Expression of a mineral phosphate solubilizing gene from *Erwinia herbicola* in two rhizobacterial strains

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Abstract

A genetic construction was carried out using the broad host range vector pKT230 and plasmid pMCG898, which encodes the *Erwinia herbicola* pyrroloquinoline quinone (PQQ) synthase, a gene involved in mineral phosphate solubilization (mps). The final construction was transformed and expressed in *Escherichia coli* MC1061, and the recombinant plasmids were transferred to *Burkholderia cepacia* IS-16 and *Pseudomonas* sp. PSS recipient cells by conjugation. Clones containing recombinant plasmids produced higher clearing halos in plates with insoluble phosphate as the unique (P) source, in comparison with those of strains without plasmids, demonstrating the heterologous expression of the *E. herbicola* gene in the recipient strains. This genetic manipulation allowed the increase in mps ability of both strains, enhancing their potentialities as growth promoters of agricultural crops. These results represent the first report on the application of the recombinant DNA methodology for the obtainment of improved phosphate solubilizing ability from rhizobacterial strains for biofertilization purposes. © 2000 Published by Elsevier Science B.V.

Keywords: Phosphate solubilization; PGPR; Genetically engineered microorganisms; GEM; Biofertilization

1. Introduction

Rhizosphere bacteria can enhance plant growth by different mechanisms (Glick, 1995a). One of these is the dissolution of insoluble phosphates from soil, making phosphorous (P) available for plant assimilation. In fact, a considerable P component in soil is in the form of insoluble mineral phosphates or organic matter (Goldstein, 1994). Free inorganic phosphate is released from organic P compounds by enzymatic processes (Rossolini et al., 1998), while the production of organic acids, such as gluconic acid, by soil microorganisms is the principal mechanism for mps (Rodríguez and Fraga, 1999).

Genetic manipulation is considered the most promising way to create more effective plant growth promoting rhizobacteria (PGPR) (Glick and Bashan, 1997). The pSL775 derived vector

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pMCG898 carries an insert from an *E. herbicola* genomic library, selected by its ability to solubilize mineral phosphate (Goldstein and Liu, 1987). Plasmid pMCG898 contains a 1.8 kb region encoding the PQQ synthase enzyme (Liu et al., 1992). The metabolic function of the PQQ synthase enzyme is the biosynthesis of the cofactor PQQ, necessary for the assembly of the glucose dehydrogenase (GDH) holenzyme, which acts in the oxidation of glucose to gluconic acid (Goldstein, 1994, 1995). A mineral phosphate solubilizing gene from *Pseudomonas cepacia* has also been cloned and expressed in *E. coli*, and its involvement in gluconic acid production was also demonstrated (Babu-Khan et al., 1995).

Burkholderia cepacia IS-16 strain is being used in Cuba as a biofertilizer in several important crops (Martínez A., et al., personal communication), and the improvement of its plant growth promoting capacity would be an important contribution to enhance results in the agricultural practice. On the other hand, *P. sp.* PSS (Villa et al., 1997) has the ability to inhibit the growth of microorganisms by producing antibiotics and siderophores, indirectly stimulating plant growth. It would be of a great value to complement this ability with an efficient solubilization of soil P.

In this paper the transfer of the *E. herbicola* PQQ synthase gene to both IS-16 and PSS bacterial strains is reported, using the broad host range vector pKT230. The expression of this trait in recipient cells was evaluated in relation with the enhancement of mps ability.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured in Luria Bertani (LB) broth and maintained in LB agar at 37°C. *Pseudomonas* and *Burkholderia* strains were cultivated at 30°C in nutrient broth (NB) and nutrient agar (NA) (Sambroock et al., 1989). Cultures were incubated in an orbital shaker at 175 rpm. The antibiotics used for selection were (μg ml^{-1}): Ampicillin (Amp), 100; streptomycin (Str), 100; spectinomycin (Sp), 100; tetracycline (Tet), 25; kanamycin (Km), 50.

For the assay of mps, the strains were cultivated in a medium based in that described by Illmer and Schinner (1992), with the following composition (g l^{-1}): glucose, 10; NH_4NO_3, 0.373; $MgSO_4$, 0.41; KCl, 0.295; NaCl, 0.2; $FeCl_3$, 0.003; Ca_3HPO_4, 0.7. Agar (20 g l^{-1}) was added for plate cultures. For kinetic studies, 1000 ml Erlen-

Table 1
Bacterial strains and plasmids[a]

Strain or plasmid	Characteristics	Reference
Strain		
Escherichia coli MC1061	F$^-$ araD139 (ara-leu)7696 galE15 galK16 (lac)X74 rpsL (Str^r) hsdR2 ($r_k - m_k$) mcrA mcrB1	Sambroock et al., 1989
Burkhordelia cepacia IS-16	Wild type, Ap^r, Sp^r	Martínez et al., pers.com.
P. sp. PSS	Wild type, Ap^r, Km^r, Sp^r	Villa et al., 1997
Plasmid		
pMCG898	Contains the PQQ synthase gene involved in mps, Ap^r	Goldstein & Liu, 1987
pKT230	Broad host range vector for Gram negative bacteria, Str^r, Km^r	Bagdasariam et al., 1981
pRK2013	Helper plasmid, Km^r	Figurski & Helinski, 1979

[a] Str, Streptomycin, Ap, Ampicillin, Sp, Spectinomycyn, Km, Kanamycin, Tet, Tetracycline.

meyer flasks with 200 ml of medium were used, with a rate of inoculation of 5%. Samples were withdrawn at different time intervals and the soluble P concentration was estimated in the supernatant after centrifugation of the samples at $10\,000 \times g$. The experiments were carried out in triplicate.

2.2. Determinations

Growth was estimated by the measurement of the absorbance at 600 nm. Samples from cultures grown in medium with tricalcium phosphate were previously diluted 1:1 (v/v) using 1N HCl to dissolve the residual insoluble phosphate and measured against a blank identically treated. Soluble P was determined in the supernatant according to Chen et al. (1956). Mineral phosphate solubilization (mps) was assayed by measuring the halo size in plates containing tricalcium phosphate as the only P source. Exponentially growing cultures were adjusted to an optical density of 0.5 (at 600 nm), 1 µl of these samples was inoculated in the plates and the halos measured after 24 h of incubation at 30°C.

2.3. Recombinant DNA techniques

Plasmid extraction using the alkaline lysis method, agarose gel electrophoresis, preparation of competent cells and *E. coli* transformation were essentially as described by Sambroock et al. (1989). Restriction enzymes (New England Biolab), T_4 ligase (New England Biolab) and alkaline phosphatase (Sigma), were used according to the manufacturer's instructions.

2.4. Genetic constructions

The subcloning strategy is shown in Fig. 1. The pKT230 vector (IncQ, RSF 1010 replicon, Bagdasariam et al., 1981) was digested with BamHI, dephosphorylated and re-ligated with plasmid pMCG898 (Goldstein and Liu, 1987) previously digested with BamHI and the ligation mixture was transformed in *E. coli* MC1061. Transformants were selected for the expression of the expected antibiotic resistance profile (Ap, Km and Str) in LB-agar plates.

2.5. Conjugation

Plasmids were transferred from *E. coli* to *Burkholderia* and *Pseudomonas* strains by a triparental mating, using the pRK2013 plasmid as a helper (Figurski and Helinski, 1979). Matings were performed overnight on NA plates, using 1:10:1 and 1:1:1 rates from overnight cultures of donor, recipient and helper strains, respectively. The resultant growth was plated on NA medium containing Str, Km and Sp. The Sp resistant trait, harbored by IS-16 and PSS strains, was used to select against the donor *E. coli* strain.

2.6. Plasmid stability in the recombinant strains

To analyze the stability of the recombinant pL230 plasmid in the host rizobacterial strains, single colonies of the transconjugants grown in NA plus Str and Km were inoculated in NB medium without antibiotics. After 8 h of growth, the cultures were diluted 1000 fold in non-antibiotic medium. The dilution procedure was repeated several times until the cells had gone through 50 generations, on the absence of selective (antibiotic) pressure. The cultures were sampled and plated on NA in both the presence and absence of Km and Str as selective antibiotics. The stability was determined by comparing the number of colonies present on selective and non-selective plates.

3. Results and discussion

When the broad host range vector pKT230 was transferred by conjugation to both recipient strains, frequencies of 8×10^{-4} (transconjugants per recipient cell) for the 1:1 recipient to donor strain ratio and of 5×10^{-4} for the 1:10 proportion, were obtained for *B. cepacia* IS-16, while 4×10^{-4} for the 1:1 ratio and 7×10^{-3} for the 1:10 proportion were found for *P. sp.* PSS. This indicates that, for both strains, a higher frequency was obtained when the recipient to donor strain ratio was increased 10 fold. Therefore, this ratio was selected for further conjugations.
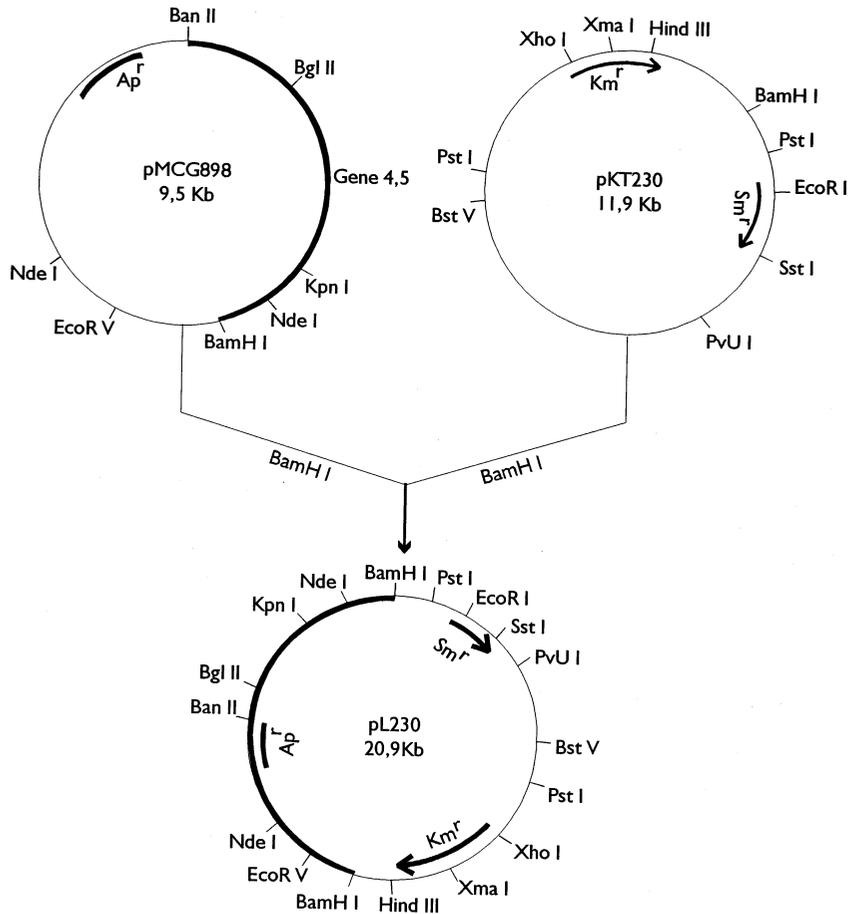
Fig. 1. Genetic construction for the transfer of the PQQ synthase gene from *E. herbicola* to vector pKT230. Km[r]: resistance to kanamycin; Ap: resistance to ampicillin; Str: resistance to streptomycin.

Ligation from the experimental strategy shown in Fig. 1 was transformed in *E. coli*. The frequency of transformation of pL230 plasmid was 3.5×10^2 (transformants per μg DNA). Some clones were selected for further characterization.

The expression of phosphate solubilization ability from the selected *E.coli* transformants was assayed in plates with insoluble phosphate as the unique P source. Clones containing recombinant plasmids formed clearing halos with greater diameter than those of strains without plasmids (Fig. 2).

Representative clones were transferred to both recipient rhizobacterial strains by conjugation and transconjugants were selected by antibiotic-resistance criteria. Twenty-eight of the putative transconjugants (14 from each strain), harboring the pL230 plasmid, were further characterized for halos formation on insoluble phosphate medium. Table 2 shows the results of halo size on tricalcium phosphate plates from the transconjugants resulting in increased phosphate solubilization. The wild type strains PSS and IS-16 can solubilize inorganic phosphate at a basal level, so both of them produced clearing halo when grown on tricalcium phosphate medium. The significant increase of the halo size in transconjugants with pL230 constructions, in comparison with the strains without plasmid, was indicative of the expression of the cloned gene, which allowed the
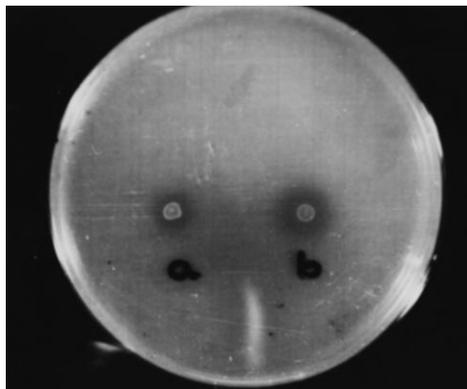
Fig. 2. Clearing halos formation on medium containing tricalcium phosphate as the sole P source by the bacterial cultures. (a) *E. coli* MC 1061 strain. (b) Strain MC 1061 harboring plasmid pL230 with the PQQ synthase gene from *E. herbicola*.

enhancement of mps in comparison with the wild type strains.

In order to demonstrate the presence of the recombinant plasmids, all transconjugants with increased halo formation were submitted to plasmid purification. The extracts were used to retrotransform the *E. coli* MC1061 strain. In all cases a high frequency of transformants showing the phenotype corresponding to the antibiotic resistance encoded by the plasmid (Str, Ap and Km) were obtained. On the other hand, digestions of the extracted plasmids with the appropriate enzymes produced fragments in correspondence with the size of the original plasmids (data not shown), corroborating the presence of the recombinant constructions in the obtained clones.

The presence of a foreign plasmid can produce a metabolic load in the host microorganism (Glick, 1995b). In this study, the growth of transconjugants with increased mps activity was evaluated in NB medium, in comparison with the strains without the recombinant plasmid. The growth rate and final cell density of the transconjugants were similar to that of the wild type strains (data not shown), suggesting that the recombinant plasmid did not adversely affect the growth of the host cells, at least in the assayed conditions

On the other hand, the stability of the recombinant plasmid in the host cell under laboratory growth conditions was studied for some of the obtained clones. After 50 generations of growth on non selective medium the rate of cells keeping the ability to growth on the antibiotic medium were 7, 12, 13 and 15% for clones C916–3, C916–2, C916–5 and C308S–1, respectively. This indicates that instability of the plasmid may occur in non-selective conditions. This suggests chromosomal integration of the genes as an advantageous strategy for future works.

Kinetic analysis of P concentration in the culture broth using an insoluble P source has been used by several authors to show P release capacity of microbial strains. However, a lack of correlation between substrate utilization-as measured by halo size-and P concentration in liquid cultures has been reported (Das, 1963; Ostwall and Bhide, 1972), and variations in P concentrations along the cultivation time have also been observed (Khan and Bhatarar, 1977; Babenko et al., 1984). In this study the kinetics of soluble P concentration was assayed from several transconjugants versus the recipient strain cultured in medium with an insoluble P source. Although higher P values were always detected for the transconjugants in some intervals, variations in P concentration were observed along the cultivation time for both strains. An example of this is shown in Fig. 3. Several increases and decreases of P concentration were detected along the cultivation time. Besides this, a correlation between pH increase and P drop and vice versa was observed. These results could support the hypothesis proposed by

Table 2
Diameters of clearing halos on tricalcium phosphate medium from transconjugants and wild type strains[a]

Strain	Halo size (mm)	% of increase
PSS (wild type)	4.6 ± 0.7	–
C307S-1	5.1 ± 0.5*	11
C308S-1	6.1 ± 0.6*	32
C309S-1	5.5 ± 0.6*	20
C310S-1	5.6 ± 0.6*	22
IS-16 (wild type)	4.4 ± 0.4*	–
C916-2	6.2 ± 0.6*	42
C916-5	5.5 ± 0.6*	25

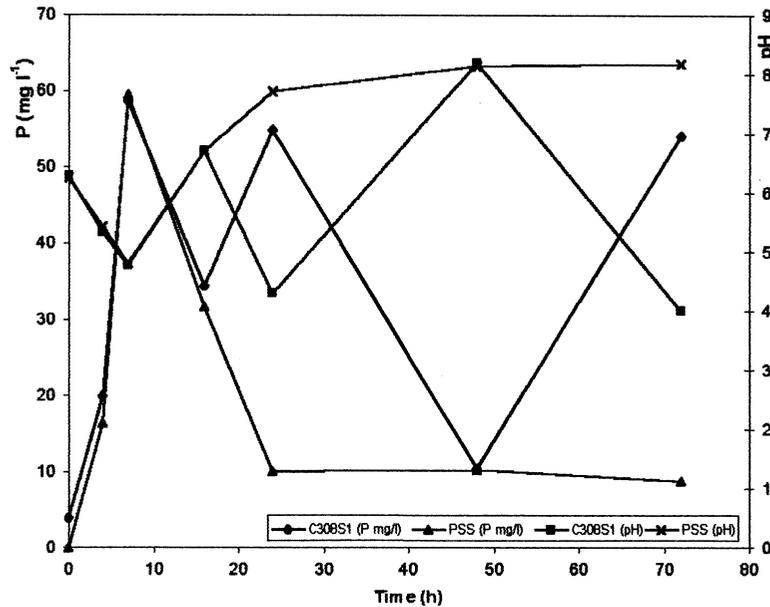[a] *, Significant difference with the wild type strain (Student's *t*-test, 95% reliability).

Fig. 3. Kinetics of soluble P and pH of cultures from the wild type strain PSS and the transconjugant C308S-1 grown in medium with an insoluble P source.

Illmer and Schinner (1992) about the probable formation of Organo-P compounds with the organic acids, which are further used as nutrient source by the bacteria. These evidences also support the idea that P concentration is not a reliable parameter for assessing phosphate solubilization capability of a microbial strain for comparative studies.

In previous works, genes involved in mps from *E. herbicola* (Goldstein and Liu, 1987) and *P. cepacia* (Babu-Khan et al., 1995) were expressed in *E. coli*, producing an increase in hydroxyapatite dissolution. The present results demonstrate that heterologous expression of a mps gene from *E. herbicola* in *B. cepacia* and *P. sp*. strains was also possible and that tricalcium phosphate was solubilized as well by the effect of this gene product. The enhancement of the mps trait obtained in this work is a relevant step, which would allow a higher efficiency of phosphate utilization by the plant, thus improving the plant growth promotion effect of both rhizobacterial strains. In future studies chromosomal insertion of the PQQ synthase gene would be advantageous, both to avoid horizontal transfer risk and to increase the stability of the new trait.

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