Isolation and identification of alkaliphile *Stenotrophomonas* species DL18 from Indian Soda Lake Lonar and analysis of F<sub>1</sub>F<sub>0</sub>ATP synthase a-subunit [v1; ref status: not approved 2, http://f1000r.es/11RhJKU]

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**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<sub>1</sub>F<sub>0</sub>ATP synthase a-subunit with reference to alkaliphile-specific domains. Although the a-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<sub>1</sub>F<sub>0</sub>-ATP synthases. The F<sub>1</sub> 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<sub>0</sub> (the subscript ‘1’ indicates that it was the first of several factors isolated from mitochondria) is involved in catalysis<sup>1</sup>. F<sub>0</sub> consists of five subunits α<sub>β</sub>γδε, with a ring of α- and β-subunits alternating around a single γ-subunit<sup>2</sup>. F<sub>0</sub> is a membrane embedded domain with subunits ab<sub>2</sub>c<sub>10-15</sub>. Out of this subunits, a-subunit is a stator and c-ring is a rotor ring through which ions (H<sup>+</sup> or Na<sup>+</sup>) are translocated<sup>3-6</sup>. Each c-chain from the ring consists of two α-helices traversing the membrane, and the polar loop extends out of the membrane to interact with the γ-and ε-subunits. A cytoplasmic F<sub>1</sub> catalytic domain is connected with a membrane-embedded F<sub>0</sub> domain by a central (γε) and peripheral (b<sub>2</sub>δ) stalk<sup>2-6</sup>.

Downhill ion translocation across the membrane through F<sub>0</sub> causes rotation of the c-ring, which induces conformational changes in the catalytic β-subunit and results in ATP synthesis. The c<sub>11</sub> ring from *Ilyobacter tartaricus* has Na<sup>+</sup> ion binding specificity, while the
Materials and methods

Isolation and culture of bacteria

Isolation and culture of bacteria 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 method. 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 to represent the evolutionary history of the taxa for 16S rRNA.

Further, the DNA sequencing of the ATP F\textsubscript{1} 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 (Arg\textsuperscript{200}) (supplementary figure 3). Moreover, other amino acids that were conserved in most of the bacteria include Leu\textsuperscript{207}, Arg\textsuperscript{208}, Leu\textsuperscript{201}, Gly\textsuperscript{217}, Asn\textsuperscript{214}, Gly\textsuperscript{216}, Gln\textsuperscript{252}, Ala\textsuperscript{253}, Phe\textsuperscript{255} (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 (Arg\textsuperscript{200} of *Stenotrophomonas* species DL18) was aligned with the expected position of the facultative alkaliphile *Bacillus pseudofirmus* OF4 (i.e. Arg\textsuperscript{207}; GenBank Accession number: YP_003426326). This positively charged Arg\textsuperscript{200} plays an elemental role in the function of the F\textsubscript{f} rotor\textsuperscript{2}. Amino acid Lys\textsuperscript{180} of *Bacillus pseudofirmus* OF4 was replaced by Gly\textsuperscript{208} in the *Stenotrophomonas* species DL 18. In addition, a glycine residue was observed at the same position in other alkaliphiles, *T. cyclicum* (Gly\textsuperscript{206}; GenBank Accession number: YP_004537849), and same amino acid family Ala\textsuperscript{290} 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_002221206), and *A. cryptum* (GenBank Accession number: YP_001233541) (Figure 3). Lys\textsuperscript{180} and corresponding amino acids were located in transmembrane helix-4 (aTMH-4) in *Bacillus pseudofirmus* OF4 and other alkaliphiles\textsuperscript{23,24}. 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* (His\textsuperscript{245}), and *Theoalkalivibrio* species K90mix (His\textsuperscript{262}), was present at position 240 (His\textsuperscript{240}) (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 His\textsuperscript{245}. It was proposed that Gly\textsuperscript{208} and Lys\textsuperscript{180} 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* OF4\textsuperscript{18}.

However, Ala\textsuperscript{198} from the *Stenotrophomonas* species DL 18 and Ala\textsuperscript{196} from *T. cyclicum* were found to correspond with Gly\textsuperscript{208} in *E. coli* and Gly\textsuperscript{170} in *Bacillus pseudofirmus* OF4 in alignment of the a-subunit as shown in Figure 2. In a similar way, Gly\textsuperscript{208} 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 His\textsuperscript{245} along with the Glu\textsuperscript{219} positioning plays a critical role in ion translocation in *E. coli*\textsuperscript{25}, while in *Stenotrophomonas* species DL 18 these were at His\textsuperscript{240} and Glu\textsuperscript{209} as shown in Figure 2.

McMillan et al\textsuperscript{45} 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 pseudoformus* OF4 α-subunit, specifically with the position of the residue at 180. These studies included mutations at Lys<sup>180</sup> position as Gly<sup>180</sup>, His<sup>180</sup> and Arg<sup>180</sup>. 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<sup>120</sup> and Lys<sup>180</sup> to Lys<sup>120</sup> and Gly<sup>180</sup> showed ATP synthase activity in *Bacillus pseudoformus* OF4. Similar studies in *E. coli* showed that the positions Gly<sup>212</sup> and His<sup>240</sup> along with Glu<sup>219</sup> had a critical interaction with Fo function<sup>20,27</sup>.

After comparison with acidophiles, neutrophiles and alkaliphiles, Leu<sup>197</sup> of the *Stenotrophomonas* species DL 18 was found to be conserved in TMH-4 (Figure 3). From alignment, Glu<sup>209</sup> of the *Stenotrophomonas* species DL 18 was conserved in alkaliphiles, neutrophiles and some acidophiles except His<sup>186</sup> *Acidithiobacter cryptum*. In addition, the position of Gly<sup>212</sup> in *Bacillus pseudoformus* OF4 corresponds to Ser<sup>212</sup> in *E. hirae* (neutrophile; GenBank Accession number: YP_006487510), His<sup>245</sup> in *E. coli* and His<sup>240</sup> in the *Stenotrophomonas* species DL 18 while glutamate in acidophiles, Glu<sup>222</sup> in *A. ferrooxidans* and Glu<sup>225</sup> 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, alkaliphiles and neutrophiles. Hence, these residues are found to be critical in channel formation. In the same scenario, the Gly<sup>208</sup> and His<sup>240</sup> may be form the proton translocation channel in *Stenotrophomonas* species DL 18. Thus the basic side chain residue His<sup>240</sup> 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 DL.18 ATP synthase α-subunit derived by homology modeling: The amino acid sequence of α-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.

B. pseudofirmus OF4 153 FLLFFKIIIEEFANTLTLGMRFLFGNYAEEKLILLVLTAGTSGLIGM 198
B. clausii KSM-K16 153 FLFFFKIIEEFANTLTLGMRFLFGNYAEEKLILLVLTAGTSGLIGM 198
B. megaterium DSM 319 150 FLFFFKIIIEEFANTLTLGMRFLFGNYAEEILILLVLTAGTSGLIGM 195
E. hirae ATCC5790 152 FMFMKLIIEEFTNLTLALLRLFGNYAEEVLVILIAANLNNLGVWS 197
Stenotrophomonas sp. DL18 181 FNNLLINIVELSKPSIGRRMLFLFGNYAEEIFVILVWLGAGGFFGA 226
Stenotrophomonas malophilia 181 FNNLLINIVELSKPSIGRRMLFLFGNYAEEIFVILVWLGAGGFFGA 226
E. coli K12 191 VNLLEGVSSLKPSGIRFLFGNYAGEFLILLFLGELFLFILFLAGLFL 231
Thioalkalivibrio sp. K90mix 203 INFALIENLAKPAALFRLFLGNYLAAEFLIGLNVYSGAILIG 248

B. pseudofirmus OF4 199 FGAFLPL-IVWCAFGGLFGLGAIQYAYFAMLAMVYMAHVE- 238
B. clausii KSM-K16 199 IFAPFVL-IVWCAFGGLFGLGAIQYAYFAMLAMVYMAHVE- 238
B. megaterium DSM 319 196 IGAIAFM-LLWQGSFSGVAIQAFITMFLVYLSHKVSSDH 236
E. hirae ATCC5790 198 LPLAIFMVLNFASFLDSIQAQVAVTLSVMYLSHKVIESE 239
Stenotrophomonas sp. DL18 227 INGAAGFLGMLMLHHLLVIFLQAFIMHLISYVYSLSED- 266
Stenotrophomonas malophilia 227 INGAGVF-LGMLMLHHLLVIFLQAFIMHLISYVYSLSED- 266
E. coli K12 232 WSCWILN-VPWAIHFLILTIQAFIMHLISYVYSLSED- 271
Thioalkalivibrio sp. K90mix 249 LAGGVLH-VGWAIFHILVIFLQAFIMHLISYVYSLSED- 289

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.

B. pseudofirmus OF4 157 FKIIIEEFANTLTLGMRFLFGNYAEEKLILLVLTAGTS-- 193
B. clausii KSM-K16 157 FKIIIEEFANTLTLGMRFLFGNYAEEKLILLVLTAGTS-- 193
B. megaterium DSM 319 154 LKIIIIEDEFANTLTLGMRFLFGNYAEEKLILLVLTAGTS- 190
E. hirae ATCC5790 156 MLKIEFNLMTALRLYNYAGEVTLIINANMN- 192
E. coli K12 195 LEGRSLSLKLFLGMLFLFGNYAGELILFIL------- 225
Thioalkalivibrio sp. K90mix 207 LEFILNLAKPAALRFLGNYLAAEFLILGNYLS- 242
Stenotrophomonas sp. DL18 185 INLEWVLSDKPAALFRLFLGNYLAAEFLILGNYLS- 220
Stenotrophomonas malophilia 185 INLEWVLSDKPAALFRLFLGNYLAAEFLILGNYLS- 220
A. ferrooxidans ATCC53993 173 MLKIEEIEFLPLALRFLGNYLAAEFLILGNYLS- 207
T. cyclicum ALM1 183 MTRIEELAKPAALRFLGNYLAAEFLILGNYLS- 221
Acidiphilium cryptum 162 IEELVSSLKPAALFRLFLGNYLAAEFLILGNYLS- 200

B. pseudofirmus OF4 194 GLIQMGFGALPL-IVWCAFGGLFGLGAIQYAYFAMLAMVYMAHVE 237
B. clausii KSM-K16 194 AVNGDFFVFVFL-IVWCAFGGLFGLGAIQYAYFAMLAMVYMAHVE 237
B. megaterium DSM 319 191 GLFSTGGAAILM-LWQGSFSGVAIQAFITMFLVYLSHKVSSDH 234
E. hirae ATCC5790 193 LOWSVSLLPAFLNMLGMLFLSFGLIAQVFEITLWGMYLMKIEE 237
E. coli K12 226 AGLLLLPNQVINLYWPAHFIMALLQLAFIMHLISYVYSLSSE- 269
Thioalkalivibrio sp. K90mix 243 AGLILGGLAGLGVHVAIFHILVIFLQAFIMHLISYVLDSYSE- 287
Stenotrophomonas sp. DL18 221 AGFFGAVAGAAGFLGMLMLFLHLLVIFLQAFIMHLISYVLDSYSE- 264
Stenotrophomonas malophilia 221 AGFFGAVAGAAGFLGMLMLFLHLLVIFLQAFIMHLISYVLDSYSE- 264
A. ferrooxidans ATCC53993 208 --WG--GLVNGMVWSLLFILITQITFTLVTLYGLMNQ 247
T. cyclicum ALM1 225 AAALVAFPLAIDLGLWAIFHILITQIFMHLISYVLDSNAD 269
Acidiphilium cryptum 206 VGVVAAALPGLLADLFALEFVAFQAYVFAIFLTCLHLHDA1HM 250

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 prepared 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.

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References

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Version 1

Referee Report 18 January 2013
doi:10.5256/f1000research.231.r712

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.

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.

Competing Interests: No competing interests were disclosed.

Referee Report 09 January 2013
doi:10.5256/f1000research.231.r654

Thomas Meier
Department of Structural Biology, Max-Planck Institute of Biophysics, Frankfurt, 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.

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.

*Competing Interests:* No competing interests were disclosed.