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Rhizobium meliloti exoG and *exoJ* Mutations Affect the ExoX-ExoY System for Modulation of Exopolysaccharide Production

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R. meliloti **Rm1021 normally produces an acidic Calcofluor-binding exopolysaccharide, called succinoglycan or EPS I, which is required for successful nodulation of alfalfa by this strain. At least 13 loci affecting production of EPS I were previously mapped to a cluster on the second of two symbiotic megaplasmids in Rm1021, pRmeSU47b. A putative regulatory region was originally defined by the *exoG* and *exoJ* mutations. *exoG* and *exoJ* mutants produced less exopolysaccharide than wild-type strains and induced nitrogen-fixing nodules on alfalfa with reduced efficiency compared with the wild type. These mutants appeared to produce only a low-molecular-weight form of EPS I. Mutations called *exoX* cause an increase in exopolysaccharide production and map in the same region as the *exoG* and *exoJ* mutations. The DNA sequence of this region reveals that it contains two open reading frames, called *exoX* and *exoY*, which have homologs in other *Rhizobium* species. Interestingly, the *exoG* insertion mutations fall in an intergenic region and may affect the expression of *exoX* or *exoY*. The *exoJ* mutation falls in the 3′ portion of the *exoX* open reading frame and is probably an allele of *exoX* that results in altered function. *exoG* and *exoJ* mutations limit EPS I production in the presence of *exoR95* or *exoS96* mutations, which cause overproduction of EPS I. Gene regulation studies suggest that ExoX and ExoY constitute a system that modulates exopolysaccharide synthesis at a posttranslational level. The deduced sequence of ExoY is homologous to a protein required for an early step in xanthan gum biosynthesis, further suggesting that the modulatory system may affect the exopolysaccharide biosynthetic apparatus.**

The symbiotic bacterium *Rhizobium meliloti* induces the formation of nodules on the roots of alfalfa plants, invades these nodules, and fixes nitrogen once inside (29, 30, 40, 43). *R. meliloti* Rm1021 synthesizes an acidic exopolysaccharide, called succinoglycan or EPS I (1), which can be stained with the fluorescent laundry whitener Calcofluor. Mutants that fail to make EPS I have been identified by their lack of fluorescence under UV light when grown on Calcofluor-containing agar plates. These mutants were deficient in invasion of alfalfa nodules they induced, showing that EPS I is required for the invasion of alfalfa nodules by this strain (16, 27, 32).

EPS I consists of a repeating polymer of an octameric subunit composed of seven glucose residues, one galactose residue, and succinyl, acetyl, and 1-carboxyethylidene (pyruvate) modifications (1). A number of genes necessary for its synthesis have been identified and include *exoB*, encoding UDP-galactose epimerase (8, 9), *exoC*, encoding glucose-6-phosphate isomerase (48), and a number of other *exo* genes. All of these lie in a cluster of *exo* genes on the second symbiotic megaplasmid, pRmSU47b, except for *exoC*, which maps to the chromosome (14, 17, 32). Mutations in the *exoA*, *exoB*, *exoF*, *exoL*, *exoM*, *exoP*, *exoQ*, *exoT*, or *exoY* genes abolish EPS I production (16, 27, 32, 39, 51), while mutations in the *exoH* gene cause the bacteria to produce EPS I lacking succinyl modifications (26). In addition, a number of mutations mapping in the *exo* cluster cause increased or decreased EPS I production (22, 23, 32, 51). These include the *exoG*, *exoJ*, *exoK*, and *exoN* mutations which cause decreased EPS I production (32), and *exoX* mutations which cause increased EPS I production (51). Mutations possibly corresponding to the *exoJ* and *exoX* mutations have also been reported by others (22, 23). In multiple copies, the *exoX* gene causes decreased EPS I production (51), and *exoX* has been postulated to be a regulatory gene. Multiple copies of the *exoY* gene, which maps close to *exoX*, can compensate for the decrease in EPS I synthesis caused by multiple copies of *exoX*, and on this basis *exoY* has been postulated to be part of the same putative regulatory system as *exoX* (19, 51).

Chromosomal mutations that affect the amount of EPS I produced have also been reported. Mutations in *exoR* and *exoS* cause overproduction of EPS I (13), an effect mediated at the level of gene expression (36, 39). Mutations in the *exoD* gene result in increased or decreased EPS I production depending on culture conditions (25, 37). However, the effect of *exoD* mutations on EPS I production appears to be indirect (37).

We previously reported that strains carrying the *exoG* or *exoJ* Tn*5* insertion mutations fluoresce only slightly on plates containing Calcofluor and produce lowered amounts of exopolysaccharide, predominantly of low molecular weight (32). In addition, they invade alfalfa nodules with reduced efficiency (32). In the present work, we describe additional Tn*5*-generated mutations which map close to the *exoG* and *exoJ* insertions and, in contrast to the *exoG* and *exoJ* mutations, cause overproduction of EPS I. DNA sequencing reveals that this region includes the *R. meliloti exoX* and *exoY* genes. These show interesting homologies to other bacterial genes involved in the synthesis of polysaccharides. The Tn*5* insertions in this region that affect EPS I production fall in the *exoX* gene or in an intergenic region between the *exoX* and *exoY* genes.

Finally, we report experiments aimed at gaining an under-

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TABLE 1. *Rhizobium meliloti* strains

Strain	Genotype	Source or reference
Rm1021	SU47 Sm^r	35
Rm8002	Rm1021 *pho-1*	18
Rm8264	Rm8002 *exoA264*::Tn*phoA*	39
Rm8265	Rm8002 *exoF265*::Tn*phoA*	39
Rm8271	Rm8002 *exoP271*::Tn*phoA*	39
Rm8274	Rm8002 *exoT274*::Tn*phoA*	39
Rm8302	Rm1021 *exoG302*::Tn*5*	32
Rm8319	Rm1021 *exoX(J)319*::Tn*5*	32
Rm8321	Rm1021 *exoG321*::Tn*5*	32
Rm8330	Rm1021 *exoX330*::Tn*5*	32
Rm8350	Rm1021 Ω-*350*::Tn*5*	32
Rm8357	Rm1021 Ω-*357*::Tn*5*	32
Rm8363	Rm1021 *exoX363*::Tn*5*	This work
Rm8385	Rm8002 *fix-385*::Tn*phoA*	31
Rm8395	Rm1021 *exoR95*::Tn*5-233*	13
Rm8396	Rm1021 *exoS96*::Tn*5-233*	13
Rm8552	*exoG302*::Tn*5* *exoR95*::Tn*5-233*	This work
Rm8553	*exoX(J)319*::Tn*5* *exoR95*::Tn*5-233*	This work
Rm8554	*exoN416*::Tn*5* *exoR95*::Tn*5-233*	This work
Rm8555	*exoX363*::Tn*5* *exoR95*::Tn*5-233*	This work
Rm8556	*exoG302*::Tn*5* *exoS96*::Tn*5-233*	This work
Rm8557	*exoX(J)319*::Tn*5* *exoS96*::Tn*5-233*	This work
Rm8558	*exoN416*::Tn*5* *exoS96*::Tn*5-233*	This work
Rm8559	*exoX363*::Tn*5* *exoS96*::Tn*5-233*	This work

standing of the behavior of this EPS I-modulatory system. We find that multiple copies of *exoX* cause a decrease in the amount of EPS I produced, as previously reported (51), and provide evidence that this modulation occurs at a posttranslational level. In addition, we have investigated the interactions of *exoG*, *exoJ*, and *exoX* mutations with mutations in *exoR* and *exoS*.

MATERIALS AND METHODS

Strains and media. Bacterial strains are listed in Table 1. Bacteria were grown in Luria-Bertani medium (33), with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB-MC) added for *R. meliloti* cultures. Nitrogen-free M9 medium for exopolysaccharide production was as previously described (26). The following antibiotics were used at the indicated concentrations: for *R. meliloti*, gentamicin (20 μg/ml), neomycin (200 μg/ml), spectinomycin (100 μg/ml), streptomycin (500 μg/ml), and tetracycline (10 μg/ml); for *Escherichia coli*, gentamicin (5 μg/ml), kanamycin (20 μg/ml), tetracycline (10 μg/ml), spectinomycin (100 μg/ml), and chloramphenicol (20 μg/ml).

Genetic techniques. Plasmid matings, transductions, and transposon replacements were as previously described (11, 12, 15, 21, 42). Insertion 363 was transferred to cosmid pD56 by mating pD56 from strain Rm8363 into *E. coli* HB101 and selecting for cotransfer of the cosmid Tc^r and transposon Km^r markers.

Exopolysaccharide detection. Insertion mutants were screened on Luria-Bertani agar plates containing 0.02% Calcofluor White M2R (Cellufluor, Polysciences, Warrington, Pa.) buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), using a hand-held long-wavelength UV lamp. Exopolysaccharide production in liquid medium was assayed by resuspending bacteria in M9 medium with or without ammonia and containing 1% mannitol as a carbon source to an optical density at 600 nm of approximately 0.5. Cells were incubated at 30°C for 60 to 75 h, and exopolysaccharide was assayed by the anthrone method (28). High- and low-molecular-weight EPS fractions of culture supernatants were from 1-week-old cultures grown in M9–1% mannitol–0.1% glutamate. The fractions were separated by rotary ultrafiltration with a Benchmark filtration system and an MX-100 filter (Membrex, Inc., Garfield, N.J.). ^{1}H-nuclear magnetic resonance (NMR) spectra were obtained as described previously (37) with a 500-MHz NMR machine operated by the Francis Bitter National Magnet Lab at the Massachusetts Institute of Technology.

Plant nodulation tests. *R. meliloti* strains were screened for their nodulation phenotypes on *Medicago sativa* cv. Iroquois seedlings as described by Leigh et al. (27).

Subcloning and sequencing. We previously described the subclone pEX80, containing an 8.0-kb *Eco*RI fragment of *R. meliloti* DNA from the part of the *exo* region contained on cosmid pD56 (27, 32). We subcloned a 4.5-kb *Cla*I fragment from pEX80 covering the *exoG*, *exoJ*, *exoF*, and *exoQ* insertions into the *Pst*I site of the broad-host-range vector pRK404 (12) to make plasmid pEX458. The same 4.5-kb fragment was inserted into plasmid Bluescript KS$^+$ (Stratagene, San Diego, Calif.) to make pEX45. pEX45 was cut with *Hin*dIII and religated to make plasmid pEX14, containing a 1.6-kb *Cla*I-*Hin*dIII fragment in Bluescript KS$^+$. This plasmid was used to construct nested sequencing deletions (see below). The 1.6-kb *Cla*I-*Hin*dIII fragment was cloned into the *Pst*I site of pRK404 to make pEX146. The 2.7-kb *Cla*I-*Bgl*II genomic fragment was subcloned by cloning the equivalent 2.7-kb *Bam*HI-*Bgl*II fragment from pEX458 into the *Bam*HI site of pRK404 to make plasmid pEX270. Nested unidirectional sequencing deletions were made by using exonuclease III and exonuclease VII according to the suppliers' instructions. Inserts of selected sequencing deletion plasmids were cut out of Bluescript KS$^+$ by digesting with *Sac*I-*Kpn*I and recloned into the *Pst*I site of pRK404. For sequencing Tn*5* insertion junctions, fragments extending from the *Bgl*II site in Tn*5* to the *Bgl*II site at the end of pEX270 were cloned from the pD56::Tn*5* derivative of each mutation into the *Bam*HI site of Bluescript SK$^+$ (Stratagene). We failed to obtain such a clone for the *exoJ319* insertion, so as a sequencing template we used a 0.6-kb DNA fragment that included the rightward Tn*5* junction of insertion *exoJ319*, obtained by polymerase chain reaction amplification of DNA from strain Rm8319 (34). All of these junctions were sequenced by using an oligodeoxynucleotide primer complementary to the inverted repeat of Tn*5*. In cases where fragment ends were not complementary, ends were blunted with T4 DNA polymerase or the Klenow fragment of DNA polymerase I prior to ligation.

Nucleotide sequence accession number. The sequences reported here have been submitted to GenBank and have the accession number M61751.

RESULTS

Mutations in the *exoG-J* region. We have previously described the Tn*5*-generated mutations *exoG302*, *exoG321*, and *exoJ319*, which cause reduced fluorescence on plates containing Calcofluor and are complemented by the previously described cosmid pD56 (27, 32) (Fig. 1). In a screen for random Tn*5* insertions conferring altered phenotypes on Calcofluor plates, we isolated one, *exo-363*, which caused especially bright fluorescence. When cosmid pD56 was introduced into strain Rm8363, which carries insertion *exo-363*, the resulting strain fluoresced about as much as the

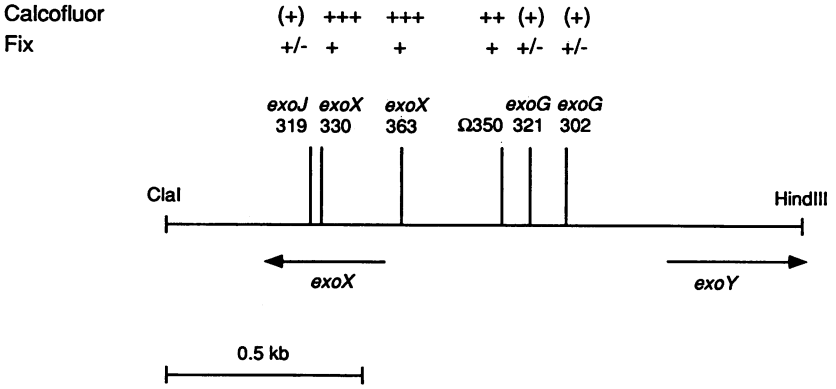

FIG. 1. Tn*5* insertion mutations in the *exoG-J* region. Positions are based on sequencing the rightward Tn*5* insertion junctions (Fig. 4). *exoX* and *exoY* open reading frames (Fig. 4) are indicated by arrows. Formation of nitrogen-fixing nodules (+) or non-nitrogen-fixing nodules (−) on alfalfa and degree of fluorescence on Calcofluor plates are indicated. For Calcofluor fluorescence, ++ indicates wild-type fluorescence, +++ indicates especially bright fluorescence, and + indicates dim fluorescence. The *Cla*I-*Hin*dIII fragment indicated is cloned in plasmid pEX146 (Fig. 3). All of these insertions were reported previously (32), except for insertion *exoX363*. *Eco*RI restriction sites (32) are located 2.8 kb to the left of the *Cla*I site shown, and 3.3 kb to the right of the *Hin*dIII site.

wild-type strain, suggesting that *exo-363* was located in the DNA corresponding to that contained on this cosmid. Southern hybridization analyses confirmed this supposition (data not shown). In addition, we discovered that a strain carrying the Tn*5* insertion Ω330, which maps close to insertion *exoJ319* (32), appears to fluoresce more than the wild-type strain on Calcofluor plates, similarly to the strain carrying insertion *exo-363*. Previously, we had described this strain as having wild-type fluorescence (32). In light of our reexamination of this strain, we have renamed the insertion in it *exo-330*. A map of the region in which these insertions lie is shown in Fig. 1. The *exo-330* insertion falls slightly to the right of the *exoJ319* insertion. The *exo-363* insertion mutation falls between the *exo-330* insertion on the left and insertion Ω350 (which has no phenotype) on the right. The two *exoG* insertions map farther to the right (Fig. 1). All of these insertions fall within about 1 kb of each other.

Previously, we reported that strains carrying either *exoG* insertion invaded alfalfa nodules with reduced efficiency, and that the *exoJ319* insertion caused still less efficient nodule invasion. Other previously isolated insertions in this region, including *exo-330*, had no effect on nodulation. We have also tested the effect on nodulation of *exo-363*. We found that, in contrast to strains carrying *exoG* or *exoJ* insertions, and similarly to the *exo-330* mutant, strains carrying the *exo-363* insertion mutation nodulated alfalfa plants effectively and efficiently.

In an attempt to detect any anomalies in the exopolysaccharide produced by strains carrying insertions in this region, we analyzed high- and low-molecular-weight polysaccharide fractions from supernatants of nitrogen-limited cultures of strains carrying *exo-363*, *exoJ319*, or *exoG321* mutations. To simplify analysis of low-molecular-weight material, we used strains also mutant in the *ndvB* locus, required for production of a cyclic β-(1,2)-glucan. High- and low-molecular-weight fractions were separated by rotary ultrafiltration (see Materials and Methods), and low-molecular-weight fractions were further purified by passage over a P4 gel filtration column.

The *exo-363* strain, Rm8363, produced roughly 3.5 times as much anthrone-positive material as its wild-type parent, Rm1021. At least 86% of this material was of high molecular weight, as judged by precipitability with 2 volumes of ethanol. An ^{1}H-NMR spectrum of this material was identical to that of EPS I of normal structure (Fig. 2). We did not analyze low-molecular-weight material from supernatants of strain Rm8363 because we were unable to separate sufficient quantities of low-molecular-weight material from contaminating high-molecular-weight material. As we reported previously (32), the *exoJ319* strain produced a small amount of high-molecular-weight anthrone-positive material, about 1%

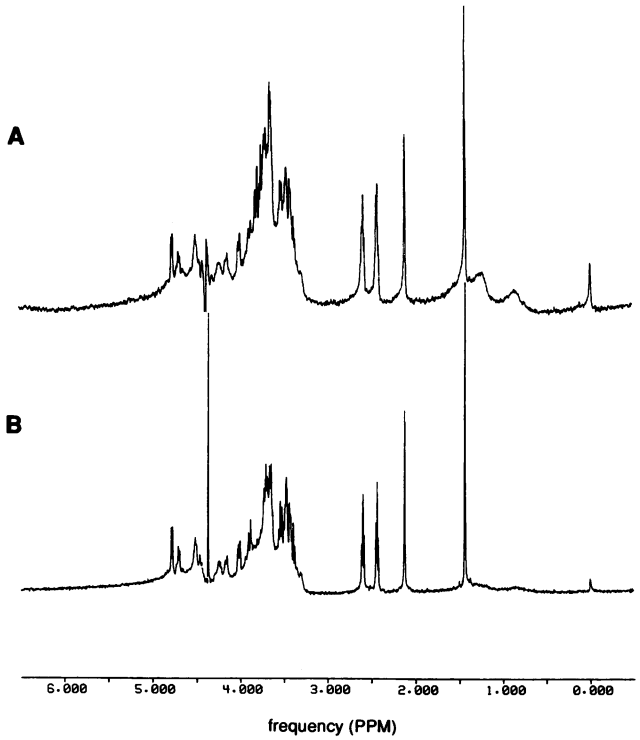

FIG. 2. ^{1}H-NMR spectra of high-molecular-weight EPS I from wild-type strain Rm1021 (A) and strain Rm8363 (*exoX363*) (B).

TABLE 2. Calcofluor fluorescence phenotypes of merodiploids carrying insertions in the *exoG-J* region

Cosmid	Relative fluorescence[a] of strain						
	Rm1021 (wild type)	Rm8319 *exoJ319*	Rm8330 *exoX330*	Rm8363 *exoX363*	Rm8350 Ω-*350*	Rm8321 *exoG321*	Rm8302 *exoG302*
pLAFR1	++	−	++++	++++	+	−	−
pD56	+++	++	+++	++	+++	+++	+++
pD56 *exoJ319*	+++	++	+++	+++	+++	+++	+++
pD56 *exoX330*	++++	++++	++++	++++	++++	++++	++++
pD56 *exoX363*	++++	++++	++++	++++	++++	++++	++++
pD56 *exoG321*	+	+	++	+	+	+	+

[a] Key: −, no fluorescence on Calcofluor plates; +, dim fluorescence; ++ or +++, bright fluorescence; ++++, very bright fluorescence and a slightly mucoid phenotype. Some of these complementations were also done previously (32).

as much as the isogenic *exo*$^+$ strain. In addition, we were able to isolate similarly low amounts of high-molecular-weight material from the *exoG321* strain and from an *exoA32* strain. The anthrone-positive material in these fractions was not EPS I, however, since the ^{1}H-NMR spectra of these fractions did not contain peaks corresponding to the characteristic acetyl, succinyl, or 1-carboxyethylidene substituents of the wild-type exopolysaccharide (data not shown). These findings suggest that the *exoJ319* insertion as well as the *exoG321* insertion eliminate production of high-molecular-weight EPS I. Preliminary analysis of low-molecular-weight samples from both the *exoJ319* and the *exoG321* strains have indicated that they consist of a mixture containing material related to EPS I, since the ^{1}H-NMR spectra of these fractions had upfield peaks characteristic of the EPS I substituents, in addition to peaks from contaminating material (data not shown). We have not identified the non-EPS I material also present in these fractions.

Genetics of the *exoG-J* region. The *exoG* and *exoJ* insertions behave anomalously in complementation tests (32). First, both *exoG* insertions were partially dominant when present on a cosmid. Thus, a wild-type strain containing a derivative of cosmid pD56 carrying either the *exoG302*::Tn*5* or the *exoG321*::Tn*5* mutation was less bright on Calcofluor plates than the wild-type strain carrying the vector pLAFR1. On the other hand, the genomic (single-copy) versions of these insertion mutations were recessive to the wild-type DNA on cosmid pD56. Second, the introduction of a derivative of pD56 carrying the *exoJ319* insertion into an *exoJ319* strain had the anomalous effect of increasing the fluorescence of this strain on Calcofluor plates to the level of the wild-type strain (Table 2). Insertion mutation *exoJ319* thus appeared to complement itself in merodiploids. Finally, whereas a derivative of pD56 carrying insertion *exoJ319* was able to restore wild-type fluorescence to *exoG302* or *exoG321* strains, derivatives of pD56 carrying either *exoG* mutation failed to complement the dim fluorescence of an *exoJ319* strain. Thus, neither the *exoG*::Tn*5* insertion mutations nor the *exoJ*::Tn*5* insertion mutation behaved as would be expected of recessive mutant alleles of a gene.

To assess whether the *exo-330* or *exo-363* mutations might also exhibit partial dominance or anomalous complementation, we introduced derivatives of pD56 carrying these mutations into the wild-type strain Rm1021 and the other strains carrying Tn*5* insertion mutations in the region. For this purpose, insertion *exo-363* was crossed onto cosmid pD56 as described in Materials and Methods. In addition, we introduced derivatives of pD56 carrying mutations *exoG321* and *exoJ319* into *exo-330* and *exo-363* mutants. The fluorescence of the resulting strains on Calcofluor plates is summarized in Table 2. When plated on Calcofluor, the wild-type strain containing the pD56 *exo-330* cosmid or the pD56 *exo-363* cosmid fluoresced more than the wild-type strain containing cosmid pD56. Insertions *exo-330* and *exo-363* therefore behave similarly to the two *exoG* insertions in that they are dominant when present at a slightly elevated copy number but recessive when in single copy. In complementation tests, insertions *exo-330* and *exo-363* behaved as a distinct locus, being unable to complement themselves or each other for wild-type fluorescence on Calcofluor and being complemented by cosmids carrying each of the other insertions (Table 2). As elaborated below, insertions *exo-330* and *exo-363* fall in a gene denoted *exoX*, and we therefore refer to these insertions henceforth as *exoX330* and *exoX363*.

The variety of phenotypes conferred by Tn*5* insertions in this relatively small region, as well as the partial dominance of some of these insertions, suggested that the region might contain regulatory elements. In addition, our failure to isolate high-molecular-weight EPS I from *exoG* and *exoJ* mutants suggested that the mutations in these strains might specifically affect production of the high-molecular-weight form of EPS I. Either model might explain the decreased nodule invasion efficiency of strains carrying the *exoG* and *exoJ* insertions. Subsequent experiments were designed to investigate the molecular nature of the Tn*5* insertions so as to understand the basis of their effects.

DNA from the *exoG-J* region causes decreased EPS I production when present in multiple copies. In order to clarify the arrangement of potential genes in this region, we first constructed various subclones of the *exoG-J* region and tested these for their ability to complement the Calcofluor phenotypes of *exoG*, *exoJ*, and *exoX* strains. First, we subcloned a 4.5-kb *Cla*I fragment spanning all of these insertions into the broad-host-range vector pRK404 to make the plasmid pEX458. This plasmid complemented the mutant Calcofluor phenotypes of strains carrying the *exoG302*, *exoG321*, *exoJ319*, and *exoX363* insertions (Table 3), as well as the Calcofluor-dark phenotype of mutant *exoF55* (26, 32; data not shown). Similarly, plasmid pEX270, containing a 2.7-kb *Cla*I-*Bgl*II fragment, complemented these same *exoG*, *exoJ*, and *exoX* mutations (Table 3 and Fig. 3). We then subcloned a 1.6-kb *Cla*I-*Hin*dIII fragment, also spanning all of the *exoG* and *exoJ* insertions, to make the plasmid pEX146 (Fig. 3). Curiously, when we introduced this subclone into strains carrying Tn*5* insertions in this region, we found that it caused all strains to fluoresce less on Calcofluor plates. Thus, *exoG* and *exoJ* strains lost fluorescence entirely, and the *exoX363* strain exhibited greatly reduced fluorescence (Table 3).

Moreover, when we introduced plasmid pEX146 into the

TABLE 3. Effects of subclones on Calcofluor fluorescence of various strains

Plasmid	Relative fluorescence[a] of strain				
	Rm1021 (wild type)	Rm8319 *exoJ319*	Rm8363 *exoX363*	Rm8321 *exoG321*	Rm8302 *exoG302*
pRK404	++	−	++++	−	−
pEX458	+++	+++	+++	+++	+++
pEX270	+++	+++	+++	+++	+++
pEX146	−	−	−/+	−	−
pEX119	−/+	−	+	−	−
pEX126	++	−	++++	−	−
pEX127	++	−	++++	−/+	−
pEX426	−	−	−/+	−	−
pEX427	−	−	−/+	−	−

[a] Number of + signs indicates relative fluorescence on Calcofluor plates, as described in Table 2, footnote *a*. − indicates no fluorescence, and −/+ indicates very slight fluorescence. Plasmids are described in Fig. 3.

wild-type parent strain Rm1021, it also exhibited greatly reduced fluorescence on Calcofluor plates (Fig. 3). In addition, we introduced plasmid pEX146 into previously described EPS I-overproducing strains carrying mutation *exoR95* or *exoS96* (13). In these backgrounds as well, pEX146 caused greatly reduced fluorescence on Calcofluor plates (data not shown). We tested whether this reduction of fluorescence reflected a decrease in exopolysaccharide production by measuring anthrone reactive material in supernatants of nitrogen-starved liquid cultures. We found that plasmid pEX146 caused the wild-type strain to produce roughly one-fourth as much exopolysaccharide as the wild-type strain carrying the vector plasmid (data not shown).

We constructed more subclones of this region in the course of sequencing the 1.6-kb *Cla*I-*Hin*dIII fragment from plasmid pEX146 (see below). As part of our sequencing strategy, we made a number of deletions of this fragment from each end. We subcloned some of these deletions into the broad-host-range vector pRK404 and tested the effect of the resulting constructs on the Calcofluor fluorescence of the wild-type strain and of the *exoG*, *exoJ*, and *exoX* strains. As shown in Table 3 and Fig. 3 and 4, plasmids pEX426 and pEX427, carrying 916 and 1,201 nucleotides of the left end of the 1.6-kb fragment, caused decreased fluorescence in all strains just as plasmid pEX146 did. The rightward boundary of the activity that causes decreased EPS I production must therefore lie to the left of nucleotide 916. Deletions from the other direction helped localize the leftward boundary. Plasmid pEX119, missing 367 nucleotides from the left end of the 1.6-kb *Cla*I-*Hin*dIII fragment, caused decreased fluorescence. However, the plasmids pEX127 and pEX126, which were shortened by 456 and 596 nucleotides relative to plasmid pEX146 (Fig. 3 and 4), appeared to have no effect on the Calcofluor fluorescence of any strain (Table 3). It thus appears that the left boundary of the activity that causes decreased exopolysaccharide production falls between nucleotides 367 and 456 of the 1.6-kb *Cla*I-*Hin*dIII fragment.

We were interested in the mechanism by which plasmid pEX146 decreased exopolysaccharide synthesis. We therefore tested the level of expression of four gene fusions of *exo* genes to *phoA*, the gene encoding alkaline phosphatase, in the presence of several plasmids. Introduction of the *exoR95* or *exoS96* mutations into strains containing these fusions has previously been shown to lead to increased alkaline phosphatase synthesis (39). In addition to plasmid pEX146, we used the vector pRK404 and plasmid pEX458, which contains all of the DNA sequences present on pEX146 as well as

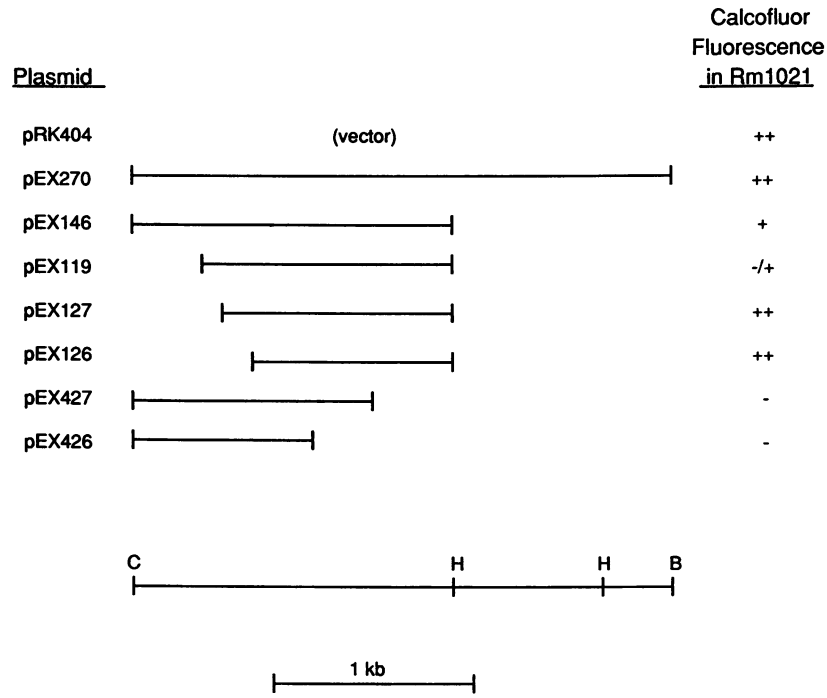

FIG. 3. Plasmid subclones of the *exoG-J* region and their effects on Calcofluor fluorescence of the wild-type strain Rm1021. DNA cloned in plasmid pEX458 (not shown) extends another 1.8 kb to the right of the *Bgl*II site. Restriction sites: C, *Cla*I; H, *Hin*dIII; B, *Bgl*II. Calcofluor fluorescence: −, no fluorescence; +, intermediate fluorescence; ++, wild-type fluorescence.

TABLE 4. Effect of pEX146 on expression of *exo*::Tn*phoA* fusions

Strain	Alkaline phosphatase activity from plasmid[a]		
	pRK404	pEX458	pEX146
Rm8002 (*pho-1*)	1.0	3.6	3.9
Rm8385 (*fix-385*::Tn*phoA*)[b]	16	12	14
Rm8265 (*exoF*::Tn*phoA*)	47	40	35
Rm8274 (*exoT*::Tn*phoA*)	8.8	9.2	7.4
Rm8271 (*exoP*::Tn*phoA*)	19	18	15
Rm8264 (*exoA*::Tn*phoA*)	20	16	15

[a] Strains were grown in Luria-Bertani LB-MC, and alkaline phosphatase assays were performed as described previously (39). Values are expressed as [(A_{420} from alkaline phosphatase assay) × 1,000]/[(optical density at 600 nm of culture prior to harvesting) × (duration of assay in minutes)] and are means from two assays per strain. Plasmids are described in the text.

[b] This strain, included as a control, contains a Tn*phoA*-generated fusion to an uncharacterized gene.

an extra 2.8 kb of sequences to the right of those cloned in plasmid pEX146 (see above). Whereas pEX146 causes a decrease in Calcofluor fluorescence and EPS I production, plasmids pRK404 and pEX458 have no effect on Calcofluor fluorescence. Neither pEX146 nor pEX458 had a significant effect on the levels of expression of these translational fusions compared with their expression levels in the presence of the vector pRK404 (Table 4). The slight effects seen would seem to be insufficient to explain the more substantial drop in exopolysaccharide production caused by plasmid pEX146. It therefore seems likely that plasmid pEX146 affects exopolysaccharide synthesis at a posttranslational level.

The *exoG-J* region does not affect EPS II synthesis. *R. meliloti* Rm1021 has a cryptic capacity to produce a second exopolysaccharide called EPS II, distinct in structure from EPS I (18). In order to determine whether the effect of plasmid pEX146 on exopolysaccharide synthesis was specific to EPS I, we tested its effect on production of EPS II. We introduced pEX146 into an *exoA32 expR101* strain, which produces EPS II but is blocked for EPS I synthesis by the *exoA32* mutation. We found that the *exoA32 expR101* strain produced the same amount of exopolysaccharide when it carried pEX146 as when it carried the vector pRK404 or the larger plasmid pEX458 (data not shown). This result establishes that pEX146 does not affect the level of EPS II production.

We also obtained genetic evidence that the mutations in this region do not affect EPS II function as they do EPS I. EPS II has been found to be able to substitute for EPS I in nodulation. Thus, the nodule invasion defect of an *exoA* strain, which does not make EPS I, is suppressed by the *expR101* mutation, which causes production of EPS II (18). Since *exoG* and *exoJ* strains exhibit reduced nodulation efficiency compared with the wild-type strain, we could test whether these mutations affect EPS II function in invasion by assessing the ability of the *expR101* mutation to suppress the reduced nodule invasion phenotype caused by *exoG* and *exoJ* mutations. We found that *exoG302 expR101* and *exoJ319 expR101* strains were able to nodulate alfalfa plants with the same efficiency as an *exoA32 expR101* strain (data not shown). This result supports the notion that the region in which these mutations lie is important specifically for the synthesis and symbiotic role of EPS I but not of EPS II.

Sequence of the *exoG-J* region. We sequenced the 1.6-kb *Cla*I-*Hin*dIII fragment spanning the insertion mutations. The sequence is shown in Fig. 4. In the whole length of the sequence, we found just two plausible open reading frames. The first starts at nucleotide 557 and reads leftward for 98 codons to nucleotide 261. The second starts at nucleotide 1273 and continues rightward for 131 codons to the *Hin*dIII site at the end of the fragment. The second open reading frame has another potential ATG initiation codon at position 1327, which would make the open reading frame to the *Hin*dIII site 113 codons long. As described below, we refer to the first and second open reading frames as *exoX* and *exoY*, respectively. Potential ribosome-binding sites are present between 9 and 17 nucleotides upstream of the start of the *exoX* open reading frame and between 9 and 12 nucleotides upstream of the second ATG codon of the *exoY* open reading frame (Fig. 4). On the basis of this potential ribosome-binding site, we have tentatively assigned the second ATG in the *exoY* open reading frame as its probable start codon.

When we searched the GenBank data base with this sequence, we found that the entire region was highly homologous to sequences encoding exopolysaccharide regulatory genes from the broad-host-range *Rhizobium* sp. strain NGR234. This sequence, similarly to ours, contained a short (96-amino-acid) open reading frame reading from right to left, called *exoX*. To the right of *exoX*, a region without obvious open reading frames stretches for 765 nucleotides. Finally, two more open reading frames extend from left to right, at the right end of the sequenced DNA. The first is 226 amino acids long and has been named *exoY*. The second is at least 321 amino acids long and has provisionally been called ORF1 (19). We found that the deduced amino acid sequence of our leftmost open reading frame, *exoX*, is 74% identical to the 96 amino acids of NGR234 *exoX* (Fig. 5). The 113-amino-acid deduced sequence of our incomplete *exoY* open reading frame at the right end of the sequence is 79% identical to the first 113 amino acids of the *exoY* sequence from NGR234 (Fig. 6). Finally, the intergenic region is also highly homologous, showing 60% DNA sequence identity over its length. This homology is particularly strong in the putative promoter regions identified by Gray et al. (19) (Fig. 4) but includes other segments as well. Interestingly, the segments of the intergenic region that are most conserved between the two species all appear to contain AT-rich stretches (data not shown).

Our data base search also revealed that our deduced *exoX* sequence was 19% identical over 86 amino acids to the *psi* gene of *Rhizobium leguminosarum* bv. phaseoli (Fig. 5) (5). Furthermore, our *exoY* N-terminal deduced sequence was homologous to the *pss2* gene from *R. leguminosarum* bv. phaseoli (3). The identity in this case was 28% over 76 amino acids (Fig. 6). Homology of NGR234 ExoX to *R. leguminosarum* bv. phaseoli Psi has previously been shown (19), and homology between *R. meliloti* ExoY and *R. leguminosarum* bv. phaseoli Pss2 has also been reported (22).

The sequence of a cluster of genes from *Xanthomonas campestris* required for xanthan gum biosynthesis has recently been determined, and biochemical functions for some of the genes has been assigned (10). We compared our deduced ExoX and ExoY sequences with each of these xanthan *gum* genes and found that ExoY was homologous to the C-terminal portion of GumD. The same portion of GumD sequence was also homologous to the *R. leguminosarum* bv. phaseoli Pss2 and *Rhizobium* sp. strain NGR234 ExoY sequences. The GumD sequence is 27% identical over 99 amino acids to the *R. meliloti* ExoY N-terminal sequence,

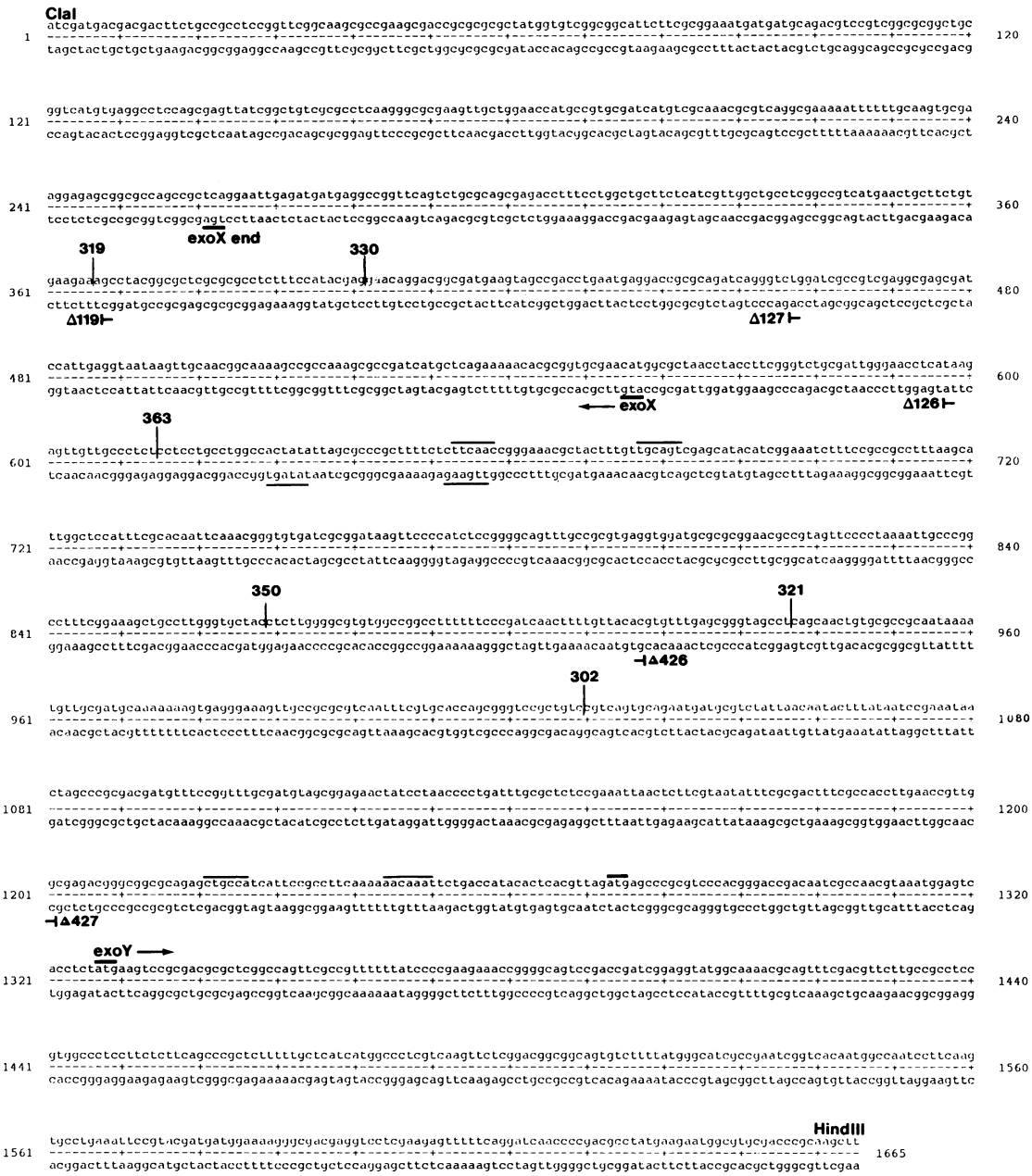

FIG. 4. Sequence of the *exoG-J* region. Shown are positions of start and stop codons for the putative *exoX* and *exoY* open reading frames, positions of the rightward junctions of Tn*5* insertions in the region, and endpoints of subclones shown in Fig. 3. Also indicated are putative promoter elements, on the basis of homology to sequences in *Rhizobium* sp. strain NGR234 (19).

33% identical over 212 amino acids to the NGR234 ExoY sequence, and 41% identical over 197 amino acids to the *R. leguminosarum* Pss2 sequence. Alignment of these sequences is shown in Fig. 6. This homology is discussed below.

Locations of Tn*5* insertions. To understand the phenotypes of the Tn*5* insertion mutations in this region better, we determined the precise positions of the rightward junction of each of them (Fig. 1 and 4). We found that the *exoG302* insertion falls in the intergenic region at nucleotide 1029, 298 nucleotides to the left of the putative translational start of *exoY*; that insertion *exoG321* falls slightly to the left of *exoG302*, at nucleotide 936; that insertion Ω350, which has no obvious phenotype, also falls in the intergenic region, at nucleotide 868; that insertion *exoX363*, which causes overproduction of EPS I, falls at nucleotide 614, between the putative promoter of *exoX* and its translational start; that insertion *exoX330*, which causes overproduction of EPS I, falls in the latter half of the *exoX* open reading frame at nucleotide 401; and that insertion *exoJ319*, which causes underproduction of EPS I, falls at nucleotide 366, just 35 nucleotides to the left of insertion *exoX330*. (We had previously mapped insertion *exoJ319* to the right of insertion *exoX330* [32].) Finally, in the course of this work, we also

```
R.l. Psi                    MHQRCF GLRASLSIFK AFAVTLAASV FLQVVYFLSL
NGR ExoX     MFAPRFVVSM LGALAAFAIA TYFLTGSIAS TAVQTLLCAV LIQVGYFLAV
R.m. ExoX    MFAPRVFLSM IGALAAFAVA TYYLNGSLAS TAIQTLICAV LIQVGYFIAV
                                        .       ..  .  .. ..

R.l. Psi     LFMSFRPTRE SDRSIHSGTR QADQPQKRDR DKTEQSNVPK LDPRRKRRTP*
NGR ExoX     LFLVWKEARD R-RKLSPG-- QLPADPTNDE KQTGKLSLRR LNRPPHFNS*
R.m. ExoX    LFLVWKEARE R-RRLSSQKQ FMTAEAANDE KQPGKVSLRR LNRPHHLNS*
```

FIG. 5. Alignment of *R. leguminosarum* bv. phaseoli Psi, *Rhizobium* sp. strain NGR234 ExoX, and *R. meliloti* ExoX deduced amino acid sequences. Identities to the *R. meliloti* sequence are shown in boldface, and dots indicate matches among all three sequences.

found that our previously reported insertion Ω357, which appears to fluoresce less brightly and with a slightly different hue than the wild-type parental strain on Calcofluor plates (38), falls beyond the end of the *exoX* open reading frame, 18 nucleotides to the left of the *Cla*I site.

The positions of the various Tn*5* insertions in this region raise questions about the mechanisms whereby they affect exopolysaccharide production. The *exoX330* and *exoX363* insertions would appear from their positions to eliminate expression of the *exoX* gene, consistent with their recessive character and with the overexpression of EPS I that they cause. The positions of the two *exoG* insertions, at some distance from either reading frame, suggest that this region has some other importance, perhaps as a regulatory region or a non-protein-coding transcript. The *exoJ319* insertion falls in the 3′-terminal portion of the *exoX* gene, despite causing a different phenotype from the *exoX330* and *exoX363* insertions. We therefore conclude that *exoJ319* is an allele of *exoX* that results in an alteration of the action of ExoX. We have therefore renamed this insertion *exoX(J)319*.

Interactions with other regulatory mutations. The *exoR95*:: Tn*5* and *exoS96*::Tn*5* mutations cause overproduction of exopolysaccharide (13). This overproduction arises at least in part as a consequence of increased expression of other *exo*

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X.c. GumD    MLLADLSSAT YTTSSPRLLS KYSAAADLVL RVFDLTMVVA SGLIAYRIVF

X.c. GumD    GTWVPAAPYR VAIATTLLYS VICFALFPLY RSWRGRGLLS ELVVLGGAFG

X.c. GumD    GVFALFAVHA LIVQVGEQVS RGWVGLWFVG GLVSLVAART LLRGFLNHLR

X.c. GumD    TQGVDVQRVV VVGLRHPVMK ISHYLSRNPW VGMNMVGYFR TPYDLAVAEQ

X.c. GumD    RQGLPCLGDP DELIEYLKNN QVEQVWISLP LGERDHIKQL LQRLDRYPIN

R.l. Pss2                                              MDL VLKRAFDIFS
X.c. GumD    VKLVPDLFVF GLLNQSAEQI GSVPVINLRQ GGVDRDNYFV VAKALQDKIL
NGR ExoY                   MKSATRS ATT-AFFIPQ ETGAIRPIGG ISKRSFDVLI
R.m. ExoY                  MKSATRS ASS-PFFIPE ETGAVRPIGG MAKRSFDVLA
                             ..                                . .

R.l. Pss2    SLSALLVLAP FLLFVALLIK LDSPGPVLFK QTRWGKNCKA IKVYKFRSMR
X.c. GumD    AVIALMGLWP LMLAIAVGVK MSSPGPVFFR QRRHGLGGRE FYMFKFRSMR
NGR ExoY     AILALIALSP LFLLVMGLVK FSDGGSIFYG HRRIGHNGQT FKCLKFRTMM
R.m. ExoY    ASVALLLFSP LFLLIMALVK FSDGGSVFYG HRRIGHNGQS FKCLKFRTMM
               ..   .  .     .    .      . .        ... .

R.l. Pss2    TDLCDVSGVA ---------- ----QTVKND PRITRIGAIL RRTNVDELPQ
X.c. GumD    VHDDHGTTIQ ---------- ----QATKND TRITRFGSFL RRSSLDELPQ
NGR ExoY     ENGDRVLQEF FKSNPAAYEE WRTTRKLQDD PRVTVVGSVL RKLSLDELPQ
R.m. ExoY    EKGDEVLEEF FRINPDAYEE WRATRKL
                                          .  .  .  .  .  .    .....

R.l. Pss2    LLNVLLGHMS VVGPRCHAIG MRAGGMLYEE LVPEYHQRHA MRPGMTGLAQ
X.c. GumD    IFNVLGGSMS IVGPRPHAAQ HNTH---YEK LINHYMQRHY VKPGITGWAQ
NGR ExoY     LLNIIRGEMS IVGPRPVVED ELE---LYDS AAEFYL-R-- SRPGLTGLWQ
               .  . ..  ....            .      . .     .. .. .

R.l. Pss2    MRGLRGPTDR PAKARARIAS DLYYVGNFSI VMDMRIIFGT VVSELTRGKG F*
X.c. GumD    VNGFRGETPE LRTMKKRIQY DLDYIRRWSL WLDIRIIVLT AVRVLGQKTA Y*
NGR ExoY     ISGR-----N DVSYATRVAF DTHYVQNWSL LADLVIVFKT IPAVCLSRGS Y*
               .            .  .  .  .  .  .  .
```

FIG. 6. Alignment of *R. leguminosarum* bv. phaseoli Pss2, *X. campestris* GumD, *Rhizobium* sp. strain NGR234 ExoY, and *R. meliloti* ExoY deduced amino acid sequences. Identities with the NGR234 ExoY sequence are shown in boldface, and dots indicate positions where residues in all of the sequences are the same.

TABLE 5. Accumulation of anthrone-positive material in culture supernatants with and without nitrogen

Genotype	Anthrone reactivity of culture supernatant grown with or without NH_4Cl[a]	
	+ NH_4Cl	− NH_4Cl
Wild type	0.19 ± 0.01	2.2 ± 0.33
exoG302	0.19 ± 0.04	0.14 ± 0.05
exoX(J)319	0.19 ± 0.05	0.16 ± 0.03
exoN416	0.17 ± 0.02	0.50 ± 0.03
exoX363	1.5 ± 0.5	4.2 ± 0.4
exoR95	33 ± 2.6	9.1 ± 1.7
exoG302 exoR95	1.9 ± 1.1	0.15 ± 0.04
exoX(J)319 exoR95	0.70 ± 0.14	0.26 ± 0.08
exoN416 exoR95	21 ± 1.0	6.8 ± 0.2
exoX363 exoR95	34 ± 0.5	12 ± 2.0
exoS96	3.6 ± 2.1	5.9 ± 1.1
exoG302 exoS96	0.15 ± 0.05	0.18 ± 0.15
exoX(J)319 exoS96	0.15 ± 0.04	0.29 ± 0.06
exoN416 exoS96	1.2 ± 0.7	2.1 ± 0.15
exoX363 exoS96	14 ± 1.5	8.3 ± 1.3

[a] Values are expressed as A_{620} (from anthrone test)/ml/optical density at 600 nm of culture ± standard deviations. Cultures were grown for 60 to 75 h in M9–1% mannitol medium with or without 0.1% ammonium chloride as a nitrogen source.

genes (36, 39), in contrast to the ExoX-Y system, which appears to affect EPS I production at a posttranslational level. A strain carrying the *exoR95* mutation failed to invade nodules effectively unless it accumulated suppressors that decreased the amount of exopolysaccharide made (13). Furthermore, the high level of production of EPS I by the *exoR95* strain was not increased further in response to ammonia starvation as was wild-type EPS I production (13). Strains carrying the *exoS96* mutation, on the other hand, nodulated effectively despite a higher basal level of EPS I production than the wild-type strain. Like the wild-type strain, the *exoS96* strain produced even more EPS I in the absence of ammonia than in its presence (13). In order to ascertain how *exoG*, *exoX(J)*, and *exoX* mutations might interact with the regulatory pathways defined by the *exoR95* and *exoS96* mutations, we constructed double mutant strains containing either the *exoG302*, *exoX(J)319*, or *exoX363* mutation; and the *exoR95* or *exoS96* mutation. We then assayed the amount of exopolysaccharide produced by these strains in liquid cultures in the presence and absence of nitrogen. The results are presented in Table 5.

Several observations emerge from examination of this data. As previously found (13), cultures of the wild-type strain produced very little exopolysaccharide in the presence of ammonia but produced substantially more when starved for nitrogen. Cultures of the *exoG302* and *exoX(J)319* strains produced the same small amount of anthrone-positive material in the supernatant as did the wild-type in the presence of ammonia but, interestingly, did not show a further increase in response to nitrogen starvation. These results suggest that the *exoG302* and *exoX(J)319* mutations may affect control of EPS I synthesis in response to nitrogen availability. Alternatively, the results may reflect a limitation of EPS I production by these mutations at some step subsequent to nitrogen control. The *exoX363* strain overproduced anthrone-positive material in both the presence and absence of nitrogen and, like the wild-type strain, produced more in the absence of nitrogen. As previously observed, the *exoR95* and *exoS96* strains overproduced anthrone-positive material in the presence or absence of nitrogen. We found that the *exoR95* strain actually produced significantly more EPS I in the presence of nitrogen than in its absence. The *exoS96* strain responded to nitrogen starvation by increasing its exopolysaccharide production (Table 5).

In the *exoR95* and *exoS96* backgrounds, the *exoX(J)319* mutation severely limited EPS I production, although levels were slightly higher than they were for a strain carrying mutation *exoX(J)319* alone. These results are consistent with the interpretation that the *exoX(J)319* mutation limits the amount of EPS I that strains carrying it can synthesize. Similarly to the *exoX(J)319* insertion, the *exoG302* insertion appeared to limit the amount of EPS I made in an *exoS96* background. It thus appears that the EPS I overproduction caused by the *exoS96* mutation depends on the integrity of the *exoG* region. In an *exoR95* background, however, the effect of the *exoG302* insertion depended on the medium. In the absence of nitrogen, the *exoG302 exoR95* strain made the same small amount of anthrone-positive material as the *exoG302* single mutant. In the presence of ammonia, however, the double mutant produced substantially more anthrone-positive material than the *exoG302* single mutant, although less than the *exoR95* single mutant. These data suggest that the overproduction caused by the *exoR95* mutation is completely eliminated by the *exoG302* mutation in nitrogen-free medium but only partially eliminated in medium containing ammonia. This conclusion supports our previous suggestion that the primary function of ExoR is to repress *exo* gene expression in nitrogen-rich medium (13). A more detailed understanding of ExoR control awaits a molecular analysis of transcripts of this putative promoter region.

The *exoX363* insertion caused increased exopolysaccharide production in the *exoR95* background in the absence of nitrogen but not in the presence of ammonia. Since the latter level of anthrone-positive material is already over 10 times that seen in cultures of the wild-type strain under any conditions, the *exoR95 exoX363* double mutant may be limited by the exopolysaccharide synthesizing capacity of the bacteria. The *exoX363* mutation caused an increase in exopolysaccharide production in an *exoS96* background in both the presence and absence of ammonia (Table 5).

On alfalfa plants, *exoG302 exoR95* and *exoX(J)319 exoR95* strains induced Fix^+ nodules. Roughly two-thirds of the plants inoculated by each of these strains appeared to have at least one Fix^+ nodule. However, these Fix^+ nodules could have arisen if mutations that suppressed the nodulation deficiency caused by the *exoR95* mutation had occurred during nodule invasion. When analyzing bacteria recovered from Fix^+ nodules, we customarily identify strains in which an *exoR* strain has acquired a suppressor mutation (13) by assessing their Calcofluor phenotype. *exoR* bacteria from such nodules generally fluoresce much less than the starting strain used to inoculate (13). The *exoG302 exoR95* and *exoX(J)319 exoR95* strains fluoresce so weakly, however, that we did not feel confident that we could detect the presence of additional suppressor mutations by this method. Therefore, we did not determine whether bacteria within nodules induced by these strains had acquired suppressor mutations, and further work will be required to rigorously establish the nodulation phenotypes of *exoG302 exoR95* and *exoX(J)319 exoR95* strains.

Effects of mutation *exoN416* on exopolysaccharide production. We have previously described Tn*5* mutations in the *exoN* locus, which cause reduced EPS I production but have no effect on nodulation of alfalfa (32). An *exoN416* mutation produced both high- and low-molecular-weight exopolysac-

charide (32) having ^{1}H-NMR spectra similar to those of wild-type EPS I (38). In order to compare an *exoN* mutation with the *exoG* and *exoX(J)* mutations, we tested the effect of mutation *exoN416* on exopolysaccharide production in wild-type, *exoR95*, and *exoS96* backgrounds. As shown in Table 5, the *exoN416* mutation decreased the amount of anthrone-positive material in culture supernatants in all three backgrounds. The extent of this effect was similar in both the presence and absence of ammonia, so that the overall pattern of production of anthrone-reactive material in the *exoN416* background is the same as in a wild-type background, except that roughly one-half as much material was produced by *exoN416* strains. The effects of the *exoN416* mutation on EPS I production thus appear much easier to explain than the effects of the *exoG302* and *exoX(J)319* mutations. Perhaps, for example, *exoN* mutations simply decrease the expression of other *exo* genes.

On alfalfa plants, the *exoN416 exoR95* double mutant formed Fix$^+$ nodules, but bacteria recovered from these nodules fluoresced substantially less than the starting *exoN416 exoR95* strain. Therefore, despite the somewhat reduced amount of EPS I produced by the double mutant compared with the *exoR95* single-mutant strain, it appears that the *exoR95* mutation still has a deleterious effect on nodulation when in combination with an *exoN416* mutation.

DISCUSSION

We previously described Tn*5*-generated *exoG* and *exoJ* insertion mutations in *R. meliloti* Rm1021 which appeared to affect the balance of high- and low-molecular-weight EPS I produced (32). In this work, we report new Tn*5*-generated insertion mutations that cause overproduction of EPS I and show that all of these mutations are located in a region of DNA encoding a system that modulates exopolysaccharide production. This system exists in other *Rhizobium* species and consists of the *exoX* and *exoY* genes. Other workers have found that the components of the ExoX-Y system affect the amount of exopolysaccharide made (4, 19, 51), and in *R. leguminosarum* bv. phaseoli, this putative regulation has been proposed to be important in symbiosis (4). We have sequenced the *exoX* gene of strain Rm1021, and part of the *exoY* gene, as well as the insertion junctions of the Tn*5* mutations we had in this region. Our data suggest models for how the ExoX-Y system might affect exopolysaccharide production and how the insertion mutations in the region might influence the distribution of high- and low-molecular-weight EPS I produced.

The existence of the *exoX* and *exoY* genes in *R. meliloti* had been reported by Zhan and Leigh, who found cross-complementation between *R. meliloti* and NGR234 *exoX* and *exoY* genes (50, 51). In fact, the sequence presented here reveals that the *R. meliloti exoX* and *exoY* genes are highly homologous to the corresponding genes in strain NGR234 (19), and somewhat less homologous to the *psi* and *pss2* genes from *R. leguminosarum* bv. phaseoli (3, 5). In all three species, the genes also share functional homologies. For example, multiple copies of *exoX* (or *psi*) cause a decrease in production of the corresponding exopolysaccharide, and multiple copies of *exoY* or *pss2* can compensate for the effect of multicopy *exoX* (5, 19, 51). Extra copies of *exoY* without *exoX* cause an increase in exopolysaccharide production. Mutations in *exoY* or *pss2* eliminate exopolysaccharide synthesis, suggesting that this gene encodes or is upstream of an exopolysaccharide biosynthetic gene (2, 19, 51).

Previous studies of these genes have stressed their putative roles in regulating the amount of exopolysaccharide produced (4, 19, 51). However, the phenotypes of the Tn*5* mutations we have described in this region suggest that the functions of these genes may be more complex than simply regulating the amount of exopolysaccharide made. In particular, the observation that *exoG* and *exoX(J)* strains apparently produce low-molecular-weight material related to EPS I without concomitant synthesis of high-molecular-weight EPS I suggests that the ExoX-Y system may play a role in determining the molecular weight distribution of the exopolysaccharides produced (see below). Alternatively, the skewed size distribution of EPS I from cultures of *exoG* and *exoX(J)* strains could reflect an inherent tendency of the EPS I biosynthetic apparatus to produce low- rather than high-molecular-weight EPS I when the overall synthesis rate is low.

In *Rhizobium* sp. strain NGR234 and *R. leguminosarum*, insertion mutations in *exoX* or *psi* generally cause overproduction of exopolysaccharide. Our *exoX330* and *exoX363* insertion mutations appear to be of this type, since they cause overproduction of EPS I, disrupt the *exoX* gene, and behave as recessive alleles when in single copy. Our observation that the *exoX330* and *exoX363* mutations behave as dominant alleles when present on cosmid pD56, causing an increase in fluorescence on Calcofluor plates when derivatives of pD56 carrying either mutation are introduced into the wild-type strain, is best explained by the presence of *exoY* on this cosmid. That is, the extra copies of *exoY* on pD56 *exoX*::Tn*5* derivatives cause EPS I production to be higher than if the wild-type *exoX* gene were also present in multiple copies. The cumulative results of studies of mutations in *exoX* and with multicopy plasmids carrying *exoX* suggest that the putative ExoX protein attenuates exopolysaccharide synthesis. Our finding that expression of fusions to other *exo* genes is at best only slightly affected by multiple copies of *exoX* suggests that the attenuation occurs post-translationally. The finding that EPS II synthesis is unaffected by multicopy *exoX* is also consistent with ExoX having a specific effect at the level of EPS I synthesis, rather than acting through some possibly more general effect on metabolism.

The results of the deletion analysis presented here show that the region responsible for the multicopy attenuation of EPS I synthesis includes most of the *exoX* gene but suggest that only part of the 98-amino-acid ExoX protein is necessary for this phenotype. Deletion AC19 extends into the open reading frame from the C terminus as far as amino acid 63 but appears not to eliminate the exopolysaccharide-inhibitory phenotype of multicopy *exoX*. Deletion AC27 extends a further 29 amino acids to amino acid 34 and eliminates the exopolysaccharide-inhibitory phenotype. These results show that between 34 and 63 N-terminal amino acids of ExoX are sufficient for the decreased exopolysaccharide production phenotype caused by multiple copies of the *exoX* gene. The finding that insertion *exoX330*, which causes overproduction of EPS I, is located after codon 51 of the *exoX* open reading frame shows that in single copy more than 51 amino acids are necessary for ExoX attenuation of EPS I synthesis. Together, these data suggest that between 51 and 63 amino acids of the ExoX protein may be sufficient for its inhibitory activity. Interestingly, the N-terminal half of ExoX includes the hydrophobic domain, which is the part that is most conserved between the NGR234 (and *R. meliloti*) ExoX and the *R. leguminosarum* bv. phaseoli Psi sequences (19).

The predicted *R. meliloti* ExoY N-terminal sequence

reveals it to be a member of a family of related putative gene products that includes ExoY from strain NGR234, Pss2 from *R. leguminosarum* bv. phaseoli, and GumD from *X. campestris*. Xanthan gum and many other bacterial exopolysaccharides are synthesized on isoprenoid lipid carriers as oligosaccharide subunits which are subsequently polymerized (44, 45). Strains mutant in *gumD* fail to add glucose, the first sugar residue in xanthan gum subunit biosynthesis, to the lipid carrier (10). Presumably, GumD either catalyzes the transfer of glucose from UDP-Glc to the isoprenoid lipid carrier or catalyzes some other step prior to, or essential for, this transfer. The homology between GumD and ExoY suggests that ExoY might perform a similar function. Similarly to xanthan gum biosynthesis, the subunit of EPS I of *R. meliloti* is synthesized on an isoprenoid lipid carrier (46), but the first residue transferred to the lipid is galactose rather than glucose (47). Seven glucose residues are subsequently transferred to the first galactose residue (46). Thus, on the basis of its homology to GumD, ExoY might conceivably be (i) the first glycosyltransferase in subunit biosynthesis, which would transfer galactose to the lipid carrier; (ii) a glucosyl transferase, which might transfer a glucose residue from UDP-Glc to the growing subunit; or (iii) a protein carrying out some function prior to the first galactosyltransferase reaction, for example charging the isoprenoid lipid carrier with phosphate. In contrast to the situation with *R. meliloti*, it is not known which sugar is added first in the syntheses of exopolysaccharides of *R. leguminosarum* bv. phaseoli and NGR234, the other strains known to possess an ExoY-homologous gene.

In any case, the homology to GumD suggests that ExoY may encode an enzyme needed at or near the first step in EPS I subunit biosynthesis. This possibility is consistent with the role proposed for ExoY in regulating EPS synthesis (19, 20, 51). In the model previously proposed for NGR234, ExoX and ExoY form a protein complex whose stoichiometry affects the amount of EPS made. High ratios of ExoX to ExoY lead to decreased exopolysaccharide synthesis, whereas low ratios lead to high synthesis. It seems appropriate that regulators of exopolysaccharide production should affect an early step in the pathway, as has been observed in the majority of regulated biosynthetic pathways studied in bacteria.

Our *exoG* insertions are interesting in that they cause a decrease in EPS I production despite falling in no obvious open reading frame. The model suggested by Gray and Rolfe (20), whereby the balance of ExoY gene product relative to ExoX determines the amount of exopolysaccharide made, could explain this finding if the *exoG* insertions cause a decrease in expression of the putative *exoY* gene. This would result in increased production of ExoX relative to ExoY, which could in turn cause a decrease in exopolysaccharide synthesis. The partially dominant Calcofluor phenotype of the *exoG* insertions when carried on cosmid pD56 could also be explained if they cause a relative decrease in *exoY* expression. In NGR234, Gray et al. found two transcription starts upstream of *exoY*, one 65 nucleotides upstream of the putative *exoY* start of translation, and the other 591 nucleotides farther upstream. If the *R. meliloti exoY* gene is transcribed from two promoters, our *exoG* insertions might be expected to disrupt the transcript originating farther upstream. Alternatively, the *exoG* insertions might disrupt control sequences involved in *exoY* expression or might affect a transcript having a function other than as messenger RNA. More detailed studies of the putative promoter region, which will address these models, are in progress. Interpretation of the complex regulatory phenotype of the *exoG302 exoR95* double mutant might also benefit from such an analysis.

Insertion *exoX(J)319* falls after codon 63 of the *exoX* coding sequence and is thus an allele of *exoX*. In contrast to mutations *exoX330* and *exoX363*, however, it causes a decrease in EPS I production, suggesting that it may alter the function of ExoX. Moreover, the failure of *exoX(J)319* strains to increase their production of EPS I under different culture conditions, or in *exoR95* or *exoS96* backgrounds, suggests that the *exoX(J)319* mutation limits EPS I production at a point downstream of the action of ExoR, ExoS, or nitrogen availability. This conclusion is consistent with the apparent posttranslational effect of multiple copies of *exoX* on EPS I production.

We have previously found that the symbiotic defect of an *exoX(J)319* strain is more severe than that of either of the *exoG* mutants (32). In addition, we have observed that an *exoX(J)319* mutant is clearly Fix^- on the alternative plant host *Melilotus alba*, whereas the *exoG* strains and the wild-type parent are all Fix^+ (38). This result shows that the *exoX(J)319* mutation causes a more absolute symbiotic defect than the *exoG* mutations, consistent with our interpretation that the *exoX(J)319* mutation may affect EPS I production by altering the function of a protein, whereas the *exoG* mutations more likely affect the level of some gene product.

Production of other bacterial exopolysaccharides is known to require a number of gene products in addition to those required for the enzymology of exopolysaccharide chain synthesis. In *Acetobacter xylinum*, a single catalytic subunit of cellulose synthase is sufficient to synthesize cellulose in vitro. However, three additional genes are required in vivo. These may encode functions involved in transport or crystallization of the cellulose fibrils (49). Similarly, the syntheses of *E. coli* and *Haemophilus influenzae* capsular polysaccharides require a number of genes whose functions are to transport or secrete the polysaccharides. Like *exoX* and *exoY*, these transport or secretion genes are common among strains making polysaccharides of quite different structures (for a review see reference 6). *E. coli* strains mutant in these genes accumulate polysaccharide inside the cells or in the periplasm (24).

These considerations raise the possibility that the ExoX and/or ExoY gene products actually act in some structural capacity rather than simply regulating the amount of EPS I made. For example, they might be involved in determining the size of exopolysaccharide chains, or the structures of EPS I chain termini. It is also possible that the two components perform different functions in the EPS I biosynthesis pathway. For example, ExoX may be required for termination and release of EPS I chains, whereas ExoY may be an enzyme limiting for synthesis of the EPS I octameric subunit. In this model, the ExoX and ExoY proteins need not interact physically, but might interact functionally by affecting the amount of lipid-linked EPS I subunit available for polymerization, ExoX by removing subunits from the pool and ExoY by synthesizing more. Alternatively, ExoX or ExoY might be required for transport of EPS I intermediates out of the cell or periplasm. Our inability to detect high-molecular-weight EPS I in culture supernatants of *exoG* or *exoX(J)* strains might be explained if the ExoX-ExoY system normally participates in or modulates one of these functions. Thus, notwithstanding the ability of the ExoX-ExoY stoichiometric model to explain the effects of different gene dosages of *exoX* and *exoY* on EPS production, it is uncertain

whether the role of ExoX in the wild-type cell is to regulate the amount, the higher-order structure, or the localization of the exopolysaccharide produced.

Lastly, it is not known what signals or metabolic conditions this putative regulatory system might respond to. The proportions of high- and low-molecular-weight EPS I made by *R. meliloti* have been found to be affected by the osmolarity of the medium (7, 25), low-molecular-weight EPS I being produced in greatest abundance at very low osmolarity (7). Possibly, the ExoX-Y system responds to low osmolarity by increasing production of low-molecular-weight EPS I molecules that could act as osmoprotectants. In *A. xylinum*, cellulose synthesis is activated posttranslationally by cyclic diguanylic acid (41), raising the possibility that second messengers may be involved in regulation of EPS I production as well.

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