Alkaliphile-specific motif analysis of *Stenotrophomonas* species DL18 F_1F_o-ATP synthase *c*-subunit isolated from Indian alkaline Soda Lake, Lonar

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The membrane-associated F_1F_o-ATP synthase of bacteria plays a vital role in the production of energy molecule, i.e. adenosine triphosphate (ATP). However, under alkaline conditions, ATP synthesis in bacteria is not thermodynamically feasible due to external high pH. Various studies reported motifs in ATP synthase *c*-subunit as alkaliphile-specific features for adaptation under alkaline condition. Some conserved residues in alkaliphiles were observed in *Stenotrophomonas* species DL18 isolated from Indian alkaline Soda Lake, Lonar, which has pH 10.5. The above-mentioned specific amino acid features in the studied alkaliphile may involve proton translocating mechanism for ATP synthesis. The studied motifs of F_1F_o-ATP synthase *c*-subunit of *Stenotrophomonas* species DL18 have G*X*G*X*G*X*A in the inner helix and G*XX*D*XX*F in the outer helix. The overall interacting residues of the *c*-subunit structure may be responsible for the ATP synthesis in particular pH conditions.

Keywords: Alkaliphile, ATP synthase, electrochemical ion gradient, motif analysis.

ADENOSINE triphosphate (ATP) acts as energy currency that allows energy to be utilized in a variety of biochemical processes in living cells. ATP molecules are synthesized by ATP synthase complex using the electrochemical ion gradient of H^+ or Na^+ across the phospholipid membranes[1]. ATP synthase, also called ATPase complex, comprises two different domains: the water-soluble F_1 and the membrane-embedded F_o motor. Therefore, they are known as F_1F_o-ATP synthases of bacteria and perform two critical functions. They catalyse the synthesis of ATP from ADP and inorganic phosphate (P_i) utilizing the electrochemical ion gradient. ATP synthase functions as ATPase by generating the electrochemical ion gradient at the expense of ATP under conditions of low driving force by rotating in the opposite direction, i.e. ATP hydrolysis. The membrane potential ($\Delta\psi$) and a transmembrane ion concentration gradient (ΔpH/ΔpNa) are alike in ion transport through F_o rotor[1,2]. The cytoplasmic F_1 catalytic subunit consists of $\alpha_3\beta3\gamma\delta\varepsilon$ subunit complex, whereas membrane-embedded F_o consists of ab_2c_{10-15} subunits. F_1 is in contact with F_o by a central ($\gamma\varepsilon$) and peripheral ($b_2\delta$) stalk[3]. The *c*-subunits of the rotor are in direct contact with the *a*-subunit of F_o. Each *c*-subunit has two α-helices connected by a loop region, which is in contact with the γ- and ε-subunits. Under alkaline condition, the cytoplasmic pH is maintained below 1.5–2.3 pH units than the external environment. Hence, the reversed ΔpH poses a major thermodynamic problem for ATP synthesis[3]. This is circumvented by adaptations in the *a*-subunit and *c*-subunits. Despite extensive studies of F_1F_o-ATP synthase *c*-subunit, essential steps of the proton transport mechanism have remained unclear, especially the proton binding as well as release mechanism. In the present study, attempts were made to analyse F_1F_o-ATP synthase *c*-subunit of *Stenotrophomonas* species DL18, which was isolated from Indian alkaline Soda Lake, Lonar.

Briefly, isolation and 16S rRNA-based identification of facultative alkaliphile was carried out by initial culture studies at pH 9.5, followed by pure culture maintenance and growth studies of orange pigmented bacterium, i.e. *Stenotrophomonas* species DL18 (GenBank accession number: JN995612) at different pH conditions (pH 7–12). ATP synthase gene was amplified using *pfu* polymerase and the *c*-subunit was sequenced by primer walking method. The respective sequence was deposited in the NCBI database (GenBank accession number: JN995615). The blastx program was used to get a total of 105 amino acid sequences of the *c*-subunit (Protein ID: AET99166).

BLAST analysis of ATP synthase F_0 *c*-subunit of *Stenotrophomonas* species DL18, which was isolated from Indian alkaline (pH 10.5) Soda Lake, Lonar, suggests 98% identity at nucleotide level (283 identical nucleotides out of total 289 nucleotides from query coverage) and 100% at amino acid level (90 amino acids identical out of 90 amino acids from 85% of query coverage) in comparison with *Stenotrophomonas maltophilia*. Some of the analysed conserved residues in alkaliphiles were observed in *Stenotrophomonas* species DL18 as Ala^{27}, Glu^{44}, Arg^{48}, Gln^{49}, Pro^{50}, Glu^{51}, Leu^{56}, Phe^{61}, Ile^{62}, Leu^{66}, Ile^{73}, Phe^{83} (*Stenotrophomonas* species DL18 numbering system for ATP synthase *c*-subunit; Figure 1). In addition, loop sequence (RQPE), which interacts with subunits γ and ε of F_1, connecting the inner (helix-1) and outer (helix-2) α-helices was conserved among the alkaliphiles (Figure 2). In majority of bacteria, glutamate (Glu^{54} in *Bacillus pseudofirmus* OF4) is conserved in the C-terminal helix of *c*-subunit near the centre of the membrane. However, some of the bacterial ATP synthases are found with aspartate (Asp^{61} in *Escherichia coli*). Hence,

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1 MYFAVLTNFAQIQSSTALAVGIMIGLAALGAGLGLAIMAGKFLESAARQPE
LIPVLQVRMFITAGLIDAAFIISVAVGLLLAFANPLSAASPVPSPQGFRQALAG 105
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Figure 1. Some alkaliphilic conserved residues of *Stenotrophomonas* species DL18 shown in bold.

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1 MYFAVLTNFAQIQSSTALAVGIMIGLAAL**GAGLGLA**IMAGKFLESAA**RQPE**LIPVLQVRMFITA**GLIDAAF**IISVAVGLLLAFANPLSAASPVPSPQGFRQALAG 105

Figure 2. Helix-1 and helix-2 from *Stenotrophomonas* species DL18 are shown as bold and underlined. The conserved loop region is shown in a box.

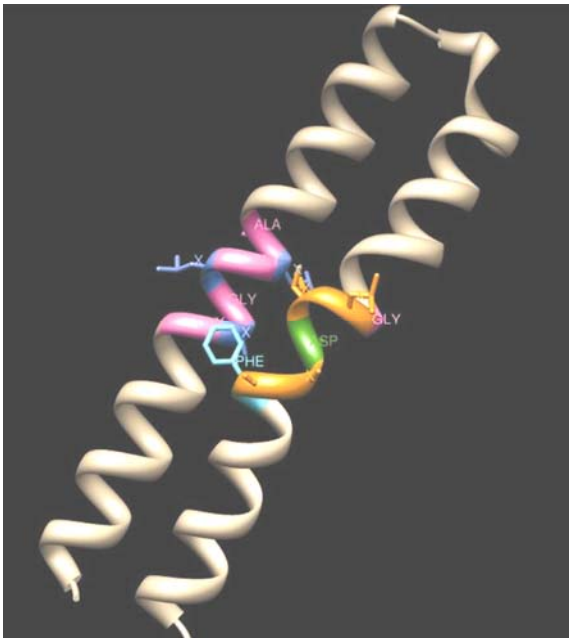

Figure 3. *In silico* model of *Stenotrophomonas* DL 18 F_1F_o-ATP synthase *c*-subunit. The predicted protein structure was obtained using on-line Swiss model workspace and visualized using USCF chimera 1.6 rc. The specific amino acid residues, i.e. G*X*G*X*G*X*A motif in the inner helix-1 and G*XX*D*XX*F in the outer helix-2 are labelled.

proton translocating ATP synthases have either glutamate or aspartate at this position. The protein structure prediction was performed using Swiss model on-line web server and the model was generated as shown in Figure 3. The carboxylic side chain of these amino acids play a critical role in ion binding (E/D pathway)[4]. Studies have shown that dicyclohexylcarbodiimide (DCCD) picks up a proton, which is being accepted by the carboxylate group of Glu or Asp, in the binding site[5]. In our study, the amino acid with carboxylic side chain was observed at the 67th position, i.e. Asp[67] in *Stenotrophomonas* species DL18.

Alkaliphilic *Bacillus* species consists of A*X*A*X*A*X*A in the inner helix and P*XX*E*XX*P in the outer helix, from which P[51] is considered as alkaliphile-specific[3,6]. However, *Stenotrophomonas* species DL18 was observed with a GAGLGLA motif in the inner helix and GLIDAAF motif in the outer helix. Similarly, in other alkaliphiles, *Thioalkalivibrio* species K90mix and *Thioalkalimicrobium cyclicum* ALM1, helix-1 motif was GAAIGVG (G*X*A*X*G*X*G) and GSAIGWG (G*X*A*X*G*X*G), respectively. *Bacillus halodurans* C-125 has GGAIAVA (G*X*A*X*A*X*A), while *Bacillus clausii* has GGAIGVA (G*X*A*X*G*X*A) in helix-1 of *c*-subunit (Figure 4). However, these are alkaliphiles. From the alignment, the amino acid residues from *T. cyclicum* ALM1 and *Acidithiobacillus ferrooxidans* were identical in helix-1 (GSAIGWG) and helix-2 (GLMESFP). But *T. cyclicum* ALM1 is alkaliphile, which grows above pH 9 and *A. ferrooxidans* is acidophile, which grows in the pH range 1.3–4. Therefore, the helix-1 and helix-2 residues may influence the functional properties of carboxylate of helix-2. This was shown by DCCD-resistant ATP synthesis and inactivation due to mutation at Ala[24] and Ile[28] in *E. coli*[7].

In *I. tartaricus* Na^+ is translocated through each *c*-subunit, which has glutamate as acidic residue. The Na^+ ion is coordinated by four residues residing in three helices consisting of Glu[65] from one *c*-subunit, while the other subunit residues include Ser[66], Thr[67] and Gln[32], which have polar and uncharged side chain from the C- and N-terminals respectively. Proton translocating ATP synthase does not have such polar residues[4,8]. Such residues are absent in *Stenotrophomonas* species DL18. This shows that the bacterium may utilize proton translocating mechanism for ATP synthesis (Figure 5). In addition, several high-resolution structures showed the presence of water molecules within the hydrophobic core of the protein[4,5,8]. The proton from hydronium ion, which is coordinated by backbone carbonyls, would be in the binding site of the acidic residue. The transient linear water chain may facilitate proton transfer, specifically within the protein. In protein function, bound water molecules may be actively involved in the proton transfer mechanism. Moreover, proton hopping, also called as Grotthuss mechanism might be involved in proton translocation, since the process is efficient to transfer proton over long distances[9,10].

Net charge on the rotor to that of a dipole is reduced by an ion from the stator and the motor moves through the hydrophobic part, while the arginine attracts the next empty rotor site[4]. Difference in pKa of side chains of amino acids affects protonation and deprotonation. Briefly, amines are easily protonated if protons are available. Carboxylic acid is pretty good source of protons and because protons bind to amines pretty well, it seems that the proton transfers from one site to the other site (the acidic or basic sites, i.e. side chains of amino acids in proteins). Aspartate (D) (present in outer helix G*XX*D*XX*F) contains carboxylate side chain, as the pH increases, the carboxylic acid is deprotonated. As the pH rises above isoelectric point, a proton is removed from the ammonium group to generate the anionic form of the amino acid. Thus, the overall charge of the amino acid changes as a function of pH. This shows that the overall interacting residues of *c*-subunit structure may be responsible for

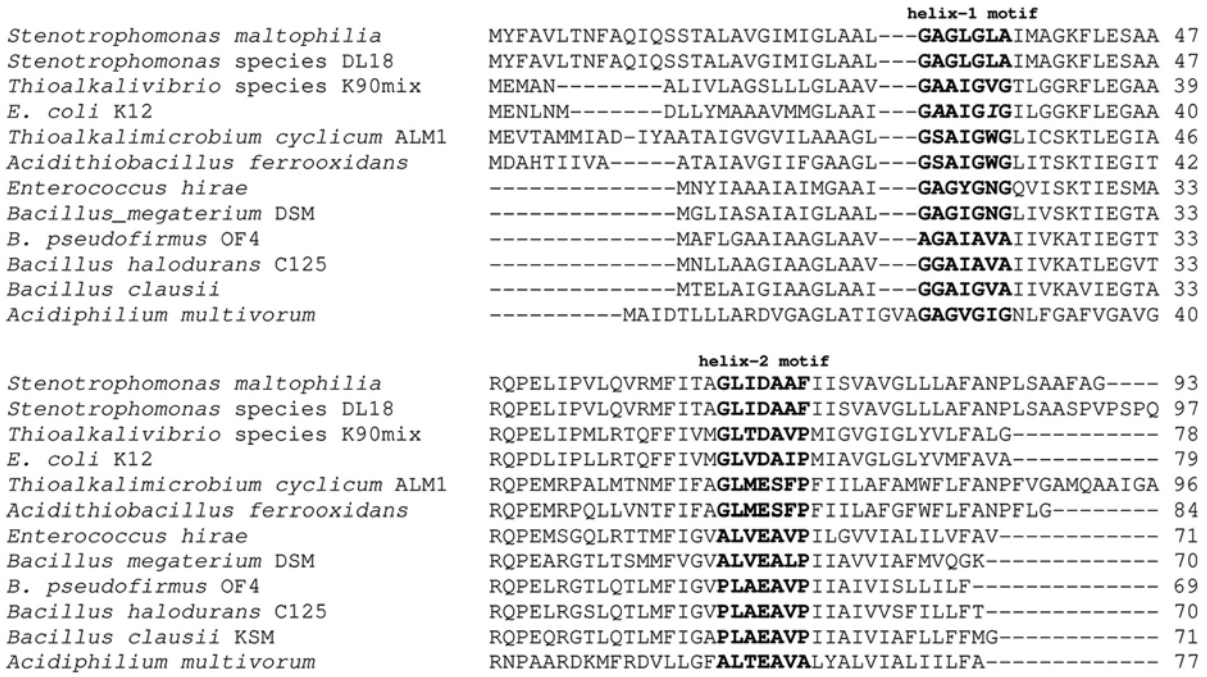

Figure 4. Alignment of F_1F_o-ATP synthase *c*-subunit of acidophiles, neutrophiles and alkaliphiles. The helix-1 and helix-2 motifs are shown in bold.

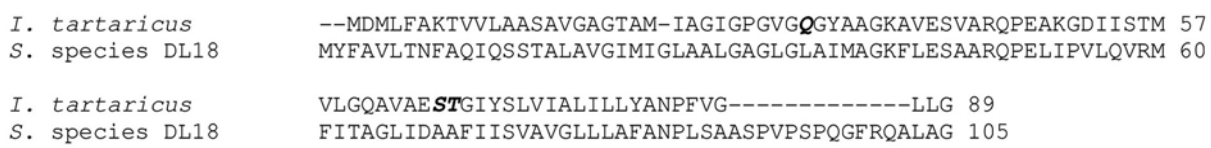

Figure 5. The alignment of *c*-subunit of *Ilyobacter tartaricus* and *Stenotrophomonas* species DL18. The residues of *I. tartaricus* shown in bold and italics are those involved in Na^+ ion translocation.

ATP synthesis under particular pH conditions. Under the same scenario *Stenotrophomonas* species DL18, which grows in high alkaline pH, has G*X*G*X*G*X*A motif in helix-1 and G*XX*D*XX*F in helix-2, which differ from known alkaliphile specific motifs of *B. pseudofirmus* OF4.

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