

Research Note

Expression of a *Serratia marcescens* Chitinase Gene in *Sinorhizobium fredii* USDA191 and *Sinorhizobium meliloti* RCR2011 Impedes Soybean and Alfalfa Nodulation

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A gene encoding chitinase from *Serratia marcescens* BJL200 was cloned into a broad-host-range vector (pRK415) and mobilized into *Sinorhizobium fredii* USDA191. Chitinolytic activity was detected in *S. fredii* USDA191 transconjugants that carried the *S. marcescens* *chiB* gene. Chitinase-producing *S. fredii* USDA191 formed nodules on soybean cultivar McCall. However, there was a delay in nodule formation and a marked decrease in the total number of nodules formed by the chitinase-producing *S. fredii* in comparison with the wild-type strain. Expression of chitinase in *S. meliloti* RCR2011 also impeded alfalfa nodulation. Thin-layer chromatography of ¹⁴C-labeled Nod factors from chitinase-producing *S. fredii* USDA191 revealed hydrolysis of lipochitooligosaccharides.

Pesticides are widely used to control plant diseases. However, most pesticides are hazardous to the health of humans and animals. They also have deleterious effects on ecological systems. Biological control promises to be a useful alternative approach in the control of plant pathogens (Weller 1988). Chitin, a β -1,4-linked polymer of *N*-acetyl glucosamine (GlcNAc), is an important structural component of insects, fungi, and nematodes (Monreal and Reese 1969). Several organisms, including higher plants, fungi, and bacteria, produce chitinases (EC 3.2.1.14) that cleave the glycosidic bonds in the chitin chain by either an endolytic or exolytic mechanism (Monreal and Reese 1969). It has been shown that application of chitin can reduce plant disease caused by certain soil fungi and nematodes (Mian et al. 1982; Shapira et al. 1989). This beneficial effect has been attributed to proliferation of organisms that secrete chitin-degrading enzymes (Mankau and Das 1969; Mercer et al. 1992; Miller and Sands 1977). Based on

the potential role of chitinase as an anti-fungal agent (Schlumbaum et al. 1986; Broglie et al. 1991), attempts have been made to express chitinase genes in rhizosphere-colonizing soil bacteria to control pathogenic fungi (Fuchs et al. 1986; Sitrit et al. 1993).

Serratia marcescens, a gram-negative bacterium, is very efficient in the degradation of chitin because of its ability to produce different chitinolytic enzymes. Two chitinase genes (*chiA* and *chiB*) have been isolated from *S. marcescens* (Jones et al. 1986; Brurberg et al. 1995). Inactivation of one of the chitinase genes greatly reduced the ability of this bacterium to inhibit the growth of *Fusarium oxysporum* f. sp. *pisi* (Fop), a fungal pathogen (Jones et al. 1986). Attempts have also been made to express the *S. marcescens* chitinase in *Pseudomonas* spp. and plant symbiotic bacteria such as *Rhizobium* spp. to control soil pathogens (Fuchs et al. 1986; Sitrit et al. 1993). It was proposed that, since *Rhizobium* spp. can efficiently colonize the roots of hosts and nonhosts in large numbers, they could serve as a powerful biocontrol agents. Sitrit et al. (1993) expressed the *chiA* gene from *S. marcescens* in *R. meliloti* and have demonstrated that the nodule extracts from *chiA* expressing alfalfa plants caused lysis of *Rhizoctonia solani* hyphal tips. Interestingly, chitinase-producing *R. meliloti* strains also had no effect on alfalfa symbiosis. This observation is intriguing since rhizobium-induced nodule formation in legumes is initiated by Nod factors, which are modified oligomers of *N*-acetylglucosamine (GlcNAc). Nod factors can be hydrolyzed by chitinases (Staelin et al. 1994; Minic et al. 1998). Hence, the expression of chitinase genes in *R. meliloti* may lead to the breakdown of the Nod factors that will lead to defective symbiosis. We therefore decided to examine the consequences of expressing the chitinase gene in *Sinorhizobium fredii* USDA191 on soybean symbiosis.

A plasmid (pMAY2-10) containing the *chiB* gene of *S. marcescens* BJL200 (Sundheim et al. 1988) as a 1.8-kb *EcoRI/MluI* fragment in pGEM-7f (+) was obtained from May Brurberg (Agricultural University of Norway). This plasmid was digested with *EcoRI* and *HindIII* and recloned into pRK415, a broad-host mobilizable vector (Keen et al. 1988), digested with the same enzymes. The resulting plasmid was named pSMC1 and mobilized into *S. fredii* USDA191 by tri-

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parental mating with the helper plasmid pRK2013. Transconjugants, which were resistant to tetracycline, were grown in YEM (Vincent 1970) media. *S. fredii* USDA191(pRK415) and USDA191(pSMC1) exhibited similar growth characteristics and had identical doubling time. In order to see whether the chitinase gene was being expressed in *S. fredii* USDA191, we performed a highly sensitive silver stain method for detecting chitinolytic activity after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Marek et al. 1995). Three-day-old, 5-ml cultures of *S. fredii* USDA191 containing *chiB* and pRK415 were centrifuged and the pellet was resuspended in 500 μ l of SDS-sample buffer without β -mercaptoethanol. The samples were boiled for 5 min and 10 μ l-aliquots were resolved by SDS-PAGE (Laemmli 1970). The resolving gel contained 0.01% glycol chitin. After electrophoresis, the chitinolytic activity was detected essentially as described earlier (Marek et al. 1995). Proteins with chitinolytic activity appeared as clear zones on a dark brown background. Extracts from *S. fredii* USDA191 carrying *chiB* revealed chitinase activity associated with a 56-kDa protein (Fig. 1). Addition of genistein, an isoflavonoid that induces the expression of nodulation genes of *S. fredii*, had no effect on the chitinase activity (Fig. 1, lane 3). Protein extracts from *S. fredii* carrying the vector pRK415 alone revealed no chitinase activity (Fig.1, lane 1). This result confirmed that the *S. marcescens chiB* was being expressed in *S. fredii*. Subcellular fractionation of the cell extracts from *S. fredii* USDA191 expressing *chiB* revealed that the chitinase activity was associated with the cytosol and the periplasm (data not shown). The estimated molecular mass of the chitinase was 56 kDa, which is the same size as the one expressed in *S. marcescens* (Brurberg et al. 1995).

We also investigated the consequence of expressing the chitinase in *S. fredii* on soybean nodulation. Seeds of soybean

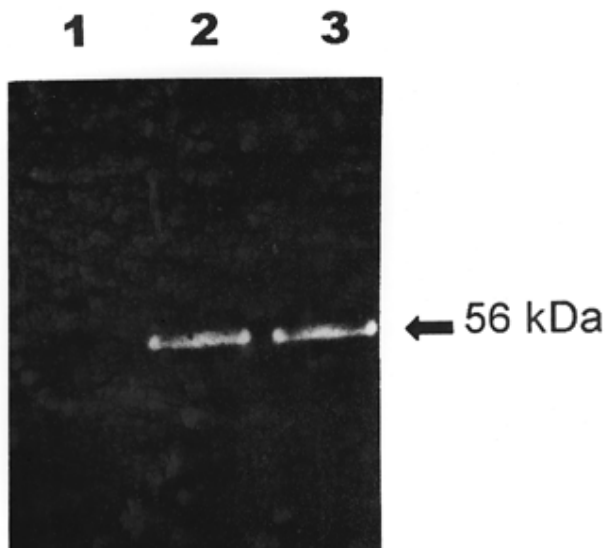


Fig. 1. Expression of *Serratia marcescens* chitinase in *Sinorhizobium fredii* USDA191. Total proteins were resolved on a 13.5% sodium dodecyl–polyacrylamide electrophoresis gel and the chitinolytic activity was detected by silver stain. The resolving gel contained 0.01% (wt/vol) glycol chitin. Lane 1: USDA191 (pRK415); lane 2: USDA191 (pSMC1); and lane 3: USDA191 (pSMC1) grown in the presence of 1 μ M genistein. β -Mercaptoethanol was omitted in all protein samples.

(*Glycine max* (L.) Merr.) cultivar McCall were surface sterilized and germinated on water agar plates at 30°C. The roots of 3-day-old soybean seedlings were dip inoculated with cells of USDA191 containing either *chiB* or the cloning vector pRK415 (10^8 cells per ml). The seedlings were transferred to autoclaved plastic growth pouches that had been prewetted with distilled water. Soybean plants were grown in a growth chamber and the appearance of the nodules was monitored from day 5 after inoculation and alternate days thereafter until day 20. The kinetics of nodulation and final nodule numbers induced by USDA191 (pSMC1) were drastically different from those of USDA191 (pRK415). Chitinase-producing USDA191 exhibited delayed nodulation, with the nodules first visible at 9 days after inoculation (Fig. 2). At day 20, McCall soybean inoculated with USDA191 (pRK415) had 32 nodules per plant, while the chitinase-producing strain had only 12 nodules per plant. We also measured the nodule acetylene-reduction rates by the method of Schwinghamer et al. (1970). The nitrogenase activity expressed on a nodule fresh weight basis was similar between these two treatments (data not shown).

S. fredii synthesizes an array of Nod factors consisting of a series of β -1,4-linked oligomers of *N*-acetyl-D-glucosamine, with degrees of polymerization ranging from three to five (Bec-Ferte et al. 1994 and 1996). Chitinase readily digests the Nod factors of *S. fredii* and breaks down into chitobiose and chitotrioses (Bec-Ferte et al. 1996). In order to examine whether expressing chitinase in *S. fredii* had any effect on the Nod factors, we labeled the USDA191 cells with 14 C-acetate in the presence and absence of 1 μ M genistein. Supernatant from the labeled cultures was loaded on a C18 reversed-phase SepPak cartridge (Waters Corp., Milford, MA). The column

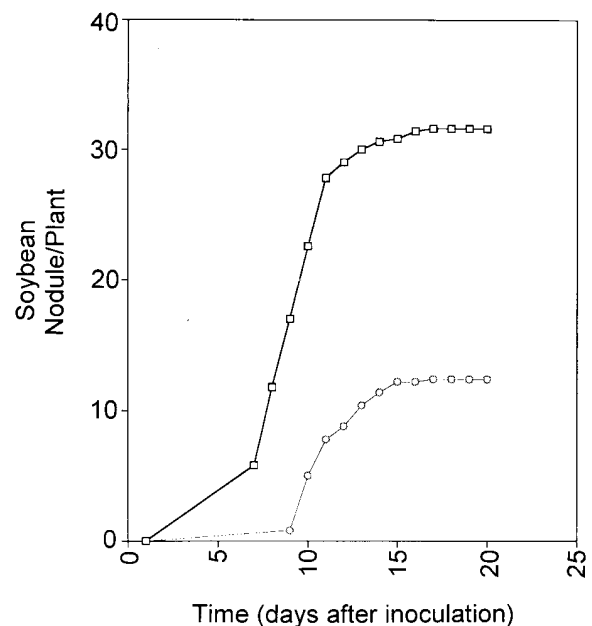


Fig. 2. Nodulation of cv. McCall soybean by *Sinorhizobium fredii* USDA191 expressing *Serratia marcescens* chitinase. Seedlings were inoculated with either USDA191 (pRK415) (circles) or USDA191 (pSMC1) (squares). Each point represents the mean nodule number from 32 plants from two independent experiments. Standard errors were less than 9% of means.

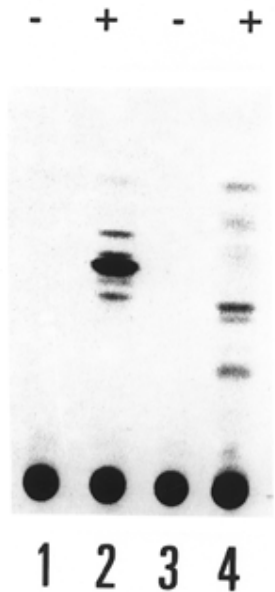


Fig. 3. Thin-layer chromatography (TLC) analysis of radiolabeled compounds produced by *Sinorhizobium fredii* USDA191. Bacteria were grown in presence of ^{14}C -acetate in the presence or absence of $1\ \mu\text{M}$ genistein. Radiolabeled compounds were resolved on octadecyl silica reversed-phase TLC plates and subjected to autoradiography. Samples: lane 1, *S. fredii* USDA191(pRK415), lane 2, *S. fredii* USDA191 (pRK415) grown in the presence of $1\ \mu\text{M}$ genistein; lane 3, *S. fredii* USDA191(pSMC1); lane 4, *S. fredii* USDA191(pSMC1) grown in the presence of $1\ \mu\text{M}$ genistein.

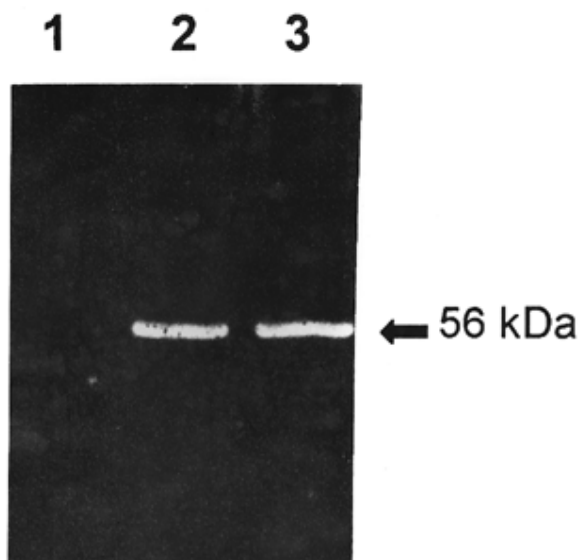


Fig. 4. Expression of *Serratia marcescens* chitinase in *Sinorhizobium meliloti* RCR2011. Total proteins were resolved on a 13.5% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and the chitinolytic activity was detected by silver stain. The resolving gel contained 0.01% (wt/vol) glycol chitin. Lane 1: RCR2011 (pRK415); lane 2: RCR2011 (pSMC1); lane 3: RCR2011 (pSMC1) grown in the presence of $1\ \mu\text{M}$ luteolin. β -Mercaptoethanol was omitted in all protein samples.

was washed with water and the Nod factors were eluted with methanol and concentrated to about $100\ \mu\text{l}$. Nod factors were resolved on octadecyl silica reversed-phase thin-layer chromatography (TLC) plates as described earlier (Bec-Ferte et al. 1994). Labeled Nod factors were visualized by autoradiography. Figure 3 shows at least four ^{14}C -labeled spots from the culture of USDA191 that had been grown in the presence of genistein (Fig. 3, lane 2). These radioactive spots were absent in uninduced cultures of USDA191 (Fig. 3, lane 1). The chitinase-producing USDA191 also revealed several ^{14}C -labeled spots, but their R_f values were different from those of the non-chitinase-producing strain. One heavily ^{14}C -labeled spot seen in USDA191 (Fig. 3, lane 2) was missing in the chitinase-producing strain; instead, few additional spots were evident (Fig. 3, lane 4). These results indicated that the Nod factors were being hydrolyzed by *S. marcescens* chitinase, resulting in altered Nod factors.

Earlier, Sitrit et al. (1993) reported *R. meliloti* expressing *S. marcescens* chitinase did not influence nodule formation, plant growth, or nitrogen fixing ability of alfalfa. In addition, these authors showed that protein extracts from nodules initiated by chitinase-producing *Rhizobium* spp. were able to degrade the hyphal tips of *R. solani*. We also tested the extracts from soybean nodules initiated by chitinase-producing and non-producing strains of USDA191, and found that both extracts were able to lyse *R. solani* hyphal tips. The protein extracts from soybean root nodules contained multiple chitinase activity as visualized by the SDS-PAGE/silver chitinolytic activity stain (data not shown). We believe that the lysis of *R. solani* hyphal tips was probably mediated by plant chitinases. We also wanted to test whether expressing the *chiB* gene in *S. meliloti* had any adverse effect on alfalfa nodulation. We

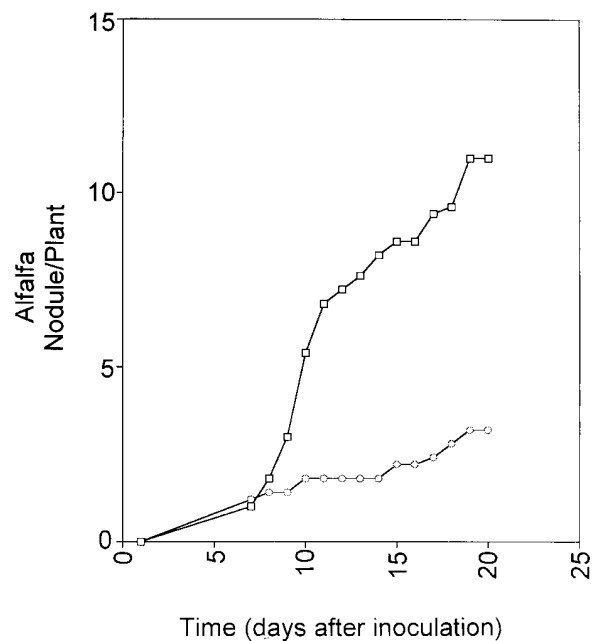


Fig. 5. Nodulation of alfalfa cv. Nitro by *Sinorhizobium meliloti* RCR2011 expressing *Serratia marcescens* chitinase. Seedlings were inoculated with either RCR2011 (pRK415) (circles) or RCR2011 (pSMC1) (squares). Each point represents the mean nodule number from 32 plants from two independent experiments. Standard errors were less than 7% of means.

therefore mobilized the *chiB* construct into *S. meliloti* RCR2011 and verified whether the transconjugants can produce chitinase. SDS-silver stain chitinolytic activity gels clearly established that the transconjugants were able to produce chitinase of about 56 kDa (Fig. 4). Chitinase-producing RCR2011 also exhibited delayed nodulation and reduced nodule numbers on alfalfa cv. Nitro (Fig. 5). Therefore, it is evident that expressing the *chiB* gene in RCR2011 does affect nodulation. This result differs from the earlier study (Sitrit et al. 1993). This could be due to the fact that we expressed *chiB*, while the other study expressed *chiA*. *chiA* encodes the most abundant chitinase in *S. marcescens* and it is secreted in culture media. *chiB*, on the other hand, is localized in the periplasm and is found in the culture medium only after prolonged culturing times, presumably due to cell lysis (Fuchs et al. 1986; Jones et al. 1986; Brurberg et al. 1995). It is most likely that the subcellular location of the chitinase could have a direct influence on the breakdown of the Nod factors. Since Nod factors accumulate primarily in the membranes (Orgambide et al. 1995), the periplasmic-located chitinase may be best suited to act on these signal molecules.

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