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SHORT COMMUNICATION

Transconjugation studies in *Azospirillum* sp. negative to mineral phosphate solubilization

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Abstract It has been previously shown that certain gram-negative bacteria do not have the ability to solubilize insoluble phosphates due to the lack of pyrroloquinoline-quinone synthesis genes (*pqq*). PQQ is required as cofactor for the assembly of the glucose dehydrogenase (GDH) holoenzyme, which acts in the oxidation of glucose to gluconic acid. In this context the transconjugation and expression of *pqq* genes in *Azospirillum* sp. was studied using the construct pMCG 898. pMCG 898 containing *pqq* gene/s was mobilized into an *Azospirillum* strain negative to mineral phosphate solubilization by biparental mating. The presence of the construct was also confirmed by minipreps of the transconjugants. The transconjugants were able to solubilize dicalcium phosphate while the wild type was not able to do so. The nitrogen-fixing ability of the transconjugants was also examined and they retained the ability to fix nitrogen. Further detailed studies are required to confirm the utility of such strains in releasing inorganic P from fixed phosphates in soil.

Keywords *Azospirillum* sp. · pMCG 898 · Pyrroloquinoline quinone · Transconjugation

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Introduction

Phosphorus is one of the major essential plant nutrients and plays an important role in higher crop yields. Microbes as well as their activities are concentrated in the rhizosphere (Killham 1994). The rhizosphere, being rich in microbial diversity, harbours phosphate solubilizers, nitrogen fixers and plant growth-promoting rhizobacteria in addition to the general flora. Among them phosphate-solubilizing bacteria play an important role in dissolution of insoluble phosphates from soil, making phosphorus available for plant assimilation. Among various mechanisms involved in mineral phosphate solubilization (MPS), the production of organic acids by soil microorganisms is the principal mechanism (Rodriguez and Fraga 1999).

The most promising way to create more effective plant growth-promoting rhizobacteria (PGPR) is by genetic manipulation (Glick and Bashan 1997). Shotgun cloning experiments using *Erwinia herbicola* DNA enabled Goldstein and Liu (1987) to clone gene(s) to convert MPS^- *E. coli* to MPS^+ phenotype. Transposon mutagenesis of a cosmid construct pMCG 898, carrying a 4.5 kb insert showed that the essential gene was a 1.8 Kb region (Liu et al. 1992). They showed by sequence comparison and minicell analysis that the gene codes for synthesis of pyrroloquinoline quinone

(PQQ), which is a cofactor for the enzyme glucose dehydrogenase (GDH). The quinoprotein GDH controls a unique step in direct oxidation of glucose (Duine et al. 1979). The full functional complement of GDH enzyme for direct oxidation of glucose is not present in all bacteria. Thus Goldstein and Liu (1987) proved that the GDH-mediated dissimilatory bypass system involving direct oxidation of glucose to gluconic acid in the periplasmic space was responsible for mineral phosphate solubilization in *Erwinia herbicola*. The glucose dehydrogenase (GDH) holoenzyme plays an important role in the oxidation of glucose to gluconic acid (Goldstein 1995). In *Pseudomonas cepacia*, a different cofactor, encoded by the *gabY* gene was sufficient to produce gluconic acid and induce solubilization of insoluble phosphate in *E. coli* (Babu Khan et al. 1995). Rodriguez et al. (2000) have also been successful in the expression of mineral phosphate-solubilizing gene from *Erwinia herbicola* in two rhizobacterial strains, *Burkholderia cepacia* IS-16 and *Pseudomonas* sp. PSS. They observed an increase in the *mps* ability of both strains, enhancing their potentialities as growth promoters of agricultural crops.

Bacteria of the genus *Azospirillum* are nitrogen-fixing soil bacteria that associate with the roots of many plants including important crops such as wheat, rice and maize (Tarrand et al. 1978). It is known that certain gram-negative bacteria lack the ability to solubilize insoluble phosphates since they lack the *pqq* genes. PQQ is required as cofactor for the activity of apo-glucose dehydogenase which is responsible for the production of gluconic acid. Therefore an attempt was made in this study to transconjugate a MPS-negative *Azospirillum* sp using the construct pMCG 898 (Liu et al. 1992).

Materials and methods

Strains used

The strains used in the study viz., *Escherichia coli* DH5α and *Azospirillum* sp (Amp^R) were from the culture collection of Department of Agricultural Microbiology, UAS, Dharwad. The construct pMCG 898 containing *pqq* synthase gene was obtained from Dr. A.H. Goldstein, Alfred University, USA.

Preparation of competent cells of *Escherichia coli*

The method followed was the one given by Chakrabarty et al. (1975) with some modifications. *E. coli* DH5α was grown in 100 mL Luria Bertani (LB) broth for 12 h at 37 °C. Reinoculation using 0.5 mL of this culture was done into 10 mL fresh LB and allowed to grow for about 4 h. It was chilled on ice for 30 min. The cells were harvested by pelleting at 5300 × *g* for 8 min at 4 °C in a refrigerated microfuge and resuspended in 5 mL of 100 mM $CaCl_2$. The cells were further chilled on ice for 10 min and pelleted down at 4400 *g* for 8 min at 4 °C. The cells were then resuspended in 1 mL of 100 mM $CaCl_2$ and stored at –20 °C after adding 0.2 mL of 100% glycerol.

Transformation of *Escherichia coli* DH5α

To 100 μL of competent cells in an Eppendorf tube 10 μL of plasmid DNA was added and then chilled on ice for 1 h. A brief heat shock was given at 42 °C in a water bath for 2 min and brought back to ice for 5 min. To the above preparation, 3 mL of LB was added and incubated at 37 °C for 2 h. The cells were pelleted down at 4400 × *g* for 5 min, resuspended in 100 μL of fresh LB and spread plated on LA (Luria Bertani agar) containing ampicillin at 100 μg/mL.

Transfer of *pqq* genes into *Azospirillum*

The mobilization of pMCG898 containing *pqq* gene/s conjugally was attempted through biparental mating. The recipient *Azospirillum* strain in LB and the donor *E. coli* DH5α containing the plasmid pMCG 898 in LB supplemented with 100 μg/mL ampicillin were grown overnight. The cells were pelleted down at 5300 *g* for 5 min and resuspended in 100 μL fresh LB. Fifty microlitre of each of the two strains were mixed and patch mated on Luria agar. They were then replica plated on M9G minimal agar supplemented with ampicillin 100 μg/mL and incubated for 36 h at 28 °C. The transconjugants that came up were purified and single colonies picked up for further studies. The ability of *pqq* gene/s to allow *Azospirillum* to perform MPS activity was visualized through the formation of a solubilization zone on modified Sperber's agar medium (Krishnaraj 1996).

Nitrogen fixation by transconjugants

To assess the effect of the transformed construct containing the *pqq* genes on the nitrogen-fixing efficiency of wild type *Azospirillum*, the in vitro nitrogen fixation was assessed in N-free semi-solid sodium malate medium essentially according to the protocol given by Jackson (1973). To confirm the ability of

transconjugants to have the dual properties of nitrogen fixation and phosphate solubilization, they were grown separately in two sets of N-free semi-solid sodium malate medium. In one set, the wild type and transconjugants were inoculated to the normal N-free semi-solid sodium malate medium containing dipotassium monohydrogen phosphate (K_2HPO_4). In the second set, they were inoculated to N-free semi-solid sodium malate medium in which entire K_2HPO_4 was replaced with 5 g of tricalcium phosphate (TCP)/L to serve as sole source of phosphorus. The inoculated tubes were incubated at 30 ± 1 °C in an incubator and growth in the form of sub surface pellicle formation was recorded after seven days of incubation.

The ability of the transconjugants to solubilize dicalcium phosphate and the amount of nitrogen fixed by them were measured. Four replicates were maintained for both dicalcium phosphate solubilization and nitrogen fixation by the transconjugants.

Isolation of plasmid DNA

To detect the presence of the construct in the transconjugants, the plasmid DNA was isolated following the procedure of Sambrook et al. (1989). Ten microlitre of plasmid DNA isolated following the procedure of Sambrook et al. (1989) was mixed with 2 μL of 6× loading dye. It was allowed to run on 0.8% agarose gel along with λ/*Eco*RI + *Hin*dIII double digest as DNA molecular marker and visualized on a u.v. transilluminator.

Results

Transconjugation and expression of *pqq* genes in *Azospirillum* sp.

The experiment was aimed at mobilizing the construct pMCG 898 carrying genes responsible for synthesis of pyrroloquinoline quinone (PQQ). The construct was mobilized into *Azospirillum* by biparental mating. Ampicillin resistant *Azospirillum* colonies were picked up as exconjugants with the *pqq* genes. The ability of transconjugants to solubilize dicalcium phosphate was studied by spotting them on modified Sperber's medium. It was noticed that the transconjugants were able to solubilize dicalcium phosphate while the wild type was not able to do so (Fig. 1). To detect the presence of the construct in the transconjugants, the plasmid DNA was isolated and the presence of the construct was also confirmed by making minipreps of the transconjugants. It was noticed that the transconjugants harboured pMCG898 (Fig. 2).

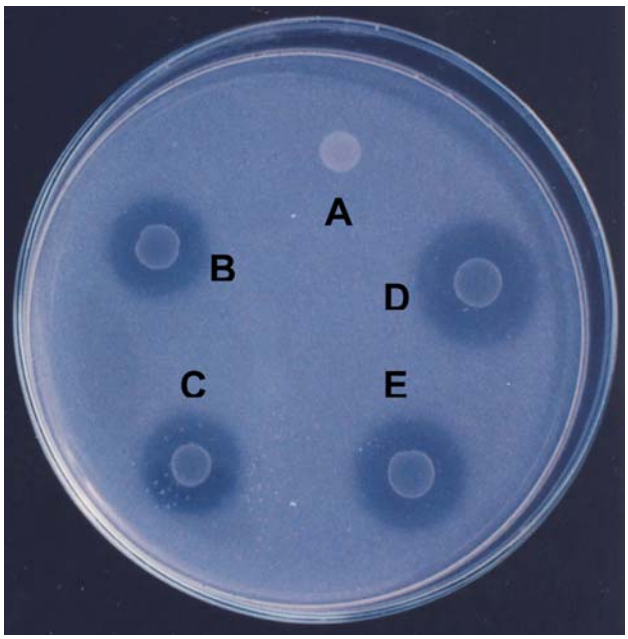

Fig. 1 Formation of solubilizing zones on modified Sperber's agar by the transconjugants. (**A**) *Azospirillum* wild type, (**B**) Transconjugant No. 8, (**C**) Transconjugant No. 18, (**D**) Transconjugant No. 19, (**E**) Transconjugant No. 77

The transconjugants were grown in semi-solid N-free malate medium with tricalcium phosphate (TCP)/dipotassium monohydrogen phosphate (K_2HPO_4) as sole source of P. When TCP was used as the only P source, the wild type *Azospirillum* did not grow, whereas the transconjugants were able to grow (Fig. 3). However, when K_2HPO_4 was used a sole source of P, the wild type and the transconjugants were able to grow forming pellicle (Fig. 4). The nitrogen-fixing ability of the transconjugants was also examined. All the transconjugants retained the ability to fix nitrogen. The amount of nitrogen fixed by the transconjugants was same as that of the wild type strain. The amount of nitrogen fixed by the transconjugants and the wild type *Azospirillum* sp. were of the order of 19.6 mg per gram of carbon source used.

Discussion

It has been previously shown that certain gram-negative bacteria lack the ability to solubilize insoluble phosphates since they lack the *pqq* genes. PQQ is required as cofactor for the activity of apo-glucose dehydrogenase which is responsible for the production of gluconic acid. Hence the construct pMCG 898 containing the *pqq* gene/s was used to transform an MPS^- strain of *Azospirillum* sp. By biparental mating the

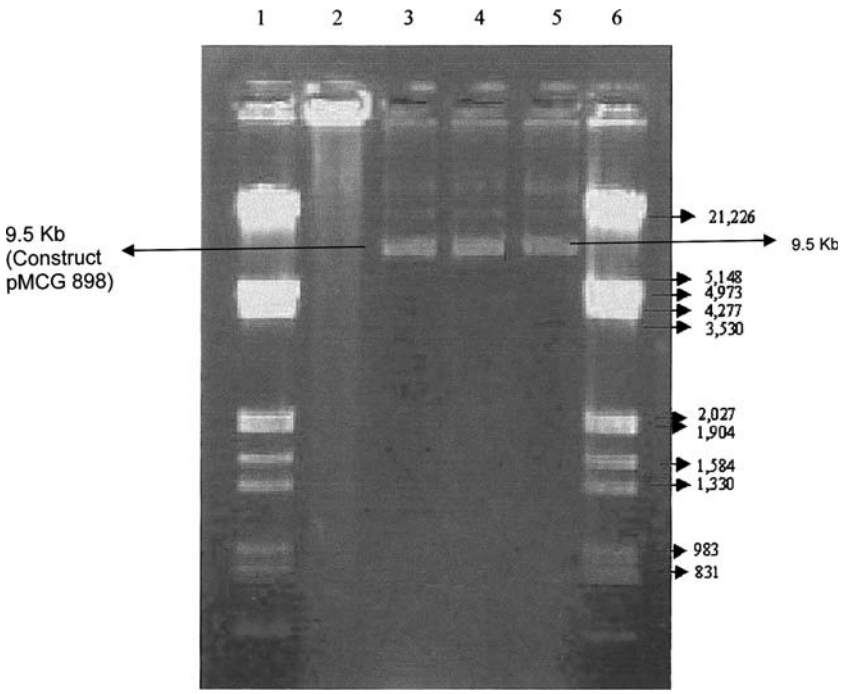

Fig. 2 Plasmid profile of the transconjugants, Lane 1 and lane 6: λ *Eco*RI + *Hin*dIII double digest, Lane 2: Plasmid preparation from *Azospirillum* wild type, (Note: No plasmids were observed), Lane 3: pMCG 898 [isolated from *Escherichia coli* (pMCG 898)], Lane 4: Plasmid preparation from Transconjugant No.8, Lane 5: Plasmid preparation from Transconjugant No.18

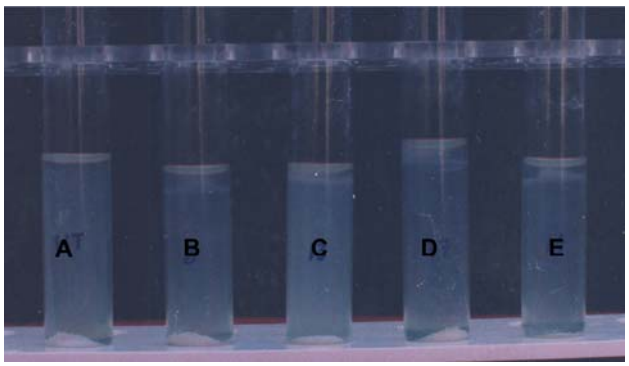

Fig. 3 Growth and pellicle formation by transconjugants and wild type *Azospirillum* in nitrogen semi-solid sodium malate medium with tricalcium phosphate as the sole source of phosphorus (Note no growth and pellicle of wild type). (**A**) *Azospirillum* wild type, (**B**) Transconjugant No. 8, (**C**) Transconjugant No. 18, (**D**) Transconjugant No. 19, (**E**) Transconjugant No. 77

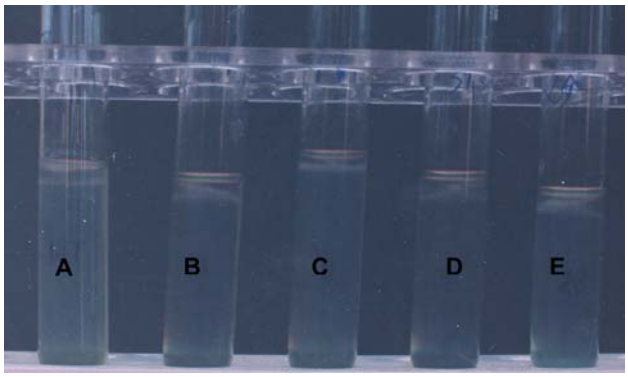

Fig. 4 Growth and pellicle formation by transconjugants and wild type *Azospirillum* in nitrogen semi-solid sodium malate medium with dipotassium monohydrogen phosphate as the sole source of phosphorus. (**A**) *Azospirillum* wild type, (**B**) Transconjugant No. 8, (**C**) Transconjugant No. 18, (**D**) Transconjugant No. 19, (**E**) Transconjugant No. 77

construct was mobilized into *Azospirillum* and then they were tested for their ability to solubilize dicalcium phosphate by spotting them on modified Sperber's medium. The transconjugants obtained proved positive by forming clear zones on the medium. The expression of phosphate solubilization ability from the selected *E. coli* transformants was studied using insoluble phosphate as the unique P source (Rodriguez et al. 2000). They observed that clones containing recombinant plasmids (*pqq* gene/s) formed clearing halos. To confirm the presence of construct in the transconjugants, the plasmid DNA was isolated and then to run on 0.8% agarose gel. The bands on the agarose gel showed that the transconjugants harboured pMCG898. Based on these results it was inferred that *Azospirillum* strain used in the study contains the *apo-GDH* gene and requires PQQ to show MPS activity. Successful expression of *mps* genes in *E. coli* from *E. herbicola* (Goldstein and Liu 1987) and *P. cepacia* (Babu-Khan et al. 1995) have been reported earlier.

Further, the transconjugants when inoculated to N-free semi-solid malate medium with either TCP or K_2HPO_4 as sole source of phosphorus, were able to

grow and form a pellicle whereas the wild type *Azospirillum* was able to grow and form a pellicle with K_2HPO_4 as the sole source of phosphorus but not with TCP. These results clearly indicate that the *Azospirillum* transconjugants with pMCG 898 had acquired the MPS activity and still retained the N_2-fixing activity. The nitrogen-fixing ability of the transconjugants did not differ from that of the wild type indicating that the transconjugation of *Azospirillum* with pMCG 898 did not cause any metabolic load on the wild type. Such observations have been made earlier by Krishnaraj (1996) who observed no change in the MPS activity when *Pseudomonas* sp. 201 was transformed with a construct containing cry1A. This observation differs from the observation of Glick et al. (1986) who reported that genetic transformation of *Azotobacter vinelandii* by the introduction of broad host range plasmid DNA (pRK2501) caused a number of physiological changes. The capacity for nitrogen fixation, mean cell size and synthesis of siderophores were also decreased. Similarly, Rodriguez et al. (2000) demonstrated that heterologous expression of an mps gene from *Erwinia herbicola* in *Burkholderia cepacia* and *Pseudomonas* sp. strains was also possible and that tricalcium phosphate (TCP) was solubilized as well by the effect of this gene product and also did not effect the growth of host cells. Extensive work is required to confirm the utility of such strains in releasing P_i from fixed phosphates in soil. The transconjugation experiments performed also indicated the possibility of developing transgenic bacteria possessing multiple characters.

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