Meier et al., 2009).

In the view of several reports on the sodium dependence of energy-coupled reactions in cyanobacteria (Willey et al., 1987; Skulachev, 1988; Pogoryelov et al., 2003), we have searched cyanobacterial genomes for the Na\(^+\)-translocating ATPases. We found several apparently Na\(^+\)-dependent cyanobacterial ATPases, but always as second copies, in addition to the H\(^+\)-translocating ATP synthases. Here, we report the common properties of these ATPases, which are encoded in apparently highly mobile operons and have a set of specific traits that qualify them as a separate subfamily of the F-type ATPases. Since these ATPases, besides forming a novel subfamily, are always encoded next to the typical rotary ATPases and are predominantly Na\(^+\)-dependent, we refer to them hereafter as N-ATPases.

2 METHODS
Phylogenetic distribution of the N-ATPase operons was deduced from protein BLAST (Altschul et al., 1997) searches against the NCBI’s RefSeq database (Prutt et al., 2009) (last searched February 1, 2010) and verified by examining gene neighborhoods of the retrieved ORFs and by checking for the presence of the N-ATPase-specific subunit AtpR. Phylogenetic trees were constructed using the neighbor-joining algorithm with MEGA (Kumar et al., 2008). Multiple alignments were constructed from BLAST outputs with manual editing. Transmembrane segments were predicted using TMHMM (Krogh et al., 2001). Sequence logos were drawn with WebLogo (Crooks et al., 2004).

3 RESULTS
Search of the NCBI’s RefSeq database (Prutt et al., 2009) for cyanobacterial \(c\) (proteolipid) subunits that would have a full set of Na\(^+\)-binding ligands (Mulikidjianian et al., 2008a; Meier et al., 2009) returned five hits, all coming from marine cyanobacteria (Supplementary Fig. S1a). In each case, the operon encoding the
Na\textsuperscript{+}-binding c subunit was present in the genome along with another ATPase operon, encoding an H\textsuperscript{+}-translocating F-type ATPase. As depicted below for Synechococcus sp. PCC 7002, an alignment of the Na\textsuperscript{+} - and H\textsuperscript{+}-binding c-subunits (top and bottom, respectively) from the same cyanobacteria revealed a Glu substitution of the Gin residue (shown in blue) that serves as a Na\textsuperscript{+} ligand (uncharged residues of the transmembrane segments are highlighted in yellow, Na\textsuperscript{+} and H\textsuperscript{+} ligands are labeled with asterisks). Among the 4084

![Diagram](https://academic.oup.com/bioinformatics/article-abstract/26/12/1473/287511)

3.1 Always the second: conservation and distinctive properties of the N-ATPase

A search of the complete genome database identified homologous N-ATPase operons in some representatives of the bacterial phyla Aquificae, Chlorobi and Planctomycetes, in certain members of α-, β-, γ- and δ-subdivisions of Proteobacteria, and in two archaea, Methanosarcina barkeri and M.acetivorans (see Supplementary Fig. S2). All these operons had the same atpDCI-genotype and were characterized by the fact that these operons are always present in the genomes as second copies alongside operons that encode typical ESTxxY

Na\textsuperscript{+}-binding motif (Supplementary Fig. S1). The Na\textsuperscript{+}-dependent ATPase operons in all cyanobacteria had a similar gene order, with a single gene insertion in Acaryochloris marina and a two-gene insertion in the two strains of Cyanothecae sp. (Supplementary Fig. S2).

3.2 Lateral transfer of N-ATPase genes

Presence of the N-ATPase operon in the genomes of M.barkeri and M.acetivorans, but not in the closely related M.mazei, suggested that this operon had been acquired via lateral gene transfer. This suggestion is consistent with the gene neighborhoods of the atpDCQRFEG operons in M.barkeri and M.acetivorans (Fig. 2) and the absence of these operons in any other archaeal genomes sequenced so far. Gene neighborhoods of the N-ATPase operons in various bacteria are also consistent with the insertion of this operon (Supplementary Fig. S7). The widespread presence of the N-ATPase genes among diverse bacteria deprecates the historical designation of these enzymes as ‘archaeobacterial F1F0-ATPases’. The strict conservation of the gene order and co-linearity of the phylogenetic trees for distinct N-ATPase subunits suggests that the whole operon is being transferred as a single unit. However, the GC content of the N-ATPase operons shows a good correlation with the average...
While the A-type ATP synthase was apparently H⁺-translocating in at least two cyanobacteria. The respective genes could be plasmid-borne, as is the case of N-ATPases. (Swingley et al., 2008). They also disputed the idea that these enzymes do not clearly fit into any of the described families. McInerney et al. (2003; McInerney et al., 2007) noted that the c-subunits encoded in these operons clustered like F-ATPase operon was repeatedly noted in bacterial genomes. (Glöckner et al., 2007), the presence of a ‘set of ATP synthase genes’ in M. mazei Gs1. While the A-type ATP synthase was apparently H⁺-dependent, the second, F-type ATPase appeared to be Na⁺-translocating (Becher and Müller, 1994; Pisa et al., 2007). Although this second ATPase has not been found in the sequenced genome of M. mazei (Fig. 2), the respective genes could be plasmid-borne, as is the case of N-ATPases in at least two cyanobacteria.

4 DISCUSSION

Following the description of an ‘archaeobacterial F₁/Fₒ-ATPase’ in M. burkertii (Sami et al., 1997), the presence of a ‘Methanosarcina-like’ F-ATPase operon was repeatedly noted in bacterial genomes (Glöckner et al., 2003; McInerney et al., 2007; Swingley et al., 2008), although the exact function(s) of these enzymes and their cation specificity remained obscure. McInerney et al. (2007) noted two F-type ATPases encoded in the genome of Syntrophus aciditrophicus and suggested that both of them were Na⁺-translocating (incidentally, the F-type ATPase of this organism is definitely H⁺-specific, whereas the cation specificity of its N-ATPase is unknown; it might be specific for Na⁺, see below). In their analysis of the A. marina genome, Swingley et al. (2008) noted the presence of a plasmid-encoded ‘set of ATP synthase genes that were arranged into a unique operon … conserved with full synteny in a remarkable array of organisms, including cyanobacteria, archaea, planctomycetes, chlorobi and proteobacteria’. The authors noted that the α-subunits encoded in these operons clustered together on a phylogenetic tree and suggested that these enzymes formed a separate new family of ATP synthases. However, they slightly overstated their case by claiming that its ‘individual proteins do not clearly fit into any of the described families’ (Swingley et al., 2008). They also disputed the idea that these enzymes were Na⁺-translocating. In addition, the key observation by Daniel Haft that these enzymes always ‘represent a second F₁/Fₒ ATPase system’ has only been published online in the JCVI’s Comprehensive Microbial Resource (Davidson et al., 2010).

As a result, there still exists a significant confusion as to the phylogenetic distribution, organization and the functional role(s) of these enzymes.

The alignment in Supplementary Fig. S1 shows that, despite the doubts of Swingley et al. (2008), the c subunit of the A. marina N-ATPase has a full set of Na⁺-binding ligands, including the recently recognized additional Thr residue of the ESTxxY motif (Mulkidjanian et al., 2008a; Meier et al., 2009). While this residue is missing in c-subunits of several N-ATPases, including the one from S. aciditrophicus, a Glu residue is present instead of the Na⁺-coordinating Gln residue in the first transmembrane helix of the c subunit of nearly all N-ATPases (Supplementary Fig. S1). As has been noted previously (Meier et al., 2009; Saum et al., 2009), this Glu residue could potentially provide two ligands for the Na⁺ ion and thereby complete the Na⁺-coordinating shell. If so, all these N-ATPases would end up being capable of binding Na⁺ ions.

A hallmark of the N-ATPase operons is the presence of the atpR gene. Because of the low dielectric permittivity of the membrane, the strategic positioning of two Arg residues of AtpR in the hydrophobic core of the membrane (Supplementary Fig. S5) implies the presence of negatively charged residues in their vicinity. Given the absence in the N-ATPase operons of the atpR gene, whose product was recently shown to interact with the c-ring (Suzuki et al., 2007), we suggest that the product of the AtpR gene serves essentially the same function, regulating N-ATPase assembly and/or its activity. Just like ApR assists c-ring assembly by directly interacting with the c-subunits (Suzuki et al., 2007), AtpR could do that through the interaction of its two Arg residues with N-ATPase-specific c-subunits, most of which carry two Glu residues in the middle of their transmembrane helices (Supplementary Fig. S1).

The observation that N-ATPases are always found alongside typical F- or A-type ATPases suggests that the N-ATPases cannot functionally replace those enzymes in their role as ATP synthases. Indeed, in M. acetivorans, deletion of the N-ATPase operon had no visible effect on cell growth or ATP synthesis, whereas a mutant lacking the A-ATP synthase genes could not be obtained (Saum et al., 2009). We conclude that, similarly to the eukaryotic V-ATPases, the N-ATPases do not catalyze ATP synthesis, which leaves ATP-driven ion pumping as the most plausible function for these enzymes. By analogy with V-ATPases, the N-ATPase c-oligomer ring can be expected to consist of a smaller number of c-subunits than the c-oligomer ring of F-type ATP synthases.

Acquisition of an operon capable of extrusion of Na⁺ ions would be beneficial to the marine bacteria and other organisms living in high-salt environments. Accordingly, many N-ATPase-encoding bacteria are either marine organisms or grow in the presence of salt. Since Na⁺-translocating ATPases can also translocate protons (von Ballmoos et al., 2008), the N-ATPases could in principle function as outward proton pumps in low-sodium and/or acidic environments. A typical N-ATPase operon (with untranslated AtpR) has been found in an industrial strain of Pseudomonas veronii growing on 2-butanone (Onaka et al., 2007). The presence of N-ATPase genes in such pathogens as Burkholderia mallei appears to be inherited from their free-living relatives and might be related to their survival in blood.

Using a larger set of sequences than the one used by Swingley et al. (2008), we have confirmed that N-ATPases branch separately from other F-type ATPases (Fig. 1, Supplementary Fig. S3).
This separate branching suggests a possible early divergence of the N-ATPases, which is compatible with the following, supposedly ancestral traits of these enzymes:

(i) Both AtpF/A subunit of the N-ATPase and the E subunit of the peripheral stalk of the V/A-type ATPases correspond to a fusion of b- and a-subunits of the typical F-ATPases (Pallen et al., 2000).

(ii) The presence of the second membrane-embedded Glu residue is consistent with the evolution of the c-subunit from a duplication of an amphiphilic helix that contained a Glu residue in the middle (Davis, 2002). Similar two-Glu c-subunits are found in the A-type ATPases of methanogens and F-type ATPases of Thermotogae.

(iii) Outward pumping of Na⁺ ions, the predicted function of N-ATPases, appears to be an ancient trait and has been previously suggested as a function of the common ancestor of the F- and V-type ATPases (Mukidjanian et al., 2007; 2008a, b; 2009).

All these features, which N-ATPases share either with V/A-ATPases—or with the putative common ancestor of all rotary ATPases, suggest that N-ATPases represent a distinct early-diverging family of rotary ATPases. Thus, the Na-transporting common ancestor of all F-type ATPases apparently gave rise to two different families of ATPases: (i) the reversible ATPases/ATP synthases (‘genuine’ F-ATPases) and (ii) ATP-driven ion pumps (N-ATPases).

In conclusion, the N-ATPases (until now usually referred to as ‘archaeobacterial F₁F₀-ATPases’) are encoded in an apparently highly mobile operon that, most likely, confers on its host the ability of ATP-driven outward pumping of Na⁺ ions, which complements the H⁺ specificity of the native chromosome-encoded F-ATPase (or A-ATPase). We predict that, similarly to the eukaryotic V-ATPases, the evolutionary divergence of these two functions may be explained by the second membrane-embedded Glu residue in the middle (Davis, 2002). Similar two-Glu c-subunits are found in the A-type ATPases of methanogens and F-type ATPases of Thermotogae.

REFERENCES

Aßhfeld, G. et al. (1997) Gapped BLAST and PSI-BLAST—a new generation of protein database search programs. Nucleic Acids Res., 25, 3389–3402.

Booth, D. and Miller, V. (1994) ΔψNa⁺ drives the synthesis of ATP via a ΔψNa⁺-translocating F₁F₀-ATP synthase in membrane vesicles of the archaeon Methanosarcina acetivorans. J. Bacteriol., 176, 2543–2550.