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QUARTERLY

Homology of genes for exopolysaccharide synthesis in *Rhizobium leguminosarum* and effect of cloned *exo* genes on nodule formation*

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A 5.4 kb *Bam*HI fragment of *R. leguminosarum* bv. *trifolii* TA1 was found to carry genes involved in exopolysaccharide synthesis (*exo* genes). This fragment was strongly hybridized to the total DNA from *R. l.* bv. *viciae* and bv. *phaseoli* digested with *Eco*RI. No homology was found with total DNA of *R. meliloti* and *Rhizobium* sp. NGR 234. The *exo* genes from *R. l.* bv. *trifolii* TA1 conjugally introduced into *R. l.* bv. *viciae* 1302 considerably affected the symbiosis: the nodules induced on vetch were abortive and did not fix nitrogen. On the other hand, *Phaseolus* beans infected with *R. l.* bv. *phaseoli* harbouring *R. l.* bv. *trifolii exo* genes formed the nitrogen-fixing nodules. It can be concluded that additional copies of *exo* genes introduced into wild type *Rhizobium leguminosarum* strains can disturb the synthesis of acidic exopolysaccharides and affect symbiosis of the plants forming indeterminate nodules, but do not affect symbiosis of the plants forming the determinate nodules.

Extracellular polysaccharides (EPS)[1] are produced by all species of symbiotic nitrogen-fixing rhizobia and play an essential role in symbiosis. To establish the function of EPS in symbiosis, numerous *Rhizobium* mutants have been isolated. The *Exo*⁻ mutants of *R. meliloti, R. l.* bv. *trifolii* and *viciae* form ineffective nodules on their host plants [1 - 6]. On the other hand, mutants of *R. l.* bv. *phaseoli* defective in acidic exopolysaccharide synthesis are symbiotically effective on *Phaseolus* beans [7 - 9]. It has been therefore concluded that EPS are essential for the infection of indeterminate nodule-type legumes but are not essential for the infection of determinate nodule-type plants.

The acidic EPS produced by *R. leguminosarum* strains is a polymer composed of octasaccharide subunits that contains galactose, glucose and uronic acids in a molar ratio of 1:5:2, as well as noncarbohydrate acyl groups such as pyruvate, acetate and butyrate [10 - 14]. The chemical structure of EPS is different from that of the succinoglucan synthesized by *R. meliloti* [15] and EPS of *Rhizobium* sp. NGR 234 [11].

Several genes involved in synthesis or regulation of EPS have been identified in *R. meliloti, Rhizobium* sp. NGR 234 and *R. l.* bv. *phaseoli*, and the general organisation of the *exo* genes has been described [6 - 9, 16 - 20]. The activity of some of these genes is required for nodulation and/or nitrogen fixation [7 - 9]. Detailed analysis of the *exo* genes of *R. meliloti* and *Rhizobium* sp. NGR 234 led to define some common genes which are homologous and functionally interchangable in the synthesis of both polysaccharides [18]. The regulatory genes such as *psi*

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[1]Abbreviations: EPS, extracellular polysaccharides.

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(*R. leguminosarum*) and *exo* X (*R. meliloti*, *Rhizobium* sp. NGR 234) showed functional equivalence in inhibiting EPS production, although there was no hybridization between them [6, 18].

We have previously described the recombinant cosmid pARF136, which corrected *exo* mutation in *R. l.* bv. *trifolii* 93. The DNA fragment carrying *exo* genes from *R. l.* bv. *trifolii* TA1 was located on the non-symbiotic 300 kb megaplasmid [21, 22]. In this report, we showed by cross species hybridization analysis, the homology between the *R. l.* bv. *trifolii* TA1 *exo* region and the total DNA of *R. l.* bv. *viciae* and bv. *phaseoli*. We were not able to detect the homology with the total DNA of *R. meliloti* and *Rhizobium* sp. NGR 234.

R. l. bv. *trifolii exo* genes present as extra copies in *R. leguminosarum* strains which formed indeterminate nodules, markedly disturbed the symbiosis, but did not influence the symbiosis of *R. l.* bv. *phaseoli*, forming determinate nodules on *Phaseolus*.

MATERIALS AND METHODS

Strains, plasmids and media. Bacterial strains and plasmids are listed in Table 1. *Rhizobium* strains were grown on mannitol-yeast-extract agar medium (79 CA), as described by Vincent [27]. For *E. coli* cultures, the LB medium was used [28]. Concentration of antibiotics were as described previously [21].

Genetic techniques. Cosmids were transferred conjugatively in triparental matings, as described before [22]. The transconjugants Tc^rRif^r of *R. l.* bv. *viciae* and bv. *phaseoli* were purified several times by single colony isolations and used for nodulation assay.

Nodulation assay. Seeds of vetch (*Vicia sativa* L.cv. Jaga) and beans (*Phaseolus vulgaris* L. cv. Hara) were surface sterilized and germinated on agar plates containing nitrogen free R medium [27]. Vetch seedlings were transferred onto R medium slants and inoculated with *R. l.* bv. *viciae*. Bean seedlings were grown in 300 ml flasks containing 200 ml of the agar R medium. One germinated seed was transferred to each flask and inoculated with 1 ml cell suspension of the appropriate *R. l.* bv. *phaseoli* strain. The plants were cultured in standard conditions (21°C, relative humidity 70 - 80%, day/night 12/12 h, irradiance 40 W m^{-2} white fluorescent lamps, POLAM, Poland) for 28 days. Nitrogenase activity was measured as described previously [2].

DNA manipulation. Total DNA from *Rhizobium* strains was isolated according to Sambrook *et al.* [28]. Routine manipulations for

Table 1
Bacterial strains and plasmids

Strains or plasmids	Relevant properties	References
Strains		
R. meliloti L5 30	$Nod^+Fix^+Sm^r$	[23]
R. leguminosarum bv. *viciae* RBL 1302	Nod^+Fix^+pJB5JI	[24]
R. leguminosarum bv. *viciae* RS 3	Nod^+Fix^+	[25]
R. leguminosarum bv. *phaseoli* F4	Nod^+Fix^+	[25]
R. leguminosarum bv. *trifolii* 24	Nod^+Fix^+	IUNG Puławy
Rhizobium sp. NGR 234	Nod^+Fix^+	[4]
Plasmids		
pRK 2013	Nm^r ColE1 replicon with RK2 *tra* genes	[26]
pARF 136	pLAFR3 containing 19 kb *Bam*HI insert complementing *exo* mutation	[21]
pARF 1368	pRK7813 vector containing 5.4 kb *Bam*HI fragment of pARF 136	[21]
pARF 25	pRK7813 containing 4.5 kb *Bam*HI/*Hin*dIII fragment of pARF 1368	[21]

plasmid isolation, agarose gel electrophoresis nick translation, Southern blotting, and hybridization were carried out as described previously [21].

RESULTS AND DISCUSSION

Homology between the *exo* region of *R. l.* bv. *trifolii* and DNA of other *Rhizobium* species

The recombinant cosmid pARF 136 isolated previously, corrected *Exo*⁻ mutation in *R. l.* bv. *trifolii* 93 [21, 22]. The restriction map of pARF136 and its several subclones in the broad host range vector pRK7813 are shown in Fig. 1. The cosmids containing 5.4 kb *Bam*HI (pARF 1368) and 4.5 kb *Bam*HI-*Hin*dIII (pARF 25) overlapping fragments retained the ability to complement of *Exo*⁻ mutation in *R. l. bv. trifolii* strain 93. We have tested the DNA homology between the cloned *exo* region from *R. l.* bv. *trifolii* TA1 and DNA of other *Rhizobium* strains: *R. l.* bv. *trifolii* 24, *R. l.* bv. *viciae* RS 3, *R. l.* bv. *phaseoli* F4, *R. meliloti* L5.30 and *Rhizobium* sp. strain NGR 234. Total DNA from these rhizobia were digested with *Eco*RI, blotted and hybridized to a nick-translated 5.4 kb *Bam*HI fragment of pARF 1368 (Fig. 2).

The results of hybridization indicated a strong homology between the 5.4 kb *Bam*HI fragment and 8.0 kb *Eco*RI of *R. l.* bv. *phaseoli* F4 and *R. l.* bv. *trifolii* 24, and 6.5 kb *Eco*RI fragment of *R. l.* bv. *viciae* RS 3 [Fig. 2]. The 8.0 kb *Eco*RI fragment fully overlaps the 5.4 kb *Bam*HI fragment in cosmid pARF 136 (Fig. 1). We could also observe some extent of restriction polymorphism in the homologous region of *R. l.* bv. *viciae;* the *Eco*RI fragment homologous to 5.4 kb *Bam*HI fragment from *R.l.* bv. *trifolii* was smaller (6.5 kb) than in the other biovars (8.0 kb) of *R. leguminosarum.*

On the other hand, we did not observe any homology between the *exo* region from strain TA1 and either *R. meliloti* L5.30, or *Rhizobium* sp. strain NGR 234 (Fig. 2).

Symbiotic properties of the different rhizobia carrying the cloned *exo* region

We reported previously, that recombinant plasmids pARF 1368 and pARF 25 conjugatively introduced into *R. l.* bv. *trifolii* 93 restored the ability of this mutant to synthesize EPS. *R. l.* bv.

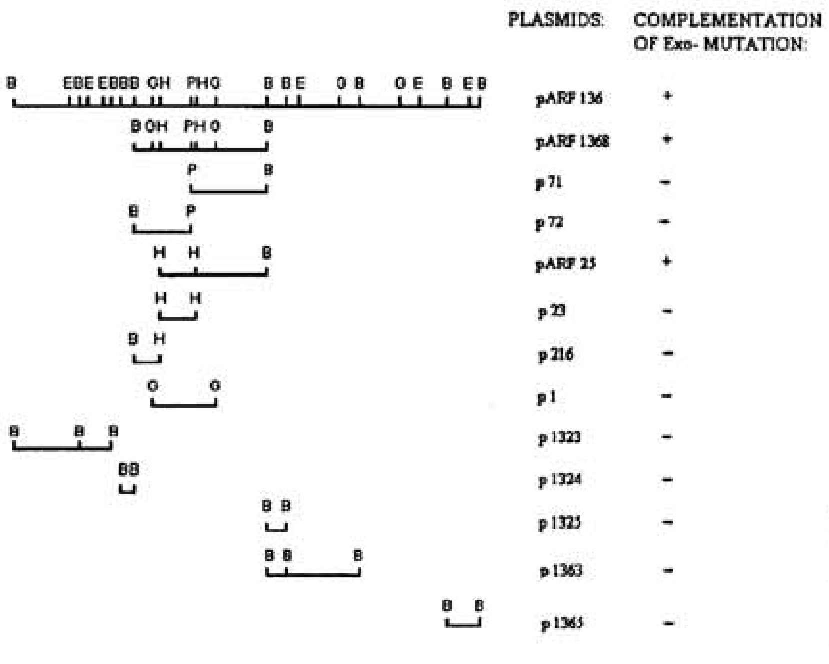

Fig. 1. Restriction map of the exo region of R. l. bv. trifolii TA1 cloned in plasmid pARF 136. (B, BamHI; E, EcoRI; G, BglII; H, HindIII; P, PstI); +, –, refer to production of EPS in R. l. bv. trifolii 93 Exo⁻ strain carrying respective plasmids.

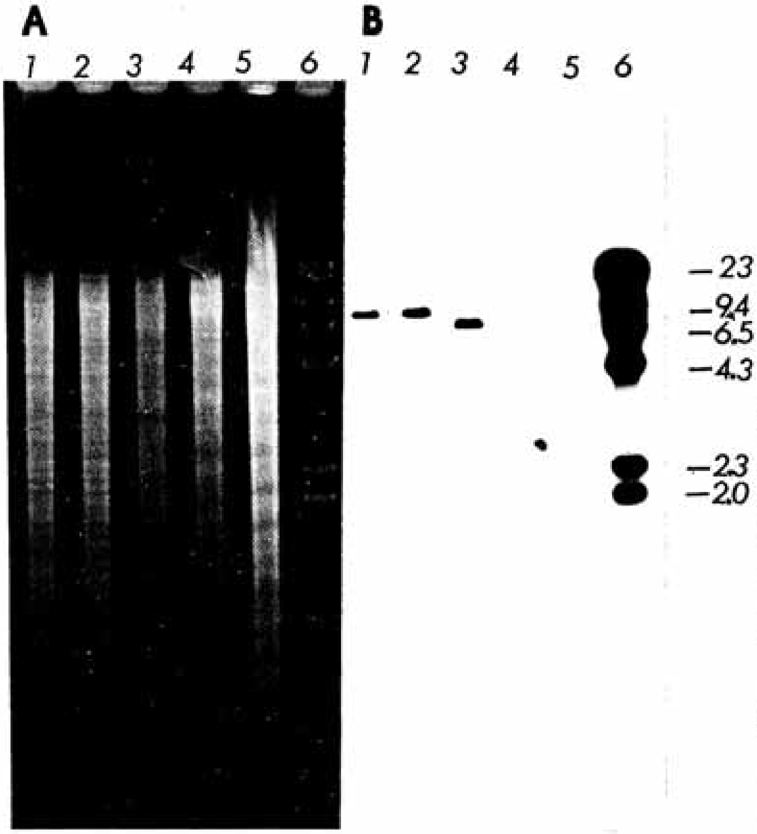

Fig. 2. Agarose gel stained with ethidium bromide (A) and autoradiogram of a Southern blot of EcoRI-digested total DNA (B) from R. l. bv. phaseoli F4 (1), R. l. bv. trifolii 24 (2), R. l. bv. viciae 3 (3), R. meliloti L5 30 (4) and Rhizobium sp. strain NGR 234 (5) and lambda DNA digested with HindIII (6) probed with ^{32}P-labelled 5.4 kb BamHI fragment of pARF 1368 carrying exo region from R. l. bv. trifolii TA1 and ^{32}P-labelled lambda/HindIII DNA. The numbers refer to molecular markers HindIII-digested phage lambda DNA.

trifolii wild-type strain 24 harbouring the recombinant plasmids produced an altered exopolysaccharide containing less noncarbohydrate substitutions, and nodules induced by this strain were ineffective [22].

To test the effect of the *exo* region from *R. l.* bv. *trifolii* TA1 on the symbiosis of *R. l.* bv. *viciae* with *Vicia sativa* and *R. l.* bv. *phaseoli* with the *Phaseolus* bean, the plasmids pARF 1368 and pARF 25 were conjugatively introduced into *Rhizobium* strains. The transconjugants were tested on respective host plants for symbiotic phenotypes (Table 2). The mucoid transconjugants *R. l.* bv. *viciae* 1302 pARF 1368 infected only 47% of the tested vetch and the plants were yellow with small, abortive nodules. *R. l.* bv. *viciae* 1302 pARF 25 were non-nodulating because they lost, for unknown reasons, the symbiotic plasmid (pJB5JI). *R. l.* bv. *phaseoli* F4 pARF 1368 and F4 pARF 25 were mucoidal, nodulated effectively nearly all tested *Phaseolus* beans, and the plants were green with the no-

Table 2

Effect of R. l. bv. trifolii TA1 exo region on symbiotic properties of R. leguminosarum bv. viciae and bv. phaseoli

Strains	No of nodulated /no of tested plants[a]	No of nodules /plant	Nitrogenase activity μM C_2H_4/h per plant
R. l. bv. *viciae*			
RBL 1302	50/50	5.8	0.46
RBL 1302 pARF 1368	33/70	1.8	0.0
RBL 1302 pARF 25	0/35	0.0	NT
R. l. bv. *phaseoli*			
F4	11/13	18	0.88
F4 pARF 1368	13/13	25	2.25
F4 pARF 25	12/13	20	0.52

[a]The plants were tested after 28 days of growth; NT, not tested.

dules looking like those induced by the F4 wild-type strain (Table 2). The *R. l.* bv. *phaseoli* reisolated from the bean nodules were Tc^rSm^r, indicating relative stability of the recombinant cosmids inside the plants.

The molecular basis of the role of EPS in the symbiosis has not been elucidated. It is known that properly modified acidic EPS appears to be required for the establishment of effective, indeterminate nodules like those of alfalfa, clover, and pea [14, 16, 22]. In contrast, determinate nodules, like those of *Phaseolus* or *Lotus* are effective even when induced by *exo* mutants or a *Rhizobium* strain with altered EPS [1, 8, 29]. Our results are in agreement with these reports. The *exo* genes of *R. l.* bv. *trifolii* TA1, although homologous to those of other biovars, disturb the synthesis of the acidic EPS when present as additional copies, and lead to ineffectiveness of indeterminate nodules formed by these rhizobia on clover or vetch. We can suppose that the conjugally introduced *exo* genes from *R.l.* bv. *trifolii* TA1 produce improperly modified EPS of the host bacteria and affect some stages of infection in the indeterminate-type nodules. In the case of determinate nodules formed on *Phaseolus* by *R. l.* bv. *phaseoli*, the modifications of EPS are not critical and nodules are fully effective.

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