BioS, a Biotin-Induced, Stationary-Phase, and Possible LysR-Type Regulator in *Sinorhizobium meliloti*

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Sinorhizobium meliloti 1021 produces biotin required for growth, but it also responds to external biotin signals from alfalfa plants through the *bioS* regulatory locus. Mutation of *bioS* increases biotin uptake, extends stationary phase in the presence of biotin, and impairs competitive growth in the presence of biotin. New data supporting the relevance of this gene to plant-microbe interactions show that a *bioS-gusA* reporter fusion is expressed by bacteria on plant roots, by bacteria in alfalfa root nodules, and more generally by any stationary-phase bacterial cells in the presence of biotin. Significant transcription of *bioS-gusA* was detected at 50 pM biotin, and a 16-fold induction was measured in stationary-phase cells with 1 nM biotin. A positive autoregulation of *bioS* is suggested by the fact that, in the presence of 10 nM biotin, a *bioS-gusA* fusion in wild-type cells was transcribed at more than twice the rate of the same reporter fusion in a *bioS* mutant background. Analyses of primer extension products and expression studies with three different *bioS-gusA* promoter fusions defined major characteristics of the *bioS* promoter. Expression of *bioS* in *Escherichia coli* resulted in the production of a 35-kDa BioS protein with characteristics of an LysR-type regulator. Those traits included a deduced amino acid sequence containing possible helix-turn-helix DNA-binding structures and three T-N₍₁₁₎-A motifs upstream from the *bioS* gene. A regulatory role for BioS protein was supported by tests showing that a 321-bp DNA fragment containing the *bioS* promoter altered the electrophoretic mobility of recombinant BioS and of a corresponding band in protein extracts from *S. meliloti* 1021. The absence in BioS of any significant homology to known amino acid sequences was consistent with the fact that DNA hybridization tests with seven genera of bacteria detected *bioS* only in *Sinorhizobium*.

Additional keywords: *Medicago sativa*, rhizosphere.

Basic facts about biotin are well established: It is a classic vitamin cofactor that binds CO₂ as bicarbonate in various carboxylase enzymes, including acetyl CoA carboxylase (Li and Cronan 1993) and pyruvate carboxylase (Dunn et al. 1996; Dunn 1998); it is produced by microorganisms and plants, but not by animals; and it regulates its own synthesis at very low con-

centrations, as biotinoyl-AMP, through the *birA* gene (Cronan 1989). The *birA* gene product is a key regulatory protein that functions both as a repressor of the *bio* operon (Barker and Campbell 1981) and as a biotin-protein ligase that binds biotin into acetyl-CoA carboxylase (Barker and Campbell 1981). Despite the well-known biological activity of low biotin concentrations, no other biotin-regulated bacterial genes are well characterized, and no biotin-uptake system has been genetically defined.

Sinorhizobium meliloti is an agriculturally important bacterium that forms N₂-fixing root nodules on alfalfa (*Medicago sativa*). When alfalfa plants are no longer present, the number of *S. meliloti* cells in the soil declines markedly but, as new alfalfa seeds germinate, the bacteria multiply and form new nodules (Bottomley 1992). Recent data show that plant-derived biotin is an important factor that stimulates growth of *S. meliloti* in the rhizosphere (Streit et al. 1996). A larger, rapidly growing population of *S. meliloti* contributes immediately to root nodule formation and, ultimately through N₂ fixation, to superior plant growth. Early studies established that biotin is not essential but promotes growth of (*Sino*)*Rhizobium* spp. in culture (West and Wilson 1939).

The molecular mechanism by which external biotin from the alfalfa plant stimulates growth of *S. meliloti* cells is unclear. One possible mechanism involves the biotin-dependent carboxylases present in *S. meliloti* (Dunn 1998). However, another possible mechanism involves *bioS*, a biotin-inducible gene in *S. meliloti* that affects competitive growth and biotin uptake (Streit and Phillips 1997). Three phenotypic traits are associated with *bioS*: (i) cells mutated in *bioS* compete equally with wild-type cells in biotin-free medium but poorly when 40 nM biotin is present; (ii) *bioS* mutants show an increased lag phase; and (iii) stationary-phase *bioS* mutant cells accumulate 30 to 50% more ¹⁴C-biotin than wild-type cells. A possible role for *bioS* in stationary-phase *S. meliloti* cells is further suggested by the position of the *bioS* locus among three genes showing homology to survival operons of other bacteria. The present study, which was initiated to characterize further the role of *bioS* in *S. meliloti*, establishes that the BioS protein has traits similar to LysR-type regulators and probably is involved in sensing plant-exuded biotin.

RESULTS

Expression of *bioS* reporter fusions.

The *bioS-gusA* reporter fusion constructed in Rm1021-WS7G for this study was significantly more sensitive than the *bioS-nptII* in Rm1021-B5, which was first used to prove that

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biotin induces *bioS* (Streit and Phillips 1997). For example, whereas 400 pM was the lowest concentration of biotin that markedly induced the *bioS-nptII* reporter, significant ($P \leq 0.05$) β -glucuronidase (GUS) activity was induced in Rm1021-WS7G by 50 pM biotin (Fig. 1). Expression of *bioS* increased strongly with the amounts of biotin present in the medium. In the pres-

Fig. 1. Effect of low biotin concentrations on transcription of *bioS* in *Sinorhizobium meliloti*. Expression of a *bioS-gusA* reporter fusion was measured in stationary-phase Rm1021-WS7G cells after growth in GTS medium (Kiss et al. 1979) containing different biotin concentrations. Data represent mean $\pm$ SD of four replicate cultures.

Fig. 2. Transcription of *bioS* in *Sinorhizobium meliloti* cells during growth in 10 nM biotin. Expression of a *bioS-gusA* fusion measured *bioS* transcription in Rm1021-WS7G cells growing in GTS medium (Kiss et al. 1979).

ence of 1 nM added biotin, a 16.2-fold induction of the *bioS-gusA* reporter fusion was measured, compared with cells that had been growing in the absence of added biotin.

Transcription of *bioS* in Rm1021-WS7G was greatest in stationary phase cells (Fig. 2). In total, there was a 9.2-fold increase in *bioS* expression between early log phase cells and those entering stationary phase. These results were confirmed with Rm1021-WSGB19, which carries Tn5-B22, a transposable promoter probe containing *lacZ* as a reporter. In the presence of 40 nM biotin this mutant showed an 8.3-fold induction of the *bioS-lacZ* fusion when stationary phase cells were compared with cells in early log phase (Table 1). A weak expression of the *bioS* gene also was observed in cultures without added biotin. However, β -galactosidase activities measured in those cultures were significantly lower than in the corresponding cultures containing 40 nM of added biotin. Correctness of the *gusA* and *lacZ* insertions in both Rm1021-WS7G and Rm1021-WSGB19 was verified by polymerase chain reaction (PCR) tests and Southern hybridizations to predicted restriction fragments. In addition, the location of the promoter probe in Rm1021-WSGB19 was confirmed by DNA sequencing (Fig. 3).

To examine a possible autoregulation by the BioS protein, transcriptional activity of *bioS-gusA* was measured in *bioS* mutant Rm1021-WS44G, which was constructed by homologous recombination with the *bioS-gusA* fusion into *bioS*. This mutant showed a relationship between *bioS* induction and growth qualitatively similar to that measured in Rm1021-WS7G and Rm1021-WSGB19. However, the quantitative levels of *bioS* expression were generally two- to threefold lower than those detected in Rm1021-WS7G. For one such experiment in which cells grew 96 h in the presence of 10 nM biotin, Rm1021-WS7G produced 3,250 relative GUS units while Rm1021-WS44G yielded 1,250 relative GUS units. The observation that *bioS* expression under the control of its own promoter was elevated in the *bioS* background is consistent with the concept that BioS regulates its own transcription.

Tests with alfalfa seed rinses, i.e., effusates that might be produced naturally during germination, supported the conclusion that biotin is the primary, perhaps only, plant-derived inducer of *bioS*. In those experiments, Rm1021-WSGB19 showed no induction of the *bioS-lacZ* in plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) indicator and methanolic or water-soluble fractions from alfalfa seed effusates when streptavidin (6.2 mU) was present to bind any available biotin (data not shown).

To investigate *bioS* induction in experimental alfalfa rhizospheres, seeds were sterilized and inoculated with small numbers of Rm1021-WS44G cells carrying a *bioS-gusA* fusion. Staining 10- to 14-day-old plants with X-Gluc (5-bromo-

Table 1. The effect of biotin on transcription of a *bioS-lacZ* reporter fusion in *Sinorhizobium meliloti* RM1021-WSGB19

Growth phase	β -galactosidase activity (Miller units) ^a	
	No biotin added	40 nM biotin added
Early log	0.0 $\pm$ 0.0	3.1 $\pm$ 1.2
Early stationary	0.5 $\pm$ 0.2	12.6 $\pm$ 0.3
Stationary	2.1 $\pm$ 0.3	19.8 $\pm$ 2.5
Late stationary	ND ^b	26.1 $\pm$ 0.1

^a Data represent the mean $\pm$ SD from three experiments.

^b Not determined.

4-chloro-3-indolyl- β -D-glucuronic acid) indicator revealed that the *bioS* gene was strongly induced in bacteria growing on the alfalfa root (Fig. 4A). In addition, those bacteria that were growing on the agar near the root or the seed also showed *bioS-gusA* transcription (Fig. 4B), presumably suggesting that plant biotin was diffusing into the agar. No *bioS-gusA* transcription was observed in rhizobial colonies growing at a greater distance from the plant (Fig. 4B). This result complements a previous study that showed biotin is present in alfalfa root exudates (Rovira and Harris 1961). Finally, strong *bioS-gusA* expression also was observed in young alfalfa root nodules (Fig. 4C), thus suggesting a role for the BioS protein in symbiosis.

Identification of the *bioS* promoter.

In order to study the activity of the region identified by sequencing as the possible *bioS* promoter, three different *bioS-gusA* fusions (Fig. 3) were mobilized into Rm1021, and *bioS* transcription was assayed at different biotin concentrations. The overall *bioS-gusA* transcription with the *bioS-gusA-X* fusion in extra copies in *S. meliloti* in pMB393 was significantly higher than the *bioS* transcription measured in strains Rm1021-WSG7 and Rm1021-WS44G, which carry only a single copy of the *bioS-gusA* fusion. The *gusA* activity observed for the *bioS-gusA-H* fusion was 11.3-fold lower at the 40 nM biotin concentration tested, and the transcription of the

bioS-gusA-N was 15.1-fold lower than the activities observed for the *bioS-gusA-X* fusion (Table 2). A similar result was observed when 4 nM biotin were present in the assay. However, the *bioS* transcription of the *bioS-gusA-H* was only 7.4-fold lower and the transcription measured for the *bioS-gusA-N* fusion was only 8.7-fold lower than *bioS* transcription observed for the *bioS-gusA-X* fusion. The increased biotin concentration present in the medium resulted in a 2.2-fold increase in *bioS* transcription in the Rm1021*bioS-gusA-X* strain (Table 2). In contrast, the *bioS* transcription in the two strains carrying the *bioS-gusA-N* and the *bioS-gusA-H* fusion was only weakly affected by the increased amounts of biotin present in the test. In contrast to expression studies in which single copies of the *bioS-gusA* fusions were employed (Fig. 1), we observed already a relatively high *bioS-gusA* expression with the different *bioS-gusA* fusions supplied as extra copies when no biotin was added (Table 2). Taken as a whole, these results with the *bioS-gusA* fusions in pMB393 indicate that the 129-bp region upstream from the ATG codon is important for promoter activity (Fig. 3; Table 2). However, the 444-bp DNA region upstream of the *HindIII* restriction site also is required for strong *bioS* promoter activity. When *bioS-gusA* activity was analyzed in the *Escherichia coli* background in the biotin auxotrophic strain EcWS-*bioS-gusA-X* in the presence of 40 nM biotin, *bioS* transcription was only slightly above background. This

Fig. 3. Construction of *bioS-gusA* fusions in the medium copy vector pMB393 for a *bioS* promoter analysis. Horizontal arrow indicates direction of transcription in *bioS*. **A**, Vertical arrows indicate location of *bioS* transposon mutants and mutants created by homologous recombination used in this study. **B**, Construction of *bioS-gusA* promoter fusions *pbioSgusA-X*, *pbioSgusA-H*, and *pbioSgusA-N* in pMB393. P indicates *bioS* promoter as determined by expression studies and primer-extension experiments. DNA fragments were inserted into *XhoI* or *HindIII* restriction sites in pMB393. Correct orientation of insertions in pMB393 was confirmed by restriction analysis. Vertical lines mark restriction sites H, *HindIII*; N, *NdeI*; E, *EcoRI*; X, *XhoI*.

low *bioS* transcription in the *E. coli* auxotrophic strain was independent from the biotin present in the medium (data not shown). However, this finding suggests that the *bioS-gusA* activity measured in *S. meliloti* with the same *bioS-gusA* fusion was indeed the result of the active promoter upstream of the *bioS* gene.

To further characterize the *bioS* promoter primer extension studies were performed. In these tests total RNA of Rm1021-*bioSgusA-X* was isolated and hybridized with an excess of primer PE1 labeled with fluorescein prior to the reverse transcriptase reaction. In those experiments a transcriptional start site was identified as located 20/21 bp upstream of the ATG, as shown in Figure 5. A consensus ribosomal binding site was found 6 bp upstream of the ATG codon (Fig 5). Surprisingly, we identified a 7-bp TCCCCAT-motif homologue to the -10 region of the *mucR* -10 region within the *bioS* -10 region and an 8-bp GCATTGTC-motif homologue to the 5' region of the *bdhA* -10 region. This finding suggests that the three genes might underlie common regulatory principles.

Overexpression and isolation of BioS protein.

More than 95% of the *S. meliloti* BioS protein produced in *E. coli* BL21-EH13 was found as an insoluble pellet following centrifugation. When BioS was purified from the pellet under denaturing conditions, the protein was recovered in the manufacturer's buffer E, which is specified as normally eluting dimers or tetramers. Analysis of the purified BioS on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels revealed an apparent molecular mass of 35 kDa (Fig. 6A). Protein blot

analyses with polyclonal antiserum produced in response to BioS protein indicated the BioS serum was quite specific for BioS protein with only minor cross-reactivity with other proteins in *E. coli* and *S. meliloti* (data not shown). As expected, BioS-specific bands detected in protein blots made with *E. coli* BL21-EH13 cell extracts and Rm1021 cell extracts were of equal sizes (Fig. 6B).

BioS DNA and amino acid sequence analysis.

Previous analyses established the absence of any significant homology in *bioS* to other bacterial genes for either nucleotide sequence or amino acid sequence of proteins resulting from any of six possible reading frames (Streit and Phillips 1997). The molecular mass of 35 kDa established here for BioS protein (Fig. 6) contrasts with the previously predicted mass of 22 kDa, which was estimated with the one predicted start codon that would allow expression of the inserted *nptII* reporter gene (Streit and Phillips 1997). The validity of that particular start

Table 2. The effect of biotin on transcription of *bioS-gusA* reporter fusions in *Sinorhizobium meliloti*

<i>S. meliloti</i> strain	β -glucuronidase activity ^a		
	No biotin added	4 nM biotin added	40 nM biotin added
Rm1021- <i>bioS-gusA-X</i>	9,822 (755)	10,439 (707)	21,294 (1,617)
Rm1021- <i>bioS-gusA-H</i>	1,408 (300)	1,409 (148)	1,869 (75)
Rm1021- <i>bioS-gusA-N</i>	1,221 (17)	1,195 (118)	1,403 (36)

^a Data represent the mean $\pm$ SD from three experiments.

Fig. 4. Induction of *bioS* in Rm1021-WS44G cells by plant-exuded biotin. **A**, Magnification ($\times 50$) of alfalfa root after staining with X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid). Control roots colonized and nodulated by Rm1021 were not stained by X-Gluc. **B**, Rhizobial colonies that had been growing in the rhizosphere (RH) of a developing alfalfa root or close to an alfalfa seed coat (SC). Alfalfa seedling was removed from plate prior to staining with the β -glucuronidase substrate to prevent diffusion of blue color into the agar. Former position of seedling on plate indicated by a dotted line. **C**, Expression of a *bioS-gusA* reporter fusion in an alfalfa root nodule.

codon is supported by analyses in the current study (Fig. 5). The 35-kDa *E. coli* protein in the current study was judged not to be a species artifact because the *lacZ* reporter in Rm1021-WSGB19, which inserted downstream from the initially identified translational stop codon, shows the same biotin-dependent phenotype as any of the other *bioS* reporter mutants (Table 1,

Fig. 3). In addition, DNA analyses from two commercial laboratories did not identify a frame shift in the DNA sequence, which would result in a significantly larger open reading frame. Postulating an alternative codon usage for the first possible stop codon (TGA), which is located 597 nucleotides from the ATG codon, in the *bioS* open reading frame (GenBank no. U89162),

Fig. 5. The 5' DNA region of the *bioS* gene. **A**, DNA sequence containing the *bioS* promoter. Putative translational start codon is boxed; SD indicates position of consensus Shine-Dalgarno sequence (in bold italic letters). Transcriptional start site corresponds to a TG (circled, bold); bold T/A segments indicate the three T-N₍₁₁₎-A motifs. Motifs are also marked by a number (1–3) and underlined by a dotted (motif 1), solid (motif 2), or dashed (motif 3) line. Dyad structures corresponding to the different motifs are indicated by short horizontal arrows with dotted, solid, or dashed lines, respectively. * = the dyad of the first T-N₍₁₁₎-A motif is incomplete. **B**, BioS gel-retardation studies. Lane 1, BioS recombinant protein with DNA; lane 2, control reaction lacking DNA; lane 3, Rm1021 crude lysate with DNA; lane 4, control reaction lacking DNA; Lanes 1–2 and 3–4 represent two individual experiments analyzed on different gels; approximately 25 to 35 µg of rhizobial protein extract and 5 µg of BioS protein were used in gel-retardation studies. Primers 7022 and *bioS40*-Dig (**A**) were used to amplify the 321-bp, digoxigenin-labeled DNA fragment employed in the gel-shift assays. **C**, Mapping of the 5' end of *bioS* by a primer-extension reaction. Fluorogram (upper panel) shows primer-extension reaction, which produced a significant peak (vertical arrow) that corresponds to a T/G 20/21 bp upstream of the ATG codon as measured in a DNA sequencing reaction with the same primer (lower panel fluorogram). No such peak was detected in a control reaction (minus reverse transcriptase, data not shown). Reverse transcriptase reaction and sequencing reactions were performed with nonradioactively labeled primer PE1 (shaded horizontal arrow in **A**) and analyzed by automated sequencing. Primer extension experiment was conducted three times with the same result. TCCCCAT-motif homologue to the –10 region of the *mucR* (Bertram-Drogatz et al. 1997) –10 region is boxed and dark shaded; GCATTTC-motif homologue to the 5' region of the *bdhA* (Aneja and Charles 1999) –10 region is boxed and light shaded (**A**).

predicts a 34.5-kDa protein consisting of 324 amino acids, a value similar to that observed in SDS polyacrylamide electrophoresis and immunological analyses (Fig. 6).

Several structural motifs in the DNA sequence upstream of the *bioS* gene and in the deduced amino acid sequence indicate that the BioS protein may belong to the LysR-type regulatory protein family: (i) The *bioS* promoter contains three nucleotide sequences consisting of a T-N₍₁₁₎-A motif (Fig. 5A); (ii) Within these regions, dyadic nucleotide sequences possibly involved in protein binding and often associated with LysR-type receptor proteins are present (Fig. 5A); and (iii) The deduced protein sequence contains homologies to the three different possible helix-turn-helix (HTH) DNA-binding motifs found in most LysR-type receptor proteins. Two of the motifs (I, II) were identified toward the N terminal region of the protein and only one in the C terminus (III). All three motifs were in the same reading frame. Both motifs detected in the N-terminal region of BioS were considered to be significant matches by the program (OWL25.3) used to identify the different motifs. Possible HTH motifs identified were NAAASGDGLGN (8) for motif I, TPRISLSADAL (49) for motif II, and LAFRQNGAAFNR (293) for motif III. Numbers in parentheses indicate the position of the first amino acid of the three different possible HTH motifs in the deduced BioS amino acid sequence.

BioS-DNA binding studies.

The hypothesis that BioS protein interacts with the LysR-type regulatory motifs in the promoter region of *bioS* was supported by results from BioS-DNA binding experiments. The electrophoretic mobility of a 321-bp, digoxigenin-labeled DNA fragment containing the N-terminal region of the *bioS*

gene and the promoter region (Fig. 5A) was definitely altered by the presence of BioS protein (Fig. 5). A low mobility BioS-DNA complex was formed in the presence of extracts from cells that had been harvested in stationary phase from Rm1021 (Fig. 5B, lane 3). Recombinant BioS protein purified from *E. coli* BL21-EH13 also formed a low mobility complex with the *bioS* DNA (Fig. 5B, lane 1). These results support the hypothesis that the BioS protein in *S. meliloti* interacts with the 321-bp fragment used in the gel shift assay.

Tests for *bioS* homologues in other bacteria.

Heterologous DNA hybridization experiments showed that *bioS* is present in many *S. meliloti* strains but in few other bacteria tested (Table 3). Indeed, the *bioS* gene was detected only in the closely related species *Sinorhizobium fredii*. Tests with a DNA sequence specific for the putative *S. meliloti* survival operon surrounding *bioS* in *S. meliloti* detected strong hybridization in all *S. meliloti* strains and *S. fredii*. In addition, weak homologies were present in *Rhizobium* sp. strain NGR234 and *Rhizobium* sp. strain OR191. No DNA homologies were observed for either *bioS* or the putative survival operon in any of the other Gram-negative or Gram-positive soil microorganisms tested.

DISCUSSION

Results from these experiments establish that *bioS* is expressed most strongly in stationary-phase *S. meliloti* cells (Fig. 2, Table 1) and document that transcription of this gene is induced by biotin concentrations between 10 and 50 pM (Fig. 1). These observations are consistent with the fact that open reading frames surrounding *bioS* in *S. meliloti* show high

Fig. 6. Expression and immunological detection of a BioS histidine-tagged fusion protein. **A**, Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis analysis of recombinant BioS protein produced in *Escherichia coli* BL21-EH13 and crude cell extracts from *Sinorhizobium meliloti*. Cellular proteins were extracted and resolved on polyacrylamide gels. Lane M, protein size marker; lane 1, *E. coli* BL21; lane 2, *E. coli* BL21-EH13 prior to induction with isopropyl-beta-D-thiogalactopyranoside (IPTG); lane 3, *E. coli* BL21-EH13 3 h after induction with 1 mM IPTG; lane 4, 3 μ g of recombinant BioS protein after denaturing Ni-NTA purification. **B**, Immunological analysis of BioS. Lanes 1–4 as in **A**; lane 5, Crude extract of *S. meliloti* 1021 cells.

homologies to genes found in prokaryotic survival operons and with the three phenotypes related to stationary phase that are observed in *bioS* mutants: (i) *bioS* mutants accumulate increased levels of biotin in stationary-phase cells; (ii) stationary-phase *bioS* mutants show an extended lag before resuming growth; and (iii) stationary-phase *bioS* mutants compete poorly with wild-type cells in fresh medium under low-biotin conditions but grow equally well in the absence of biotin (Streit and Phillips 1997). The clear relevance of these phenotypes to successful growth in the rhizosphere and the demonstrated expression of *bioS* on roots (Fig. 4A,B) reinforce the importance of biotin for *S. meliloti* colonization of alfalfa roots (Streit et al. 1996) and justify examination of the *bioS* promoter for a possible role in sensing plant-exuded biotin. The significance of *bioS* expression by bacteria in root nodules (Fig. 4C), which emphasizes a potential additional role for this gene in plant-microbe interactions, can be clarified only in future studies.

Several lines of evidence from this study support an initial conclusion that BioS is a regulatory protein involved in a transduction system that detects external biotin molecules. First, the finding that expression of a *bioS-gusA* fusion in the wild-type background (strain Rm1021-WS7G) was more than twice as high as that of the same reporter fusion in a *bioS* mutant background (strain Rm1021-WS44G) is consistent with the BioS protein functioning as a positive autoregulator of its own expression. Second, primer-extension experiments defined a promoter region that interacts with the BioS protein in gel-retardation studies (Fig. 5B) and thus are consistent with autoregulation of this locus. Third, analyses of the recombinant BioS protein showed many characteristics associated with an LysR-type transcriptional activator (Fig. 5A).

Expression studies with three *bioS-gusA* fusions (Fig. 3B) show clearly that the *bioS* promoter is located on a 573-bp fragment upstream of the ATG codon (Table 2). Primer-extension analysis confirmed that interpretation and identified a transcriptional start site 20/21 bp upstream of the ATG codon. Furthermore, DNA-gel retardation studies with BioS protein indicate that this protein interacts with a DNA region flanking the translational start codon of *bioS* (Fig. 5B). Thus, both structural and functional details of the *bioS* promoter are consistent with autoregulation of *bioS* by the BioS protein.

Structural evidence supporting a similarity between BioS and an LysR-type transcriptional activator comes from the DNA motifs identified in the *bioS* promoter region (Fig. 5A) and from the deduced amino acid sequence of BioS (Fig. 5C). In addition, most LysR-type receptors consist of approximately 300 amino acids, which is comparable to the size of the recombinant BioS protein expressed in *E. coli* and to the native protein in *S. meliloti* (Fig. 6). In the data bases used to locate possible helix-turn-helix DNA-binding motifs in BioS, 111 LysR-type regulatory proteins are identified (Henikoff et al. 1988), including many *nodD* and *syrM* homologues in rhizobia (Kondorosi et al. 1991; Barnett et al. 1996). Most (93) of the 111 true LysR-type regulators contain three possible HTH motifs, and 18 contain two of the three binding motifs. The conserved HTH motifs consist of 11 or 12 amino acids completely encompassing the HTH motif (prosite pattern HTH-LysR-Family). All three possible DNA-binding motifs are present in BioS. Therefore, we speculate that the BioS protein may belong to the family of HTH-LysR-type receptors. The identification of three different T-N₍₁₁₎-A motifs and corresponding dyad structures in the *bioS* promoter

region provides further evidence that BioS probably belongs to the LysR-type family of transcriptional regulators. Although it is believed that most LysR-type receptors interact with their co-repressor in the C-terminal domain, in no specific case has direct binding of the transcriptional regulator and the co-repressor molecule been shown (Schell 1993). Indeed, although it is reasonable to speculate that BioS binds biotin, we have no direct data supporting that possibility.

Several observations suggest that a more detailed examination of *bioS* may be informative. First, the *bioS* DNA locus apparently is the first biotin-regulated gene with no demonstrated link to biotin synthesis. This statement is based on the fact that cells grow well on defined medium without added biotin when they are mutated in the *bioS* locus studied here (Streit and Phillips 1997). Second, data base searches show no significant homologies for the *bioS* gene, and DNA hybridizations with a *bioS*-specific DNA probe suggest that the *bioS* gene is restricted to a very narrow range of bacteria (Table 3). Taken together, these observations suggest that *bioS* is an unusual gene that evolved in a small group of bacteria closely related to *S. meliloti*. Such uniqueness offers one possible explanation for the discrepancy between the previously postulated *bioS* open reading frame (Streit and Phillips 1997) and the actual size of the BioS protein measured in this work.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Microbiological materials used in the present work are listed in Table 4. *E. coli* was grown at 37°C on Luria-Bertani

Table 3. DNA homologies of the *bioS* gene and the possible *Sinorhizobium meliloti* survival operon with total DNA isolated from 21 different bacteria

Bacterial strain ^a	S. meliloti DNA probe specificity ^b	
	<i>bioS</i> gene	Survival operon
<i>Agrobacterium tumefaciens</i> C58	—	—
<i>Escherichia coli</i> K12*	—	—
<i>Corynebacterium glutamicum</i> ATCC1302*	—	—
<i>Mesorhizobium loti</i>	—	—
<i>Pseudomonas putida</i>	—	—
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> VF39	—	—
<i>R. leguminosarum</i> bv. <i>viciae</i> TAL182	—	—
<i>R. leucaena</i>	—	—
<i>R. etli</i> CNF1500	—	—
<i>R. tropici</i> CIAT899	—	—
<i>R. galegae</i>	—	—
<i>Rhizobium</i> sp. strain NGR234	—	(+)
<i>Rhizobium</i> sp. strain OR191	—	(+)
<i>Sinorhizobium fredii</i>	+	+
<i>S. meliloti</i> 1021*	+	+
<i>S. meliloti</i> BS48	+	+
<i>S. meliloti</i> BS65	+	+
<i>S. meliloti</i> GR4	+	+
<i>S. meliloti</i> RM65	+	+
<i>S. meliloti</i> MV7	+	+
<i>S. meliloti</i> BS34	+	+
<i>S. meliloti</i> BS51	+	+
<i>S. meliloti</i> BS11	+	+
<i>S. saheli</i>	—	—

^a * = With the exception of the indicated strains, all bacteria were gifts from M. Keller, Universität Bielefeld, Germany.

^b —, no signal detected; +, strong signal detected; (+), weak signal detected.

(LB) medium (Sambrook et al. 1989) supplemented with appropriate antibiotics. Rhizobia were cultured at 28°C on GTS (Kiss et al. 1979) or TY (Sambrook et al. 1989) medium, and growth was monitored as absorbance at 600 nm.

DNA manipulations, hybridization, and analyses.

DNA was isolated and cloned by standard methods (Sambrook et al. 1989). DNA hybridizations were done with a nonradioactive digoxigenin DNA hybridization kit (Boehringer, Mannheim, Germany). The *bioS*-specific probe was derived as previously specified (Streit and Phillips 1997), and *pbioS-SalI* was used as a probe specific for the possible *S. meliloti* survival operon. In heterologous DNA analyses, hybridizations were done at 68°C for *S. meliloti* DNA and 62°C for all other species. DNA sequencing used protocols standard for ABI 377 automated DNA sequencers. Possible helix-turn-helix DNA-binding motifs were identified with the OWL 25.3 search software from the Biochemistry Department, University College London.

Construction of *bioS* reporter strains.

Transposon mutagenesis to make Rm1021-WSGB19 was performed by standard methods (Simon et al. 1989). The mutant was identified in PCR tests with Tn5-*out* and 7022 primers (Streit and Phillips 1997). Approximately 5,000 mutants were picked and analyzed. Putative mutants were confirmed by further PCR tests.

Plasmids for inserting *gusA* into *bioS* to make Rm1021-WS7G and Rm1021-WS44 were constructed in the *sacB* positive selection suicide vector pJQ200mp18 (Quandt and Hynes 1993). A 3.3-kb *SalI* fragment containing *bioS* was subcloned in pJQ200mp18 to form pWS7. A 2.0-kb fragment containing *gusA* from pCAM140 (Wilson et al. 1995) was excised with *EcoRI* and, after partial digestion of pWS7, inserted into the vector to produce pWS7G. The plasmid pWS7G was mobilized into Rm1021 in a triparental mating with pRK2013. Single recombinant clones were selected on GTS medium containing 40 nM biotin, gentamicin (50 mg/liter), streptomycin (500 mg/liter), and X-Gluc (30 mg/liter). Double-recombinant clones were selected on the same medium in the presence of 5% sucrose. Insertion of the *gusA* gene in both mutant strains was analyzed by PCR with primers 7022 (5' CCG TGC AAG CTT CCC GCT TTA GG 3') and *gusA-B* (5' ACG TGC GTT CAT CCG GCG GGC CCT G 3').

Two of the three *bioS-gusA* promoter fusions in pMB393 (Fig. 3) were constructed by excising the *HindIII* or a *XhoI* fragment from pWS7G and inserting it into the corresponding sites in pMB393 to form *pbioS-gusA-H* or *pbioS-gusA-X*, respectively. For construction of *pbioS-gusA-N* the plasmid *pbioS-gusA-X* was restricted with *NdeI* and *SmaI* restriction enzyme, and the protruding 5' end of the *NdeI* restriction site was treated with S1 nuclease prior to religation. All constructs were mobilized into *R. meliloti* 1021 as described above and

Table 4. Microbiological materials

Material	Relevant trait	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>recA1</i> , Δ <i>lacZ</i>	GibcoBRL, Karlsruhe, Germany
VCS257	Host strain for cosmid banks	Stratagene, La Jolla, CA
HB101	pRK2013 helper strain for matings	Stratagene
K12	Reference strain containing bio operon	ATCC
BL21(pLysS)	Host strain for protein expression	Novagen, Madison, WI
BL21-EH1	<i>bioS</i> gene in pBSK+ in DH5 α	This work
S17-1-pCAM140	Promoterless <i>gusA</i> gene in mini Tn5	Wilson et al. 1995
BL21-EH13	<i>bioS</i> gene in pet19b (pet19b- <i>bioS</i>) in BL21(pLysS)	This work
ATCC 33767	<i>bio</i> operon deleted from λ att-gal	ATCC
ECWS-pMB393	pMB393 in <i>E. coli</i> ATCC33767, control strain	This work
ECWS- <i>bioS-gusA-X</i>	<i>bioS-gusA-X</i> in <i>E. coli</i> ATCC33767	This work
S17-1	Modified RP4 plasmid integrated into genome	Simon et al. 1983
<i>Sinorhizobium meliloti</i>		
Rm1021	2011, Sm ^r	Meade et al. 1982
Rm1021-B5	<i>bioS</i> ::Tn5-B30 (nptII) mutant of Rm1021	Streit and Phillips 1997
Rm1021-WSGB19	<i>bioS</i> ::Tn5-B22 (<i>lacZ</i>) mutant of Rm1021	This work
Rm1021-WS7G	<i>bioS-gusA</i> mutant of Rm1021	This work
Rm1021-WS44G	<i>bioS-gusA</i> mutant of Rm1021, double recombinant of Rm1021-WS7G	This work
Rm1021- <i>bioS-gusA-H</i>	<i>pbioS-gusA-H</i> in Rm1021	This work
Rm1021- <i>bioS-gusA-X</i>	<i>pbioS-gusA-X</i> in Rm1021	This work
Rm1021- <i>bioS-gusA-N</i>	<i>pbioS-gusA-N</i> in Rm1021	This work
Plasmids		
pBSK+	pBluescript SK+ multicopy cloning vector	Stratagene
pRK2013	pRK212.2 derivative for matings	Figurski and Helinski 1979
Tn5-B22	pSUP102 with transposable <i>lacZ</i> promoter probe, Gm ^r	Simon, et al. 1989
pet19B	Expression vector	Novagen
pMB393	Broad host range vector, Spc ^r	Gage et al. 1996
<i>pbioS-gusA-H</i>	<i>bioS-gusA</i> fusion in pMB393 (Fig. 4)	This work
<i>pbioS-gusA-X</i>	<i>bioS-gusA</i> fusion in pMB393 (Fig. 4)	This work
<i>pbioS-gusA-N</i>	<i>bioS-gusA</i> fusion in pMB393 (Fig. 4)	This work
<i>pbioS-SalI</i>	<i>bioS</i> gene on a 3.3 kb <i>SalI</i> fragment in pBSK+	Streit and Phillips, 1997
pJQ18mp200	pACYC184ori, mob, <i>sacB</i> , Gm ^r	Quandt and Hynes, 1993
pWS7	pJQ18mp200 with <i>bioS</i> gene	This work
pWS7G	pJQ18mp200 with <i>bioS</i> as <i>gusA</i> fusion	This work
pEH1	<i>bioS</i> gene in pBSK+, in <i>EcoRV</i> site	This work
pEH13	<i>bioS</i> gene pet19B, in <i>NdeI</i> site	This work

resulting strains were named Rm1021-*bioS-gusA*-N, Rm1021-*bioS-gusA*-H and Rm1021-*bioS-gusA*-X.

Cloning and expression of BioS as a histidine-tagged fusion protein.

The *bioS* gene was amplified from *S. meliloti* chromosomal DNA with *Pfu* DNA polymerase in 35 cycles with primers *bioS*36a (5' GGG CAT ATG CAG ATC GAG AAC AGG TTG AAC GCT 3') and *bioS*36b (5' GGG GCA TAT GTT AAA GCG CAT TAG AGT TAC CGC 3'). Assuming that the *bioS* open reading frame (GenBank no. U89162) initially measured might not correlate with the size of the BioS protein, primers were designed to allow cloning of a much longer open reading frame consisting of 972 bp. The oligonucleotides were designed to introduce an *NdeI* site at both ends of the gene. The PCR fragments were ligated into pBSK⁺ to make pEH1 and then excised with *NdeI*. This secondary fragment was ligated into pet19b to form pEH13. The expected nucleotide sequence of the PCR fragment in pet19b was confirmed by DNA sequencing. It revealed in this case that 2-bp exchanges had occurred in pEH13 at positions 15 and 16 downstream of the putative translational start codon that had no influence on the actual reading frame.

Freshly transformed *E. coli* BL21(pLysS) cells carrying pEH13 were used to overproduce the BioS protein. In cultures that had reached an optical density at 600 nm of 0.5 at 37°C, production of the recombinant protein was induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After 3 h, cells were harvested, then disrupted in a French pressure cell, and BioS protein was purified from the soluble fraction and the pellet. Extracts were loaded on 1-ml Ni-nitrilotriacetic agarose (Ni-NTA) columns (Qiagen, Hilden, Germany), and chromatography was done as specified by Qiagen. Resulting protein fractions were analyzed by 7.5% SDS polyacrylamide gel electrophoresis.

Primer extension studies.

The *bioS* transcriptional start site was identified by automated fluorescent primer extension (Katzen et al. 1996) with a fluorescein-labeled primer (PE1) complementary to the 5' terminus of the *bioS* gene. We used *pbios-gusA*-X as a control for the sequencing reaction and Rm1021-*bioS-gusA*-X for RNA isolation. Total RNA was isolated with the RNeasy kit (Qiagen) following the manufacturer's instructions.

BioS antibody preparation and immunological detection.

BioS-specific antibodies were obtained from Goetek (Göttingen, Germany). Purified BioS protein tagged with histidine at the N-terminal end was used for the injections. For Western blotting (immunoblotting) crude cell extracts and purified BioS protein were prepared, separated on 7.5% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Sartorius, Göttingen, Germany). Primary rabbit antibodies were detected with alkaline-phosphatase-labeled secondary antibodies (Sigma, Heidelberg, Germany) with NBT (nitroblue tetrazoliumchloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) for colorimetric detection.

BioS-DNA binding studies.

Possible BioS-DNA interactions were examined in an electrophoretic mobility assay with a 321-bp DNA fragment containing the putative *bioS* promoter, which carried a nonradio-

active label. Different dilutions of cell extracts or purified, histidine-tagged BioS protein were added to the digoxigenin-labeled DNA fragment (0.5 µg/µl) in a reaction buffer containing 0.4 mg of bovine serum albumin (BSA) per ml, 50 mM Tris HCl, pH 8.0, 100 mM NaCl, 0.05 mg of sonicated herring testes DNA per ml, and 5% glycerol. After incubation at 25°C for 30 min, the reaction was loaded on a 5% polyacrylamide gel and electrophoresed 2 to 3 h at 40 to 50 V in 1× TAE buffer (Sambrook et al. 1989). The separated DNA and DNA-protein complexes were transferred to nylon membranes (Schleicher & Schuell, Dassel, Germany) by vacuum blotting for 1 h. Colorimetric detection of the digoxigenin-labeled DNA fragments was performed according to the protocol devised by Boehringer Mannheim. Primers used to amplify the 321-bp oligonucleotide for the nonradioactive band-shift tests were *bioS*40-Dig (5' CCG GCC GAG ATA GAG CAG CGC 3') and 7022. Positions of these primers are indicated in Figure 5. A PCR to amplify the 321-bp fragment from *S. meliloti* chromosomal DNA was run at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. Reactions were done in standard PCR buffer in 50 µl. Following PCR amplification, the digoxigenin-labeled DNA fragment was electrophoresed on a 1.2% agarose gel and purified with the Qiaex II (Qiagen) gel extraction kit. Cell extracts of Rm1021 were prepared from stationary-phase cultures with a French pressure cell; recombinant BioS was purified as described below.

β-Galactosidase and GUS assays.

Activity of β-galactosidase and GUS was measured in cells harvested from cultures in GTS medium. When *bioS* transcription was measured at pM biotin concentrations, cells were transferred at least three times into fresh GTS medium without additional biotin prior to the experiment. All cultures were grown in 5-ml tubes, except for measurement of *bioS* transcription at different growth stages and *bioS* autoregulation, when 30-ml cultures in flasks were employed. All measurements were repeated at least three times by standard methods for β-galactosidase (Miller 1972) and GUS (Wilson et al. 1995) assays.

Preparation of alfalfa seed effusates.

Seed effusates were produced by soaking 50 g of alfalfa seeds (*Medicago sativa* cv. Europe) in 50 ml of distilled water or methanol. After 2 days of soaking, the aqueous supernatant was filter sterilized and used in induction assays.

Root colonization tests.

bioS-gusA activity in alfalfa rhizospheres was assayed as described previously (Streit et al. 1992). Alfalfa plants (*Medicago sativa* cv. Moapa 69) for these tests were grown on agar plates containing one-half strength Hoagland medium (Hoagland and Arnon 1951) without nitrogen. Prior to inoculation, seeds were surface sterilized (Streit et al. 1996). In tests in which *bioS-gusA* activity of bacteria directly associated with the root surface was monitored, 50 µl of bacterial cultures of Rm1021-WS44G was applied to each seed. When bacteria growing close to a developing root or germinating seed were assayed for *bioS-gusA* activity, 100 µl of bacterial suspensions in fresh GTS medium containing approximately 1,000 CFU per ml was spread on the plates. In those tests, plants were removed from the plates prior to being stained with X-Gluc to prevent diffusing of the blue dye into the agar.

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