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RESEARCH ARTICLE

Induction of Osmotolerance by *Staphylococcus sciuri* HP3 *in Lens esculenta Var. Masoor 93 under NaCl Stress*

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ABSTRACT

Agricultural output is greatly reduced in saline soil. Bacterial adaptation to salt stress in saline soil can be helpful for reducing the detrimental effects of salts in saline soil. This study was conducted to assess the impact of salt stress on efficiency of isolate HP3 *Staphylococcus* (*Staph.*) *sciuri* (previously isolated from histoplane of maize plant) in counteracting the adverse effect of salinity. The response of bacterial growth in the presence or absence of osmolytes (proline, glycine betaine and choline) at varying salt stress (0, 0.5, 1, 1.5, 2 and 2.5 molar NaCl) in LB-broth and M9 medium, bacterial osmolyte accumulation, biofilm formation and plant growth promoting potential was determined. Plant growth was measured in terms of seedling length, total soluble proteins and sugars contents of inoculated *Lens esculenta* Var. Masoor 93 seedlings in laboratory conditions under salt stress. The results showed that bacterial growth decreased with higher salinity (1.5-2.5 M NaCl). The biofilm formation of strain was visible as gummy colonies (0.5 M NaCl). Crystal violet ring assay showed a tendency of HP3 for biofilm formation at 0.5 to 2.0 M NaCl stress but no biofilm ring was observed at 0 or 2.5 M NaCl stress. However, highest on glass slides at 2.0 M NaCl stress. In the microtitre plate assay, biofilm was generally high at 1-1.5 M NaCl stress. However, exogenous osmolytes (especially proline) favoured bacterial growth and biofilm at high salt stress with little exceptions. In plant growth experiments, there was a significant reduction ($P<0.05$) in seedling length (56 %) at high NaCl stress. Bacterial inoculations significantly improved the seedling length, Protein (31 %) and total soluble sugars (81 %) of seedlings. Accumulation of osmolytes and biofilm formation at salinity actually shows the promising strategies of stress tolerance that helped the *Staph. sciuri* to survive and improving plant growth under salt stress.

INTRODUCTION

Salinity is one of the abiotic stress that reduces the agriculture yield. In saline soil, presence of indigenous bacteria showed their innate ability to survive under salt stress. *Staphylococcus* (*Staph.*) *sciuri* is a microorganism reported to be present in a wide varieties of environment, animal waste (Stepanovic et al., 2001; Nagase et al., 2002), various food products, environmental reservoirs like soil, sand, water, and marsh grass (Nunes et al., 2016). The ubiquitous nature of *Staph. sciuri* strains in different environments may enable these bacteria to adapt and give clues about the survival and osmoadaptation strategies. Many researchers have cited for the significance of *Staph. sciuri* towards biofilm formation, however, very little has been described in terms of the plant growth promotion. The present study is aimed to give an insight about the survival strategy, biofilm formation and plant growth promoting response of this bacterium in rhizosphere under the salt stress.

Salinity is an important environmental stress and to counteract, bacteria show complex mechanisms for example, membrane flexibility in *Desulfovibrio vulgaris* has been reported to increase under higher salinity, thus helping the cells to tolerate salt stress (Zhou et al., 2013). Intracellular osmolytes accumulation and cell aggregation embedded in a sheath of polysaccharides as biofilm are commonly manifested phenomena of bacterial owe to salt stress

conditions (Ameur et al., 2011; Karunakaran and Biggs, 2011). Intracellular osmolytes accumulation in the bacteria also helps them to maintain perturbed osmotic balance under salinity. Different accumulated osmolytes in bacteria and archaea include trehalose, proline, glutamate, ectoine, glycine betaine, and carnitine, amino acids and their derivatives (Kempf and Bremer, 1998; Murdock et al., 2014). Although these osmolytes are accumulated inside the cells, however, bacteria can also accumulate these favorably from the outer environment (Bremer and Kraemer, 2000; Wood, 2015). Bacteria predominately exist as structured community i.e., a biofilm under adverse natural conditions. The biofilm can be formed on different living or nonliving surfaces (Salta et al., 2013; Tremblay et al., 2013). When compared to planktonic stage, bacteria in a biofilm have more survival abilities against different stress factors like salinity (Palomino et al., 2013), predators (Chavez-Dozal et al., 2013), antibiotics (Wang et al., 2005), sonication (Kobayashi et al., 2009), heat shock (Chung and Toh, 2014) and nutrient limitation (Burmolle et al., 2006; Torres et al., 2011; Moryl et al., 2014). The impact of salinity on bacterial osmolytes accumulation and biofilm formation seem to play a significant role in natural conditions. Both of these two phenomena are variously influenced by salinity levels. To alleviate the negative impact of saline soil on plant growth, inoculation of indigenous bacteria having ability to tolerate salt, accumulate osmolytes and biofilm formation can be useful to increase agricultural yield. The purpose of this research is to check the biofilm formation and intracellular osmolytes accumulation in *Staph. sciuri* at different salinity levels. Moreover, this study is an effort to check the potential of *Staph. sciuri* in improving plant growth. These properties in bacteria will be useful to evaluate their potential in saline soil.

MATERIALS AND METHODS

Strain HP3 (*Staphylococcus sciuri* Accession No. JN542714) previously isolated from histoplane of *Zea mays* by Mirza et al., 1998 was used in this research. The strain was routinely maintained in LB-Agar plates (Gerhardt et al., 1994) containing 0.5 M NaCl.

Effect of varying NaCl concentrations and exogenous osmolytes on the growth and endogenous osmolyte accumulation in *Staphylococcus sciuri*

Bacterial cells were grown in LB and M9 broth, supplemented with NaCl (0, 0.5, 1, 1.5, 2, 2.5 M) and exogenous osmolytes (L-proline, glycine betaine and choline at concentrations of 0 and 1 mM) for 24 hours at 37ºC at shaking incubator. From these cells, intracellular osmolytes i.e., proline (Tonon et al., 2004), glycine betaine and choline (Grieve and Grattan, 1983) were extacted following same modifications as previously described (Qurashi and Sabri, 2013). For these osmolyte quantification, standard curves (µg ml^{-1}) of L-proline, glycine betaine and choline were used, respectively.

Qualitative assays for biofilm formation and Microscopic analysis

The *Staphylococcus Sciuri* (HP3) was grown on LB agar plates added with 0.5 M NaCl in the absence or presence of glucose (20 %). The plates were incubated at 37°C for 48 hours. Colonies were scored for gummy or mucoidy on the basis of appearance after 48 hours of culture incubation. These experiments were performed in triplicate. The biofilm ring assay was carried out with some modification as previously reported (Christensen et al., 1985). LB broth (10 ml) media containing different NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5 M) was added in each 12 mm diameter glass tubes. Each tube was inoculated with 100 µL with fresh culture (cultured at 37°C for 24 h in LB broth) at final cell density 10^{8} CFU/ml, and incubated at 37°C for 24 hours with no shaking. Finally after taking optical density of the culture (planktonic cells), the culture was discarded and to each empty tube, 10 ml of 0.01 % crystal violet was added to stain the cells of established biofilm as ring. Each tube was allowed to stand for about 20 minutes at room temperature. After 20 minutes, crystal violet solution was discarded and empty tubes were washed using sterile distilled water. Tubes were placed in an inverted position and air dried. Test tubes were examined and the biofilm assay results were positive when a visible violet ring appeared on the air, liquid interface and the tube bottom. Due to the possibility that the bacterial cells switch from planktonic stage to sessile stage under osmotic stress, pattern of biofilm development was studied by establishing biofilm on glass slides. The experiment was performed in flasks containing (250 ml LB) medium supplemented with different salt concentrations. Neat and clean, sterilized glass slides (1-1.2 mm thickness: 25.4 x 76.2 mm length) were inserted at a slant position (half slide submerged in media) against the wall of flask containing media before autoclaving. The sterilised medium containing glass slides was inoculated with fresh bacterial cultures (final cell density 10^{8} CFU/ml) and flasks were incubated at 37°C without agitation for 48 hours (optimized conditions). After incubation, slides were aseptically removed, rinsed and stained with 0.01 % aqueous crystal violet. Finally air dried glass slides were observed in the light microscope at 100 X using an oil immersion lens.

Quantitative assay

For biofilm quantification, the procedure suggested by Fujishige et al. (2006) was followed with some modifications as previously discussed (Qurashi and Sabri, 2012). The biofilm formation was checked in LB

broth and M9 media, supplemented with varying salt stress (0, 0.5, 1, 1.5, 2, 2.5 M NaCl) and exogenous osmolytes (L-proline, glycine betaine and choline at final concentrations of 0 and 1 mM). The results are reported as a normalized biofilm value (OD 570 $_{nm}$ / OD 590 $_{nm}$).

Plant Growth experiments

For plant growth experiments, same procedure and conditions as previously described (Qurashi and Sabri, 2012) were followed. Thoroughly washed, surface sterilized seeds (0.1 % $HgCl_2$ solution for 10 minutes) certified and healthy seeds of *Lens esculenta* Var. Masoor 93 (from Punjab seed corporation, Lahore, Pakistan) were used. Seeds were grown for 15 days in plastic pots containing autoclaved and dried garden soil (120 g pot $^{-1}$) using these concentrations of NaCl (0, 50, 100 and 200 mM) per gram weight of soil in the department of Microbiology and molecular Genetics, university of the Punjab under control conditions of laboratory. Finally after 15 days of growth, seedlings were harvested. The plant growth was determined in terms of seedling length (cm), total soluble protein content (µg per gram fresh weight) following (Afrasayab et al., 2010) and total soluble sugar contents (mg per gram fresh weight) following Dubois et al., (1956).

Statistical analysis

Each experiment was carried out in three replicates and statistical data analysis was carried out from mean values, standard errors of the means were calculated and shown in all the figures as error bars. Analysis of variance and difference between the means was tested using the least significant difference test ($P<0.05$) (Steel and Torrie, 1981).

RESULTS

Effect of salinity and exogenous osmolytes on the growth and endogenous osmolyte accumulation

Staphylococcus Sciuri (HP3) showed a general decreasing response in cell densities at high salt (1.5-2.5 M) stress in both tested media. When no osmolytes were added in medium, bacterial growth was slightly higher in M9 as compared to respective LB. However, with the addition of osmolytes in LB (except in choline supplemented LB at 1.5 and 2.5 M NaCl stress), increase in cell densities was recorded. The response of cell densities in M9 was variable depending on the salt concentrations. In the absence of NaCl stress, the cell densities declined (Fig 1). The effect of respective exogenous osmolytes and NaCl on osmolyte accumulation at intracellular level was recorded (Fig. 2). Results showed that in non proline supplemented media, higher proline accumulation was recorded in the presence of 1 M NaCl stress (in LB) or to 1.5 M NaCl stress in M9 media. In the presence of exogenous

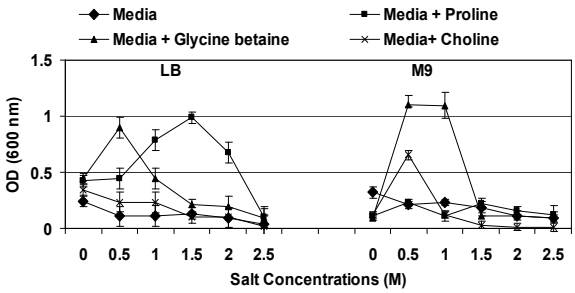

Fig. 1: Effect of different osmolytes under varying NaCl concentrations on bacterial growth in LB and M9 broth after 24 hours incubation. Means of three replicates and standard errors of the means are drawn.

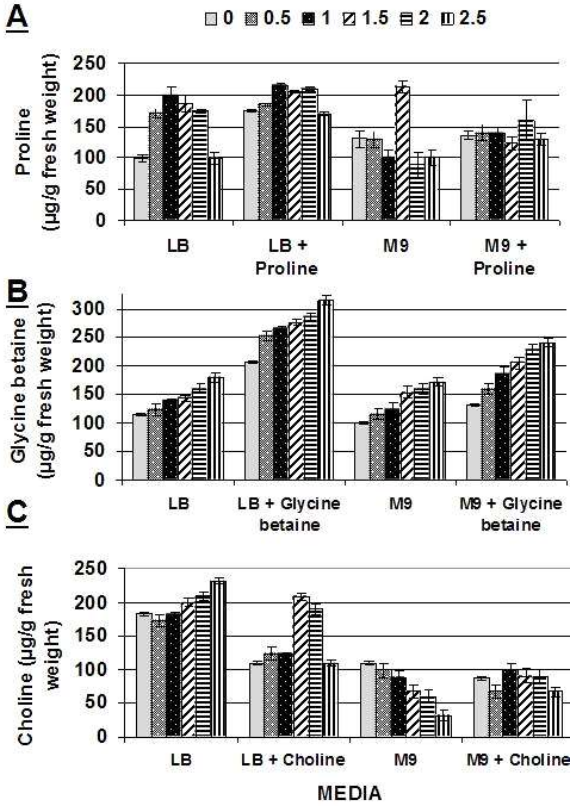

Fig.: 2: Effect of different osmolytes under varying NaCl concentrations on endogenous osmolytes accumulation in LB and M9 broth. Means of three replicates and standard errors of the means are drawn.

proline in LB media, highest increase (72 %) in proline accumulation was recorded at 2.5 M NaCl stress. When proline was added in M9 medium, highest (82 %) increase in proline accumulation was recorded at 2.0 M NaCl stress as compared to non proline supplemented control (Fig 2A). The accumulation of endogenous glycine betaine increased in all media when exogenous

osmolytes were added (Fig. 2 B). The accumulation of the endogenous glycine betaine was maximum at 2.5 M NaCl stress in both medium. Generally accumulation of glycine betaine was higher in LB than M9 media with few exceptions. In non-choline supplemented media, maximum choline accumulation was recorded at 2.5 M NaCl in LB and 0 M in M9 media as compared to rest of salt concentrations (Fig. 2 C). The accumulation of endogenous choline level decreased when exogenous choline was present in the media with few exceptions (1.5 M NaCl stress in LB and 1-2.5 M NaCl in M9 media).

Effect of exogenous osmolytes and salt stress on biofilm

The likelihood that presence of gummy phenotype in bacterial colonies can contribute to biofilm formation, analysis of the gummy appearance of bacterial colonies as an initial qualitative assay was made. A positive response of strain HP3 was recorded for colony gummy morphology under salt stress (0.5 M NaCl concentration) whether 20 % glucose was added or not in the medium. Biofilm ring assay is another qualitative approach used to study biofilm formation. Positive results were recorded when strain HP-3 was incubated for 48 hours at different NaCl stress (0.5-2 M NaCl stress), however, no biofilm ring was formed either in the absence of NaCl or at 2.5 M NaCl stress (Fig. 3). Strain HP3 showed a tendency towards the biofilm formation on glass slides. The dense clumps and aggregation of bacterial cells were recorded at all salinity levels (0-2.5 M NaCl stress). The microscopic images clearly showed that at a lower concentrations (0-1 M NaCl stress) (Fig. 4 A-C), cells form clump and showing a tendency towards aggregate. As NaCl level increases (Fig. 4 D-E), the bacterial aggregation and biofilm formation becomes pronounced. This clumping of cells was much more pronounced at 2 M NaCl stress (Fig. 4 E), however, at 2.5 M NaCl stress visible clumps were reduced in size (Fig. 4 F). The results of the quantitative assay of biofilm formation using microtitre plate assay, (Fig. 5) showed that, generally the biofilm formation was high in LB media than M9 media in the absence of NaCl stress. At higher salt stress (1.5-2.5 M NaCl stress), biofilm formation was high in M9 media. The highest biofilm formation was recorded at 1.5 M NaCl stress in LB and 2-2.5 M NaCl stress in M9 media in non osmolytes supplemented media. The addition of osmolytes, decreased the level of biofilm formation in most of the cases as compared to non-osmolytes supplemented media (Fig. 5). In LB with exogenous proline, maximum biofilm development was recorded at 2.5 M NaCl stress, however, when medium was supplemented with glycine betaine and choline, biofilm formation was not favoured. With the addition of osmolytes, biofilm formation in M9 was relatively higher at 2.5 M NaCl stress than other salt concentration.

STRAIN	BIOFILM RING ASSAY					
	SALT CONCENTRATIONS (M)					
	0	0.5	1	1.5	2	2.5
HP3	-VE	+VE	+VE	+VE	+VE	-VE
RESULTS OF RING ASSAY : -VE means NO Ring +Ve means PURPLE Ring is formed						

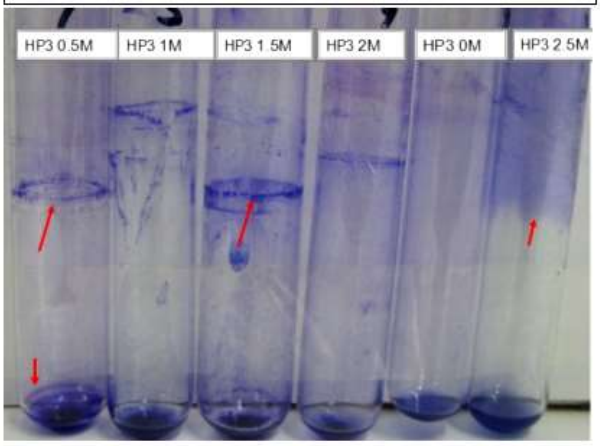

Fig. 3: Biofilm ring formtion: Arrows indicate the purple color ring developed on glass tubes at different salt concentrations. No ring was recorded at 0 M NaCl or at 2.5 M NaCl stress

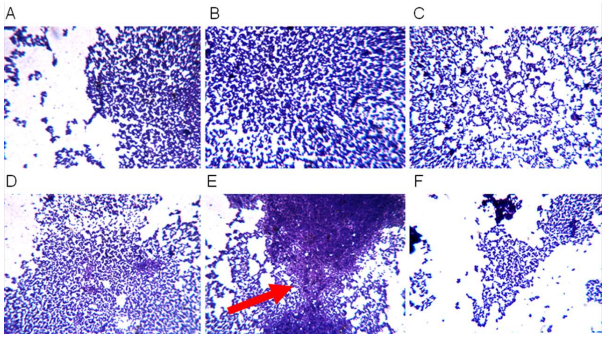

Fig. 4: Biofilm formation: Biofilm formation on glass slides at different NaCl concentrations (A. 0 M B. 0.5 M C. 1 M D. 1.5 M E. 2 M F. 2.5 M). Arrows indicate dense clumping on glass slides

Effect of salt stress on the plant growth

In non inoculated seedlings significant reduction in seedling length (56 % at 200 mM NaCl stress) was recorded, however, significant increase was recorded in seedling length (11 % at 50 mM NaCl stress) of inoculated plants (Fig. 6 A). Protein (31 %) and total soluble sugars (81 %) of non inoculated *Lens esculenta* Var. Masoor-93 seedlings was augmented at 100 mM NaCl stress (Fig. 6 B-C) when compared to 0 mM NaCl stress. However, inoculation significantly increased the total soluble Protein (11 % at 200 mM NaCl stress) and total soluble sugars (60 % at 200 mM NaCl stress) contents of inoculated plants when compared to non inoculated plants at salt stress (Fig. 6).

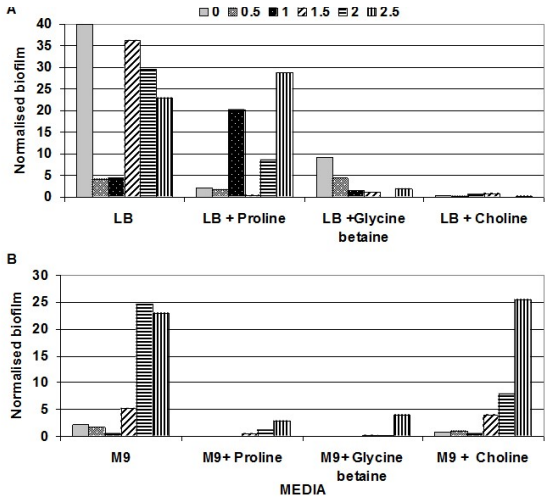

Fig. 5: Normalized biofilm recorded at different NaCl stress (0, 0.5, 1, 1.5, 2 and 2.5 M) and exogenous osmolytes (Proline, Glycine betaine and Choline-1mM) in LB and M9 media.

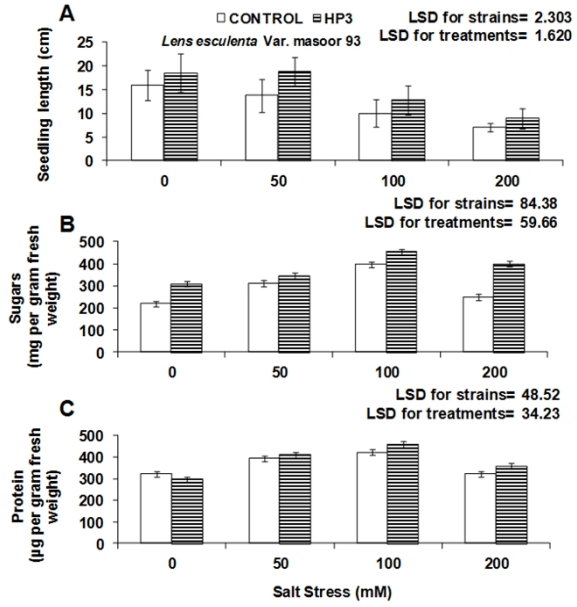

Fig. 6: Effects of HP3 strain on (A) Seedling length (B) sugars (mg per gram fresh weight) (C) Protein (µg per gram fresh weight) of *L. esculenta* Var. masoor 93 under different salt stresses.

DISCUSSION

This paper reports the effect of different salt stress and exogenous osmolytes on the bacterial growth response and biofilm formation of *Staphylococcus sciuri* (HP3) in two different media. Cell growth of *Staphylococcus sciuri* (HP3) decreased at a higher level of NaCl concentrations (1.5-2.5 M) in both the media. Without exogenous osmolytes, bacterial cell density was slightly higher in M9 media as compared to LB. This shows the nutritional requirement of bacterial strains affected by salinity. Under nutrient or salt stress, bacteria switch towards sessile stage and this situation results in increased striving of bacteria for survival and protection. This is in line with previous findings (Fujishige et al., 2006). Under higher salt stress, exopolysaccharide is also produced by bacteria that help bacteria in attachment and offering protection by retaining a water layer around the cells (Dimkpa et al., 2009, Deng et al., 2015). The use of M9 medium in this study is helpful for effective evaluation of the bacterial growth under distinguishing effects of media components. Exogenous osmolytes in LB medium stimulated the bacterial growth at high salt stress (1.5-2.5 M) in LB, however, effect of exogenous osmolytes in M9 media was not pronounced. These osmolytes might be synthesised or accumulated in cells to cope with stress in the form of salt or nutrient deprived conditions. This is in line with the findings of Ameur et al. (2011) who reported the variable growth response of two strains *Streptomyces* sp. MADO2 and *Nocardiopsis* sp. MADO3 in different medium and osmoprotection of glycine betaine and proline increases under high salinity. Similar observations were recorded from study of Sartori et al. (2012) indicating the osmoprotective and regulatory role of these osmolytes under inhibitory growth conditions in the form of stress. In the presence of exogenous proline and glycine betaine, improvement in gram positive bacterial growth has also been reported previously (Morikawa, 2010). These results showed the likelihood that availability of osmolytes like proline, glycine betaine and choline in natural environment can contribute in bacterial growth improvements at high osmotic stress (Tsai et al., 2011). Exogenous glycine betaine has been found to stimulate the growth of free living bacteria and N_2 fixing root symbionts under salt stress (Talibart et al., 1997). Contrary to the results of growth experiments, there was a general response of high level of proline accumulation in LB medium with salinity. This response was further augmented with exogenous proline in the media. LB media contains yeast extract that is a source of trehalose and glycine betaine (Santos and Costa, 2002) and helps in osmotic adjustment. Comparatively low level of accumulation in M9 medium can be justified for the less rich composition that only contains glucose as source of carbon. Intracellular glycine betaine accumulation was elevated with high salinity irrespective of media used and exogenously supplemented osmolytes in the medium showing that they can alleviate growth inhibition under salt stress. A concomitant increase in the intracellular accumulation of glycine betaine has been reported under high osmotic pressure in surrounding (Ameur et al., 2011) suggesting their role as metabolite that is

accumulated in cell when bacteria face inhibitory growth conditions. Generally, the level of choline accumulation was higher in non-supplemented LB; however, it decreased when exogenous choline was present in the medium with few exceptions. In the absence of osmotic stress, osmolytes like glycine betaine, choline and other osmolytes have been found to enhance their catabolism in S. *meliloti* and *Pseudomonas aeruginosa* (Talibart et al., 1997). The development of a biofilm has also been reported as a stress response shown by bacterial cells (Jefferson, 2004; Gravesen et al., 2005). To study the response of bacterial cells for biofilm formation as an osmoadaptation response, the biofilm was checked using qualitative and quantitative assays. In the initial qualitative biofilm assay, positive results for gummy colonies were obtained from *Staph. Sciuri* (HP3) in the presence of salt and 20% glucose. Similar results were reported by Agarwal et al. (2011) where high concentration of glucose and sodium chloride increases the biofilm formation in *Staph. aureus* species, irrespective of their pathogenesis. The assay for biofilm ring test showed that the ring development was recorded when the salinity level was raised from 0.5 M to 2 M NaCl stress. However, in the absence of salinity or in the presence of 2.5 M NaCl stress, biofilm ring formation was absent. The nutrient and stress conditions might have influenced the matrix of biofilm. Enriched growth medium has high nutrients that result in more polysaccharides and biofilm (Moryl et al., 2014). The bacterial biofilm ring is mainly composed of cells and exopolysaccharides (Pan et al., 2010; Tahmourespour and Kermanshahi, 2011). Microscopic results of biofilm were obtained using light microscopy. Biofilm was established on glass slides, showed dense bacterial aggregation on slides. The variable pattern of cell clumping and dense aggregation was observed to be dependent on level of salinity. These results were inline with the hypothesis that bacteria have tendency to develop biofilm under salinity. The results showed that development initiated with adhesion of cells to surfaces (Fig. 4). It led to the development of biofilm clumps in different patterns. These clumps were actually the cells that accumulated under the salt stress. It is notable that the rise in salt concentrations, the cell grouping was also increased up to 2 M NaCl stress. However, at 2.5 M NaCl stress, cell clumps were reduced much in size. This might be due to detachment of cells at high NaCl concentration (2.5 M NaCl). The development of biofilm is initiated by two steps involving adhesion and the creation of multiple cell layers (Garrett et al., 2008). In previous studies, capsular polysaccharide has been found to help the *Staphylococcus* strain in attachment process (Chan et al., 2014). EPS production can also affect bacterial adaptation under salinity by adjusting the membrane fluidity (Qiu et al., 2012). Decho, (2010) reported that though not essential, but presence of EPS helps to provide the structural firmness to microbial assemblies in nature. In previous studies, level of exopolysaccharides have been found to be influenced by the environmental conditions that in turn can stimulate the detachment process in biofilms of *Staphylococcus Sciuri* isolated from the floors of cooking place (Leriche and Carpentier, 2000) and marine *Pseudomonas* sp. S9 (Wrangstadh et al., 1988) resulting in decreases biofilm formation. The role of shear forces in detachment of cells from biofilm have also been reported previously (Salta et al., 2013). The qualitative and quantitative assays were performed to relate the phenotypic characteristic of bacterial strain to physiological behaviour under salt stress conditions. The need for biofilm quantification using a microtitre plate assay raises due to its trustworthy and accurate results (Agarwal et al., 2011). The level of biofilm formation improved in the presence of 1 mM proline in LB (at 2.5 M NaCl stress). This observation is same in the study of Goh et al., (2013) where the addition of exogenous amino acids, especially proline favoured biofilm development. Salinity and exogenous glycine betaine and choline, reduced the biofilm formation in LB. The less biofilm formation in nutrient rich media (LB) supplemented with exogenous osmolytes suggests low level of stress to bacterial cells. It further points out the protective role of biofilm formation under stress. It can also be the possibility that bacteria don't need to synthesise osmolytes when osmolytes are already present in medium. However, in minimal media biofilm formation significantly improved at 2.5 M NaCl. These results are of particular importance in the sense that biofilm formation in S. *sciuri* strains when cultured in a nutritionally deficient medium and high salinity are representatives natural stress conditions (Stepanovic et al., 2001; Kostaki et al., 2012). According to some reports, the biofilm development was better in the nutrient deficient medium in *E.coli* (Zalewska-Piatek et al. 2013). Nutrient limitation induces stress, which leads to bacterial cells aggregation, biofilm formation and production of polysaccharides (Poole, 2012), while addition of exogenous osmolytes significantly favored the development of biofilm in the presence of salt stress. The presence of biofilm under high salinity seems to offer additional protection under osmotic stress to bacterial strains. This enables the cells to survive in adverse natural environment bacteria in biofilm are more opposed to effect of biocides, antibiotics host defense than free living stages (Boles and singh, 2008). Stepanovic et al. (2001) reported the effect of 2.5 % of NaCl more pronounced on biofilm formation of *S. sciuri* strains. The presence of proteins and polysaccharides are involved in the development of biofilm formation (Beauregard et al., 2013). The recruitment of cells from planktonic phase to sessile

stage (Resser et al., 2007) to form a biofilm matrix is also a shift in life style to overcome salt stress. The development of biofilm shows a tendency of cells to thrive for successful survival under osmotic stress. The enhanced resistance to desiccation in biofilm results in high metabolic cooperation between cells (Hansen et al., 2007). The development of biofilm offer competitive advantage to bacteria existing in the rhizosphere by protecting from the water deprivation effect, present under high salt stress (Bogino et al., 2013). Taken together, findings in this study help to understand the osmoadaptational strategies taken by bacteria under stress. Both these strategies help the halophiles to lessen the osmotic stress and successfully survive in salt stress.

In plant growth experiments, salinity showed the significant negative affects on plant growth. The negative consequences of salinity have been reported in various non leguminous (Sakr et al., 2012) as well as leguminous crops (Kafi et al., 2012; Katerji et al., 2012). However, plant growth was significantly improved when inoculated with bacterial strain. Total soluble sugar and protein contents significantly increased in inoculated plants in control conditions. Accumulation of these osmolytes have been reported to play a role in osmotic adjustments in plant tissues under stress (Mohamed and Ismail, 2011). With increasing salinity, change in metabolites like soluble sugars, proteins and lipids have been reported offering osmoprotection (Chen and Murata, 2011: Aly et al., 2012). These phenomenon seem positively helpful for plants growing at different salinity levels. This is in line with findings in crops like tomatoes and barley (Khosravinejad et al., 2009) showing higher soluble sugar contents at high salinity. Increase in intracellular protein accumulation under salt stress has been reported in plants when inoculated with *Azospirillium* (Hamida and shaddad, 2010). Considering the previous findings showing carbohydrates, proline, protein and soluble sugars very helpful in osmotic adjustments in plant tissues (Amini and Ehsanpour., 2006; Hayat et al., 2012), these strains were found helpful for plant inoculation.

Conclusion

Findings of the study corroborates the view that bacterial osmoadaptation in terms of biofilm formation and osmolytes accumulation is supportive for its successful survival under salt stress. These strategies assist the cells to overcome salt stress. These two strategies might have helped the cells to increase plant growth in saline soil.

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Authors' contribution

All authors contributed equally in preparing this manuscript.

REFERENCES

Afrasayab S, M Faisal and S Hasnain, 2010. Comparative study of wild and transformed salt tolerant bacterial strains on Triticum aestivum growth under salt stress. Brazilian Journal of Microbiology, 41: 946-955.

Agarwal RK, S Singh, KN Bhilegaonkar and VP Singh, 2011. Optimization of microtitre plate assay for the testing of biofilm formation ability in different Salmonella serotypes. International Food Research Journal, 18: 1493–1498

Aly A, A Ahmed, F Khafaga and GN Omar, 2012. Improvement the adverse effect of salt stress in egyptian clover (*Trifolium alexandrinum* l.) by asa application through some biochemical and RT-PCR markers Journal of Applied Phytotechnology in Environmental Sanitation, 1: 91-102.

Ameur H, M Ghoul and J Selvin, 2011. The osmoprotective effect of some organic solutes on Streptomyces sp. MADO2 and Nocardiopsis sp. MADO3 growth. Brazilian Journal of Microbiology, 42: 543-553.

Amini F and AA Ehsanpour, 2006. Response of tomato (Lycopersicon esculentum Mill.) cultivars to MS, water agar and salt stress in in vitro culture. Asian Journal of Plant Sciences, 9: 170-175.

Beauregard PB, YV Chai, H lamakis, R Losick and R Kolter, 2013. *Bacillus subtilis* biofilm induction by plant polysaccharides. Proceedings of the National Academy of Sciences of the United States of America, 110: E1621–E1630.

Bogino P, A Abod, F Nievas and W Giordano, 2013. Water-limiting conditions alter the structure and biofilm-forming ability of bacterial multispecies communities in the alfalfa rhizosphere. PLOS ONE: 8: e79614.

Boles BR and PK Singh, 2008. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proceedings of the National Academy of Sciences of Sciences of the United States of America, 105: 12503-12508.

Bremer E and R Kramer, 2000. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible

solutes in bacteria. In: Storz G., Hengge-Aronis R. (Eds.), Bacterial Stress Responses, Chapter 6, 79-97, ASM Press, Washington, DC, USA, pp. 501.

Burmolle TM, JS Webb, D Rao, LH Hansen, SJ Sorensen and S Kjelleberg, 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Applied and Environmental Microbiology, 72: 3916–3923

Chan YG, HK Kim, O Schneewind and D Missiakas, 2014. The Capsular Polysaccharide of Staphylococcus aureus Is Attached to Peptidoglycan by the LytR-CpsA-Psr (LCP) Family of Enzymes. Journal of Biological Chemistry, 289: 15680-15690.

Chavez-Dozal A, C Gorman, M Erken, PD Steinberg, D McDougald. and MK Nishiguchia, 2013. Predation response of *Vibrio fischeri* biofilms to bacterivorus protists. Applied and Environmental Microbiology, 79: 553–558.

Chen, THH and N Murata, 2011. Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological application space. Plant, Cell and Environment, 34: 1–20 doi: 10.1111/j.1365-3040.2010.02232.x

Christensen GD, WA Simpson, JJ Younger, LM Baddour, FF Barrett, DM Melton and EH Beachey 1985. Adherence of Coagulase negative Staphylococci to plastic tissue culture plates: a quantitative model for the adherence of Staphylococci to medical devices. Journal of Clinical Microbiology, 22: 996-1006.

Chung PY and Toh YS, 2014. Anti-biofilm agents: recent breakthrough against multi-drug resistant *Staphylococcus aureus*. Pathogens and Disease, 70: 231–239. doi: 10.1111/2049-632X.12141

Decho AW, 2010. Overview of biopolymer-induced mineralization: What goes on in biofilms? Ecological Engineering, 36: 137–144.

Deng J, EP Orner, JF Chau, EM Anderson, AL Kadilak, RL Rubinstein, GM Bouchillon, RA Goodwin, D J Gage and LM Shor, 2015. Synergistic effects of soil microstructure and bacterial EPS on drying rate in emulated soil micromodels. Soil Biology and Biochemistry, 83: 116-124. doi:10.1016/j.soilbio.2014.12. 006.

Dimkpa C, T Weinand and F Asch, 2009. Plant–rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell. Environment. 32: 1682–1694.

Dubois M, KA Gilles, JK Hamilton, PA Reberts and F Smiths, 1956. Colorimetric method for determination of sugars and related substances. Annals of Chemistry, 3: 350-356.

Fujishige NA, NN Kapadia, PL De Hoff and AM Hirsch, 2006. Investigations of Rhizobium biofilm formation, FEMS Microbiology Ecology 56: 195–206.

Garrett TR, M Bhakoo and Z Zhang, 2008. Bacterial adhesion and biofilms on surfaces. Progress in Natural Science, 18: 1049-1056.

Gerhardt GG, RGE Murray, WA Wood and NR Kreig, 1994. Methods for general and molecular bacteriology, ASM, 1325. Massachusetts Ave., NW Washington, DC, 2005.

Goh SN, A Fernandez, SZ Ang, WY Lau, DLE Ng and ESG Cheah, 2013. Effects of different amino acids on biofilm growth, swimming motility and twitching motility in *Escherichia Coli* BL21. Journal of Biology and Life Science, 4: 2157-6076

Gravesen A, C Lekkas and S Knochel 2005. Surface attachment of Listeria monocytogenes is induced by sublethal concentrations of alcohol at low temperatures. Applied and Environmental Microbiology, 71: 5601–5603.

Grieve CM and SR Grattan 1983. Rapid assay for determination of water soluble quaternary ammonium compounds. Plant and Soil, 70: 303-307.

Hamdia MA and MAK Shaddad, 2010. Review: Salt Tolerance Of Crop Plants, Journal of Stress Physiology & Biochemistry, 6: 64-90.

Hansen S, P Rainey, J Haagensen and SR Molin, 2007. Evolution of species interactions in a biofilm community. Nature, 445: 533–536

Hayat S, Q Hayat, MN Alyemeni, AS Wani, J Pichtel and A Ahmad, 2012. Role of proline under changing environments: A review. Plant Signaling and Behavior, 7: 1456–1466.

Jefferson KK 2004. What drives bacteria to produce a biofilm? FEMS Microbiology Letters, 236: 163–173.

Kafi M, M Goldani and MHS Jafari, 2012. Effectiveness ofnutrient management in managing saline agro-ecosystems: a case study of lens culinaris medik. Pakistan Journal of Botany, 44: 269-274.

Kahn M, R Kolter, CThomas, D Figurski, R Meyer, E Remaut and DR Helinski, 1979. Plasmid cloning vehicles derived from plasmids ColE 1, F, R6K and RK2. Methods in Enzymology, 268-280.

Karunakaran E and CA Biggs, 2011. Mechanisms of Bacillus cereus biofilm formation: an investigation of the physicochemical characteristics of cell surfaces and

extracellular proteins. Applied Microbiology and Biotechnology, 89: 1161–1175.

Katerji N, M Mastrorilli, FZ Lahmer and T Oweis, 2012. Emergence rate as a potential indicator of crop salt-tolerance. European Journal of Agronomy, 38:1-124

Kempf B and E Bremer, 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Archives of Microbiology, 170: 319–330

Khosravinejad F, R Heydari and T Farboodnia, 2009. Effect of salinity on organic solutes contents in barley. Pakistan Journal of Biological Sciences, 12: 158-162.

Kim YS, JH Seo, HJ Cha, 2003. Enhancement of heterologous protein expression in Escherichia coli by co-expression of nonspecific DNA-binding stress protein, Dps. Enzyme and Microbial Technology, 33: 460-465.

Kloos WE, 1980. Natural populations of the genus Staphylococcus. Annual Review of Microbiology, 34: 559–59

Kobayashi H, M Oethinger, MJ Tuohy, GW Procop and TW Bauer, 2009. Improved Detection of Biofilm-formative Bacteria by Vortexing and Sonication: A Pilot Study. Clinical Orthopaedics and Related Research, 467: 1360–1364.

Kostaki M, N Chorianopoulos, E Braxou, GJ Nychas and E Giaouris, 2012. Differential biofilm formation and chemical disinfection resistance of sessile Cells of *Listeria monocytogenes* Strains under Monospecies and Dual-Species (with *Salmonella enterica*) Conditions. Applied and Environmental Microbiology, 78: 2586–2595.

Leriche V and B Carpentier, 2000. Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms. Journal of Applied Microbiology, 88: 594–605.

Mirza TZ, AN Sabri and S Hasnain, 1998. Salt tolerant bacteria from rhizosphere, rhizoplane, histoplane and phylloplane of Mazus plant inhabitant of salt range. Science International, 10: 151-156.

Mohamed AN and MR Ismail, 2011. Changes in organic and inorganic solutes of in vitro tomato cultivars under NaCl stress. Australian Journal of Crop Science, 5: 939-944

Morikawa M, 2010. Dioxygen activation responsible for oxidation of aliphatic and aromatic hydrocarbon compounds: current state and variants. Applied Microbiology and Biotechnology, 87: 1596–1603.

Moryl M, A Kaleta, K Strzelecki, S Różalska and A Różalski, 2014. Effect of nutrient and stress factors on polysaccharides synthesis in *Proteus mirabilis* biofilm. Acta Biochimica Polonica, 61: 133-9.

Murdock L, T Burke, C Coumoundouros, DE Culham, CE Deutch, J Ellinger, CH Kerr, SM Plater, E To, G Wright and JM Wood, 2014. Analysis of Strains Lacking Known Osmolyte Accumulation Mechanisms Reveals Contributions of Osmolytes and Transporters to Protection against Abiotic Stress. Applied and Environmental Microbiology, 80: 5366–5378. http://doi.org/10.1128/AEM.01138-14

Nagase N, A Sasaki, K Yamashita, A Shimizu, Y Wakita, S Kitai and J Kawano, 2002. Isolation and species distribution of staphylococci from animal and human skin. Journal of Veterinary Medical Science, 64: 245-50.

Nunes RS, CP Souza, KS Pereira, EM Aguila and VM Paschoalin, 2016. Identification and molecular phylogeny of coagulase-negative staphylococci isolates from Minas Frescal cheese in southeastern Brazil: Superantigenic toxin production and antibiotic resistance. Journal of Dairy Science, 99: 2641-2653.

Palomino, MM, MC Allievi, A Grundling, C Sanchez-Rivas and SM Ruzal. 2013. Osmotic stress adaptation in Lactobacillus casei BL23 leads to structural changes in the cell wall polymer lipoteichoic acid. Microbiology, 159: 2416-2426.

Pan X J, Liu, D Zhang, X Chen, L Li, W Song and J Yang, 2010. A comparison of five extraction methods for extracellular polymeric substances (EPS) from biofilm by using three-dimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy. Water, 36: 111-116.

Poole K, 2012. Bacterial stress responses as determinants of antimicrobial resistance Review Journal of Antimicrobial Chemotherapy. 67: 2069-2089.

Qiu X, H Wang, D Liu, L Gong, X Wu and X Xiang, 2012. The physiological response of synechococcus elongates to salinity: a potential biomarker for ancient salinity in evaporative environments Geomicrobiology Journal, 29: 477–483.

Qurashi AW and AN Sabri, 2012. Bacterial exopolysaccharide and biofilm formation stimulate chickpea growth and soil aggregation under salt stress. Brazilian Journal of Microbiology, 43: 1183-1191.

Qurashi AW and AN Sabri, 2013. Osmolyte accumulation in moderately halophilic bacteria improves salt tolerance of chickpea. Pakistan Journal of Botany, 45: 1011-1016.

Reeser RJ, RT Medler, SJ Billington, BH Jost, LA Joens, 2007. Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. Applied and Environmental Microbiology 73: 1908–1913.

Ren D, JS Madsen, SJ Sørensen and M Burmølle, 2014. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. The ISME Journal ISME J, 9: 81-89.

Sakr MT, NM El-Sarkassy and MP Fuller, 2012. osmoregulators proline and glycinebetaine counteract salinity stress in canola. Agronomy for Sustainable Development, 32: 747-754.

Salta M, JA Warton, Y Blache, KR Stokes and JF Briand, 2013. Marine biofilms on artificial surfaces: structure and dynamics. Environmental Microbiology, 15: 2879–2893.

Santos H and MS da Costa, 2002. Compatible solutes of organisms that live in hot saline environments. Mini-review Environmental Microbiology, 4: 501–509.

Sartori M, A Nesci, N Magan and M Etcheverry, 2012. Accumulation of the betaine and ectoine in osmotic stress adaptation of biocontrol agents against *Fusarium verticillioides* in maize. Agricultural Sciences 3: 83-89.

Steel RGD and JH Torrie, 1981. In: Principles and procedures of statistics, a biometrical approach (2nd Edn). McGraw Hill International Book Company, USA.

Stepanovic S, V Dimitrijevic, D Vukovic, I, Dakic, B Savic, and M Svabic-Vlahovic, 2001. *Staphylococcus sciuri* as a part of skin, nasal and oral flora in healthy dogs. Veterinary Microbiology, 82:177-185.

Tahmourespour A and RK Kermanshahi, 2011. The effect of a probiotic strain (*Lactobacillus acidophilus*) on the plaque formation of oral Streptococci. Bosnian Journal of Basic Medical Sciences. 11: 37-40

Talibart R, M Jebbar, K Gouffi, V Pichereau, G Gouesbet, C Blanco, T Bernard and J Pocard, 1997. Transient accumulation of glycine betaine and dynamics of endogenous osmolytes in salt-stressed cultures of *Sinorhizobium meliloti*. Applied and Environmental Microbiology, 63: 4657–4663

Tonon G, C Kevers, O Faivre-Rampant, M Graziani. and T Gaspar, 2004. Effect of NaCl and mannitol iso-osmotic stresses on proline and free polyamine levels in embryogenic Fraxinus angustifolia callus. Plant Physiology, 161: 701-708.

Torres CE, G Lenon, D Craperi, R Wilting, and A Blanco, 2011. Enzymatic treatment for preventing biofilm formation in the paper industry. Applied Microbiology and Biotechnology, 92: 95–103.

Tremblay YD, C Lévesque, RP Segers and M Jacques 2013. Method to grow *Actinobacillus pleuropneumoniae* biofilm on a biotic surface. BMC Veterinary Research, 9: 213.

Tsai M, RL Ohniwa, Y Kato, SL Takeshita, T Ohta, S Saito, H Hayashi and K Morikawa, 2011. *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. BMC Microbiology, 11: 13.

Wang X, AK Dubey, K Suzuki, CS Baker, P Babitzke, and T Romeo, 2005. CsrA post-transcriptionally represses pgaABCD, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. Molecular Microbiology, 56: 1648–1663.

Wood JM, 2015. Bacterial responses to osmotic challenges. The Journal of General Physiology, 145: 381-388.

Wrangstadh M, PL Conway, and S Kjelleberg 1988. The role of an extracellular polysaccharide produced by the marine Pseudomonas sp. S9 in cellular detachment during starvation. Canadian Journal of Microbiology, 35: 309-312.

Zalewska-Piątek B, S Wilkanowicz, P Bruździak, R Piątek and J Kur, 2013. Biochemical characteristic of biofilm of uropathogenic *Escherichia coli* Dr+ strains. Microbiological Research, 168: 367–378

Zhou A, E Baidoo, Z He, A Mukhopadhyay, J K Baumohl, P Benke, MP Joachimiak, M Xie, R Song, AP Arkin, TC Hazen, JD Keasling, JD Wall, DA Stahl and J Zhou, 2013. Characterization of NaCl tolerance in *Desulfovibrio vulgaris* Hildenborough through experimental evolution. The ISME Journal, 7: 1790-1802.