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Short Communication

Obtaining transconjugant strains for bacteria-rice interaction studies

Obtención de cepas transconjugantes para estudios de la interacción bacteria-arroz

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ABSTRACT: The objective of this work was to obtain transconjugant strains for the study of plant-bacteria interaction. The obtaining of transconjugant bacteria with the plasmid pHC60 was carry out by triparental mating with two *Escherichia coli* strains and two strains isolated from Cuban rice cultivars. Furthermore, an inoculation assay with rice plants cv. INCA LP-5 and transconjugant bacteria was carry out and confocal fluorescence microscopy were used to locate the bacteria in plant tissues. Two transconjugant strains containing *gfp* gene were obtained, since they produced fluorescent colonies in ultraviolet light, in culture medium with tetracycline and 5-nitrofurantoin. The multiplication dynamics of wild type and transconjugant strains don't showed differences in the end of bacteria growth in the medium. Two transconjugant strains were showed colonizing rice root, one of them as a putative endophyte.

Key words: *Rhizobium*, grass, microscopy.

RESUMEN: El objetivo de este trabajo fue obtener cepas transconjugantes para el estudio de la interacción planta-bacteria. La obtención de bacterias transconjugantes portadoras del plásmido pHC60 se realizó mediante apareamiento triparental con dos cepas de *Escherichia coli* y tres cepas aisladas de cultivares cubanos de arroz. Se realizó además un ensayo de inoculación con plantas de arroz cv. INCA LP-5 y bacterias transconjugantes. Se empleó microscopía confocal de fluorescencia para localizar las bacterias en los tejidos vegetales. Se obtuvieron tres cepas transconjugantes que contenían el gen *gfp*, pues produjeron colonias fluorescentes en luz ultravioleta, en medio de cultivo con tetraciclina y 5-nitrofurantoína. La dinámica de multiplicación de las cepas de tipo salvaje y transconjugantes no mostró diferencias a las 30 h de cultivo. Se visualizaron dos cepas transconjugantes de *Rhizobium* colonizando las raíces de plántulas de arroz a las 72 horas de la inoculación, una de ellas como posible endófito.

Palabras clave: *Rhizobium*, gramíneas, microscopía.

INTRODUCTION

Different tools have been development to study deeply the bacteria-plant association such as *omic* sciences, serological techniques coupled with confocal laser scanning microscopy and the method of the tagging with genes coding for a fluorescing protein (1-3). These tools have allow explore the rhizobia-rice interaction and understand the differences between this association and rhizobia-legume plants interaction, above all of infection process and bacteria distribution inside the vegetable tissue (4,5).

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Recently, it was report in Cuba for first time that *Rhizobium* is "true endophyte" of rice which was demonstrated by inoculation assays with a transconjugant strain which was visualized by confocal microscopy (6). Although in this work transconjugant cells containing *gfp* gene which coding for a green fluorescent protein (stain Rpd16pHC60 containing the plasmid pHC60) were showed colonizing the tissues of rice plant cv. INCA LP-5, no evidence of the obtaining of these bacteria was shown, even from other strains that have showed promoting growth activity in rice plant cultivar INCA LP-5. Taking into account the above, the objective of this work was to obtain effective transconjugant strains for plant-bacteria interaction studies.

MATERIALS AND METHODS

Bacteria used in this work and their main characteristics are listed in Table 1.

All they belong to bacteria collections of the National Institute of Agricultural Science, Cuba. Three of them; *Rhizobium* sp. Rpd16, *Rhizobium* sp. Rpr11 and *Rhizobium* sp. 5P1 were studied deeply in previous investigations (6,7). The strain *Pseudomonas* sp. S5-38 is less studied and it is putative endophytic bacteria of rice seed cv. INCA LP-5.

Obtaining transconjugant bacteria containing the plasmid pHC60

Transconjugant bacteria of the strains Rpr11, 5P1 and S5-38 containing the plasmid pHC60, were obtained according the methodology reported previously with the strain Rpd16. Similarly, the strains *E. coli* DH5 α(pRK2013) and *E. coli* DH5 α(pHC60) were used and triparental mating was carry out. The process efficacy was checking cultured the transconjugants strains on Tryptone-yeast extract (TY) with tetracycline (10 μg mL^{-1}) and 5-nitrofurantoine (50 μg mL^{-1}), incubated at 30 °C during 48 h. The plates were exposed to UV and fluorescent colony were cultured in the same medium and maintained at 4 °C (6).

Several multiplication dynamics were carried out to check out if the plasmid presence affects the growth of strains S5-38, Rpd16, Rpr11 and 5P1. The strains were incubated in a thermostated shaker (HEIDOLPH-UNIMAX-2010, Schwabach, Germany) at 150 rpm and 30 °C for 24 h. The OD (λ = 600 nm) was measured every 2 h for 30 h. Five replicates of each strain were used.

Bacteria-rice plant interaction

An inoculation assay was carried out to study the bacteria-rice interaction reported previously (6). Only the transconjugants Rpd16pHC60 and Rpr11pHC60 were used. One hundred microliters of the strains inoculants (10^8 CFU mL^{-1}) were used to inoculate pre germinated rice seed cv. INCA LP-5. The seedlings were maintained in half-strength Hoagland's plant growth medium and incubated in controlled conditions during 72 hours post inoculation (hpi). The roots were cut, inmoviliced (agarose 4 %) and sectioned and then the bacterial colonization of seedling roots was visualized in a confocal laser microscope (Zeiss, LSM 800 AiryScan, Jena-Germany).

Statistical analysis

Absorbance values obtained in the multiplication dynamics were subjected to the normality test (Bartlett test) and homogeneity of variance (Kolmogorov-Smirnov test). A simple classification analysis of variance was applied with the Tukey HSD mean comparison tests for $p<0.05$. The Statgraphic Plus program version 5.0 was used for statistical processing of the data and Microsoft Excel 2010 for its representation.

RESULTS AND DISCUSSION

Previously, it was demonstrate that strain *Rhizobium* sp. Rpd16 is endophyte of rice, since it was showed in intercellular spaces of the parenchyma of root and leaf sheath by confocal microscopy. For this, a transconjugant strain containing the plasmid pHC60 was obtained (Rpd16pHC60) (6). Similarly, the triparental mating with *E. coli* strains allowed obtain strains containing this plasmid which was confirmed when the strains Rpr11pHC60, 5P1pHC60 and S5-38pHC60 grown in TY medium with tetracycline and 5-nitrofurantoine, and fluorescent colonies were showed in presence of UV light (Figure 1).

Table 1. Strains used in this work

Strain	Main features	Reference
Rhizobium sp. Rpr11 (MT387213)	Strain isolated from rice rhizosfere cv. INCA LP-5, characterized account plant growth promoting trait. It promotes the rice cv. INCA LP-5 growth	(6)
Pseudomonas sp. S5-38 (MT808971)	Strain isolated from rice seed cv. INCA LP-5	Bacteria collection of National Institute of Agricultural Science (INCA)
Rhizobium sp. 5P1 (MT759831)	Strain isolated from rice rhizosphere cv. INCA LP-7 that promoted the rice growth in controlled, greenhouse and field conditions.	(7)
Rhizobium sp. Rpd16pHC60	Rpd16 strain containing the plasmid pHC60	(6)
Escherichia coli DH5α	*sup*E44Δ*lac*U169(Φ80*lacZ*ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1	(8)
Escherichia coli pRK2013	ColE1 replicon with RK2 *tra* genes. Used for mobilizing incP and incQ plasmids (Km^r)	(9)

*Fractions in parentheses represent the access number of consensus sequences deposited in GenBank

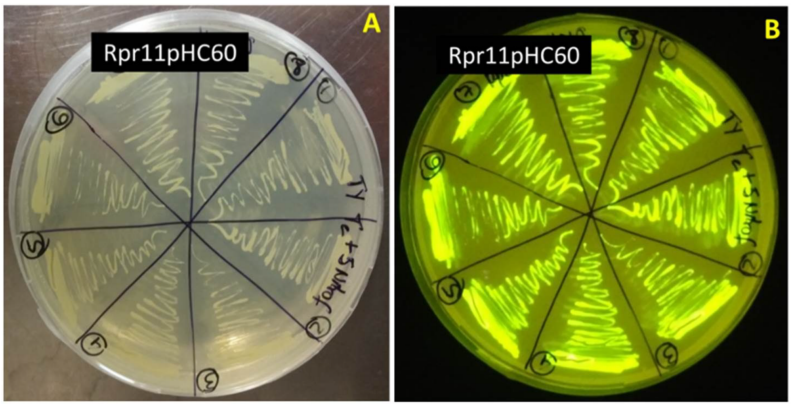


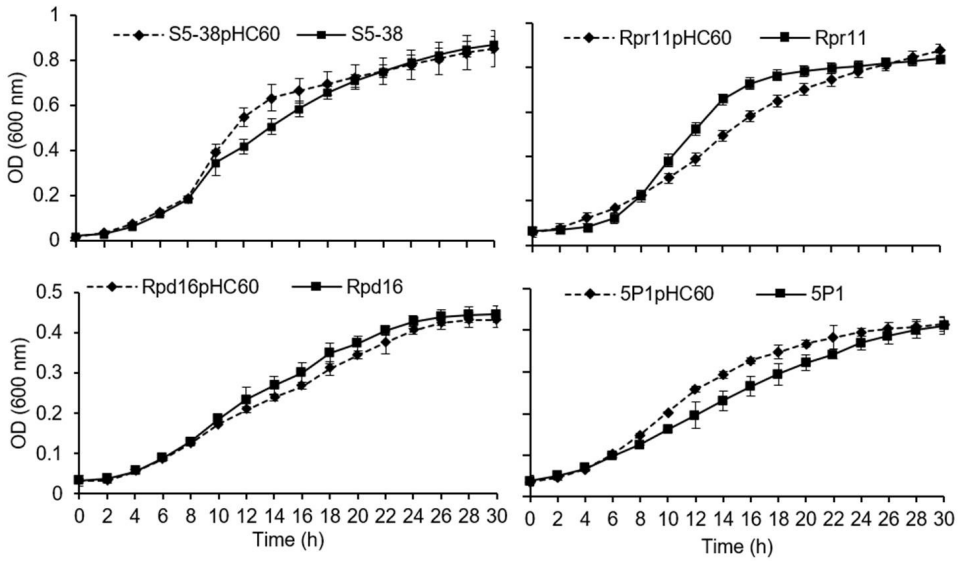
Figure 1. Transconjugant strain Rpr11 (Rpr11pHC60) containing the plasmid pHC60 in TY medium with tetracycline (10 µg mL^{-1}) and 5-nitrofurantoine (50 µg mL^{-1}), and exposed to white light (A), UV light (B)

Multiplication dynamics showed some differences in the strains growth. However the optical density of four wild type strains and their transconjugant strains were similar at the end of experiment (Figure 2). The effect of plasmid pHC60 in the transconjugant strains growth could be not significative.

No significative differences were showed in the strain Rpd16pHC60 growth and its wild type strain in all evaluation moments. This behavior was also reported when the effect of GFP-tagging on an diazotrophic and endophytic strain *Paenibacillus polymyxa* was studied (10). Probably, it is other reason to think why the plasmid pHC60 did not affect the ability of strain Rpd16 to colonize plant tissues, as was reported in previous works (6). Only significative differences were showed at 12-14 h between strains S5-38 and S5-38pHC60, and at 10-20h between Rpr11 and Rpr11pHC60, and between 5P1 and 5P1pHC60. In those moments, the transconjugant strains S5-38pHC60 and 5P1pHC60 had greater OD than theirs wild type strains.

In the other hand, it is know that in bacteria-plant interaction are distinguished associations with different degrees of complexity and the rhizosphere, phyllosphere and endosphere have been the main sceneries for the study of particularities of this interaction. The knowledge of association levels between bacteria and plants is essential for understand the how the microorganism colonizes the plant which is necessary to design and validate effective's inoculants in the field (1,2). In order to study the interaction bacteria-plant, rice seedlings cv. INCA LP-5 was inoculated only with the strains Rpd16 pHC60 and Rpr11pHC60. The selection was based on previous studies that suggesting that their wild type strains are promising to bio-fertilize rice crop (7).

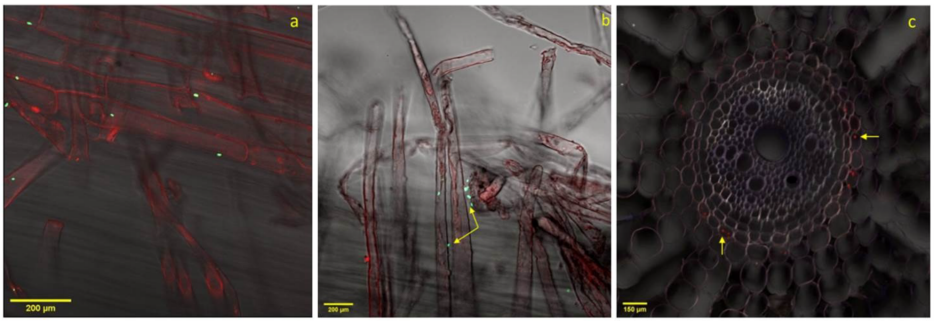
Although there was already evidence about the endophytic behavior of Rpd16pHC60 strain of rice, it was used on this occasion to delve into the forms of association with rice plants and as a comparison model with the Rpr11 strain.



The data points and bars represent the means and standard errors of the mean from three replicates at each sampling time (Tukey HSD $P<0.05$, n = 5). OD: optical density, µ: growth specific velocity

Figure 2. Multiplication dynamics in medium TY of strains S5-38, Rpd16, Rpr11, 5P1 and their transconjugants containing the pHC60 plasmid

Cross sections of rice roots were analyzed by confocal microscopy at 72 hpi and the results showed individual bacterial of the strain Rpd16pHC60 in the intercellular spaces of the cortical root parenchyma, similar to reported previously (Fig. 3a) (6). However, transconjugant cells were also visualized attached to the root hair which was not showed in micrographs reported before (Figura. 3b).



(a, b) Seedlings inoculated with the strain Rpd16pHC60. Transconjugant cells are displayed in green, red color: autofluorescence of plant tissue. (c) Root cross section of seedlings inoculated with the strain Rpr11pHC60. Transconjugant cells are displayed in red. Yellow arrows: presence of the transconjugant strains

Figure 3. Fluorescence confocal microscopy images showing root tissue sections of 72 h old rice seedlings cultivar INCA LP-5

The strain Rpr11pHC60 was localized colonizing the intercellular spaces of root parenchyma. This is new evidence that suggest other strain belonging *Rhizobium* genus as endophyte of rice cv. INCA LP-5. However, it is necessary to study more deeply their interaction with rice to conclude that it is a "true endophyte". Previously, it is reported studies about rhizobia-pinach (*Spinacia oleracea*) and rhizobia-sugarcane (*Saccharum officinarum*) interactions by the method of the tagging with genes coding for a fluorescing protein (11,12). Previous investigations confirmed that inoculation of rice plants cv. INCA LP-5 with strain Rpr11 increased the potassium content and root dry weigh in greenhouse conditions (7). Thus, if Rpr11 have an endophytic behavior, it is an attribute that also would allow explaining its plant growth promoting activity.

CONCLUSIONS

The employ of transconjugant strains and the confocal microscopy for plant-bacteria interaction studies is an effective and novel tool. Here, three transconjugant strains containing the pHC60 plasmid were obtained and one of these is a putative endophyte of rice cv. INCA LP-5. The fact that the wild type strains used came from of different rice cultivars and association levels with the plant could give us more knowledge about the particularly of their interaction with rice plant.

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