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Extracellular Proteins Involved in Soybean Cultivar-Specific Nodulation Are Associated with Pilus-Like Surface Appendages and Exported by a Type III Protein Secretion System in *Sinorhizobium fredii* USDA257

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Several gram-negative plant and animal pathogenic bacteria have evolved a type III secretion system (TTSS) to deliver effector proteins directly into the host cell cytosol. *Sinorhizobium fredii* USDA257, a symbiont of soybean and many other legumes, secretes proteins called Nops (nodulation outer proteins) into the extracellular environment upon flavonoid induction. Mutation analysis and the nucleotide sequence of a 31.2-kb symbiosis (*sym*) plasmid DNA region of USDA257 revealed the existence of a TTSS locus in this symbiotic bacterium. This locus includes *rhc* (rhizobia conserved) genes that encode components of a TTSS and proteins that are secreted into the environment (Nops). The genomic organization of the TTSS locus of USDA257 is remarkably similar to that of another broad-host range symbiont, *Rhizobium* sp. strain NGR234. Flavonoids that activate the transcription of the *nod* genes of USDA257 also stimulate the production of novel filamentous appendages known as pili. Electron microscope examination of isolated pili reveals needle-like filaments of 6 to 8 nm in diameter. The production of the pili is dependent on a functional *nodD*1 and the presence of a *nod* gene–inducing compound. Mutations in several of the TTSS genes negate the ability of USDA257 to elaborate pili. Western blot analysis using antibodies raised against purified NopX, Nop38, and Nop7 reveals that these proteins were associated with the pili. Mutations in *rhcN*, *rhcJ*, *rhcC*, and *ttsI* alter the ability of USDA257 to form nodules on *Glycine max* and *Macroptilium atropurpureum*.

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Nucleotide and amino acid sequence data for the USDA257 TTSS locus is available in the GenBank database under accession number AF229441.

Many legumes have the ability to establish a symbiotic association with certain soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Rhizobium* (collectively identified as rhizobia). This association leads to the formation of specialized organs called nodules. Bacteria within the nodules differentiate into nitrogen-fixing bacteroids, which reduce nitrogen to ammonia for use in plant growth and development. In exchange, the plant provides a specialized environment and carbohydrates to the rhizobia.

The induction of nodules on legume roots requires at least three sets of symbiotic signals to be exchanged between the partners (Broughton et al. 2000). The first signals are flavonoids, plant-derived molecules that interact with NodD and activate the transcription of several key nodulation (*nod*) genes in the microsymbiont. The products of some of these genes are involved in the synthesis of a second set of signal molecules, Nod-factors, that play a crucial role in developmental initiation of nodules on legume roots. A third group of signal molecules comprising extracellular polysaccharides, lipopolysaccharides, and flavonoid-induced extracellular proteins, also play important but not fully understood roles in infection thread formation, nodulation efficiency, and nitrogen fixation (Krishnan 2002; Leigh et al. 1985; Sutton et al. 1994; Viprey et al. 1998).

Sinorhizobium fredii USDA257 (hereafter called USDA257) forms nodules on soybean in a cultivar-specific manner. Nitrogen-fixing nodules are produced on the soybean cultivar 'Peking' but not on agronomically advanced cultivar 'McCall' (Heron and Pueppke 1984). Random transposon mutagenesis confirmed that a plasmid-borne locus, *nolXWBTUV*, is involved in regulating soybean cultivar specificity (Meinhardt et al. 1993). Most of the genes located in this locus are induced by flavonoid signals, and their protein products reveal significant sequence homology to components of a type III secretion system (TTSS) (Meinhardt et

al. 1993). USDA257 secretes several extracellular proteins, designated as signal responsive (SR) proteins, into the rhizosphere upon flavonoid induction (Krishnan and Pueppke 1993). N-terminal amino acid sequence determination of one of the SR proteins indicated export without N-terminal processing, a hallmark of proteins that are secreted by TTSS. Disruption of the *nolXWBTUV* locus also abolished the secretion of the SR proteins (Krishnan et al. 1995). These observations indicate that SR proteins may be transported through TTSS, and some products of the *nolXWBTUV* may code for the components of TTSS in USDA257. Proteins secreted by TTSS in *Yersinia* species are called Yops (*Yersinia* outer proteins), and Marie and associates (2001) have proposed Nops (nodulation outer proteins) for *Rhizobium* proteins that are secreted by TTSS. Based on this proposal, previously identified TTSS-dependent secreted proteins, such as NolX and y4xL, have been renamed as NopX and NopL (Marie et al. 2001).

Several plant and animal pathogenic bacteria employ the TTSS to transfer virulence proteins directly into the host cells (Brown et al. 2001; Casper-Lindley et al. 2002; Galan and Collmer 1999; Lee 1997). These systems have been extensively characterized, and some common features of TTSS can be derived. The TTSS, which is genetically and morphologically similar to the bacterial flagellum, is activated by host cell contact in vivo (Pettersson et al. 1996). The secretion signal is localized in the 5′ region of the mRNA or at the amino terminus of the secreted protein, and often the TTSS is associated with filamentous appendages (Anderson and Schneewind 1997; Lloyd et al. 2002). The presence of a macromolecular complex, termed a needle complex, that spans both the inner and outer bacterial membranes with a needle-like projection that projects outward from the bacterial membrane has been described in *Salmonella typhimurium* (Kubori et al. 1998). These structures are about 120 nm long and are composed of at least three proteins, InvG, PrgH, and PrgK (Kubori et al. 1998). In addition to needle complexes, filamentous appendages, which vary in size from 8 to 50 nm in diameter, may also be involved in transport of virulence factors into the host cells (Ginocchio et al. 1994; Jin and He 2001; Roine et al. 1997; Van Gijsegem et al. 2000).

Unlike animal and plant bacterial pathogens, very little is known about TTSS in symbiotic rhizobia. Here, we report the genetic organization and sequence of a 31.2-kb DNA region that includes the entire type III protein secretion machinery of USDA257. The TTSS of USDA257 also elaborates filamentous appendages upon induction with soybean root exudates. Biochemical analysis reveals the presence of several Nops in the purified filamentous appendages.

RESULTS

Type III secretion genes in *S. fredii* USDA257.

Screening an USDA257 genomic library with a ^{32}P-labeled 8.0-kb *Eco*RI fragment known to contain the *nolXWBTUV* locus resulted in the identification of four positive cosmid clones. Cosmid clone pHBK447, with a total insert size of 28.8 kb, contained 10 *Eco*RI fragments. The complete nucleotide sequence of one of the *Eco*RI fragments (pRFDH410) has been reported earlier and contains several open reading frames (ORFs), some of which show strong sequence homology to *hrc* (hypersensitive response and pathogenesis conserved) genes of plant and pathogenic bacteria (Meinhardt et al. 1993). Two ORFs, ORF4 and *nolV*, located downstream of *nolU*, were identified in this region (Meinhardt et al. 1993). These two ORFs show significant homologies to the N- and C-terminals of *nolV* from NGR234, *S. fredii* HH103, *B. japonicum* USDA110, and *M. loti* MAFF303099. This led us to resequence this region in USDA257. The earlier published sequence had one base pair deleted, resulting in introduction of a stop codon and truncating the *nolV* sequences. We have now corrected this error, and the revised version of the sequence (GenBank accession number AF229441) codes for NolV of a comparable size as from other rhizobia.

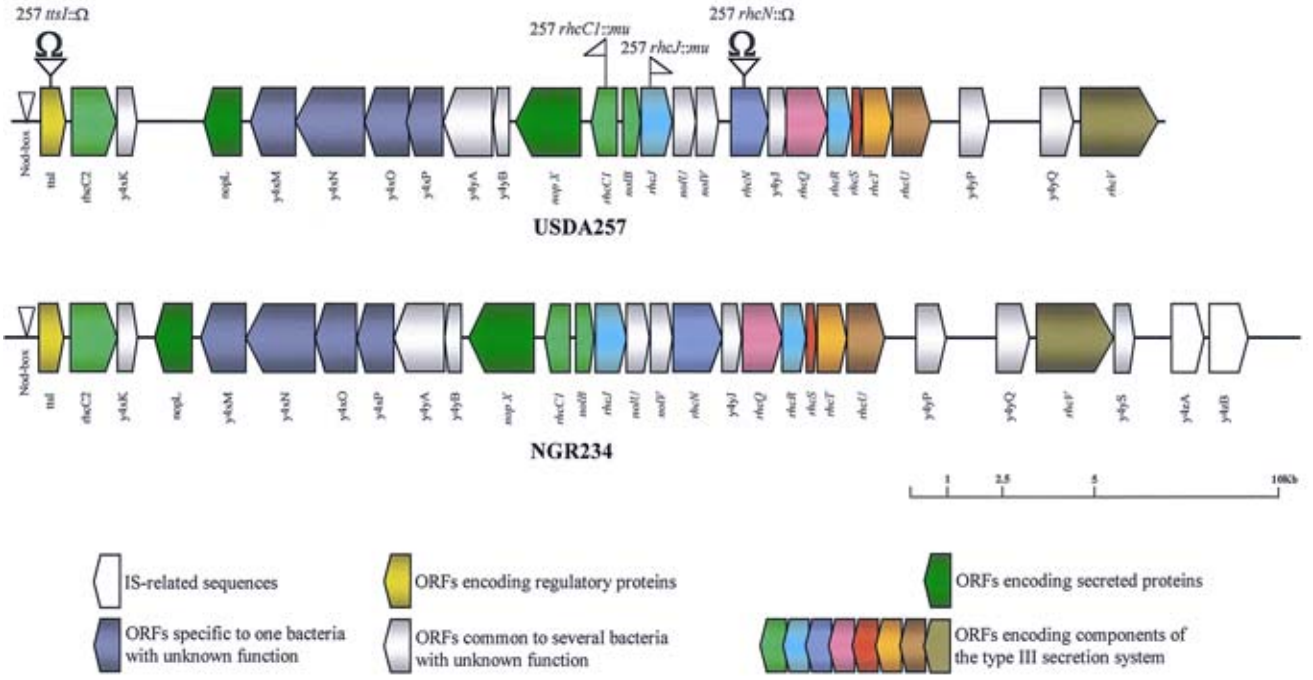

Fig. 1. Genetic organization of the type III secretion system genes of *Sinorhizobium fredii* USDA257 and *Rhizobium* sp. strain NGR234. Predicted open reading frames (ORFs) are shown as arrow-shaped boxes, oriented according to their direction of transcription (Marie et al. 2001). The positions of mini-Mu insertion and omega interposon in USDA257 are also shown. Similar coloring represents homologous ORFs. Accession numbers are: USDA257, AF229441 and NGR234, AE000108.

Recent sequence information has confirmed the presence of a TTSS on the symbiotic plasmid of *Rhizobium* sp. strain NGR234 (hereafter termed NGR234) (Freiberg et al. 1997) and *R. elti* CFN42 (NCBI database, accession number U80928) and in the genome of *M. loti* MAF303099 (Kaneko et al. 2000) and *B. japonicum* USDA110 (Göttfert et al. 2001). The TTSS in these rhizobia are clustered within regions of 35 to 47 kb (Marie et al. 2001). These clusters include genes that encode

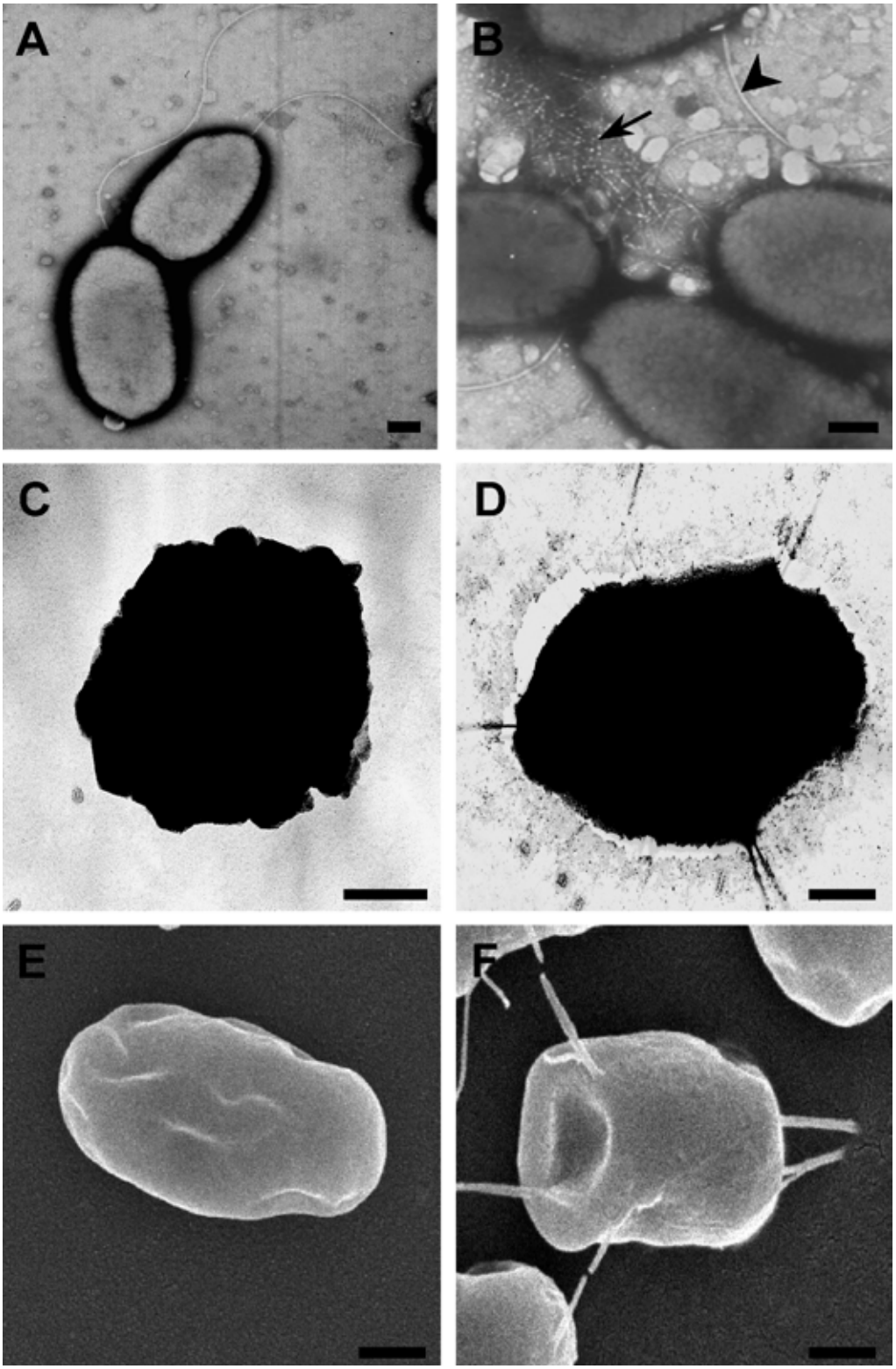

Fig. 2. Electron micrographs of surface appendages produced by USDA257. Cells of *Sinorhizobium fredii* USDA257 grown in yeast extract-manitol (YEM) agar plates were negatively stained with 1% phosphotungstic acid (pH 6.5). **A,** Cells grown in the absence of genistein elaborate flagella of 10 to 12 nm in diameter. **B,** Addition of genistein results in the production of pilus-like filaments (indicated by arrows) that are thinner than flagella (arrowhead). **C and E,** USDA257 cells grown in the absence of genistein reveal no surface appendages, while **D** and **F,** cells grown in the presence of genistein produce pili-like appendages. In D and F, chemical fixation of USDA257 cells grown in YEM liquid media improves the visualization of pili (indicated by arrows). Micrographs of negatively stained USDA257 cells are shown in C and D. The micrographs shown in E and F were obtained with a cold field emission scanning electron microscope. Bars = 0.25 μm.

the secretion apparatus as well as proteins that are secreted to the rhizosphere. To examine whether a similar physical organization of TTSS also occurs in USDA257, we sequenced all of the remaining nine *Eco*RI fragments of pHBK447. We extended the sequence by sequencing a 3-kb DNA region from another overlapping cosmid (pRK1489).

The genetic organization of the TTSS of USDA257 is strikingly similar to that of NGR234, a strain that nodulates more than 110 genera of legumes (Pueppke and Broughton 1999). The sequenced region revealed 26 predicted ORFs delineated by y4xI and y4yR (Fig. 1). Nine of the ORFs (y4yD, y4yF, y4yM, y4yL, y4yN, y4yR, y4yO, y4yI, and y4yK) showed extensive homology to *hrc* genes that encode the components of the TTSS apparatus of plant and animal pathogenic bacteria. To distinguish the TTSS components of symbiotic rhizobia from plant pathogenic bacteria, Viprey and associates (1998) proposed to name the 'Hrc' protein homologues as 'Rhc' (rhizobia conserved). To maintain a uniform nomenclature, we have also assigned the Rhc designation for all the components of the TTSS machinery of USDA257 (Fig. 1). Based on the structure of the TTSS apparatus of *P. syringae* (Baker et al. 1997), it is suggested that RhcC1 and RhcC2, which encode the N- and C-terminal domains of HrcC, code for the outer membrane protein. RhcJ codes for an outer membrane lipoprotein and RhcR, RhcS, RhcT, RhcU, and RhcQ all code for inner membrane proteins. Two cytoplasmic proteins, RhcQ and RhcN, are also part of the TTSS machinery of NGR234 and USDA257 (Fig. 1). A similar gene arrangement is also found in *M. loti*, except that y4yP, y4xP, y4xN, and y4xM are absent in the TTSS of this organism (Marie et al. 2001). In *B. japonicum*, the y4x1-rhcC2-y4xK and y4yQ-rhcV-y4yS cluster is divergently oriented when compared with NGR234, USDA257, and *M. loti* counterparts (Marie et al. 2001). In addition, the TTSS of *B. japonicum* USDA110 contains several ORFs that are not present in other symbiotic bacteria (Göttfert et al. 2001). The complete nucleotide sequence of *Rhizobium etli* CFN42 symbiotic plasmid p42d has been recently elucidated (accession number U80928). Sequence analysis indicates that *R. etli* also contains a TTSS. Some of the components of the TTSS, such as *rhcC*, were not detected in this region. In addition, the organization of the *rhc* genes was also different when compared with other symbiotic bacteria. In spite of these dif-

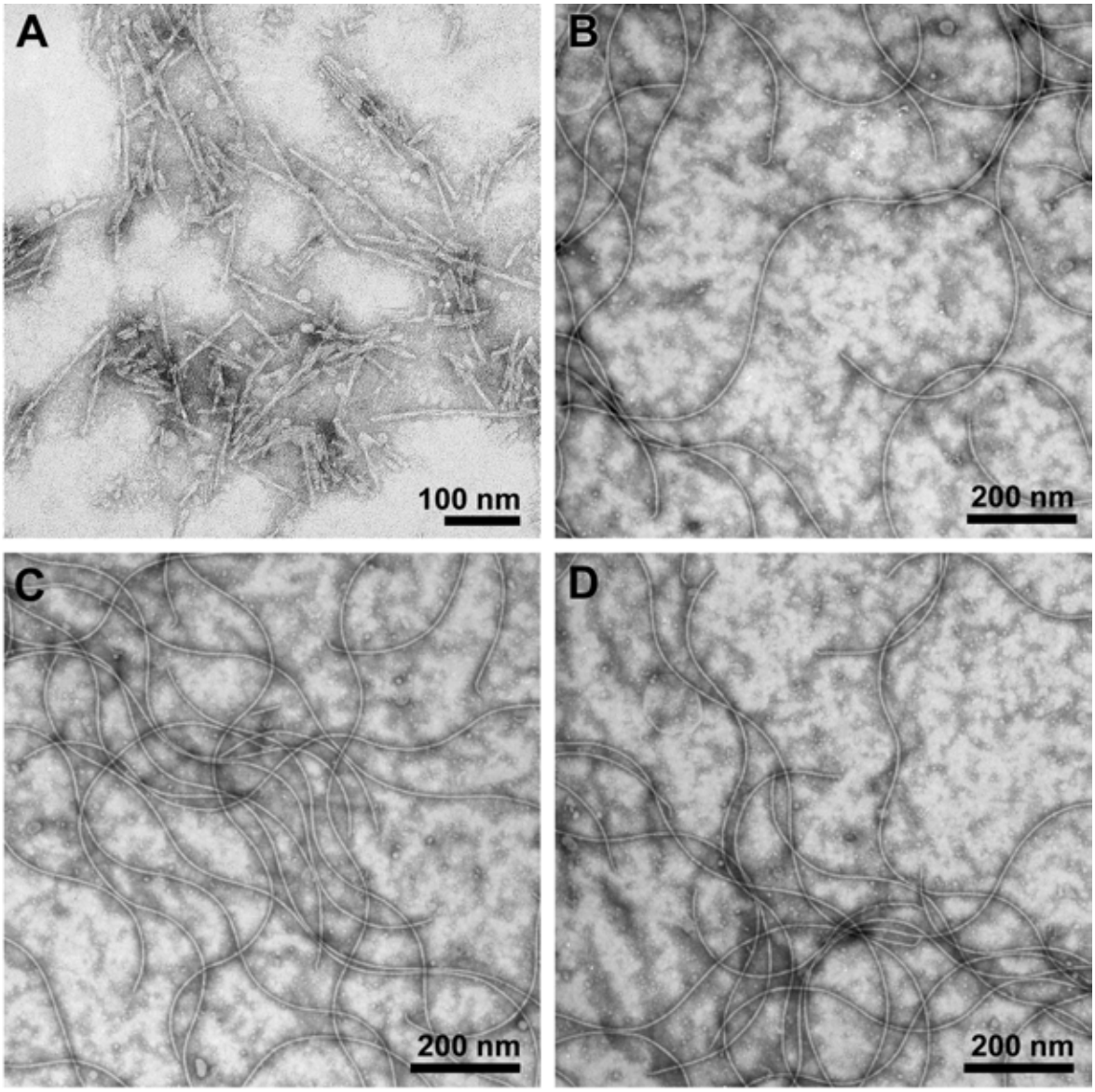

Fig. 3. Electron micrographs of the isolated surface appendages of *Sinorhizobium fredii* USDA257. **A,** Surface appendages from USDA257, **B,** *rhcN*, **C,** *ttsI*, and **D,** *nodD* mutants grown in yeast extract-mannitol liquid media containing genistein were examined with a transmission electron microscope after staining with 1% phosphotungstic acid (pH 6.5). Note that *rhcN*, *ttsI*, and *nodD*1 mutants do not elaborate pili.

ferences, the *rhc* genes appear to be conserved among all the symbiotic bacteria that contain a TTSS.

Flavonoids stimulate USDA257 to produce pilus-like surface appendages.

Electron-microscope observation of USDA257 cells grown in yeast extract-mannitol (YEM) agar plates revealed the presence of several flagella (Fig. 2A). Isolated flagella filaments have a diameter of approximately 10 to 12 nm. In contrast to the complex flagella of *S. meliloti* (Krupski et al. 1985), the USDA257 flagella filaments are structurally simple. Addition of the isoflavone genistein, a potent inducer of *nod* genes of USDA257, to the culture media resulted in the production of a second type of appendage. These pilus-like appendages are much thinner than flagella and are elaborated in abundance (Fig. 2B). However, the pili-like appendages were not directly associated with the bacteria. Since the specimens were not subjected to chemical fixation before they were examined by electron microscope, we suspected that the pili could have been dislodged from the bacteria. In order to better visualize these surface appendages, we first fixed the bacteria in glutaraldehyde followed by negative staining. This procedure enabled us to visualize the pili radiating from the surface of the bacteria (Fig. 2D). The presence of pili became more apparent when these specimens were subjected to critical point drying and examined by cold field emission scanning electron microscope (Fig. 2F). The production of pili appears to be dependent on genistein, since USDA257 grown in its absence failed to produce these surface appendages (Fig. 2C and E). We also evaluated the ability of several other flavonoids to promote the formation of pilus-like surface appendages. Daidzein, apigenin, and luteolin, compounds that have been previously shown to activate the *nodABC* genes of USDA257 (Krishnan and Pueppke 1991), stimulate production of the pili. Noninducers of *nodABC* genes of USDA257, including biochanin A and umbelliferone, were unable to elaborate pili. Soybean seed exudates from cultivars McCall and Peking also induced USDA257 to produce pili (data not shown).

We also isolated the surface appendages from USDA257 grown in YEM liquid cultures with inducers and examined them by transmission electron microscopy (Fig. 3). The isolated surface appendages from USDA257 grown in the presence of genistein consisted mostly of narrow filamentous appendages (pili) of about 6 to 8 nm in diameter (Fig. 3A). A few flagella were also seen, but their abundance was relatively low when compared with that of pili. The production of the pili appears to be dependent on TTSS, since *rhcJ*- and *rhcC*1-negative mutants failed to elaborate pili. However, these mutants retained the ability to produce flagella (Fig. 3B and C). Similarly, a *nodD*1-negative mutant of USDA257 was unable to elaborate pili upon flavonoid induction (Fig. 3D).

Identification of proteins associated with USDA257 flagella and pili.

Polyacrylamide gel analysis of isolated flagella of USDA257 revealed two abundant proteins of 34 and 36 kDa (Fig. 4). These two abundant proteins appear to be flagellin subunits. This observation was confirmed by Western blot analysis using antibodies raised against the purified *S. meliloti* RMB7201 flagellin. The antibodies specifically cross-reacted against the 34- and 36-kDa polypeptides (data not shown). Isolated pili from genistein-induced USDA257 cells, when resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), showed several abundant proteins, prominent among them were 64-, 38-, 25-, 18-, and 7-kDa proteins (Fig. 4). These proteins were absent in the flagella preparations, indicating that they are specifically associated with pili. In addition, these proteins were absent from *rhcN*-, *rhcC*1-, and *rhcJ*-negative mutants. The 34- and 36-kDa flagellin subunits were not readily detected in the pili preparations (Fig. 4). This may be due to the fact that the genistein-induced cultures of USDA257 contain a much greater abundance of pili relative to flagella. We further purified the pili by sucrose density centrifugation (Roine et al. 1997) and used this purified preparation to identify the proteins that are associated with it. Western blot analysis using antibodies raised against NopX, Nop38, and Nop7 gave positive reactions with all the three antibodies, confirming that these three proteins were associated with USDA257 pili (Fig. 5).

TTSS play an important role in nodulation.

We examined the symbiotic properties of USDA257 TTSS mutants by inoculating them on four legume hosts (Table 1). All the TTSS mutants that we examined formed a lower number of nodules on soybean cultivar Peking in comparison with the wild-type parent USDA257. Similar reduction in the nodulation was also observed with *Macroptilium atropurpureum*. However, the TTSS mutants were not affected in their ability to nodulate *Vigna unguiculata* and produced similar number of nodules as USDA257. The USDA257 *ttsI* mutant was unable to nodulate soybean cultivar McCall and behaved exactly like USDA257, producing only rudimentary swellings on soybean roots.

DISCUSSION

The complete nucleotide sequence of the TTSS has been reported previously for *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997), *M. loti* (Kaneko et al. 2000), *B. japonicum* USDA110 (Göttfert et al. 2001), and *R. etli* CFN42 (NCBI database, accession number U80928). Although the sequence reported here is the first for a cultivar-specific symbiont, the genetic organization of the TTSS of USDA257 and NGR234 is

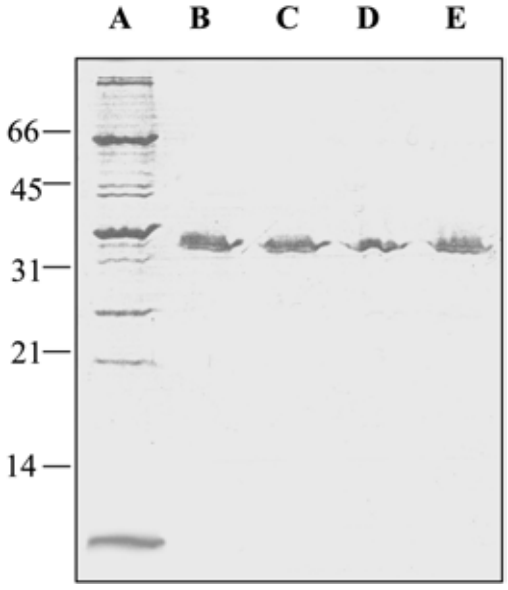

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the isolated surface appendages of *Sinorhizobium fredii* USDA257. Surface appendages from wild-type USDA257 (lane A), *rhcN* (lane B), *rhcC*1 (lane C), *rhcJ* (lane D), and *ttsI* (lane E) mutants grown in yeast extract-mannitol media containing genistein were suspended in SDS sample buffer and were resolved on a 15% SDS-PAGE gel. The resolved proteins were visualized by staining the gel with Coomassie Brilliant Blue. The sizes of the molecular weight markers in kDa are also shown.

98% identical. In addition to the TTSS genes, the nucleotide sequences of several other nodulation genes of these two organisms also reveal remarkable similarity with each other (Relić et al. 1994). DNA subtraction-hybridization studies have shown that NGR234 and USDA257 share most of the genomic background and are closely related phylogenetically (Perret et al. 1994). These observations suggest a common ancestry for these two broad-host range symbionts. Interestingly, the TTSS of USDA257 and *B. japonicum* USDA110, two symbionts of soybean, shows only limited sequence homology, and their genetic organization is also quite different. In addition to the absence of NopX, a soybean-cultivar specificity protein, the TTSS of *B. japonicum* USDA110 contains several other ORFs with unknown functions (Göttfert et al. 2001).

USDA257 nodulates 72 genera of legumes and the nonlegume *Parasponia andersonii* (Pueppke and Broughton 1999). NGR234 has even broader host range than USDA257 and nodulates 112 genera (Pueppke and Broughton 1999). The exceptionally broad host range of NGR234 has been attributed to its ability to elaborate a diverse array of Nod factors (Price et al. 1992), but Nod factors alone cannot fully explain the basis for broad host range. For example, NGR234 produces the same set of Nod factors as does USDA257 but still cannot nodulate soybeans. This implies that, in addition to Nod factors, other signal molecules such as the extracellular proteins delivered by the TTSS may also play a crucial role in regulating nodulation. Our results show that proteins secreted via the TTSS control the nodulation on soybean cultivars McCall and Peking. Similarly NGR234 *rhc* mutants show altered nodulation phenotype on *Pachyrhizus tuberosus* and *Tephrosia vogelii* (Viprey et al. 1999). These observations are consistent with the suggestion that flavonoid-inducible TTSS proteins along with flavonoids and Nod factors are the three important symbiotic signals required for successful nodulation (Broughton et al. 2000).

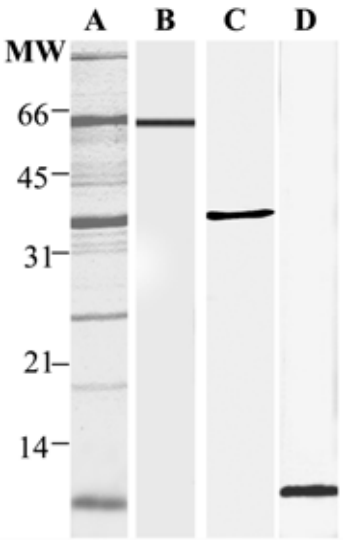

Fig. 5. Immunological identification of proteins associated with sucrose-gradient purified surface appendages of *Sinorhizobium fredii* USDA257. Purified pili proteins from USDA257 were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lane A) and were electrophoretically transferred to a nitrocellulose membrane and probed with antibodies raised against the following proteins: lane B, NopX, lane C, Nop38, and lane D, Nop7. The sizes of the molecular weight markers in kDa are also shown (lane A).

We have demonstrated that USDA257 produces extracellular appendages (pili) when grown in the presence of *nod* gene-inducing compounds. In plant and animal pathogenic bacteria, the TTSS is used to inject effector proteins into the plasma membrane and cytosol of the host cells (Galan and Collmer 1999). The delivery of the *Salmonella typhimurium* effector proteins into the host cells is believed to be mediated by a macromolecular complex (needle complex) that spans both the inner and outer bacterial membranes (Kubori et al. 1998). The needle complexes may serve as hollow conduits through which type III secreted proteins are transferred to the eukaryotic cells. In addition to the needle complexes, other supramolecular structures have also been reported. For example, *E. coli* and *S. typhimurium* produce filamentous appendages that are about 50 nm in diameter (Ginocchio et al. 1994; Knutton et al. 1998). In contrast, the plant pathogen *Pseudomonas syringae* produces Hrp pili that are much thinner (6 to 8 nm in diameter) (Roine et al. 1997) similar to those of USDA257.

The function of the type III secreted proteins of animal pathogens such as *Shigella* and *Yersinia* is to subvert the mammalian immune system (Cornelis and Wolf-Watz 1997). *Yersinia* spp. secrete several proteins (Yops) after direct contact with eukaryotic host cells. Some of these Yops are targeted into the eukaryotic cytosol, where they carry out distinct functions that ultimately lead to the death of the host cells. In plant pathogenic bacteria, the *hrp* genes, which encode the components of TTSS, are essential for elicitation of the hypersensitive response in resistant plants and of disease in susceptible plants (He 1998). Mutations in the *hrp* genes eliminate the virulence of the bacteria. Plant pathogenic bacteria secrete two groups of proteins through the Hrp secretion system (He 1998). The first group of proteins (avirulence proteins) is directly targeted into the host cells. The second group of proteins, such as harpin, that elicit HR response when infiltrated into the intercellular space of plant leaves does not appear to be secreted into the host cell. In the case of symbiotic bacteria such as USDA257 and NGR234, the type III secreted proteins appear to play an important role in regulating nodulation on legume roots. Interestingly, the *rhc* mutants, which were unable to secrete Nops, had varying symbiotic phenotypes on their hosts. In the case of USDA257, the abolition of protein secretion results in drastic phenotypic effect on soybean nodulation. Unlike the wild-type USDA257, the *rhc* mutants have acquired the ability to form nitrogen-fixing nodules on soybean cultivar McCall. However, the absence of the type III secreted proteins appears to have no effect on cowpea nodulation. Based on the current study and that of Viprey and associates (1998), it is apparent that the type III secreted proteins can either positively or negatively affect the nodulation process in a host-dependent manner.

Table 1. Symbiotic response of legume species to inoculation with *Sinorhizobium fredii* USDA257 and the type III secretion system mutants

	Number of nodules[a]			
Strain	**Soybean cv. Peking**	**Soybean cv. McCall**	**Siratro**	**Cowpea**
USDA257	21 ±1.03	0	13 ± 0.98	13 ± 0.99
RhcC mutant	8 ± 1.5	8 ± 0.92	5 ± 0.51	10 ± 0.83
RhcJ mutant	8 ± 0.75	10 ± 1.30	4 ± 0.46	12 ± 0.74
RhcN mutant	8 ± 0.70	6 ± 0.64	7 ± 1.16	11 ± 1.18
TtsI mutant	12 ± 1.24	0	10 ± 1.03	9 ± 1.51

[a] Data represent mean number of nodules per plant (n = 16 plants per treatment). Nodules were counted 25 days after inoculation.

Biochemical analysis of the USDA257 pili preparations reveals the association of several Nops with the purified pili. The most prominent among them are Nop7, Nop38, and NopX. Even though Western blot analysis indicates that these proteins are localized on the pili, we cannot exclude the possibility that this association may have resulted due to an artifact of the purification procedure. Electron microscopy studies using intact bacteria may provide conclusive evidence for the association of Nop7, Nop38, and NopX with the pili. Our repeated attempts to localize the Nops on the intact bacterial cells have not been successful, due to technical problems. We also do not know if the type III secreted proteins of the symbiotic bacteria interact at the root surface or enter into the host cells. We are in the process of generating type III secreted proteins that have been tagged with green fluorescent protein (GFP). We intend to use the GFP-tagged proteins to monitor the delivery of such proteins into the host cells.

MATERIALS AND METHODS

Microbiological techniques.

Bacterial strains and plasmids are listed in Table 2. *Rhizobium* and *E. coli* strains were maintained as stock cultures in 25% glycerol at –70°C. *Rhizobium* strains were cultured in YEM broth (Vincent 1970) at 30°C, and *E. coli* strains were grown in Luria-Bertani medium (Sambrook et al. 1989) at 37°C. When appropriate, antibiotics were added at the following concentrations (μg per ml): ampicillin, 50; kanamycin, 50; tetracycline, 10; and spectinomycin, 50.

Molecular methods.

Plasmid isolation, restriction digestion, DNA ligation, Southern hybridization, and other procedures with nucleic acids followed the general protocols of Sambrook and associates (1989).

Construction of *ttsI* and rhcN mutants.

A 2.4-kb *Pst*I/*Eco*RI fragment from pGEm-7zf6RinodD2 was inserted into pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.) to produce pBS24*ttsI*. This plasmid was digested with *Sac*II, and the ends were converted to blunt ends by addition of Klenow fragment. A spectinomycin-resistant 2.0-kb Ω cassette was then cloned into blunt ends of the above plasmid to yield pBS24*ttsI* Ω. A 4.4-kb fragment was excised from pBS24*ttsI* Ω by digestion with *Pst*I and *Eco*RI and was treated with Klenow fragment. The insert was purified by agarose gel electrophoresis and was cloned into the *Sma*I site of pJQ200ucl (Quandt and Hynes 1993) to produce pJQ200uc124*ttsI* Ω. This plasmid was introduced into USDA257 by triparental mating, with pRK2013 as a helper plasmid (Figurski and Helinski 1979). Marker exchange was forced by selection on minimal media plates containing 5% (wt/vol) sucrose. USDA257ΩrhcN was obtained using constructs originally made for generating the corresponding mutant in NGR234 (Viprey et al. 1998). USDA257 *rhcC*1 and *rhcJ* mutants have been described earlier (Meinhardt et al. 1993).

Isolation and purification of USDA257 surface appendages.

Bacterial surface-associated appendages were isolated from USDA257 cultures grown in 100 ml of liquid YEM. Bacteria were removed from the culture medium by centrifugation at 11,300 × *g* for 30 min. The clear supernatant was subjected to ultracentrifugation in a Beckman SW 50.1 rotor at 150,000 × *g* for 90 min. The pellet was resuspended in a small volume of sterile water and was designated as isolated surface appendages. Aliquots of this fraction were used for transmission electron microscopy or gel electrophoresis.

Purification of the bacterial appendages from 100-ml cultures of USDA257 was carried out according to Roine and associates (1997). The surface appendages obtained as described above were resuspended in 10 mM Tris-HCl (pH 7.5) and were subjected to another round of ultracentrifugation in a 10:60 (wt/wt) percent sucrose gradient in a Beckman SW 50.1 rotor at 80,000 × *g* for 20 h. After centrifugation, the gradient was separated into 10 fractions and was analyzed by SDS-PAGE (Laemmli 1970). Transmission electron microscope examination revealed that fraction 10 contained the purified pili. This fraction was used for Western blot analysis.

Electrophoresis and Western blot analysis.

Proteins associated with surface appendages from USDA257 and *rhc* mutants were resolved by SDS-PAGE with the discontinuous buffer system of Laemmli (1970). The slab gel (10 × 8 × 0.75 cm) consisted of separation and stacking gels of 15 and 4% acrylamide, respectively. Electrophoresis was performed at a 20-mA constant current per gel until the tracking dye reached the bottom of the gel. The proteins were visualized by staining with Coomassie Brilliant Blue. Western blot analyses using sucrose-gradient purified surface appendages were performed, essentially as described by Burnett (1981). The production of antibodies to NopX, Nop38, and Nop7, which were previously referred to as SR1, SR3, and SR5, respectively, has been described earlier (Krishnan 2002; Krishnan et al. 1995). The antibodies were diluted 1:3000 in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH7.5, 500 mM NaCl) containing 5% (wt/vol) nonfat dried milk. Immunoreactive polypeptides were identified by following the horseradish peroxidase color development procedure provided by the manufacturer (Bio-Rad Laboratories, Inc., Richmond, CA, U.S.A.).

Table 2. Bacterial strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source/Reference
Escherichia coli DH5α	ϕ80*lacZ*ΔM15Δ(*lacZYA-argF*), U169 *hsdR*17 *recA*1 *endA*1 *thi*-1	Gibco BRL, Gaithersburg, MD, U.S.A.
Sinorhizobium fredii USDA257	Broad host range, Nod^+ on soybean	Heron and Pueppke 1984
USDA257Ω*rhcN*	USDA257 derivative Containing an Ω insertion in *rhcN*, Sp^r	This study
USDA257Ω *ttsI*	USDA257 derivative Containing an Ω insertion in *ttsI*, Sp^r	This study
USDA257mm34	USDA257 derivative Containing a miniMu insertion in *rhcJ*, Kn^r	Meinhardt et al. 1993
USDA257mm17	USDA257 derivative Containing a miniMu insertion In *rhcC*1, Kn^r	Meinhardt et al. 1993
Plasmid		
pGEM-7zf(+)	Ap^r	Promega, Madison, WI, U.S.A.
pBluescript II SK (+)	Ap^r	Stratagene, La Jolla, CA, U.S.A.
pGEM-T easy	Ap^r	Promega
pJQ200uc1	Gm^r	Quandt and Hynes 1993
pHBK447	Tc^r, pLAFR1 cosmid carrying TTSS genes from USDA257	This study
pRK1489	Tc^r, *nodD*2 containing cosmid of USDA257 in pLAFR1	Krishnan and Pueppke 1991
pRFDH410	Ap^r, 8.0-kb *Eco*RI fragment containing *nolBTUVWX* in pTZ19u	Meinhardt et al. 1993

Transmission electron microscopy.

USDA257 cells grown in the presence or absence of 1-μM genistein were spun down, washed three times in distilled water, resuspended in sterile water, and placed on carbon-coated copper grids. The bacteria on the grids were fixed in 2% gluteraldehyde and 2% paraformaldehyde for 15 min at room temperature. Fixed colonies were washed briefly in distilled water and stained with 1% phosphotungstic acid (pH 6.5). The grids then were examined with a JOEL JEM electron microscope at 100 kV. Pili and flagella were also observed with transmission electron microscope by pacing drops of isolated bacterial surface appendages on carbon-coated copper grids followed by staining with 1% phosphotungstic acid (pH 6.5).

Cold field emission scanning electron microscopy.

USDA257 cells were placed on poly-L-lysine-coated (1 mg/ml) cover slips and allowed to settle for 15 min. Colonies on the cover slips were fixed for 30 min at room temperature, using 2% gluteraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. Fixed colonies were washed briefly in distilled water and then postfixed for 30 min with 1% osmium tetroxide. The specimens were dehydrated in a graded series of ethanol, subjected to critical point drying, and sputter-coated with lead. The specimens were examined with a S-4700 cold field emission scanning electron microscope (Hitachi, Japan) at 5.0 kV and 10 μA.

Plant assays.

Legume seeds were surface-sterilized and were germinated at 28°C on water-agar plates for three days. Seedlings were dipped in a suspension of inoculating bacteria of a known concentration and were planted directly into aseptically prepared plastic growth pouches premoistened with nitrogen-free nutrient solution. Plants were placed in a growth chamber that was set at a constant temperature of 28°C with light intensity of 500 μE m^{-2} s^{-1} under a 12-h day and night cycle. Plants were harvested 25 days after inoculation, and nodulation responses were assessed visually.

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