Expression of *Serratia marcescens* Chitinase Gene in *Rhizobium meliloti* During Symbiosis on Alfalfa Roots

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Chitin is a major structural cell-wall component of many plant pathogenic fungi. The polymer is degraded by chitinase and the latter's substantial role in biological control has been well established. We introduced the chitinase gene from *Serratia marcescens* into the plant symbiont *Rhizobium meliloti*, which colonizes the root nodules of alfalfa (*Medicago sativa*). *Rhizobium* colonies harboring the plasmid with the chitinase gene were identified by the clear halos of degraded chitin surrounding them. Functional analysis showed nitrogenase activity to be similar in the transconjugants and wild-type bacteria. Plants infected with either bacteria exhibited similar growth, with no symptoms of nitrogen deficiency. Western blot of proteins extracted from nodules infected with the transconjugants revealed a 58-kDa band corresponding to *S. marcescens* chitinase. The antifungal activity of *R. meliloti* during symbiosis on alfalfa roots was verified by lysis of *Rhizoctonia solani* hyphal tips treated with cell-free nodule extracts. The role of chitinase in plant defense mechanisms is discussed.

Additional keyword: biological control.

The increasing use of hazardous pesticides to control plant diseases has threatened the stability of many ecological systems. Most of the pesticides are nonselective and may therefore become hazardous to humans, animals, and other organisms, among them the natural enemies of plant pathogens. A promising alternative to the use of hazardous chemical pesticides is that of biological control (Sivan and Chet 1992; Weller 1988). A major mechanism involved in the biological control of plant pathogens is parasitism. Several cell wall-degrading enzymes, such as chitinase, are involved in this process (Elad *et al.* 1982). Chitinase is a glucanohydrolase that degrades the chitin polymer, a major cell-wall constituent of many fungi, into short dimers of *N*-acetylglucosamine (Jeuniaux 1966). The role of chitinase in the biological control of plant diseases has been well established (Broglie *et al.* 1991; Roberts and Selitrennikoff 1988). Shapira *et al.* (1989) have shown that irrigation with chitinase reduces disease incidence in plants grown in soil infected with pathogenic fungi which contain chitin as one of their cell-wall components.

Potential agents for controlling plant diseases are the soil bacteria that colonize the rhizosphere and are capable of promoting plant growth (Bull *et al.* 1991; Burr *et al.* 1978; Schroth and Hancock 1982). Plant symbiotic bacteria are also excellent root colonizers, and this suggests their use as biocontrol agents. To date, however, they have not been employed as such because they lack the ability to control plant diseases. The introduction of genes involved in the biocontrol mechanism into plant-symbiotic bacteria could generate new and powerful biocontrol agents.

Bacteria of the genus *Rhizobium* establish a symbiosis with leguminous plants, resulting in the formation of root nodules. The bacteria are present inside the nodules as bacteroids that fix atmospheric nitrogen and provide legume plants with ammonia. Although the induction of nodule formation is host specific, *Rhizobium* can attach to roots of nonhost plants in the same way they attach to the host (Chen and Phillips 1976; Terouchi and Syono 1990). This phenomenon, plus the ability to colonize in large numbers the rhizosphere of a wide variety of plants makes *Rhizobium* spp. the obvious choice for experiments aimed at generating new biocontrol agents.

In this work we describe the expression of *Serratia marcescens* chitinase gene in *Rhizobium meliloti*, and the antifungal activity of proteins extracted from the alfalfa nodules infected with the *Rhizobium* transconjugants.

RESULTS

Chitinase expression in *R. meliloti*.

The plasmid pYZ291 (Fig. 1), carrying the *S. marcescens* chitinase gene, was transferred from *E. coli* strain O25 to *R. meliloti* 1021 by triparental mating. Tetracyclin-resistant transconjugants were selected on plates containing chitin. *R. meliloti* colonies harboring the chitinase gene were identified by a clear halo of degraded chitin. The levels of chitinase activity, detected in both *E. coli* and *R. meliloti* carrying the plasmid, indicated that the *tac* promoter allows efficient expression of the chitinase gene in *R. meliloti* (Table 1). However, during symbiosis, chitinase activity in the nodules was about 40% that detected in free-growing *Rhizobium* (Table 1). Chitinase activity in

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the nodules was calculated per microgram of protein, including bacterial and nodular proteins. The protein concentration in the nodule extract was about twice that in the bacterial protein extract, which explains the difference between chitinase activity in free-growing *Rhizobium* and that recorded during *Rhizobium* symbiosis on alfalfa roots (data not presented). Southern blot analysis of nodule extracts, carried out 45 days after inoculation, verified the presence and stability of the plasmid pYZ291 in the *Rhizobium* cells during the symbiotic stage (Fig. 2).

The expression of the chitinase gene by *Rhizobium* inside the alfalfa nodules was detected by Western blot analysis. Alfalfa nodules were ground and crude protein extracts were run on SDS-PAGE. Proteins were then transferred to nitrocellulose paper. An immunoblot was performed with polyclonal antibodies raised against *S. marcescens* chitinase. A single band of 58 kDa appeared, corresponding to the chitinase band of *S. marcescens* (Fig. 3).

To determine whether the transfected chitinase is functionally expressed in the nodules, crude protein extracts were run on native PAGE. The substrate used in this assay enables differentiation between the plant chitinases and the introduced chitinase, since this substrate is suitable only for bacterial chitinases. The chitinase activity was detected only in protein extract of alfalfa nodules infected

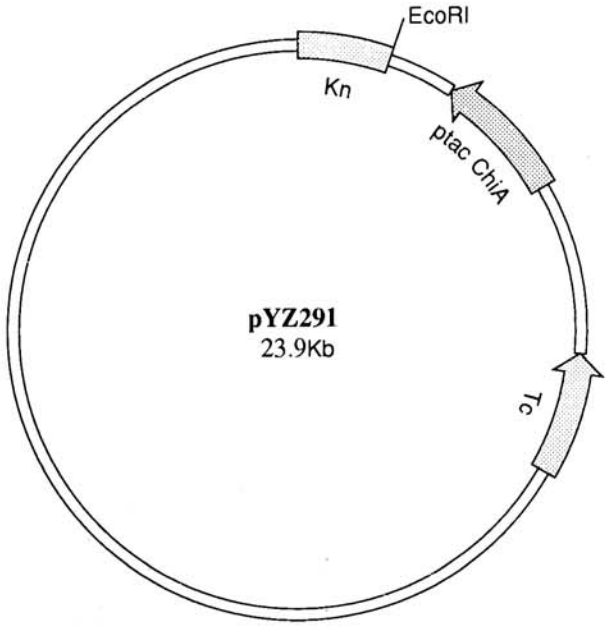

Fig. 1. Schematic representation of plasmid pYZ291 constructed as described in text. Arrows indicate the direction of transcription.

Table 1. Chitinase activity in *E. coli*, free-growing *Rhizobium meliloti*, and extracts of alfalfa nodules infected with *Rhizobium* carrying plasmid pYZ291 or wild-type *Rhizobium*

Strain	Chitinase activity (Units[a])
E. coli 025	1.15
R. meliloti 1021, free-living	0
Nodule extract[b] infected with *R. meliloti* 1021	0.40
R. meliloti 026, free-living	0.83
Nodule extract infected with *R. meliloti* 026	0.72

[a] 1 U of chitinase is defined as μmole of pnp/μg of protein per hour released from pnp-chitobiose.
[b] Alfalfa plants inoculated with strain 026 were harvested after 44 days and crude protein extracts from the nodules were prepared for chitinase activity assay.

Fig. 2. Southern blot of DNA extracted from alfalfa nodules infected with *Rhizobium meliloti* carrying the plasmid pYZ291 or wild-type *R. meliloti* 1021. Lane 1, plasmid pYZ291. Lane 2, DNA extracted from nodules infected by the wild-type *R. meliloti* 1021. Lanes 3–7, DNA extracts of nodules infected with *R. meliloti* carrying plasmid pYZ291 strains 001, 005, 006, 011, and 026, respectively.

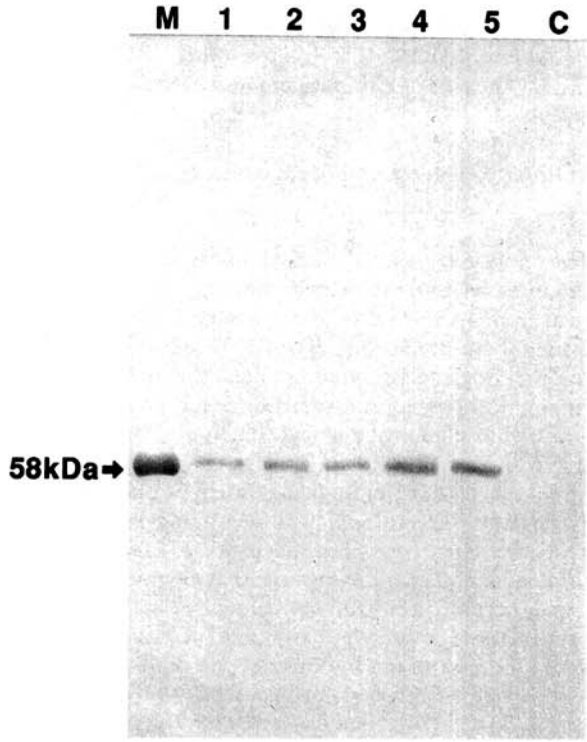

Fig. 3. Western blot analysis of proteins extracted from alfalfa nodules. Lanes: M, chitinase marker; 1–5, crude protein extracts of nodules infected with *Rhizobium meliloti* carrying plasmid pYZ291; C, proteins of control nodules infected with *R. meliloti* 1021.

with the transconjugants but not in extracts of plants infected with the wild-type bacteria (Fig. 4).

Symbiotic proficiency of the transconjugants.

Several plant growth parameters normally influenced by the symbiotic relationship were examined to verify that the expression of chitinase was not interfering with nodule formation and plant development. Nitrogenase activity in plant roots infected with the transconjugants or wild-type bacteria was similar, suggesting that the ability to maintain enzyme activity is not impaired by chitinase expression (Table 2). The efficiency of nitrogen fixation by the *Rhizobium* transconjugants was also evaluated, by monitoring chlorophyll content and plant dry weight. Both parameters were similar to those in plants infected with the wild-type bacteria (Table 3). The equally similar fresh weights of nodules from plants infected with the wild-type or the bacteria carrying plasmid pYZ291 clearly indicate that the ability to induce nodule formation is not impaired by chitinase expression (Table 3).

Fig. 4. Activity assay in polyacrylamide gel of the chitinase expressed by *Rhizobium meliloti* during symbiotic growth on alfalfa nodules. Protein extracted from alfalfa nodules were run on native PAGE and the gel was overlaid with agarose containing 4-methylumbelliferyl β-D-*N,N'*-diacetylchitobioside. Chitinase activity was detected under UV light. Lane 1, crude protein extract of nodules infected with *Rhizobium meliloti* carrying plasmid pYZ291. Lane 2, crude protein extract of nodules infected with wild-type *Rhizobium meliloti*. Lane 3, marker, *Serratia marcescens* chitinase.

Table 2. Acetylene reduction activity and chitinase activity in nodules infected with different *Rhizobium meliloti* transconjugants carrying plasmid pYZ291

Rhizobium isolate	Chitinase activity[a] (Units)	Acetylene reduction (μmole C_2H_4/hr plant)
1021	0.000	4.9
001	0.409	9.8
005	0.280	7.3
006	0.339	13.2
011	0.305	8.4
026	0.203	7.1

[a]The values represent net chitinase activity contributed by the introduction of the chitinase gene. One unit of chitinase activity is defined as μmole of GlcNac/g FW hr released from colloidal chitin.

Effect of nodule extract on hyphal tips.

Chitinase activity in the nodules against plant-pathogenic fungi was demonstrated by the nodule extract's ability to lyse *R. solani* hyphal tips. Hyphal tips immersed in a nodule extract from plants infected with the transconjugants were swollen within 10 min (Fig. 5). This was followed by spilling of the cytoplasmic contents, indicating lysis of the fungal tips. Cell wall lysis was not observed in hyphal tips treated with nodule extract from plants infected with the wild-type bacteria. To exclude the possibility that a plant factor from the nodules was involved in the hyphal tips lysis, the antifungal activity of free-living *Rhizobia* was determined. Treating hyphal tips with crude protein extracts of free-living *Rhizobia* expressing the chitinase gene increased up to ninefold the number of deteriorated hyphal tips. Extracts of nodules infected by the transconjugants increased the number of deteriorated tips by threefold (Table 4).

DISCUSSION

Microorganisms capable of lysing other organisms are widespread in natural ecosystems and can serve as a resource for the responsible genes (Monreal and Reese 1969). The lysis of *R. solani* hyphal tips by extracts of alfalfa

Table 3. Nodule fresh weight, chlorophyll content, and dry matter production of plants[a] inoculated with strain 026[b]

Strain	Chlorophyll (μg/g)	Nodule fresh weight (mg/plant)	Plant dry weight (mg/plant)
R. meliloti 1021	171.6	18.68	218.38
R. meliloti 026	217.7	19.66	196.72

[a]Means of 36 plants from five pots.
[b]Plants inoculated with strains 1021 or 026 were compared 44 days after inoculation of 1-day-old seedlings.

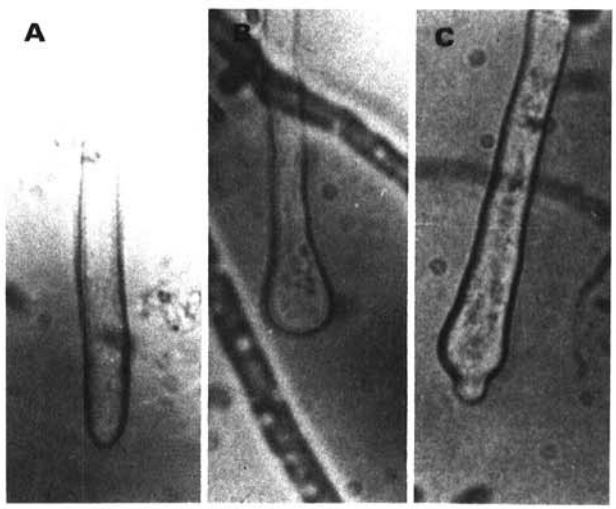

Fig. 5. Light micrographs showing the effect of alfalfa root nodule protein extract on hyphal tips of *Rhizoctonia solani*. **A,** Hyphal tip treated with crude protein extract of nodules from plants infected with the wild-type *Rhizobium meliloti* 1021. **B, C,** Hyphal tips treated with crude protein extract of nodules from plants infected with *Rhizobium meliloti* 026 carrying the chitinase gene of *Serratia marcescens* (×400).

nodules inoculated with *R. meliloti* carrying plasmid pYZ291 clearly demonstrates the feasibility of introducing genes involved in antifungal activity into symbiotic bacteria. Although the chitinase expressed in *Rhizobium* efficiently degrades hyphal tips of *R. solani,* its antifungal activity is restricted to fungi containing chitin in their cell walls. Thus, an ideal biocontrol agent would be one that employs multiple defense strategies, coordinately activated against pathogens. Cloning of several lytic enzymes into the same organism could generate biocontrol agent with broad antifungal activity.

Numerous reports have described the involvement of chitinolytic activity in the biological control of soilborne diseases by microorganisms (Elad *et al.* 1984; Inbar and Chet 1991; Jones *et al.* 1986). The plants themselves synthesize chitinases that have been shown to inhibit fungal growth, suggesting an essential function for chitinases in the induced resistance to pathogen attack (Boller *et al.* 1983; Schlumbaum *et al.* 1986). It has now been clearly established that transgenic plants expressing cloned lytic enzymes, including chitinase, show enhanced resistance to fungal pathogens (Broglie *et al.* 1991; Jach *et al.* 1992). However, prior to infection, hydrolytic enzymes are only present in the plant in small amounts, if at all. The accumulation of these enzymes appears to be a relatively late event, following invasion of the pathogen (Graham and Graham 1991). It therefore seems that immediate release of the first elicitor signal from the pathogen is crucial to the successful activation of the defense mechanisms in the plant. Constant expression of the chitinase gene in *Rhizobium* during symbiosis could contribute to an immediate reaction toward the invading pathogen. The contribution of the cloned chitinase gene to the total chitinolytic activity in the nodules was about 1.7 U/μg of protein as estimated from gel activity assays. This contribution is enough to enable efficient lysis of hyphal tips as determined in the antifungal assays. Moreover, constitutive secretion of chitinase to the rhizosphere by tree-growing *Rhizobium,* together with chitinase expression inside the nodules by symbiotic *Rhizobium* could increase plant resistance to pathogens.

Bacteria growing in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere is the "front" at which roots are protected against attacking pathogens (Schippers 1988). Other types of bacteria with promising qualities for use as biocontrol agents are symbiotic bacteria such as *Rhizobium,* which colonize the rhizosphere as well as the root nodules. As a result of their location inside the plant, they may provide more efficient protection against plant pathogens than other rhizobacteria. Until now, attempts to improve the biocontrol activity of rhizobacteria by introducing chitinase genes have failed for several reasons: 1) introduction of unstable constructs and subsequent rapid loss of the plasmids harboring the chitinase gene (Sundheim *et al.* 1988); 2) poor expression of the chitinase gene due to inefficient promoter reading (Fuchs *et al.* 1986). The plasmid pYZ291 is highly stable in the bacteroids during symbiotic growth in the nodules as indicated by the Southern blot performed 45 days after inoculation. Moreover, 99% of the *Rhizobium* colonies carried the plasmid after growing the cells in liquid culture for 100 generations without antibiotics (data not presented).

Beneficial rhizobacteria were previously shown to be very potent agents for biological control and for enhancing plant growth. Until now, plant symbiotic bacteria have been neglected as candidates for use as biocontrol agents. However, in several plants this system might constitute a convenient and efficient alternative to the expression of similar lytic enzymes in transgenic plants. This work demonstrates the potentiality and feasibility of combining the unique characteristics of plant symbiotic bacteria with the ability to control plant diseases.

Table 4. Lysis of *Rhizoctonia solani* hyphal tips by crude protein extracts of free-living *Rhizobium meliloti* or by extracts of alfalfa nodules infected with *R. meliloti* 026 expressing the chitinase gene and with wild type *R. meliloti* 1021

Strain	Deteriorated tips (number/slide[a])
R. meliloti 1021, free-living	2
R. meliloti 026, free-living	18
Nodule extract infected with *R. meliloti* 1021	4
Nodule extract infected with *R. meliloti* 026	12

[a]The number of deteriorated hyphal tips was determined by counting three slides per treatment and the experiment was repeated three times.

MATERIALS AND METHODS

Strains and culture conditions.

Strains are listed in Table 5. *Escherichia coli* and *R. meliloti* were grown in Luria-Bertani (LB) broth at 37° C and 30° C, respectively. *Rhizobium* strain 1021 is a streptomycin-resistant derivative of strain SU47 that forms an effective symbiosis with alfalfa and is therefore designated as "wild type" throughout this paper. The selective antibiotic, concentrations used in the triparental

Table 5. Bacterial strains and plasmids used in this study

Strain	Characteristics	Reference/source
E. coli		
A6006	Δ pRX, Cm^r	Strain collection
A5039	*lacI*^{Q}	Strain collection
025	A6006, pYZ291, *ptac* CHIA, Kn^r, Tc^r	This study
Rhizobium meliloti		
1021	St^r derivative of SU47, pSym$^+$	Meade *et al.* 1982
001	1021, pYZ291	This study
005	1021, pYZ291	This study
006	1021, pYZ291	This study
011	1021, pYZ291	This study
026	1021, pYZ291	This study
Plasmids		
pRK2013	ColE1::pKK2 used as helper in plasmid transfer from *E. coli* to *Rhizobium*	Ditta *et al.* 1980
pRK290	Tc^r	Ditta *et al.* 1980
pYZ291	pRK290, Tc^r, Kn^r, *ptac* ChiA	This study
pKK223-3	Amp^r, *ptac*	Brosius and Holy 1984
pLCHIA	4.7-kb *Bam*HI-*Eco*RI fragment carrying a chitinase gene of *Serratia marcescens*	Shapira *et al.* 1989

matings were (per milliliter) 50 μg of kanamycin (Kn), 200 μg of streptomycin (St), and 12.5 μg of tetracyclin (Tc).

DNA manipulations.

Standard techniques were as described by Sambrook *et al.* (1989). DNA fragments were purified from agarose gels using the Gene Clean kit (Bio-101 Inc., La Jolla, CA).

Plasmid construction.

Plasmid pYZ291 was constructed by isolating the 1.8-kb *Hin*fI DNA fragment from plasmid pLCHIA (Table 5). The fragment was made blunt-ended with the Klenow enzyme and cloned into the *Sma*I site of plasmid pKK223-3. A *Bam*HI fragment cut from plasmid pKK223-3 and carrying the CHIA gene under the *tac* promoter was cloned into the unique *Bgl*II site of pYZ290 and this plasmid was designated pYZ291. The plasmid pYZ291 is identical to plasmid pRK290 with one exception: a 1.6-kb *Eco*RI fragment carrying Kn resistance was cloned into the *Eco*RI site of plasmid pRK290 (Ditta *et al.* 1980). *E. coli* strain A5039 was the cloning host for all plasmids used here.

Conjugal plasmid transfer.

Plasmid transfer from *E. coli* to *R. meliloti* was performed by triparental mating. Stationary-phase cultures of *E. coli* carrying the plasmid to be transferred, pYZ291, a helper strain of *E. coli* carrying plasmid pRK2013, and the *R. meliloti* recipient were spun, washed, and suspended to half of their original volumes in TM (10 mM Tris, pH 7.4, 10 mM $MgSO_4$). Equal volumes (100 μl) of each culture were mixed and plated on LB plates. After overnight incubation at 30° C, bacterial growth was suspended in TM and transconjugants were selected on LB plates containing 200 μg of St per milliliter, 12.5 μg of Tc per milliliter, and 0.2% colloidal chitin.

Chitinase assay.

Chitinase (EC 3.2.1.14) activity was determined by degradation of colloidal chitin to *N*-acetylglucosamine (GlcNac) monomers in the presence of chitobiase. *N*-acetylamino sugars was estimated by the method of Reissig *et al.* (1959). Chitinase activity was also determined using *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose (pnp-chitobiose) as substrate. The reaction mixture (0.5 ml) contained 0.1 M phosphate buffer, pH 6.3, and 350 mM pnp-chitobiose, and was incubated at 37° C. The reaction was stopped with an equal volume of 1 M Na_2CO_3, and the absorbance at 410 nm was determined. A good correlation between the chitinase activities determined using the two methods was obtained. One unit of enzyme activity was defined as μmole GlcNac/g FWh released from colloidal chitin or μmole pnp/μg protein·h released from pnp-chitobiose.

Southern blot.

DNA extracts were prepared by grinding 100 mg of nodules to a fine powder with pestle and mortar in liquid nitrogen. The powder was suspended in 100 μl of cracking buffer containing 1 M NaOH, 0.5 M EDTA, 1% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.05% bromophenol blue. The mixture was incubated for 5 min and centrifuged for 10 min. The supernatant was collected and loaded on 0.8% agarose gel. DNA probe was prepared from the chitinase gene of *S. marcescens*.

Chitinase activity assay in polyacrylamide gel.

Crude protein extracts of alfalfa nodules (7 μg of protein) were separated on native 10% polyacrylamide gel. The gel was soaked in 50 mM phosphate buffer, pH 6.3, for 30 min and then overlaid with low melting agarose containing 4-methylumbelliferyl β-D-*N,N'*-diacetylchitobioside. The gel was incubated at 37° C and chitinase activity was visualized under UV light.

Symbiotic assays.

The ability of *R. meliloti,* carrying plasmid pYZ291, to form nodules on alfalfa (*Medicago sativa* 'Gilboa') plants was determined as previously described (Patterson *et al.* 1990). Alfalfa plants were grown in pots containing sandy soil and watered with modified N-free nutrient solution (Johnson *et al.* 1957). Nitrogenase activity of the nodules was determined by the acetylene reduction method following a 10-min incubation of the plants with acetylene (Sprent 1971). Chlorophyll content was determined by extracting leaves in *N,N*-dimethylformamide (Moran and Porath 1980).

Lysis of hyphal tips.

Cell-free extracts were prepared by grinding 0.5 g of fresh nodules to a fine powder with a pestle and mortar in liquid nirogen. The powder was suspended in 5 ml of 0.1 M phosphate buffer, pH 6.3, and centrifuged for 10 min at 10,000 *g*. The supernatant was concentrated and dialyzed under vacuum, and 100 μl of the concentrate containing about 80 μg of proteins was used for lysis. Agar layered onto microscope slides and bearing mycelium of *Rhizoctonia solani* was flooded with cell-free extract of alfalfa nodules. After incubation for 5 min at 30° C, hyphal tips were observed under light microscope. The antifungal activity of free-living *Rhizobium* was also determined by the hyphal tip assay. Rhizobium cells were grown, sonicated, and centrifuged to remove cell debris. The supernatant was lyophilized and the crude protein extract was dissolved in 50 mM phosphate buffer pH 6.3. The specific activity of chitinase in the assays was 2–3.6 μmole pnp/μg protein hr.

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