World Journal of Microbiology & Biotechnology (2006) 22: 603–606
DOI 10.1007/s11274-005-9077-1

Salinity-induced accumulation of poly-β-hydroxybutyrate in rhizobia indicating its role in cell protection

N.K. Arora[1,*], V. Singhal[1] and D.K. Maheshwari[2]
[1]*Department of Microbiology, Institute of Biosciences and Biotechnology, C.S.J.M. University, Kanpur, India;*
[2]*Department of Botany and Microbiology, Gurukul Kangri University, 249 404, Hardwar, Uttaranchal, India;*
*(*Author for correspondence: Tel.: +91-1334-254611; E-mail: nkarora_net@rediffmail.com)*

Received 8 August 2004; accepted 4 October 2005

Keywords: Cell protection, poly β-hydroxybutyrate, salinity, *Sinorhizobium*

Summary

Poly β-hydroxybutyrate (PHB) is an energy and carbon storage material accumulated in response to the limitation of an essential nutrient. The effect of different salt concentrations on growth and PHB accumulation of four different *Sinorhizobium* strains was examined. Irrespective of the strain, a defined trend in the accumulation of PHB inside the cells was observed. While minimum PHB content was accumulated at low or zero salinity, maximum was observed by the salt-tolerant strains at higher salt concentrations. This suggests a definite role for PHB in cell protection in saline conditions.

Introduction

Of the world's total land surface 40% can be categorized as having potential salinity problems (Lal & Khanna 1995). A composite stress like salinity having both ionic and osmotic components can be extremely detrimental for growth of soil-inhabiting root-nodulating bacteria. It has been reported that cellular adaptation in bacteria in the presence of salinity stress is achieved by accumulation of high intracellular concentrations of inorganic ions, low organic mass organic solutes or induction of stress proteins (Gouffi *et al.* 2000).

Poly β-hydroxybutyrate (PHB) is the most abundant of a general class of optically active microbial polyesters (Anderson & Dawes 1990). This microbial polyester is usually formed as intracellular inclusions during unbalanced growth. Rhizobia have been reported to produce PHB up to 55% of cell dry mass (Tombolini & Nuti 1989). It is now well established that PHB is accumulated during excess carbon in response to the limitation of an essential nutrients like N, P or O_2 and serves as an internal reserve of carbon (Mercan *et al.* 2002). The ability to synthesize and degrade PHB may influence the capability of bacterial cells to survive extended periods of starvation in the soil (Anderson & Dawes 1990). It has been suggested that intracellular PHB accumulated in the rhizosphere may be an important source of carbon and energy during the root hair infection by rhizobia (Charles *et al.* 1997). Stam *et al.* (1986) observed two possible roles of PHB, protection of nitrogenase when oxygen concentration in nodule rises and serving as carbon and energy source during starvation outside the nodule.

High salinity is one of the common stresses faced by the soil bacteria in tropical and subtropical regions. A large fraction of agricultural lands occur in regions affected by saline soil. In India alone about 7.5 million hectares of land are saline or alkaline (Kumar *et al.* 1999). Salt-tolerant rhizobial strains along with legumes can be utilized for reclamation of saline and alkaline lands. It has been reported that microorganisms accumulate intracellular inorganic ions such as potassium, amino acids, betaines or carbohydrates to equilibrate osmotic pressure (Hua *et al.* 1982). The present investigation was undertaken to assess the possible role of PHB in the stress tolerance of such agronomically important microorganisms as rhizobia. The PHB content of the rhizobial cells was determined under the stress of different salt concentrations in four different strains of *Sinorhizobium*.

Materials and methods

Strain *S. meliloti* MTCC 3402 was procured from IMTECH, Chandigarh. Three other strains, namely *S. meliloti* (RMP_5), *Sinorhizoium* (JB_1) and *Sinorhizobium* sp. (soybean nodulating) were procured from Department of Botany and Microbiology, Gurukul Kangri University, Haridwar, Uttaranchal, India. The bacterial

strains were first of all screened for the ability to tolerate different NaCl concentrations. The effect of different concentrations of salt was determined in yeast extract mannitol (YEM) broth (Vincent 1970) supplemented with different concentrations of NaCl (0–1000 mM). The salt-amended broths were inoculated and kept in an incubator shaker at 30 °C and 150 rev/min. The bacterial growth was observed in the form of absorbance at 610 nm up to 48 h using Shimadzu UV-VIS spectrophotometer (model UV-1601) (Kumar *et al.* 1999). The GI_{50} value that is the concentration of NaCl at which the growth of strains was inhibited by 50%, was determined according to Unni & Rao (2001).

For the determination of effect of different NaCl concentrations on cellular PHB content, salt-supplemented YEM broth inoculated with rhizobial strains was incubated at 30 °C and 150 rev/min for a period of 48 h. After incubation the broth was centrifuged at 18,785×*g* at 4 °C for 15 min by Remi cooling centrifuge (model C-30), to separate the cells from broth. The bacterial pellet formed was treated and PHB extracted by hot chloroform according to the method of Poindexter & Eley (1983). The acetone-insoluble, pure white residue of PHB remaining after extraction was measured directly by dry weight determination (Page & Knosp 1989). The purity of PHB was checked by converting it into crotonic acid by hot sulphuric acid treatment and taking spectra from 220 to 260 nm using a Shimadzu UV-VIS spectrophotometer (Law & Slepecky 1961).

Results and discussion

All the four bacterial strains were able to tolerate varied NaCl concentrations in YEM broth. While strains *Sinorhizobium* JB_1 and *S. meliloti* MTCC 3402 were tolerant, *S. meliloti* (RMP_5) and *Sinorhizobium* sp. (soybean) were sensitive to higher NaCl concentrations. Strain *Sinorhizobium* JB_1 was found to be the most tolerant. It could tolerate up to 900 mM NaCl concentration (Figure 1). However a 50% inhibition in growth (GI_{50}) was observed at 700 mM salt concentration. Strain JB_1 was followed by *S. meliloti* MTCC 3402, which could also tolerate 900 mM NaCl but its GI_{50} being at 600 mM NaCl concentration (Figure 1). Growth of both *Sinorhizobium* JB_1 and *S. meliloti* MTCC 3402 was even enhanced up to 300 mM NaCl concentration. Previous reports (Hua *et al.* 1982; Arora *et al.* 2000) also observed stimulation of rhizobial growth with slight increase in NaCl concentration. Strains *S. meliloti* (RMP_5) and *Sinorhizobium* sp. (soybean) showed 50% growth inhibition at 400 and 300 mM NaCl concentration, respectively (Figure 1). Earlier Kumar *et al.* (1999) observed salt tolerance up to 800 mM concentration by rhizobial strains. Singleton *et al.* (1982) even reported that rhizobia isolated from arid regions can grow in solutions with salinity as high as that of sea water.

All the four rhizobial strains showed a distinct pattern as far as PHB accumulation was concerned and maximum intracellular PHB was found at higher NaCl concentrations (Table 1). Both the salt-tolerant strains *Sinorhizobium* JB_1 and *S. meliloti* MTCC 3402 accumulated maximum PHB at higher NaCl concentrations. While *Sinorhizobium* JB_1 accumulated maximum PHB at 700 mM NaCl, *S. meliloti* MTCC 3402 did it at 600 mM. On the other hand strains *S. meliloti* RMP_5 and *Sinorhizoium* sp. (soybean) showed maximum PHB synthesis at 300 mM NaCl. All of the strains showed minimum PHB accumulation at optimum conditions that is in controls (YEM broth without salt) which increased with increase in salt concentration. There was an 89% increase in PHB accumulation by the strain JB_1 in presence of 700 mM NaCl as compared to the control, which declined steeply with further increase in salinity. Similarly strain *S. meliloti* MTCC 3402 showed an increase of 73% from 0 to 600 mM NaCl concentration (Table 1). PHB accumulation thus depended upon the ability of strains to tolerate the salts. In salt-tolerant strains, PHB accumulation reached maximum at higher salinities. Also more PHB accumulation was observed in salt-tolerant strains as compared to sensitive ones. The similarity in PHB accumulation pattern, independent of strain type indicates a certain role of PHB in cellular osmoregulation in rhizobia. It has been reported that under unfavorable conditions in which a non-carbon nutrient such as N, P, K or O_2 is limiting for growth, many bacteria accumulate PHB in the cells (Aneja & Charles 1999). During high osmotic stress due to increased salinity bacterial cells find it difficult to absorb N, K or other ions. It may be due to this reason that PHB starts accumulating in the cells. Kim *et al.* (1999) also confirmed the accumulation of PHB in bacterial cells because of potassium limitation. There can be another benefit of the accumulation of PHB in bacteria at high salt concentrations. According to Anderson & Dawes (1990), one of the major advantages in synthesis of PHB is that by doing this bacteria store large quantities of reduced carbon without significantly affecting the osmotic pressure of the cell. We suggest that by storing large quantities of reduced carbon in the form of PHB during the osmotic stress, the bacterial cells may balance the osmotic pressure imposed by the environment. However, further study is needed to confirm the role of PHB which is accumulated in rhizobial cells during high saline conditions.

Large scale use of PHB-based biodegradable thermoplastics and biopolymers has been restricted due to high production costs (Ackermann & Babel 1998; Sujatha *et al.* 2005). This work reveals one of the physical conditions which can be exploited to bring the PHB production costs down by increasing the product yield. The results suggest a certain role of PHB in the protection of bacterial cells in extremely saline conditions. Apart from this, the study also reports highly salt-tolerant rhizobial

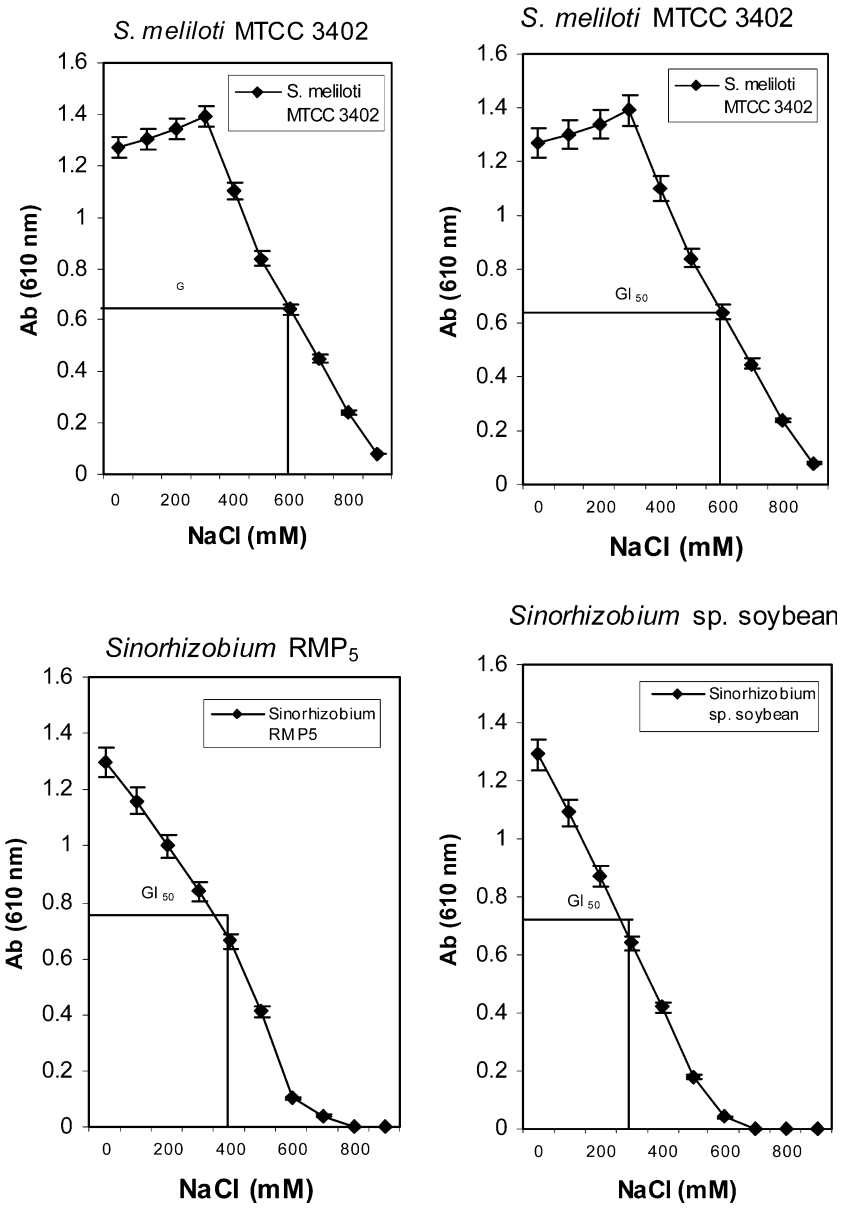

Figure 1. Effect of different salt concentrations on the growth of different *Sinorhizobium* strains. Values represented are mean of 05 replicates ± SE.

Table 1. Effect of different NaCl concentrations (mM) on the accumulation of PHB (in mg/ml) in different *Sinorhizobium* strains.

Strain	0	100	200	300	400	500	600	700	800	900
Sinorhizobium JB_1	0.72^{a}	0.73^{a}	0.75^{a}	0.81^{b}	0.96^{c}	1.15^{d}	1.27^{e}	1.36^{f}	0.78^{ab}	0.31^{g}
S. meliloti MTCC 3402	0.80^{a}	0.82^{a}	0.83^{a}	0.85^{a}	1.01^{b}	1.21^{c}	0.38^{d}	0.92^{ab}	0.39^{e}	0.08^{f}
S. meliloti RMP_5	0.86^{a}	0.91^{a}	1.04^{b}	1.15^{c}	0.87^{a}	0.49^{c}	0.18^{d}	0.03^{e}	–	–
Sinorizobium sp.	0.72^{a}	0.80^{b}	0.83^{b}	0.91^{c}	0.80^{b}	0.36^{c}	0.18^{d}	0.02^{e}	–	–

Results are mean of 05 replicates. Means in the column followed by different letter are significantly different at $P < 0.05$.

strains which can be very useful in reclamation of saline soils.

Acknowledgements

Financial assistance provided by Department of Science and Technology is gratefully acknowledged. Thanks are due to the Vice-Chancellor, CSJM University, Kanpur, providing necessary facilities and support.

References

Ackermann, J.U. & Babel, W. 1998 Approaches to increase the economy of the PHB production. *Polymer Degradation and Stability* **59**, 183–186.

Anderson, A.J. & Dawes, E.A. 1990 Occurrence, metabolism, metabolic role and industrial uses of bacterial poly hydroxyalkanoates. *Microbiological Reviews* **54**, 450–472.

Aneja, P. & Charles, T.C. 1999 Poly-3-hydroxybutyrate degradation in *Rhizobium* (*Sinorhizobium*) *meliloti*: isolation and characterization of a gene encoding 3-hydroxybutyrate dehydrogenase. *Journal of Bacteriology* **181**, 849–857.

Arora, N.K., Kumar, V. & Maheshwari, D.K. 2000 Isolation of both fast and slow growing rhizobia effectively nodulating a medicinal legume. *Mucuna pruriens*. *Symbiosis* **29**, 121–137.

Charles, T.C., Cai, G.Q. & Aneja, P. 1997 Megaplasmid and chromosomal loci for the PHB degradation pathway in *Rhizobium* (*Sinorhizobium*) *meliloti*. *Genetics* **146**, 1211–1220.

Gouffi, K., Bernard, T. & Blanco, C. 2000 Osmoprotection by pipecolic acid in *Sinorhizobium meliloti* specific effects of D and L isomers. *Applied and Environmental Microbiology* **66**, 2358–2364.

Hua, S.T., Tsai, V.Y., Lichens, G.M. & Noma, A.T. 1982 Accumulation of amino acids in *Rhizobium* sp. Strain WR 1001 in response to sodium chloride salinity. *Applied and Environmental Microbiology* **44**, 135–140.

Kim, S., Kim, P. & Kim, J. 1999 Maximization of poly-β-hydroxybutyrate accumulation by potassium limitation in Methylobacterium organophilum andits related metabolic analysis. *Journal of Bacteriology* **9**, 140–146.

Kumar, H., Arora, N.K., Kumar, V. & Maheshwari, D.K. 1999 Isolation, characterization and selection of salt tolerant rhizobia nodulating *Acacia catechu* and *A nilotica*. *Symbiosis* **26**, 279–288.

Lal, B. & Khanna, S. 1995 Selection of salt-tolerant *Rhizobium* isolates of *Acacia nilotica*. *World Journal of Microbiology and Biotechnology* **10**, 637–639.

Law, J.H. & Slepecky, R.A. 1961 Assay of poly-β-hydroxybutyric acid. *Journal of Bacteriology* **82**, 33–36.

Mercan, N., Aslim, B., Yüksegdag, Z.N. & Beyatl, Y. 2002 Production of poly-β-hydroxybutyrate (PHB) by some *Rhizobium* bacteria. *Turkish Journal of Biology* **26**, 215–219.

Page, W.J. & Knosp, O. 1989 Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. *Applied and Environmental Microbiology* **55**, 1334–1339.

Poindexter, J.S. & Eley, L.F. 1983 Combined procedure for assay of poly-β-hydroxybutyric acid and inorganic phosphate. *Journal of Microbiological Methods* **1**, 1–17.

Singleton, P.W., El Swaify, S.A. & Bohlool, B.B. 1982 Effect of salinity on *Rhizobium* growth and survival. *Applied and Environmental Microbiology* **44**, 884–892.

Stam, H., van Verseveld, H.W., de Vries, W. & Stouthamer, A.H. 1986 Utilisation of poly-β-hdroxybutyrate in free living cultures of *Rhizobium* ORS571. *FEMS Microbiology Letters* **35**, 215–220.

Sujatha, K., Mahalakshmi, A. & Shenbagarathai, R. 2005 A study on accumulation of PHB in native *Pseudomonas* isolates LDC-5 and LDC-25. *Indian Journal of Biotechnology* **4**, 216–221.

Tombolini, R. & Nuti, M.P. 1989 Poly (β-hydroxyalkanoate) biosynthesis and accumulation by different *Rhizobium* species. *FEMS Microbiology Letters* **60**, 299–304.

Unni, S & Rao, K.K. 2001 Protein and lipopolysaccharide profiles of a salt-sensitive *Rizobium* sp. and its exopolysaccharide-deficient mutant. *Soil Biology and Biochemistry* **33**, 111–115.

Vincent, J.M. 1970 *A Manual for the Practical Study of Root Nodule Bacteria (IBH Handbook 5)*, Oxford: Blackwell Scientific Publications. ISBN 0632064102.