The *Rhizobium leguminosarum* bv. *trifolii* RosR: Transcriptional Regulator Involved in Exopolysaccharide Production

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The acidic exopolysaccharide is required for the establishment of symbiosis between the nitrogen-fixing bacterium *Rhizobium leguminosarum* bv. *trifolii* and clover. Here, we describe RosR protein from *R. leguminosarum* bv. *trifolii* 24.2, a homolog of transcriptional regulators belonging to the family of Ros/MucR proteins. *R. leguminosarum* bv. *trifolii* RosR possesses a characteristic Cys₂His₂ type zinc-finger motif in its C-terminal domain. Recombinant (His)₆RosR binds to an RosR-box sequence located upstream of *rosR*. Deletion analysis of the *rosR* upstream region resulted in identification of two –35 to –10 promoter sequences, two conserved inverted palindromic pentamers that resemble the cAMP-CRP binding site of *Escherichia coli*, inverted repeats identified as a RosR binding site, and other regulatory sequence motifs. When assayed in *E. coli*, a transcriptional fusion of the cAMP-CRP binding site containing the *rosR* upstream region and *lacZ* gene was moderately responsive to glucose. The sensitivity of the *rosR* promoter to glucose was not observed in *E. coli* Δ cyaA. A *rosR* frame-shift mutant of *R. leguminosarum* bv. *trifolii* formed dry, wrinkled colonies and induced nodules on clover, but did not fix nitrogen. In the *rosR* mutant, transcription of *pssA-lacZ* fusion was decreased, indicating positive regulation of the *pssA* gene by RosR. Multiple copies of *rosR* in *R. leguminosarum* bv. *trifolii* 24.2 increased exopolysaccharide production.

Additional keywords: catabolic repression, zinc finger protein.

Rhizobium leguminosarum produces large amounts of acidic exopolysaccharide (EPS) that has been shown to be indispensable for nodule invasion and, therefore, for successful nitrogen-fixing symbioses with legumes that form an indeterminate type of nodules with persistent meristem, such as *Medicago*, *Pisum*, *Trifolium*, and *Vicia* spp. (Becker and Pühler 1998; Perret et al. 2000). EPS-deficient mutants of *Rhizobium leguminosarum* fail to nodulate their host plants (Borthakur et al. 1988) or induce formation of ineffective nodules (Rolfe et al. 1996; Skorupska et al. 1995; van Workum et al. 1997). The EPS of *R. leguminosarum* is a polymer of octasaccharide subunits, each composed of one galactose, five glucose, and two glucuronic acid residues, additionally decorated with acetyl,

pyruvyl, and hydroxy-butanoyl modifications (Hollingsworth et al. 1988). EPS production requires the expression of several *exo* or *pss* genes. Most of them are dispersed throughout the genome, except for three major EPS clusters of chromosomal genes of *R. leguminosarum* bv. *viciae* (Young et al. 2006) and *R. etli* (González et al. 2006). The largest *pss* gene cluster includes the *pss* genes encoding specific glycosyl transferases, epimerases, and deacetylases involved in the biosynthesis of the EPS repeating unit, genes encoding proteins engaged in polymerization and transport of EPS, and other genes encoding enzymes that modify EPS (González et al. 2006; Skorupska et al. 2006). Most of these genes and their specific roles in EPS production have not been studied to date.

Unlike with *Sinorhizobium meliloti*, knowledge of regulation of *pss* gene expression in *R. leguminosarum* is only fragmentary. Previously, the *psi* and *psr* regulatory genes have been described in *R. leguminosarum* bv. *phaseoli* (now *R. etli*) (Lachford et al. 1991; Mimmack et al. 1994a,b). Even though a *psiA* mutant was not altered in its ability to produce EPS, it elicited empty nodules without infection threads and bacteria on *Phaseolus* spp. (Borthakur and Johnston 1987; Lachford et al. 1991). The increased copy number of *psiA* prevented the production of EPS in *R. leguminosarum* bvs. *phaseoli* and *viciae* and abolished nodulation ability of their respective hosts. The inhibitory effect of multiple copies of *psiA* on nodulation and EPS production could be overcome in the presence of additional copies of *psr* or *pssA* encoding the first glucosyl-IP-transferase (Lachford et al. 1991; Mimmack et al. 1994a,b). An *exoR* regulatory gene with extensive similarity to *exoR* of *S. meliloti* has been identified on the chromosome of *R. leguminosarum* bv. *viciae* (Reeve et al. 1997). An *exoR* mutant produced three times as much EPS as the wild-type strain, which suggested the role of this gene in negative regulation of EPS synthesis.

An *rosR* gene encoding a transcriptional regulatory protein has been identified in *R. etli* CE3 (Araujo et al. 1994; Bittinger et al. 1997). An *rosR* deletion mutant formed mucoid, domed colonies, efficiently nodulated bean plants, and produced EPS that was identical to that produced by the wild-type strain. However, the mutant was significantly impaired with respect to nodulation competitiveness and nodule occupancy compared with the parental strain (Bittinger et al. 1997). *R. etli* RosR regulates more than 50 genes affecting the expression of many functionally diverse genes, as revealed by genome-wide genetic screening (Bittinger and Handelsman 2000).

RosR of *R. etli* shares significant identity with the Ros protein of *Agrobacterium tumefaciens* (Chou et al. 1998). Ros protein recognizes and binds the so-called *ros*-box, a 40-bp A:T-rich sequence located within the operators of *virC* and *virD* and

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upstream of the *ipt* gene. Ros negatively regulates *virC* and *virD* while positively affecting EPS production. Mutations in the *ros* gene result in derepression of *virC* and *virD* and the loss of succinoglycan production (Chou et al. 1998).

RosR of *R. etli* also displays high amino acid sequence similarity with MucR, which acts as a transcriptional repressor of galactoglucan (EPS II) production and an activator of succinoglycan (EPS I) production in *S. meliloti* 2011 (Becker et al. 1997; Keller et al. 1995). The *mucR* mutant produced EPS II, whereas only very small amounts of low molecular weight EPS I oligosaccharides were secreted (Keller et al. 1995; Zhan et al. 1989). MucR repressed *exp* genes encoding EPS II biosynthesis enzymes but had a weak or no effect on transcription of the *exo* genes that encode EPS I synthesis proteins (Bertram-Drogatz et al. 1998; Glazebrook and Walker 1989; Keller et al. 1995; Quester and Becker 2004). However, in *S. meliloti* EFB1, MucR is essential for galactoglucan production and *mucR* mutants are completely nonmucoid (Martin et al. 2000).

The C-terminal part of MucR/Ros proteins contains an amino acid sequence that reveals similarity to the Cys₂His₂-type zinc-finger motif, which was found primarily in DNA-binding proteins of eukaryotic origin (Berg and Shi 1996; Cooley et al. 1991). Four conserved residues are ligands for a central zinc ion that stabilizes small globular domain (i.e., the finger domain) (Pabo and Sauer 1992). Eukaryotic transcription factors using this motif typically contain tandem arrays of such C₂H₂ zinc fingers that bind to neighboring sites on the DNA. Each finger has a conserved $\alpha\beta\beta$ structure and the amino acids on the surface of the α -helix contact the bases in the major groove. MucR/Ros proteins contain only one zinc-finger motif. In Ros of *A. tumefaciens*, the peptide loop spacing of the zinc finger is 9 amino acids (aa) as opposed to the invariant 12 aa in the classical C₂H₂ motif (Chou et al. 1998).

In this study, we present the results of *rosR* gene identification in *R. leguminosarum* bv. *trifolii* strains 24.2 and TA1. The *rosR* gene undergoes a complex transcriptional control, as evidenced by mapping of several regulatory sites in the upstream region of *rosR* and deletion analysis of this region. The *rosR* gene is subject to negative autoregulation by its own protein product. Using an electrophoretic mobility shift assay (EMSA), we assayed binding of the purified (His)₆RosR protein to an RosR-box sequence identified in the *rosR* upstream region. The *rosR* mutant formed dry, wrinkled colonies and elicited nodules on clover plants, but did not fix nitrogen.

RESULTS

Cloning of *rosR* gene and sequence analysis.

Using the genomic sequence of *R. etli* (Bittinger et al. 1997) as a guide in primer design, the *rosR* homolog was isolated from *R. leguminosarum* bv. *trifolii* 24.2 and TA1 strains by polymerase chain reaction (PCR) amplification. After amplification by PCR, *rosR* genes of 24.2 and TA1 strains were cloned into pUC19 to form the pB31 and pB35 plasmids, respectively, and the 1,174-bp insert of pB31 and 856-bp insert of pB35 were sequenced. In both cases, sequence analysis indicated the presence of a 432-bp-long open reading frame (ORF) with a putative ribosome binding site (AGGAGA) located 6 bp upstream of the potential ATG translation start codon (Fig. 1). Downstream of the TGA stop codon, a palindrome sequence of *rho*-independent transcription termination sites was found. The sequences of both *rosR* ORFs were identical; therefore, in further study, only the *rosR* gene of Rt24.2 strain was analyzed.

The predicted protein product of the *rosR* coding region was composed of 143 aa (15.7 kDa) and was highly similar to *R. etli* RosR (98% identity at the protein level), *S. meliloti* MucR (80% identity), *A. tumefaciens* Ros (80% identity), *A. radio-*

bacter RosAR (80% identity), and *Rhizobium* sp. strain NGR234 MucR (75% identity). Homologs of the *rosR* gene also have been found in other rhizobia (e.g., *Mesorhizobium loti* MAFF303099 [accession nos. BAB54066 and BAB52589] and *Bradyrhizobium japonicum* [accession number BAC48333]). Southern hybridization with the *rosR* gene as a probe showed one hybridization site, indicating a single *rosR* locus in Rt24.2 and RtTA1 strains (data not shown).

As with other proteins of the Ros/MucR subfamily, a putative zinc-finger domain was found at the carboxy-terminal half of the RosR protein of Rt24.2. It was composed of 19 aa residues, including two conserved cysteines (C) and two conserved histidines (H) in a C-N₂-C-N₉-H-N₃-H-type motif (Fig. 1). In this type of regulatory protein, one atom of Zn is tetrahedrally coordinated by the conserved C and H residues composing the finger, which interacts with short runs of guanine residues in DNA (Chou et al. 1998).

Functional analysis of *rosR* upstream region.

Computer analysis of the *rosR* upstream region revealed the presence of several regulatory sequences, including the RosR-box (position -133 to -112 bp relative to the start codon) (Fig. 1). The RosR-box is a 22-bp-long sequence containing two nine-nucleotide inverted repeats separated by four G and is the consensus sequence identified as the binding site for MucR/Ros transcriptional regulators in rhizobia (Bertram-Drogatz et al. 1997; Keller et al. 1995). This sequence is identical to the Ros-box of *R. leguminosarum* bv. *viciae* (Young et al. 2006), almost identical to *R. etli* (Bittinger et al. 1997), and similar to the Ros-box of *A. radiobacter* (Brightwell et al. 1995) and *A. tumefaciens* (Cooley et al. 1991). The sequence of the MucR-box in *S. meliloti* (Keller et al. 1995) is most divergent (Fig. 2).

Upstream of the *rosR* coding region, a putative promoter with a score of 0.95 (position -355 to -278 bp) was identified. In the in silico predicted sequence, two putative promoters (P1 and P2), highly similar to the eubacterial sigma factor (σ^{70}) binding site (Wösten 1998), were found: one -35 and -10 consensus sequence with an optimal spacing of 17 bp (5'-TTGGC GN₁₇TATTTG), and another -35 and -10 putative promoter with longer spacing between two hexamers (5'ATGCAAN₂₀TA CAAT) (Fig. 1). Apart from the promoters, at position -169 to -125 relative to the start codon, three repeats of a conserved T-(N₁₁)-A element were found. The element comprises a LysR motif present in many promoters regulated by LysR-type transcriptional regulators (Schell 1993), among them the NodD protein that positively controls the synthesis of the nodulation factor in rhizobial symbioses (Long 2001) and SyrM protein that belongs to the family of transcriptional activators of EPS I production in *S. meliloti* (Dusha et al. 1999). The third T-(N₁₁)-A repeat partially overlaps the RosR-box.

To functionally analyze the putative *rosR* promoter, a set of plasmids with 5'-end (pEP0-pEP7) and 3'-end deletions (pEP8-pEP12) of the promoter region fused to a promoterless *lacZ* gene were constructed (Fig. 3). *E. coli* DH5 α cells were transformed with these plasmids, which subsequently were transferred conjugally to the wild-type Rt24.2 strain. β -galactosidase activities in DH5 α and Rt24.2 strains carrying plasmids with different *lacZ* fusions were assayed (Fig. 3). The pEP0 plasmid containing the longest promoter fragment revealed a significantly lower activity in DH5 α and slightly lower activity in Rt24.2 when compared with the next transcriptional fusion (pEP1). Starting from -357 bp (pEP2), the progressive 5'-end deletions reduced the *rosR* expression level in both genetic backgrounds (Fig. 3). In *E. coli*, a nearly three-fold reduction of LacZ activity was observed for the -281 fusion (plasmid pEP3) relatively to the -403 fusion (pEP1), and then a gradual decrease of activity up to the -219 fusion

(pEP4). In Rt24.2, the reduction of LacZ activity for the -281 fusion (pEP3) was more drastic (approximately 6.5 times) and, for the -235 fusion (pEP3C), the LacZ activity was practically at the background level. Overall, deletion of the two putative promoters containing the -35 to -10 consensus elements (Fig. 1)

resulted in complete reduction in β -galactosidase activity in both backgrounds.

When the *rosR* upstream sequence (Fig. 1) was further analyzed, two sequences highly similar to cAMP-CRP binding sites in *E. coli* (Ebright et al. 1989) were identified. Specific

Fig. 1. Nucleotide sequence of 1,174-bp fragment containing *rosR* gene of *Rhizobium leguminosarum* bv. *trifolii* 24.2 with the upstream region. The deduced amino acid sequence of *rosR* is given in the single-letter code. The putative C₂H₂ motif in RosR protein, RosR-box, and cAMP-CRP binding sites are shaded in light gray. P1 and P2 -35 and -10 promoter sequences are marked by dark-gray shaded boxes. Transcription start sites are marked by an angled arrows. Putative promoters and palindromic terminator sequences are underlined. The ribosome binding site (rbs), translation start codon, and short, direct repeats (DR) are boxed. Long inverted repeats (IR1 and IR2) are overlined with arrows. LysR motifs are underlined and marked with stars. Overlined short arrows indicate location of primers used for polymerase chain reaction amplification of DNA fragments of the upstream *rosR* region. Shaded box in +177 position of *rosR* indicates deletion of C nucleotide resulting in a frame-shift mutation.

binding sites for cAMP-CRP usually are located upstream of the promoters and are essential for transcriptional activation or repression. In the case of *rosR*, the first imperfect inverted palindromic sequence, GC-TGTGA-N₇-TTGCT-GG, was located upstream of the P1 promoter. The second cAMP-CRP binding site (i.e., AT-TGTGA-N₇-TCGTA-CT) was found downstream of the P1 promoter and partially overlapped the -10 sequence of P2 (Fig. 1). It was identical (first pentamer) or highly similar (second pentamer) to the consensus sequence AAN-TGTGA-N₆-TCACA-T, which is responsible for binding the CRP dimer in complex with cAMP in *E. coli*, resulting in the regulation of transcription (Kolb et al. 1993).

To assess the significance of both the cAMP-CRP sequences in *rosR* expression and their probable responsiveness to the carbon source, Rt24.2 strain derivatives carrying either i) plasmid pEP1 with the entire *rosR* upstream sequence; ii) plasmid pEP3A lacking the entire P1 promoter and -35 hexamer of P2 but with the second, more conserved AN-TGTGA-N₇-TCGTA-NT element; iii) plasmid pEP3B lacking the first pentamer of the second cAMP-CRP binding site; and iv) plasmid pEP3C with promoters and both cAMP-CRP binding sites deleted were examined. The strains were grown in M1 minimal medium supplemented with glycerol or glucose as sole carbon source and LacZ activity was measured (Fig. 4A). In strains carrying pEP1 and pEP3A plasmids, 40 and 32% reduction, respectively, in LacZ activity was observed in the presence of glucose compared with the activity in the presence of glycerol. In Rt24.2 derivative carrying pEP3C with both cAMP-CRP binding sites deleted (but also no promoters present), β -galactosidase activities were very low regardless of the carbon source (Fig. 4A). These results indicate that *rosR* expression depends on the nature of the carbon source and is moderately affected by glucose. Both P1 and P2 promoters (Fig. 1) and at least the downstream putative cAMP-CRP binding site are essential for the transcriptional activation of *rosR* (Fig. 4A).

To further examine probable cAMP-CRP-mediated activation of *rosR* transcription, pEP constructs bearing the truncated promoter region fused to promoterless *lacZ* were transferred into *E. coli cyaA* mutant (VH1000cyaA) and a wild-type strain. *E. coli* strains harboring mutations of the *cya* gene (encoding adenylate cyclase) do not synthesize cAMP and fail to respond to catabolite repression by glucose. The *E. coli* derivatives were grown in the presence of glycerol or glucose and LacZ activities were measured (Fig. 4B and C). We observed

that *rosR* expression was moderately reduced in the presence of glucose in an *E. coli* wild-type strain carrying pEP1 to pEP3A plasmids (Fig. 4B). The level of repression did not exceed 50% in the wild-type strain. In the case of pEP3B fusion lacking the first pentamer, the responsiveness of *rosR* transcription to glucose disappeared (Fig. 4B).

In the *E. coli cyaA* mutant carrying the same fusions as the *rosR* upstream region, LacZ activity was practically invariable, regardless of the carbon source (Fig. 4C), indicating that cAMP is important for the responsiveness of *rosR* transcription to glucose. Moreover, the level of LacZ activity in the *E. coli cyaA* mutant bearing fusions pEP1 to pEP3A was lower (approximately 30 to 35%) when grown in the presence of glycerol compared with the *E. coli* wild-type strain. This suggested positive regulation of *rosR* by cAMP-CRP.

Inverted repeats IR1 (CCTCAGTTT) and IR2 (AAACTGAGG) and two directly repeated short CAAGT sequences that might function as potential regulator binding sites also were detected in the *rosR* upstream region (Fig. 1).

The study of the *rosR* upstream region was supplemented by analysis of β -galactosidase activity in *E. coli* and Rt24.2 strains bearing transcriptional fusions of the 3'-end deleted promoter region with promoterless *lacZ* gene (Fig. 3). In both backgrounds, deletion of the -35 to -10 consensus of P1 and P2 elements (pEP12) resulted in total lack of LacZ activity. The highest LacZ activity was observed in strain Rt24.2 carrying pEP11 plasmid that contained the entire P1 promoter but was lacking the cAMP-CRP downstream element. LacZ activity of pEP11 was threefold higher compared with the pEP10 plasmid additionally bearing the P2 promoter and cAMP-CRP binding site. In pEP9, increase in LacZ activity (by a factor of 1.58) was observed in comparison with pEP10, indicating stimulatory effect of the IR1 and IR2 inverted repeats on *rosR* transcription. A low level of LacZ activity in pEP8 might be explained by the presence of the Ros-box bound by RosR protein, which would result in decreased *rosR* transcription. This indicated *rosR* autoregulation by its own protein product. It also is possible that the decrease in *rosR* transcription in pEP8 versus pEP9, in both genetic backgrounds, was caused by interaction of global regulatory LysR-type proteins with the LysR motif, synergistically with the effect of autoregulation (Fig. 3).

In conclusion, transcription of the *rosR* gene in strain Rt24.2 is directed from the P1 and P2 promoters. In the presence of glucose, transcriptional activity of *rosR* is reduced, indicating

Fig. 2. Comparison of sequences that show similarity to *Rhizobium leguminosarum* bv. *trifolii* RosR binding site in the *rosR* upstream region. Nucleotides conserved in all sequences are shaded. Arrows indicate inverted repeats.

rosR regulation by catabolic repression. Moreover, additional regulatory elements, first and foremost the RosR-box, modulate *rosR* transcription.

Mapping of the start site of *rosR* transcription by primer extension analysis.

To determine the transcription start site of *rosR*, fluorescent primer extension analysis was carried out with a 5'-FAM-labeled oligonucleotide complementary to the 5' end of the *rosR* gene. Two primer extension products displaying similar abundance were detected (Fig. 5). One of them was 298 bp in length and the first nucleotide to be transcribed was an adenine (A) located -273 nucleotides (nt) upstream of the proposed *rosR* translation start codon and 8 nt downstream of the -10 (TATTTG) sequence of the P1 promoter (Fig 1). The second primer extension product was 265 bp in length and the first transcribed nucleotide was a guanine (G) that was located -240

nt upstream of the *rosR* translation start codon and 6 nt downstream of the -10 (TACAAT) sequence of the predicted P2 promoter. Moderate LacZ activity in pEP3, pEP3A, and pEP3B fusions and complete lack of LacZ activity in the pEP3C fusion with both promoters deleted confirms the transcriptional activity of the P2 promoter and the presence of the second -240-nt transcription start site (Figs. 1 and 3).

Overproduction of RosR protein in *E. coli*.

To overproduce Rt24.2 RosR as a recombinant N-terminally His-tagged protein, plasmid pQE450 was used (details below). A substantial portion of the (His)₆RosR protein was produced in an insoluble aggregated form. The soluble fraction of the protein was subjected to purification by Ni-NTA affinity chromatography. We have not observed the deleterious effect of (His)₆RosR overproduction on *E. coli* growth described in the case of MucR (Bertram-Drogatz et al. 1997) and Ros protein

Fig. 3. A, Physical and genetic map of Rt24.2 region encompassing *rosR* gene. Abbreviations: B, *Bam*HI; P, *Pst*I; H, *Hind*III; S, *Sph*I; N, *Not*I. **B**, Transcriptional activity of *rosR* putative promoter assayed in *Escherichia coli* DH5 α and Rt24.2 strains carrying pMP220 plasmid with fragments of the *rosR* upstream region fused with promoterless *lacZ* gene. Lengths of polymerase chain reaction products in particular plasmids are shown as horizontal lines. *E. coli* strains were grown in Luria-Bertani medium; Rt24.2 strains were grown in 79CA medium with mannitol as carbon source. β -Galactosidase activities for the pEP0 to pEP12 plasmid fusions are given as the averages from at least three or four independent experiments.

(Cooley et al. 1991). Purified (His)₆RosR was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a major band of approximately 18 kDa was detected, in accordance with the deduced molecular weight of 15.7 kDa for the *rosR* gene product and accounting for the presence of the N-terminal His₆-tag (Fig. 6A). The identity of (His)₆RosR was confirmed by Western blotting with anti-His₅-tag antibodies (Fig. 6B). In the SDS-PAGE analysis of RosR protein, in addition to a major 18-kDa band, a band of approximately 38 kDa was detected.

Considering the possibility of self-association of RosR, in vitro chemical cross-linking with glutaraldehyde was performed.

After treating RosR with even low (0.01%) concentrations of glutaraldehyde, dimeric and trimeric forms were detected by Western blotting with anti-His₅ antibodies (Fig. 6C). In the presence of an increasing concentration of glutaraldehyde, increased amounts of multimeric forms of RosR appeared at the expense of the monomeric one (Fig. 6C). This implies that RosR protein could function at least as a dimer.

Binding of (His)₆Ros protein to the RosR-box.

We performed EMSA to study DNA binding of purified (His)₆RosR fusion protein. For these experiments, we used a PCR-amplified 185-bp digoxigenin (DIG)-labeled DNA frag-

Fig. 4. Effect of carbon source on transcriptional activity of the upstream region fragments of the *rosR* gene assayed in **A**, Rt24.2 and in **B**, *Escherichia coli* wild-type and **C**, *cyaA* mutant strains. Strains were grown in M1 medium supplemented with Tc and 1% glucose or 1% glycerol for 18 h, and β -galactosidase activities were measured. Data shown are the mean of $\pm$ standard deviation of four replicates.

ment I (encompassing the region from –193 to –9 nt), matching the predicted RosR-box site in the *rosR* upstream region and increasing concentration of the purified (His)₆RosR (Fig. 7A). The observed retardation was dependent on protein concentration with an increasing amount of protein, the molecular weight of the DNA-(His)₆RosR complex became visibly larger (Fig. 7A). However, (His)₆RosR at 2 ng μL^{-1} was sufficient to shift both the total amount of DNA fragment I and the shorter DNA 78-bp fragment II (region from –158 to –81 nt) present in the samples (Fig. 7B).

To examine the DNA binding specificity of (His)₆RosR, electrophoretic mobility shift competition assays with unlabeled oligonucleotide competitor DNA fragments I to IV were carried out (Fig. 8). A DIG-labeled DNA RosR-box containing fragment I was used as a target for (His)₆RosR and oligonucleotides I to IV as competitors in all reactions (Fig. 8A through D). DNA fragment I (185 bp) had to be in a 100-fold excess over labeled DNA in order to effect full competition, as may be seen by the absence of a visibly shifted band (Fig. 8A). The shorter fragment II (78 bp), comprising nucleotides –158 to –81 upstream of *rosR*, was more effective in competition, because a 10-fold excess of DNA was sufficient for full competition (Fig. 8B). This probably might be explained by a higher diffusion rate of smaller fragments (Bertram-Drogatz et al. 1997). When DNA fragment III (160 bp) containing the Ros-box of *A. tumefaciens* was used as a competitor, it had to be in a 500-fold excess to sufficiently compete with DNA fragment I (Fig. 8C). Furthermore, DNA fragment IV (158 bp) containing the MucR-box even in 500-fold excess did not compete with labeled DNA (Fig. 8D). These data indicated high specificity of (His)₆RosR binding to the RosR-box in fragment II and lower specificity binding with fragment I. In competition assays with heterologous fragments III and IV, the binding was less specific.

rosR mutant and complementation analysis.

To assess the function of RosR in *R. leguminosarum* bv. *trifolii* and its possible engagement in EPS biosynthesis regulation, as was done in the case of Ros/MucR proteins (Bertram-Drogatz et al. 1997; Cooley et al. 1991; Keller et al. 1995), we have attempted to construct a *rosR* deletion mutant lacking the entire ORF. A construct in which the *rosR* gene was replaced by a spectinomycin cassette was obtained and introduced into the Rt24.2 wild-type strain in order to generate the null mutation. However, we were not able to select desirable transconjugants. Thus, we subcloned the *rosR* ORF from pB31 plasmid into suicide vector pK19mobGII and the construct was used to transform *E. coli* S17-1 (discussed below). Verification of insert correctness of several recombinant plasmids by sequence analyses revealed that the *rosR* ORF of one of the plasmids, named pEPB12, had one nucleotide deletion (ΔC in position +177 nt) (Fig. 1). The deletion caused a frame shift mutation starting with the 60th amino acid residue and created a nonsense codon (TGA) in position +277, which resulted in synthesis of a shorter putative RosR protein (92 instead of 143 aa). In addition,

we identified nucleotide substitutions resulting in L38P and D41E mutations of RosR. We used pEPB12 plasmid to construct the *rosR* mutant by homologous recombination. pEPB12 was transferred from *E. coli* S17-1 to *R. leguminosarum* bv. *trifolii* 24.2 by conjugation and the clone, named Rt2440, with altered, dry, wrinkled colony morphology was isolated (Fig. 9A). The Rt2440 mutant strain induced nodules on clover but inoculated plants appeared yellowish, indicating inefficient symbiosis (Table 1). The amount of EPS produced by the Rt2440 mutant was 35% of wild-type strain Rt24.2. Symbiotic and EPS deficiency defects in the Rt2440 mutant were complemented by the wild-type *rosR* allele (Table 1).

The function of Rt24.2 RosR also was studied by complementation of *rosR* mutation in the closely related species *R. etli* CE3 Δ *rosR*. The mutant forms very characteristic domed colonies on agar plates because of their highly hydrophobic surface, whereas the parental strain CE3 produces flat colonies with hydrophilic cell surface (Bittinger et al. 1997). To study the possibility of restoring wild-type morphology of the CE3 Δ *rosR* mutant by *rosR* of Rt24.2, plasmid pRC24 was introduced by conjugation. As a result, flat, mucoid wild-type colony morphology was restored in mutant CE3 Δ *rosR* (Fig. 9B). EPS production was measured in the exconjugants CE3 Δ *rosR*(pRC24) and compared with the level of EPS produced by mutant CE3 Δ *rosR* and wild-type CE3 strains. Nearly two times more EPS was produced in CE3 Δ *rosR*(pRC24) (glucose equivalents at 1.55 $\mu\text{g}/\mu\text{g}$ of protein) than in wild-type strain CE3 (glucose at 0.93 $\mu\text{g}/\mu\text{g}$ of protein) and three times more than in the mutant strain CE3 Δ *rosR* (glucose equivalents at 0.54 $\mu\text{g}/\mu\text{g}$ of protein).

Complementation tests in which plasmid pRC24 was conjugally transferred into the *mucR* mutant of *S. meliloti* (Keller et

Fig. 5. Primer extension analysis of *rosR* promoter. Fluorogram obtained from a primer extension assay with 20 μg of total RNA and a FAM-labeled primer corresponding to position +25 to –2 bp of the *rosR* sequence. Length of cDNA primer extension products is given above the peaks. Two high peaks are the GeneScan-500 ROX internal lane standards with the size of 247 and 300 bases.

Table 1. Complementation of the *Rhizobium leguminosarum* bv. *trifolii* 2440 *rosR* mutant by pRC24 carrying the *rosR* gene^a

Strain, plasmid	Nodule no. per plant (days after infection)					Shoot wt (mg plant ⁻¹) ^b	EPS (mg mg ⁻¹) ^c
	5	15	21	30	35		
Rt2440	0.13	2.5	4.2	7.1	7.7	45.8 $\pm$ 11.2 (62.7%)	0.34 $\pm$ 0.03 (35%)
Rt2440(pRC24)	0.35	4.6	7.9	12.4	13.7	66.5 $\pm$ 12.8 (91%)	1.15 $\pm$ 0.08 (121%)
Rt24.2	0.5	6.9	9.5	15.3	16.8	73.0 $\pm$ 14 (100%)	0.95 $\pm$ 0.07 (100%)
Uninoculated clover	...	...	...	...	...	30.2 $\pm$ 7.5 (41.4%)	...

^a Given values ($\pm$ standard deviation) are averages of two independent experiments with 30 plants for each treatment.

^b Shoot fresh weight.

^c Exopolysaccharide (EPS) production (Glc equivalents in mg/mg⁻¹ of protein).

al. 1995) or *ros* mutant of *A. tumefaciens* (Cooley et al. 1991) did not yield positive results, signifying that the *rosR* gene of *R. leguminosarum* bv. *trifolii* 24.2 did not complement *mucR* or *ros* mutations in the respective mutant strains.

To assess the effect of multiple copies of *rosR* on EPS production and symbiosis with clover, plasmid pBR24 was conjugally transferred into strain Rt24.2. The exconjugants Rt24.2 (pBR24) produced increased amount of EPS (128% of the wild-type Rt24.2 strain) and induced more nodules on clover (data not shown). These results suggested that RosR positively regulates EPS synthesis in *R. leguminosarum* bv. *trifolii* 24.2.

Regulatory function of RosR.

To study regulation of *rosR* transcription, we introduced pEP1 fusion, where expression of a promoterless *lacZ* gene was under the control of the entire upstream region of *rosR* into wild-type Rt24.2 and Rt2440 mutant strains (Table 2). Activity of β -galactosidase was twofold higher in the Rt2440 *rosR* mutant than in the wild-type strain, which indicated auto-regulation of *rosR*. In the wild-type background, LacZ activity

was decreased in the presence of both the pEP1 fusion and pBR24 with the *rosR* gene, confirming the repressive effect of RosR on its own transcription.

The effect of RosR on transcription of the *pssA* gene encoding the first IP-glucosyltransferase in the pathway of *R. leguminosarum* EPS repeating unit synthesis (Pollock et al. 1998) was examined. In the upstream region of the *pssA* gene of

Table 2. Analysis of regulatory function of the *rosR* gene

Strain and plasmids	β -Galactosidase activity (Miller units) ^a	Fold
Rt24.2 (pEP1)	2,896 $\pm$ 113	1
Rt2440 (pEP1)	5,854 $\pm$ 241	2
Rt24.2 (pEP1, pBR24)	1,917 $\pm$ 102	0.66
Rt24.2 (pSF2)	273 $\pm$ 20	1
Rt2440 (pSF2)	164 $\pm$ 14	0.6
Rt24.2 (pSF2, pBR24)	408 $\pm$ 32	1.49

^a Given values ($\pm$ standard deviation) are averages of three independent experiments.

Fig. 6. A, Purification of $(\text{His})_6\text{RosR}$ protein overproduced in *Escherichia coli* M15 harboring pQE450. Soluble proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lane 1, proteins from uninduced *E. coli* M15(pQE450) cells; lane 2, proteins after induction with 1 mM IPTG; lane 3, purified $(\text{His})_6\text{RosR}$ protein eluted from Ni-NTA agarose, and lane 4, proteins molecular mass standards (kDa). B, Western-blotting analysis with anti- His_5 antibodies of soluble proteins from *E. coli* M15(pQE450) producing $(\text{His})_6\text{RosR}$, after 3 h of induction with 1 mM IPTG, lane 2, $(\text{His})_6\text{RosR}$; and lane 1, purified $(\text{His})_6\text{RosR}$. Positions monomers and dimers are indicated. C, Western-blotting analysis of purified $(\text{His})_6\text{RosR}$ protein with anti- His_5 antibodies after treatment with increasing concentration of glutaraldehyde. Positions of monomers, dimers, trimers, and multimers are indicated. Molecular weight standards (kDa) are indicated at the right.

strain Rt24.2, an RosR-binding site putatively able to bind RosR was identified earlier (Janczarek and Skorupska 2004). Plasmid pSF2 containing a transcriptional fusion of the *pssA* upstream region with promoterless *lacZ* gene was introduced into Rt24.2 and the Rt2440 *rosR* mutant. Absence of RosR activity in the mutant background resulted in a decrease of β -galactosidase activity (by a factor of 0.6), indicating an apparent positive effect of the *rosR* gene product on *pssA* transcription (Table 2). In the wild-type strain, in the presence of additional copies of *rosR* (plasmid pBR24), the level of *pssA* transcription was increased by a factor of 1.5. Overall, *rosR* gene transcription was negatively autoregulated by RosR but the *pssA* gene encoding a key enzyme in EPS synthesis was positively regulated.

DISCUSSION

In the present study, the *R. leguminosarum* bv. *trifolii* 24.2 *rosR* gene was identified and overexpressed in *E. coli* as an N-terminally His₆-tagged fusion protein containing a C-terminal conserved zinc-finger DNA-binding domain, as predicted in silico. The Ros/MucR subfamily of C₂H₂ zinc-finger proteins comprises global positive or negative regulators of transcription of genes involved in diverse functions in prokaryotes, one being the regulation of EPS synthesis in *S. meliloti*, *A. tumefaciens*, and *A. radiobacter* (Bittinger and Handelsman 2000, Chou et al. 1998; D'Souza-Ault et al. 1993, Rüberg et al. 1999). RosR of Rt24.2 is very conservative in its amino acid composition and the C₂H₂ zinc-finger motif. Transcriptional regulators that bind DNA usually act as dimers, and the presence of RosR of Rt24.2 as dimeric and multimeric structures was demonstrated in this study. Binding of the (His)₆RosR protein to the RosR-box was evidenced by retardation of 185- and 78-bp DNA fragments, both matching a 22-bp-long consensus sequence. The specificity of (His)₆RosR binding to fragments I and II was demonstrated by competition experiments. Fragment III containing the Ros-box of *A. tumefaciens* (Cooley et al. 1991; D'Souza-Ault et al. 1993) competed with low affinity, because a 500-fold excess of the competitor over fragment I was needed to achieve competition. The RosR-box sequence of Rt24.2 is identical to the RosR-box of *R. legumi-*

nosarum bv. *viciae*, almost identical to the Ros-box of *R. etli*, and similar to the RosR-box of *A. radiobacter* and *A. tumefaciens*. The sequence of the MucR-box of *S. meliloti* is most divergent (Bertram-Drogatz et al. 1998; Keller et al. 1995) and MucR protein did not bind the Ros-box of *A. tumefaciens*, indicating a high-specificity interaction between MucR and its DNA target (Bertram-Drogatz et al. 1997).

In addition to the RosR binding sequence, several regulatory sequences have been mapped in the upstream region of Rt24.2 *rosR*, indicating a complex transcriptional control of *rosR*. A deletion analysis of the region upstream of *rosR* revealed a dependence of *rosR* transcription on P1 and P2 promoters containing conserved σ^{70} -dependent -35 or -10 motifs. Primer extension mapping detected two transcription start sites. Based on the primer extension data and sequence analysis of the upstream promoter region, separate -35 and -10 consensus sequences could be assigned for each transcript. Based on analyses of the LacZ activities, one could conclude that the P1 promoter is stronger than the P2 promoter, but an insignificant difference in the amount of both cDNA fragments in the primer extension assay indicated a comparable level of the two transcripts. Further experiments are needed to elucidate the activity of these promoters under different conditions.

These promoter sequences also were active in *E. coli* strains, where we observed a two-fold higher LacZ activity of plasmid fusions compared with native Rt24.2 background, indirectly indicating autoregulation of the *rosR* gene. Negative autoregulation of the *rosR* transcription also was evidenced by increased *rosR* expression in the absence of RosR in the Rt2440 mutant background. The *rosR* expression was visibly depressed in the presence of additional copies of *rosR*. Similarly, negative autoregulation of the *ros* gene in *A. tumefaciens* (Cooley et al. 1991) and *mucR* in *S. meliloti* (Keller et al. 1995) has been reported.

Plasmid fusions carrying 5'- and 3'-end deletion fragments of the *rosR* upstream region with the reporter *lacZ* gene allowed us to examine the significance of cAMP-CRP binding sites in activation of *rosR* transcription. The 5'-end deletions of both promoters reduced transcription to the background level in both Rt24.2 and *E. coli*. Transcription assays of *rosR* in the *E. coli* mutant lacking *cyaA*, the gene encoding adenylate cyclase, showed that, in the absence of cAMP, the *rosR* promoter ceased

Fig. 7. A, Electrophoretic mobility shift analysis with purified (His)₆RosR of increasing concentration. Digoxigenin-labeled DNA **A**, fragment I and **B**, fragments I and II matching a consensus RosR-box were added to all reactions to give a final concentration of 0.1 ng μl^{-1} . Protein concentration in **B** was 2 ng μl^{-1} .

to be responsive to glucose. We conclude that cAMP-activated CRP protein is able to recognize specific DNA sites and positively regulates *rosR* expression. Unexpectedly, a 3'-end deletion construct containing both the promoters and cAMP-CRP binding site (pEP10 fusion) showed lower transcription than pEP11 fusion. This could be explained by the fact that bacteria were cultured in complete medium and, under these conditions, the cAMP-CRP complex probably was not formed. This effect was not observed in *E. coli* background where LacZ activity increased with increasing length of the upstream region.

Computer search for cAMP-CRP binding sites in the *rosR* promoter region of *R. etli* revealed a sequence identical to the one found in *R. leguminosarum* bv. *trifolii* 24.2, located at the

same distance (–244 nt) from the *RosR* start codon. The cAMP-CRP binding site located upstream of P1 promoter also was nearly identical (two substitutions). In the cases of *S. meliloti* and *A. radiobacter*, conservative cAMP-CRP binding sites (*Sm*: GT-TGTGA-N₆-TCCGT-TG; *Ar*: CG-TGTCA-N₆-TCACT-AA) downstream of the main promoter were found to be highly similar to *E. coli* consensus sequence AAN-TGTGA-N₆-TCA CA-T. The cAMP-CRP binding sites located upstream from the main promoter were much more divergent in comparison to consensus.

Formation of the cAMP-CRP complex in *E. coli* and related bacteria is a prerequisite to transcriptional activation of catabolic genes. Specific binding sites for the complex usually are located upstream of these promoters; however, CRP-dependent promoters (class III promoters) regulated by multiple sites or by CRP in combination with one or more additional protein factors also have been described (Harman 2001). Although the mechanism of cAMP-CRP complex function in rhizobia remains unknown, multiple *cya* genes encoding adenyl cyclases have been identified and some of them have been cloned and characterized (Sharypova et al. 1999; Tellez-Sosa et al. 2002). In addition, a hypothetical ORF, Crp1 (accession number DQ384109), with homology to the effector domain of the CAP family of transcription factors, was identified recently in the genome of *R. leguminosarum* bv. *trifolii* TA1.

Because *RosR* is considered to be an activator of acidic EPS biosynthesis in *R. leguminosarum* bv. *trifolii* and its intracellular concentration might be regulated by glucose, a possibility exists of a regulatory linkage between carbon metabolism and EPS production. There are several lines of evidence indicating the dependence of EPS production in *R. leguminosarum* bv. *trifolii* on the identity of the carbon source (Zevenhuizen 1986). Carbon source-dependent regulation of expression of *pss* genes in *R. leguminosarum* bv. *trifolii* could be directed by the *rosR* gene product that might function as a positive transcriptional regulator of the *pssA* gene. Possible regulatory effect of *RosR* protein on other *pss* genes remains to be elucidated.

In addition to the regulatory sites discussed above, several other elements putatively involved in *rosR* regulation were identified upstream of *rosR*, among them a three-times-repeated LysR-binding motif. LysR-type transcriptional regulators containing helix-turn-helix DNA-binding domain, such as SyrM protein, are involved in regulation of EPS production in *S. meliloti* (Barnett and Long 1997, Dusha et al. 1999). Under nitrogen limitation, NtrC and SyrM proteins act as positive regulators of succinoglycan production. The significance of LysR elements in *rosR* expression and of other, long and short inverted repeats remains to be elucidated. In conclusion, expression of the *rosR* gene encoding a transcriptional regulator requires complex control on the transcriptional level, possibly involving interaction of different proteins.

Numerous attempts to mutate the *rosR* gene in strain Rt24.2 eventually resulted in the construction of the Rt2440 mutant, which formed colonies with characteristic wrinkled, dry morphology and produced less EPS compared with the wild-type strain. The phenotype of this mutant and the fact that additional copies of *rosR* in strain Rt24.2(pBR24) caused an increase in the amount of EPS in culture supernatant indirectly confirmed positive regulation of EPS synthesis by *RosR*. The Rt24.2(pBR24) strain induced more pink nodules on clover roots than the parental strain and the weight of green part of plants was significantly higher compared with control plants (data not shown). A similar phenomenon was observed in the case of *R. etli* Δ *rosR* complemented by the *rosR* gene, which was more competitive than the parental strain (Bittinger et al. 1997). These data indicate a possible transcriptional activation of several symbiosis-related genes by the overproduced *RosR* protein.

Fig. 8. Binding specificity of purified (His)₆RosR protein with RosR-box studied by electrophoretic mobility shift assays. Digoxigenin (DIG)-labeled DNA fragment I was added to the reactions to give a final concentration of 0.1 ng μ l⁻¹. Protein was added in a final concentration 2 ng μ l⁻¹. Unlabeled competitor oligonucleotides (I to IV) were added in increasing amounts from 2- to 500-fold excess over the DIG-labeled DNA fragment I. **A**, DNA fragment I (185 bp) and **B**, DNA fragment II (78 bp) contain the RosR-box of *Rhizobium leguminosarum* bv. *trifolii*, **C**, DNA fragment III (160 bp) contains the Ros-box of *Agrobacterium tumefaciens*, and **D**, DNA fragment IV (158 bp) matches the MucR-box of *Sinorhizobium meliloti*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used in the present study are listed in Table 3. *R. leguminosarum*, *R. etli*, and *A. tumefaciens* strains were grown in 79CA medium with 1% mannitol or 1% glycerol as carbon source at 28°C (Vincent 1970). *E. coli* strains typically were grown in Luria-Bertani (LB) medium at 37°C (Sambrook et al. 1989). To study the expression of *rosR* in the *Rhizobium* spp. or *E. coli*, the strains were cultured for 24 h in minimal M1 medium (Sambrook et al. 1989) supplemented with 1% mannitol or glucose, and vitamin solution at 2 ml/liter, prepared according to Brown and Dilworth (1975). Antibiotics were supplemented as required at the following final concentrations: for *E. coli*, kanamycin at 25 or 40 µg ml⁻¹, ampicillin at 100 µg ml⁻¹, tetracycline at 10 µg ml⁻¹, and gentamicin at 10 µg ml⁻¹; and for *Rhizobium* and *Agrobacterium* spp., streptomycin at 200 µg ml⁻¹, nalidixic acid at 40 µg ml⁻¹, tetracycline at 10 µg ml⁻¹, gentamicin at 10 µg ml⁻¹, and kanamycin at 40 µg ml⁻¹.

Complementation tests were carry out by triparental conjugation (Figurski and Helinski 1979) using pRC24 plasmid bearing *rosR* of Rt24.2 as donor and *R. etli* CE3Δ*rosR*, *S. meliloti* 3131 *mucR*, or *A. tumefaciens* NTR1 *rosR* mutant strains as recipients. Exconjugants were selected on 79CA medium supplemented with the appropriate antibiotics.

DNA methods.

Standard techniques were used for plasmid isolation, restriction enzyme digestion, ligation, DNA cloning, transformation, and agarose gel electrophoresis (Sambrook et al. 1989). Sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and the ABI Prism 310 sequencer. Database searches were done with the BLAST and FASTA programs available from the National Center for Biotechnology Information (Bethesda, MD, U.S.A.) and European Bioinformatic Institute (Hinxton, U.K.). The putative promoter sequences were predicted using Neural Network Promoter prediction at the Berkeley Drosophila Genome Project.

Cloning of *rosR* gene.

Genomic fragments of Rt24.2 and RtTA1 containing the entire *rosR* ORF were amplified using the following primers:

forward, RosA 5'-GCGGATCCGCGACTTTACCAGATTTA-3' for Rt24.2; forward, RosB 5'-GTCACGCTCTTCGGAATT CAGGGGT-3' for RtTA1; and reverse, RosD 5'-TCGGATCCT GTCGGCAAAGCATAAGA-3' for both Rt24.2 and RtTA1 strains. The introduced *Bam*HI and *Eco*RI recognition sites are underlined. The 1,174-bp PCR product for Rt24.2 and 856-bp fragment for RtTA1 were cloned into the respective sites of pUC19 vector resulting in pB31 and pB35 plasmid, respectively. The inserts of pB31 and pB35 containing the entire *rosR* genes were sequenced. *rosR* Rt24.2 and RtTA1 sequences are available in GenBank database under accession numbers AY683453 and AY683454, respectively.

Construction of plasmids with nested deletions of the *rosR* promoter region.

To construct the plasmids with deletions of the *rosR* promoter region, plasmid pMP220 carrying a promoterless *lacZ* gene was used. For each 5'-end deletion fusion, an upstream primer for the putative promoter region of *rosR*, with introduced *Eco*RI restriction site, and the common reverse primer RR1, which annealed within the coding sequence of *rosR* with introduced *Xba*I restriction site, were used to amplify DNA fragments from pB31. For construction of 3'-end deletion fusions, common pEP1 primer and a set of reverse (pEP8 to pEP12) primers were used (Table 4). The PCR products were digested with *Eco*RI and *Xba*I restriction enzymes and inserted between the *Eco*RI and *Xba*I sites of pMP220 plasmid, resulting in plasmids pEP0 to pEP12 containing nested deletions of *rosR* promoter fused to a promoterless *lacZ* gene. The constructed plasmids were transferred from *E. coli* DH5α into *R. leguminosarum* bv. *trifolii* 24.2 by triparental conjugations. β-galactosidase activities were determined by the method of Miller (1972) and expressed in Miller units. The reported values are averages of three to six independent measurements.

Mutagenesis of *rosR*.

The 1,100-bp fragment of pB31 plasmid was PCR amplified using pEP1 and RosD primers. PCR product was digested with *Eco*RI/*Bam*HI enzymes, cloned into the respective sites of the suicide vector pK19mobGII, and *E. coli* S17-1 cells were transformed with this construct. Inserts of several recombinant plasmids were sequenced and, in the insert of the plasmid

Fig. 9. Complementation of the A, *Rhizobium leguminosarum* bv. *trifolii* 2440 and B, *R. etli* Δ*CE3* mutants by pRC24 plasmid containing the *rosR* gene of Rt24.2. Colonies were grown for 7 and 12 days on 79CA medium.

named pEPB12, one nucleotide deletion (Δ C in position +177 nt) was found which caused a frameshift mutation starting with the 60th amino acid residue. The nonsense codon (TGA) in position +277 was created which resulted in synthesis of a shorter putative RosR protein (92 instead of 143 aa) (Fig. 1). In addition, nucleotide substitutions resulting in L38P and D41E mutations of RosR were identified. pEPB12 was transferred from *E. coli* S17-1 to *R. leguminosarum* bv. *trifolii* 24.2 by conjugation and transconjugants were selected on 79CA medium with kanamycin and nalidixic acid. We isolated a clone, named Rt2440, with altered, wrinkled colony morphology. Recombination between pEPB12 and the *R. leguminosarum* bv. *trifolii* 24.2 *rosR* gene was verified by PCR amplification using pEP1 and RosD primers with Rt2440 as a template and *Pfu* polymerase (Roche, Branchburg, NJ, U.S.A.) followed

by sequencing of PCR product. Sequencing of the Rt2440 PCR fragment confirmed the presence of the mutations mentioned above.

Construction of *rosR* containing expression plasmid.

The *rosR* gene was amplified by PCR from the pB31 plasmid as a template using forward primer EK1-5'-CCAGGAG AAAAAGGATCCCGGATA-3' with an introduced *Bam*HI recognition site (underlined) and the reverse primer EK2-5'-GTCGTCGACGCTTTCGAAAAAGCT-3' with an introduced *Sal*I recognition site (underlined) and Ready Mix *Taq* PCR Reaction Mix (Sigma-Aldrich, St. Louis). The PCR product was digested with *Bam*HI and *Sal*I restriction enzymes and inserted into the corresponding sites in pUC19, yielding the pEK450 plasmid. The sequence of PCR product was verified by se-

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Sources or reference
<i>Rhizobium leguminosarum</i>		
bv. <i>trifolii</i>		
Rt24.2	Wild type, Rif ^r , Nx ^r	Skorupska et al. 1995
RtTA1	Wild type, Rif ^r , Sm ^r	Chakravorty et al. 1982
Rt2440	Rt24.2 derivative carrying <i>rosR</i> with one nucleotide deletion	This work
<i>R. etli</i>		
CE3	Spontaneous Sm ^r derivative of CFN42; Sm ^r , Nx ^r	Noel et al. 1984
CE3 Δ <i>rosR</i>	CE3 with <i>rosR</i> deleted; Sm ^r , Nx ^r	Bittinger et al. 1997
<i>Sinorhizobium meliloti</i>		
Rm2011	Wild type, Sm ^r	Casse et al. 1979
Rm3131	Rm2011 <i>mucR31::Tn5</i> , Sm ^r , Km ^r	Keller et al. 1995
<i>Agrobacterium tumefaciens</i>		
NT1REB	Flagellum-free derivative of C58, Sm ^r	Lai and Kado 1998
NTR1	NT1REB with <i>ros</i> mutation, Sm ^r	Cooley et al. 1991
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook et al. 1989
M15	Lac ⁻ , Ara ⁻ , Gal ⁻ , Mtl ⁻	Qiagen
VH1000	Derivative of MG1655, <i>lacZ</i> , <i>lacI</i> , <i>pyrE</i> ⁺	Gaal et al. 1997
VH1000 <i>cyaA</i>	Derivative of VH1000, Δ <i>cyaA::Km</i>	Gaal et al. 1997
Plasmids		
pUC19	Cloning and sequencing vector, Ap ^r	Sambrook et al. 1989
pRK2013	Tra ⁺ helper plasmid, Km ^r	Figurski, Helinski 1979
pRK7813	IncP, <i>mob</i> , Tra ⁻ cosmid vector, Tc ^r	Stanley et al. 1987
pBBR1MCS-5	Mob ⁺ , <i>lacZ</i> α , Gm ^r	Kovach et al. 1995
pREP4	<i>ori</i> p15A, Km ^r , <i>lacI</i> ^q	Qiagen
pK19mobGII	<i>Mob</i> , <i>lacZ</i> α , <i>gusA</i> , Km ^r	Katzen et al. 1999
pQE-32	<i>ori</i> ColE1, Ap ^r , expression vector	Qiagen
pMP220	IncP, <i>mob</i> , promoterless <i>lacZ</i> , Tc ^r	Spaink et al. 1987
pB31	pUC19 with 1,174-bp <i>Bam</i> HI fragment containing <i>rosR</i> of Rt24.2	This work
pB35	pUC19 with 856-bp <i>Eco</i> RI- <i>Bam</i> HI fragment containing <i>rosR</i> of RtTA1	This work
pBR24	pBBR1MCS-5 with 1,174-bp <i>Bam</i> HI fragment containing <i>rosR</i> of Rt24.2	This work
pRC24	pRK7813 with 1,174-bp <i>Bam</i> HI fragment containing <i>rosR</i> of Rt24.2	This work
pEPB12	pK19mobGII with 1,100-bp <i>Eco</i> RI- <i>Bam</i> HI fragment containing <i>rosR</i> of 24.2 with one nucleotide deletion	This work
pEP0	pMP220 carrying the -450-bp upstream region to +243 bp of the <i>rosR</i> coding region	This work
pEP1	pMP220 carrying the -403-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP2	pMP220 carrying the -357-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP3	pMP220 carrying the -281-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP3A	pMP220 carrying the -263-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP3B	pMP220 carrying the -250-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP3C	pMP220 carrying the -235-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP4	pMP220 carrying the -219-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP5	pMP220 carrying the -159-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP6	pMP220 carrying the -96-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP7	pMP220 carrying the -41-bp upstream region to +243-bp fragment of <i>rosR</i> coding region	This work
pEP8	pMP220 carrying the -403-bp to -32 bp of <i>rosR</i> upstream region	This work
pEP9	pMP220 carrying the -403-bp to -185 bp of <i>rosR</i> upstream region	This work
pEP10	pMP220 carrying the -403-bp to -232 bp of <i>rosR</i> upstream region	This work
pEP11	pMP220 carrying the -403-bp to -268 bp of <i>rosR</i> upstream region	This work
pEP12	pMP220 carrying the -403-bp to -348 bp of <i>rosR</i> upstream region	This work
pSF2	pMP220 with one 1,084-bp <i>Sac</i> I fragment carrying promoter region of <i>pssA</i>	Janczarek and Skorupska 2004
pEK450	pUC19 with 450-bp <i>Bam</i> HI- <i>Sal</i> I fragment containing <i>rosR</i> of Rt24.2, Ap ^r	This work
pQE450	pQE32 with 450-bp <i>Bam</i> HI- <i>Sal</i> I fragment containing <i>rosR</i> of Rt24.2, Ap ^r	This work

^a Rif^r, Nx^r, Sm^r, Ap^r, Tc^r, Gm^r, and Km^r = resistant to rifampicin, nalidixic acid, streptomycin, ampicillin, tetracycline, gentamicin, and kanamycin, respectively.

quencing. The insert then was subcloned into the expression vector pQE-32 (QIAexpress; Qiagen, Hilden, Germany), generating plasmid pQE450 for the overproduction of a recombinant RosR carrying an N-terminal His₆-tag.

Primer extension analysis.

Total RNA was isolated from *R. leguminosarum* bv. *trifolii* 24.2 cells using the Trizol reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA was quantitated by determining the absorbance at an optical density at 260 nm (OD₂₆₀) and stored at -80°C. The *rosR* transcriptional start site was identified by an automated fluorescent primer extension method as was described by Lloyd and associates (2005). A 5'-FAM-labeled oligonucleotide primer (5'-CATTGCCGGTCGCTATATCCGTCATA-3') complementary to a region near the 5' end of the *rosR* gene (from -2 to +25 bp) was used. RNA was treated with DNase I (Roche) before use. Primer extension reactions were carried out where FAM-labeled primer at a final concentration of 10 pmol was added to 20 µg of total RNA in the final volume of 20 µl. The cDNA synthesis was performed in one step using AMV RT enzyme (Promega Corp., Madison, WI, U.S.A.) (Lloyd et al. 2005). Samples of FAM-labeled cDNA were separated on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) capillary electrophoresis instrument using techniques and parameters suggested by the manufacturer. The ROX-500 size standards (Applied Biosystems) were included in each run to determine fragment lengths.

Overproduction and purification of RosR protein.

E. coli M15 carrying the plasmid pQE450 was grown in 200 ml of LB medium supplemented with ampicillin (100 µg ml⁻¹)

and kanamycin (25 µg ml⁻¹) at 30°C. When the culture had reached an OD₆₀₀ of approximately 0.4, the expression of *rosR* was induced by addition of isopropyl-β-D-thiogalactopyranoside (final concentration 1 mM). The cells were harvested 3 to 4 h postinduction and lysed in lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and lysozyme at 1 mg ml⁻¹) on ice for 30 min. Following this, the samples were sonicated on ice using six 10-s bursts at 200 to 300 W with a 10-s cooling period between each burst. Crude cell lysates were clarified by centrifugation at 10,000 × *g* for 40 min. The supernatant was applied to a nikel-nitrilotriacetic acid resin (Ni-NTA) (Qiagen) and the (His)₆RosR protein was eluted from the column with 200 mM imidazole. Column fractions containing the (His)₆RosR protein were pooled, two times dialyzed against buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10% vol/vol glycerol) and stored at -20°C. Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay (BioRad Laboratories, München, Germany). The purification yield was approximately 1.5 mg of (His)₆RosR protein per 200 ml of *E. coli* M15 culture.

EMSA.

For EMSA, DNA fragments of *rosR* promoter matching the RosR-box sequence were amplified by PCR using plasmid pB31 as a template. The primers used for PCR amplification are listed in Table 5. The fragments were labeled at 3' ends with DIG-11-ddUTP and terminal transferase using DIG Gel Shift Kit (Roche), according to the manufacturer's instruction. Unlabeled specific competitor fragments were obtained by PCR amplification using the same primers. In a routine assay, DIG-labeled DNA fragments I and II were mixed with purified (His)₆RosR in reaction buffer containing 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol (DTT), 0.2% wt/vol Tween 20, 30 mM KCl, poly-L-lysine at 5 µg ml⁻¹, and poly[d(I-C)] at 0.5 µg ml⁻¹ to a total volume of 20 µl. The reaction mixture was incubated for 15 min at 25°C. In competition assays, (His)₆RosR protein was added to DIG-labeled DNA fragments in the presence of various concentrations of specific competitor DNA fragments. After incubation, the reaction mixtures were loaded onto a nondenaturing 5% polyacrylamide gel with 10% glycerol and resolved in Tris-glycine buffer (25 mM Tris, 190 mM glycine) supplemented with 5 mM MgCl₂, 1 mM EDTA, and 0.1 mM DTT at 7 V/cm for 3 h. DNA protein complexes were electrotransferred onto a nylon membrane and visualized by chemiluminescent detection according to the instruction manual of DIG Gel Shift Kit (Roche).

Chemical cross-linking.

For chemical cross-linking, 1 µg of RosR protein in 50 mM potassium phosphate buffer (pH 7.6) was incubated for 15 min at room temperature with increasing concentration of glutaraldehyde (0.01 to 0.25%, vol/vol). The reactions were stopped by addition of loading buffer, heated (5 min at 95°C), and analyzed by SDS-PAGE.

Table 4. DNA primers used in the construction of semi-nested deletion of the *rosR* upstream region fused with promoterless *lacZ* in plasmid pMP220^a

Name of primer	Sequence of primers (5'-3')
Forward	
pEP0	GCGAATTCGCGACTTTACCAGATT
pEP1	ATGCAAGAATTCGCACAGGAAGC
pEP2	AGCCCCTGAATTCCTTCATCTGTCA
pEP3	GGTATTTGGAATTCGAAGTAGAGTTCT
pEP3A	GCAAGTAGAGTTGGAATTCGAATTAC
pEP3B	GTGAATTAGAATTCCTACTGCTTCCAAG
pEP3C	TACTGCTTCGAATTCCTCAGTTT
pEP4	GTCCCTCAGTTTGAATTCCTAATGTC
pEP5	CGGTACGGAATTCGAAGAACAGGT
pEP6	TCGAAACAGGAATTCGATTCTCTGC
pEP7	GCGTGGAATTCAAAGGCCGTTTGA
Reverse	
RR1	CGCATTCTAGACATGTGATCTGCT
pEP8	AACGGCTCTAGACTGACACGCCAAA
pEP9	TCATGCTCTAGACGATGGCCTCAGT
pEP10	GAGGGATCTAGAACGAGTACGATTGT
pEP11	CAGAACTCTAGATGCATTTTCAAATACCATCG
pEP12	TGACAGATCTAGAAATTGAGGGGCTCT

^a Sequences for the *EcoRI* and *XbaI* restriction sites are underlined.

Table 5. Primers used for polymerase chain reaction (PCR) of the DNA fragments used in electrophoretic mobility shift assays

DNA	Name of primer	Sequence of primers (5'-3')	Length of PCR product (bp)
Fragment I with RosR-box of Rt24.2	M2	TCGCCTGGATCCTGACGTAGTGGT	185
	M4	TCCTGGTAGGATCCTCAAACGGCCT	...
Fragment II with RosR-box of Rt24.2	M5	CAGTATAGGATCCGGTTACGCT	78
	M6	CGCAGGGATCCACCTTCTGTT	...
Fragment III with Ros-box of <i>Agrobacterium tumefaciens</i> NT1REB	AT1	GGTTGATCAGACCTTTGTTGGCCATG	160
	AT2	ATGAACGCCGCATCGAGCGATGTGAA	...
Fragment IV with MucR-box of <i>Sinorhizobium meliloti</i> 2011	SM1	AGTCTCCATCGCGAGATAGCGGTCCGTT	158
	SM2	GAGTTCATTGCTCGTACCGAGCGAAGTC	...

Western immunoblotting.

SDS-PAGE-separated proteins were electroblotted onto Immobilon P (polyvinylidene difluoride) membranes (Millipore, Bedford, MA, U.S.A.), and probed with mouse anti-His₅ tag antibodies (Qiagen), followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin antibodies (Sigma-Aldrich). Bands were visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich).

EPS isolation.

For EPS isolation, 5-ml cultures of rhizobia were grown in 79CA medium with 0.5% glycerol for 3 days at 28°C in a rotary shaker. EPS was precipitated from culture supernatants with 10 volumes of 96% ethanol. The precipitate was collected by centrifugation, lyophilized, resolved in water, and analyzed for carbohydrates according to Loewus (1952). Total sugars content was calculated in glucose equivalents.

Plant tests.

Red clover (*Trifolium pratense* cv. Ulka) seed were surface sterilized, germinated, and grown as described previously (Skorupska et al. 1995). The clover plants were inspected for root nodule formation and, after 5 weeks, plants were harvested and green matter production was estimated by weighing the shoots.

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