The *bio* operon on the acquired symbiosis island of *Mesorhizobium* sp. strain R7A includes a novel gene involved in pimeloyl-CoA synthesis

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The symbiosis island of *Mesorhizobium* sp. strain R7A is a 500 kb chromosomal genetic element that upon transfer converts nonsymbiotic mesorhizobia to symbionts able to nodulate and fix nitrogen with *Lotus corniculatus*. Four genomic species of nonsymbiotic mesorhizobia have been isolated. All were auxotrophic for thiamin and biotin and three were auxotrophic for nicotinate, whereas derivatives of the strains containing the symbiosis island were prototrophic for all three vitamins. In this work, a 13-2 kb region of the island that converts the nonsymbiotic to nicotinate and biotin prototrophy was characterized. The region contained orthologues of the *Escherichia coli bioBFD* and *A* genes arranged in an operon with a novel gene, *bioZ*, a *nadABC* operon, the nitrogen-fixation regulatory gene *nifA*, and a homologue of the pantothenate biosynthesis gene *panD*. The *bioZ* gene product was similar to *β*-ketoacyl-acyl carrier protein synthase III (*FabH*). *bioZ::Tn5* mutants grew poorly in the absence of biotin and the *bioZ* gene complemented an *E. coli bioH* mutant, suggesting that its product is involved in the synthesis of pimeloyl-CoA. The *bio* operon was not required for symbiosis, as only mutants in the *nifA* gene were impaired in symbiosis, and a *bioA::Tn5* mutant was not impaired in rhizosphere colonization. The rationale for the vitamin biosynthetic loci being located on an acquired genetic element that is absent from nonsymbiotic mesorhizobia remains to be determined.

**Keywords:** biotin biosynthesis, *β*-ketoacyl-acyl carrier protein synthase III, horizontal gene transfer

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**INTRODUCTION**

In *Mesorhizobium* sp. strain R7A, the majority of the symbiosis genes are contained on a 500 kb chromosomal genetic element termed a symbiosis island. The island transfers to nonsymbiotic mesorhizobia present in the environment and converts them to symbionts able to nodulate *Lotus corniculatus* and *Lotus japonicus*. The island integrates into a phe-tRNA gene, reconstructing the gene at the left end and forming a 17 bp direct repeat of the 3′ end of the gene at the right end (Sullivan et al., 1995; Sullivan & Ronson, 1998).

The symbiosis island was discovered in a population of mesorhizobia nodulating a stand of *L. corniculatus* planted 7 years earlier. The stand was established with a single inoculant *Mesorhizobium* strain, ICMP 3153, in tussock grassland devoid of naturalized rhizobia capable of nodulating the plant. Genetically diverse mesorhizobia recovered from nodules from the stand all contained identical symbiotic DNA, suggesting that they arose through transfer of symbiotic DNA from the inoculant strain to nonsymbiotic rhizobia present at the site. The symbiotic DNA was located on the chromosome of the strains (Sullivan et al., 1995). Subsequently, seven nonsymbiotic strains that belonged to four
genomic species of mesorhizobia were isolated from the site where the diverse symbionts were discovered. Phenotypic characterization of the nonsymbionts revealed that they were auxotrophic for biotin and thiamin and all but one were auxotrophic for nicotinate. In contrast, ICMP 3153, R7A, which is a field reisolate of ICMP 3153, and the diverse symbionts were prototrophic for all three vitamins, suggesting that the island contained genes required for vitamin synthesis (Sullivan et al., 1996). Transfer of the symbiosis island from R7A to nonsymbionts in the laboratory was subsequently demonstrated using selection for prototrophy (Sullivan & Ronson, 1998).

Little is known about vitamin synthesis and metabolism in rhizobia. Individual strains of some species require one or more of pantothenate, thiamin and biotin for growth, and Azorhizobium cauliformans also requires nicotinate (Dreyfus et al., 1983; Graham, 1963). Loci required for thiamin synthesis are located on plasmids in Sinorhizobium meliloti and Rhizobium etli (Finan et al., 1986; Miranda-Rios et al., 1997). An early study of the biotin requirements of several rhizobial species suggested that rhizobia could be divided into prototrophs, auxotrophs and bradytrophs, strains which grow poorly in continuous serial culture in biotin-free media (Wilson & Wilson, 1942). Recent studies carried out using S. meliloti suggested that both synthesis and uptake of biotin promote rhizosphere colonization (Streit & Phillips, 1996). The association of vitamin loci with acquired genetic elements, some of which contain known symbiotic genes, together with the results of Streit et al. (1996) suggest that the loci may play an important role in the plant–microbe interaction. However, the fact that some wild-type symbiotic rhizobia are vitamin auxotrophs counters this suggestion.

Here we present the analysis of a 13.2 kb region of the Mesorhizobium loti symbiosis island that contains operons required for the syntheses of nicotinic acid and mononucleotide (NMN) and biotin. The bio operon included a novel gene, designated bioZ, which was implicated in the synthesis of the biotin precursor pimeloyl-CoA. The region also contained a mifA gene required for nitrogen fixation. The vitamin loci were not required for symbiotic nitrogen fixation or for rhizosphere colonization.

METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Escherichia coli strains were cultured at 37°C in either Luria–Bertani (LB) medium or M9 minimal medium supplemented with thiamin at 1 μg ml⁻¹ and avidin at 1 μg ml⁻¹ when required. Mesorhizobial strains were grown at 28°C in TY (Beringer, 1974) or RDM media (Ronson et al., 1987) with 10 mM glucose (G/RDM) and supplemented with 1 μg thiamin ml⁻¹, 1 μg nicotinic acid ml⁻¹ and 20 ng biotin ml⁻¹. For vitamin auxotrophy studies, G/RDM was solidified with Noble agar (15%, w/v; Difco) and supplemented with combinations of the three vitamins. In experiments to assess the growth requirements for biotin, avidin (10 ng ml⁻¹ for mesorhizobia; 1 μg ml⁻¹ for E. coli) was also added. Media were supplemented with antibiotics as required at the following concentrations: for E. coli, 100 μg ampicillin ml⁻¹, 50 μg kanamycin ml⁻¹, 25 μg gentamicin ml⁻¹ and 15 μg tetracycline ml⁻¹; for mesorhizobia, 200 μg neomycin ml⁻¹, 50 μg gentamicin ml⁻¹ and 2 μg tetracycline ml⁻¹.

DNA manipulations. Mesorhizobium genomic DNA was prepared as previously described (Sullivan et al., 1995). Plasmid DNA extraction, agarose gel electrophoresis, cloning and electroporation were carried out using established methods (Sambrook et al., 1989). Southern blotting was carried out by capillary transfer. DNA probes were labelled by random priming and hybridized using standard conditions (Sambrook et al., 1989; Sullivan et al., 1995). Following hybridization, membranes were washed sequentially at 65°C in 2 x SSC/0.1% SDS, 1 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS.

DNA sequencing. EcoRI and PstI fragments of p637 were subcloned into pUC19, and the plasmid templates were sequenced using universal M13 forward and reverse sequencing primers and custom primers. Other primers used included a Tn5-specific sequencing primer, 5'-CGTTCAGGACGCTACTT-3'. Sequencing was carried out using thermal cycle sequencing and an Applied Biosystems model 377 autosequencer. The sequence was assembled using the Seqman package (DNASTAR, Madison, WI). Open reading frames (ORFs) likely to encode genes were identified using Genemark.hmm utilizing a heuristically derived model (Besemer & Borodovsky, 1999), and through database searches using blast version 2.0 (Altschul et al., 1997).

Tn5 mutagenesis. An E. coli HB101 clone containing cosmid p637 was mutagenized with Tn5 using phage λ467 (de Bruijn & Lupski, 1984). After phage infection, Tn5 insertions in p637 were selected by preparing cosmid DNA from pooled Km' Tc' mutants, transforming this DNA by electroporation into HB101 and selecting for Km' Tc' transformants. The positions of Tn5 insertions were mapped by restriction endonuclease analysis and by cloning BamHI or EcoRI–BamHI fragments containing the arms of Tn5 into pUC19 and sequencing these clones using the Tn5-specific primer. Marker exchange of Tn5 insertions into strain R7A was forced by plasmid incompatibility using pPH1J1 (Ruvkun & Ausubel, 1981). To confirm that the exchange had occurred, genomic DNA was prepared from the putative R7A:: Tn5 mutants, digested with EcoRI, blotted and probed successively with p637 and p239.

Bacterial crosses. Crosses were carried out by triparental spot matings on TY plates using the helper plasmid pRK2013 in HB101 (Ditta et al., 1980) and using HB101 donor strains. Mesorhizobium transconjugants were selected on G/RDM media supplemented with appropriate antibiotics.

Nodulation tests and symbiotic phenotype. Nodulation tests were carried out using L. corniculatus as described by Vincent (1970). Uninoculated plants and plants inoculated with strain R7A were used as controls. After 2 weeks, plants were scored for the presence of nodules. After 6 weeks, shoot length and shoot wet weight were measured. No significant difference in shoot weight or shoot length in comparison to uninoculated controls indicated an ineffective (Fix-) symbiosis.

Rhizosphere colonization studies. Inocula were prepared from 48 h cultures grown in G/RDM media supplemented with 1 ng biotin ml⁻¹. The cells were washed and diluted, and viable counts were performed as described by Streit et al. (1996). L. corniculatus seeds were surface-sterilized, germinated as described by Vincent (1970). Individual 2-d-old seedlings were transferred into modified Leonard jars containing vermiculite and 0.25 × Hoagland's solution, inocu-
Identification of genes required for nicotinate mononucleotide and biotin syntheses

We have previously reported that all seven nonsymbiotic mesorhizobial strains isolated (CJ1–CJ7) were auxotrophic for biotin and thiamin and all but CJ5 were auxotrophic for nicotinate (Sullivan et al., 1996). A clone, p637, from a pLAFR1 cosmid library of strain R7A (Sullivan & Ronson, 1998) was identified that complemented the nonsymbiotic *Mesorhizobium* strain CJ3 for the requirements for nicotinate and biotin but not thiamin. Hybridization of the cosmid DNA to symbiotic and nonsymbiotic strains showed that it contained DNA from the symbiosis island (data not shown).

The nucleotide sequence of the insert in p637 was determined, and a 13·2 kb region containing potential vitamin loci was analysed (Fig. 1). Of 12 ORFs encoding proteins larger than 60 amino acids identified, 11 showed similarity to sequences in the databases (Table 2). The deduced amino acid sequences of *nadA*, *B*, and *C* were similar to those of the *nadA*, *B*, and *C* gene products which constitute the pathway for NMN synthesis in *E. coli* (Penfound & Foster, 1996). Genes required for biotin synthesis identified on the basis of the similarity of their products to *E. coli* orthologues were *bioB*, *F*, *D* and *A*. The p637 genes were coupled by overlapping stop and start codons, suggesting that they are trans-
Table 2. Sequence similarities to predicted gene products

<table>
<thead>
<tr>
<th>ORF</th>
<th>Coordinates</th>
<th>Length (aa)</th>
<th>Degree of similarity*</th>
<th>Organism†</th>
<th>Putative function</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifA</td>
<td>233–2044</td>
<td>603</td>
<td>65/76; 27–601</td>
<td>Bradyrhizobium japonicum</td>
<td>nif-specific regulator</td>
<td>CAA29530</td>
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<tr>
<td>ORF1</td>
<td>2618–3577</td>
<td>319</td>
<td>46/62; 22–316</td>
<td>Neisseria meningitidis</td>
<td>Unknown</td>
<td>AAF40835</td>
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<tr>
<td>nadA</td>
<td>3642–4616</td>
<td>324</td>
<td>32/50; 1–322</td>
<td>E. coli</td>
<td>Quinolinate synthetase</td>
<td>BAA35409</td>
</tr>
<tr>
<td>nadB</td>
<td>4613–6154</td>
<td>513</td>
<td>38/51; 13–481</td>
<td>E. coli</td>
<td>t-Aspartate oxidase</td>
<td>BAA20446</td>
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<tr>
<td>nadC</td>
<td>6156–7037</td>
<td>293</td>
<td>41/58; 19–280</td>
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<td>Nicotinate-nucleotide pyrophosphorylase</td>
<td>AAC36922</td>
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<tr>
<td>ORF2</td>
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<td>Unknown</td>
<td>Biotin synthetase</td>
<td>AAC73862</td>
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<tr>
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<td>7542–8537</td>
<td>331</td>
<td>59/75; 18–325</td>
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<td>7-Keto-8-aminolargonic acid synthase</td>
<td>AAA23516</td>
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<tr>
<td>bioF</td>
<td>8534–9673</td>
<td>379</td>
<td>36/53; 28–372</td>
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<td>55; 1–322</td>
<td>AAA23518</td>
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<tr>
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<td>9670–10305</td>
<td>212</td>
<td>31/47; 1–158</td>
<td>E. coli</td>
<td>Dethiobiotin synthase</td>
<td>AAD19178</td>
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<tr>
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<td>38/54; 3–413</td>
<td>E. coli</td>
<td>55; 1–322</td>
<td>AAD19178</td>
</tr>
<tr>
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<td>327</td>
<td>41/55; 1–322</td>
<td>Streptomyces coelicolor</td>
<td>Pr. β-ketoacyl-acyl carrier protein synthase III</td>
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<tr>
<td>panD</td>
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<td>34/54; 5–325</td>
<td>E. coli</td>
<td>β-Ketoacyl-acyl carrier protein synthase III</td>
<td>AAA23749</td>
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</table>

* Percentage identity/percentage similarity over given range of amino acids.
† Similarities to E. coli orthologue given where available.

Tn5 mutagenesis

To confirm the function of the genes on p637 identified by sequence analysis, several Tn5 mutants of R7A were created by marker exchange using a selection of p637::Tn5 insertions. The precise location of the insertions was determined by sequence analysis using a Tn5-specific primer (Fig. 1). Mutants in nadd (Tn5–26) and nadC (Tn5–29) were nicotinate auxotrophs while mutants in bioA (Tn5–5) and bioZ (Tn5–27 and Tn5–33) grew poorly on solid media lacking biotin. Addition of avidin, a protein that binds biotin with high affinity, to solid media at 10 ng ml–1 completely abolished growth of mutants Tn5–5, Tn5–27 and Tn5–33, whereas growth of R7A was only slightly impaired. These results indicate that mutants Tn5–5, Tn5–27 and Tn5–33 were biotin auxotrophs. Insertions in ORF1 and nifA had no effect on bacterial growth, while insertions were not obtained in nadA, ORF2, bioBDF or panD. All of the Tn5 mutants were Nod– Fix–, with the exception of the nifA mutants Tn5–4 and Tn5–32, which were Fix+.

Complementation studies with M. loti bio genes

The Bio− phenotype of the R7AbioZ::Tn5 strains implies that bioZ is required for synthesis of the biotin precursor pimeloyl-CoA, as only the bioA, B, D and F genes are required for the synthesis of biotin from pimeloyl-CoA (Demoll, 1996). In E. coli, the bioC and bioH genes are involved in pimeloyl-CoA synthesis.
The bioW not the bioC alone complemented the bioZ encoded a pimeloyl-CoA synthetase that utilizes pimelic acid as a substrate, and the gene is able to complement E. coli bioH and bioC mutants in the presence of neutralized pimelic acid at 30 μg ml⁻¹ (Bower et al., 1996; Gloeckler et al., 1990). To determine if BioZ has a similar activity to BioW, the E. coli bioC mutant was transformed with p1013 and plated on media lacking biotin and containing 30 μg neutralized pimelic acid ml⁻¹. Plasmid pBIO403 containing the B. subtilis bioW gene was also used. pBIO403 but not p1013 complemented the bioC mutant in the presence of pimelic acid (Table 3).

In order to further confirm a role for bioZ in biotin synthesis in R7A, the bioZ gene was subcloned from p1013 as a BamHI–HindIII fragment into the broad-host-range expression vector pML122 to produce plasmid p1015 (Table 1). This plasmid restored the ability of R7A::Tn5-27 to grow on biotin-free G/RDM containing avidin.

Plasmids p637::Tn5-5, p637::Tn5-27 and p637::Tn5-
33 did not convert CJ3 to biotin prototrophy, confirming that bioA and bioZ are required for biotin synthesis in M. loti. To determine whether ORF2 or panD, which flank the bio operon, were also required, regions of p637 were first cloned into pBluescript II KS and subsequently subcloned into pJ3200 using suitable restriction sites to produce p995, p996 and p997 (Table 1; Fig. 1). These plasmids converted CJ3 to biotin prototrophy, indicating that neither ORF2 nor panD is required for biotin synthesis in CJ3.

**Growth-room rhizosphere colonization studies**

Five rhizosphere competition experiments, each with five replicates, were carried out using modified Leonard jars placed in a growth room. Treatments comprised R7A alone, R7A::Tn5-5 (bioA::Tn5) alone, and a 1:1 mix of the two strains. Inocula were prepared by washing and diluting 48 h cultures 100-fold to give 20–120 c.f.u. per treatment. Between 10^6 and 10^7 bacteria were recovered from each root harvested 10 d post-inoculation, indicating that the bacteria had undergone 12–18 doublings. No differences in the abilities of the two strains to colonize the L. corniculatus rhizosphere were detected (R7A, log10 4.7 ± 1.2; R7AbioA::Tn5, log10 3.3 ± 1.1; 1:1 mix, log10 5.4 ± 1.0, with each strain equally represented).

**DISCUSSION**

We have shown that an acquired genetic element required for symbiosis, the M. loti symbiosis island, contains structural operons required for the biosyntheses of biotin and NMN closely linked to nifA, a symbiotic activator of nif and fix genes (Fischer, 1994). The bio operon contains a novel gene, bioZ, that provides insight into a poorly understood step in the pathway of biotin synthesis. In addition, the location of the bio operon on an acquired genetic element may explain the observed diversity of rhizobia with respect to biotin auxotrophy.

Biotin is an essential cofactor for carboxylase enzymes and in E. coli and B. subtilis is synthesized from pimeloyl-CoA via four enzymic steps involving the products of the bioA, B, D and F genes. Steps involved in pimeloyl-CoA synthesis are less well-characterized but differ between the two organisms. The functions of the symbiosis island bioA, B, D and F genes were confirmed by their ability to complement respective E. coli bio mutations. However, orthologues of bioZ are not found amongst the known bio genes of other organisms.

Variation in the growth of complemented E. coli bio mutants that was dependent on the particular plasmid used for complementation was observed. For example, p961 complemented the E. coli bioA, bioD and bioF mutants to wild-type growth; however, the E. coli bioB mutant was Bradytrophic when complemented with this plasmid. In contrast, the E. coli bioB mutant was fully complemented by p964 containing the entire bio operon fused to the E. coli lacZ promoter, but p964 did not complement the bioA, D or F mutants. Plasmid p964 was also the only plasmid containing the entire symbiosis island bio operon that complemented the bioH mutant, albeit to Bradytrophic growth, whereas p1013 restored the mutant to wild-type growth. Problems with complementation and/or viability associated with the expression of bioB have been observed in other organisms, including E. coli and Serratia marcescens (Ifuku et al., 1995; Levy-Schil et al., 1993; Sakurai et al., 1993). Moreover, S. meliloti strains expressing the E. coli bio operon show reduced viability (Streit & Phillips, 1996).

Pimeloyl-CoA is a seven-carbon thioester that is the first dedicated intermediate in the biotin biosynthetic pathway. B. sphaericus and B. subtilis both contain a pimeloyl-CoA synthetase, encoded by bioW, which condenses pimellic acid with CoA to form pimeloyl-CoA. bioW complements both E. coli bioC and bioH mutants in the presence of pimellic acid (Bower et al., 1996; Gloeckler et al., 1990). In E. coli, pimeloyl-CoA synthesis does not involve pimellic acid as an intermediate. Instead, a synthetic mechanism similar to a fatty acid synthase reaction involving the condensation of three malonyl-CoA molecules has been suggested based on radiolabelling studies (Ifuku et al., 1994; Sanyal et al., 1994). The symbiosis island bioZ gene cannot complement an E. coli bioC mutant, even in the presence of free pimellic acid, and therefore its product does not appear to act as a pimeloyl-CoA synthetase; however, it does complement bioH mutants.

The strong sequence similarity of BioZ to β-ketoacyl-acyl carrier protein synthase III (FabH) suggests that BioZ is a fatty acid synthase. FabH forms part of the dissociated (type II) fatty acid synthase system in bacteria. It catalyses the first step in fatty acid synthesis, the condensation of an acetyl-CoA starter unit with malonyl-ACP to produce fatty acids with odd numbers of carbon atoms (Heath & Rock, 1996), may mediate. Instead, a synthetic mechanism similar to a condensation of an acetyl-CoA starter unit with malonyl-ACP to form acetosacetyl-ACP. The enzyme also has an acetyl-CoA:ACP transacylase activity which involves the interconversion of acetyl-ACP and acetyl-CoA (Tsay et al., 1992). Condensing enzymes such as FabH function via an acyl–enzyme intermediate attached as a thioester to an active cysteine residue. The active-site cysteine of FabH is conserved in BioZ (Fig. 2). It seems possible that BioZ is involved in a condensation reaction perhaps involving incorporation of a thioester with an odd number of carbon atoms leading to synthesis of pimeloyl-ACP, and also in its transacylation to pimeloyl-CoA. E. coli FabH, which has been shown to carry out a condensation reaction of propionyl-CoA with malonyl-ACP to produce fatty acids with odd numbers of carbon atoms (Heath & Rock, 1996), may carry out similar steps to BioZ but have a narrower specificity for its transacylation reaction, accounting for the requirement for BioH in E. coli. In this model, BioC would be required for the synthesis of one of the substrates for the initial condensation reaction. It of course remains to be determined whether mesorhizobia contain a bioC orthologue elsewhere in their genome.

The presence of vitamin synthesis genes on the symbiosis island suggests that they may be required for, or augment, the symbiotic interaction. However, mutants...
with Tn5 insertions in the NMN and biotin synthesis operons were unaffected in nodulation or nitrogen fixation, and the bioA mutant was unaffected in rhizosphere colonization. These results are consistent with the findings that some rhizobia are natural biotin auxotrophs (Graham, 1963) and that nonsymbiotic mesorhizobia were present in the rhizosphere of L. corniculatus grown in the field (Sullivan et al., 1996). However, they are contrary to a previous study which reported that S. meliloti biotin auxotrophs competed very poorly with the parent strain (Streit et al., 1996). It is possible that the difference in the results reflects the different host plants or rhizobial species used.

Despite the lack of an obvious symbiotic role, the presence of vitamin synthesis operons on the island strongly suggests that they can contribute to a successful plant–microbe interaction. Conversely, the lack of the operons may benefit the nonsymbiotic mesorhizobia. Acquired genes usually confer a selective advantage only under specific conditions and may be prone to loss under other environmental conditions. The vitamin synthesis genes may have been lost from the nonsymbionts if they were detrimental to the rhizobia for maintaining a saprophytic lifestyle in the absence of the host. For example, vitamin starvation may enable the bacteria to adopt a stress-resistant dormant state, which would only be alleviated in the presence of an environment conducive to growth. In this regard, the nonsymbiotic rhizobia may use the presence of vitamins as an indicator of the presence of a plant root. Certainly the presence of the bio and nad genes on the symbiosis island extends the range of ‘house-keeping’ genes known to be encoded on accessory genetic elements.

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