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Sinorhizobium meliloti ExoR Is the Target of Periplasmic Proteolysis

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Sinorhizobium meliloti **ExoR regulates the production of succinoglycan and flagella through the ExoS/ChvI two-component regulatory system. ExoR has been proposed to inhibit the ExoS sensor through direct interaction in the periplasm. To understand how ExoR suppression of ExoS is relieved, which is required for the expression of ExoS/ChvI-regulated symbiosis genes, we characterized wild-type ExoR and ExoR95 mutant proteins. In addition to the previously identified precursor and mature forms of ExoR (designated $ExoR_p$ and $ExoR_m$, respectively), we detected a 20-kDa form of ExoR (designated $ExoR_{c20}$) derived from the wild-type ExoR protein, but not from the ExoR95 mutant protein. $ExoR_{c20}$ was isolated directly from *S. meliloti* periplasm to identify its N-terminal amino acids and the site of the proteolysis, which is highly conserved among ExoR homologs. $ExoR_{c20}$ retains the C terminus of the wild-type ExoR. When expressed directly, $ExoR_{c20}$ did not complement the *exoR95* mutation, suggesting that $ExoR_{c20}$ does not function directly in the ExoR-ExoS/ChvI regulatory pathway and that $ExoR_m$ is the functional form of ExoR. A single-amino-acid change (ExoRL81A) at the site of ExoR periplasmic proteolysis resulted in the reduction of the amount of $ExoR_m$ and the loss of the regulatory function of the ExoR protein. These findings suggest that $ExoR_m$ is a target of periplasmic proteolysis and that the amount of $ExoR_m$ could be reduced through effective proteolysis to relieve its suppression of ExoS.**

The Gram-negative soil bacterium *Sinorhizobium meliloti* establishes a nitrogen-fixing symbiosis with its plant host, alfalfa (*Medicago sativa*), through a set of complex and reciprocal signal exchanges in the absence of fixed nitrogen sources (11, 17, 27, 32, 48). The formation of an infection thread inside alfalfa root hairs is an essential step in the early stage of this symbiosis that requires the presence of *S. meliloti* exopolysaccharides, succinoglycan (SG), exopolysaccharide II (EPSII), or capsular polysaccharide (KPS). *S. meliloti* SG has been shown to be much more effective than the other two *S. meliloti* polysaccharides, EPSII and KPS, at eliciting the formation of infection threads (3, 7, 23, 29, 39). The structure and biosynthetic pathway of succinoglycan have been well documented, although its precise role in eliciting the formation of infection threads remains unknown (20–22, 30, 43, 51).

Succinoglycan production is inversely coregulated with flagellum production by a single signal transduction pathway consisting of the *S. meliloti* ExoR protein and the ExoS/ChvI two-component regulatory system (55) and the EmmABC system (37). While the transcription of succinoglycan biosynthesis genes is upregulated by the mutations *exoR95*::Tn*5* and *exoS96*::Tn*5*, transcription of the flagellum biosynthesis genes is downregulated (55). This coordinated regulation is consistent with the switch from free-living to invasion-ready cells that is required at this stage of symbiosis. It also suggests that the ExoR protein and the ExoS/ChvI two-component system play crucial roles in controlling the overall changes needed for *S. meliloti* cells to switch from free living to symbiosis inside the root nodules.

The *S. meliloti exoR* gene was initially identified through isolation of the *exoR95*::Tn*5* mutation, which was later identified and sequenced (10, 41). The *exoR* gene encodes a 268-amino acid ExoR protein with a conserved signal peptide for exporting the protein to the bacterial periplasm, as confirmed in recent findings (53). In addition to regulating succinoglycan and flagellum production, ExoR has been shown to be involved in regulating biofilm production and lipopolysaccharide modifications (16, 28). The ExoR protein has been found to regulate the expression of a large number of gene functions in very different metabolic pathways, suggesting that ExoR plays other important roles (53). ExoR homologs have been found and characterized in *Rhizobium leguminosarum* and *Agrobacterium tumefaciens*, where they also function in regulating polysaccharide, flagellum, and biofilm production (42, 47). Many additional ExoR homologs have been discovered in recent genome-sequencing efforts, but little is known about their functions.

The *S. meliloti* ExoS and ChvI proteins form a typical bacterial two-component signal transduction system (8, 38). The *S. meliloti* ExoS protein consists of a large periplasmic domain and a cytoplasmic kinase domain, and it has been shown to phosphorylate *S. meliloti* ChvI directly (8). Recent analysis of *exoS* and *chvI* deletion mutants has shown that the ExoS/ChvI system is essential for symbiosis and that these two proteins regulate the expression of a variety of genes involved in carbon metabolism and many other functions (2, 50). These findings are consistent with the results of a transcriptome analysis of the *exoS96* mutant (53). Collectively, these findings suggest that the ExoS/ChvI system plays an essential role in preparing *S. meliloti* cells for their transformation from free-living to nitrogen-fixing cells inside the root nodules. The importance of the *S. meliloti* ExoS/ChvI system was further highlighted by the finding that two of its close homologs are essential for host infections in *Brucella abortus* and *A. tumefaciens* (4, 15, 24, 31, 45).

Recent genetic and biochemical data suggest that ExoR, ExoS, and ChvI form a single signal transduction pathway (5, 53). The

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TABLE 1 Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant properties	Reference or source
E. coli strains		
DH5α	General-purpose strain	25
BL21(DE3)(pLysS)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* λ(DE3)pLysS T1R Cm^r	Novagen
MT616	MT607; pRK600; Cm^r	13
S. meliloti strains		
Rm1021	Wild type; Sm^r	29
Rm7095	Rm1021 *exoR95*::Tn*5*; Nm^r	10
Rm8002	Rm1021 Pho^-	19
SmHC12	Rm8002 *exoR95*::Tn5; Nm^r	This work
SmHC18	Rm1021 *exoR108*::pK19mob2ΩHMB; *chvI109*	This work
Plasmids		
pET-16b	Expression vector containing His tag; Ap^r	Novagen
pMB393	Expression vector; Sp^r	18
pHC93	Expression vector; pBBR1MCS3; Tc^r	Laboratory stock
pK19mob2ΩHMB	Suicide plasmid; Km^r	34
pRK600	Helper plasmid; Cm^r	13
pSW213	Cloning vector; *lacI*^{q} *lacp lacZ*^{a} Tc^r	35
pHC508	pK19mob2ΩHMB; nucleotides 88 to 433 of *exoR* ORF; Km^r	This work
pHC510	pMB393; *exoRp exoR*	This work
pHC518	pHC93; *lacp exoR*	This work
pHC615	pET16b; His *tag-exoR*	This work
pHC630	pMB393, *exoRp exoR-exoC*-Flag tag	This work
pHC641	pHC93; *lacp* $ExoR_m$ *exoR*$_{\Delta SP}$ (ExoR without its signal peptide)	This work
pHC528	pSW213; *exoR-phoA* fusion under an inducible *lac* promoter; *lacp exoR*::*phoA*	This work
pHC555	pSW213; *exoR*$_m$*-phoA* fusion under an inducible *lac* promoter; *lacp exoR*$_{\Delta SP}$::*phoA*	This work
pHC533	pSW213; ExoR signal peptide (*exoR*$_{SP}$)-*phoA* fusion under an inducible *lac* promoter; *lacp exoR*$_{SP}$::*phoA*	This work
pHC567	pMB393; fusion of ExoR signal peptide (aa[b] 1–30) with $ExoR_{c20}$ (aa 81–268) under *exoR* native promoter *exoRp* (SP-*exoR*$_{c20}$)	This work
pHC571	pMB393; *exoR* ORF with L81A mutation under *exoR* native promoter; *exoRp exoR*(*L81A*)	This work
pHC572	pMB393; *exoR* ORF with W79A mutation under *exoR* native promoter; *exoRp exoR*(*W79A*)	This work
pHC573	pMB393; *exoR* ORF with Y87A mutation under *exoR* native promoter; *exoRp exoR*(*Y87A*)	This work
Phage		
M12	Generalized transducing phage for *S. meliloti*	12

[a] *lacp lacZ*, the *lacZ* gene under *lac* promoter control.
[b] aa, amino acids.

ExoR protein has been localized to the periplasm of *S. meliloti* cells (53), as was confirmed by our unpublished data. ExoR has been found to exist in two forms, the 29-kDa full-length precursor form ($ExoR_p$) and the 26-kDa mature form without its predicted signal peptide ($ExoR_m$), in wild-type *S. meliloti* cells (5). Coimmunoprecipitation of ExoR and ExoS suggested that they form protein complexes (5). Increased expression of the *exoS* gene also led to accumulation of $ExoR_m$, suggesting that ExoS stabilizes ExoR in the ExoR-ExoS complex. The ExoR-ExoS interaction was interrupted by single-amino-acid changes in either the ExoR protein or the periplasmic domain of ExoS (5). Taken together, these findings led to a proposed model in which ExoR interacts with ExoS to form a protein complex that keeps ExoS in the off state, resulting in conditions favoring free living, with higher levels of flagellum production and lower levels of succinoglycan production (5).

Expanding on this suggested model, our recent data from genetic analyses suggest that ExoR autoregulates its own expression through the ExoS/ChvI two-component system (33). Loss of functional ExoR protein in the *exoR95* mutant leads to upregulation of *exoR* gene expression, along with that of succinoglycan biosynthetic genes (33). This upregulation can be suppressed by single-amino-acid mutations in the ExoS sensing domain, which is consistent with direct ExoR-ExoS interactions. This raises the possibility that ExoR autoregulation through the ExoS/ChvI system is used to modulate the expression of the genes regulated by the ExoS/ChvI system in response to host or environmental signals.

While the current model can explain how ExoR turns off the ExoS/ChvI system, it does not explain how the ExoS/ChvI system is turned on during symbiosis in response to environmental or plant signals. Our new findings, presented here, provide a better understanding of the molecular basis for the ExoR protein's ability to turn the ExoS/ChvI two-component regulatory system on and off.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains, phages, and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C, and *S. meliloti* strains were grown in LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$

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(LB-MC) at 30°C (29). LB-MC agar containing 0.02% (wt/vol) calcofluor white M2R (Blue Brighter 28; Sigma) was buffered to pH 7.4 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) and used to examine succinoglycan production on agar media (29). The following antibiotics were used at the concentrations indicated: ampicillin, 100 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; neomycin, 200 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}, and streptomycin, 500 μg ml^{-1}.

Motility assay. Bacterial cell motility was examined using swarming plates containing 0.3% agar as described previously, with some modifications (52). Briefly, fresh cell cultures were prepared and diluted to an optical density at 600 nm (OD_{600}) of 0.1. Then, 2 μl of each diluted culture was inoculated into an LB-MC soft-agar plate and incubated for 2 to 3 days to determine colony size.

Plant nodulation test. Alfalfa nodulation assays were carried out on plates as previously described, with slight modifications (29). A set of eight alfalfa seedlings were planted in a square petri dish, and all plants were grown inside the petri dishes. The plants were examined after 4 weeks for the number of nodules to determine overall symbiotic efficiency.

Expression and purification of *S. meliloti* ExoR-His for antibody production. The *S. meliloti exoR* open reading frame (ORF) was obtained by PCR, using Rm1021 genomic DNA as the template and two PCR primers: exoRfNdeI-1 and exoRrXhoI-1. The PCR product was digested with NdeI and XhoI and then cloned into pET-16b between NdeI and XhoI restriction sites to generate plasmid pHC615. The His-tagged ExoR protein was purified from BL21(DE3)(plysS, pHC615) with 1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) induction and using an Ni-nitrilotriacetic acid (NTA) purification system under denaturing conditions (Invitrogen). Purification of the His-tagged ExoR protein was confirmed using Coomassie blue-stained SDS-polyacrylamide gels and sent to the Pacific Immunology Company (Ramona, CA) to raise ExoR-specific polyclonal antibodies in rabbits. The specificity of the ExoR-specific polyclonal antibodies was confirmed by Western blotting.

Immunoblotting. For immunoblotting, total cellular proteins or purified protein was resolved by SDS-PAGE; transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad); detected with primary antibodies, including ExoR-specific polyclonal antibodies and FLAG-specific monoclonal antibody coupled with alkaline phosphatase (AP) (Novagen); and treated with NBT-BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate) (Sigma) to detect AP activities.

Construction of the *exoR108-chvI109* double mutant. The DNA fragment consisting of nucleotides 88 to 433 of the *exoR* ORF was generated by PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers: exoRdfHindIII-2 and exoRdrBsrGI. The PCR product was digested with HindIII and BsrGI and cloned into suicide plasmid pK19mob2ΩHMB (34) to produce pHC508. The resulting plasmid was then conjugated into *S. meliloti* Rm1021 using helper MT616 in a triparental mating, and a few conjugants were isolated after 6 days. The interrupted *exoR* gene was transduced from the conjugants into Rm1021, which yielded only one transductant, the *exoR108* mutant. Insertion of the suicide plasmid into the genome was confirmed by PCR and by sequencing the site of insertion. The *chvI* gene was amplified from the genome of the *exoR108* transductant using primers chvIf-27 and chvIr759 and sequenced using primers chvIf293 and chvIr419. The *exoS* gene was similarly amplified from the genome of the *exoR108* transductant using primers exoSf-61 and exoSr1918 and sequenced using primers exoSr407, exoSr829, exoSf653, exoSf1098, and exoSr1912. One single mutation was found in the *chvI* gene, but no mutations were found in the *exoS* gene. The *exoR108* transductant was thus designated the *exoR108-chvI109* double mutant. The construct was confirmed by sequencing. The primers used in cloning and sequencing are listed in Table 2, along with all other plasmid constructions described in this paper.

Constructs expressing ExoR and ExoR without signal peptide. The DNA fragment containing the *exoR* promoter and ORF was generated in a PCR using *S. meliloti* genomic DNA as the template and primers exoRfHindIII-1 and exoRrXhoI-2 and cloned between the HindIII and XhoI sites of a medium-copy-number plasmid, pMB393, to generate pHC510. Similarly, the *exoR* gene was amplified by PCR using primers exoRfNdeI-3 and exoRrXhoI-3 and cloned into pHC93 to generate pHC518, expressing the wild-type ExoR from the *lac* promoter on the plasmid. The region of the *exoR* gene without the signal sequence was amplified using primers exoRfNdeI-2 and exoRrXhoI-3, cloned into plasmid pHC93 to generate pHC641, and used to express ExoRm, the form of ExoR lacking its signal peptide.

Construction and analysis of ExoR-PhoA fusions. A set of three ExoR-PhoA fusions were constructed. To construct the fusion of PhoA with the full-length ExoR, an XbaI/KpnI DNA fragment containing the *E. coli phoA* gene (lacking its signal sequence) was prepared by PCR using the genomic DNA of the *S. meliloti exoF265*::Tn*phoA* mutant as the template and primers phoAfXbaI and phoArKpnI. An XhoI/XbaI DNA fragment containing the complete *exoR* ORF except the stop codon was obtained by PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers, exoRfXhoI and exoRrXbaI. The two DNA fragments were individually treated with either XhoI/XbaI or XbaI/KpnI and ligated with an XhoI- and KpnI-treated low-copy-number plasmid, pSW213, to generate pHC528. To construct the fusion of PhoA and ExoR without signal peptide, the region of the ExoR-PhoA fusion in pHC528 was amplified without the ExoR signal peptide region using two primers, exoRmfXhoI and phoArKpnI, and recloned into the same region of pSW213 to create pHC555. To create the fusion of PhoA with ExoR signal peptide, the coding region of the ExoR signal peptide was amplified in a PCR using pHC528 as the template and primers exoRfXhoI and exoRsp-phoAr. The region for PhoA was amplified using primers exoRsp-phoAf and phoArKpnI. The two fragments were joined together in an overlapping PCR using primers exoRfXhoI and phoArKpnI. The product of this overlapping PCR was cloned into pSW213 to create pHC533. All three fusions were expressed from an inducible *lac* promoter on the plasmid with 0.8 mM IPTG.

Construction and analysis of FLAG-tagged ExoR proteins. The unique 8-amino-acid FLAG tag (DYKDDDDK; Sigma) was inserted into the C terminus of the ExoR protein to help track it. To construct the C-terminally tagged ExoR, ExoR-CF, a DNA fragment containing ExoR and the FLAG tag, was generated by PCR using *S. meliloti* genomic DNA as the template and primers exoRfHindIII-1 and exoRcflagrXhoI, which introduced the FLAG tag. This DNA fragment was cloned into pMB393 to generate pHC630 and express ExoR-CF.

To track FLAG-tagged ExoR proteins, cells of *S. meliloti* strains expressing the ExoR-CF fusion were collected from exponential-phase cultures; washed with coimmunoprecipitation (CoIP) buffer (36); resuspended in 3 ml CoIP buffer with 1% (wt/vol) Triton X-100, 10 mM $MgCl_2$, 30 mg lysozyme, and 30 units of DNase I at 4°C for 30 min; and sonicated (550 Sonic Dismembrator; Fisher Scientific) twice on ice (level 2; 4.5 min each time). Cell lysates were centrifuged at 4°C and 13,000 rpm in a microcentrifuge for 5 min. The supernatants were collected, mixed with 20 μl anti-FLAG M2-agarose (Sigma), and incubated overnight at 4°C. The M2-agarose was collected from the reaction mixture by centrifugation at 5,000 rpm and 4°C for 3 min, washed three times with CoIP washing buffer, resuspended in 75 μl of 100 ng μl^{-1} FLAG peptide, incubated at 4°C for 1 h, and centrifuged at 13,000 rpm for 5 min to collect the supernatant for storage at −20°C. Alternatively, the washed M2-agarose was mixed with 75 μl loading buffer, boiled at 100°C for 5 min, and stored at −20°C.

Direct isolation of FLAG-tagged $ExoR_{c20}$-CF from *S. meliloti* periplasm. An overnight culture of the *S. meliloti exoR95* (pHC630) strain was diluted 1:100 in 1 liter of fresh LB-MC–spectinomycin and further incubated at 30°C with shaking to an OD_{600} of 0.3. Cells were collected, washed twice in Tris-buffered saline (TBS) (20 mM Tris, 0.2 mM NaCl, pH 7.5), resuspended in TBS with 0.5 M sorbitol for 10 min at room temperature, collected by centrifugation, resuspended in sterile water for 10 min on ice, and removed by two consecutive centrifugations (10 min

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TABLE 2 Oligonucleotide primers used for cloning and sequencing

Primer	Oligonucleotide nucleic acids[a]
exoRfNdeI-1	5′-GGAATTCCATATGATGCGCGCGGGTGAATTGAAGTC-3′
exoRrXhoI-1	5′-CCGCTCGAGTCAATCGTCGTCGTTCTGCAGATGCA-3′
exoRcflagrXhoI	5′-CCGCTCGAGTCATCA**TTTGTCATCGTCGTCCTTGTAGTC**ATCGTCGTCGTTCTGCAGATG-3′
exoRfHindIII-1	5′-CCCAAGCTTCATCGGCCACAAGTTCAACG-3′
exoRrXhoI-2	5′-CCGCTCGAGTCATCAATCGTCGTCGTTCTGC-3′
exoRdfHindIII-2	5′-GCCAAGCTTTCGATCCCGGAGC-3′
exoRdrBsrGI	5′-GGCTGTACAGGTCGGACCTTACCG-3′
exoRfNdeI-3	5′-GGCATATGCGCGCGGGTGAATTGAA-3′
exoRrXhoI-3	5′-GGGGTACCTATCAATCGTCGTCGTTCTGC-3′
exoRfNdeI-2	5′-GGAATTCCATATGTTCGATCCCGGAGCCGGCGT-3′
exoRfXhoI	5′-CCGCTCGAGTAACGAAAGAAACTGATTGTTATG-3′
exoRrXbaI	5′-GCTCTAGAATCGTCGTCGTTCTGCA-3′
phoAfXbaI	5′-GCTCTAGACCTGTTCTGGAAAACCGGG-3′
phoArKpnI	5′-GGGGTACCTTATTTCAGCCCCAGAGCGGC-3′
exoRmfXhoI	5′-CCGCTCGAGTAACGAAAGAAACTGATTGTTATGTTCGATCCCGGAGCCGGCGTGA-3′
exoRsp-phoAr	5′-CCGGTTTTCCAGAACAGGAGCCCGTGCTGGCCCGGC-3′
exoRsp-phoAf	5′-GCCGGGCCAGCACGGGCTCCTGTTCTGGAAAACCGG-3′
exoRL81Ar	5′-GCGTACATATTGGCTGCCGCCCAGCGAGAGCC-3′
exoRL81Af	5′-CTCGCTGGGCGGCAGCCAATATGTACGCCTATG-3′
exoRW79Ar	5′-CGTACATATTGGCGAGCGCCGCGCGAGAGCCCGTGTGCC-3′
exoRW79Af	5′-GGCACACGGGCTCTCGCGCGGCGCTCGCCAATATGTAC-3′
exoRY87Ar	5′-CCGCGACCCCATCGCCAGCGGCGTACATATTGGCGAGG-3′
exoRY87Af	5′-CTCGCCAATATGTACGCCGCTGGCGATGGGGTCGCGGA-3′
exoR90w241r	5′-GGCGTACATATTGGCGAGAGCCCGTGCTGGCCCG-3′
exoR90w241f	5′-CCAGCACGGGCTCTCGCCAATATGTACGCCTATG-3′
chvIf-27	5′-CCGGGCCTCGAAACGGAGTA-3′
chvIr759	5′-GGCCACGAGTGATCCGCAAT-3′
chvIf293	5′-TCATCACCAAGCCCTTCTCACAG-3′
chvIr419	5′-GGGAAGGCGTGTCGGCATT-3′
exoSf-61	5′-ACGGGCGAATGCGACAGG-3′
exoSr1918	5′-CCCCGGTGCCTCACTTCC-3′
exoSr407	5′-CGCTCCTGAATGATCGGGAAT-3′
exoSr829	5′-CGGCATGGACGATCTTGTCG-3′
exoSf653	5′-CGATCTATCCGGAGGTGATGAAC-3′
exoSf1098	5′-GAGCCACGAACTCAAGAACCC-3′
exoSr1912	5′-TGCCTCACTTCCGAAAGACAG-3′

[a] The DNA sequence encoding FLAG peptide is in boldface.

each; 13,000 rpm). The cell-free supernatant was mixed with HEPES buffer (40 mM HEPES, 500 mM NaCl, 4% Triton X-100, pH 7.0), mixed with 60 μl agarose beads covalently linked to FLAG-specific monoclonal antibody (Sigma A2220) following the manufacturer's instructions, and incubated at 4°C overnight with gentle inversion of the microcentrifuge tube. The agarose beads were collected at 10,000 rpm for 1 min, washed six times with TBS buffer (50 mM Tris, 0.2 mM NaCl, pH 7.5), rinsed once with 0.5 ml 0.1 M glycine-HCl (pH 3.5), resuspended in 30 μl SDS-PAGE loading buffer, boiled for 5 min, and run on a 15% SDS-polyacrylamide gel at a constant 150 V for 1.5 h using the Bio-Rad gel system.

Protein N-terminal determinations by mass spectrometry (MS)-based peptide mapping (performed by Sheng Zhang, Proteomics and Mass Spectrometry, Cornell University, Ithaca, NY). (i) In-gel digestion of excised gel bands. Following visualization of the gel, the protein band of interest was excised and placed into a 0.5-ml microtube for subsequent in-gel digestion with trypsin and manual extraction, both performed following a previously described protocol (44). All gel-extracted supernatants were combined and evaporated to dryness in a Speedvac.

(ii) Protein identification and peptide mapping by nano-LC–tandem-MS (MS-MS) analysis. The trypsin-digested samples were reconstituted in 15 μl of 0.1% (vol/vol) formic acid with 2% (vol/vol) acetonitrile prior to MS analysis. Nano-liquid chromatography (LC) was carried out in an LC Packings Ultimate integrated capillary high-performance liquid chromatography (HPLC) system equipped with a Switchos valve-switching unit (Dionex, Sunnyvale, CA). The gel-extracted peptides were injected using a Famous autosampler (Dionex, Sunnyvale, CA) onto a C_{18} PepMap trap column (Dionex, Sunnyvale, CA) for on-line desalting and then separated on a PepMap C_{18} RP nanocolumn and eluted in a 60-min gradient of 5% to 40% acetonitrile in 0.1% formic acid at 275 nl min^{-1}. The nano-liquid chromatograph was connected in line to a hybrid triple-quadrupole linear ion trap mass spectrometer, 4000 Q Trap, from ABI/MDS Sciex (Farmingham, MA) equipped with a Micro Ion Spray Head II ion source.

Data acquisition from the MS was performed using Analyst 1.4.2 software (Applied Biosystems) in positive ion mode for information-dependent acquisition (IDA) analysis. For the IDA analysis, after each survey scan from *m/z* 375 to *m/z* 1,550 and an enhanced-resolution scan, the three highest-intensity ions with multiple charge states were selected for MS-MS, with rolling collision energy applied for detected ions based on different charge states and *m/z* values.

(iii) Data analysis. The MS-MS data generated from nano-LC/electrospray ionization (ESI)-based IDA analysis were submitted to Mascot 2.2 for database searching using an in-house-licensed Mascot local server, and a search was performed to query the Swiss-Prot database (taxonomy, *Proteobacteria*) with one missed cleavage site by semitrypsin allowed. The peptide tolerance was set to 1.5 Da, and the MS-MS tolerance was set to 0.6 Da. Carbamidomethyl modification of cysteine and oxidation of a methionine were set as variable modifications. Only significant scores for the peptides

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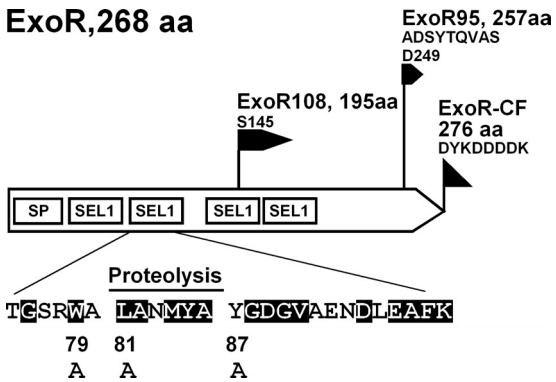

FIG 1 Schematic representation of the ExoR protein showing the signal peptide (SP), four putative Sel1 protein-protein interaction domains (SEL1), a FLAG-tagged ExoR protein (ExoR-CF), two mutated ExoR proteins (ExoR95 and ExoR108), the proteolysis region of ExoR, the amino acids that are conserved (in black-shaded letters) (Fig. 6) around the proteolysis region, and the positions of the 3 amino acids that were mutated to alanine (A) for functional analyses. The amino acid sequences of the C-terminal FLAG tag (CF), the ExoR95 C terminus, and the target region (81 to 87) of ExoR proteolysis are shown.

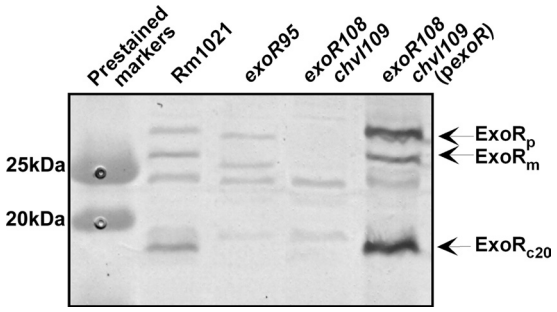

FIG 2 Western blot showing ExoR protein profiles of the *S. meliloti* wild-type Rm1021, the *exoR95* mutant, the *exoR108-chvI109* double mutant, and the *exoR108-chvI109* (pHC510) double mutant, which is labeled as a p*exoR* mutant expressing the wild-type *exoR* gene. Prestained proteins (Bio-Rad catalog number 161-0373) were used as size markers. The three forms of the ExoR protein are precursor (ExoR$_p$), mature (ExoR$_m$), and the main cleavage product (ExoR$_{c20}$). The *exoR95* mutant expresses two smaller ExoR proteins, ExoR95$_p$ and ExoR95$_m$, but no detectable cleavage products.

defined by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM) greater than "identity" were considered for the peptide identification.

Construction of an ExoR$_{c20}$-expressing plasmid. To construct a translational fusion of the *exoR* signal peptide and the ExoR C-terminal proteolytic product (ExoR$_{c20}$), the *exoR* signal peptide coding region, along with the *exoR* promoter region and the ExoR$_{c20}$ (amino acids 81 to 268) coding region, was obtained individually from two PCRs. The primers used to amplify the coding region for the *exoR* promoter and the ExoR signal peptide were exoRfHindIII-1 and exoR90w241r, and those used to amplify the coding region for ExoR$_{c20}$ were exoR90w241f and exoRrXhoI-2. After PCR and purification, the two fragments were ligated together via recombinant PCR using primers exoRfHindIII-1 and exoRrXhoI-2. This PCR product was then purified and digested with restriction enzymes HindIII and XhoI and ligated with the vector pMB393 digested with the same enzymes to generate plasmid pHC567.

Construction of plasmids expressing ExoR protein with single-amino-acid mutations. Single-amino-acid mutations of ExoR were introduced via PCR site-directed mutagenesis. To construct pHC571 with the ExoR L81A mutation, two separate PCRs were run using primer pairs exoRfHindIII-1/exoRL81Ar and exoRL81Af/exoRrXhoI-2 and plasmid pHC510 DNA as the template. Primers exoRL81Ar and exoRL81Af introduced a codon change resulting in an L-to-A mutation at position 81 of the ExoR protein. The two PCR DNA fragments were ligated together via recombinant PCR using primers exoRfHindIII-1 and exoRrXhoI-2. This PCR product was then purified and digested with restriction enzymes HindIII and XhoI and ligated with vector pMB393 digested with the same enzymes to generate plasmid pHC567.

Using a similar approach, plasmid pHC572 was constructed with the W79A mutation created by primers exoRW79Ar and exoRW79Af, and pHC573 was constructed with the Y87A mutation created by primers exoRY87Ar and exoRY87Af.

RESULTS

Extra protein bands in ExoR profiles. The ExoR protein autoregulates its own expression through the ExoS/ChvI system (33), and it has been shown to exist in a 29-kDa precursor form (ExoR$_p$) and a 26-kDa active mature form (ExoR$_m$) without signal peptide (5) (Fig. 1). The ExoR$_m$ form most likely interacts directly with the sensing domain of ExoS in the periplasm to regulate ExoS sensor activity. This suggests that the amount of ExoR$_m$ is linked to the activity of ExoS and that this amount needs to be tightly regulated, perhaps by converting it into an inactive form. To find other possible forms of ExoR, ExoR polyclonal antibodies were prepared by using gel-purified N-terminally labeled His-ExoR. When total cellular proteins from *S. meliloti* wild-type strain Rm1021 were resolved and probed with our ExoR polyclonal antibodies, three extra prominent protein bands of 20, 21, and 24 kDa were detected, in addition to the known 29-kDa ExoR$_p$ and 26-kDa ExoR$_m$ (Fig. 2). To determine the origin of the 20-, 21-, and 24-kDa proteins, the ExoR profile of the wild-type strain was compared to that of the loss-of-function *exoR95* mutant.

The *exoR95* mutant is a transposon Tn*5* insertion mutant; the site of the Tn*5* insertion has been genetically mapped to the end of the *exoR* gene (41). To determine the size and amino acid sequence of the ExoR95 protein, the site of the transposon insertion was determined. Tn*5* was found to be inserted between codons 248 and 249, replacing the original 20 C-terminal amino acids of ExoR (DDRRVAITMSQNMHLQNDDD) with a new set of 9 amino acids (ADSYTQVAS) in the ExoR95 protein. This suggested that if the ExoR95 protein is synthesized and exported to the periplasm like the wild-type ExoR, it should exist in 28-kDa ExoR95$_p$ and 25-kDa ExoR95$_m$ forms.

Western blot analysis showed that the *exoR95* mutant not only has the predicted 28-kDa ExoR95$_p$ and 25-kDa ExoR95$_m$ forms (Fig. 2), but also the 21- and 24-kDa proteins that were found in the wild-type Rm1021 cells. It is also interesting that the 20-kDa protein is missing from the *exoR95* mutant. The 21- and 24-kDa proteins are smaller than the 25-kDa ExoR95$_m$, which makes it possible in theory for them to be derivatives of the 25-kDa ExoR95$_m$ protein. Therefore, a strain with a smaller, or no, ExoR protein was needed to help identify the origins of the 20-, 21-, and 24-kDa proteins.

Construction of an *exoR* mutant for biochemical analysis. After multiple attempts, we were able to obtain only one *exoR* mutant—the *exoR108* mutant, resulting from the insertion of plasmid pHC508 carrying part of the *exoR* ORF. The ExoR108 protein is predicted to be a fusion of 144 N-terminal amino acids from ExoR and 51 C-terminal amino acids from the inserted suicide plasmid (Fig. 1). The *exoR108* mutant overproduced succi-

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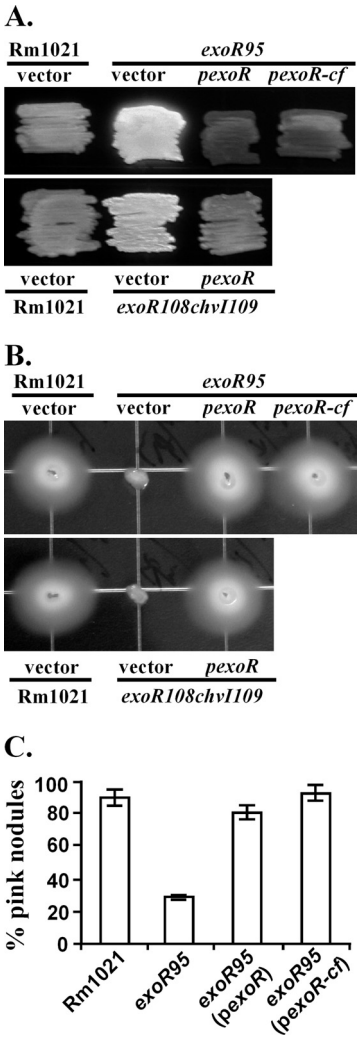

FIG 3 Functional analyses of the wild-type ExoR protein and the C-terminally FLAG-tagged ExoR proteins. (A and B) Each protein was expressed in the *exoR95* background and in the *exoR108-chvI109* double-mutant background to determine its function in regulating succinoglycan production, as determined by the levels of colony brightness on calcofluor-containing medium (A), and flagellum production, as determined by the sizes of swimming colonies (B). (C) The functions of these modified ExoR proteins in nodulation were measured according to the percentage of pink nodules in the *exoR95* mutant background. The plasmids for the expression vector, p*exoR*, and p*exoR-cf* are pMB393, pHC510, and pHC630, respectively.

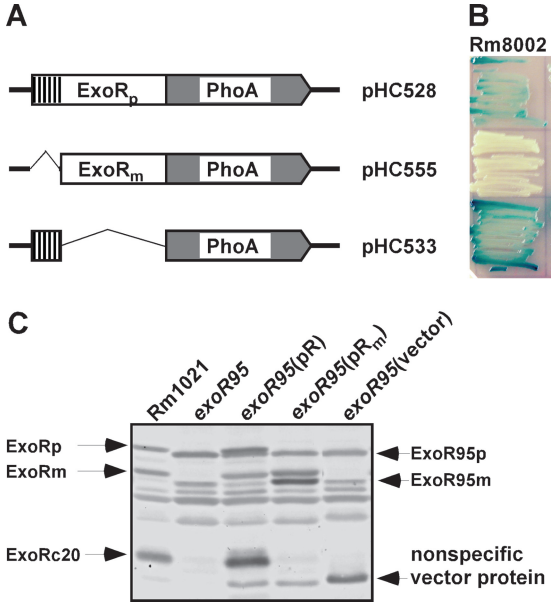

FIG 4 Cellular location of ExoR protein and site of proteolysis. (A and B) Schematic representation of three different ExoR-PhoA fusions (A) and their corresponding phosphatase activities in the *S. meliloti* Rm8002 background (B). The vertically hatched boxes represent the ExoR signal peptide. (C) ExoR protein profiles were determined by Western blotting for wild-type ExoR protein expressed from pHC518 (pR) and the ExoR protein without signal peptide, $ExoR_m$, expressed from plasmid pHC641(pR_m) in the *exoR95* mutant background.

noglycan and formed nonswimming colonies, similar to the *exoR95* mutant. These *exoR108* mutant phenotypes were complemented by the plasmid pHC510 expressing wild-type ExoR protein, suggesting that the *exoR108* mutation is a loss-of-function mutation (Fig. 3A and B).

The difficulties in obtaining an *exoR* mutation, and prior findings of *exoR* suppressor mutations in the *exoS* and *chvI* genes, prompted us to check *exoS* and *chvI* in the *exoR108* mutant. Interestingly, while there was no mutation in the *exoS* gene, there was a single G-to-A nucleotide change at position 325 in the *chvI* gene in the *exoR108* mutant. This mutation results in a change in amino acid 109 of the ChvI protein from V to M, so the *exoR108* mutant is in fact an *exoR108-chvI109* double mutant. It was possible to transduce the *exoR108* mutation from the *exoR108-chvI109* mutant into wild-type Rm1021 expressing the wild-type *exoR* gene from plasmid pHC510, but not into Rm1021 itself. This raised the possibility that the *exoR* gene can only be interrupted to generate the *exoR108* mutation in the presence of either a plasmid-borne copy of the *exoR* gene or the *chvI109* mutation. These findings suggest that the possibility that the *chvI109* mutation could contribute to the phenotype of the *exoR108-chvI109* double mutant cannot be ruled out, and the mutant could therefore only be used for biochemical analyses of ExoR proteins in this study.

A novel 20-kDa form of the ExoR protein. Three proteins at 29, 26, and 20 kDa stood out clearly in the ExoR protein profiles of the *exoR108-chvI109* double mutant with plasmid pHC510 expressing wild-type ExoR protein, while the intensities of the 21- and 24-kDa bands remained the same as for the *exoR108-chvI109* double mutant without plasmid pHC510 (Fig. 2). The positions of the 29-, 26-, and 20-kDa proteins matched those of the three protein bands in wild-type strain Rm1021 (Fig. 2). Since the 21- and 24-kDa proteins were present in wild-type Rm1021, as well as in the *exoR95* and *exoR108* mutants, they are most likely nonspecific proteins. Together, these findings suggest that the ExoR protein exists in three different forms, 29, 26, and 20 kDa. The finding of the previously unknown 20-kDa ExoR protein suggests that ExoR could be the target of proteolysis.

$ExoR_m$ is digested in the periplasm. The 20-kDa ExoR protein, designated $ExoR_{c20}$, could be the proteolysis product of either $ExoR_p$ in the cytoplasm or $ExoR_m$ in the periplasm. To find a way to restrict ExoR in either the cytoplasm or periplasm, the role of its signal peptide was further examined. The entire ExoR protein ($ExoR_p$), ExoR without the signal peptide ($ExoR_m$), and the ExoR signal peptide were separately fused to PhoA (Fig. 4A). The

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presence of the signal peptide was sufficient for the ExoR-PhoA fusion and PhoA to be exported to the periplasm, where it displayed phosphatase activity, as indicated by the blue color of the colonies on medium containing XP (5-bromo-4-chloro-3-indolylphosphate) (Fig. 4B). In the absence of signal peptide, the $ExoR_m$-PhoA fusion protein remained inside the cells, as indicated by the white color of the colonies (Fig. 4B).

To help determine the site of $ExoR_m$ proteolysis, wild-type ExoR and $ExoR_m$ were expressed in the *exoR95* mutant from plasmids pHC518 and pHC641 to deliver $ExoR_m$ to the periplasm or cytoplasm, respectively. When wild-type ExoR was expressed in the *exoR95* mutant from plasmid pHC518, all three forms of the wild-type ExoR—$ExoR_p$, $ExoR_m$, and $ExoR_{c20}$—were clearly visible, in addition to $ExoR95_p$ and $ExoR95_m$ (Fig. 4C). In contrast, when $ExoR_m$ was expressed in the *exoR95* mutant from plasmid pHC641, the $ExoR_m$ protein, as well as $ExoR95_p$ and $ExoR95_m$, was found, but very little $ExoR_{c20}$ protein was detected (Fig. 4). This suggests that $ExoR_m$ is very ineffectively processed inside the cytoplasm. Taken together, these findings suggest that $ExoR_m$ is digested efficiently in the periplasm to yield the 20-kDa form. This conclusion was further confirmed by direct isolation and sequencing of the ExoR proteolysis product from the periplasm, as described below.

Functional analyses of C-terminally FLAG-tagged ExoR protein. To determine whether the 20-kDa form of ExoR shares the same C terminus with $ExoR_m$, ExoR-CF, an ExoR protein with an 8-amino-acid C-terminal FLAG tag (DYKDDDDK), was generated by fusing the FLAG tag to the ExoR C terminus (Fig. 1). ExoR-CF was expressed from plasmid pHC630 with the native *exoR* promoter. Our results showed that ExoR-CF was as effective at complementing the succinoglycan overproduction phenotype of the *exoR95* mutant as the wild-type ExoR protein expressed from plasmid pHC510 (Fig. 3A). Similarly, ExoR-CF was as effective as the wild-type ExoR in restoring the swimming activity (Fig. 3B) and nodulation of alfalfa plants of the *exoR95* mutant, as determined by the percentage of pink nodules (Fig. 3C). These findings suggest that the biochemical analysis of ExoR-CF should reflect the properties of the wild-type ExoR protein.

The C terminus of the 20-kDa form of ExoR. The C terminus of the 20-kDa form of ExoR could be determined by identifying which form of ExoR protein retains the FLAG tag. Total proteins from the *exoR95* mutant expressing ExoR and ExoR-CF proteins from plasmids pHC510 and pHC630, respectively, were prepared, immunoprecipitated with FLAG-specific monoclonal antibody, and probed with either our ExoR polyclonal antibodies (Fig. 5A) or the FLAG-specific monoclonal antibody (Fig. 5B). Wild-type ExoR protein without a FLAG tag could not be precipitated with the FLAG-specific antibody, so it was not detected by either antibody. Our analysis of C-terminally FLAG-tagged ExoR-CF showed that all three different forms of ExoR were detected by both ExoR and FLAG antibodies (Fig. 5). This suggests that $ExoR_p$, $ExoR_m$, and $ExoR_{c20}$ were all carrying the FLAG tag. Altogether, these findings suggest that the 20-kDa form of ExoR derives from the C-terminal side of the ExoR protein (hence its designation, $ExoR_{c20}$).

The N terminus of the 20-kDa ExoR fragment and the site of ExoR proteolysis. To identify the N-terminal amino acid of $ExoR_{c20}$, which is also the site of ExoR proteolysis, the C-terminally FLAG-tagged ExoR-CF proteins were isolated directly from the periplasm of *S. meliloti* cells using osmotic shock and immunoprecipitation with commercial monoclonal FLAG tag antibody. $ExoR_{c20}$-CF, $ExoR_{c20}$ with a C-terminal FLAG tag, was first isolated directly from a crude preparation of periplasmic proteins by immunoprecipitation and then resolved by SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie blue. A piece of membrane containing $ExoR_{c20}$-CF was used to identify its N-terminal amino acid by peptide mapping.

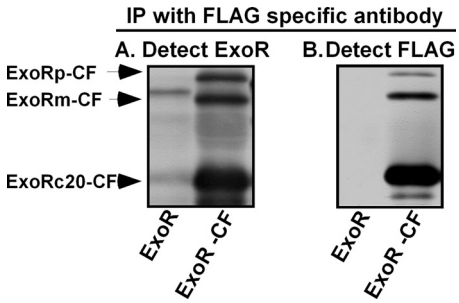

FIG 5 Western blot protein profiles of the *exoR95* mutant carrying plasmid pHC510 and pHC630 expressing wild-type ExoR and ExoR-CF, respectively. The proteins were immunoprecipitated (IP) with FLAG antibody and detected with either ExoR polyclonal antibodies (A) or FLAG antibody (B).

The results of the peptide mapping showed that the N-terminal amino acid of $ExoR_{c20}$-CF is L and that it is amino acid 81 in the ExoR protein. The confidence in this finding was measured by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM). A Mascot value of 59 represents 99% confidence. The Mascot value for L as the N-terminal amino acid was 94, indicating more than 99% confidence in the identification. Interestingly, amino acids 84, 85, 86, and 87 were also identified as N-terminal amino acids, with Mascot values between 85 and 111. This suggested that ExoR proteolysis could take place between amino acids 80 and 87 of the ExoR protein to yield a mixture of 181- to 188-amino-acid-long peptides (Fig. 1). These peptides could be the result of a single digestion between amino acids 80 and 81 followed by additional digestion, or they could be the result of a single random digestion within the region of amino acids 80 to 87. The preparation method for peptide mapping used here precluded us from determining which fragment was the dominant form. Taken together, these findings suggest the strong likelihood of $ExoR_m$ protein being further digested between amino acids 80 and 87 to yield $ExoR_{c20}$, a set of 181- to 188-amino-acid (19.9- to 20.6-kDa) peptides.

A large number of ExoR orthologs have been discovered through genome sequencing, although few of them have been characterized in detail. Sixteen ExoR orthologs were aligned based on their overall amino acid identity, ranging from 97% to 51% (Fig. 6). Despite the dramatic decrease in the overall amino acid identity, however, the amino acid sequence around the ExoR proteolysis sites remains highly conserved among the ExoR orthologs (Fig. 6). This raises the possibility that ExoR proteolysis is conserved among other ExoR orthologs and that proteolysis could be a common molecular signaling mechanism for ExoR proteins in different bacteria.

$ExoR_{c20}$ does not function in the ExoS/ChvI system. The discovery that both $ExoR_m$ and $ExoR_{c20}$ are stably maintained in the wild-type Rm1021 cells, and that $ExoR_{c20}$ is absent in the loss-of-function *exoR95* mutant, raised the possibility that $ExoR_{c20}$ is the functional form of the protein. To examine the function of

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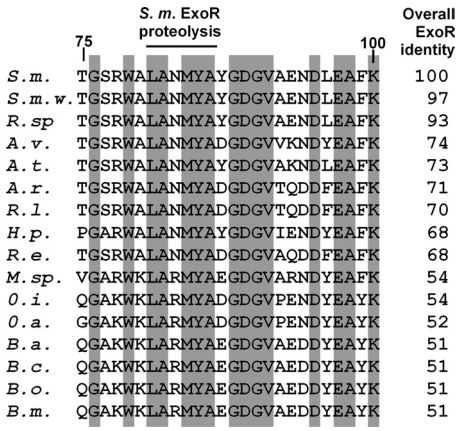

FIG 6 Amino acid sequence alignment of ExoR proteolysis regions from 16 ExoR orthologs. Conserved amino acids are shaded. The overall amino acid identities between *S. meliloti* ExoR and its orthologs are listed. The positions of the amino acids and the site of proteolysis are marked based on the *S. meliloti* ExoR protein. The following species are shown: *S. meliloti* Rm1021 (*S. m.*), *Sinorhizobium medicae* WSM419 (*S. m. w.*), *Rhizobium* sp. strain NGR234 (*R. sp.*), *Agrobacterium vitis* S4 (*A. v.*), *Agrobacterium tumefaciens* strain C58 (*A. t.*), *Agrobacterium radiobacter* K84 (*A. r.*), *Rhizobium leguminosarum* bv. trifolii WSM2304 (*R. l.*), *Heoflea phototropica* DFL-43 (*H. p.*), *Rhizobium etli* CIAT652 (*R. e.*), *Mesorhizobium* sp. strain BNC1 (*M. sp.*), *Ochrobactrum intermedium* LMG 3301 (*O. i.*), *Ochrobactrum anthropi* ATCC 49188 (*O. a.*), *Brucella abortus* strain 2308 A (*B. a.*), *Brucella ceti* strain Cudo (*B. c.*), *Brucella ovis* ATCC 25840 (*B. o.*), and *Brucella melitensis* 16 M (*B. m.*).

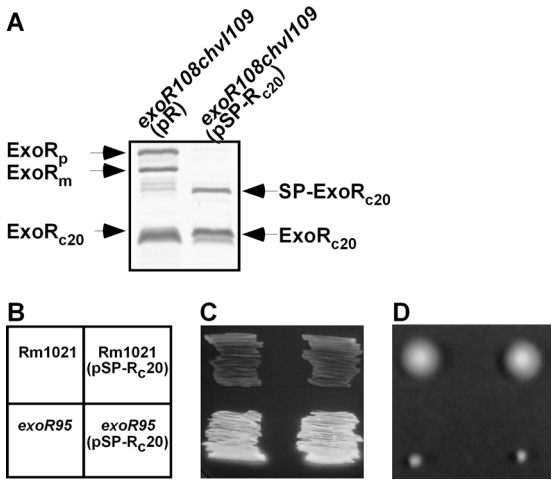

FIG 7 (A) Biochemical and functional analysis of the ExoR proteolysis product $ExoR_{c20}$, which was expressed from pHC510 (pR) and pHC567 (pSP-R_{c20}) in the *exoR108-chvI109* mutant for ExoR and SP-ExoR and probed in a Western blot. (B to D) The effects of the presence of $ExoR_{c20}$ on succinoglycan production was measured by calcofluor fluorescence (B and C), and its effects on swimming ability were measured by the sizes of the colonies (D).

$ExoR_{c20}$ directly, the longest form of $ExoR_{c20}$, with 188 amino acids, was fused to the 30-amino-acid ExoR signal peptide to generate SP-$ExoR_{c20}$ expressed from pHC567. When total proteins from the *exoR108-chvI109* mutant expressing SP-$ExoR_{c20}$ were probed with ExoR polyclonal antibodies, both SP-$ExoR_{c20}$ and $ExoR_{c20}$ were detected (Fig. 7A). This suggests that SP-$ExoR_{c20}$ is expressed, processed, and stably maintained without its signal peptide in the periplasm.

To test the function of $ExoR_{c20}$, the SP-$ExoR_{c20}$ protein was expressed in the wild-type strain Rm1021 and the *exoR95* mutant. The presence of the SP-$ExoR_{c20}$-expressing plasmid pHC567 did not change either the succinoglycan or the swimming phenotype for either wild-type Rm1021 or the *exoR95* mutant (Fig. 7C and D). These results, along with the finding that the wild-type ExoR protein was able to complement the phenotypes of the *exoR95* mutant (Fig. 2), suggest that the ExoR proteolysis product, $ExoR_{c20}$, does not function in regulating the production of succinoglycan or flagella.

ExoR proteolysis and regulatory functions are altered by point mutations. Our finding of functional $ExoR_m$ and nonfunctional $ExoR_{c20}$ coexisting in the periplasm raised the possibility that the amount of $ExoR_m$ is maintained at a certain level and that any changes to the level of $ExoR_m$ alter the function of the ExoR protein. To test this possibility, two highly conserved amino acids at positions 79 and 81 and one nonconserved amino acid at position 87 were changed individually to A (alanine) (Fig. 1). When these three mutant ExoR proteins, ExoRW79A, ExoRL81A, and ExoRY87A, were expressed from plasmids pHC571, pHC572, and pHC573, respectively, in the *exoR108-chvI109* double mutant, the level of the $ExoR_m$ form was significantly reduced for the ExoRL81A mutant and slightly reduced for the ExoRW79A mutant but not changed for the ExoRY87A mutant (Fig. 8A). This suggests that the L81A mutation may have reduced the amount of the $ExoR_m$ form of ExoR mutant protein in the cells.

The regulatory functions of the three mutated ExoR proteins were examined by assessing their abilities to complement the succinoglycan-producing (represented by calcofluor brightness) and nonswimming phenotypes of the *exoR95* mutant (Fig. 8C and D). Compared to the wild-type ExoR protein, the ExoRL81A protein was not able to complement either the succinoglycan-producing or nonswimming phenotype of the *exoR95* mutant, and it is therefore a loss-of-function mutation. Both the ExoRW79A and ExoRY87A proteins partially complemented both the succinoglycan-producing and nonswimming phenotypes of the *exoR95* mutant. ExoRY87A was more effective than ExoRW79A in complementing the succinoglycan-producing phenotype. These results suggest that both ExoRW79A and ExoRY87A are functional and that ExoRY87A is more effective than ExoRW79A but less effective than wild-type ExoR.

The combined biochemical and functional analyses of the three mutant ExoR proteins suggest that the level of $ExoR_m$ is linked to the regulatory function of the ExoR protein. This raises the possibility that ExoR proteolysis can be modulated by environmental or plant signals to regulate the production of succinoglycan, flagella, and many other cellular products required for symbiosis.

DISCUSSION

Recent publications have shown that the *S. meliloti* ExoR protein most likely functions as the repressor of the ExoS sensor, as part of the ExoR autoregulation pathway, which allows it to indirectly regulate the expression of a large number of genes required for host invasion and symbiosis (5, 33, 53). Both genetic and biochemical data appear to support the model that ExoR interacts with the ExoS periplasmic sensing domain directly to suppress ExoS, keeping it in an off state. It follows that ExoR suppression of ExoS would have to be relieved during nodulation so that the expression of the invasion and symbiosis genes could be turned on

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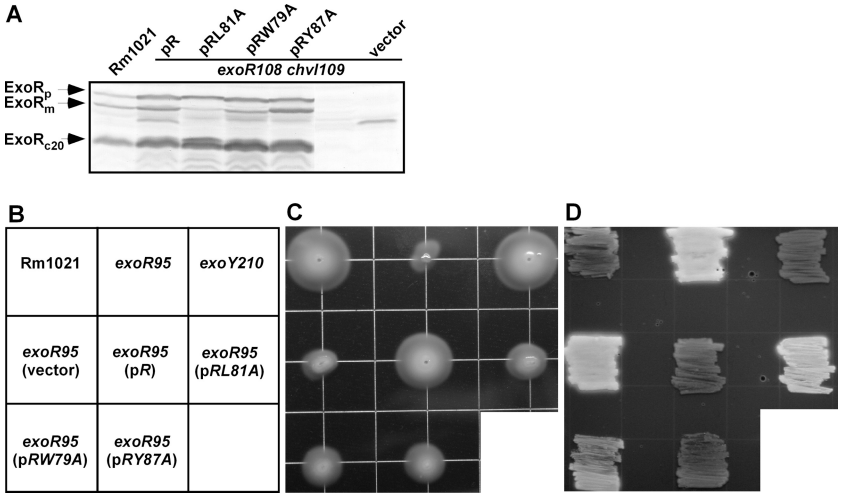

FIG 8 Biochemical and functional analysis of ExoR proteins with single-amino-acid mutations, ExoRL81A, ExoRW79A, and ExoRY87A, expressed from plasmids pHC571, pHC572, and pHC573, respectively, in the *exoR108-chvI109* mutant for Western blotting (A) and in the *exoR95* mutant for their effects on succinoglycan production (B and C) and swimming ability (D).

to support the nodulation. However, the molecular mechanism mediating the relief of ExoR suppression of ExoS is not clear.

One simple and attractive model for the relief of ExoR suppression is a reduction in the amount of ExoR protein in its mature and active form, ExoR$_m$, by changing it to an inactive form through proteolysis or modification. To monitor such changes in ExoR$_m$ on a Western blot, antibodies that can recognize the entire ExoR protein and an *S. meliloti* strain to provide the genetic background with no ExoR protein are required. While generating ExoR-specific polyclonal antibodies was relatively straightforward, engineering an *S. meliloti* strain with no ExoR proved to be challenging.

Two complementary approaches were followed to find a clean background for analysis of the ExoR protein. The first was to determine the status of ExoR in the original loss-of-function *exoR95* mutant. Our analysis showed that the mutated ExoR95 protein can be easily found in two forms, ExoR95$_p$ and ExoR95$_m$. This makes it more difficult to monitor the changes in wild-type ExoR protein, since these two forms are just slightly smaller than the wild type, as predicted based on the site of the *exoR95* mutation. The second approach, which was to generate an ExoR deletion mutation, turned out to be much more difficult. The only new *exoR* mutation generated from this effort was the *exoR108-chvI109* double mutant with a plasmid insertion in the *exoR* gene. The *exoR108* mutation can be transduced from the *exoR108-chvI109* double mutant into wild-type strain Rm1021(pHC510) expressing the *exoR* gene from plasmid pHC510, but not into Rm1021 alone. This suggests that the presence of a *chvI109* mutation is likely to be essential for the existence of the *exoR108* mutation in the *S. meliloti* genome. This led us to decide that even though the succinoglycan and flagellum production phenotypes of the *exoR108* mutant can be complemented by the wild-type *exoR* gene, the *exoR108-chvI109* mutant would only be used for biochemical analysis of the ExoR protein in this study.

Our comparative analysis of the ExoR protein profiles in a different genetic background led to the discovery of a new form of ExoR protein, ExoR$_{c20}$, while confirming the existence of the ExoR$_p$ and ExoR$_m$ forms. Two lines of evidence suggest that ExoR$_{c20}$ is the periplasmic proteolysis product of ExoR$_m$. The first is that when ExoR$_m$ was sequestered inside the cytoplasm by removing its signal peptide, no ExoR$_{c20}$ was detected. The second is the direct isolation of ExoR$_{c20}$ from the periplasm. While ExoR$_{c20}$ has a single C terminus, which is the same as that of wild-type ExoR, it appears to have multiple N termini. When a gel-purified single ExoR$_{c20}$ protein band was used for N terminus mapping, the results demonstrated N termini at positions 81, 84, 85, 86, and 87 of the ExoR ORF. These fragments may be produced by one or multiple enzymes in parallel or produced by one specific endoprotease digestion followed by multiple exoprotease digestions. While further analysis of these proteolysis products is important to identify the protease involved in ExoR$_m$ proteolysis, the more important finding of this study is the fact that ExoR$_m$ is the subject of proteolysis. This could serve as the molecular mechanism that reduces the active ExoR$_m$ and relieves suppression of the ExoS sensor.

The presence of stably maintained ExoR$_{c20}$ in wild-type Rm1021, but not in the loss-of-function *exoR95* mutant, raised the question of whether ExoR$_{c20}$ functions in the ExoR-ExoS/ChvI signal transduction pathway. To address this question, the longest form of ExoR$_{c20}$ was fused directly to the ExoR signal peptide and expressed in the wild-type Rm1021 and the *exoR95* mutant. The presence of ExoR$_{c20}$ in both strains was confirmed by Western blotting. The presence of ExoR$_{c20}$ did not alter the succinoglycan and swimming phenotypes of either the wild type or the *exoR95* mutant. This suggests that ExoR$_{c20}$ does not play any significant role in regulating succinoglycan or flagellum production. More importantly, it also suggests that digesting ExoR$_m$ to ExoR$_{c20}$ might function as a molecular mechanism regulating the amount of ExoR$_m$ in the periplasm.

For proteolysis of ExoR$_m$ to serve as a mechanism regulating the function of ExoR, modulation of the amount of ExoR$_m$ should change the regulatory function of ExoR. To test this hypothesis, the highly conserved amino acid leucine (L) at the site of proteolysis was changed to alanine (A), generating ExoRL81A. In addition, similar site-directed mutations were generated for another conserved amino acid, giving ExoRW79A, and a nonconserved

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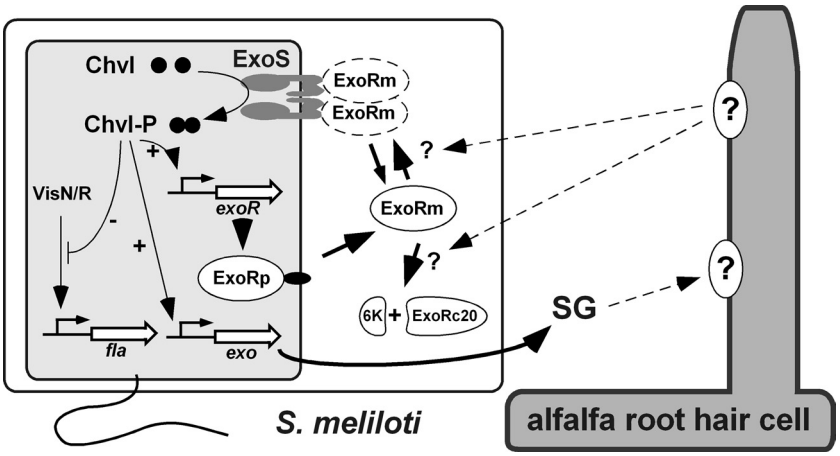

FIG 9 Model of the ExoR-ExoS/ChvI signal transduction pathway. It is hypothesized that the active mature form of ExoR, $ExoR_m$, is digested via slow proteolysis in wild-type cells. An increase in ExoR proteolysis triggered by environmental or plant signals or mutations in the *exoR* genes reduces the amount of $ExoR_m$ binding to the ExoS sensing domain, leading to the activation of ExoS sensor and the activation or suppression of expression of the genes regulated by the ExoS/ChvI two-component regulatory system.

amino acid, giving ExoRY87A. The mutation of the nonconserved amino acid 87 showed no detectable change in the levels of $ExoR_m$ protein or the regulatory function of ExoR. Compared to the wild-type ExoR, the ExoRW79A mutation of the conserved W (tryptophan) slightly decreased the $ExoR_m$ level and slightly reduced the regulatory function of ExoR. The ExoRL81A mutation, which changes the proteolysis site (amino acids 80 and 81), dramatically decreased the amount of $ExoR_m$ protein and completely abolished the regulatory function of the ExoR protein. Altogether, these findings link reduction of $ExoR_m$ and the regulatory function of the ExoR protein. In addition, our finding of a single-amino-acid change, the L81A mutation, enhancing $ExoR_m$ proteolysis raises the possibility that the ExoR proteolysis sequence is fine tuned for optimum control of proteolysis instead of efficient proteolysis. Better control of $ExoR_m$ proteolysis allows *S. meliloti* cells to sense and react more effectively to the presence of yet-to-be-identified host or environmental signals.

A model is proposed to integrate the new findings from this study with those from previous work (Fig. 9). We propose that the newly synthesized precursor form of ExoR, $ExoR_p$, is directed to the membrane and secreted into the periplasm without its signal peptide to form the mature $ExoR_m$. The amount of periplasmic $ExoR_m$ in free-living *S. meliloti* cells is kept in equilibrium by both interaction with ExoS and its steady proteolysis, forming $ExoR_{c20}$. This model is supported by previous results (5, 33, 53, 55) and our finding that the point mutation L81A reduces the level of $ExoR_m$ and disables the regulatory function of the ExoRL81A protein. The ExoR95 mutant protein might have an altered conformation so that it is resistant to proteolysis, and thus, no proteolytic product, $ExoR95_{c19}$, equivalent to $ExoR_{20}$, is detectable or the resulting $ExoR95_{c19}$ is not stable.

Our model predicts that plant or environmental signals would alter the rate of $ExoR_m$ proteolysis, thereby changing the level of ExoR suppression of ExoS. This would allow *S. meliloti* cells to modulate the production of succinoglycan and flagella, as well as the expression of many other ExoR-ExoS/ChvI pathway-regulated genes, to support the establishment of symbiosis in response to the presence of plant host or environmental signals. Based on this prediction, we are currently screening for conditions that can change the level of succinoglycan production by the wild-type Rm1021.

Close homologs of ExoR, as well as of ExoS and ChvI, of the ExoR-ExoS/ChvI signal transduction pathway have been found in the genomes of more than 40 different bacterial species. The regulatory mechanisms and the genes regulated by these systems are, with a few exceptions, unknown. When ExoR homologs are aligned to the *S. meliloti* ExoR protein in order of decreasing overall homology from 93% to 50%, the levels of homology around the region of proteolysis remain unchanged. The high levels of conservation of the protein sequence around the $ExoR_m$ proteolysis region raise the possibility that ExoR proteolysis is a common molecular mechanism mediating bacterial sensing in the presence of their hosts or of changes in their environment.

Bacterial periplasmic proteases have been shown to participate in bacterial sensing of environmental signals in several systems. An *E. coli* membrane-bound protease, DegS, which is activated by unassembled outer membrane porins, cleaves the periplasmic domain of the membrane-anchored regulator RseA (1, 49). This triggers further cleavage of RseA by a metalloprotease, YaeL, to release σ^E, which normally attaches to RseA (49). The free σ^E turns on the expression of stress-related genes (49). In the case of a polarity determinant of *Caulobacter*, PodJ, the periplasmic domain of PodJ is also regulated through its cleavage into small fragments by a periplasmic protease, PerP (6). The example that is closest to *S. meliloti* ExoR is the function and regulation of the *E. coli* periplasmic adaptor protein, CpxP, which is involved in sensing pH variations to regulate membrane lipid composition (9, 26, 40, 54). The CpxP protein interacts with and inhibits the periplasmic sensing domain of the CpxA protein, the sensor of the CpxA/CpxR two-component system (14, 46, 54, 56). The periplasmic serine protease DegP is activated by general envelope disruptions, including pH changes, and cleaves the CpxP protein, thereby removing CpxP from the CpxA sensor (26). This results in the activation of the CpxA sensor and the expression of its regulated genes (26, 54). Our finding that ExoR is the subject of proteolysis in the periplasm suggests a regulatory role similar to those of RseA, PodJ, and CpxP.

While it has been well documented that ExoR, ExoS, and ChvI

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play essential roles in symbiosis, it is not known what environmental signals are transmitted through the ExoR-ExoS/ChvI signal transduction pathway. The factors that might function upstream of ExoR are also unknown. Our finding of ExoR proteolysis in the periplasm suggests a protease(s) as a key factor upstream of ExoR. The proteolysis of ExoR might be regulated by other proteins in the periplasm through protein-protein interactions or by other enzymes that modify ExoR. These possibilities will be further investigated to gain more insight into the regulation of ExoR and the function of the ExoR-ExoS/ChvI signal transduction pathway. The results of our analysis of ExoR will no doubt be helpful in understanding and combating the pathogenicities of *A. tumefaciens* and *B. abortus*, as well as many other host-interacting bacteria that rely on homologs of the ExoR-ExoS/ChvI signal transduction pathway.

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