Journal of Applied & Environmental Microbiology, 2017, Vol. 5, No. 2, 79-85
Available online at http://pubs.sciepub.com/jaem/5/2/4

DOI:10.12691/jaem-5-2-4

Isolation and Characterization of Moderately Halophilic and Halotolerant Bacteria from the Freshwater Al-Asfar Lake, Al Ahsa, Saudi Arabia

I. Alshubaith[1], D. J. Gilmour[2,*]

[1]P.O. Box 60066 Hofuf, Al Ahsa, Saudi Arabia
[2]Department of Molecular Biology and Biotechnology, Firth Court, Western Bank, University of Sheffield, Sheffield S10 2TN, UK
*Corresponding author: D.J.Gilmour@Sheffield.ac.uk

Abstract A range of water samples from the Al-Asfar lake, Al Ahsa, Saudi Arabia were enriched with LB medium (plates and liquid medium) containing a range of salt concentrations up to 2 M NaCl. Three strains of halotolerant or moderately halophilic bacteria were isolated and identified by 16S rDNA sequencing as belonging to the genera *Staphylococcus, Halobacillus* and *Halomonas.* The first two organisms (*S. warneri* and *Halobacillus* sp.) were further charcaterized to understand their ability to grow at high salinities up to 3 M NaCl. *Halobacillus* sp. was shown to be moderately halophilic (optimum growth between 0.17 and 1 M NaCl), whereas *S. warneri* was shown to be halotolerant, optimum growth at 0.17 M NaCl. Nuclear magnetic resonance (NMR) was used to determine the compatible solutes accumulated by the two strains. Betaine was accumulated by both organisms and *Halobacillus* sp. also utilized glutamate at low salt concentrations. This work demonstrates the presence of moderately halophilic and halotolerant bacteria in a freshwater lake.

Keywords: *moderate halophiles, freshwater, Halobacillus, Staphylococcus*

Cite This Article: I. Alshubaith, and D. J. Gilmour, "Isolation and Characterization of Moderately Halophilic and Halotolerant Bacteria from the Freshwater Al-Asfar Lake, Al Ahsa, Saudi Arabia." *Journal of Applied & Environmental Microbiology*, vol. 5, no. 2 (2017): 79-85. doi: 10.12691/jaem-5-2-4.

1. Introduction

Moderately halophilic bacteria can be easily isolated from a range of saline environments [1] including salterns [2] and salt lakes [3]. Slightly halophilic or halotolerant organisms require 1-6% (0.2-1M) NaCl for optimum growth, while moderate halophiles require 6-15% (0.5-2.5 M) NaCl [4,5]. However, despite these salt requirements, it has also been shown that moderately halophilic (and often alkaliphilic) bacteria can also be isolated from non-extreme environments such as garden soil and fields, where they are not expected to grow [6]. Following this initial publication, a range of novel moderately halophilic and alkaliphilic bacteria have been isolated from garden and forest soils in Japan [7,8,9,10]. In parallel, novel slightly halophilic bacteria were isolated from forest soils in China [11,12].

Clearly, moderately halophilic bacteria can survive in non-saline soil environments and all of the slightly and moderately halophilic bacteria isolated are endospore-formers suggesting that they survive in non-saline habitats in the form of spores. In the present study, the previous observations were extended by sampling a non-saline water body (Al-Asfar Lake, Al Ahsa, Saudi Arabia) and enriching the water samples with highly saline complex media as a way of selecting for halotolerant or moderately halophilic bacteria. Using this method, three strains of halotolerant or moderately halophilic bacteria were isolated and subsequently identified as species belonging to the genera *Halobacillus, Halomonas* and *Staphylococcus*.

2. Materials and Methods

2.1. Isolation and Selection of Microorganisms

The sampling site was Al-Asfar Lake which is situated 13 kilometres (8.1 miles) to the east of the centre of the Al Ahsa region, and is one of the most important shallow wetland lakes in the Eastern Province of Saudi Arabia as well as the Gulf area. Al Ahsa is the largest oasis in the world, being approximately 20,000 hectares in size, as well as being one of the largest and oldest agricultural centres in the Arabian Peninsula. An irrigation system was put in place in 1971 and delivers 328,000,000 cubic metres of spring water to about 22,000 farms, with additional water supplied by treated wastewater from Al-Hofuf sewage station. The excess drainage water is collected by a drainage network and discharged into two evaporation lakes which are called Al-Asfar and Al-Uyoun. The lake has good wetlands, sabkhas and sand dunes as well as large expanses of open water. Salt tolerant vegetation is present in some of the sabkha areas and huge stands of *Phragmites* reeds occur around much of the lake. The habitat is very important for wildlife and

birds in particular and is not something you would expect to find in a large desert.

A total of 24 x 250 ml sterile flasks were filled with 50 ml of LB medium (5 g yeast extract, 10 g tryptone in 1 litre of distilled water plus different concentrations of NaCl from 0.17 to 2 M) and inoculated with 10 ml of water samples which were collected from the Al-Asfar lake and drainage canals. The sampling sites are described in Table 1. All flasks were incubated at 37°C with shaking at 250 rpm. To isolate moderately halophilic bacteria, serial sub-culture and streak plating techniques were used repeatedly with LB media (liquid medium and solid agar plates produced by the addition of 15 g bacteriological agar No.1 (Oxoid) per litre.). A mixture of samples was prepared some in liquid only cultures, some after filtering the water sample on to 0.45 µm filters and incubating the filter on the surface of the agar plate.

Eventually single colonies were selected according to the best growth demonstrated at high concentrations of NaCl. Cells were examined microscopically using Gram stain reaction and motility test respectively to find out more information about the bacterial isolates.

Table 1

Sample Number	Description of Sampling Site
1	Collected from treated water used for agriculture (Fudhol Station point)
2	Collected from the canal of treated water next to treatment station (Alluwaimi point)
3	Taken from the canal of treated water for agriculture purposes (Bani Ma'an adjacent point)
4	Al Asfar drainage canal 3-4 km prior to reaching the main lake near Alomran Reservoir
5	Al Asfar drainage canal 2 km prior to reaching the main lake
6	Al Asfar lake (western point)
7	Al Asfar lake (northern point)
8	Al Asfar lake (eastern point)

Eight water samples were aseptically collected into sterile medical tubes.

2.2. Molecular Biology Techniques

Genomic DNA Extraction: DNA was extracted using an Anachem Key Prep kit following the manufacturer's instructions. For *S. warneri*, lysostaphin (Sigma – Aldrich) was used for cell wall lysis prior to using the Key Prep kit.

Polymerase Chain Reaction (PCR) Amplification of 16S rRNA: Following extraction of genomic DNA, polymerase chain reaction (PCR) was carried out in order to amplify the 16S rRNA gene, the primers used to amplify the 16S rRNA gene were two universal bacterial primers (synthesised by Eurofins MWG GmbH): Forward primer (F: 5' CCG AAT TCG TCG ACA ACA GAG GAT CCT GG 3') and Reverse primer (R: 5' CCC GGG ATC CAA GCT TAC GGC TAC CTT GT 3') designed to target the conserved regions of the 16S rRNA gene [13]. PCR mix was used either as a master mix (Fermentas) or the reaction mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl Distilled Water, 5 µl 10x Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1 µl genomic DNA and 0.5 µl Taq polymerase (Bioline). Amplifications were carried out in a MyCycler thermocycler (BioRad) and began with an initial denaturation step consisting of 94°C for 3 min followed by 30 cycles consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C followed by a final extension at 72°C for 5 minutes. PCR reactions were cleaned up using an Anachem Key Prep Purification kit as per the manufacturer's protocols.

Gel Electrophoresis: Following PCR and purification processes, gel electrophoresis was used to check and confirm the correct gene had been amplified (16S rRNA is 1.5 kbp). The gel was made by adding 2 ml of 50X TAE into a conical flask, add distilled water up to 100 ml and add 1 g of agarose (ICN Biomedicals Inc.) to produce a 1% gel, This mixture was then heated in a microwave until the agarose had melted, after which it was allowed to cool whilst being stirred, and 5 µl of ethidium bromide (Biorad #161-0433) was added prior to pouring into a Biorad Subcell GT electrophoretic tank with a 30 well comb. Once the gel had set, it was covered with 1X TAE buffer and run at 90-100 V using a Biorad PowerPack 300. PCR products were loaded on the gel as follows, 2 µl of the PCR reaction was added to 2 µl of loading dye and analysed on a 1% agarose gel against 1 µl of 1 kb GeneRuler ladder (Fermentas). Gels were visualised using the Uvitec "Uvidoc" mounted camera system.

Ligation, Transformation and Digestion: A TOPO 10 cloning kit (ThermoFisher) was used to ligate the 16S rRNA gene into the vector, competent *E. coli* cells were transformed with the vector containing the 16S rRNA insert. Ligation reaction and transformation were carried out following the TOPO 10 cloning reaction protocol. A digestion step was used to confirm if the plasmid had the correct insert or not.

Sequencing: The results are checked using gel electrophoresis and samples containing the correct insert were sent to the University of Sheffield Medical School for sequencing. The 16S rDNA sequences were then compared to similar sequences using the NCBI Blast web site.

2.3. Determination of Compatible Solutes Using Nuclear Magnetic Resonance Spectroscopy (NMR)

Samples (5 ml) of stationary phase cultures of *Halobacillus* sp. and *S. warneri* strains were centrifuged at 3000 *g* for 10 minutes then the supernatant was discarded. The pellets were kept in a -80°C freezer. When the analysis was due, the pellets were thawed, resuspended in 1 ml of distilled water and vortexed for 1 minute at room temperature. Then the samples were sonicated (2 x 20 seconds) and centrifuged at 6000 *g* in the microfuge for 10 minutes. The supernatants were transferred into two 1.5 ml microcentrifuge tubes, placed in a -80°C freezer for 2 hours and then freeze dried for two days. Freeze dried samples were prepared for Nuclear Magnetic Resonance (NMR) analysis by dissolving them in 500 µl of D_2O in a microcentrifuge tube and then 5 µl of trimethyl syle propionate (TSP) were added. Next, the dissolved sample was transferred into an NMR tube and run in the NMR as described by [14].

3. Results

3.1. Isolation and Selection of Microorganisms

The aim of this part of the work was to isolate and characterise moderately halophilic bacteria from fresh water samples collected from Al Ahsa, Saudi Arabia. After the enrichment process in liquid and solid medium of salinities up to 2 M NaCl, four strains were isolated, two from sample 4 (4cFLTR and 4M6) and two from sample 6 (6aFLTR and 6aFLSK). Sample 4 was taken from a drainage canal prior to entering the lake and sample 6 was taken from the lake itself (Table 1).

3.2. Identification of Strains Using 16S rDNA Sequencing

As described in the methods, genomic DNA was extracted, followed by PCR amplification and purification of 16S rRNA gene. The sequences were subjected to computer software analyses in which they were compared with other sequences using the NCBI GenBank library using BLAST programme.

4cFLTR strain was shown to match *Staphylococcus warneri* at 99-100% identity. Strain 4M6 was identified as a species of *Halobacillus* with the closest species match to *Hb. blutaparonensis* and *Hb. dabanensis*. Strains 6aFLTR and 6aFLSK were very closely related (99.8% identity) and were both identified as species of *Halomonas* with closest matches to *H. venusta, H. campaniensis* and *H. alkaliphila*. Since the genus *Halomonas* has been very well studied [15], it was decided not to study the 6aFLTR and 6aFLSK in further detail. The two other strains, *Halobacillus* sp and *S. warneri* were the subject of further characterisation as described below.

3.3. Growth of *Halobacillus* sp. at Different Salinities

Growth curves were carried out for *Halobacillus* sp. to observe the effect of different salinities (0.17 M, 1 M, 2 M, 3 M and 4 M NaCl) on growth rate in rich LB medium. Bacterial growth was quantified and monitored using direct optical density (OD) measurements at 600 nm (Figure 1) and *Halobacillus* sp. grew very well up to 1 M NaCl and good growth was also found at 2 and 3 M NaCl, but it was decreased compared to growth at 1 M NaCl. No growth was found at 4 M NaCl (Figure 1). On the basis of these growth curves, *Halobacillus* sp. is classified as a moderately halophilic bacterium.

3.4. Growth of *S. warneri* at Different Salinities

Growth curves were carried out for *S. warneri* to find out the effect of different concentrations of NaCl (0.17 M, 1 M, 2 M, 2.5 M and 3 M) on growth rate in rich LB medium. (Figure 2). Unlike, *Halobacillus* (Figure 1), *S. warneri* growth decreased at all salinities above 0.17 M NaCl. However, good growth takes place up to 2.5 M NaCl, but at 3 M NaCl there was only very weak growth (Figure 2). On the basis of the growth curves, *S. warneri* is classified as a halotolerant bacterium.

3.5. Determination of Compatible Solutes (Osmolytes) by Nuclear Magnetic Resonance Spectroscopy (NMR)

The vast majority of halotolerant and moderately halophilic bacteria respond to increasing salinity by accumulating compatible solutes – small molecular weight organic compounds, which do not interfere with cell metabolism even when present in very high concentrations [16,17]. In order to identify the compatible solutes accumulated by *Halobacillus* sp. and *S. warneri,* their cells were grown in different salinities and the compatible solutes were detected by NMR.

The NMR spectra of *Halobacillus* sp. cells contain signals from normal cellular metabolites (e.g. signals in the range of 0.5 – 3 ppm) (Figure 3). There was some change in these signals with NaCl concentration: signals between 2 and 2.5 ppm decreased gradually in intensity from 1 to 2.5 M NaCl. These were mainly from glutamate, showing that glutamate is being used as an osmolyte at low salt concentrations. The most obvious signals in the spectrum were from betaine, which is present at 2 M NaCl and increases in 2.5, 3 and 3.5 M NaCl. Therefore, the glutamate is being replaced as an osmolyte by betaine as the salt concentration increases. This is a common observation for many moderately halophilic bacteria.

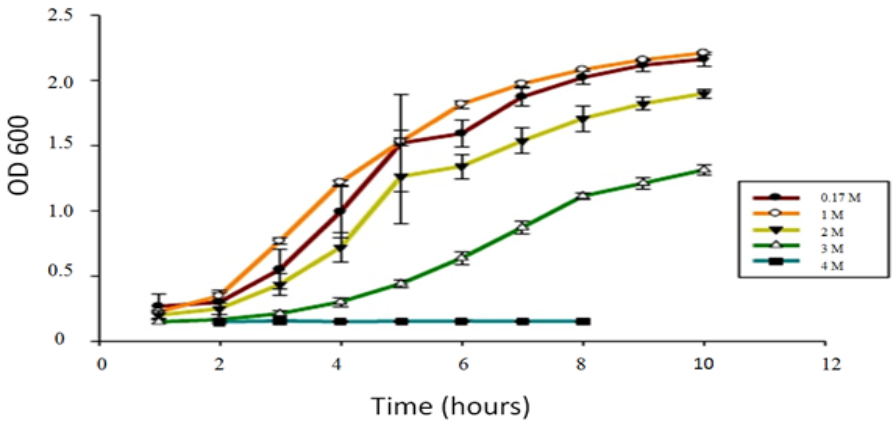

Figure 1. Growth curves for *Halobacillus* sp. Cells were grown in LB medium at different salinities (0.17 M, 1 M, 2 M, 3 M and 4 M NaCl) and incubated at 37ºC on an orbital shaker at 250 rpm. The OD was measured at 600 nm against a medium blank. Data points are the means of three replicates plus or minus standard deviation

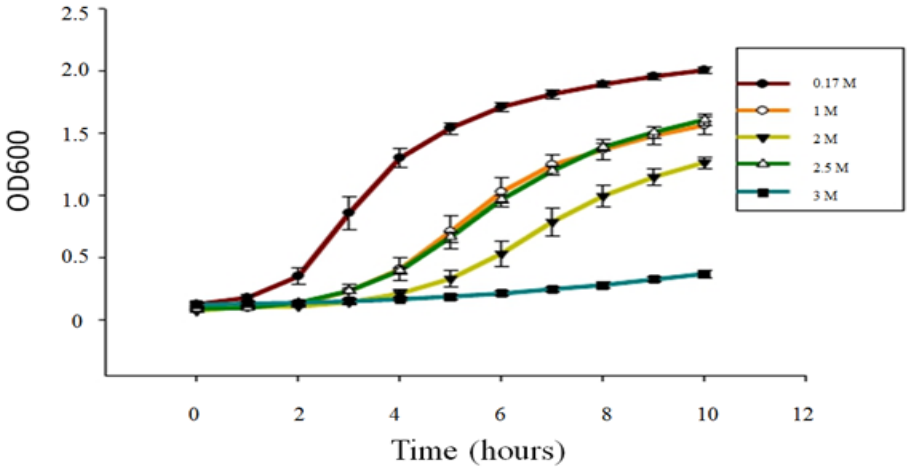

Figure 2. Growth curves for *Staphylococcus warneri*. Cells were grown in LB medium at different salinities (0.17 M, 1 M, 2 M, 2.5 M and 3 M NaCl) and incubated at 37ºC on an orbital shaker at 250 rpm. The OD was measured at 600 nm against a medium blank. Data points are the means of three replicates plus or minus standard deviation

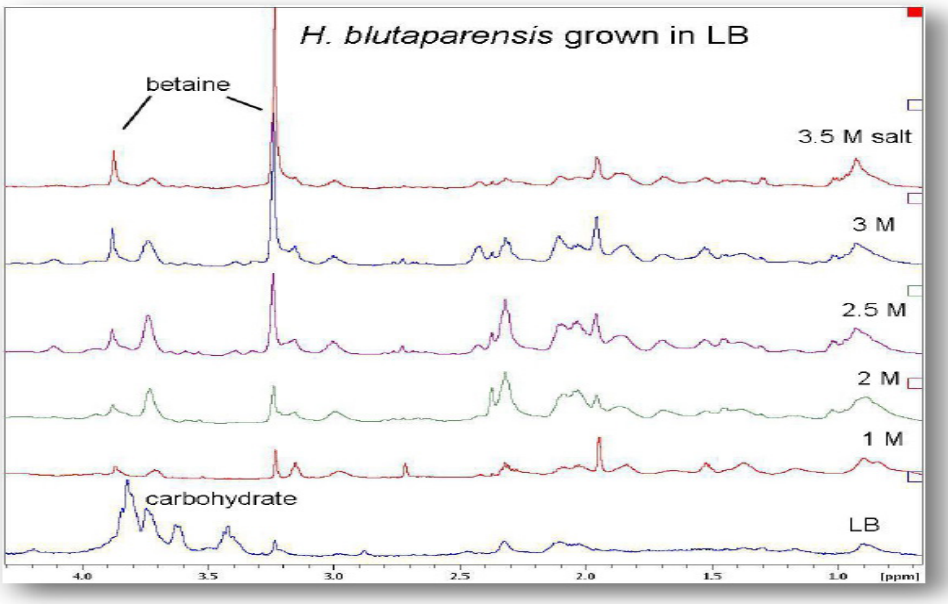

Figure 3. One-dimensional 1H-NMR spectra of cell extracts derived from *Halobacillus* sp. Cells were grown in LB medium with different concentrations of NaCl from 0.17 M (normal LB medium) to 3.5 M NaCl

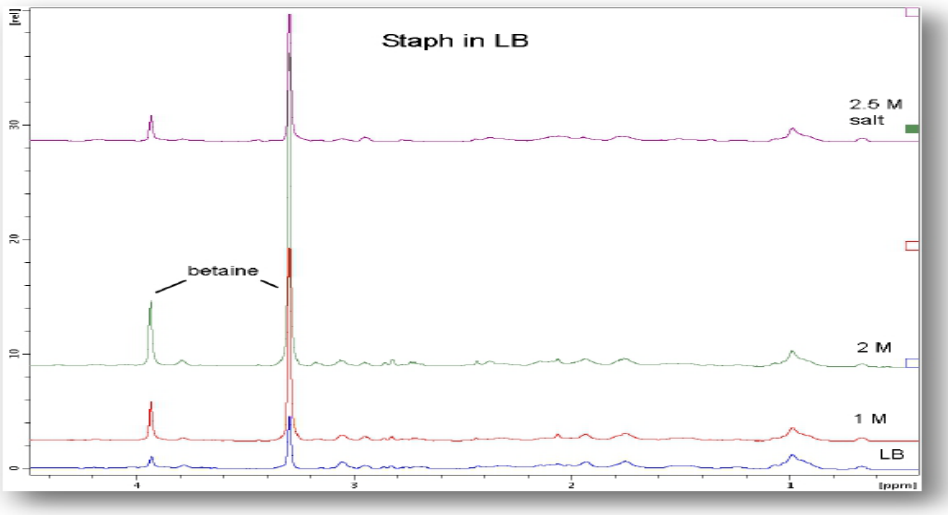

Figure 4. **One-dimensional 1H-NMR spectra of cell extracts derived from *Staphylococcus warneri***. Cells were grown in LB medium with different concentrations of NaCl from 0.17 M (normal LB medium) to 2.5 M NaCl

Figure 4 shows that *S. warneri* also accumulated betaine in response to increasing salinity up to 2 M NaCl, but the level of betaine decreased at 2.5 M NaCl, probably indicating that this was nearing their maximum salt tolerance, which agrees well with the growth data (Figure 2). There are signals from normal cell metabolites (0.5 – 3 ppm) which did not change much with NaCl concentration, and there is no evidence for any other compatible solutes being present. It is concluded that betaine is the only compatible solute that increased with increasing salinity in *S. warneri* cells (Figure 4).

4. Discussion

The work described above shows that a moderately halophilic bacterium belonging to the genus *Halobacillus* was isolated from freshwater samples – in this case from the Al-Asfar Lake and surrounding drainage canals in Al Ahsa, Saudi Arabia. Figure 1 shows that *Halobacillus* sp. is moderately halophilic, because growth at 1 M NaCl is virtually identical to growth in normal LB medium i.e. 0.17 M NaCl. *Halobacillus* sp. could grow at 3 M NaCl, but no growth was found at 4 M NaCl (Figure 1). The genus *Halobacillus* was proposed in 1996 and initially consisted of three species (*Hb. litoralis, Hb. trueperi* and *Hb. halophilus*) isolated from the Great Salt Lake, Utah, USA [18]. The 16S rDNA sequencing results from the current study clearly indicated that our Hassa strain was a member of the *Halobacillus* genus with most matches being to strains only identified to the genus level (see supplementary material). However, within the group of matches showing 99% identity there were 6 matches to *Hb. trueperi,* 4 matches to *Hb. blutaparonensis* and 1 match to *Hb. dabanensis.* As mentioned above, *Hb. trueperi* was originally isolated from the Great Salt Lake [18]. The original description of Hb. blutaparonensis was by [19] who isolated the strain from the roots of the *Blutaparon portulacoids* plant - a succulent herb found on sand dunes and beaches of the Atlantic coast of Brazil. *Halobacillus dabanensis* was originally isolated from a salt lake in Xinjiang, China [20]. It should be noted however, that the closest matches to these three species of *Halobacillus* are for strains isolated from India and China emphasising the world-wide presence of *Halobacillus* species (see supplementary material). We can conclude that strain 4M6 isolated from the Al-Asfar lake definitely belonged to the genus *Halobacillus*, but further work is required to identify it at the species level.

Using, NMR analysis, *Halobacillus* sp was shown to accumulate the compatible solute betaine at high salinities when grown in LB medium (Figure 3). Betaine is a common compatible solute and is often used by microorganisms grown in rich LB medium where choline (a component of LB medium) can be transported into the cells at high salinities and converted to betaine by a two-enzyme pathway [21]. This is a very efficient way of accumulating compatible solute and allowing growth at high salinity. In M9 minimal medium, many moderate halophiles switch to ectoine as their compatible solute and most organisms will accumulate some ectoine at high salinities even when grown in LB medium [22]. However, *Halobacillus* sp. does not accumulate ectoine at high salinities in LB medium (Figure 3) and it does not grow in minimal medium at high salinity (data not shown). This suggests that *Halobacillus* sp. cannot synthesise ectoine and is thus dependent on choline-driven betaine synthesis for growth at high salinities. It thus differs from another species of *Halobacillus*, *Hb. halophilus*, which is known to synthesise ectoine [23].

The other strain isolated from Al-Asfar lake and further characterised was clearly identified as *S. warneri* (see supplementary material), and is of interest due to its potential pathogenicity. It has been described as a dangerous nosocomial pathogenic agent in hospitals [24]. However it was believed for a long time that S. warneri bacteria are harmless common commensals inhabiting the skin and nasal cavities of humans and animals [25]. High risk of *S. warneri* infection was found in immunocompromised patients [26] and a draft genome sequence of *S. warneri* isolated from a neonate blood sepsis patient has been published [27]. The distribution of *S. warneri* is thought to be widespread and a recent study showed its presence as an endophyte in apples and oranges sourced in Tamil Nadu, India [28]. In the present study, *S. warneri* was shown to be halotolerant rather than moderately halophilic, because the best growth was at 0.17 M NaCl (Figure 2). Only very slow growth was seen at 3 M NaCl. NMR analysis showed that *S. warneri* also accumulated betaine as its compatible solute at high salinities (Figure 4). It is known that *S. aureus* accumulates betaine and proline as its main compatible solutes [29]. In the current work, only betaine was found to be accumulated by *S. warneri* (Figure 4).

To conclude, a moderately halophilic bacterium and a halotolerant bacterium were isolated from the freshwater Al-Asfar lake, confirming the widespread presence of moderately halophilic bacteria in non-saline habitats.

Acknowledgements

We would like to thank Professor Mike Williamson for running and analysing the NMR samples.

References

[1] Ventosa, A., Marquez, M. C., Garabito, M. J., and Arahal, D. R. (1998). Moderately halophilic gram-positive bacterial diversity in hypersaline environments, *Extremophiles 2*, 297-304.

[2] Jose Leon, M., Martinez-Checa, F., Ventosa, A., and Sanchez-Porro, C. (2015). Idiomarina aquatica sp nov., a moderately halophilic bacterium isolated from salterns, *International Journal of Systematic and Evolutionary Microbiology 65*, 4595-4600.

[3] Xue, Y., Ventosa, A., Wang, X., Ren, P., Zhou, P., and Ma, Y. (2008). Bacillus aidingensis sp nov., a moderately halophilic bacterium isolated from Ai-Ding salt lake in China, *International Journal of Systematic and Evolutionary Microbiology 58*, 2828-2832.

[4] Gilmour, D. J. (1990). Halotolerent and halophilic microorganisms *In Microbiology of Extreme Environments. Ed. Edwards, C., Open University Press, Milton Keynes, U.K.*, 147-177.

[5] Oren, A. (2008). Nomenclature and taxonomy of halophilic archaea - comments on the proposal by DasSarma and DasSarma for nomenclatural changes within the order *Halobacteriales*, *Int J Syst Evol Micr 58*, 2245-2246.

[6] Echigo, A., Hino, M., Fukushima, T., Mizuki, T., Kamekura, M., and Usami, R. (2005). Endospores of halophilic bacteria of the family *Bacillaceae* isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm), *Saline Systems 1*, 8.

[7] Echigo, A., Fukushima, T., Mizuki, T., Kamekura, M., and Usami, R. (2007). Halalkalibacillus halophilus gen. nov., sp nov., a novel moderately halophilic and alkallphilic bacterium isolated from a non-saline soil sample in Japan, *International Journal of Systematic and Evolutionary Microbiology 57*, 1081-1085.

[8] Usami, R., Echigo, A., Fukushima, T., Mizuki, T., Yoshida, Y., and Kamekura, M. (2007). Alkalibacillus silvisoli sp nov, an alkaliphilic moderate halophile isolated from non-saline forest soil in Japan, *International Journal of Systematic and Evolutionary Microbiology 57*, 770-774.

[9] Echigo, A., Minegishi, H., Mizuki, T., Kamekura, M., and Usami, R. (2010). Geomicrobium halophilum gen. nov., sp nov., a moderately halophilic and alkaliphilic bacterium isolated from soil, *International Journal of Systematic and Evolutionary Microbiology 60*, 990-995.

[10] Echigo, A., Minegishi, H., Shimane, Y., Kamekura, M., and Usami, R. (2012). Natribacillus halophilus gen. nov., sp nov., a moderately halophilic and alkalitolerant bacterium isolated from soil, *International Journal of Systematic and Evolutionary Microbiology 62*, 289-294.

[11] Chen, Y.-G., Hao, D.-F., Chen, Q.-H., Zhang, Y.-Q., Liu, J.-B., He, J.-W., Tang, S.-K., and Li, W.-J. (2011). Bacillus hunanensis sp nov., a slightly halophilic bacterium isolated from non-saline forest soil, *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 99*, 481-488.

[12] Chen, Y.-G., Zhang, Y.-Q., Chen, Q.-H., Klenk, H.-P., He, J.-W., Tang, S.-K., Cui, X.-L., and Li, W.-J. (2011). Bacillus xiaoxiensis sp nov., a slightly halophilic bacterium isolated from non-saline forest soil, *International Journal of Systematic and Evolutionary Microbiology 61*, 2095-2100.

[13] Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study, *Journal of Bacteriology 173*, 697-703.

[14] Frings, E., Kunte, H. J., and Galinski, E. A. (1993). Compatible solutes in representatives of the genera, *Brevibacterium* and *Corynebacterium*: occurrence of tetrahydropyrimidines and glutamine, *FEMS Microbiol. Lett. 109*, 25-32.

[15] Arahal, D. R., Vreeland, R. H., Litchfield, C. D., Mormile, M. R., Tindall, B. J., Oren, A., Bejar, V., Quesada, E., and Ventosa, A. (2007). Recommended minimal standards for describing new taxa of the family Halomonadaceae, *International Journal of Systematic and Evolutionary Microbiology 57*, 2436-2446.

[16] da Costa, M. S., Santos, H., Galinski, E. A., and Antranikian, G. (1998). An overview of the role and diversity of compatible solutes in Bacteria and Archaea, *Advances in Biochemical Engineering Biotechnology; Biotechnology of extremophiles*, 117-153.

[17] Empadinhas, N., and da Costa, M. S. (2008). Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes, *International Microbiology 11*, 151-161.

[18] Spring, S., Ludwig, W., Marquez, M. C., Ventosa, A., and Schleifer, K. H. (1996). Halobacillus gen nov, with descriptions of Halobacillus litoralis sp nov and Halobacillus trueperi sp nov, and transfer of Sporosarcina halophila to Halobacillus halophilus comb nov, *International Journal of Systematic Bacteriology 46*, 492-496.

[19] Barbosa, D. C., Bae, J. W., von der Weid, I., Vaisman, N., Nam, Y. D., Chang, H. W., Park, Y. H., and Seldin, L. (2006). Halobacillus blutaparonensis sp nov., a moderately halophilic bacterium isolated from Blutaparon portulacoides roots in Brazil, *Journal of Microbiology and Biotechnology 16*, 1862-1867.

[20] Liu, W. Y., Zeng, J., Wang, L., Dou, Y. T., and Yang, S. S. (2005). Halobacillus dabanensis sp nov and Halobacillus aidingensis sp nov., isolated from salt lakes in Xinjiang, China, *International Journal of Systematic and Evolutionary Microbiology 55*, 1991-1996.

[21] Cummings, S. P., and Gilmour, D. J. (1995). The effect of NaCl on the growth of a *Halomonas* species: accumulation and utilization of compatible solutes, *Microbiology 141*, 1413-1418.

[22] Kunte, H. J. (2006). Osmoregulation in bacteria: compatible solute accumulation and osmosensing, *Environmental Chemistry 3*, 94-99.

[23] Saum, S. H., and Müller, V. (2008). Regulation of osmoadaptation in the moderate halophile *Halobacillus halophilus*: chloride, glutamate and switching osmolyte strategies, *Saline Systems 4*.

[24] Kamath, U., Singer, C., and Isenberg, H. (1992). Clinical significance of *Staphylococcus warneri* bacteremia, *J Clin Microbiol 30*, 261-264.

[25] Kassem, I. I. (2009). Detection and characterization of staphylococcal pathogens in the environment: a community approach, PhD thesis, The University of Toledo.

[26] Ivić, I., Karanović, J., and Pavičić-Ivelja, M. (2013). Sepsis with multiple abscesses caused by *Staphylococcus warneri*: a case report, *Central European Journal of Medicine*, 1-3.

[27] Kropp, K. A., Lucid, A., Carroll, J., Belgrudov, V., Walsh, P., Kelly, B., Smith, C., Dickinson, P., O'Driscoll, A., Templeton, K., Ghazal, P., and Sleator, R. D. (2014). Draft Genome Sequence of a Staphylococcus warneri Strain Isolated from a Preterm Neonate Blood Sepsis Patient at the Royal Infirmary, Edinburgh, Scotland, *Genome announcements 2*.

[28] Phukon, M., Sahu, P., Srinath, R., Nithya, A., and Babu, S. (2013). Unusual Occurrence of Staphylococcus warneri as Endophyte in Fresh Fruits along with Usual Bacillus spp, *Journal of Food Safety 33*, 102-106.

[29] Miller, K. J., Zelt, S. C., and Bae, J.-H. (1991). Glycine betaine and proline are the principal compatible solutes ofStaphylococcus aureus, *Current Microbiology 23*, 131-137.

Appendix

Partial 18S rDNA Sequences for Strains 4M6 and 4cFLTR

Partial 18S rDNA Sequence for Strain 4M6 – Length = 548 bp

ACACGTGGGCAACCTGCCTGTAAGATCGGGATAACTCCGGGAAACCGGGGCTAATACCGGGTAATACTTTC
TTTCGCATGAAGGAAAGTTGAAAGATGGCTTCTCGCTATCACTTACAGATGGGCCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC
GGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCG
TGCGAATAGAGCGGTACCTTGACGGTACCTAACGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGA
TGTGAAAGCCCACGGCTCNACCGTGGAGGGTCATTGGAAACTGGGGAACTTGA

Partial 18S rDNA Sequence for Strain 4cFLTR – Length = 747 bp

CCGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCTCCT
TTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGAAACCTACCTATAAGACTGGGATAACTTCGGGAAA
CCGGAGCTAATACCGGATAACATATTGAACCGCATGGTTCAATAGTGAAAGGCGGCTTTGCTGTCACTTAT
AGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCT

GTTATCAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTGATCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCG
CGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAA
CTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACC
CAGTGGCGAAGGCGACTTTCTGGTCTGTAACTACCGCTATT