Symbiosis, 13 (1992) 139–145
Balaban, Philadelphia/Rehovot

Differential Complementation of *Rhizobium meliloti* 7027: Isolation of a Second *ExoC* Locus from *Azospirillum brasilense* Sp7

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Received October 20, 1991; Accepted March 5, 1992

Abstract

Azospirillum brasilense SP7 cosmid clones which complemented binding of the fluorescent dye calcofluor by *Rhizobium meliloti 7027 exoC* mutants were identified. One class of cosmids was also found capable of restoring wild-type exopolysaccharide production and β-1,2-glucan synthesis. A restriction endonuclease map of the *A. brasilense* DNA insert from complementing clones demonstrated no homology with a previously identified *exoC* locus. The new (*exoC2*) locus was further mapped to the *A. brasilense* chromosome.

Keywords: *Azospirillum*, exopolysaccharide, glucan, calcofluor, *Rhizobium*, *exoC*

1. Introduction

Bacteria of the genus *Azospirillum* colonize plant roots and can exert beneficial effects on plant growth. However, the mechanisms of this interaction is poorly understood. Because the *Azospirillum*-plant association lacks a clear diagnostic phenotype (such as root nodule formation in the case of *Rhizobium*) most attempts to characterize genes involved in this interaction with plants

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0334-5114/92 /$03.50

have relied upon complementation of defined *Rhizobium* or *Agrobacterium* mutants. The *exoC* locus is required for plant interactions by both genera. Mutations in this locus result in the inability to synthesize both succinoglucan and β-1,2-glucan (Marks et al., 1987; Cangelosi et al., 1987; Leigh et al., 1985); and, in the case of *R. meliloti*, *exoC* mutants synthesize an abnormal lipopolysaccharide (Leigh and Lee, 1988).

Previous attempts to identify genes involved in exopolysaccharide (EPS) production and plant association in *A. brasilense* led to the cloning of an *exoC* locus which restored EPS production to *R. meliloti* 7027, resulting in a Calcofluor bright phenotype (Michiels et al., 1988). However, β-1,2-glucan production was not restored, and the EPS synthesized was not wild-type. In this study we report the identification of a second *exoC* locus which more fully complements the *exoC* mutation.

2. Materials and Methods

The sources and maintenance of bacterial cultures used in this study were identical to those described earlier (Eyers, 1990). An *A. brasilense* SP7 cosmid library was screened by conjugative transfer to Rm7027 as previously described (Michiels et al., 1988). Complementing cosmids were initially selected on the basis of restoration of fluorescence on Calcofluor containing media when viewed under UV light (Cal^+). Subsequently, Rm7027 harboring cosmid isolates were assayed for the additional ability to form nitrogen-fixing nodules when inoculated on alfalfa as determined by the acetylene reduction method (Meade et al., 1982).

Plasmid DNA was purified from complemented Rm7027 cultures by an alkaline lysis procedure (Birnboim and Doly, 1979). Restriction enzymes were obtained from Boehringer Mannheim and used according to the supplier's directions.

EPS was purified from complemented Rm7027 grown in N-free M9 medium in which glucose was replaced by fructose (Eyers, 1990). Following removal of the bacteria by centrifugation at 10,000×g for 20 min, the supernatant was dialysed extensively with H_2O using dialysis tubing with a Mr cutoff of 2000 Da. The supernatants were then concentrated by freeze drying. For proton-magnetic resonance analysis, EPS samples of 15 mg were exchanged several times with deuterium oxide before dissolving in 99.98% D_2O (Sigma Chemical Co.). NMR spectra were then recorded using a Bruker WM500 instrument, and the chemical shifts measured relative to an external tetramethylsilane standard.

Motility was analysed on yeast extract-mannitol medium supplemented with 0.3% agar by measuring the diameter of colonies formed after 48 hr. The presence of β-1,2-glucan was analysed by gel chromatography of trichloroacetic acid extracts of cell pellets using Bio-Gel P4 columns (Geremia et al., 1987).

3. Results

Cosmid DNA from Cal$^+$, Fix$^+$ Rm7027 cultures was isolated and subjected to restriction endonuclease analysis. All four of the cosmids clones tested produced identical *EcoR*I maps, indicating the integrity of the library. The restriction map of the entire 26 kb *A. brasilense* DNA insert of one of these clones (pCal134) is shown in Fig. 1. The restriction map clearly distinguishes

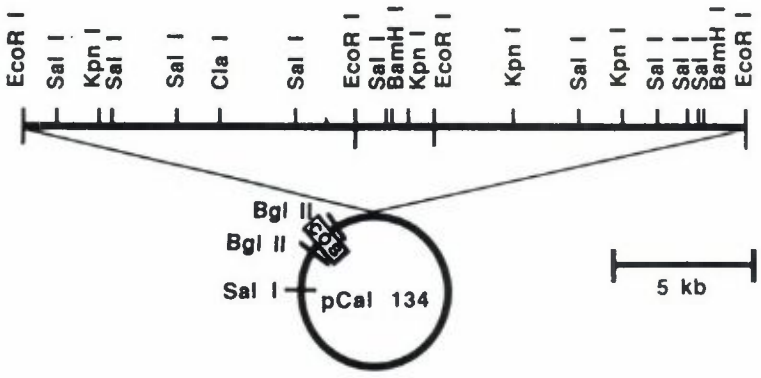

Figure 1. Restriction endonuclease map of pCal134 containing the *exoC2* locus. pCal134 consists of 26 kb of *A. brasilense* chromosomal DNA inserted into the *EcoR*I site of pLAFR1.

the new *exo*-complementing locus (*exoC2*) from the previously identified *exoC1* encoded by pCal112 (Michiels et al., 1988). Hybridization of radiolabeled *exoC2* plasmid DNA to *A. brasilense* DNA isolated by the method of Kado and Liu (1981) localized the *exoC2* locus to the chromosome, not the p90 plasmid containing the *exoC1* locus (data not shown). Furthermore, no cross-hybridization was observed between pCal134 and either pD15, containing the Rm1021 *exoC* gene, (Leigh et al., 1985) or pCal112, confirming the second locus was not the result of a nuclear excision/duplication event.

The ability of the *exoC2* locus to complement the mot$^-$ phenotype of Rm7027 was also analysed. Comparisons of colony diameters of Rm7027 and Rm7027 (pCal134) clearly demonstrated the restoration of motility of the latter when plated on 0.3% agar plates.

Proton-NMR analysis of EPS isolated from Rm7027 harboring pCal112 or pCal134 demonstrated the restoration of wild-type EPS synthesis by pCal134 (Fig. 2). The Cal$^+$ binding EPS produced by Rm7027 (pCal112) is clearly lacking the prominent pyruvate and two succinate moiety peaks of Rm1021, while both classes of substituents are restored in Rm7027 (pCal134) (see Fig. 2). β-1,2-glucan production was also evidenced by chromatography of TCA extracts of Rm7027 (pCal134) on Bio-Gel P4 columns (data not shown) with an elution profile corresponding to that of wild-type Rm1021 (Geremia et al., 1987).

4. Discussion

The restoration of nitrogen-fixation capacity, as well as EPS and glucan synthesis abilities in Rm7027 firmly establishes that the new *A. brasilense exoC2* locus can also complement the *exoC* mutation. Still, the existence of two *exoC* loci is surprising and the physiological role of each locus remains to be identified. As shown in Table 1, all *Rhizobium* mutants or transconjugants devoid of β-1,2-glucan have a *Fix*$^-$ phenotype. Although a role for β-1,2-glucan in osmolarity control has been proposed (Miller et al., 1986; Dylan et al., 1990), our results strongly suggest that glucans play a more direct role in *Rhizobium*-plant interaction as restoration of β-1,2-glucan production restores nitrogen-fixation ability. This conclusion is confirmed by more recent reports that *Agrobacterium chvB* mutants lacking periplasmic β-1,2-glucan are avirulent regardless of the osmolarity of the growth medium (Cangelosi et al., 1990).

It has been suggested that the pleiotropic *exoC* phenotype is the consequence of loss of phosphoglucomutase activity (Uttaro et al., 1990), which would result in the absence of UDP-glucose necessary for EPS, LPS, and glucan biosynthesis. However, the differential complementation ability of *exoC* clones supports a regulatory role for *exoC*. Clearly, *exoC1* does not restore phosphoglucomutase activity, for no β-1,2-glucan is produced and the EPS which is synthesized is not wild-type. Furthermore, since glucan synthesis has not been detected in *A. brasilense* (Altalbe et al., 1990), it is unlikely that pCal134 encodes a structural gene for this production pathway. Future experiments will focus on the characteristics of *A. brasilense exoC1* and *exoC2* mutants constructed by site-directed mutagenesis using the cloned genes. Characterization of the

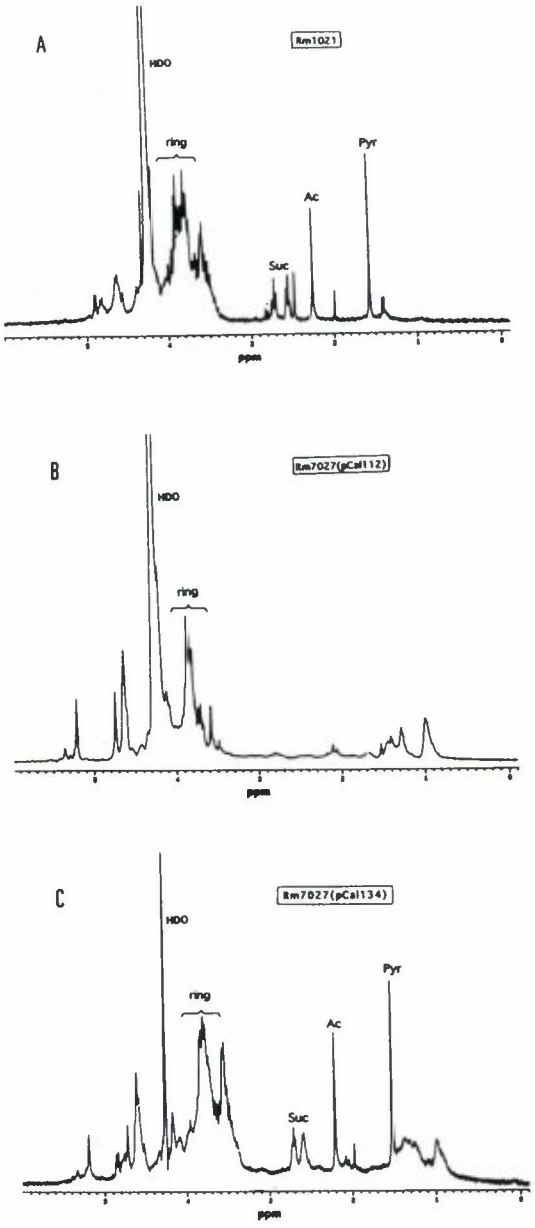

Figure 2. Proton NMR specta of EPS produced by *R. meliloti* strains. Panel A: Rm1021, wild-type; B:Rm7027 (pCal112), *exoC1*; C: Rm7027 (pCal134), *exoC2*. The positions of several signal peaks corresponding to ring substituent moeities which are restored by *exoC2* are labeled. Abbreviations used: HDO, hemideuterated water; ring, ring protons of the sugars; Suc, methyl protons of the succinyl groups; Ac, methyl protons of the acetyl groups; Pyr, methyl protons of the pyruvyl groups.

Table 1. Phenotypic characteristics of *R. meliloti exoC* mutants

Strain	Cal	EPS	glucan	motility	Plant phenotype
Rm1021	+	WT	+	+	Nod^+Fix^+
Rm7027	–	–	–	–	Nod^+Fix^-
Rm7027 (pCal112)	+	not WT	–	+	Nod^+Fix^-
Rm7027 (pCal134)	+	WT	+	+	Nod^+Fix^+

protein product of *exoC2* may also provide an answer to the functional roles of β-1,2-glucan and EPS in plant interactions.

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