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 nonsymbionts 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

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

Abbreviation: NMN, nicotinic acid mononucleotide.

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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 caulinodans* 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 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 *nifA* 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 (1.5%, 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 × SSC/0.1% SDS, 1 × SSC/0.1% SDS and 0.1 × 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'-CGTTCAGG-ACGCTACTT-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^r Tc^r mutants, transforming this DNA by electroporation into HB101 and selecting for Km^r Tc^r transformants. The positions of Tn5 insertions were mapped by restriction endonuclease analysis and by cloning *Bam*HI or *EcoRI*-*Bam*HI 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 pPH1JI (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 and 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-

Table 1. Strains and plasmids

Strain or plasmid	Relevant properties	Reference
Strains		
Mesorhizobia		
R7A	Field reisolate of ICMP 3153	Sullivan <i>et al.</i> (1995)
CJ3	Nonsymbiotic <i>Mesorhizobium</i> strains	Sullivan <i>et al.</i> (1996)
<i>E. coli</i>		
HB101	<i>pro leu thi gal lacY recA str hsdD hsdM</i>	Boyer & Roulland-Dussoix (1969)
R872	<i>bioF3</i>	Cleary & Campbell (1972)
R875	<i>bioB17</i>	Cleary & Campbell (1972)
R877	<i>bioD19</i>	Cleary & Campbell (1972)
R878	<i>bioC18</i>	Cleary & Campbell (1972)
R879	<i>bioA24</i>	Cleary & Campbell (1972)
BM7086	$\Delta(mal-bioH)$ <i>gal</i>	Hatfield <i>et al.</i> (1969)
Plasmids		
pLAFR1	Broad-host-range cosmid, Tc ^r	Friedman <i>et al.</i> (1982)
pIJ3200	Broad-host-range cosmid, Tc ^r	Liu <i>et al.</i> (1990)
pRK2013	<i>rep</i> (colE1), Mob ⁺ Tra ⁺ Km ^r	Ditta <i>et al.</i> (1980)
pPH1JI	IncP plasmid, Gm ^r	Hirsch (1979)
pML122	Broad-host-range expression vector, Gm ^r	Labes <i>et al.</i> (1990)
pBluescript II KS	<i>lacZα</i> Ap ^r	Stratagene
pUC19	<i>lacZα</i> Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
p239	pUC19 containing the <i>nptII</i> gene from Tn5	This study
p637	pLAFR1 containing a 23.14 kb region of the R7A symbiosis island	This study
p961	pBluescript II KS containing a 6.82 kb <i>PvuII</i> fragment of p637	This study
p962	pBluescript II KS containing the insert of p961 in the opposite orientation	This study
p963	pBluescript II KS containing a 5.90 kb <i>AatII-PvuII</i> fragment of p637	This study
p964	pBluescript II KS containing the insert of p963 in the opposite orientation	This study
p995	pIJ3200 containing insert from p961	This study
p996	pIJ3200 containing a 5.90 kb <i>AatII-PvuII</i> fragment of p637	This study
p997	pIJ3200 containing a 6.24 kb <i>SpeI-PvuII</i> fragment of p637	This study
p1013	pUC19 containing a <i>BglII</i> (end-filled)- <i>BamHI</i> fragment of p637	This study
p1015	pML122 containing a <i>HindIII-BamHI</i> fragment from p1013	This study
pBIO131	pUC9 containing the <i>B. subtilis bioI</i> gene	Bower <i>et al.</i> (1996)
pBIO403	pCL1921 containing the <i>B. subtilis bioW</i> gene	Bower <i>et al.</i> (1996)

lated with 20–90 c.f.u. and incubated under controlled conditions as described by Streit *et al.* (1996). Roots were harvested 10 d post-inoculation and ground in 1 ml sterile distilled water with a Dounce homogenizer. Rhizobia were enumerated by plating dilutions on G/RDM media. For dual inoculation experiments where R7A was compared to R7A::Tn5-5, 50 colonies were patched from G/RDM plates used for total enumeration onto G/RDM containing neomycin to determine the ratio of mutant to wild-type.

RESULTS

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-

Fig. 1. Map of the 13.2 kb region. The locations and relative orientations of the ORFs are shown above the map. Plasmids containing cloned fragments of the region are shown below the map. The positions of various Tn5 insertions are indicated on the map by $\bigcirc$ symbols. Only restriction sites used for cloning are shown: A, AatII; B, BamHI; Bg, BglII; P, PvuII; S, SpeI.

Table 2. Sequence similarities to predicted gene products

Percentage identities and similarities were derived from deduced gene products of ORFs using BLAST 2.0 (Altschul *et al.*, 1997).

ORF	Coordinates	Length (aa)	Degree of similarity*	Organism†	Putative function	Accession no.
<i>nifA</i>	233–2044	603	65/76; 27–601	<i>Bradyrhizobium japonicum</i>	<i>nif</i> -specific regulator	CAA29530
ORF1	2618–3577	319	46/62; 22–316	<i>Neisseria meningitidis</i>	Unknown	AAF40835
<i>nadA</i>	3642–4616	324	32/50; 1–322	<i>E. coli</i>	Quinolinate synthetase	BAA35409
<i>nadB</i>	4613–6154	513	38/51; 13–481	<i>E. coli</i>	L-Aspartate oxidase	BAA02446
<i>nadC</i>	6156–7037	293	41/58; 19–280	<i>E. coli</i>	Nicotinate-nucleotide pyrophosphorylase	AAC36922
ORF2	7048–7395	115	None detected		Unknown	
<i>bioB</i>	7542–8537	331	59/75; 18–325	<i>E. coli</i>	Biotin synthetase	AAC73862
<i>bioF</i>	8534–9673	379	36/53; 28–372	<i>E. coli</i>	7-Keto-8-aminopelargonic acid synthase	AAA23516
<i>bioD</i>	9670–10305	212	31/47; 1–158	<i>E. coli</i>	Dethiobiotin synthase	AAA23518
<i>bioA</i>	10305–11570	421	38/54; 3–413	<i>E. coli</i>	7,8 Diaminopelargonic acid aminotransferase	AAD19178
<i>bioZ</i>	11567–12550	327	41/55; 1–322	<i>Streptomyces coelicolor</i>	Pr. β -ketoacyl-acyl carrier protein synthase III	CAA16179
			34/54; 5–325	<i>E. coli</i> FabH	β -Ketoacyl-acyl carrier protein synthase III	AAA23749
<i>panD</i>	12609–13061	150	40/67; 1–111	<i>E. coli</i>	Aspartate 1-decarboxylase	P31664

* Percentage identity/percentage similarity over given range of amino acids.

† Similarities to *E. coli* orthologue given where available.

cribed as a single operon. The start codon of a fifth ORF, *bioZ*, overlapped the stop codon of *bioA*. The deduced amino acid sequence of *bioZ* showed similarity to β -ketoacyl-acyl carrier protein synthases (KAS IIIs) from several bacterial species, including the *fabH* gene product of *E. coli* (Table 2; Fig. 2). The deduced amino acid sequences of *nifA* and *panD* showed similarity with the NifA protein required for regulation of several *nif* and *fix* genes in nitrogen-fixing bacteria, and the PanD protein required for pantothenate biosynthesis, respectively. The translated product of ORF1 showed strong sequence similarity over its entire length with the product of a gene of unknown function from *Neisseria meningitidis* that is flanked by *nadA* and *nadC* in that organism (Table 2).

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 *nadB* (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, *bioBFD* 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 R7A*bioZ*::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

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MBioZ  MSRSRVLGFGHHAPSRRKVENPFIENRLGLEPGWIERRTGIRSRFWATDQTLISGLATQAGDMALANAGIDRSITGLLLL 80
ScFabH  MTRASVLTGLGSLPSRQVINAELERTMOTSDIEWIRARTGIAQRYVAEEGTLTSDLVGGAERALKSARLTPOETDAVIV 80
EcFabH  MY--TKIIGTGSYLEQVRINADLEKMDTSDEWIVTRTGIRERHIAAPNEIVSIMGFEAATRAIEMAGIEKDOIQLIV 78

MBioZ  ATSTPDHLLIPSAPLVAKHLGICRACAVLITGACAGFTYALMFADGF-TRLHGASLVIAANILSRFRINAEKASSVLFA 159
ScFabH  ATTPDRPCATAPITVAARLGTCPVPAPFDVSAVCSGFLYGLATGSGLIASGAERVLVIGAEETSRIILNEQDRSTSVIFG 160
EcFabH  ATTSATIAFBSAACQIQSMIGIKGCPAFDVAACAGFTYALSVADQYVKSQAVLYALVVGSDVLAFTCDITDRCTIIFG 158

MBioZ  DAAGALVIGACDDDLGLCA-SVDSGGSRYGLTQIPAGGSSIPFHDDLDLGQTRMTMTDCREVFARAVEMMTDCSTSA 238
ScFabH  DGAGAVVLRAGEPGETCALGPLRLGSDGTGVDLITVPAGGPPREGAAAFDLADRYFTMECKRVFWLAVQRMGECABSVL 240
EcFabH  DGAGAVVLAASERF--GIIST-HIADGSYGEILILNADRVNENSIH-----LTMAQNEVEKVAVTELAHIVDET 228

MBioZ  AVAGVRPQDIDREVPQOANRIFDAVGRNLGIADBAIVKTIAYGNSAAITPLSLSLAHRAPFRPGEKVLAAACAGL 318
ScFabH  DRAGWRVADVVDLVSHQANRITARLADEIGIPRERSVSNIAEVGNTAAASIPALDHAHARGTLRPGDRVLLTAFGGGL 320
EcFabH  AANNLDRSQLDWLVPHQANLRITISATAKKLGMSMDNVVWLDHRGNTSAAVFCALDEAVRDGRIKPGQVLLTBAFGGGF 308

MBioZ  SCGATVVG-----I 327
ScFabH  TWGAALITWPAVDPV 335
EcFabH  TWGSALV-----RF 317

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Fig. 2. Comparison of the deduced amino acid sequence of the symbiosis island *bioZ* gene (MBioZ) with the sequence of the *fabH* gene products from *Streptomyces coelicolor* (ScFabH) (accession no. CAA16179) and *E. coli* (EcFabH) (accession no. AAA23749). The putative active site cysteine is marked with an asterisk.

Table 3. Complementation analysis of *E. coli bio* mutants with plasmid clones containing symbiosis island and *B. subtilis bio* genes

See Fig. 1 for map of clones and Table 1 for plasmid descriptions and strain designations of *E. coli* mutants. All strains were grown on M9 minimal medium containing $1 \mu\text{g}$ avidin ml^{-1} . + + +, Prototrophic; +, bradytrophic; –, auxotrophic; NT, not tested.

Plasmid	Insert*	<i>plac</i> †	<i>bioA24</i>	<i>bioB17</i>	<i>bioD19</i>	<i>bioF3</i>	<i>bioC18</i>	Δ <i>bioH</i>
p961	ORF2– <i>panD</i>	+	+ + +	+	+ + +	+ + +	–	–
p962	ORF2– <i>panD</i>	–	+	–	+	+	–	–
p963	<i>bioB</i> – <i>panD</i>	–	+	–	+	+	–	–
p964	<i>bioB</i> – <i>panD</i>	+	–	+ + +	–	–	–	+
p1013	<i>bioZ</i>	+	NT	NT	NT	NT	–‡	+ + +
p1015	<i>bioZ</i>	–	NT	NT	NT	NT	–	–
pBIO131	<i>B. subtilis bioI</i>		NT	NT	NT	NT	+ + +	+ + +
pBIO403	<i>B. subtilis bioW</i>		NT	NT	NT	NT	+ + +‡	+ + +‡

* First complete gene and last gene in plasmid insert.

† Orientation of plasmid insert relative to *lac* promoter.

‡ Strains grown in the presence of neutralized pimelic acid.

(Demoll, 1996; Eisenberg, 1987). Complementation studies were carried out to determine if *bioZ* could complement *E. coli bioC* and *bioH* mutants, and to confirm the function of the R7A *bioBFDA* genes. Plasmid pBIO131 containing the *Bacillus subtilis bioI* gene was used as a positive control for the complementation of *bioH* and *bioC* mutants (Bower *et al.*, 1996). Several plasmids that differed in their symbiosis island gene and the orientation of the genes with respect to the vector *lac* promoter were constructed (Fig. 1; Table 1). The results suggested that variations in the level of expression of the R7A *bio* operon from the various constructs influenced their ability to complement the *E. coli bio* mutants. *E. coli bioA*, *B*, *D*, *F* and *H* mutants were complemented by at least one construct containing the entire symbiosis island *bio* operon, while *bioZ* alone complemented the *E. coli bioH* mutant but not the *bioC* mutant (Table 3).

The *bioW* gene from *Bacillus sphaericus* and *B. subtilis* encodes 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 $30 \mu\text{g ml}^{-1}$ (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 $30 \mu\text{g}$ 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 *Bam*HI–*Hind*III 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 pIJ3200 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 10^5 -fold to give 20–120 c.f.u. per treatment. Between 10^5 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, $\log_{10} 4.7 \pm 1.2$; R7A*bioA*::Tn5, $\log_{10} 5.3 \pm 1.1$; 1:1 mix, $\log_{10} 5.4 \pm 1.0$, 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* mutants. 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 pimelic acid with CoA to form pimeloyl-CoA. *bioW* complements both *E. coli bioC* and *bioH* mutants in the presence of pimelic acid (Bower *et al.*, 1996; Gloeckler *et al.*, 1990). In *E. coli*, pimeloyl-CoA synthesis does not involve pimelic 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 pimelic 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 form acetoacetyl-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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