Isolation and identification of alkaliphile *Stenotrophomonas* species DL18 from Indian Soda Lake Lonar and analysis of $F_1F_o$ ATP synthase $\alpha$-subunit [v1; ref status: not approved 2, http://f1000r.es/11RhJKU]

Devendra Lingojwar$^{1,2}$, Ravikant Jadhav$^2$ and Kachru Gawai$^1$

$^1$ Department of Chemistry, University of Pune, Ganeshkhind, Pune 411007, India
$^2$ ATG LAB, Ganesh Nagar, Pimple Nilakh, Pune 411027, India

Correspondence to Kachru Gawai: krgawai@chem.unipune.ac.in

Abstract Lonar Lake, an Indian Soda Lake, is well known for its biodiversity of extremophiles including alkaliphiles. Most of the molecular studies on Lonar Lake alkaliphiles are based on molecular identification by 16S ribosomal RNA along with numerous applications in the biotechnology industry. However, molecular basis of adaptation of these alkaliphiles to high alkaline conditions is incompletely understood. Attempts were made to isolate and identify alkaliphiles from their naturally occurring original habitat, i.e. Lonar Lake, India with high alkaline conditions of pH 10.5. One of the facultative alkaliphile, *Stenotrophomonas* species DL18, was studied for $F_1F_o$ ATP synthase $\alpha$-subunit with reference to alkaliphile-specific domains. Although the $\alpha$-subunit of *Stenotrophomonas* DL18 showed significant similarity with neutrophiles, the isolated bacteria is an alkaliphile and optimally grows at pH 10.5.

Introduction

ATP is the molecular currency for a living cell, which is not only merely growing and dividing but also continuously responding to external environmental stimuli. To sustain life in extreme conditions, microorganisms devise specific mechanisms for their adaptation. Along with other transporter proteins, ATP synthase is widely considered as one of the key molecules for adaptation at alkaline conditions.

Hydrolysis of nucleoside tri-phosphates, specifically ATP, provides the chemical energy to drive a wide variety of cellular reactions. ATP synthases are central to ATP production during oxidative phosphorylation. These are energy-coupling factors and hence called $F_1F_o$-ATP synthases. The $F_1$ integral membrane protein complex (the subscript ‘o’ denotes its inhibition by the drug oligomycin) provides a transmembrane pore for protons, whereas the peripheral protein $F_o$ (the subscript ‘1’ indicates that it was the first of several factors isolated from mitochondria) is involved in catalysis$^1$. $F_o$ consists of five subunits $\alpha\beta\gamma\delta\epsilon$, with a ring of $\alpha$- and $\beta$-subunits alternating around a single $\gamma$-subunit$^2$. $F_o$ is a membrane embedded domain with subunits $\alpha\beta$, $\gamma\delta\epsilon$. Out of this subunits, $\alpha$-subunit is a stator and $c$-ring is a rotor ring through which ions (H$^+$ or Na$^+$) are translocated$^{3,4}$. Each $c$-chain from the ring consists of two $\alpha$-helices traversing the membrane, and the polar loop extends out of the membrane to interact with the $\gamma$-and $\epsilon$-subunits. A cytoplasmic $F_i$ catalytic domain is connected with a membrane-embedded $F_o$ domain by a central ($\gamma\delta\epsilon$) and peripheral ($\beta\delta$) stalk$^{2,4}$.

Downhill ion translocation across the membrane through $F_o$ causes rotation of the $c$-ring, which induces conformational changes in the catalytic $\beta$-subunit and results in ATP synthesis. The $c_{11}$ ring from *Ilyobacter tartaricus* has Na$^+$ ion binding specificity, while the
c₁ ring from *Bacillus pseudofirmus* OF4 and the c₆ ring from *Spirulina platensis* has H⁺ ion binding specificity. Ion coordination geometry and distances determine the ion specificity. Translocated ions bind to conserved carboxylate of aspartate or glutamate (D/E) in the outer α-helices of c-rings. However, ions are further coordinated by a network of residues. The inner and outer α-helices are in a staggered position. Out of these, the inner helices are hydrophobic in nature and in contact with the phospholipids, while outer helices are hydrophilic with a-subunit interaction for ion translocation. The transmembrane electric potential controls the kinetics of rotary motion, which seems to be independent of the ionic gradient. However, alkaliphiles grow at high environmental pH, which poses the thermodynamic problem of synthesizing ATP with ATP synthase. For this, there are some crucial amino acid residue adaptations in the a- and c-subunit solving the problem of proton capture from an alkaline environment and subsequent translocation to the binding sites on the c-ring.

Various studies based on the mechanism of proton binding, through hydronium ion proton retention and transportation for ATP synthesis in the bacterial system including alkaliphiles suggest the presence of alkaliphilic specific conserved amino acid motifs in transmembrane helix-4 (TMH-4) and TMH-5 of the a-subunit and the inner and the outer helix of the c-subunit of the ATP synthase F₆ subunit as well as Na⁺/H⁺ antiporter and other cation binding proton transporters including multiple drug transporters. The presence of the above mentioned alkaliphilic specific sequences of ATP synthase F₆ subunit along with Na⁺/H⁺ antiporters and other multiple drug transporters are the major strategies for pH homeostasis of extremely alkaliphilic species.

Most studies have focused on the proton translocation channel in the a-subunit of ATP synthase. The arginine residue of the a-subunit, which transfers proton to the c-subunit, is conserved in almost all bacterial species. Recent developments in molecular studies of facultative alkaliphiles suggest the presence of highly conserved AXAXAXA motif in the amino terminal helix and a PXXE XP motif in the carboxy terminal helix of the ATP synthase c-subunit in *Bacillus pseudofirmus* OF4, an established facultative alkaliphilic species. However, similar experimental evidence from other geographic locations such as highly alkaline soda lakes need further exploration to understand pH homeostasis in facultative alkaliphiles. This study explores the comparison of the ATP synthase a-subunit of facultative alkaliphilic aerobes isolated from Lonar Lake with established and reported alkaliphiles. The present study deals with isolation, identification and analysis of alkaliphile specific amino acid motifs in the a-subunit of ATP synthase.

**Materials and methods**

**Isolation and culture of bacteria**

Underwater sediment soil samples were collected from 350 meters away from Kamalaja Devi Temple end, Lonar Lake, Buldhana, Maharashtra, India. The initial screening was performed at pH 9.5. After mix culture was obtained by the spread plate method, pure culture of each type of colony was maintained for further studies. Then isolates were further studied in the range of pH 7 to pH 12.

**DNA extraction and Polymerase Chain Reaction (PCR)**

Bacterial genomic DNA was isolated by the DNAzol method. DNA quantization and quality control for protein contamination was carried out by spectrophotometric absorbance at A₂₆₀ and A₂₈₀. The small subunit ribosomal RNA (16S rRNA) PCR for identification of bacterium was performed with forward primer (16S20F: 5’TATGGTGATCATGGCTCA3’) and reverse primer (16S1540R: 5’AAGGAGGT-GATCCAACCGCA 3‘). Briefly, master mix was prepared for 16S rRNA PCR: 10x PCR Rnx Buffer without MgCl₂ (Invitrogen, P/N Y02028B Lot no. WK1B1b, USA), 1mM MgCl₂ (Invitrogen, P/N Y02016B Lot no. WK2B1a, USA), 200 µM dNTP mix (Merck, India), 100 picomoles of each reverse and forward primers (Integrated DNA technologies, USA), 2.5U of Taq Polymerase enzyme (Invitrogen, USA, 11615-010 Lot no. VKRB1E) and nuclease free water (Merck, India) was added to make up a final volume of 100 µl. Following thermal cycling conditions were used for PCR: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 2 min. Thirty cycles of PCR were followed by final extension at 72°C for 5 min following by cooling at 4°C.

ATP synthase F₆ amplification primers were designed based on *S. maltophilia* K279a as follows: forward primer Steno a-p1F: 5’TCTGGCCGCAGTCTGCTCCG 3’ and reverse primer Steno a-p1R: 5’TACGGAGGCTGGAGGCTGACT 3’. Briefly, PCR reaction mixture of 100 µl was prepared as 10x PCR Rnx Buffer (Invitrogen), 50mM MgCl₂ (Invitrogen) 1.8 µl, 10 mM dNTP mix 3.0 µl (Merck), 100 picomoles of each forward and reverse primers (Integrated DNA technologies, USA), 5U Taq DNA polymerase (Invitrogen) with pfu (Chromous biotech, India) and bacterial genomic DNA templates 200 ng, with remaining nuclease free water (Merck, India). The thermal cycling conditions were: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, primer annealing at 55°C for 30s and primer extension at 72°C for 3 min. Final extension was carried out at 72°C for 5 min and stored at 4°C. Results of 16S rRNA and ATP synthase F₆ amplicons were visualized on 1% agarose gel with 200 ng/ml ethidium bromide (sd fine, India) and results were observed and analyzed with the help of Bio-Rad gel documentation system XR with Bio-Rad Quantity-One 4.6.5 software.
DNA sequencing and analysis
The 16S rRNA PCR product sequencing was carried out by modified Sanger's dideoxy chain termination cycle sequencing method²¹. Electropherogram was read by an automated DNA sequencer (Applied Biosystems ABI3500 XL Genetic Analyzer, Big Dye Terminator version 3.1 Cycle sequencing kit) for 1.5 kb amplicon of the isolate. The resulting final DNA sequence of isolate was subjected to BLAST analysis on the NCBI web server. The phylogenetic tree of 16S rRNA of the isolate was constructed by using a neighbor-joining (NJ) method with 1000 replicates of bootstrap in MEGA4.1 software. The bootstrap consensus tree inferred from 1000 replicates was selected carried out with the help of MEGA4.1. The blastx was used to represent the evolutionary history of the taxa for 16S rRNA sequence analysis.

Further, the DNA sequencing of the ATP Fₐ subunit of selected isolates was performed by primer walking method. The NCBI Blast server was used for the identification of the ATP synthase a-subunit and the phylogenetic analysis of the a-subunit was carried out with the help of MEGA4.1. The blastx was used to get the amino acid sequence of the a-subunit. The a-subunit of isolate was compared with that of established alkaliphiles, acidophiles and neutrophiles with the help of ClustalW.

Results and discussion
Bacterial identification
Across the pH gradient, growth of morphologically different bacterial colonies was observed. Out of these, some bacterial colonies were found with pink and orange pigmentation along with no pigmentation i.e. white colonies. The orange pigmented bacterium was identified as Stenotrophomonas species based on BLAST analysis of 16S rRNA gene sequence and titled as Stenotrophomonas species DL18 (GenBank Accession number: JN995612). Stenotrophomonas species DL18 optimally grow at the pH 9.0 to 10.0. However, the pH range of growth was pH 7.0 to 12.0 (Supplementary figure 1). The Stenotrophomonas species DL18 is known to be an aerobic, facultative alkaliphile curved rod (Supplementary figure 2).

ATP synthase a-subunit of Stenotrophomonas species DL18
BLAST analysis of the ATP synthase a-subunit of the Stenotrophomonas species DL 18 suggests maximum identity at the amino acid level (259 identical amino acids from a total of 266 amino acids of Stenotrophomonas species SKA14; GenBank Accession number: ZP_05136035). The amino acid residue arginine, which was found to be conserved in almost all bacterial species in the a-subunit, was observed at position 200 (Arg²⁰⁰) (supplementary figure 3). Moreover, other amino acids that were conserved in most of the bacteria include Leu²⁰⁷, Arg²⁰⁸, Leu²¹¹, Gly²¹⁷, Asn²¹⁴, Gly²¹⁸, Gln²³², Ala²⁵³, Phe²⁵⁵ (E. coli numbering system for ATP synthase a-subunit) in TMH-4 and TMH-5 and the corresponding amino acids were also found in the Stenotrophomonas species DL 18 in alignment (Figures 1, 2).

Comparison of a-subunit of Stenotrophomonas species DL18 with acidophiles, alkaliphiles and neutrophiles
It was observed that the most conserved arginine residue of the a-subunit (Arg²⁰⁰ of Stenotrophomonas species DL18) was aligned with the expected position of the facultative alkaliphile Bacillus pseudofirmus OF4 (i.e. Arg²⁰⁷; GenBank Accession number: YP_003426326). This positively charged Arg²⁰⁰ plays an elemental role in the function of the Fₐ rotor. Amino acid Lys¹⁸⁰ of Bacillus pseudofirmus OF4 was replaced by Gly²⁰⁸ in the Stenotrophomonas species DL 18. In addition, a glycine residue was observed at the same position in other alkaliphiles, T. cyclicum (Gly²⁰⁷; GenBank Accession number: YP_004537849), and same amino acid family Ala²⁰⁸ in Theoalkalivibrio species K90mix (GenBank Accession number: YP_003461818) as shown in Figure 2. On the other hand, glycine was also at the same corresponding position in alignment for the acidophiles, A. ferrooxidans (GenBank Accession number: YP_0022221206), and A. cryptum (GenBank Accession number: YP_001233541) (Figure 3). Lys¹⁸⁰ and corresponding amino acids were located in transmembrane helix-4 (αTMH-4) in Bacillus pseudofirmus OF4 and other alkaliphiles²²,²³. In the Stenotrophomonas species DL 18, a histidine residue, which is conserved in other reference species of the same genus (S. maltophilia K279a GenBank Accession number: YP_001973793; and S. sp. SKA14) and other alkaliphiles T. cyclicum (His²⁴⁴), and Theoalkalivibrio species K90mix (His²⁶²), was present at position 240 (His²⁴⁰) (Figure 2). However, E. coli K12 DH10B (GenBank Accession number: YP_001732559), considered as neutrophile, can adapt to slightly alkaline conditions i.e. up to pH 8.0 and this may be due to the presence of His²⁴⁰. It was proposed that Gly¹²⁰ and Lys¹⁸⁰ form a channel residing within the proton uptake pathway of the a-subunit through which protons pass onto the neighboring c-subunit in Bacillus pseudofirmus OF4²⁴.

However, Ala¹⁹⁸ from the Stenotrophomonas species DL 18 and Ala¹⁹⁶ from T. cyclicum were found to correspond with Gly²⁰⁸ in E. coli and Gly²¹⁰ in Bacillus pseudofirmus OF4 in alignment of the a-subunit as shown in Figure 2. In a similar way, Gly²⁰⁸ was observed in Stenotrophomonas species DL 18, while in this respective position alanine was found in alkaliphiles and neutrophiles. But both amino acids correspond from the same amino acid family. However, one of the studies reported the His²⁴⁵ along with the Glu²¹⁹ positioning plays a critical role in ion translocation in E. coli²⁵, while in Stenotrophomonas species DL 18 these were at His²⁴⁰ and Glu²¹⁹ as shown in Figure 2.

McMillan et al.²⁶ showed the importance of the residue with a basic side chain along with its pKa value in ATP synthesis linked with alkaline environment. However, these studies were
Supplementary material figure 1  Growth of *Stenotrophomonas* species DL18 at neutral to alkaline conditions i.e. pH 7.0 to 12.0.

Supplementary material figure 2  Scanning Electron Microscope image of *Stenotrophomonas* species DL18. Left panel 50,000 times magnification, right panel 100,000 times magnification.
carried out with a mutation study in the *Bacillus pseudofirmus* OF4 $\alpha$-subunit, specifically with the position of the residue at 180. These studies included mutations at Lys$^{180}$ position as Gly$^{180}$, His$^{180}$ and Arg$^{180}$. These mutations showed the ATP synthesis at neutral, neutral to alkaline, and only at alkaline conditions (>pH 8.5), respectively i.e. residues with a strong base, such as lysine or arginine, was ideally appropriate to function at alkaline pH. Since, it was particularly marked for histidine, which has a pKa in a neutral range, it showed significant ATP synthesis activity at pH 9.0. However, exchange mutations at Gly$^{120}$ and Lys$^{180}$ to Lys$^{120}$ and Gly$^{180}$ showed ATP synthase activity in *Bacillus pseudofirmus* OF4. Similar studies in *E. coli* showed that the positions Gly$^{212}$ and His$^{240}$ along with Glu$^{219}$ had a critical interaction with Fo function$.^{26,27}$

After comparison with acidophiles, neutrophiles and alkaliophiles, Leu$^{197}$ of the *Stenotrophomonas* species DL 18 was found to be conserved in TMH-4 (Figure 3). From alignment, Glu$^{209}$ of the *Stenotrophomonas* species DL 18 was conserved in alkaliophiles, neutrophiles and some acidophiles except His$^{186}$ *Acidiphilium cryptum*. In addition, the position of Gly$^{212}$ in *Bacillus pseudofirmus* OF4 corresponds to Ser$^{212}$ in *E. hirae* (neutrophile; GenBank Accession number: YP_006487510), His$^{245}$ in *E. coli* and His$^{240}$ in the *Stenotrophomonas* species DL 18 while glutamate in acidophiles, Glu$^{222}$ in *A. ferrooxidans* and Glu$^{225}$ in *A. cryptum* as shown in Figure 3. This showed that the channel formation may involve a glycine residue along with other residues, specifically acidic, basic and neutral side chain, which play vital roles in ATP synthesis in acidophile, alkaliophile and neutrophiles. Hence, these residues are found to be critical in channel formation. In the same scenario, the Gly$^{208}$ and His$^{240}$ may be form the proton translocation channel in *Stenotrophomonas* species DL 18. Thus the basic side chain residue His$^{240}$ and other amino acid residues may be responsible for growth of the *Stenotrophomonas* species DL 18 at high alkaline pH.

**Supplementary material** figure 3 *Stenotrophomonas* species DL18 ATP synthase $\alpha$-subunit derived by homology modeling: The amino acid sequence of $\alpha$-subunits was subjected to NCBI blastp and related known structure was obtained. Further, the homology modeling was carried out by using SWISS MODEL using respective reference PDB structure and the modeled structure was visualized by Pymol.
Figure 2 Alignment of α-subunit of ATP synthase from neutrophiles and alkaliphiles. Conserved sequences are underlined and significant variant residues for channel formation are shown in bold. These residues are mainly from TMH-4 and TMH-5. Bacillus megaterium DSM 319 (GenBank Accession number: YP_003600289) grows strictly in neutral pH environments. However, Bacillus clausii DSM K-16 (GenBank Accession number: YP_177349) is a reported alkaliphile.

Figure 3 Alignment of α-subunit of ATP synthase from acidophiles, neutrophiles and alkaliphiles. Conserved sequences from TMH-4 and TMH-5 are underlined and significant variant residues for channel formation are shown in bold.

Author contributions
Devendra Lingojwar contributed to the conception and design of the project, acquired, analyzed and interpreted the data, review of literature, and prepared the manuscript. Ravikant Jadhav helped prepare the manuscript, contributed to data collection and conducted bioinformatic analysis. Kachru Gawai provided overall guidance for the project, helped prepare the manuscript and revised it critically for intellectual content. All authors approved the final version of the article.

Competing interests
No competing interests were disclosed.

Grant information
Specific facilities related to molecular biology were kindly provided by ATG LAB. No specific grant was available for this project. The entire project was carried out with the support from the ATG LAB as an intramural project.
Acknowledgements
Authors are thankful to the Department of Chemistry, University of Pune, India for providing the place of research and infrastructure and ATG LAB for providing laboratory facilities whenever needed. The authors are also thankful to Mrs. Sarita Lingojwar, Admin Head and Laboratory Manager, ATG LAB, for providing kind help and support for laboratory facilities during this project.

References

Open Peer Review

Current Peer Review Status: ❌ ❌

Version 1

Reviewer Report 18 January 2013
https://doi.org/10.5256/f1000research.231.r712

© 2013 Cook G. This is an open access peer review report distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Gregory Cook
Department of Microbiology, University of Otago, North Dunedin, New Zealand

I don't see this study has much relevance to the field as it adds nothing to the literature in this area and is not great science e.g. the homology model is not based on a structure but just a model. The authors should have also included a c subunit analysis to check for the presence/absence of Na+-binding motifs. Overall the work is not of a standard in which others in the field can benefit.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 09 January 2013
https://doi.org/10.5256/f1000research.231.r654

© 2013 Meier T. This is an open access peer review report distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Thomas Meier
Department of Structural Biology, Max Planck Institute of Biophysics, Frankfurt, 60438, Germany

- The overall content of this work is very poor. It contains: growth of bacteria in medium, PCR, sequencing, sequence alignment and a homology model. It is what is done today in a student practicum.
- The homology model (structure of subunit α, Supplementary Figure 3) is a homology model based on a model (this was NO real structure), which basically also means, one could just take 4 random alpha helices and put them together in a bundle.
- The authors should mention in the methods section which genes from F_o were amplified by PCR.
- Supplementary material figure 1: The window ledge and the arm of the photographer is visible on the picture.
- Supplementary material figure 2: The quality of the left EM micrograph is poor, the picture is not in focus. The picture on the right side is distorted, I cannot see anything there.
- Page 5 line 1: The Cook lab did not use Bacillus pseudofirmus OF4 but rather Bacillus TA2 A1
- Figure 2 and Figure 3: The alignment should contain Bacillus TA2 because the authors refer to it in the text on Reference 25 (McMillan et al.).
- Figures 2 and 3 appear to be very similar. It may have made more sense to include all the information in one figure to avoid redundancies and save space.

The scientific value of this work is questionable. It is a good start to investigate the α-subunit from Stenotrophomonas but it is too early to publish as such.

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com