Vector for chromosomal integration of the *phoC* gene in plant growth-promoting bacteria

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Abstract

This work describes the subcloning of the gene encoding the PhoC acid phosphatase from *Morganella morganii* (*phoC* gene) in a vector that permits stable chromosomal integration of this gene in plant growth-promoting bacteria (PGPB). A plasmid was constructed using the suicide delivery vector pJMT6 (a pUT/mini Tn*5* derivative vector) and the plasmid pLR1, the latter harboring the *phoC* gene. The recombinant construction pLF17, which contains a non-antibiotic resistance selection marker, was transformed and expressed in *Escherichia coli* CC118λ*pir*. A transformant clone, *E. coli* CC118λ*pir* F17 was selected and further characterized, showing *phoC* gene expression through an histochemical assay and zymograms developed to detect phosphatase activity. With this technique, it was possible to detect, in the whole cell extract, the 25-kDa polypeptidic component responsible for acid phosphatase activity. Acid phosphatase activity was quantified in the whole cell and in the supernatant of the culture as being higher in the transformant *E. coli* CC118λ*pir* F17 than in *E. coli* CC118λ*pir* without plasmids along the cultivation time.

Introduction

Phosphorus is an essential element for plant growth. However, a considerable portion of organic and inorganic phosphate is in a poorly soluble state in soil (Goldstein, 1996). The capacity of some microorganisms to solubilize mineral and organic phosphorus in soil, making this compound available for plant growth, has been a focus of research for many years (Rodríguez and Fraga, 1999). Particularly, organic phosphates can be found in the humus in soil, and the solubilization of part of this phosphorus can be carried out by means of phosphatase enzymes produced by certain rhizobacterial strains.

Plant growth-promoting bacteria (PGPB) are bacteria that can exert a beneficial impact on plant growth and development in a direct or indirect way. The direct promotion generally entails providing the plant with a compound that is produced by the bacterium or facilitating the uptake of nutrients from the environment (Glick, 1995). This is the case of phosphate-solubilizing bacteria. Genetic manipulation by means of recombinant DNA technology seems to offer feasible approaches for obtaining improved phosphate-solubilizing strains (Fraga et al., 2001; Rodríguez and Fraga, 1999). The objective of this work was the subcloning of the *phoC* gene from *Morganella morganii* that codes for PhoC acid phosphatase in a mini-Tn5 derivative transposon-vector in order to achieve stable chromosomal integration of this gene in the recipient strain. Advantages of this delivery system are: stability without selection,

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non-antibiotic selection markers, prevention of risk of metabolic load, small size, and minimal horizontal transfer of cloned genes to indigenous microorganisms.

Materials and methods

Plasmids, bacterial strains and cultivation conditions

Escherichia coli strains were grown in LB broth (in g L^{-1}: Bacto-tryptone, 10; yeast extract, 5; NaCl, 10), at pH 7, and in LB agar (LB supplemented with 1.5% agar). Ampicillin (Ap) was used at 100 μg ml^{-1} and potassium tellurite (K_2TeO_3) was used at 60 μg ml^{-1}. Growth was carried out at 37 °C in an orbital shaker at 175 rpm. Growth was followed by the measurement of absorbance at 600 nm. Statistical and regression analysis were carried out with Statgraphics Statistical Graphics System, version 5.0, with a 95% level of significance. Plasmids and bacterial strains are listed in Table 1.

Recombinant DNA techniques

Plasmids were purified using the alkaline lysis method, essentially as described by Sambrook and Russell (2001). Restriction enzymes and T_4 DNA ligase (New England Biolab, Ltd., Ontario, Canada) were used according to the manufacturer's instructions. Preparation of competent cells and transformation were carried out according methods described by Sambrook and Russell (2001).

Cloning strategy for the construction of the integrating plasmid

To construct the delivery vector for the integration of the *phoC* gene, plasmid pPM12R was digested with *Eco*RI and the released fragment cloned at the *Eco*RI site of vector pUC18*Not* to flank the *phoC* gene with the restriction site *Not*I. The ligation product was transformed in *E. coli* MC1061 and plated on LB medium supplemented with Ap. This construction (pLR1) was then digested with *Not*I to release the *phoC* gene, and this fragment was ligated to the vector pJMT6, previously digested with *Not*I, to generate a minitransposon harboring the *phoC* gene and with potassium tellurite resistance (Tel^r) as the only selection marker (carried by pJMT6) (Figure 1). The ligation product (pLF17) was transformed in *E. coli* CC118λpir and plated on LB medium supplemented with Ap and K_2TeO_3.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli MC1061	F' *araD139 (ara-leu)7696 galE15 galK16 (lac)X74 rpsL* (Str^r) *hsdR2 (r_k-m_k) mcrA mcrB1*	Sambrook and Russell (2001)
E. coli CC118λpir	Δ*(ara-leu) araD* Δ*lacX74 galE galK phoA thi-1* *rpsE rpoB argE*(Am) *recA1* lysogenized with λ*pir* phage	Herrero et al. (1990)
Plasmids		
pPM12R	Ap^r, 4.158 kb; derivative of pBluescript SK +/− (Stratagene), harboring a 1.2-kb fragment from a library of *M. morganii* that codes for the PhoC acid phosphatase	Thaller et al. (1994)
pUC18*Not*	Ap^r, as pUC18 but multiple cloning site flanked by *Not*I sites	Sánchez-Romero et al. (1998)
pLR1	Ap^r, 3.8 kb; derivative of pUC18*Not* with the *phoC* gene ligated to the *Eco*RI site	This study
pJMT6	Ap^r, Tel^r, 8.2-kb pUT/mini-Tn*5* Tel (*Not*I site free)	Sánchez-Romero et al. (1998)
pLF17	9.4 kb, identical to pJMT6 but with the *phoC* gene ligated to the *Not*I site	This study

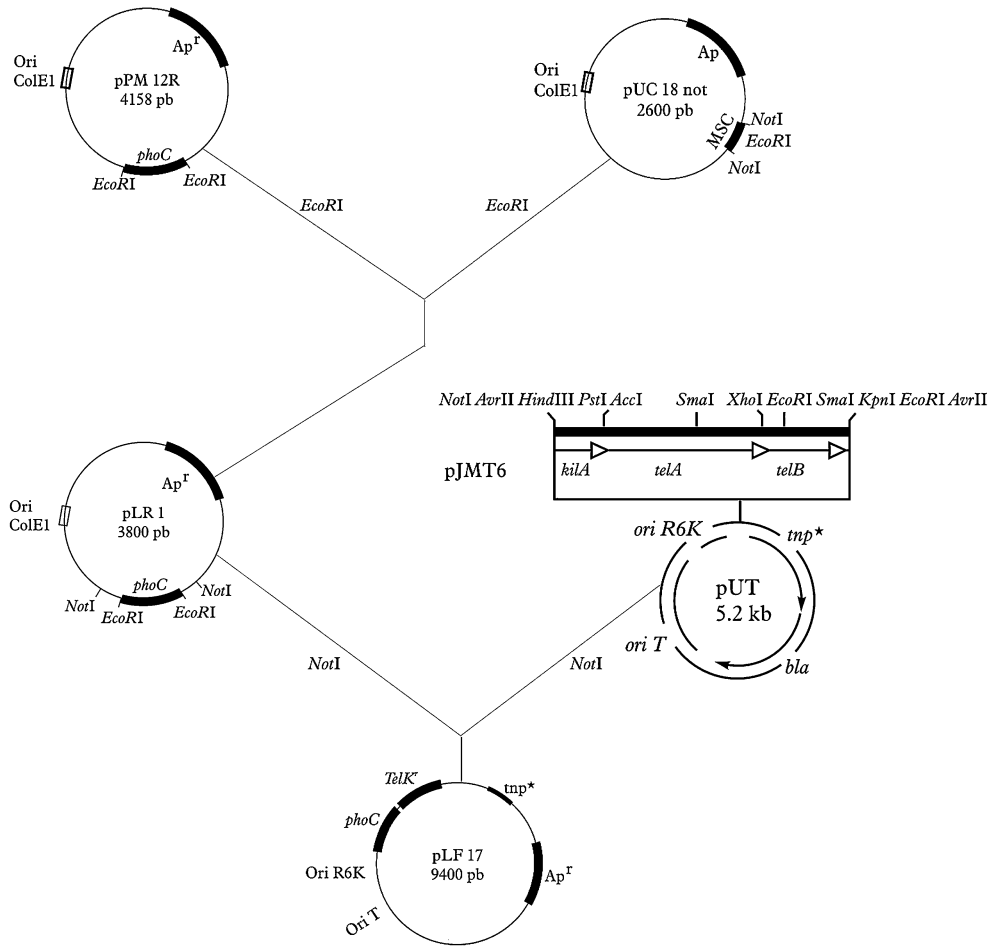

Figure 1. Cloning strategy for the construction of the integrating vector (pLF17).

Detection of acid phosphatase activity and SDS-PAGE (Zymogram)

To detect the expression of the *phoC* gene on plates, a modification of the phosphatase indicator medium, based on an histochemical detection system developed by Thaller et al. (1994), was used. This medium was LB agar supplemented with phenolphthalein diphosphate 0.2% (PDP, disodium salt, Sigma) as substrate for the enzyme and methyl green 0.005% (MG, Sigma) as stain. For the electrophoretic separation, the intact cells were washed with normal saline solution and resuspended in this solution to an optical density (OD) (600 nm) of 40. An aliquot of 40 μl of this suspension was mixed with 10 μl of the loading buffer and 20 μl from that were submitted to SDS-polyacrylamide gel electrophoresis (15%) (SDS-PAGE), according to the method of Laemmli (1970). To visualize the bands corresponding to the total proteins, the gels were stained with Coomassie Brilliant Blue R-250. For the detection of bands with phosphatase activity (Zymogram), the technique described by Thaller et al. (1994), was used.

Phosphatase activity from liquid cultures was evaluated in intact cells and supernatant fractions as described by Fraga et al. (2001).

Results and discussion

*Expression of the phoC gene in E. coli CC118*λpir

Characterization of transformant clones

Some putative transformant clones were selected for further characterization. Plasmids were ex-

tracted and digested with different restriction enzymes (Figure 2). One of the transformants, designated F17, harbored a plasmid (pLF17), which showed the expected size for the construction resulting from the union of vector pJMT6 and the *Not*I *phoC* fragment (9.4 kb). After digestion with *Not*I, plasmid pLF17 was split in two corresponding elements: a fragment of approximately 8.2 kb (pJMT6) and a 1.2 kb (*phoC* gene) fragment. After digestion with *Sma*I, pLF17 yielded a fragment of approximately 7.8 kb and a 1.6-kb fragment. This confirmed the presence of the expected recombinant plasmid in the selected transformant.

Phosphatase activity

After the growth on plates with the phosphatase indicator medium, a dark green color was observed in the F17 recombinant strain, showing the *pho*$^+$ phenotype, in contrast to the white-yellowish color of *E. coli* CC118λpir (data not shown). This qualitative method indicated that the *M. morganii phoC* gene was expressed in the host *E. coli* CC118λpir.

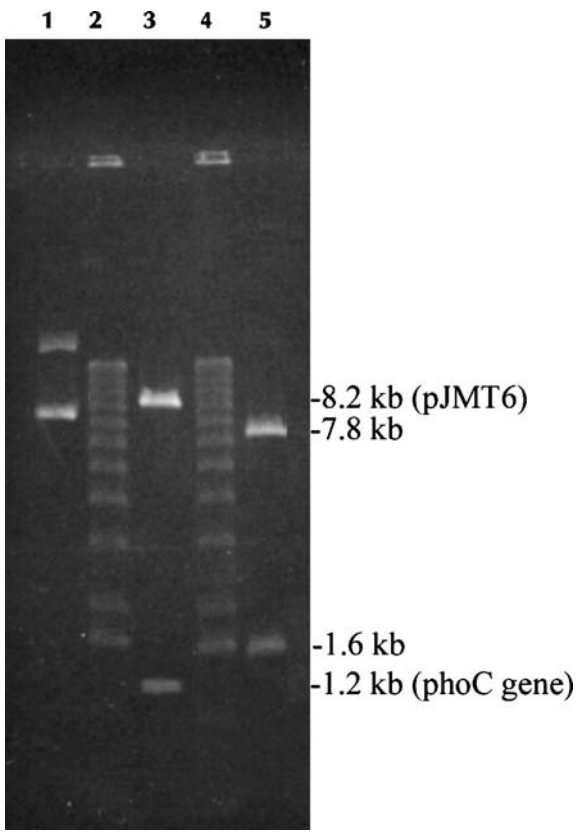

Figure 2. Restriction pattern of the plasmid pLF17 digested with different restriction enzymes. Lane 1: pLF17 not digested. Lane 3: pLF17 digested with the restriction enzyme *Not*I. Lane 5: pLF17 digested with the restriction enzyme *Sma*I. Lanes 2 and 4: DNA molecular weight markers (Marker X).

Phosphatase activity in whole cells and supernatant fractions of the recombinant clone F17 is shown in Figures 3 and 4, in comparison with the *E. coli* CC118λpir strain without any plasmid. During the exponential phase of growth, a much higher level of acid phosphatase activity was detected in intact cells compared with the supernatant fraction (Figure 3). This is consistent with the periplasmic localization of the PhoC enzyme reported by Thaller et al. (1994).

Escherichia coli CC118λpir showed a significantly smaller level of cell-bound acid phosphatase activity, in comparison with the F17 recombinant strain. This basal level of phosphatase activity in the host *E. coli* CC118λpir could be related to a low expression rate of the gene *aphA*, encoding the class B acid phosphatase/phosphotransferase reported for *E. coli* MG 1655 by Thaller et al. (1997). However, the high level of acid phosphatase activity detected in the F17 strain shows the expression of the *phoC* gene present in pLF17, and that the gene is being expressed under its own promoter.

The F17 clone showed increased levels of activity in the culture supernatant after the stationary phase of growth (Figure 4), probably a result of

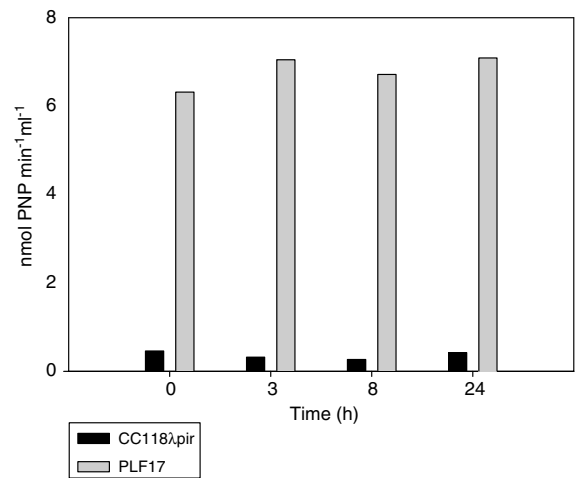

Figure 3. Acid phosphatase activity (PNP production, nmol min^{-1} ml^{-1}) associated with whole cells of the selected transformant F17 in comparison with *E. coli* CC118λpir.

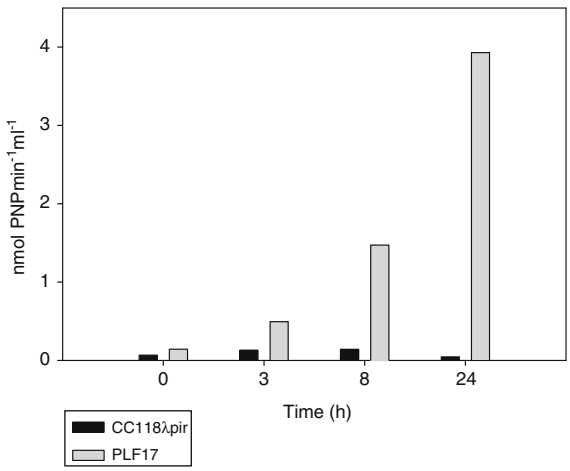

Figure 4. Acid phosphatase activity (PNP production, nmol min^{-1} ml^{-1}) associated with the supernatant of the selected transformant F17 in comparison with *E. coli* CC118λpir.

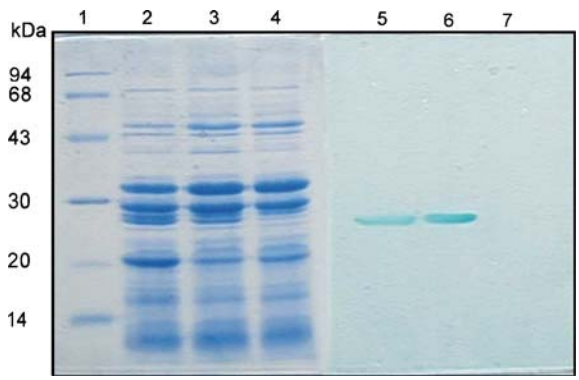

Figure 5. SDS-PAGE analysis of proteins and zymogram for phosphatase activity from *E. coli* transformants. Lane 1: Protein size markers in kDa. Lanes 2–4: Coomassie Blue-stained whole cell protein preparation of *E. coli* MC1061 PM12R (pPM12R), *E. coli* CC118λpir F17 (pLF17), and *E. coli* CC118λpir without plasmids. Lanes 5–7: Zymogram developed for phosphatase activity against PDP at pH 6.0 of *E. coli* MC1061 PM12R (pPM12R), *E. coli* CC118λpir F17 (pLF17), and *E. coli* CC118λpir without plasmids.

the cellular lysis typical of this stage of growth. The same behavior was reported by Thaller et al. (1994) for *E. coli* DH5α PM12R harboring and expressing the *phoC* gene originally cloned. Without plasmids, strain *E. coli* CC118λpir showed very low detectable activity in the supernatant.

Figure 5 shows the result of the SDS-PAGE of total proteins, as well as the zymogram pattern for the detection of the phosphatase activity. The high intensity of the color bands from *E. coli* MC1061 PM12R (pPM12R) and *E. coli* CC118λpir F17 (pLF17) (Figure 5, lanes 5 and 6) suggests that these are the product of the expression of the *phoC* gene in both cases.

Strain F17 was able to produce a band of approximately 25 kDa, which corresponds to the PhoC band of *E. coli* MC1061 (pPM12R). This result corroborates that the *M. morganii* DNA sequences located upstream of the *phoC* gene promote transcription of the *phoC* gene in *E. coli* CC118λpir F17. As expected, no band of phosphatase activity was detected in *E. coli* CC118λpir without plasmid.

Conclusions

- An integrating suicide vector (pLF17), harboring a gene encoding the PhoC acid phosphatase of *M. morganii* was constructed.
- A transformant clone (*E. coli* CC118λpir F17) harbouring the integrating vector pLF17 and expressing the gene *phoC*, was obtained.

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