Research Note

A Biotin-Regulated Locus, *bioS*, in a Possible Survival Operon of *Rhizobium meliloti*

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A novel biotin-inducible DNA locus, *bioS*, was found in *Rhizobium meliloti* 1021 by mutation with a transposable promoter probe. The promoterless *nptII* reporter responded to 40 nM biotin with a sixfold increase in NPTII protein. Stationary-phase mutant cells accumulated 50% more ¹⁴C-biotin than corresponding wild-type cells. The prototrophic mutant competed equally with the wild type in biotin-free medium but poorly when 40 nM biotin was present. The 588-bp *bioS* is located among three genes showing homology to survival operons of other bacteria, and it may be part of a system that *R. meliloti* uses to respond to plant biotin signals.

Additional keywords: *Medicago sativa*, rhizosphere ecology.

Low numbers of *Rhizobium meliloti* cells (<10⁴ CFU per g of soil) occur in soils where alfalfa (*Medicago sativa* L.) and other *Medicago* spp. have grown (Bottomley and Jenkins 1983; Brockwell et al. 1991). Little is known about how those small populations succeed in colonizing their normal host plants, but signal molecules released by the root may play an important role (Phillips and Streit 1996). Biotin is one molecule that is exuded by alfalfa roots (Rovira and Harris 1961) and promotes colonization by *R. meliloti* (Streit et al. 1996). It is not known if *R. meliloti* has a unique mechanism for responding to plant-derived biotin.

Biotin is a vitamin best known for its function as a cofactor binding CO₂ in enzymes, such as pyruvate carboxylase in *Rhizobium etli* (Dunn et al. 1996). Classical studies established that exogenous biotin generally is not essential but often promotes growth of *Rhizobium* in pure culture (West and Wilson 1939). Presumably that response is linked to the CO₂ requirement for growth of *Rhizobium* and *Bradyrhizobium* (Lowe and Evans 1962). Biotin affects transcription of *Escherichia coli* genes required for biotin synthesis (Cronan 1989), but there are no reports of other prokaryotic genes being regulated by this molecule. Rhizosphere bacteria responding to plant-derived biotin with increased growth may have a competitive advantage over non-responsive cells and thereby occupy favorable niches on root surfaces. Thus, one could postulate that

external biotin may induce a biotin-uptake system or other genes involved in converting *R. meliloti* cells from a resting or slow-growing state in soil to a fast-growing rhizosphere bacterium. To explore whether external biotin regulates any unique *R. meliloti* genes, a mutant bank was created in *R. meliloti* 1021 (Rm1021) (Meade et al. 1982) with transposable promoter probe Tn5-B30(*nptII*) (Simon et al. 1989). We offer here an initial report on a novel biotin-inducible DNA locus found with this approach.

Of 3,000 mutants carrying the promoterless *nptII* reporter, one was identified, Rm1021-B5, that showed biotin-enhanced growth on agar plates containing kanamycin (200 µg/ml). Growth tests and NPTII protein measurements confirmed that expression of the *nptII* marker in Rm1021-B5 was regulated by biotin and correlated with the increased growth on kanamycin (Fig. 1). Doubling time in liquid medium containing kanamycin decreased markedly in the presence of 0.4 nM biotin and declined more than 13-fold with 40 nM biotin (Fig. 1A). NPTII protein increased significantly with 0.4 nM biotin in the medium, and a sixfold increase in NPTII protein was observed with 40 nM biotin (Fig. 1B). Cells for these experiments were grown to stationary phase in GTS medium (Kiss et al. 1979) without biotin, centrifuged, resuspended in fresh GTS, supplemented with various concentrations of biotin, and incubated 5 h at 28°C to induce the *nptII* gene. Bacteria were harvested by centrifugation and lysed, and NPTII protein was measured with an NPTII enzyme-linked immunosorbent assay kit (5Prime-3Prime, Boulder, CO). Because Tn5-B30 is a transcriptional fusion, these data indicate the mutated locus is biotin-induced. It is possible, however, that the biotin regulation occurs indirectly through some other biotin-dependent event.

Growth tests with biotin-depleted cells inoculated from stationary phase into GTS medium containing 4 nM to 4 µM biotin showed that wild-type cells began growing 6 to 8 h sooner than the mutant (data not shown). This phenotype is associated with the site of insertion and not simply with transcription of *nptII* because other Tn5-B30 mutants of Rm1021 showed no extension of the normal lag period (Streit et al. 1996). There were no differences in viability between Rm1021 and Rm1021-B5 cells after 3 and 6 days in stationary phase following growth with either 0 or 40 nM biotin. Thus, taken as a whole, these findings suggest the mutated locus affects resumption of growth in resting cells but has no effect on survival per se in stationary phase under the conditions tested.

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In dual-culture tests, wild-type cells outcompeted the mutant in GTS medium containing 40 nM biotin (Fig. 2). In one experiment the mutant increased from 6.0×10^5 to 1.3×10^8 CFU/ml during 48 h, but wild-type cells in co-culture increased from 6.5×10^5 to 7.55×10^8 CFU/ml over the same period. The resulting decline in mutant/wild type ratio was highly significant ($P \leq 0.05$) and served as a phenotypic test for complementing the mutation. No significant change in the mutant/wild type ratio was observed either in the absence of biotin (Fig. 2) or when 40 μ M biotin was present (data not shown).

The mutation in Rm1021-B5 greatly enhanced accumulation of 14 C-biotin in biotin-depleted stationary-phase cells (Fig. 3). These experiments were conducted with cells grown 4 days in GTS medium containing no added biotin. On day 3, avidin (1 mU/ml) was added to remove all traces of biotin, and 24 h later bacteria were pelleted, washed twice with avidin-free GTS, adjusted to $OD_{600} = 0.5$, and placed in GTS containing 60 nM D-carboxyl- 14 C-biotin (8.12 MBq of biotin per mg; Amersham, Buckinghamshire, UK). The 14 C in bacteria was measured in 1-ml aliquots of cells harvested by filtration on nylon membranes (0.2 μ m pore size, 22 nm diameter; MSI, Westboro, MA), washed three times with GTS, dried, and quantified in Scintiverse RD solution (Fisher, Pittsburgh, PA) with a scintillation counter (LS8000, Beckman, Fullerton, CA). Values were corrected for background 14 C, and disinte-

grations were converted to ng 14 C-biotin by using an external standard. After 60 min, 30 to 50% more 14 C was measured reproducibly in the mutant than in Rm1021 ($P \leq 0.05$). A subsequent 40-min treatment in excess 12 C-biotin decreased cellular 14 C content no more than 15 to 20% (data not shown). Thus, most 14 C measured was in bacterial cells and was not bound as exchangeable biotin on the cell surface. No differ-

Fig 1. Biotin effects on expression of the *bioS::Tn5-nptII* locus in Rm1021-B5. **A**, Biotin-dependent growth in GTS medium containing 220 μ g of kanamycin per ml. **B**, Biotin-dependent production of NPTII protein from the promoterless *nptII*. All values are means ($\pm$ standard error) from three replicate cultures.

Fig 2. Biotin effects on competitive growth of Rm1021 and Rm1021-B5. Similar ratios of stationary-phase cells (mutant/wild type) were inoculated into GTS medium without supplemental biotin ($\bullet$) or with 40 nM biotin ($\blacksquare$). Symbols denoting mean values of viable cells from three replicate cultures obscure the smaller standard error bars.

Fig 3. Biotin uptake by Rm1021 ($\bullet$) and Rm1021-B5 ($\blacksquare$) cells. Cells grown under biotin-free conditions to stationary phase were supplied with 14 C-biotin at time 0. Mean value symbols from three replicate cultures obscure the smaller standard error bars.

ence in exchange of ^{12}C - for ^{14}C -biotin was observed between Rm1021 and mutant strain Rm1021-B5.

Cosmid clone pCosRmB5, which carries Rm1021 DNA in pSUP205 (Simon et al. 1983), was isolated for complementation and structural studies of the mutated DNA locus. To locate this cosmid clone in wild-type DNA, an appropriate digoxigenin-labeled probe (Boehringer Mannheim, Mannheim, Germany) was made from Rm1021-B5 DNA. After *EcoRI* restriction and religation of the mutant DNA, Tn5-specific primer *Tn5out* (Streit et al. 1996) was used in a 35-cycle polymerase chain reaction to amplify a 1.5-kbp DNA fragment. A single band was detected on a 1.2% agarose gel and isolated for use as a probe. This DNA probe formed strong hybridization signals with a 1.5-kb fragment of Rm1021 DNA and with a 7.8-kb piece of Rm1021-B5 DNA corresponding to the mutated DNA locus. A partial restriction map of pCosRmB5 (Fig. 4A) was generated with DNA modifying enzymes (Promega, Madison, WI). DNA fragments identified by that map were subcloned in pBSK+ (Stratagene, La Jolla, CA), and both strands were sequenced automatically (ABI377, Applied Biosystems Perkin-Elmer, Foster City, CA) to define open reading frames (ORFs) surrounding the Tn5 insertion (Fig. 4A). Putative transcriptional start sites were identified by the PC/GENE DNA analysis software (IntelliGenetics, LO-

CATION). Sequence data from a 4,153-bp fragment containing ORFs I, II, III and IV were submitted to the GenBank (NCBI) data base as accession number U81296.

The transposon-mutated ORF III (Fig. 4A), which we term *bioS* and identify as a 588-bp sequence, contained a putative ribosome binding site and a probable translational start site (data available as accession number U81296). The deduced amino acid sequence for ORF III contains 195 residues. Searches of NCBI data banks showed no significant homology of *bioS* to any bacterial gene for either amino acid or nucleotide sequences in six possible reading frames. However, DNA adjacent to *bioS* contained three other ORFs with notable homologies to previously reported bacterial genes (Table 1).

Conservative comparisons for identical amino acids across the entire protein, as well as the more specific local alignment tests (Altschul et al. 1990), offered convincing evidence that Rm1021 ORFs I (771 bp), II (615 bp), and IV (1,539 bp) correspond to known bacterial genes. Homologies of the ORFs, which are based on identification of a putative start site in each ORF, suggest that in *R. meliloti* the *surE* homologue (ORF I) codes for a 256-amino-acid protein, the *pcm* homologue (ORF II) codes for a 204-amino-acid methyltransferase, and the *lppB/nlpD* homologue (ORF IV) codes for a 512-amino-acid protein. Organization of the *surE*, *pcm*, and *lppB*/

Fig 4. Location of a biotin-regulated DNA locus in *Rhizobium meliloti* 1021. **A**, Four open reading frames (horizontal arrows) and the Tn5 insertion site in Rm1021-B5 (vertical arrow) were defined by nucleotide sequencing. A solid line under the restriction map (S: *SalI*; H: *HindIII*; E: *EcoRI*; P: *PstI*) corresponds to GenBank accession number U81296; dotted lines show adjacent regions examined for homologies. WS7022 and WS7090 are primer sites within *bioS*. **B**, Genes identified near *bioS* by deduced amino acid sequences.

nlpD homologues in *R. meliloti* follows the same patterns described for the survival operons of *E. coli* (Li et al. 1994) and *P. aeruginosa* (Fujita et al. 1994) (Fig. 4B). In a bacterium containing 3,000 genes, the probability that three genes would occur in such an identical order as a result of random events is exceedingly small ($P \leq 3.7 \times 10^{-11}$). Local alignment analyses of ORFs I and IV also showed significant identities to the *Haemophilus influenzae* genes *surE* ($P \leq 7.5 \times 10^{-49}$) and *lppB* ($P \leq 2.9 \times 10^{-14}$), which are not organized in a single operon (Fleischmann et al. 1995). Taken together, these results suggest that the novel biotin-regulated DNA locus, *bioS*, in *R. meliloti* is located in a survival operon. Data from this study showed no strong effect of *bioS* on currently recognized stationary-phase functions. Indeed, because disruption of the *E. coli nlpD* gene impairs survival (Ichikawa et al. 1994), the observation here that Rm1021-B5 showed normal viability in stationary phase suggests that *bioS* has no effect on transcription of the *nlpD* homologue in *R. meliloti*. At the same time, however, the ^{14}C -biotin uptake tests showed a major effect of the *bioS* in stationary-phase cells (Fig. 3) but no effect on log-phase cells (data not shown). These results suggest that *bioS* is related in some way to stationary-phase functions and that biotin plays a role in that process.

In *E. coli* and *P. aeruginosa* the *lppB/nlpD* gene is followed immediately by *rpoS* (Fujita, et al. 1994; Ichikawa et al. 1994; Ivanova et al. 1992; Mulvey and Loewen 1989). Sequencing 2,274 bp downstream of *R. meliloti lppB/nlpD* showed no significant homology to *rpoS* sequences available in the data bases.

Hybridization studies with genomic DNA suggest that Rm1021 has a second locus closely related to *bioS* (Fig. 5). In those experiments with *EcoRI*-digested DNA of Rm1021 and Rm1021-B5, a *bioS*-specific DNA probe, which was generated with polymerase chain reaction primers WS7022 (5'CCG TGC AAG CTT CCC GCT TTA GGT3') and WS7090 (5'CTG TTC TGA TCC TTC CGG GGC GAA G3') (Fig. 4A), detected one 1.5-kb fragment in Rm1021 and a corresponding 7.8-kb fragment containing Tn5-B30 in Rm1021-B5. In addition, under stringent hybridization conditions (68°C), an 11-kb DNA fragment was detected in both strains (Fig. 5).

Mutant Rm1021-B5 was complemented successfully with pCosRmB5, which contained the wild-type *bioS* locus from Rm1021. Transconjugants were recovered on GTS medium with appropriate antibiotics (tetracycline, 2 µg/ml; chloramphenicol, 50 µg/ml) and were grown twice to stationary phase in biotin-free GTS medium. To test if the impaired competition phenotype of the mutant (Fig. 2) was corrected, trans-

conjugant cells were diluted and inoculated together with an equal number of Rm1021 cells into GTS medium containing 40 nM biotin. Under these conditions the transconjugant/wild type ratios remained near 1.0 for 72 h after inoculation. In one test with Rm1021-WS13 (Rm1021-B5pCosRmB5) the trans-

Fig 5. Southern blot analysis of genomic *Rhizobium meliloti* DNA after complete *EcoRI* digestion. Gel blots were probed with a *bioS*-derived fragment labeled with digoxigenin. The 7.8-kb signal reflects *bioS* (1.5 kb) plus 6.3 kb from Tn5-B30. Lane 1: Rm1021; lane 2: Rm1021-B5.

Table 1. Comparisons of deduced amino acids in known genes with Rm1021 open reading frames (ORFs)

Species	Gene	Rm1021 ORF ^a	Method of analysis			
			Complete protein ^b		Local alignment ^c	
			Identity (%)	Similarity (%)	Identity (%)	P
<i>Escherichia coli</i>	<i>surE</i>	I	38	54	40	1.3×10^{-57}
	<i>pcm</i>	II	29	51	29	9.2×10^{-30}
	<i>nlpD</i>	IV	18	30	38	1.2×10^{-18}
<i>Pseudomonas aeruginosa</i>	<i>pcm</i>	II	36	56	36	3.0×10^{-7}
	<i>nlpD</i>	IV	26	40	36	2.2×10^{-25}

^a ORFs from Figure 4A.

^b Percentage of identical or functionally similar amino acids in the entire protein.

^c Percentage of identical amino acids found by local alignment of oligopeptide fragments (Altschul et al. 1990) and the probability of nonrandomness.

conjugant/wild type ratio was 1.2 at the time of inoculation and 1.1 after 72 h. In the same experiment original mutant Rm1021-B5 was inoculated together with Rm1021 at a ratio of 1.1, and a mutant/wild type ratio of 0.4 was measured after 72 h. Polymerase chain reaction analyses of Rm1021-WS13 with primers WS7022 and WS7090 (Fig. 4A) to amplify the wild-type *bioS* gene confirmed the presence of the predicted 509-bp DNA fragment in the transconjugant strain and its absence in mutant Rm1021-B5.

Microbiologically controlled tests for root colonization of alfalfa plants (Streit et al. 1996) detected no phenotypic difference between Rm1021 and Rm1021-B5 (data not shown). Experiments comparing growth and colonization by Rm1021 and Rm1021-B5 cells supplied separately and together at low cell numbers (<200 cells per plant) repeatedly showed no differences between the two strains after 2 and 6 days on the root. In all cases, mutant and wild-type cells were equally competitive when they were inoculated from cultures grown to early log phase or late stationary phase under various biotin concentrations. Such results may reflect the presence of a second, structurally related DNA locus (Fig. 5).

The most striking result from this work is the demonstration that *R. meliloti* 1021 contains a novel biotin-inducible DNA locus, *bioS*, that responds to external biotin. Cells mutated in this locus accumulated 40 to 50% more biotin than wild-type cells after 60 min in 60 nM biotin (Fig. 3). One explanation for this result would be that biotin uptake is poorly regulated in the mutant, but a change in biotin turnover or metabolism could produce the same result. Exactly how *bioS* might be involved in responding to plant-derived biotin in the rhizosphere is unclear at this time. Cells mutated in *bioS* were equally competitive with wild-type cells in the rhizosphere even though they were less competitive under low biotin conditions in laboratory culture (Fig. 2). The intriguing presence of a second DNA fragment, structurally related to *bioS* (Fig. 5), suggests that a strain mutated in both loci may help define the role of *bioS*. Discussions of biotin as a regulatory molecule in bacteria have centered on how this molecule controls biotin synthesis and on its use as a carboxylase cofactor (Cronan 1989). Within that context, *bioS* is the first biotin-regulated DNA locus identified in prokaryotes outside those genes involved in biotin synthesis, and defining its function may help clarify roles for biotin signals in rhizosphere ecology.

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