IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 1 Issue 7, September 2014.
www.ijiset.com
ISSN 2348 – 7968

Molecular characterization of Phosphate Solubilizing Bacteria (PSB) and Plant Growth Promoting Rhizobacteria (PGPR) from pristine soils

David Paul Raj R. S., Linda R. and Rhema Susan Babyson

Department of Biotechnology, School of Biotechnology and Health Sciences, Karunya Institute of Technology and Sciences, Coimbatore – 641114.

Abstract

Plant growth-promoting bacteria (PGPB) are associated with many plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere. Free-living PGPR have shown promise as biofertilizers. In addition, phosphorous solubilizing bacteria (PSB) are important for crop plants as they increase phosphorous uptake and play a crucial role as PGPR in the Crop growth enhancement. A total of 53 isolates were isolated from forest soils of Siruvani Hills. The plant growth promoting and phosphate solubilizing efficacy of these isolates was determined under *in-vitro* conditions. Ribotyping of the 16S rDNA of the promising PSB isolates was performed and the genetic variability within the strains was analyzed by comparing the sequence through BLAST homology and CLUSTAL W2 analysis.

Keywords: Plant Growth Promoting Rhizobacteria, Phosphate Solubilizing Bacteria, 16S rDNA sequencing.

Introduction

Increased population has led to an intensification of agricultural production over the past few decades; producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations. However, increasing use of chemical inputs causes several negative effects. Furthermore, the growing cost of pesticides and consumer demand for pesticide-free food has led to a search for substitutes for these products (Gerhardson 2002). Pesticides also pose a threat to food production and ecosystem stability worldwide (Stephane et al. 2005). Hence, biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Gerhardson 2002; de Weger et al. 1995; Postma et al. 2003).

These bio-control agents are also easy to deliver, improve plant growth, and activate resistance mechanism in the host, and increase production and yield (Nakkeeran et al. 2005). One possibility for substantially increasing plant yields without imposing environmental threats is to make use of certain microorganisms that protect plants from the deleterious microorganisms that occur in all agricultural soils (Radheshyam et al. 1990).

The rhizosphere harbors a diverse array of microorganisms including the PGPRs. PGPR exert a direct effect on plant growth by production of phytohormones, solubilization of inorganic phosphates, increased iron nutrition through iron-chelating siderophores and the volatile compounds that affect the plant signaling pathways. Additionally, by antibiosis, competition for space and nutrients and induction of systemic resistance in plants against a broad-spectrum of root and foliar pathogens, PGPR reduce the populations of root pathogens and other deleterious microorganisms in the rhizosphere, thus benefiting the plant growth (Podile and Kishore 2007).

One of the attribute of PGPR is the solubilization of mineral nutrients of which Phosphorous is the major nutrient. PSB play role in phosphorus nutrition by enhancing its availability to plants through release from inorganic and organic soil phosphate pools by solubilization and mineralization. Principal mechanism in soil for mineral phosphate solubilization is lowering of soil pH by microbial production of organic acids and mineralization of organic phosphate by acid phosphatases. Use of phosphorus solubilizing bacteria as inoculants increases P uptake (Ahmad Ali Khan et al. 2005). The primary objective of this work is to isolate and identify efficient PGPR and PSB from forest soils of Siruvani Hills, Coimbatore, Tamilnadu and to ribotype the promising PSB isolate.

Materials and methods

Sample collection and bacterial isolation

Soil samples were collected from forest soils of Siruvani Hills, Coimbatore and brought to the laboratory in polythene bags. The samples were serially diluted. One millilitre of each 10^{-3} to 10^{-5} dilution was pipetted out and poured into sterile

ISSN 2348 – 7968

Petri dishes containing King's B (KB) medium (King et al. 1954) and Nutrient agar medium, spread plated and incubated at room temperature (30^0C) for 24 h. The colonies with different morphology were selected, subcultured and stored at 4^0C.

Determination of PGPR efficacy of bacterial isolates

Plant growth-promoting activity of highly morphologically distinct 10 bacterial isolates was assessed on six varieties of rice cultivated widely in Coimbatore district (C048, C049, ASD16, ADT39, ADT43 & ADT45) by the standard roll towel method (ISTA 1966). The isolated bacterial strains were grown in 100 ml of KB broth and Nutrient broth respectively for 48 h on a rotary shaker. Rice seeds were surface sterilized and dried on sterile filter paper under aseptic conditions. Rice seeds were soaked in 20 ml of bacterial suspension for 2 hours and dried overnight under aseptic conditions. These seeds were germinated as per the standard roll towel method and incubated at room temperature. The untreated seeds served as control. The root length and shoot length of individual seedlings was measured and the germination percentage of seeds were calculated. The seed vigour index was calculated using the formula (Baki and Anderson 1973).

Vigour Index = (Mean root length + Mean shoot length) x Germination (%)

Statistical analysis

Statistical analysis (ANOVA) was done. The results were obtained for the different sets of experiments using SPSS16 at 5% probability level ($P < 0.05$).

Determination of the efficacy of the PSB on Pikovskaya medium

The efficacy of phosphate-solubilization was tested qualitatively by streaking the bacterial cultures on the surface of Pikovskaya medium (Pikovskaya 1948). The presence of clearing zone around bacterial colony after incubation for 2 days was used as an indicator for positive phosphate solubilization (Husen 2003).

Determination of the efficacy of PSB in NBRIP medium and broth

The *in vitro* phosphate-solubilizing capacity of each strain was determined on NBRIP (National Botanical Research Institute's Phosphate) growth medium containing tricalcium phosphate for a period of 14 days (Nautiyal 1999).

PS bacteria were inoculated into 50 ml NBRIP broth. Uninoculated broth served as control. After incubation, pH of the medium was determined every 24 hrs for 3 days (Chen et al. 2006).

Determination of IAA (Indole Acetic Acid) produced by the PSB isolates

The isolates were inoculated in 5 ml NB and kept for overnight incubation at 37^0C. About 20 µl aliquots of culture broth were transferred into another 5 ml NB supplemented with L-tryptophan at a final concentration of 500 µg ml^{-1} (from 2-mg ml^{-1} stock prepared in warm water). After incubation for 72 hrs, the culture broth was centrifuged at 3500 rpm for 10 mins and the supernatant was collected. One ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent using a vortex mixer, incubated at room temperature for 20 min and the absorbance was measured at 535 nm in a UV-Visible spectrophotometer. The uninoculated Trp-containing medium mixed with the Salkowski reagent was used as blank. The concentration of IAA in each culture medium was determined by comparison with standard IAA curve (Noura Raddadi 2007).

DNA isolation, PCR and 16S rDNA sequencing

Promising PSB bacterial isolates were cultured in 50 ml of Nutrient broth overnight at 30^0C. The bacterial cells were harvested by centrifugation and genomic DNA was extracted and purified (Sambrook and Russel 2007). 16S rRNA gene of the DNA was amplified by using the universal primers. The amplification reaction was performed in a thermal-cycler (Eppendorf thermocycler) programmed for an initial cycle of 95^0C for 5 min, followed by 35 cycles of 95^0C for 30s, 52.5^0C for 30s, 72^0C for 2 min, followed by a final extension at 72^0C for 7 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). These isolates were subjected to sequencing and the sequences were obtained.

Results and Discussion

Isolation of the bacterium

A total of 53 isolates were isolated from 6 different soil samples. The isolates were named as S5.1 to S5.10, S6.1 to S6.11, A1.1 to A1.8, A2.1 to A2.6, SI5.1 to SI5.13, SA4.1, SA4.4, SA4.5, SA4.8 and SA4.9.

Efficacy of PGPR activity

The PGPR activity was measured in terms of seed vigour index (Table 1). The isolates that showed high seed vigour index were selected as effective PGPRs. Isolates S5.7, S6.5, S5.6, S5.1 exhibited promising PGPR activity on CO48, ADT39 and ADT43, ADT45 and CO49, ASD16 respectively (Fig. 1, Fig 2) (Table 1). At the same time the isolates exhibited difference in shoot and root length promotion activity against the six varieties of rice (Table 2, 3).

Phosphate solubilizing efficacy of bacterial isolates on Pikovskaya medium

The isolates were screened for the phosphate solubilizing activity by inoculating onto

ISSN 2348 – 7968

Pikovskaya medium with dicalcium phosphate. All the isolates were screened and the results were determined based on the zone of clearance observed after 24 h. The isolate A1.3 exhibited highest zone of clearance with 10 mm radii followed by SA4.5 with zone of clearance of 9 mm radii, SA4.1 with a zone of clearance of 7 mm radii, SI5.10 with a zone of clearance of 3 mm radii and SI5.1 with zone of clearance of 2 mm radii (Fig 3).

Efficacy of PSB in NBRIP medium and broth

Although few bacterial isolates exhibited promising PS activity on Pikovskaya medium with dicalcium phosphate only one isolate SA4.1 showed a better clearance of 6 mm radii on NBRIP medium with tricalcium phosphate.

But the solubilization of tricalcium phosphate in the liquid medium by different strains was accompanied by a significant drop in pH. This was determined over a period of 3 days. All the isolates exhibited a reduction in pH which ranges from pH 4-5 from the initial pH of 7. This confirms the potentiality of the isolates to solubilize tribasic phosphate (Table 4).

Determination of IAA (Indole Acetic Acid) produced by the PSB isolates

After 72 hrs of incubation the concentration of IAA was determined for four efficient PSB isolates by comparing with the standard curve of IAA. The isolate A1.3 was found to produce highest level of IAA when compared to the other isolates (4.5 µg/5 ml), followed by isolate SA4.8 producing 4 µg/5 ml, isolate SA4.5 producing 3 µg/5 ml and the isolate SA4.1 producing 1 µg/5 ml of IAA.

DNA isolation, PCR and 16S rDNA sequencing

16S rDNA sequencing was performed (ABI 3130XL GENETIC ANALYSER) for the promising PSB isolates A1.3, SA4.1, SA4.5, SI5.1 and the gene sequence was compared with the available data in GenBank using BLAST homology search to identify the isolates at the generic level. The isolate A1.3 displayed close homology to *Bacillus thuringiensis*, SA4.1 to *Pseudomonas panipatensis*, SA4.5 to *Sinomonas atrocyanea* and the isolate SI5.1 to *Pseudomonas monteilii*. These sequences were submitted to the NCBI database and the accession numbers were obtained respectively **(Accession no: JQ389018, JQ769107, JQ769106, JQ769105)**. The Phylogenetic analyses of the obtained 16S rDNA sequences was also performed with the CLUSTAL W2 software (Fig 4, 5, 6 & 7).

The plant rhizosphere is a versatile and dynamic ecological environment of intense microbes–plant interactions for harnessing essential micro and macro-nutrients from a limited nutrient pool (Jeffries et al. 2003).

In the Present study, 53 bacterial isolates were isolated from the forest soils of Siruvani hills. Ten bacterial isolates were initially selected and their PGPR activity was tested under *in vitro* conditions. Four isolates compared to the control exhibited promising PGPR activity. The isolates also exhibited varietal difference in their growth promotional activity.

Phosphate solubilization was most frequently encountered by Bacillus isolates (80%), followed by Azotobacter, Pseudomonas and least by other isolates (Farah Ahmad 2008) and this was in accordance with our results. Out of the 53 isolates tested for Phosphate solubilizing activity, four bacterial isolates (A1.3, SA4.1, SA4.5 and SI5.1) showed promising phosphate solubilizing activity. These isolates were ribotyped and based on sequence comparison and phylogenetic analysis the isolates were confirmed to be *Bacillus thuringiensis*, *Pseudomonas panipatensis*, *Sinomonas atrocyanea* and *Pseudomonas monteilii*. This is a first report of *Sinomonas atrocyanea* exhibiting phosphates solubilisation efficacy from south India. All the other isolates are reported for the first time from the forest soils of Siruvani Hills. This study reveals that promising PGPR and PSB isolates can be isolated from the forest soils rich in natural resources which will help us to identify promising and new potentiality of the isolates compared with isolates isolated from conventional agriculture fields.

Acknowledgements

This work was supported by Karunya University's short term research grant. We would like to thank Rice Breeding Stations. TNAU, Coimbatore and The HOD, Deapartment of Genetic Engineering, SRM University for their support extended in the molecular characterization of bacterial isolates.

References

Ahmad Ali Khan, Ghulam Jilani, Mohammad Saleem Akhtar, Syed Muhammad Saqlan Naqvi, Mohammad Rasheed. 2009. Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. J. Agric. Biol. Sci. **1**(1): 48–58.

Baki AA and Anderson JD. 1973. Vigour determination in soyabean seed by multiple criteria. Crop. Sci. **31**: 630–633.

Chen, Y. P., Rekha, P. D., Arun, A. B., Shen, F. T., Lai, W. A., and Young, C. C. 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol **34**: 33–41.

de Weger L. A., van der Bij A. J., Dekkers L. C. Simons M., Wijffelman C. A., Lugtenberg B. J. J. 1995. Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. FEMS Microbiol. Ecol. **17**: 221–228.

ISSN 2348 – 7968

Farah Ahmad, Iqbal Ahmad, Khan MS. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. **163**: 173–181.

Gerhardson, B. 2002. Biological substitutes for pesticides. Trends Biotechnol. **20**: 338–343.

Husen, E. 2003. Screening of soil bacteria for plant growth promotion activities invitro. Indonesian J. Agri. Sci. **4**: 27–31.

ISTA, International rules for seed testing. 1966. Proc. Int. Seed Test Assoc. **31**: 1–152.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., and Barea, J. M. 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils **37**: 1–16.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab Clin. Med. **44**: 301–307.

Nakkeeran, S., Dilantha Fernando, W. G., and Zaki, A. S. 2005. Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. *In* PGPR: Biocontrol and Biofertilization. *Edited by* Z.A. Siddiqui, Springer, Dordrecht, The Netherlands. pp. 257–296.

Nautiyal CS. 1999. An efficient microbiological grown medium for screening phosphate solubilizing microorganisms. Fed. Europ. Materials Soc. Microbiol. Lett. **170**: 265-270.

Noura Raddadi, Ameur Cherif, Hadda Ouzari, Massimo Marzorati, Lorenzo Brusetti, Abdellatif Boudabous, Daniele Daffonchio. 2007. *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains, Annals of Microbiol. **57**(4): 481–494.

Pikovskaya, R. I. 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiol. **17**: 362–370.

Podile, A. R., Kishore, K. 2007. Plant growth-promoting rhizobacteria. *In* Plant Associated Bacteria. *Edited by* Gnanamanickam, S. S. Springer, The Netherlands, pp. 195–230.

Postma, J., Montanari, M., and Van den Boogert, P. H. J. F. 2003. Microbial enrichment to enhance the disease suppressive activity of compost. Eur. J. Soil Biol. **39**: 157–163.

Radheshyam, K Jayaswal., Marcela, F., Schroeder Ralph, G. 1990. Isolation and characterization of a pseudomonas strain that restricts growth of various phytopathogenic fungi. Appl. Environ. Microbiol. **56**(4): 1053–1058.

Sambrook, J., and Russel, W. D. 2007. Molecular cloning: A laboratory manual, 3rd edn. Cold Spring Harbour Laboratory Press. Cold Spring Harbour, N. Y.

Stephane Compant, Brion Duffy, Jerzy Nowak, Christophe Clement, and Essaid Ait Barka. 2005. Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Appl. Environ. Microbiol. **71**(9): 4951–4959.

Table 1. Details of seed vigor index exhibited by bacterial isolates on rice varieties

	CO48	CO49	ASD16	ADT39	ADT43	ADT45
S5.3	1472	1160	1225.6	1201	1155	1242
S5.6	1341	**1346**	1107.75	1043.1	1194	1059.95
S6.4	1020.8	1222.65	1073.8	1189	1224.55	1438
S6.5	1369	1226	1262.25	1527	**1637**	**1531**
S6.6	1262	922.25	1133.25	1280	1239	890.25
S5.1	1446	1148.4	**1676.7**	1251	1226	1273.95
S5.2	1454	996	1151.2	1221.7	1341	1235.9
S5.4	1400	998	660.8	1017	873	964
S5.5	1319	730.6	908.6	1189.4	1199	1169.45
S5.7	**1476**	1226	685.1	**1362.3**	1129	1169.45
C	1195	1092	1250.2	1208	1267.2	1190.7

ISSN 2348 – 7968

Table 2. Details of mean shoot length exhibited by bacterial isolates on rice varieties

	CO48	CO49	ASD16	ADT39	ADT43	ADT45
S5.3	7.52 ± 0.29	6.10 ± 0.32	9.17 ± 0.45	7.27 ± 0.18	6.24 ± 0.18	6.67 ± 0.38
S5.6	7.85 ± 0.61	6.93 ± 0.47	5.55 ± 0.45	5.21 ± 0.21	5.74 ± 0.23	5.2 ± 0.30
S6.4	8.49 ± 0.55	7.77 ± 0.58	10.33±0.29	7.49 ± 0.16	7.79 ± 0.41	6.83 ± 0.21
S6.5	7.31 ± 0.36	5.74 ± 0.48	8.92 ± 0.83	8.86 ± 0.73	8.01 ± 0.44	9.13 ± 0.74
S6.6	7.97 ± 0.32	6.69 ± 0.48	8.86 ± 0.58	7.83 ± 0.17	6.76 ± 0.15	7.05 ± 0.33
S5.1	8.73 ± 0.38	6.95 ± 0.63	10.67±0.50	6.69 ±0.21	6.26 ± 0.27	7.66 ± 0.39
S5.2	9.34 ± 0.64	5.62 ±0.26	8.79 ±0.53	7.10 ±0.17	6.88 ±0.30	8.29 ±0.38
S5.4	8.60 ±0.34	5.60 ±0.30	7.31 ±0.56	6.80 ±0.61	5.35 ±0.16	6.64 ±0.31
S5.5	8.71 ±0.40	6.06 ±0.20	8.92 ±0.50	7.19 ±0.33	6.50 ±0.27	7.10 ±0.20
S5.7	8.69 ±0.23	5.97 ±0.34	5.62 ±0.23	9.04 ±0.67	6.13 ±0.15	6.78 ±0.29
C	7.57 ±0.39	6.18 ±0.39	10.35±0.61	7.49 ±0.39	6.37±0.21	6.4 ±0.39

5% probability level (P <0.05)

Table 3. Details of mean root length exhibited by bacterial isolates on rice varieties

	CO48	CO49	ASD16	ADT39	ADT43	ADT45
S5.3	7.2 ± 0.27	5.50 ±0.93	6.15 ± 0.24	4.74 ± 0.11	5.31 ± 0.16	5.75 ±0.21
S5.6	5.56 ± 0.32	6.35 ± 0.50	5.55 ± 0.15	5.20 ± 0.35	5.74 ±0.14	5.22 ±0.18
S6.4	5.72 ± 0.51	5.10 ± 0.24	6.19 ± 0.29	4.40 ± 0.09	5.10 ± 0.28	7.55 ±0.15
S6.5	6.40 ± 0.12	6.52 ± 0.43	7.36 ±0.16	6.41 ± 0.56	8.37 ± 0.37	6.18 ±0.19
S6.6	4.65 ±0.30	4.15 ±0.25	6.25 ±0.63	4.97 ±0.25	5.63±0.18	4.82±0.18
S5.1	5.73 ±0.28	4.74 ±0.12	7.96 ±0.22	5.82 ±0.37	5.99 ±0.36	5.76 ±0.25
S5.2	5.21 ± 0.29	6.29 ±0.12	5.60 ±0.88	5.76 ±0.37	6.53 ±0.23	6.25 ±0.42
S5.4	5.4 ±0.17	5.18 ±0.18	2.13 ±0.16	3.39 ±0.50	3.36 ±0.39	2.99±0.24
S5.5	4.49 ±0.26	4.43 ±0.16	4.06 ±0.30	5.33 ±0.40	5.49 ±0.18	5.22±0.42
S5.7	6.07 ±0.27	4.34 ±0.12	2.44±0.32	5.17 ±0.52	5.16 ±0.38	5.53 ±0.43
C	4.38 ±0.24	5.81 ±0.23	7.29±0.41	4.59 ±0.43	7.71±0.41	6.72 ±0.28

5% probability level (P <0.05)

ISSN 2348 – 7968

Table 4. Details of Phosphate Solubilizing efficacy of the PSB isolates in NBRIP broth

Sample	0 hrs (pH)	24 hrs (pH)	48hrs (pH)	72hrs (pH)
Control	7.01	7.01	7.01	7.01
S5.5	7.01	6.86	6.32	6.12
S5.7	7.01	6.89	5.10	4.88
S5.9	7.01	5.44	4.84	4.69
S5.10	7.01	7.14	5.50	5.00
A1.3	7.01	6.87	6.38	5.81
A1.5	7.01	6.81	5.91	5.55
A2.6	7.01	6.61	5.53	4.81
SA4.1	7.01	6.41	5.88	5.06
SA4.4	7.01	7.20	6.31	6.30
SA4.5	7.01	6.01	5.05	4.82
SA4.8	7.01	6.64	5.40	5.07
SA4.9	7.01	6.68	6.44	5.48
SI5.1	7.01	5.59	5.03	4.10
SI5.8	7.01	5.84	5.50	4.30
SI5.10	7.01	5.59	5.43	4.51

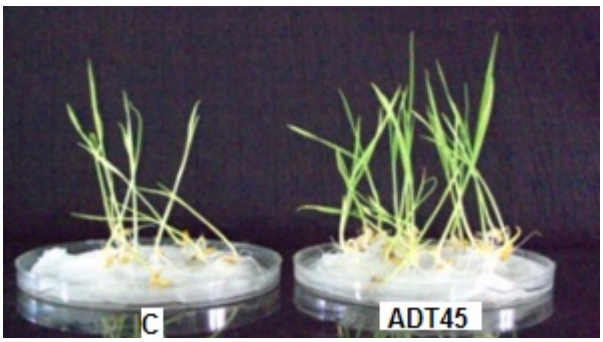

Fig 1 PGPR activity exhibited by the isolate S6.5 on rice variety ADT45 under *in-vitro* conditions

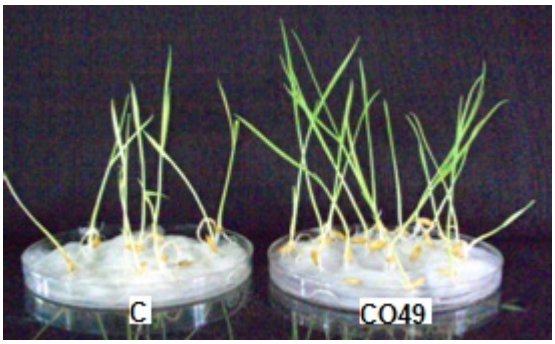

Fig 2 PGPR activity exhibited by the isolate S5.6 on rice variety CO49 under *in-vitro* conditions

ISSN 2348 – 7968

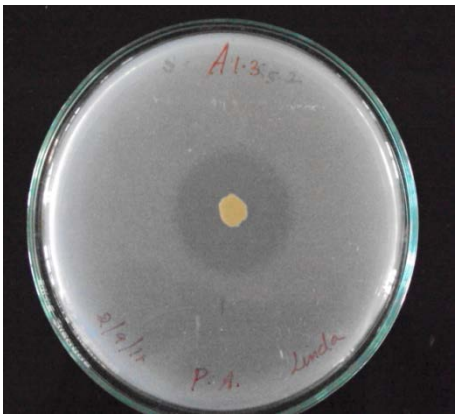

Fig 3 Zone of clearance exhibited by the isolate A1.3 on Pikovskaya medium

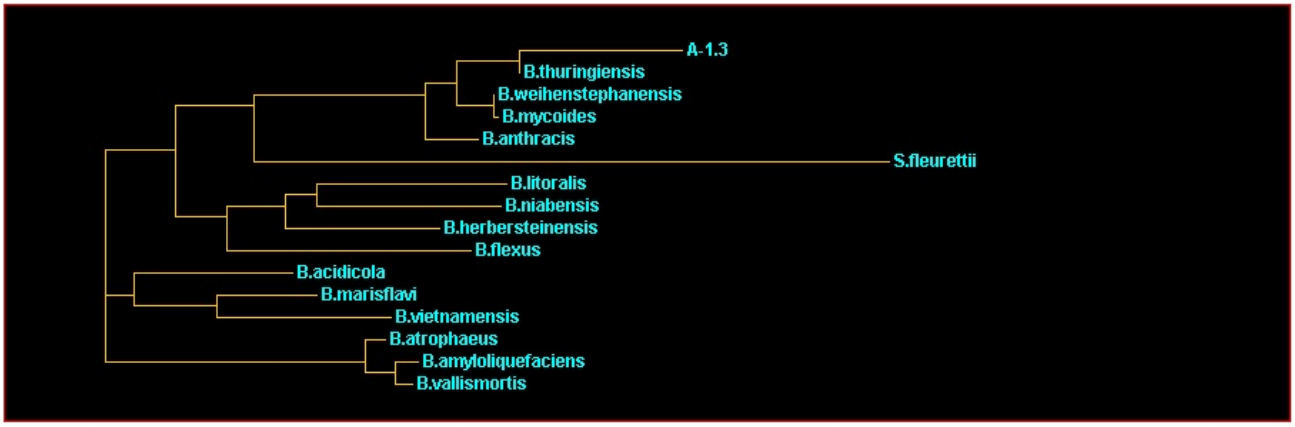

Fig 4 Phylogram of the PSB isolate A1.3

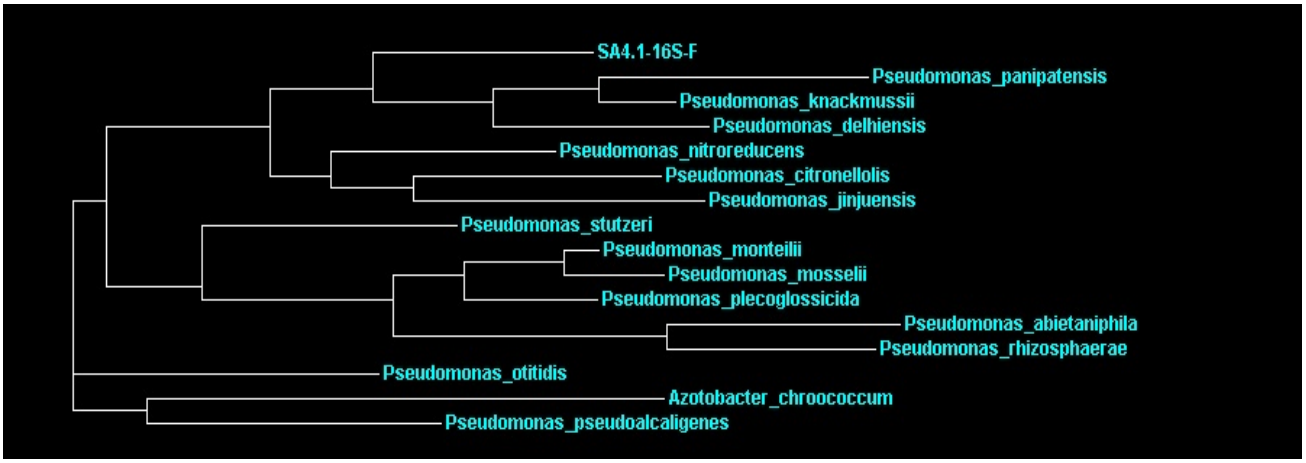

Fig 5 Phylogram of the PSB isolate SA4.1

ISSN 2348 – 7968

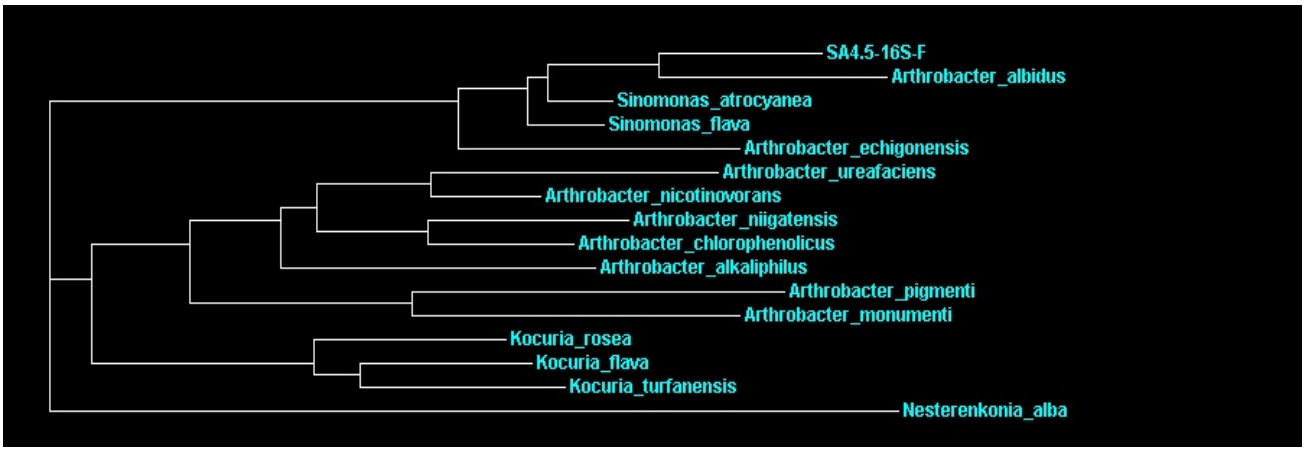

Fig 6 Phylogram of the PSB isolate SA4.5

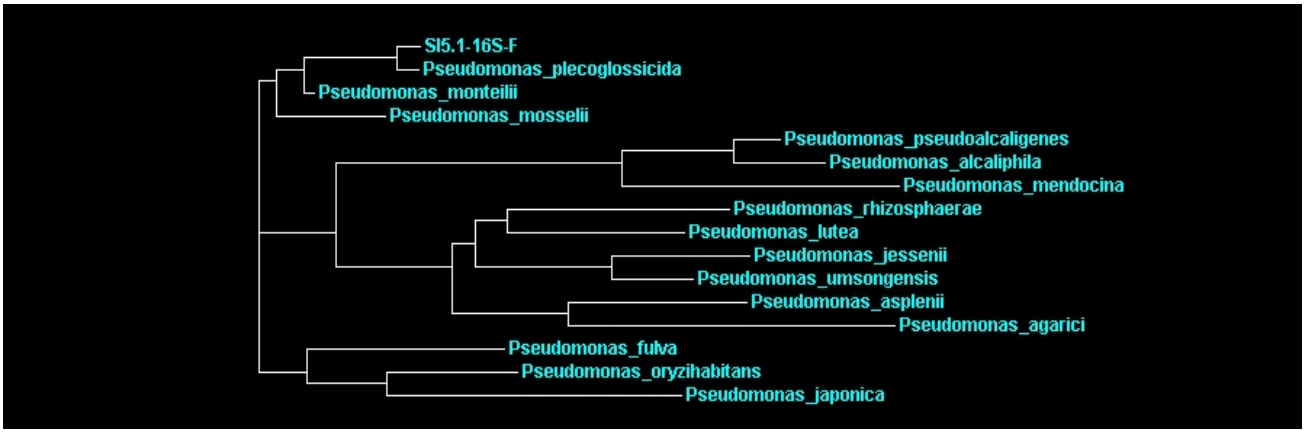

Fig 7 Phylogram of the PSB isolate SI5.1