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Distinct Cell Surface Appendages Produced by *Sinorhizobium fredii* USDA257 and *S. fredii* USDA191, Cultivar-Specific and Nonspecific Symbionts of Soybean▽†

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***Sinorhizobium fredii* USDA257 and *S. fredii* USDA191 are fast-growing rhizobia that form nitrogen-fixing nodules on soybean roots. In contrast to USDA191, USDA257 exhibits cultivar specificity and can form nodules only on primitive soybean cultivars. In response to flavonoids released from soybean roots, these two rhizobia secrete *n*odulation *o*uter *p*roteins (Nop) to the extracellular milieu through a type III secretion system. In spite of the fact that Nops are known to regulate legume nodulation in a host-specific manner, very little is known about the differences in the compositions of Nops and surface appendages elaborated by USDA191 and USDA257. In this study we compared the Nop profiles of USDA191 and USDA257 by one-dimensional (1D) and 2D gel electrophoresis and identified several of these proteins by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography-tandem MS (LC-MS/MS). Examination of the surface appendages elaborated by these two strains of soybean symbionts by transmission electron microscopy revealed distinct differences in their morphologies. Even though the flagella produced by USDA191 and USDA257 were similar in their morphologies, they differed in their flagellin composition. USDA257 pili resembled long thin filaments, while USDA191 pili were short, rod shaped, and much thinner than the flagella. 2D gel electrophoresis of pilus-like appendages of USDA191 and USDA257 followed by mass spectrometry resulted in the identification of several of the Nops along with some proteins previously undetected in these strains. Some of the newly identified proteins show homology to putative zinc protease and a LabA-like protein from *Bradyrhizobium* sp. ORS278, fimbrial type 4 assembly proteins from *Ralstonia solanacearum*, and the type III effector Hrp-dependent protein from *Rhizobium leguminosarum* bv. *trifolii*.**

Sinorhizobium fredii is a fast-growing rhizobium that was initially isolated from soils of China (16). Even though *S. fredii* is mainly known as a soybean symbiont, it can nodulate and fix nitrogen in association with a wide range of legumes (20, 33). Two strains, USDA257 and USDA191, have been intensively studied in our laboratory because of their abilities to form nodules on soybean roots, either in a cultivar-specific or nonspecific manner. USDA257 forms nodules on primitive soybean cultivars, such as the Beijing cultivar, but fails to induce nodules on agronomically improved cultivars (1). In contrast, USDA191 shows no cultivar specificity and initiates nodules on most of the tested agronomically improved North American soybean cultivars. Characterization of a Tn*5* mutant (257DH4) of USDA257 has demonstrated that a cluster of genes located on an 8.0-kb DNA fragment located on the symbiosis (*sym*) plasmid is involved in regulating the soybean cultivar specificity (17, 28). This region was designated the soybean cultivar specificity locus. Paradoxically, the same locus was also found in USDA191 (4).

It is now known that the soybean cultivar specificity locus in USDA257 and USDA191 encodes the type III protein secretion system (T3SS) (4, 21, 23). The T3SS is utilized by Gram-negative bacteria to deliver effector proteins directly into host cells through filamentous surface appendages (7, 13). In mammalian pathogens, these surface appendages are described as needle-like structures, while in phytopathogens they are referred as Hrp pili (5, 7, 11, 13). USDA257 produces two types of surface appendages, flagella and pili (1, 23). This soybean symbiont elaborates flagella when grown in yeast extract-mannitol medium, while the addition of *nod* gene-inducing compounds to the medium enables the production of pili (1, 23). The type III secretion apparatus of USDA257 is clustered within a 32-kb region (23). Both USDA257 and USDA191 utilize the TTSS to secrete several Nops (*n*odulation *o*uter *p*roteins) into the extracellular medium (19, 21, 23, 26). T3SS is under the control of the transcriptional regulator TtsI (18, 45), and putative regulatory motifs termed *tts* boxes are found upstream of *ttsI*, *nopB*, *nopC*, *nopL*, *nopP*, and *nopX* (27, 45). Some of the Nops that are secreted by T3SS include NopA, NopB, NopC, NopL, NopP, and NopX (2, 8, 9, 30, 31, 45). In the case of USDA257, abolition of Nop secretion results in an altered nodulation phenotype in a host-dependent manner. For example, the USDA257 *nopX* mutant forms nitrogen-fixing nodules on McCall soybean, while the wild-type strain is unable to nodulate this soybean cultivar (28). This observation suggests that NopX and other Nops can function as soybean cultivar specificity determinants. If this is true, then the ques-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. fredii USDA257	Nod$^+$ on soybean	USDA ARS
S. fredii USDA191	Nod$^+$ on soybean	USDA ARS
E. coli DH5α	ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
Plasmids		
pGEM-T Easy	Ap^r	Promega
pMP220	Tc^r	40
pHKnopA-P	Tc^r, 1.0-kb *S. fredii* USDA257 *nopA* promoter in pMP220	This work
pHKnopB-P	Tc^r, 164-bp *S. fredii* USDA257 *nopB* promoter in pMP220	This work
pHKnopX-P	Tc^r, 290-bp *S. fredii* USDA257 *nopX* promoter in pMP220	This work
pHKnodABC-P	Tc^r, 445-bp *S. fredii* USDA257 *nopA* promoter in pMP220	This work

tion remains how USDA191, which has a functional TTSS and produces the same set of Nops as USDA257, is able to nodulate McCall soybean. It has been suggested the presence or absence of Nops and the relative amounts of individual Nops may regulate legume nodulation in a host-dependent manner (6, 7, 9, 43). In this study we examined the differences in the Nop profiles of USDA257 and USDA191. Our study reveals not only differences in the Nop profiles but also morphological and protein compositional changes in flagella and pilus-like appendages elaborated by these two strains of soybean symbionts.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. fredii* USDA191 and USDA257 were grown in yeast extract-mannitol (YEM) medium (43) on a reciprocal shaker at 30°C. *Escherichia coli* was cultured in Luria-Bertani broth at 37°C.

Purification and analysis of bacterial surface appendages. Surface appendages from USDA191 and USDA257 were isolated by ultracentrifugation as described earlier (23) and suspended in appropriate buffers for assessment by transmission electron microscopy (TEM) or separation by polyacrylamide gel electrophoresis (PAGE).

1D SDS-PAGE. Proteins associated with the surface appendages were identified by suspending the pellet from the ultracentrifugation step directly in 100 μl of SDS sample buffer (2% SDS, 10% glycerol, 0.125 M Tris-HCl [pH 6.8], 0.1% bromophenol blue, and 5% β-mercaptoethanol). One-dimensional (1D) separation followed the method of Laemmli (24). For better resolution of low-molecular-weight proteins, we also employed high-resolution Tris-Tricine gels (37).

Western blot analysis. Purified flagella from USDA191 and USDA257 separated by 1D SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antibodies raised against *Sinorhizobium meliloti* wild-type flagella that were diluted 1:3,000 in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5], 500 mM NaCl) containing 5% nonfat dry milk. Following overnight incubation, the membrane was washed three times with TBST (TBS containing 0.3% Tween 20). The membrane was then incubated with goat anti-rabbit IgG–horseradish peroxidase conjugate which was diluted 1:10,000 in TBST containing 5% nonfat dry milk. After several rinses in TBST, immunoreactive polypeptides were identified following the horseradish peroxidase color development procedure provided by the manufacturer (Bio-Rad, Hercules, CA).

Electron microscopy. Visualization of surface appendages elaborated by USDA191 and USDA257 by electron microscopy followed the protocol described by Saad et al. (35) with slight modifications. Briefly, overnight cultures of USDA191 and USDA257 were diluted to an optical density at 600 nm of 0.2. From these starter cultures, 20-μl aliquots were removed, placed on carbon-Formvar-coated grids (300 mesh), and incubated at 30°C for 48 h. Subsequently, the rhizobia on the grids were fixed with 2% formaldehyde and 0.5% (vol/vol) glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2). Following this fixation procedure, the grids were washed with TBST and negatively stained with 1% phosphotungstic acid (pH 6.5) for 1 min at room temperature and examined with a JEM 100B electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV. Similarly, droplets of purified surface appendages were placed on carbon-coated copper grids, stained with 1% phosphotungstic acid (pH 6.5), and examined by TEM as described above.

2D SDS-PAGE. Purified surface appendages from USDA191 and USDA257 were suspended in a small volume of 7 M urea, 2 M thiourea, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 2% C7BzO, and 100 mM dithiothreitol with vortexing. Protein concentrations of the samples were determined following the method of Bradford. For standard isoelectric focusing (IEF), about 100 to 120 μg of protein was loaded per strip using passive in-gel rehydration. Linear gradient, 13-cm IPG strips (GE Healthcare, Piscataway, NJ) were brought to a rehydration volume of 250 μl with 7 M urea, 2 M thiourea, 1% CHAPS, and 2% C7BzO with the following final concentrations of reagents: 5% glycerol, 2.2% 2-hydroxyethyl disulfide (2-HED), and 0.25% 4-7 IPG buffer or 0.5% 3-10 IPG buffer. Isoelectric focusing and SDS-PAGE were performed as described previously (22).

Protein identification by mass spectrometry. A small gel piece of each protein spot for identification was excised with a 1.5-mm spot picker (The Gel Company, San Francisco, CA) from a Coomassie G-250-stained gel, washed briefly in distilled water, and then destained completely in a 50% (vol/vol) solution of acetonitrile containing 25 mM ammonium bicarbonate. After a 100% acetonitrile wash, the protein contained in the acrylamide gel was subjected to digestion using 20 μl (10 μg/ml) of modified porcine trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate. Peptides resulting from the tryptic digestion were analyzed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) or liquid chromatography-tandem MS (LC-MS/MS). MALDI-TOF MS was performed as previously described (22).

Construction of *nop* promoter-*lacZ* fusion plasmids. Promoter sequences of *nopA* (1,060 bp), *nopB* (164 bp), *nopX* (290 bp), and *nodABC* (445 bp) were amplified from the USDA257 genomic DNA by standard PCR. Primers were designed to amplify promoter sequences that also included the sequences encoding the first three amino acids following the translation start sites of these genes (Table 2). To facilitate cloning, XbaI and PstI sites were introduced in the forward and reverse primers, respectively (Table 2). The amplified PCR products were first cloned into the pGEM T-Easy vector (Promega), and the fidelities of

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′–3′)[a]	Restriction site
nopAPf	ATCTAGACCGGATTCTCGCCAGATTTGGTCGC	XbaI
nopAPr	TCTGCAGTTTAGACATGTCATTTCCCTTTCTG	PstI
nopBPf	ATCTAGACTCGTCTTGATAAACCAAATCTG	XbaI
nopBPr	TCTGCAGCAGCATCATGAGAAGGACTCGATTAC	PstI
nopXPf	ATCTAGAAATTCAGACTTTAGGATTAGCACCTC	XbaI
nopXPr	TCTGCAGGGCCGACATTGTGCATCCTCAGACG	PstI
nodABCPf	ATCTAGATTGGCGGCCAAGTCTCAGCCTGTTC	XbaI
nodABCPr	TCTGCAGAGGACGCATATCCAAAGAACTCCAC	PstI

[a] The indicated restriction enzyme site is underlined.

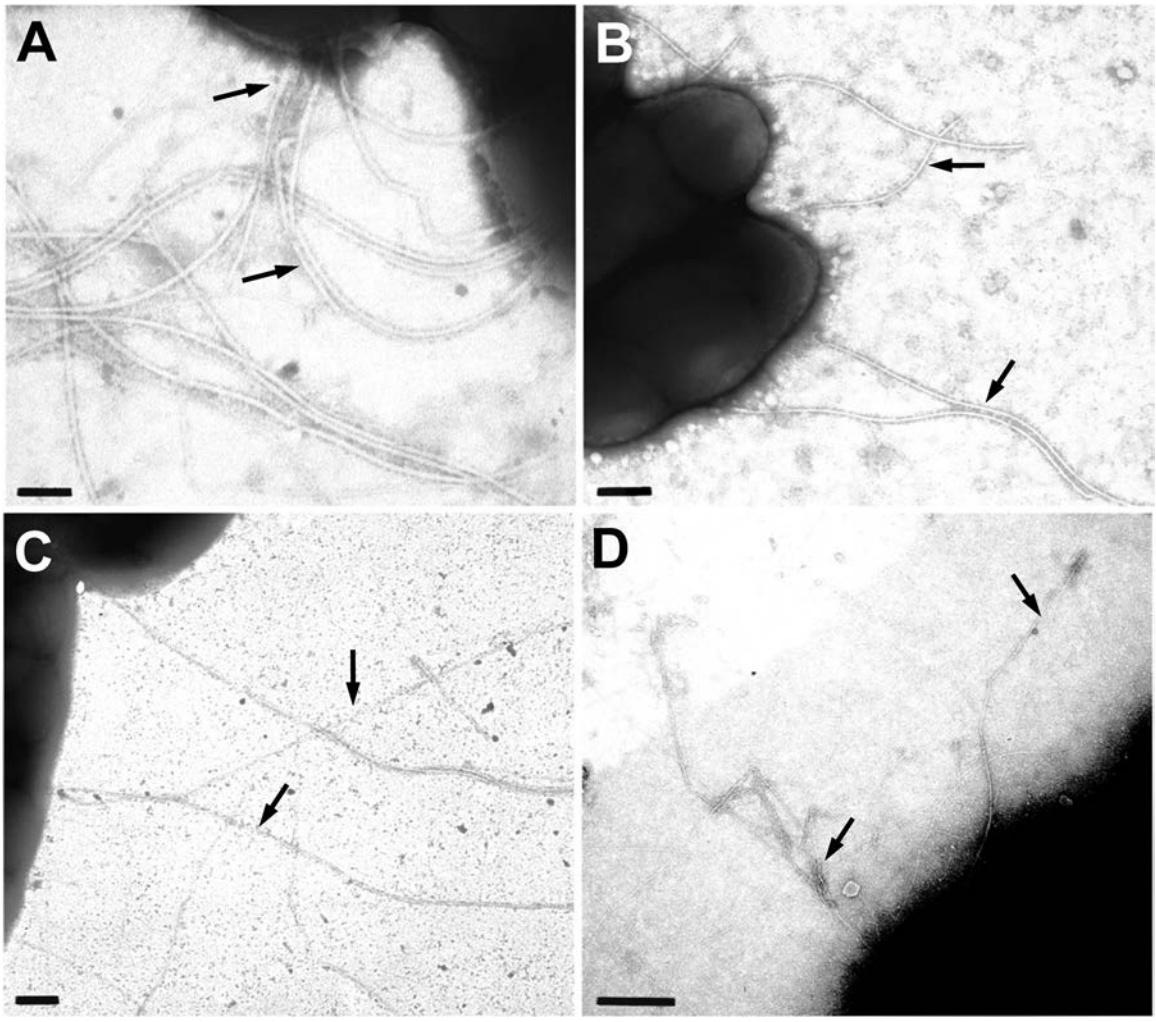

FIG. 1. Electron micrographs of surface appendages produced by USDA191 and USDA257. USDA191 and USDA257 were grown in YEM in the absence (A and B) or presence (C and D) of 1 μM apigenin on carbon-Formvar-coated gold grids at 30°C for 48 h. The bacterial cells were chemically fixed, stained with 1% phosphotungstate, and observed by transmission electron microscope. The arrows points to flagella (A and B) and to pilus-like appendages (C and D). Bars, 100 nm (A); 200 nm (B and C); 500 nm (D).

the cloned PCR products were verified by automated nucleotide sequencing. The *nop* promoters were excised from these plasmids by digesting the plasmid DNA with XbaI/PstI and ligated into XbaI/PstI-digested promoter probe pMP220 (40). These constructs were individually transferred into USDA191 and USDA257 by triparental mating with helper plasmid pRK2013 (10).

β-Galactosidase assay. Starter cultures of *Sinorhizobium fredii* USDA191 and USDA257 carrying *nop* promoter-*lacZ* fusion constructs were grown in YEM medium for 48 h. Aliquots (200 μl) of these starter cultures were added to 5 ml of fresh YEM medium containing 1 μM apigenin (dissolved in ethanol) and grown for 12 h at 30°C in an orbital shaker. Controls received an equal volume of ethanol as the treatment tubes. β-Galactosidase assays were performed as described by Miller (29). Cultures were assayed in triplicate, and the experiment was repeated twice.

RESULTS

Transmission electron microscopy observations of USDA191 and USDA257 surface appendages. Previously, we demonstrated that USDA257 produces two types of surface appendages, namely, flagella and pili, when grown in the absence or presence of *nod* gene-inducing flavonoids (23). To examine if USDA191 also produces similar appendages, we grew USDA191 on carbon-Formvar-coated grids in the presence and absence of 1 μM apigenin. For comparison, USDA257 was also grown under identical conditions. Electron microscope observation revealed the presence of filamentous structures associated with these bacteria (Fig. 1). USDA191 and USDA257 when grown in the absence of apigenin elaborated numerous flagella (Fig. 1A and B). Both strains produced curly flagella that were morphologically similar with an average diameter of 16 to 18 nm. In contrast, when these bacteria were grown in the presence of apigenin they produced morphologically distinct surface appendages. USDA257 elaborated long thin filaments (Fig. 1C), while USDA191 produced rod-shaped filaments (straight tubes) (Fig. 1D). The production of these surface appendages in both USDA191 and USDA257 was flavonoid dependent, since they were absent in cultures grown in the absence of the *nod* gene inducer (Fig. 1A and B).

Morphological differences in the surface appendages produced by USDA191 and USDA257 were further examined by

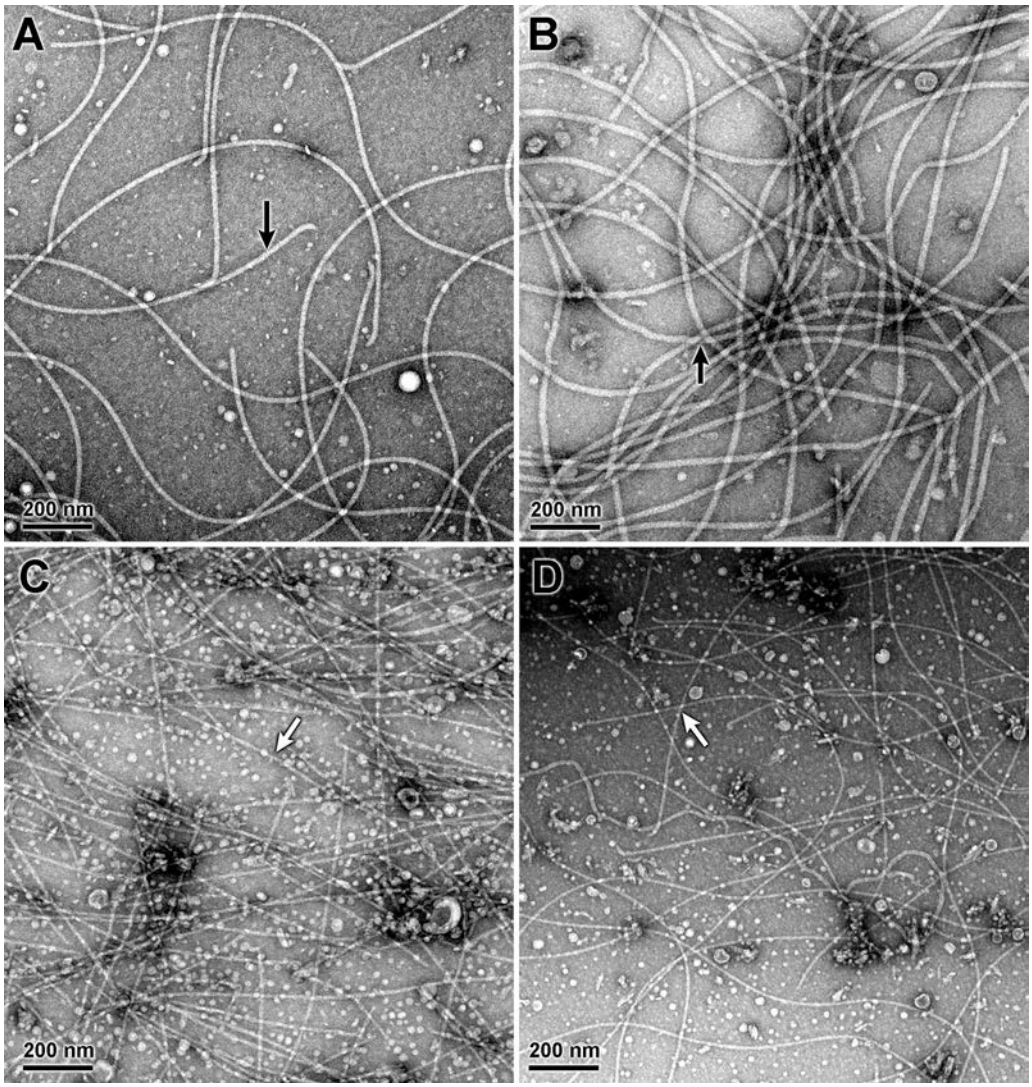

FIG. 2. Purified surface appendages isolated from USDA191 and USDA257. USDA191 and USDA257 were grown in YEM in the absence (A and B) or presence (C and D) of 1 μM apigenin for 48 h. The surface appendages were purified by ultracentrifugation. Purified surface appendages were negatively stained with 1% phosphotungstate and observed by transmission electron microscopy. The arrows points to flagella (A and B) and to pilus-like appendages (C and D). Bars, 200 nm.

transmission electron microscopy utilizing partially purified surface appendages. Electron microscopic observations of the purified flagella from 2-day cultures of USDA191 and USDA257 revealed similar morphologies (Fig. 2A and B). Both exhibited a curly morphology with an average diameter of 16 to 18 nm. In contrast to *Bradyrhizobium japonicum*, for which two morphologically distinct flagella are present (15), only one type of flagellum was found in both USDA191 and USDA257. An examination of the purified pili from apigenin-induced cultures of USDA191 and USDA257 confirmed the distinct morphological differences between the surface appendages produced by these strains. As observed with intact cells, the pili produced by USDA191 were rod shaped (straight tubes) and much thinner than the flagella, with approximate diameters of 10 to 13 nm (Fig. 2C). In contrast, electron micrographic observation of the purified pilus preparation from USDA257 revealed the presence of thin filaments (Fig. 2D). Unlike USDA191-produced surface appendages, these thin filaments were curly and had a similar appearance to flagella.

Protein composition of the purified surface appendages of USDA191 and USDA257. SDS-PAGE analysis of the purified flagella from USDA191 and USDA257 revealed two abundant bands (Fig. 3A). The flagellum from USDA191 contained two proteins with apparent molecular masses of 37 and 39 kDa. In contrast, the flagellum from USDA257 exhibited two prominent bands of 34 and 36 kDa (Fig. 3A). These abundant protein bands reacted positively in Western blot analyses against an *S. meliloti* wild-type flagellum antibody (Fig. 3B), suggesting that these proteins are flagellin subunits. We also excised the flagellin subunits from USDA191 and USDA257 and analyzed them by mass spectrometry. The mass spectral data indicated that the 39- and 37-kDa bands present in USDA191 flagella had homology to FlaA and FlaD of *S. meliloti*, respectively. In contrast, both the 36- and 34-kDa bands from USDA257

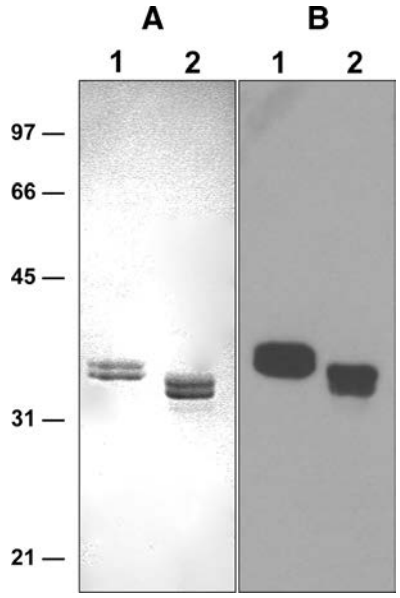

FIG. 3. 1D SDS-PAGE of flagellin proteins of *S. fredii*. Purified flagella from USDA191 (lanes 1) and USDA257 (lanes 2) were fractionated on a 12.5% SDS-PAGE and either stained with Coomassie brilliant blue (A) or transferred to a nitrocellulose membrane and probed with antibodies raised against purified *S. meliloti* flagellin (B). The immunoreactive polypeptides were visualized by chemiluminescence detection. The sizes of molecular mass markers (in kDa) are shown on the left side of the figure.

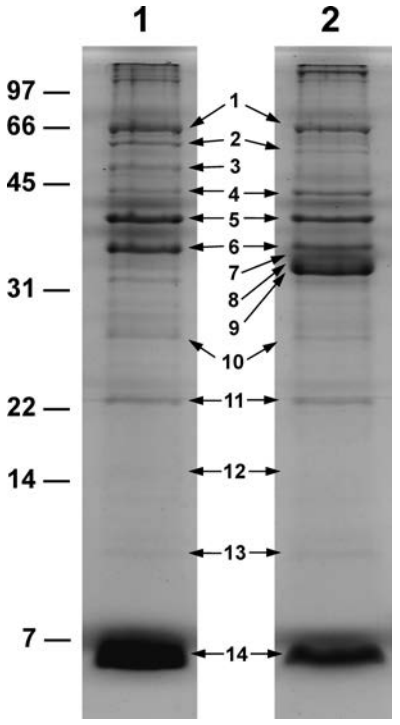

FIG. 4. Separation of pilus-associated proteins of *S. fredii* by high-resolution Tricine gels. Surface appendages isolated from apigenin-induced USDA191 (lane 1) and USDA257 (lane 2) were fractionated on a 16.5% Tricine gel and stained with colloidal Coomassie blue G-250. The numbers in the middle of the two gels correspond to the protein bands that were excised from the gel for identification by LC-MS/MS (Table 3). The positions of the protein molecular mass markers (in kDa) are shown on the left of the figure.

showed homology to FlaA from *S. meliloti*. In addition, the 36-kDa band in USDA257 also contained MS ions that showed homology to FlaB and FlaC of *S. meliloti*. However, both USDA191 and USDA257 flagellin subunits exhibited some MS properties associated with FlaA and FlaD species of *S. meliloti* as well. These data suggest that USDA191 and USDA257 flagella may consist of different amounts of FlaA, FlaB, FlaC, and FlaD. It should be noted that in *R. leguminosarum* FlaA, FlaB, and FlaC are the major components of the flagellar filament, while FlaD is a minor component (42).

In order to examine if there were any differences in the number and amount of Nops present in the purified pilus preparations from USDA191 and USDA257, we performed side-by-side comparisons of the protein compositions of the pilus preparations (Fig. 4). Pilus preparations were isolated from 2-day-old cultures of USDA191 and USDA257 grown in yeast extract-mannitol medium that was supplemented with 1 μm apigenin as described earlier (23). An examination of the protein profile clearly revealed differences in the number and the amount of Nops associated with the pilus preparations (Fig. 4). The USDA257 pilus preparation contained 14 distinct protein bands, while 11 protein bands were detected with USDA191. These protein bands were excised from the gels and subjected to mass spectrometry. NopX, NopL, NopP, Omp22, NopB, NopC, and NopA were identified in the pilus preparations from both USDA191 and USDA257 (Table 3). This observation is consistent with our earlier report, in which we demonstrated these proteins in the pilus preparation by immunoblot analysis using antibodies raised against Nops (21, 23, 26). In addition to these proteins, USDA257 also contained prominent flagellin subunits (Table 3). Interestingly, some of these flagellin subunits were not detected in the USDA191 pilus preparations. In repeated experiments, we have observed that USDA191 consistently produces larger amounts of NopA and NopX than USDA257. This is especially true for NopA, which accumulated to levels severalfold higher in USDA191 than in USDA257 (Fig. 4).

2D PAGE of USDA191 and USDA257 surface appendage proteins and their identification by mass spectrometry. Since resolution of proteins associated with the surface appendages of USDA191 and USDA257 by 1D SDS-PAGE was limited, we employed 2D PAGE for better resolution. Separation of the purified flagella from both USDA191 and USDA257 did not result in any distinct protein spots; instead, separation resulted in streaks ranging in pI value from 4 to 4.8 (Fig. 5). The poor resolution of the flagellin subunits did not appear to be an artifact, since several attempts to resolve the flagellum subunits under different IEF conditions did not improve the resolution of the protein spots. It is likely that these proteins are subjected to posttranslational modifications that result in charge heterogeneity.

Analysis of USDA191 and USDA257 pili by 2D PAGE resulted in resolution of several distinct protein spots (Fig. 6). The pilus preparations from both USDA191 and USDA257 were resolved into more than 20 protein spots. These protein spots were excised from the gels and analyzed by MALDI-TOF

TABLE 3. Pilus-associated proteins identified from *S. fredii* USDA191 and USDA257 by LC-MS/MS[a]

Band no.	Strain	Protein ID	MOWSE	No. of peptides matched	% coverage	MW E/T ratio	MS-Fit accession no. (NCBInr)
1	191	NopX (*S. fredii* NGR234)	924	37	43	66,000/64,111	gi|16520035
	257	NopX (*S. fredii* NGR234)	675	19	33	66,000/64,111	gi|16520035
2	191	NopX (*S. fredii* NGR234)	315	12	15	51,000/64,111	gi|16520035
	257	NopX (*S. fredii*)	329	7	14	50,000/51,206	gi|462734
3	191	NopX (*S. fredii*)	200	5	11	50,000/51,206	gi|462734
4	191	NopL (*S. fredii*)	74	2	5	42,000/37,108	gi|19749315
	257	NopL (*S. fredii*)	159	4	12	42,000/37,108	gi|19749315
5	191	NopL (*S. fredii*)	81	7	23	38,000/37,108	gi|19749315
	257	NopL (*S. fredii*)	73	8	24	38,000/37,108	gi|19749315
6	191	NopP (*S. fredii*)	78	10	37	37,000/31,283	gi|19749332
	257	NopP (*S. fredii*)	130	4	20	37,000/31,283	gi|19749332
7	257	Flagellin D (*S. fredii* NGR234)	188	3	12	35,000/33,906	gi|227820868
8	257	Flagellin B (*S. fredii* NGR234)	585	30	31	34,000/31,559	gi|227820866
9	257	Flagellin C (*S. fredii* NGR234)	700	51	41	33,000/31,063	gi|227820865
10	191	NopP (*S. fredii*)	631	23	38	31,000/31,283	gi|19749332
	257	NopP (*S. fredii*)	316	5	27	31,000/31,283	gi|19749332
11	191	Outer membrane protein (*S. fredii*)	196	5	25	24,000/21,859	gi|31322670
	257	Outer membrane protein (*S. fredii*)	429	13	51	24,000/21,859	gi|31322670
12	191	NopB (*S. fredii*)	106	11	64	16,000/16,879	gi|462728
	257	NopB (*S. fredii* NGR234)	776	40	76	16,000/16,885	gi|16520037
13	191	NopC (*S. fredii* NGR234)	192	3	47	10,000/9,824	gi|255767012
	257	NopC (*S. fredii* NGR234)	168	7	74	10,000/9,824	gi|255767012
14	191	NopA (*Sinorhizobium fredii*)	216	27	45	6,500/6,986	gi|55668600
	257	NopA (*S. fredii*)	228	13	45	6,500/6,986	gi|55668600

[a] Following 1D PAGE separation, protein spots were identified using peptide mass fingerprinting (LC-MS/MS). Band identification numbers correspond to the proteins labeled in Fig. 4. Molecular weight (M_r) values are given as experimental/theoretical ratios. MOWSE scores represent results from searches performed via Mascot. The number of peptides matched, percent coverage, and accession numbers within the NCBI (nonredundant) database are given. Searches were confined to microorganism databases.

MS. A comparison of the mass spectra of the peptide fragments with the database entries resulted in the identification of 17 protein spots from USDA191 and 24 protein spots from USDA257 (see Table S1 in the supplemental material). Some excised protein spots gave no significant hits, and some proteins were identified in several spots. In the case of USDA191, spots 3, 4, and 5 were identified as NopX, and spots 11, 12, and 13 were identified as NopP. Since these two groups of proteins have the same molecular mass but with different isoelectric points, this may indicate that both NopX and NopP are subjected to posttranslational modifications. Several protein spots in the USDA257 pilus preparations (spots 32, 37, 41, and 42) were identified as flagellar proteins. USDA257 also contained several distinct protein spots with molecular masses ranging from 36 to 40 kDa. The mass spectra of the peptide fragments generated from these protein spots when searched against protein database entries revealed that they are similar to the putative zinc protease (mpp-like) from

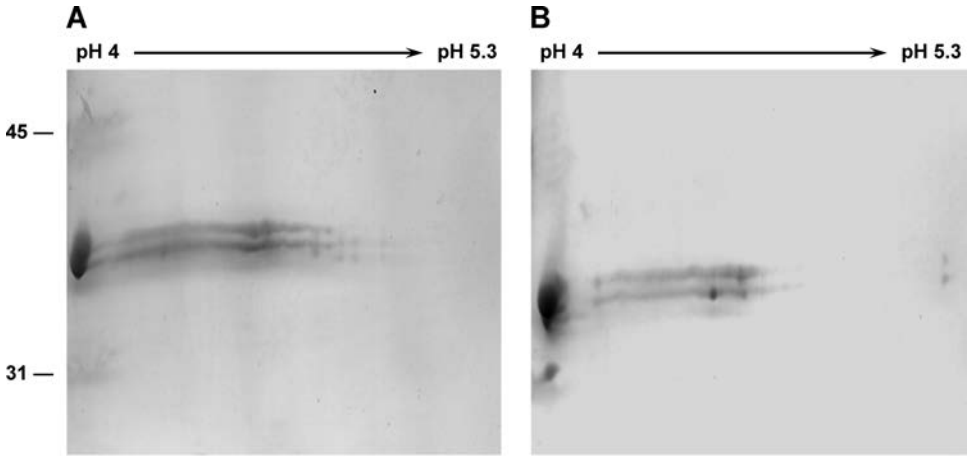

FIG. 5. 2D PAGE of flagellins of *S. fredii*. Purified flagella from USDA191 (A) and USDA257 (B) were first separated by isoelectric focusing on IPG strips (13 cm; pH 4 to 7) and then in the second dimension by SDS-PAGE on 12.5% gels. The fractionated proteins were stained with colloidal Coomassie blue G-250. The positions of the molecular mass markers ovalbumin (45 kDa) and carbonic anhydrase (31 kDa) are shown on the left of the figure.

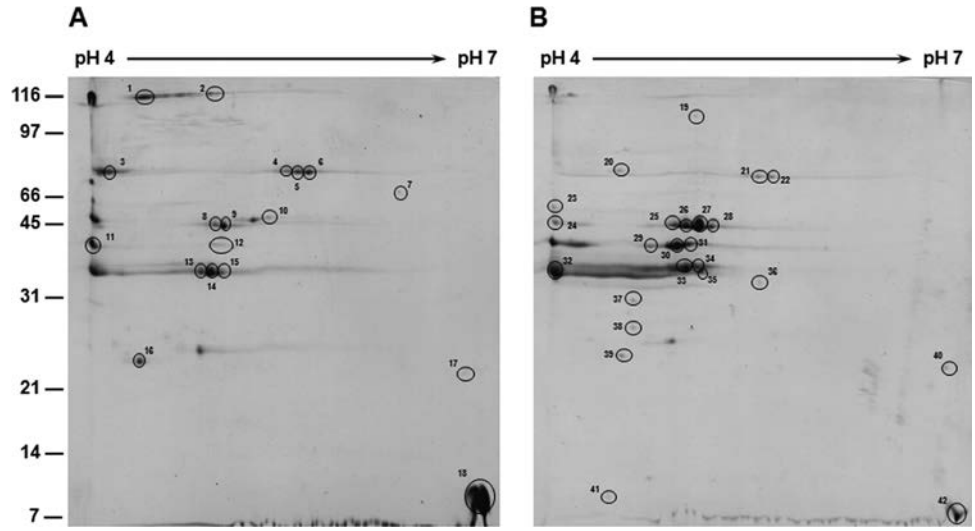

FIG. 6. 2D PAGE of pilus-associated proteins of *S. fredii.* Surface appendages isolated from apigenin-induced USDA191 (A) and USDA257 (B) were first separated by isoelectric focusing on IPG strips (13 cm; pH 4 to 7) and then in the second dimension by SDS-PAGE on 16% gels. The fractionated proteins were stained with colloidal Coomassie blue G-250. Circled protein spots were identified by MALDI-TOF MS; numbers correspond to those listed in Table S1 of the supplemental material. The positions of the protein molecular mass markers (in kDa) are shown on the left of the figure.

Bradyrhizobium sp. ORS278, type IV pilus biogenesis and competence protein PilQ from *Neisseria gonorrhoeae*, and a 34-kDa hypothetical protein from *Pseudomonas syringae* pv. Tomato strain DC3000. NopA, the most prominent protein detected in the pilus preparation by 1D SDS-PAGE, was not identified from the 2D gel excised protein spots of either USDA191 or USDA257.

Transcription levels of *nopA* and *nopX* are higher in USDA191 than in USDA257. Since the concentrations of several Nops in USDA191 were higher than in USDA257, we wanted to examine if there are any differences in the promoter activities of *nop* genes in these two rhizobia. For measuring the promoter activity of *nop* genes, we first cloned the putative promoter sequences of *nopA*, *nopB*, and *nopX* into pMP220, a promoter probe vector (40). For comparison, we also cloned the promoter region of the common *nodABC* into pMP220. These constructs were introduced into USDA191 and USDA257 by triparental mating, and the promoter activity was measured by assaying β-galactosidase activity (Table 4). The basal level of β-galactosidase activity of *nopA* was similar to that of the empty vector construct. Addition of 1 μM apigenin drastically elevated the β-galactosidase activity in both USDA191 and USDA257. The magnitude of induction was much higher in USDA191 than in USDA257 (Table 4). Similar results were also obtained for the *nopX-lacZ* fusion. Interestingly, the levels of induction of the *nopB-lacZ* fusion were not significantly different between USDA191 and USDA257. Similarly, the promoter strength of *nodABC*, a flavonoid-inducible common *nod* gene, exhibited similar promoter strength in both USDA191 and USDA257. These results indicate that *nopA* and *nopX* promoters are expressed at higher levels in USDA191 than in USDA257.

DISCUSSION

In this study we have shown that USDA191 and USDA257 produce distinct types of surface appendages. The flagella elaborated by both USDA191 and USDA257, although morphologically indistinguishable, are clearly different, since their flagellin components have distinct molecular weights. In contrast to the flagella, the pilus-like appendages produced by these strains are morphologically distinct. Unlike USDA191, which produces narrow filamentous structures, USDA257 produces long thin filaments similar to the ones described for

TABLE 4. Expression of *nop* promoters in *S. fredii* USDA191 and USDA257

Construct	β-Galactosidase activity (Miller units)[a] in the presence (+) or absence (−) of apigenin					
	USDA191			USDA257		
	−	+	Fold induction	−	+	Fold induction
pMP220	63 ± 7	44 ± 4	0	69 ± 11	80 ± 16	1.2
nopA-lacZ	17 ± 3	725 ± 21	42.6	59 ± 13	652 ± 21	11.1
nopB-lacZ	246 ± 32	1,355 ± 61	5.5	258 ± 22	1,173 ± 35	4.6
nopX-lacZ	62 ± 12	1,267 ± 41	20.4	204 ± 12	1,268 ± 96	6.2
nodABC-lacZ	55 ± 6	2,305 ± 53	41.9	44 ± 3	1,982 ± 121	45.1

[a] Activity results are reported as means ± standard errors of the means.

Rhizobium sp. NGR 234 (35). An essential component of the T3SS of pathogenic bacteria and some rhizobia is the production of pili or needle complexes associated with the cell surface (11, 13, 23, 35). These surface appendages function as conduits for the delivery of the effector proteins directly into host cells. Currently, we do not know if the different types of pilus-like appendages produced by USDA191 and USDA257 also differ in their abilities to deliver effector proteins into the soybean root cells.

The needle complexes produced by animal pathogens are mostly composed of several copies of a single small protein (5). The translocation pore, which is formed when the needle complex contacts host cells, is composed of different translocation proteins. For example, in the animal pathogen *Yersinia* spp., the needle is composed of YscF and three translocators, LcrV, YopB and YopD (5). Similarly, some plant pathogenic bacteria produce Hrp pili, which are composed of several copies of a single, small protein. They are also associated with translocation proteins, such as HrpY and PopF1 (13). Previously, we demonstrated that partially purified pili from USDA257 not only contain NopA but also NopB, NopX, NopL, and NopP (23, 26). NopX has significant homology to HrpF and PopF and is most likely a translocator, while NopB may function as a coupling protein within the pili (36). NopL and NopP have been identified as effector proteins and are involved in regulating legume nodulation in a host-dependent manner (2, 3, 39). The presence of these effector proteins in the pilus preparations of USDA191 and USDA257 is intriguing. Since the protocol used to isolate pili was solely based on an ultracentrifugation step, it is most likely that both NopL and NopP and other proteins may have copelleted with the pili. However, the possibility of protein-protein interactions between the different components of the pili and the effector proteins cannot be excluded. In this regard, it should be pointed out that NopA, NopB, and NopX, protein components of NGR234 pili, have been shown to interact directly with one another (36).

S. fredii HH103, like USDA191, is a soybean symbiont that forms nitrogen-fixing nodules on both primitive and agronomically improved North American soybean cultivars. This strain utilizes T3SS to secrete several Nops, including NopA, NopB, NopC, NopL, NopP, and NopX to the rhizosphere (9, 25). Recently, two additional proteins, NopM and NopD, were identified as type III system-secreted proteins (34). In our study, we were unable to identify NopM and NopD in either USDA191 or USDA257. This observation indicates that each *S. fredii* strain may elaborate a different subset of Nops, which could influence their host ranges. However, all these strains secrete some common Nops, such as NopA, NopB, NopC, NopL, NopP, and NopX. Even though the molecular masses of these Nops are similar between these strains, the relative concentrations of these proteins differ markedly between USDA191 and USDA257. It remains to be seen how the differences in the concentrations of Nops between these strains regulate soybean cultivar specificity. It should be pointed out that a T3SS mutant strain of USDA257 which fails to elaborate most of the Nops can efficiently form nitrogen-fixing nodules on advanced soybean cultivars. This implies that the presence of Nops alone is not sufficient for soybean nodulation; instead, it may be regulated by the correct combination and concentration of Nops.

Analysis of pilus proteins by mass spectrometry resulted in the identification of 17 proteins from USDA191 and 24 proteins from USDA257. Some of the proteins (NopX, NopP, and NopL) resolved into several protein spots of similar molecular weights but with different isoelectric points. Such a migration pattern has also been reported in *Bradyrhizobium japonicum*, and such a behavior has been attributed to phosphorylation or other posttranslational modifications (12). Interestingly, NopA, an abundant protein of the pili of both USDA191 and USDA257, was not identified by mass spectrometry from the 2D gel spots. Similarly, we were unable to identify NopC and NopB in USDA191 and NopC from USDA257 by mass spectrometry. The reason for the failure to detect these proteins is not clear, since some of these Nops are abundant and can be easily detected in a 1D gel. The most likely reason for their disappearance may be that these proteins are prone to degradation during the sample preparation process. A similar situation was also reported by Süß and others when they characterized genistein-inducible T3SS proteins of *B. japonicum* (41).

Cultivar-specific interactions of soybean with *S. fredii* have been well studied. Heron and Pueppke (14) performed a series of double inoculation experiments to examine the mechanisms of regulation of nodulation. In contrast to results obtained by Pierce and Bauer (32), who reported a rapid regulatory response that inhibits nodulation, McCall soybean nodulation was not inhibited when USDA257 was included along with USDA191. USDA257, which does not nodulate McCall soybean, however, was found to occupy nodules when inoculated with strain USDA191. Interestingly, they also reported that the addition of USDA191 culture filtrates to strain USDA257 did not enable USDA257 to initiate either infection threads or nodules on McCall. This experiment, however, did not investigate the role of Nops, since the culture filtrates were obtained from USDA191 grown in the absence of flavonoids. It will be interesting to examine if flavonoid-induced culture filtrates of USDA191 have the same effect.

Recently, we reported the positional cloning of two soybean genes, *Rj2* and *Rfg1*, that restrict nodulation of soybean (46). The *Rj2* and *Rfg1* genes restrict nodulation of soybean by *Bradyrhizobium japonicum* strain USDA122 and *S. fredii* USDA257, respectively. We have demonstrated that *Rj2* and *Rfg1* are allelic genes encoding members of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance (R) proteins. This observation indicates the presence of cognate avirulence genes in rhizobia. This appears to be the case, since some effectors (such as NopJ and NopT) have sequence homology to Avr proteins of phytopathogens (7). Therefore, it is most likely that the ability of the TIR-NBS-LRR class of plant resistance proteins to recognize these effectors will influence the fate of symbiosis. In the absence of recognition by the host R genes, the T3SS effectors may a play a positive role in nodulation, but they may function negatively if recognized by the host R gene (46).

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While names of study reagents and materials are necessary for reporting data factually, neither the University of Missouri nor USDA guarantees nor warrants the standard of product, and the use of a product by the University of Missouri or USDA implies no approval of the product to the exclusion of others that may be suitable.

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