Biol Fertil Soils (2004) 39:292–294
DOI 10.1007/s00374-004-0721-y

SHORT COMMUNICATION

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The effect of rhizobiophages on *Sinorhizobium meliloti* – *Medicago sativa* symbiosis

Received: 5 May 2003 / Accepted: 16 December 2003 / Published online: 24 January 2004

Abstract Using Tn5 mutagenesis of lysogenic *Sinorhizobium meliloti* strain L5–30 (ϕL54), three neomycin-resistant transconjugants differing in phage-resistance profiles were isolated. Two of them increased the dry weight of plants and were capable of establishing root nodules, whereas the third one was ineffective. The bacterium-phage interaction did not have observable consequences in the *Medicago sativa* - *S. meliloti* symbiosis because it did not affect the number of nodules on *M. sativa* or plant dry weight.

Keywords Alfalfa · *Medicago sativa* · Nitrogen fixation · Phage resistance · Rhizobiophages

Introduction

Nodulated legumes fix between 30 and 300 kg N ha^{-1} $year^{-1}$ depending on the plant species, microsymbiont strain, and environment (Wdowiak and Małek 2000). To increase the input of biologically fixed N_2 in agriculture, specially selected or genetically modified nodule bacteria are planned to be used as inoculants. Introduced strains must compete with highly adapted indigenous microorganisms for legume nodulation and atmospheric nitrogen fixation in specific physical and biological local soil conditions. Phages may affect the bacterial population through the selection of resistant strains and elimination of sensitive ones (Demolon and Dunez 1935; Dhar et al. 1980). Moreover, phages can act as vehicles for the dissemination of genes among related bacteria (Kowalski 1967).

Rhizobiophages are widespread in soil which contains appropriate host bacteria (Dhar et al. 1980; Wdowiak and Małek 2000; Mendum et al. 2001), although it is not clear whether they can significantly affect the bacterium number in soil, and thus the growth of legumes.

This communication describes the effect of rhizobiophages on *Medicago sativa* – *Sinorhizobium meliloti* symbiosis on the basis of the number of nodules and dry weight of the green part of plants.

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Materials and methods

Bacterial strains and phages

Bacterial strains and phages as well as their origin are shown in Table 1.

Media and growth conditions

For the growth of bacteria and propagation of phages, liquid, solidified (1.4%) and soft (0.7%) agar media 79 were used (Wdowiak and Małek 2000). Tn5-containing transconjugants and transductants were selected on BS minimal medium (Sherwood 1970). Plants were cultivated in a N-free agar medium, described by Hoagland and Arnon (1950).

Lytic specificity

Phage sensitivity profiles were determined as described by Kowalski (Kowalski 1967).

Isolation of phage-resistant mutants by Tn5-Mob mutagenesis

Phage-resistant mutants of *S. meliloti* strain L5–30 were isolated by Tn5-Mob mutagenesis. For this purpose, 18-h liquid cultures of *Escherichia coli* strain S17–1 carrying Tn5-Mob (Simon et al. 1983), and *S. meliloti* L5–30 (ϕL54) streptomycin-resistant (Str^r) lysogen carrying transducing phage ϕL54, were mixed at a ratio of 1:5. This mixture was incubated without shaking for 20 h at 30°C and plated on BS medium containing streptomycin (500 μg ml^{-1}) for *E. coli* counterselection and neomycin (100 μg ml^{-1}) to select L5–30 (ϕL54) transconjugants with Tn5 integrated into their genome. Neomycin-resistant (Nm^r) transconiugants of *S. meliloti* strain L5–30 (ϕ54) were tested for phage sensitivity with ten phages. Mutants representing three types of phage-sensitivity patterns were irradiated with UV to induce phage ϕL54. Induced

Table 1 Bacterial strains and bacteriophages used in this study. *Str*^{r} Streptomycin-resistant strain, *Nod*$^+$ *Fix*$^+$ nodule-forming N_2-fixing strain

	Relevant characteristics	Source
Bacterial strains		
Sinorhizobium meliloti L5-30 (ϕL54)	Str^{r}Nod$^+$Fix$^+$ strain lysogenic for L54 phage	Kowalski (1967)
S. meliloti L5–30	Nod$^+$Fix$^+$ wild type	
S.meliloti 776 (*chr*::Tn*5-mob*)[a]	Phage-resistant derivative of *S. meliloti* L5–30	This work
S.meliloti 73c2 (*chr*::Tn*5-mob*)	Phage-resistant derivative of *S. meliloti* L5–30	
Escherichia coli 17.1	With pSUP5011 plasmid	Simon et al. (1983)
Phages		
P6, P10, P33, P45	Virulent phages	M. Kowalski[b]
1A	Virulent phage	Staniewski[b]
M10, M12, M14, M19	Virulent phages	Werquin et al. (1988)
F4	Virulent phage	Moskalenko[c]

[a] *chr*::Tn*5-mob* mutants were isolated by Tn*5-mob* insertional mutagenesis
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[c] Institute of Microbiology Academy of Science, Moscow

Table 2 Phage sensitivity profiles determined on *S. meliloti* strain L5–30 and its derivatives. + Sensitive to phage, – resistant to phage

S. meliloti strain	Group I phages	Group II phages						Group III phages		
	1A	M10	M14	F4	P6	P10	P45	P33	M12	M19
L5–30 (ϕL54)	+	+	+	+	+	+	+	+	+	+
L5–30	+	+	+	+	+	+	+	+	+	+
776 (Class I)	–	+	+	+	+	+	+	+	+	+
R43 (Class II)	+	–	–	–	–	–	–	+	+	+
73c2 (Class III)	+	+	+	+	+	+	+	–	–	–

phage ϕL54 was used for transduction of Tn5 into *S. meliloti* strain L5–30. Transductants were selected for Nm^r and studied for phage sensitivity to confirm the dependence of the phage-resistance marker on the integration of the Tn5 transposon into the bacterial genome.

Inoculation test

M. sativa seeds were surface sterilized with 75% ethanol (3 min) and 0.1% $HgCl_2$ (2 min) and germinated on Petri dishes with N-free plant nutrient solution. In plant tests one seedling per tube with N-free medium was used. The seedlings grew for 2–4 days and then they were inoculated with 100 μl bacterial suspension (~10^7 cells ml^{-1}) plus 100 μl of the studied bacteriophage lysates which were prepared by mixing 10 μl of each of them (~10^7 phage particles ml^{-1}) directly before their application. Plants were cultivated for 6 weeks in a greenhouse at 20°C and under a 16-h light period. Plants inoculated with 100 μl of bacterial suspension (~10^7 cells ml^{-1}) were used as a control. The symbiotic effectiveness of *M. sativa–S. meliloti* associations was estimated by the number of nodules and dry weight of the green part of plants.

Each experiment, with five replicates of *M. sativa* plus the respective inoculation mixture, was repeated 3 times. The *F*-Snedecor test was used for statistical analysis. Significant differences for each pair of objects, i.e. for plants inoculated with bacteria in the presence and absence of bacteriophages, were calculated using Tukey's intervals.

Results and discussion

Using Tn5-Mob mutagenesis, three classes of phage-resistant mutants of the lysogenic strain L5–30 (ϕ54), represented by strains 776, R43 and 73c2, were isolated. Their phage-sensitivity profiles were established using ten virulent rhizobiophages (Table 2). Strain 776, resistant to 1A phage, was included in the first class. Class II was represented by strain R43 resistant to six phages, and class III by strain 73c2 resistant to three rhizobiophages.

Direct dependence of the phage-resistance phenotype on the integration of Tn5 into the *S. meliloti* genome was supported by the co-transduction of neomycin and phage-resistance markers. As expected, all transductants selected for neomycin resistance retained phage profiles of donor strains (data not presented).

S. meliloti phage-resistant mutants were studied for symbiotic properties on *M. sativa*. Number of nodules and dry matter yields of plants inoculated with two of them, i.e. R43 and 73c2, were similar to those of plants inoculated with *S. meliloti* L5–30 (ϕL54), whereas 1A resistant strain 776 was ineffective (Table 3).

To study a possible influence of rhizobiophages on the symbiotic capacities of rhizobia, *M. sativa* was inoculated with *S. meliloti* L5–30(ϕ54) strain and its phage-resistant mutants in the absence and presence of viruses as described in Materials and methods. The presence of rhizobiophages in the alfalfa inoculation mixture did not affect symbiosis (Table 3). The nodule number on *M. sativa* and dry matter yield by *S. meliloti* strains in the presence and absence of phages were similar. This result contradicts the hypotheses of Demolon and Dunez (1935) and Dhar (1980) that bacteriophages can affect a legume symbiosis by reducing the rhizobium population density. The presence of phages in soils was demonstrated for the

Table 3 Symbiotic properties of *S. meliloti* L5–30 (ϕL54) and its phage-resistant mutants in the presence and absence of phages. Data are means from three experiments; in each there were five replicates of each combination of *M. sativa* and the respective inoculation mixture. The shoot dry weight of uninoculated control plants was 2.2 mg plant^{-1}

S. meliloti strain	Effect on *Medicago sativa*	
	No. of nodules per plant[a]	Shoot dry weight per plant (mg)[a]
L5–30 (ϕL54)	9.5	14.5
L5–30 (ϕL54)+Phages[b]	11.0	15.3
776	5.0	2.4
776+Phages	4.0	2.3
73c2	7.4	11.6
73c2+Phages	8.0	11.2
R43	9.2	13.8
R43+Phages	8.3	14.9

[a] No significant differences were found for plants inoculated with bacteria in the presence and absence of bacteriophages at $P = 0.05$

[b] Plants were inoculated with each of ten phage lysates as described in Materials and methods

first time in 1935 by Demolon and Dunez (1935). They showed that phages killed rhizobia in soils where alfalfa had been cultivated for several years, resulting in widespread lucerne disease. This finding was later contradicted by Kleczkowska (1957), who indicated a correlation between the presence of phages and the presence of their hosts.

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