JOURNAL OF BACTERIOLOGY, June 1991, p. 3789–3794
0021-9193/91/123789-06$02.00/0

Vol. 173, No. 12

The *exoR* Gene of *Rhizobium meliloti* Affects RNA Levels of Other *exo* Genes but Lacks Homology to Known Transcriptional Regulators

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Received 6 February 1991/Accepted 11 April 1991

***Rhizobium meliloti* strains mutant in the *exoR* gene overproduce an exopolysaccharide called succinoglycan or EPS I. Protein fusions to several different *exo* genes required for EPS I biosynthesis are expressed at a higher level in an *exoR* strain than in a wild-type strain, showing that the overproduction of EPS I in *exoR* strains results at least in part from increased gene expression. This regulation is important to nodulation, since *exoR* mutants fail to invade alfalfa nodules unless secondary suppressor mutations that cause a decrease in EPS I production occur. Here, we show that an *exoR* strain contains higher levels of mRNA for other *exo* genes than does the wild-type parental strain. ExoR therefore most probably exerts its regulatory effect at the level of transcription. In addition, we have localized, subcloned, and sequenced the *exoR* gene. A newly constructed insertion allele of *exoR* has the same phenotype as the original mutant. The deduced sequence of ExoR is 268 amino acids long but does not show homology to other sequenced genes.**

The nodulation of legumes by bacteria of the genus *Rhizobium* is an environmentally important example of bacterial development. In this symbiotic interaction, the bacteria induce the formation of nodules on the roots of the host plant and subsequently invade these nodules through tubes called infection threads. The bacteria emerge from the infection threads into the plant cells, where a plant-derived membrane surrounds them. Then, they differentiate into bacteroids, which are the nitrogen-fixing forms of the bacteria (26, 27). In the symbiosis between *Rhizobium meliloti* strain Rm1021 and alfalfa, we have found that an acidic exopolysaccharide, called succinoglycan or EPS I, is required for the invasion of nodules by the bacteria (14, 25). Strains carrying *exo* mutations that eliminate production of EPS I are unable to invade the nodules they induce, which remain empty and fail to fix nitrogen (14, 23, 25, 28, 30). EPS I is produced in increased quantity when the bacteria are starved for nitrogen (12, 32).

We have previously described mutations, called *exoR95*::Tn*5* and *exoS96*::Tn*5*, in two genes that regulate biosynthesis of EPS I (12). These recessive mutations cause overproduction of EPS I (12, 34). On agar plates, colonies of these two strains appear very mucoid. Whereas most *exo* genes required for EPS I synthesis are located in a cluster on the second of two symbiotic megaplasmids in *R. meliloti* (15, 20), the *exoR* and *exoS* loci are located on the chromosome (12, 16). Furthermore, the *exoR95* mutation appears to uncouple the production of EPS I from regulation by the availability of ammonia, since the amount of EPS I produced no longer increases in the absence of ammonia. Strains carrying the *exoS96* mutation produce more EPS I than the wild-type strain in the presence of ammonia but, like the wild-type, still increase EPS I production in response to nitrogen starvation.

In our previous studies, we used *exo-phoA* gene fusions generated by Tn*phoA* to examine the effect of the *exoR95* and *exoS96* mutations on the expression of *exo* genes. In the free-living state, these fusions were expressed at higher levels in *exoR95* or *exoS96* mutant backgrounds (34). The increased expression of these fusions in an *exoR95* background suggested that ExoR acts, directly or indirectly, at the level of gene expression but did not distinguish among transcriptional or translational control of *exo* gene expression or control at some other level such as RNA or protein stability. On alfalfa plants, an *exoS96* mutant strain nodulated normally, but an *exoR95* mutant strain was unable to invade the nodules it induced unless suppressor mutations causing a decrease in EPS I production arose (12). This result showed that unregulated synthesis of EPS I is deleterious to nodulation and suggested that attenuation of exopolysaccharide biosynthesis by ExoR is essential at some stage in nodule invasion.

In the present work, we present evidence that the *exoR95* mutation causes an increase in *exo* gene mRNA levels. This result suggests that ExoR probably exerts its control of *exo* gene expression at the level of transcription. In addition, we describe the cloning and sequencing of the *exoR* gene. However, we find that the sequence of the putative ExoR protein lacks homology to known transcriptional regulators.

MATERIALS AND METHODS

Strains and media. *R. meliloti* strain Rm7095 is a derivative of the wild-type strain Rm1021 carrying the *exoR95*::Tn*5* mutation (12), and strain Rm8501 is a Lac$^-$ derivative of Rm1021 (17). Cosmid pM6 is a pLAFR1 derivative containing the *exoR* gene (12), and pGW2706 is a derivative of pSUP104 (36) containing the *R. meliloti trpDC* genes (2). Bacteria were grown in Luria-Bertani (LB) medium (29), with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ added for *R. meliloti* LB-MC cultures. Antibiotics were used at the following concentrations: neomycin, 200 μg/ml; streptomycin, 500 μg/ml; and tetracycline, 10 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 40 μg/ml.

Genetic manipulations. Plasmids were transferred between *Escherichia coli* and *R. meliloti* strains by mating, by using the mobilizing plasmid pRK600 as described previously (35).

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Transposon Tn*3*HoHo(Km) is a derivative of Tn*3*HoHo (37) containing a kanamycin resistance gene from Tn*903* (1). It is distinct from transposon Tn*3*HoKm (17) and is less suitable for genetic studies than the latter because the kanamycin resistance of Tn*3*HoHo(Km) is efficiently expressed only when the transposon is carried on a multicopy plasmid (1). Insertions of Tn*3*HoHo(Km) were obtained by the same method as described for Tn*3*HoKm (17). The *exoR99* allele was crossed onto the chromosome by homogenotization (21, 35).

DNA manipulations and RNA analysis. Subclones pEXR11 and pEXR12 were obtained by cloning a 2.8-kb *Eco*RI fragment from cosmid pM6 (12) into the broad-host-range vector pRK404 (10) in both orientations. Plasmid pEXR21 was derived from pEXR12 by deleting a 0.7-kb fragment extending from the *Bgl*II site in pEXR12 to a *Bam*HI site in the polylinker of the vector. Plasmids pEXR31 and pEXR32 were made by cloning a 1.55-kb *Pst*I fragment from pEXR11 into the Bluescript SK^+ vector (Stratagene, San Diego, Calif.) in both orientations. Plasmid pEXR40 carries this same 1.55-kb *Pst*I fragment in pRK404. Allele *exoR99* was constructed by ligating a cassette containing a kanamycin resistance gene from Tn*5* (13) into the *Bgl*II site in pEXR11. Deletions for sequencing of pEXR31 and pEXR32 were made with the exonuclease III-mung bean nuclease kit (Stratagene) according to the supplier's instructions. In addition, we constructed subclones with convenient restriction sites to fill in gaps not sequenced by using the serial deletions. Finally, we subcloned into Bluescript SK^+ and sequenced a 97-bp *Pst*I-*Eco*RI fragment from pEXR11 in order to obtain the sequence of the extreme C terminus of *exoR*. DNA was sequenced from primer sites in the Bluescript SK^+ vector by using Sequenase (US Biochemical Corp., Cleveland, Ohio) according to the supplier's instructions. Both DNA strands were sequenced in their entirety.

RNA was isolated from late-log *Rhizobium* cultures grown in LB-MC by hot-phenol extraction and separated from contaminating DNA by precipitation with an equal volume of 5 M LiCl. The integrity of RNA preparations was checked by running RNA samples on agarose gels, and the concentration of RNA was measured spectrophotometrically. Dot blots used equal amounts of RNA for each strain and were performed by using a dot blot apparatus made by Bio-Rad Laboratories (Richmond, Calif.). RNA was denatured with formamide-formaldehyde and blotted to GeneScreen Plus (New England Nuclear Corp., Boston, Mass.), according to the supplier's instructions. DNA probes were labeled with ^{32}P by using a nick translation kit from Bethesda Research Laboratories, Inc., (Bethesda, Md.) and were hybridized to RNA blots according to the GeneScreen Plus instructions.

Nucleotide sequence accession number. The DNA sequence reported here has been submitted to GenBank and has the accession number M61752.

RESULTS

Effects of the *exoR95* mutation on levels of mRNA from *exo* genes. We previously described several subclones carrying *R. meliloti* genes needed for synthesis of EPS I (28). Plasmid pEX41 contains the *exoH* and *exoK* genes and part of the *exoL* gene; plasmid pEX20 contains the *exoA*, *exoM*, and *exoN* genes and part of the *exoP* gene; plasmid pEX31 carries the *exoB* gene; and plasmid pEX80 contains the *exoY*, *exoF*, *exoQ*, and *exoX* genes (28, 32, 41). We tested whether the levels of RNA corresponding to these genes were increased in an *exoR95* strain by hybridizing dot blots of total RNA from LB-MC-grown *R. meliloti* with radioactively labeled probes made from these plasmids.

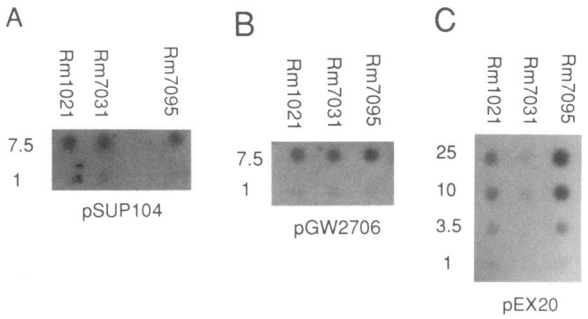

FIG. 1. Hybridization of ^{32}P-labeled vector pSUP104 (A), *trpDC* plasmid pGW2706 (B), and the *Eco*RI fragment from pEX20 containing the *exoA*, *exoM*, *exoN*, and part of the *exoP* loci (C) to total RNA from wild-type (Rm1021), *exoA31*::Tn*5* (Rm7031), and *exoR95*::Tn*5* (Rm7095) strains.

When the vector pSUP104 was hybridized to total RNA from the wild-type strain Rm1021, an $exoR^+$ *exoA31*::Tn*5* mutant strain, and an *exoR95*::Tn*5* $exoA^+$ strain, the degree of hybridization was the same for RNA from all three strains (Fig. 1A). Similarly, when a plasmid carrying the *R. meliloti trpDC* genes (pGW2706) (2) was used as a probe, all three RNA samples gave nearly the same degree of hybridization (Fig. 1B). In contrast, when the 5.8-kb insert of plasmid pEX20, which carries *exoA*, *exoM*, *exoN*, and part of *exoP*, was used as a probe, the *exoR95* $exoA^+$ strain showed substantially more hybridization than the wild-type strain (Fig. 1C). This result indicates that RNA transcripts corresponding to *R. meliloti* sequences on plasmid pEX20 are present at a higher level in the *exoR95* strain than in the wild-type parent. In the same experiment, RNA from the $exoR^+$ *exoA31*::Tn*5* strain showed only slight hybridization to the probe, showing that the transcripts being detected did indeed come at least in part from the *exoA* gene (Fig. 1C). The very low level of hybridization to RNA from the $exoR^+$ *exoA31*::Tn*5* strain suggests that the Tn*5* insertion in this strain may exert polar effects on the *exoM*, *exoN*, and *exoP* loci also located on the DNA probe used. This interpretation would be consistent with our previous hypothesis, on the basis of fusions to the *exoA* and *exoP* genes, that these loci could all be transcribed in the same direction (28). Alternatively, the *exoM*, *exoN*, and *exoP* loci might be expressed at very low levels relative to *exoA*.

Similarly, when the plasmids pEX41 and pEX80, which carry other sets of *exo* genes, were used as probes, higher levels of hybridization were seen to the RNA from the *exoR95* strain than to the RNA from the parent (Fig. 2A and B). Our previous finding that fusions to at least five different genes, *exoA*, *exoF*, *exoP*, *exoQ*, and *exoT*, are overexpressed in an *exoR95* background had suggested that the *exo* genes may be expressed together as a regulon subject to control by ExoR. The probes used in the current hybridization experiments each carry several genes, so the increase in levels of RNA that hybridize to these plasmids in the *exoR95* strain is consistent with a concurrent increase in the levels of RNA corresponding either to a subset of the genes on these plasmids or to all of them. In either case, these results suggest that the higher expression of *exo-phoA* gene fusions in an *exoR95* background (12, 34) results from increased levels of *exo* gene mRNA rather than from increased translation of *exo* transcripts. Consistent with our previous find-

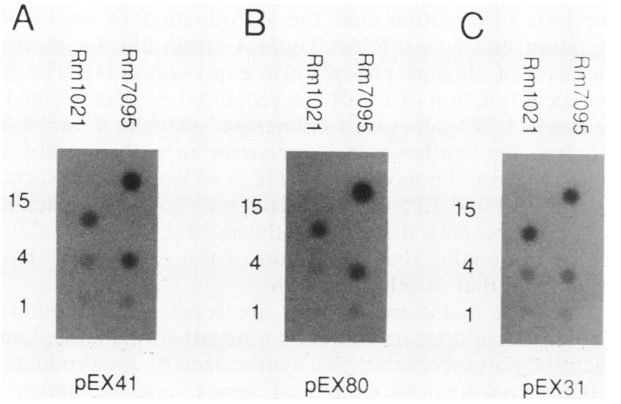

FIG. 2. Hybridization of ^{32}P-labeled plasmids pEX41 (containing the *exoH*, *exoK*, and *exoL* genes) (A), pEX80 (containing the *exoF*, *exoX*, and *exoY* genes) (B), and pEX31 (carrying the *exoB* gene) (C) to total RNA from wild-type (Rm1021) and *exoR95*::Tn*5* (Rm7095) strains. Numbers to the sides of dots refer to relative amounts of RNA loaded. Certain dots were offset so as to aid in alignment of autoradiograms.

ing that a fusion to the *exoB* gene was expressed at a low level in both wild-type and *exoR* backgrounds (34), we saw no obvious difference between the hybridization to RNA obtained from wild-type and *exoR95* strains when the *exoB*-specific plasmid pEX31 was used as a probe (Fig. 2C).

Localization of *exoR*. We had previously isolated a cosmid, pM6, containing the *exoR* gene, and had localized the *exoR95*::Tn*5* mutation to a 2.8-kb *Eco*RI fragment by Southern hybridization analysis (12) (Fig. 3). To localize the *exoR* gene further, we subcloned this fragment into the broad-host-range vector pRK404 in both orientations to make subclones pEXR11 and pEXR12. When pEXR11 and pEXR12 were introduced into the *exoR95* strain, the mucoid colony phenotype of this strain was complemented to a nonmucoid phenotype (Fig. 3). We also constructed smaller subclones of pEXR11 or pEXR12 (see Materials and Methods) and tested these for their ability to complement the *exoR95* mutation. Plasmid pEXR21, carrying a 2.1-kb *Eco*RI-*Bgl*II fragment, did not complement the *exoR95* mutation, whereas plasmid pEXR40, carrying a 1.55-kb *Pst*I fragment, did (Fig. 3).

We refined the localization of the *exoR* gene on subclone pEXR11 further by isolating four independent Tn*3*HoHo (Km) insertion mutations in it that eliminated complementation of the mucoid phenotype of an *exoR95* strain (Fig. 3). Tn*3*HoHo(Km) carries the *E. coli lacZ* gene, and creates operon fusions to *lacZ* if it inserts in a transcribed region of DNA in the appropriate orientation. We found that the derivatives of plasmid pEXR11 carrying two of these insertions, numbers 23 and 50, caused an *R. meliloti* Lac$^-$ strain to become blue on plates containing the chromogenic indicator X-Gal. In both of these insertions, the *lacZ* gene was oriented from left to right (Fig. 3). In the other two insertions that eliminated complementation of the *exoR95* mutant, numbers 41 and 55, the *lacZ* gene was oriented from right to left (Fig. 3). Introduction of plasmids carrying these latter two insertions to an *R. meliloti* Lac$^-$ strain did not give rise to a blue color on X-Gal plates. Taken together, these results imply that the *exoR* gene is transcribed from left to right in Fig. 3.

We confirmed our localization of the *exoR* gene by constructing a second allele of *exoR*. We found that we could not recombine our Tn*3*HoHo(Km) insertions onto the chromosome for technical reasons related to the nature of the drug resistance gene on the transposon (see Materials and Methods). To construct another *exoR* mutation, we inserted a cassette containing a gene for resistance to the antibiotic kanamycin (13) into the *Bgl*II site in pEXR11 (Fig. 3) and recombined this marker onto the chromosome. The resulting strain formed mucoid colonies, although these were somewhat less mucoid than those formed by an *exoR95* strain. Furthermore, also like the *exoR95* strain (12), on alfalfa

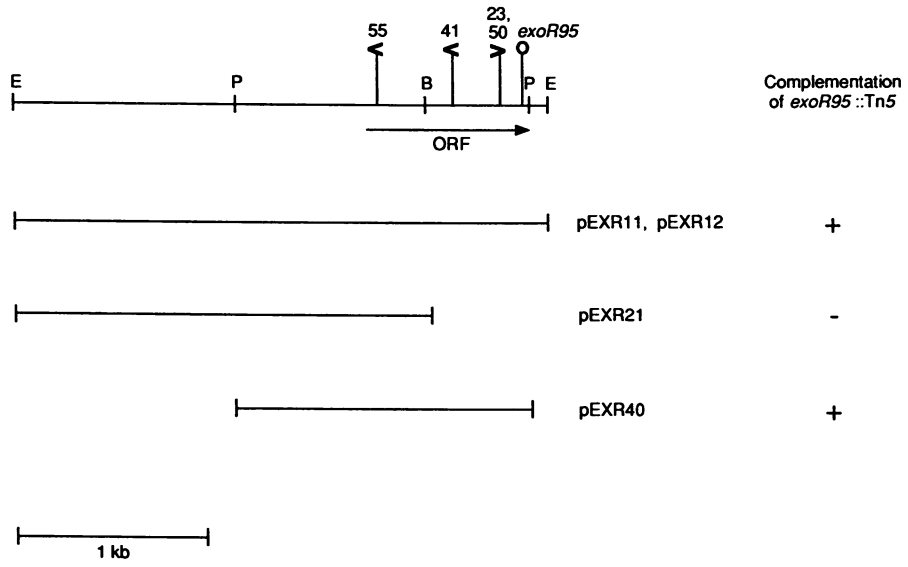

FIG. 3. Map of subclones of the *exoR* gene and the positions of Tn*3*HoHo(Km) insertions in plasmid pEXR11 that eliminate complementation of the mucoid colony phenotype of strains carrying the *exoR95* mutation. Arrowheads indicate orientations of transposon-generated operon fusions. Also shown is the position of mutation *exoR95*::Tn*5* (12). The position of the open reading frame deduced from the DNA sequence (Fig. 4) is indicated. Restriction sites: B, *Bgl*II; E, *Eco*RI; P, *Pst*I.

plants the new strain induced Fix$^+$ nodules which, however, contained bacteria that fluoresced less than did the parental strain on Calcofluor plates. These results show that the new mutation caused phenotypes similar to those caused by the *exoR95* allele and thus that we had identified the correct region for *exoR*. Moreover, the mucoid colony morphology conferred by the second *exoR* allele supports our previous conclusion that ExoR probably acts negatively to regulate exopolysaccharide production. We refer to this new mutation as *exoR99*.

Sequence of *exoR*. To determine whether ExoR might be related to any known regulatory proteins, we sequenced the *exoR* gene. We sequenced the 1.55-kb *Pst*I fragment in pEXR40 and found an open reading frame of 264 amino acids in the appropriate location and oriented in the same direction as the Tn*3*HoHo(Km) insertions that had β-galactosidase activity (Fig. 3 and 4). The open reading frame starts at nucleotide 761 and extends to the end of the DNA fragment cloned in plasmid pEXR40. This result suggested that although the plasmid pEXR40 encoded a functionally active ExoR derivative, it lacked the carboxyl terminus of the protein. We therefore sequenced more DNA to the right of the 1.55-kb *Pst*I fragment in pEXR40 (see Materials and Methods) and discovered that the open reading frame extended for four more codons, for a complete predicted polypeptide length of 268 amino acids. The *Bgl*II site at which we constructed mutation *exoR99* falls at nucleotide 1059, at amino acid 101 of the open reading frame. The open reading frame is preceded by a putative ribosome-binding site, GAAAGAAA, between 17 and 10 nucleotides upstream of the first ATG initiation codon.

DISCUSSION

The *exoR* gene was previously identified by a single mutation, *exoR95*::Tn*5*, that caused increased production of exopolysaccharide EPS I (12). The present work shows that, in cells carrying this mutation, the mRNA corresponding to several *exo* genes required for the synthesis of EPS I is present at increased levels. We have also localized, subcloned, and sequenced the *exoR* gene. The deduced sequence of the ExoR protein did not resemble any other sequence in the GenBank data base, suggesting that *exoR* encodes a previously unidentified type of regulatory protein.

In the *exoR95* mutant, we observed an increase in *exo* gene RNA levels of about fourfold, which is comparable to the two- to fivefold increase in expression of *exo-phoA* gene fusions observed previously (34). Thus, the increase in *exo* gene mRNA levels in the *exoR95* mutant can account for the increased levels of expression of the *exo-phoA* fusion proteins in this background. The increased levels of *exo* gene mRNA in an *exoR* mutant could result from either increased transcription of these *exo* genes or from increased stabilization of the transcripts of these *exo* genes. In either case, ExoR most likely acts through sequences present in the *exo-phoA* gene fusion constructions, i.e., in the promoter-proximal portions of the regulated *exo* genes. Moreover, the fusions whose levels are affected by the *exoR95* mutation fall in at least five loci (34) which represent a minimum of two transcripts (28). This latter observation implies that ExoR must act through at least two, and possibly more, sites in the *R. meliloti* genome. Additional studies designed to understand the molecular basis of ExoR action are under way.

In contrast to the results with the other genes in the *exo* cluster, the level of *exoB* mRNA is not increased in an *exoR95* background. This finding is consistent with our previous observation that the introduction of an *exoR95* mutation into an *exoB359*::Tn*phoA* strain did not increase the level of alkaline phosphatase expression (34). The biochemical function of ExoB has recently been determined to be that of UDP-galactose 4-epimerase, which in *R. meliloti* is required for synthesis of a precursor in pathways for the biosynthesis of lipopolysaccharide as well as exopolysaccharides EPS I and EPS II (5, 6). Given its role in biochemical pathways besides that for synthesis of EPS I (17, 25), it seems reasonable that regulation of the *exoB* gene should differ from that of other *exo* genes.

Since the mutations in *exoR* are recessive, the putative ExoR protein appears to act as a negative regulator. Other bacterial polysaccharides are synthesized by the products of sets of coordinately expressed genes, and a number of negative regulators of these genes have been characterized (8). For example, *E. coli* capsular polysaccharide is negatively regulated by the products of the *rcsC*, *lon*, and *ops* genes (4, 42). The sequences of RcsC and Lon do not resemble that of ExoR (7, 38). The sequence of the *ops* gene has not been reported, but *ops* mutants differ from our *exoR* mutants in that they only overproduce exopolysaccharide in minimal media (42), whereas *exoR* mutants are distinguished by their apparent uncoupling of control of exopolysaccharide production from medium composition (12). The *epsR* locus of *Pseudomonas solanacearum* also acts negatively (19), but its deduced amino acid sequence does not appear to be related to that of ExoR (22). In *Xanthomonas campestris* pv. campestris, a negative regulator of exopolysaccharide and extracellular enzyme production has been reported (39) but has not been characterized molecularly. The ExoX family of negative regulators of exopolysaccharide synthesis in *Rhizobium* strains is also unrelated by amino acid sequence to ExoR, and moreover, unlike ExoR, appears to act at a posttranslational level (3, 18, 32, 41).

The nodulation defect of *exoR* strains, and the appearance of nodulation competent derivatives of *exoR95* mutants carrying suppressor mutations that decrease the amount of EPS I produced (12), suggest that *exoR*-mediated *exo* gene regulation may be important for symbiosis. Conceivably, *exoR* may regulate *exo* genes at some stage during nodule invasion, perhaps turning off exopolysaccharide production at a crucial juncture. Studies with *exo-phoA* gene fusions have shown that several *exo* genes are expressed concurrently in the invasion zone of developing nodules (34), and coinoculation studies have also suggested that exopolysaccharide is needed during, but not after, the invasion stage of nodulation (31, 33). Taken together, these results raise the possibility that EPS I synthesis needs to be attenuated in bacterial cells as they enter the plant cells in the nodule or as they differentiate into bacteroids. This explanation has also been invoked to explain the nodulation deficiency of a mutant that overproduces exopolysaccharide in *Rhizobium leguminosarum* bv. phaseoli (3). Alternatively, ExoR control may normally be physiologically significant only in the free-living state, the symbiotic deficiency being an artifact created by the unusually high amount of EPS I produced by *exoR* strains.

The putative signal for ExoR attenuation of *exo* gene expression remains unknown. ExoR may respond to environmental conditions prevalent in the invasion zone of a developing nodule. Our previous observation that an *exoR* mutant failed to decrease its production of EPS I in response to the presence of ammonia in the growth medium (12) suggests that nitrogen availability may play a role in ExoR control. Thus, ExoR might mediate a bacterial physiological

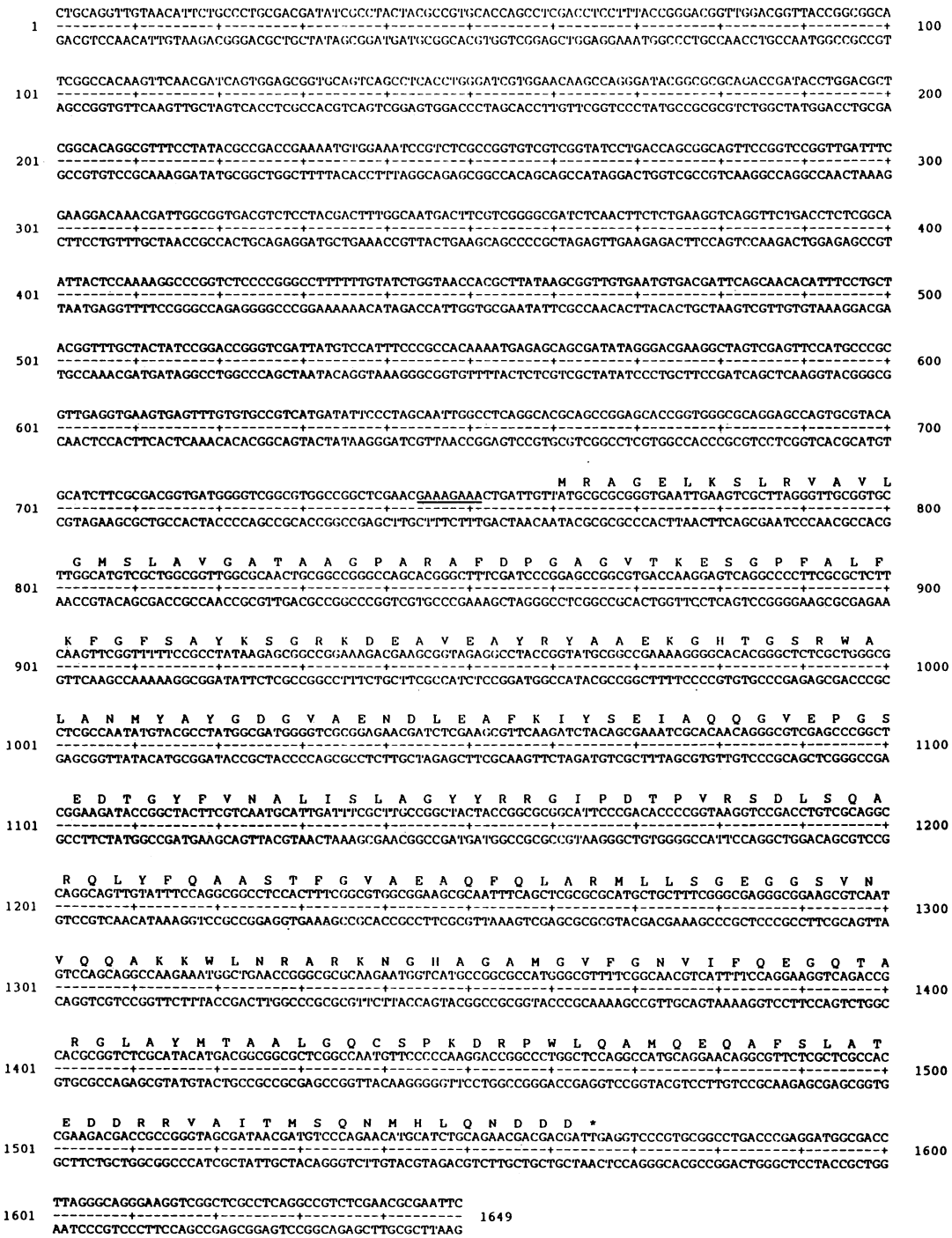

FIG. 4. Sequence of the *exoR* gene. Shown is a translation of the *exoR* open reading frame and a putative ribosome-binding site (underlined).

response that is crucial for effective nodulation. The FixL and FixJ proteins of *R. meliloti* exemplify such a response. These proteins regulate genes encoding nitrogenase and other enzymes needed for nitrogen fixation in response to low oxygen tension, an environmental condition thought to prevail within mature nodules (9, 11, 40). Studies of the regulatory effects of *exoR* mutations may lead to an understanding of the environment encountered by the bacteria as they begin to develop into bacteroids.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM31030 to G.C.W. J.G. and J.W.R. were both supported in part by National Science Foundation predoctoral fellowships.

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