Effect of salt stress on the physiology of *Frankia* sp strain CcI6

REDIET OSHONE[1], SAMIRA R MANSOUR[2] and LOUIS S TISA[1,*]

[1]*Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, USA*

[2]*Faculty of Science, Suez Canal University, Egypt*

**Corresponding author (Email, louis.tisa@unh.edu)*

Actinorhizal plants are able to overcome saline soils and reclaim land. *Frankia* sp strain CcI6 was isolated from nodules of *Casuarina cunninghamiana* found in Egypt. Phylogenetic analysis of *Frankia* sp. strain CcI6 revealed that the strain is closely related to *Frankia* sp. strain CcI3. The strain displays an elevated level of NaCl tolerance. Vesicle production and nitrogenase activity were also influenced by NaCl.

[Oshone R, Mansour SR and Tisa LS 2013 Effect of salt stress on the physiology of *Frankia* sp strain CcI6. *J. Biosci.* **38** 699–702] **DOI** 10.1007/s12038-013-9371-2

1. Introduction

Among the soil-dwelling actinobacteria, members of the genus *Frankia* are distinguished by their ability to form symbiotic nitrogen-fixing associations with a variety of woody dicotyledonous plants (termed actinorhizal plants) representing eight different plant families of angiosperms (for review, see Benson and Silvester 1993; Chaia *et al.* 2010; Wall 2000). Actinorhizal plants are ecologically important as pioneer community plants, are distributed worldwide in a broad range of ecological conditions, and have economic significance in land reclamation, reforestation, soil stabilization, landscaping and fuel. The symbiosis allows actinorhizal plants to colonize harsh environmental terrains. Actinorhizal plants have been successfully used to recolonize and reclaim industrial wastelands including lands contaminated by metalliferous mine spoils and smelter waste (Diem and Dommergues 1990; Wheeler and Miller 1990; Schwencke and Carú 2001; Ridgway *et al.* 2004).

In arid and semi-arid areas, salinization of soils and groundwater is a serious problem causing a drastic reduction in agricultural production. Over 800 million hectares of land throughout the world are salt-affected (Rengasamy 2006). Egypt, which occupies the northeast corner of Africa, belongs to arid and semi-arid climate and greatly suffers from soil salinity. By 1994, 28% of Egypt's soils had been damaged by increased salinity. A common method for dealing with salt stress problems is to reclaim saline soils with a multipurpose, fast-growing salt-tolerant tree species like actinorhizal plants (Giri *et al.* 2003). Among the actinorhizal plants, the genus *Casuarina* has been shown to have ability to grow well under such conditions, and is widely distributed in Egyptian soil.

Frankia sp. strain CcI6 was isolated from *Casuarina cunninghamiana* and shown to re-infect *C. cunninghamiana, Casuarina glauca* and *Causuarina equestifolia* (Mansour and Moussa 2005). Morphologically, the filamentous *Frankia* produces two distinct developmental structures: vesicles and sporangia. Unlike most *Frankia* strains, spore release from sporangial walls occurs spontaneously in medium lacking nitrogen. Gamma-irradiation up to 750 Gy stimulates spore germination and increases plant infectivity. The goal of this study was to test the effects of salt stress on this isolate.

2. Material and methods

2.1 *Frankia growth conditions*

Frankia sp. strain CcI6 was used in this study (Mansour and Moussa 2005). Stock cultures were grown and maintained in 5 mM propionate basal growth medium with NH_4Cl as the nitrogen source, as described previously (Tisa *et al.* 1983,

Keywords. Actinobacteria; actinorhizal symbiosis; genomes; nitrogen fixation; osmotic stress; salt stress

Published online: 1 October 2013

1999). Unless stated otherwise, *Frankia* cultures were incubated at 30°C.

2.2 *Salt sensitivity assay*

A 24-well growth assay was used to determine salt tolerance levels of *Frankia* as described previously (Furnholm *et al.* 2012). Briefly, cells were grown in propionate basal growth medium containing different concentrations of NaCl or sucrose for 14 days and growth was measured by total cellular protein content as described below. Growth yield was determined by subtracting the protein content of the inoculum.

2.3 *Protein content and dry weight determinations*

Total cellular protein content was measured by the bicinchonic acid (BCA) method (Smith *et al.* 1985) according to manufacturer's specifications (Pierce, Rockford, IL, USA) and bovine serum albumin was used as a standard. Cell solubilized in 1 N NaOH were boiled at 95°C for 10 min and centrifuged at 13,000*g* for 5 min. Triplicate measurements were made for each sample. Total cellular dry weight was determined using tarred polycarbonate membranes (Tisa *et al.* 1983)

2.4 *Vesicle induction and nitrogenase activity*

To induce vesicle development and nitrogenase activity, cells were harvested after 14 days of growth in medium containing 5.0 mM NH_4Cl and washed three times with buffer containing 20 mM morpholinepropanesulphonic acid (MOPS) and 10 mM KH_2PO_4 buffer at pH 6.8. The washed cells were inoculated into growth medium lacking a combined nitrogen sources and containing different amounts of NaCl or sucrose. The cultures were incubated at 30°C for 4 days. The vesicle numbers were determined as described previously (Tisa and Ensign 1987). Nitrogenase activity was determined by the acetylene reduction assay as described previously (Tisa and Ensign 1987).

2.5 *DNA extraction, PCR and sequencing*

Genomic DNA was isolated by the CTAB (Wilson 1989). DNA of the 16S rRNA gene was amplified by PCR with the universal primers F27 and R1492 (Weisburg *et al.* 1991) and sequenced.

2.6 *Bioinformatics analysis*

Sequences of the *Frankia* genomes were obtained from Integrated Microbial Genomes System from the Joint Genome Institute (*www.img.doe.gov*) (Markowitz *et al.* 2006). The 16S rRNA gene sequences were aligned using ClustalW (Thompson *et al.* 1997). For phylogenetic analysis, maximum parsimony and neighbour-joining trees were constructed from 1000 bootstrap replicates using MEGA 5.0 (Tamura *et al.* 2011).

3. Results

3.1 *Phylogenetic analysis of Frankia sp. strain CcI6*

The 16S rRNA gene from *Frankia* sp. strain CcI6 was amplified by PCR and sequenced. Figure 1 shows the phylogenetic relationship of CcI6 toward the sequenced *Frankia* genomes. As expected, CcI6 grouped with *Frankia* sp. strain CcI3.

3.2 *Salt tolerance levels of Frankia sp. strain CcI6*

The effect of salt and osmotic stress on the growth of *Frankia* sp. strain CcI6 was examined under nitrogen-sufficient and nitrogen-deficient conditions (figure 2). Under nitrogen-

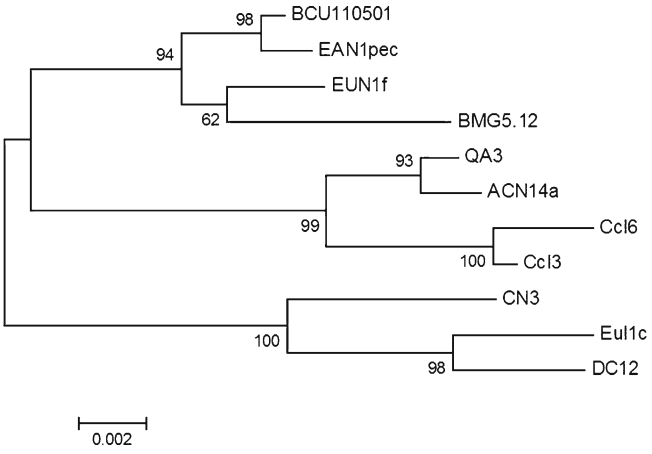

Figure 1. Neighbouring-joining tree of the 16S rRNA genes from CcI6 and sequenced *Frankia* genomes. The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.07102372 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1379 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011)

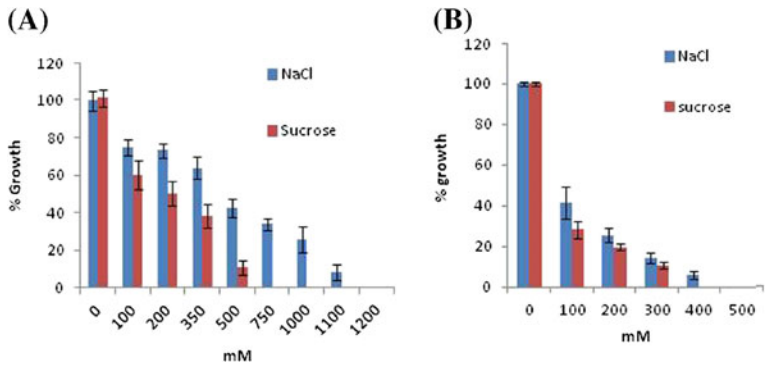

Figure 2. The effect of salt stress on the growth of *Frankia* sp. strain CcI6. (**A**) Cultures were grown in propionate basal growth medium containing 5 mM NH_4Cl under salt or osmotic stress for 14 days. (**B**) Cultures were grown under nitrogen-deficient (N_2) conditions and exposed to different salt or osmotic stress.

sufficient conditions (figure 2A), the strain was highly tolerant to NaCl, exhibiting a minimum inhibiting concentration (MIC) value of 1000 mM and a maximum tolerance concentration (MTC) value of 300 mM. The MIC and MTC values for sucrose were 600 and 50 mM, respectively. Under nitrogen-deficient conditions (figure 2B), the cultures were more sensitive to salt and osmotic stress and the MIC values of NaCl and sucrose decreased to 500 and 400 mM, respectively.

3.3 *Effect of salt stress on vesicle induction and nitrogenase activity*

The effect of salt and osmotic stress on vesicle production and nitrogenase activity was measured (figure 3). Vesicle production was inhibited by NaCl. At 400 mM NaCl, a greater than 100-fold decrease in vesicle numbers occurred and 500 mM NaCl completely blocked vesicle production. Sucrose had similar effect on vesicle production. At 300 mM sucrose, a 10-fold reduction in vesicle number and 400 mM sucrose caused a complete inhibition of vesicle production. Nitrogenase activity was inhibited by NaCl and 200 mM drastically reduced activity (15%). With sucrose, the 100 mM sucrose had no effect on activity. The increased concentrations of sucrose drastically reduced activity at 200 mM and were completely inhibited at 400 mM. On a per-vesicle basis, the effect of sucrose was more evident and showing little inhibition at concentration up to 300 mM. With NaCl, activity decreased with the values above 100 mM until it was completely inhibited at 400 mM.

4. Discussion

When compared to other *Frankia* stains (Hafeez *et al.* 1999; Tani and Saskkawa 2000; 2003), *Frankia* sp. strain CcI6 was highly tolerant to salt stress under nitrogen-sufficient conditions (figure 2A). Vesicle production and nitrogenase activity were affected by salt stress. Osmotic stress also had an effect on these activities, but was less drastic.

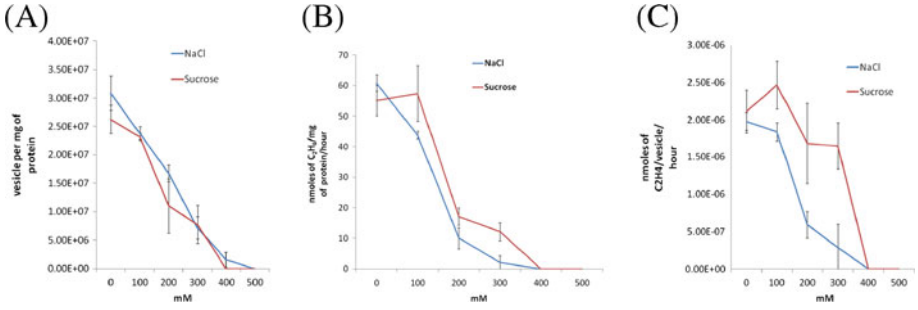

Figure 3. The effect of salt stress on the vesicle formation and nitrogenase activity by *Frankia* sp. strain CcI6. Cultures were grown under nitrogen-deficent conditions with various degrees of salt or osmotic stress. Panel (**A**) shows vesicle production. Panel (**B**) shows nitrogenase activity expressed on a protein basis, while panel (**C**) show nitrogenase activity expressed on a per-vesicle basis.

Salt stress affects enzyme activities and interferes with basic metabolisms. Bacteria employ several mechanisms to maintain homeostasis under salt stress involving osmolyte synthesis, ion transport and reactive oxygen species (ROS) scavenging. Osmolytes accumulated include amino acids such as proline, serine, and glutamine, polyamines, polysaccharides, organic solutes, soluble sugars, and inorganic cations such as K^+ (Abdelaal *et al.* 2006). We are sequencing the genome of *Frankia* sp strain CcI6 to reveal the mechanisms of salt tolerance and provide a greater understanding of the physiology of this isolate.

Acknowledgements

This work was supported in part by US-Egypt Joint Research Grant BIO13-001.

References

Abdelaal S, Julius N, Dietrich W 2006 A Proteomic Approach Towards the Analysis of Salt Tolerance in *Rhizobium etli* and *Sinorhizobium meliloti* Strains. *Curr. Microbiol*. **52** 333–339

Benson DR and Silvester WB 1993 Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Mol. Biol. Rev*. **57** 293–319

Chaia EE, Wall LG and Huss-Danell K 2010 Life in soil by the actinorhizal root nodule endophyte *Frankia.* A review. *Symbiosis* **51** 201–226

Diem HG, Dommergues YR 1990 Current and potential uses and management of Casuarinacae in the topics and subtropics; in *The biology of frankia and actinorhizal plants* (eds) CR Schwintzer and JD Tjepkema (San Diego, CA: Academic Press) pp 317–342

Felsenstein J 1985 Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39** 783–791

Furnholm T, Beauchemin N and Tisa LS 2012 Development of a semi-high-throughput growth assay for filamentous actinobacteria *Frankia*. *Arch. Microbiol.* **194** 13–20

Giri B, Kapoor R and Mukerji KG 2003 Influence of arbuscular mycorrhizal fungi and salinity on growth, biomass, and mineral nutrition of *Acacia auriculiformis*. *Biol. Fertility Soils* **38** 170–175

Hafeez FY, Hameed S and Malik KA 1999 *Frankia* and *Rhizobium* strains as inoculum for fast growing trees in saline environment. *Pak. J. Bot*. **31** 173–182

Mansour SR and Moussa LAA 2005 Role of Gamma-radiation on spore germination and infectivity of *Frankia* strains CeI523 and CcI6 isolated from Egyptian *Casuarina*. *Isotope Rad. Res.* **37** 1023–1038

Markowitz VM, Korzeniewski F, Palaniappan K, Szeto E, Werner G, Padki A, Zhao XL, Dubchak I, *et al.* 2006 The integrated microbial genomes (IMG) system. *Nucleic Acids Res.* **34** D344–D348

Rengasamy P 2006 World salinization with emphasis on Australia. *J. Exp. Bot.* **57** 1017–1023

Ridgway KP, Marland LA, Harrison AF, Wright J, Young JPW and Fitter AH 2004 Molecular diversity of *Frankia* in root nodules of *Alnus incana* grown with inoculum from polluted urban soils. *FEMS Microbiol. Ecol.* **50** 255–263

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, *et al.* 1985 Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150** 76–85

Schwencke J and Carú M 2001 Advances in actinorhizal symbiosis: host plant - *Frankia* interactions, biology, and applications in arid land reclamation. A review. *Arid. Land Res. Manag.* **15** 285–327

Saitou N and Nei M 1987 The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4** 406–425

Tamura K, Nei M and Kumar S 2004 Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* **101** 11030–11035

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S 2011 MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28** 2731–2739

Tani C and Saskkawa H 2000 Salt tolerance of *Elaeagnus macrophylla* and *Frankia* EmaI strain isolated from root nodules of *E. macrophylla*. *Soil Sci. Plant Nutr.* **46** 927–937

Tani C and Saskkawa H 2003 Salt tolerance of *Casuarina equisetifolia* and *Frankia* Ceq1 strain isolated from root nodules of *C. equisetifolia*. *Soil Sci. Plant Nutr.* **49** 215–222

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG 1997 The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25** 4876–4882

Tisa LS, Chval M, Krumholz G and Richards J 1999 Antibiotic resistance patterns of Frankia strains. *Can. J. Bot* **77** 1257–1260

Tisa L, McBride M and Ensign JC 1983 Studies on the growth and morphology of *Frankia* strains EAN1pec, EuI1c, CpI1 and $ACN1^{AG}$. *Can. J. Bot.* **61** 2768–2773

Tisa LS and Ensign JC 1987 Comparative physiology of nitrogenase activity and vesicle development for *Frankia* strains CpI1, $ACN1^{AG}$, EAN1pec and EUN1f. *Arch. Microbiol*. **147** 383–388

Wall LG 2000 The actinorhizal symbiosis. *J. Plant Growth Regul.* **19** 167–182

Weisburg WG, Barns SM, Pelletier DA and Lane DJ 1991 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173** 697–703

Wheeler CT, Miller IM 1990 Current and potential uses of actinorhizal plants in Europe; in *The biology of frankia and actinorhizal plants* (eds) CR Schwintzer and JD Tjepkema (San Diego, CA: Academic Press) pp 365–389

Wilson K 1989 Preparation of genomic DNA from bacteria; in *Current protocols in molecular biology* vol 1 (eds) FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith and K Struhl (New York, New York: Greene Publishing Associate & Wiley Interscience) pp 2.4.1–2.4.5