Isolation and identification of alkaliphile *Stenotrophomonas* species DL18 from Indian Soda Lake Lonar and analysis of F₁F_oATP synthase α -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 F₁F_oATP synthase α -subunit with reference to alkaliphile-specific domains. Although the α -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 F₁F_o-ATP synthases. The F_o integral membrane protein complex (the subscript 'o' denotes its inhibition by the drug oligomycin) provides a transmembrane pore for protons, whereas the peripheral protein F₁ (the subscript '1'

indicates that it was the first of several factors isolated from mitochondria) is involved in catalysis¹. F₁ consists of five subunits $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$, with a ring of α - and β -subunits alternating around a single γ -subunit². F_o is a membrane embedded domain with subunits $a_b_2c_{10-15}$. Out of this subunits, α -subunit is a stator and c-ring is a rotor ring through which ions (H⁺ or Na⁺) are translocated³⁻⁶. 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 F₁ catalytic domain is connected with a membrane-embedded F_o domain by a central ($\gamma\epsilon$) and peripheral ($b_2\delta$) stalk²⁻⁶.

Downhill ion translocation across the membrane through F_o causes rotation of the c-ring, which induces conformational changes in the catalytic β -subunit and results in ATP synthesis. The c₁₁ ring from *Ilyobacter tartaricus* has Na⁺ ion binding specificity, while the

c₁₃ ring from *Bacillus pseudofirmus* OF4 and the c₁₅ ring from *Spirulina platensis* has H⁺ ion binding specificity⁶. 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^{5,6}. 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^{2,6}. The transmembrane electric potential controls the kinetics of rotary motion, which seems to be independent of the ionic gradient⁵. 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-ring⁶.

Various studies based on the mechanism of proton binding^{7,8}, through hydronium ion proton retention and transportation for ATP synthesis in the bacterial system including alkaliphiles^{9,10} suggest the presence of alkaliphile 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^{3,11-14} of the ATP synthase F_o subunit as well as Na⁺/H⁺ antiporter¹⁵ and other cation binding proton transporters including multiple drug transporters^{6,16}. The presence of the above mentioned alkaliphile specific sequences of ATP synthase F_o 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 species³. Recent developments in molecular studies of facultative alkaliphiles suggests the presence of highly conserved AXAXAX motif in the amino terminal helix and a PXXEXX motif in the carboxy terminal helix of the ATP synthase *c*-subunit in *Bacillus pseudofirmus* OF4, an established facultative alkaliphile^{6,17,18}. 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 method¹⁹. 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'ATGTTGATCATG-GCTCA3') and reverse primer (16S1540R: 5'AAGGAGGT-GATCCAACCGCA 3')²⁰. Briefly, master mix was prepared for 16S rRNA PCR: 10x PCR Rxn Buffer without MgCl₂ (Invitrogen, P/N Y02028B Lot no. WK1B1b, USA), 1mM MgCl₂ (Invitrogen, P/N Y02016B Lot no. WK2B1a, USA), 200 μ M 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 μ l. 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 followed by cooling at 4°C.

ATP synthase F_o amplification primers were designed based on *S. maltophilia* K279a as follows: forward primer Steno atp1F: 5'CCTGGCGGATCCTTAGATCTCCG 3' and reverse primer Steno atp1R: 5'CAGTGAGGATCCTTAGATCTC-CGAGGCCAGCT 3'. Briefly, PCR reaction mixture of 100 μ l was prepared as 10x PCR Rxn Buffer (Invitrogen), 50mM MgCl₂ (Invitrogen) 1.8 μ l, 10 mM dNTP mix 3.0 μ l (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 F_o 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 software²². The bootstrap consensus tree inferred from 1000 replicates was selected to represent the evolutionary history of the taxa for 16S rRNA sequence analysis.

Further, the DNA sequencing of the ATP F_0 subunit of selected isolates was performed by primer walking method. The NCBI Blast server was used for the identification of the ATP synthase α -subunit and the phylogenetic analysis of the α -subunit was carried out with the help of MEGA4.1. The blastx was used to get the amino acid sequence of the α -subunit. The α -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 alkaliphilic curved rod (Supplementary figure 2).

ATP synthase α -subunit of *Stenotrophomonas* species DL18

BLAST analysis of the ATP synthase α -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 α -subunit, was observed at position 200 (Arg²⁰⁰) (supplementary figure 3). Moreover, other amino acids that were conserved in most of the bacteria include Leu²⁰⁷, Arg²¹⁰, Leu²¹¹, Gly²¹³, Asn²¹⁴, Gly²¹⁸, Gln²⁵², Ala²⁵³, Phe²⁵⁵ (*E. coli* numbering system for ATP synthase α -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 α -subunit of *Stenotrophomonas* species DL18 with acidophiles, alkaliphiles and neutrophiles

It was observed that the most conserved arginine residue of the α -subunit (Arg²⁰⁰ of *Stenotrophomonas* species DL18) was aligned with the expected position of the facultative alkaliphile *Bacillus pseudofirmus* OF4 (i.e. Arg¹⁷²; GenBank Accession number: YP_003426326). This positively charged Arg²⁰⁰ plays an elemental role in the function of the F_0 rotor³. 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* (Gly²⁰⁶; GenBank Accession number: YP_004537849), and same amino acid family Ala²³⁰ 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). Lys¹⁸⁰ and corresponding amino acids were located in transmembrane helix-4 (α TMH-4) in *Bacillus pseudofirmus* OF4 and other alkaliphiles^{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* (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 His²⁴⁵. It was proposed that Gly¹²⁰ and Lys¹⁸⁰ form a channel residing within the proton uptake pathway of the α -subunit through which protons pass onto the neighboring c -subunit in *Bacillus pseudofirmus* OF4¹⁸.

However, Ala¹⁹⁸ from the *Stenotrophomonas* species DL 18 and Ala¹⁹⁶ from *T. cyclicum* were found to correspond with Gly²⁰⁸ in *E. coli* and Gly¹⁷⁰ in *Bacillus pseudofirmus* OF4 in alignment of the α -subunit as shown in Figure 2. In a similar way, Gly²⁰⁷ 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. coli*²⁵, while in *Stenotrophomonas* species DL 18 these were at His²⁴⁰ and Glu²⁰⁹ as shown in Figure 2.

McMillan *et al*²⁵ 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.

Supplementary material figure 3 *Stenotrophomonas* species DL18 ATP synthase a-subunit derived by homology modeling: The amino acid sequence of a-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.

carried out with a mutation study in the *Bacillus pseudofirmus* OF4 a-subunit, specifically with the position of the residue at 180. These studies included mutations at Lys¹⁸⁰ position as Gly¹⁸⁰, His¹⁸⁰ and Arg¹⁸⁰. 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 Gly¹²⁰ and Lys¹⁸⁰ to Lys¹²⁰ and Gly¹⁸⁰ showed ATP synthase activity in *Bacillus pseudofirmus* OF4. Similar studies in *E. coli* showed that the positions Gly²¹⁸ and His²⁴⁵ along with Glu²¹⁹ had a critical interaction with Fo function^{26,27}.

After comparison with acidophiles, neutrophiles and alkaliphiles, Leu¹⁹⁷ of the *Stenotrophomonas* species DL 18 was found to be conserved in TMH-4 (Figure 3). From alignment, Glu²⁰⁹ of the *Stenotrophomonas* species DL 18 was conserved in alkaliphiles, neutrophiles and some acidophiles except His¹⁸⁶ *Acidiphilium cryptum*. In addition, the position of Gly²¹² in *Bacillus pseudofirmus* OF4 corresponds to Ser²¹² in *E. hirae* (neutrophile; GenBank Accession number: [YP_006487510](#)), His²⁴⁵ in *E. coli* and His²⁴⁰ in the *Stenotrophomonas* species DL 18 while glutamate in acidophiles, Glu²²² in *A. ferrooxidans* and Glu²²⁵ 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 Gly²⁰⁸ and His²⁴⁰ may be form the proton translocation channel in *Stenotrophomonas* species DL 18. Thus the basic side chain residue His²⁴⁰ and other amino acid residues may be responsible for growth of the *Stenotrophomonas* species DL 18 at high alkaline pH.

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1  MAGEALTPTSIIQHHLQNLTA PVGNGDGMFWHIHVDTF LTAVLMGLVIVVAFWLGTRKAT  60
61  AGVPGKWQAFVEICLEFVDRQAKDTYHGKSKLVTPIAITIFFWILMMNLIKMI PADFI AK  120
121  PLELAGIHYWKPVPTADV NATLGMAISVFFLMLFFALKSKGLGGFIKEFLTAPFGKWMP  180
181  FNLILNIVEWLSKPISLAMRLFGNMFGGEIVFLLI WVLGGAGFFGAIAGGAFGLGWMLFH  240
241  LLVIPLQAFIFMMLSIVYLSLSEDAH  266

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Figure 1 Complete amino acid sequence of *Stenotrophomonas* species DL18 ATP synthase a-subunit. The most conserved amino acid residues are shown as bold and underlined.

<i>B. pseudofirmus</i> OF4	153	FLLPFKIIIEEFANTLT <u>LG</u> MRLFGNVYA KE ILMILLVLTAGTSGLIGM	198
<i>B. clausii</i> KSM-K16	153	FLFPFKVIEEGSNALT <u>LG</u> MRLFGNIYA KE ILMILLVSLGTTAVYWG	198
<i>B. megaterium</i> DSM 319	150	FLFPLKIIIEEFANTLT <u>LG</u> LRLYGN IA GEILLSSLAGLATGFLGT	195
<i>E. hirae</i> ATCC9790	152	FMFPMKLIIEFTNLLT <u>LAL</u> RLYGN IA GEVLLTLIANMMNNLGWFS	197
<i>Stenotrophomonas</i> sp. DL18	181	FNLILNIVEWLSKPIS <u>LAM</u> RFGNMF GE IVFLLIWVLGGAGFFGA	226
<i>Stenotrophomonas maltophilia</i>	181	FNLILNIVEWLSKPIS <u>LAM</u> RFGNMF GE IVFLLIWVLGGAGFFGA	226
<i>E. coli</i> K12	191	VNLILEGVSLSKPVS <u>LG</u> LRLFGNMYA GE LIFILI-----AGLLPW	231
<i>Thioalkalivibrio</i> sp. K90mix	203	INFALEFINLLAKPAS <u>LAL</u> R <u>LG</u> NLYA AE MIFILIGVMYSAGLILG	248
<i>B. pseudofirmus</i> OF4	199	FGAFLPL-IVWQAF <u>GL</u> FIGAIQAYIFAMLAMVYMAHKVESH-	238
<i>B. clausii</i> KSM-K16	199	IFAFVPL-IVWQAF <u>S</u> IFIGSLQAYIFAMLAMVYMSHKVEQH-	238
<i>B. megaterium</i> DSM 319	196	IGAAIPM-LLWQGF <u>S</u> IFVGAIQAFIFTMLTMVYLSHKVSSDH	236
<i>E. hirae</i> ATCC9790	198	LPLAIPLEMVWIAF <u>SL</u> FIGSIQAFVFTLSMVYMSHKIEVEE	239
<i>Stenotrophomonas</i> sp. DL18	227	IAGGAFG-LGWMLF <u>HLL</u> VIPLQAFIFMMLSIVYLSLS-EDAH	266
<i>Stenotrophomonas maltophilia</i>	227	IAGGVFG-LGWMLF <u>HLL</u> VIPLQAFIFMMLSIVYLSLS-EDAH	266
<i>E. coli</i> K12	232	WSQWILN-VPWAF <u>H</u> ILIIITLQAFIFMVLTIIVYLSMA-SEEH	271
<i>Thioalkalivibrio</i> sp. K90mix	249	LAGGV LH-VGWAF <u>H</u> ILVVPLQAFIFMVLTVVYLDMAYESDH	289

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* KSM K-16 (GenBank Accession number: [YP_177349](#)) is a reported alkaliphile.

<i>B. pseudofirmus</i> OF4	157	FKIIIEEFANTLT <u>LG</u> MRLFGNVYA KE ILMILLVLTAGTS--	193
<i>B. clausii</i> KSM-K16	157	FKVIEEGSNALT <u>LG</u> MRLFGNIYA KE ILMILLVSLGTT--	193
<i>B. megaterium</i> DSM 319	154	LKIIIEEFANTLT <u>LG</u> LRLYGN IA GEILLSSLAGLATT--	190
<i>E. hirae</i> ATCC9790	156	MKLIEEFTNLLT <u>LAL</u> RLYGN IA GEVLLTLIANMMNN--	192
<i>E. coli</i> K12	195	LEGVSLSKPVS <u>LG</u> LRLFGNMYA GE LIFILI-----	225
<i>Thioalkalivibrio</i> sp. K90mix	207	LEFINLLAKPAS <u>LAL</u> R <u>LG</u> NLYA AE MIFILIGVMYS---	242
<i>Stenotrophomonas</i> sp. DL18	185	LNIVEWLSKPIS <u>LAM</u> RFGNMF GE IVFLLIWVLGG---	220
<i>Stenotrophomonas maltophilia</i>	185	LNIVEWLSKPIS <u>LAM</u> RFGNMF GE IVFLLIWVLGG---	220
<i>A. ferrooxidans</i> ATCC53993	173	MSLIEEITKPLS <u>LG</u> LRLFGNMF GE LVFLLAMLFP----	207
<i>T. cyclicum</i> ALM1	183	MTFIEEIAKPIS <u>LAL</u> R <u>LG</u> NIFAGELVFMLIALIGGTMA	221
<i>Acidiphilium cryptum</i>	162	IEIVSFLSRPIS <u>LS</u> VRLFANITAGHVMWEVFAGFMLMLV	200
<i>B. pseudofirmus</i> OF4	194	GLIGMFAGFLPL-IVWQAF <u>GL</u> FIGAIQAYIFAMLAMVYMAHKVES	237
<i>B. clausii</i> KSM-K16	194	AVYWGIFAFVPL-IVWQAF <u>S</u> IFIGSLQAYIFAMLAMVYMSHKVEQ	237
<i>B. megaterium</i> DSM 319	191	GFLGTIGAAIPM-LLWQGF <u>S</u> IFVGAIQAFIFTMLTMVYLSHKVSS	234
<i>E. hirae</i> ATCC9790	193	LGWFSPLAIPLEMVWIAF <u>SL</u> FIGSIQAFVFTLSMVYMSHKIEV	237
<i>E. coli</i> K12	226	AGLLPWSQWILNVPWAF <u>H</u> ILIIITLQAFIFMVLTIIVYLSMA-SE	269
<i>Thioalkalivibrio</i> sp.K90mix	243	AGLILGLAGVHLVGWAF <u>H</u> ILVVPLQAFIFMVLTVVYLDMAYSE	287
<i>Stenotrophomonas</i> sp. DL18	221	AGFFGAIAAGVFLGWMLF <u>HLL</u> VIPLQAFIFMMLSIVYLSLS-ED	264
<i>Stenotrophomonas maltophilia</i>	221	AGFFGAIAAGVFLGWMLF <u>HLL</u> VIPLQAFIFMMLSIVYLSLS-ED	264
<i>A. ferrooxidans</i> ATCC53993	208	---WWG--GLVMGEVWSLF <u>E</u> SLIIILQAFIFTVLTVVYLGMANMQ	247
<i>T. cyclicum</i> ALM1	225	AALFWAPLQAILDLGWAF <u>H</u> ILIIITLQAFIFMVLTIIVYLSMAHDE	269
<i>Acidiphilium cryptum</i>	206	GVVAAIIPGLNIALTAL <u>E</u> FLVAF <u>LQ</u> AYVFAILTCLYLHDAIHM	250

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 bio-informatic 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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Gregory Cook

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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 confirm that I have read this submission and 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

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Thomas Meier

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- 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_0 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 confirm that I have read this submission and 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.

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