

Proline auxotrophy in *Sinorhizobium meliloti* results in a plant-specific symbiotic phenotype

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In order to effectively manipulate rhizobium–legume symbioses for our benefit, it is crucial to first gain a complete understanding of the underlying genetics and metabolism. Studies with rhizobium auxotrophs have provided insight into the requirement for amino acid biosynthesis during the symbiosis; however, a paucity of available L-proline auxotrophs has limited our understanding of the role of L-proline biosynthesis. Here, we examined the symbiotic phenotypes of a recently described *Sinorhizobium meliloti* L-proline auxotroph. Proline auxotrophy was observed to result in a host-plant-specific phenotype. The *S. meliloti* auxotroph displayed reduced symbiotic capability with alfalfa (*Medicago sativa*) due to a decrease in nodule mass formed and therefore a reduction in nitrogen fixed per plant. However, the proline auxotroph formed nodules on white sweet clover (*Melilotus alba*) that failed to fix nitrogen. The rate of white sweet clover nodulation by the auxotroph was slightly delayed, but the final number of nodules per plant was not impacted. Examination of white sweet clover nodules by confocal microscopy and transmission electron microscopy revealed the presence of the *S. meliloti* proline auxotroph cells within the host legume cells, but few differentiated bacteroids were identified compared with the bacteroid-filled plant cells of WT nodules. Overall, these results indicated that L-proline biosynthesis is a general requirement for a fully effective nitrogen-fixing symbiosis, likely due to a transient requirement during bacteroid differentiation.

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INTRODUCTION

Rhizobium–legume symbiosis is a complicated and biologically important process. This symbiosis serves as the major source of fixed nitrogen for the legume hosts, allowing them to thrive in nitrogen-poor soils. Despite currently being a significant contributor of fixed nitrogen in agricultural crops, symbiotic nitrogen fixation (SNF) has not yet reached its full potential. This is due in part to commercial inoculants failing to compete well with the indigenous soil microbiome (Archana, 2010) and several key crops (i.e. cereals) being unable to enter into symbiotic relationships with rhizobia (Oldroyd & Dixon, 2014). As technologies have improved, an increasing interest has re-emerged in engineering improved inoculants and transferring symbiosis to non-legumes (Archana, 2010; Oldroyd & Dixon, 2014). These ambitious goals require a complete understanding of the symbiotic relationship. Whilst decades of

research have led to a solid understanding of the genetics and metabolism of SNF from the bacterial perspective (Dunn, 2014; MacLean *et al.*, 2007; Sadowsky *et al.*, 2013; Udvardi & Poole, 2013), our knowledge remains incomplete.

One area deserving of further attention is the role of bacterial L-proline metabolism. In contrast to L-proline catabolism, which has received a fair level of attention and is reviewed elsewhere (Dunn, 2014), the role of L-proline biosynthesis is relatively unstudied. Rhizobial L-proline biosynthesis presumably proceeds primarily from L-glutamate via ProB, ProA and ProC (Fig. 1). The expression pattern of the *Sinorhizobium meliloti* proline catabolic gene *putA* indicates that *S. meliloti* has access to L-proline throughout the infection process of alfalfa (Jiménez-Zurdo *et al.*, 1997). However, the lack of expression of *putA* in differentiated *S. meliloti* bacteroids (Jiménez-Zurdo *et al.*, 1997), the FixJ-dependent upregulation of *proB2* in *S. meliloti* (Ferrières *et al.*, 2004) and the low rate of L-proline transport by at least some rhizobial bacteroids (Glenn *et al.*, 1991; Trinchant *et al.*, 1998) may indicate a requirement for *de novo* L-proline synthesis in bacteroids. In contrast, the transcriptional downregulation

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Abbreviations: PHB, poly-3-hydroxybutyrate; SNF, symbiotic nitrogen fixation.

Three supplementary tables and three supplementary figures are available with the online Supplementary Material.

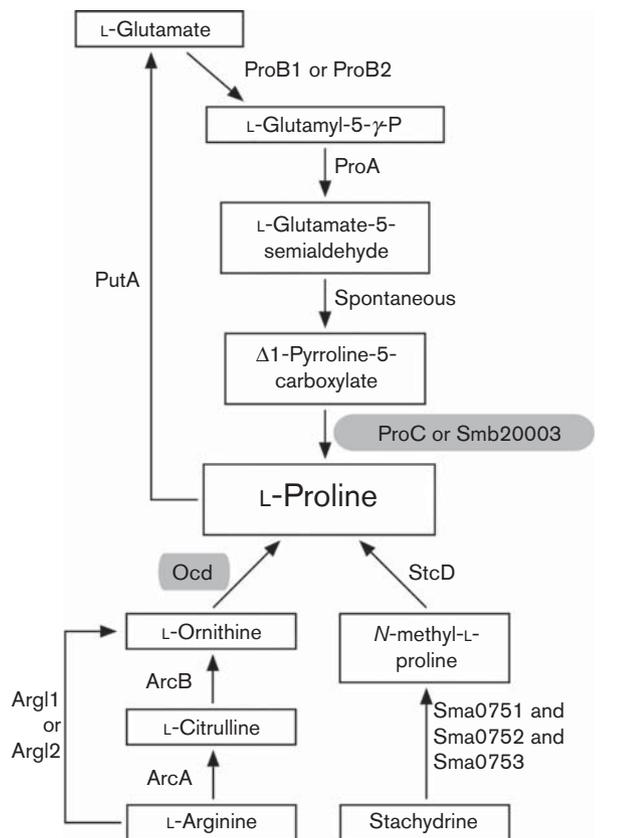


Fig. 1. Metabolism of L-proline in *S. meliloti*. Catabolism of L-proline proceeds via a bifunctional PutA enzyme, converting L-proline to L-glutamate. The primary pathway for L-proline biosynthesis begins with L-glutamate as a precursor and involves four reactions, three of which are enzyme-catalysed and one of which is spontaneous. L-Proline is also a metabolic intermediate in the catabolism of L-arginine, L-citrulline, L-ornithine and stachydrine (L-proline betaine), and synthesis of L-proline from these compounds when provided exogenously can complement mutations in the L-glutamate to L-proline biosynthetic pathway. The enzymes encoded by the *proC*, *smb20003* and *ocd* genes studied here are highlighted in grey. L-Glutamyl-5- γ -P, L-glutamyl-5- γ -phosphate.

of *proC* in bacteroids of multiple symbioses (Becker *et al.*, 2004; Karunakaran *et al.*, 2010; Li *et al.*, 2013), the higher rate of L-proline transport by some bacteroids or symbiosomes (Pedersen *et al.*, 1996; Trinchant *et al.*, 2004), proline dehydrogenase activity in *Bradyrhizobium japonicum* bacteroids in soybean nodules (Kohl *et al.*, 1988) and the ability of exogenous L-proline to stimulate nitrogenase activity in soybean nodules (Zhu *et al.*, 1992) suggest bacteroids may obtain L-proline from the plant, negating the need for *de novo* synthesis.

The only way to directly test the requirement for rhizobial L-proline biosynthesis during symbiosis is to examine the phenotype of rhizobium L-proline auxotrophs. However, few have been reported, perhaps due to genetic redundancy

in this pathway (Fig. 1) (diCenzo & Finan, 2015). Nevertheless, two rhizobial proline auxotrophs have been studied and these show divergent symbiotic phenotypes. *A. B. japonicum proC* mutant had a Fix^- phenotype (King *et al.*, 2000), whilst a genetically undefined *Rhizobium leguminosarum* proline auxotroph was Fix^+ (Chien *et al.*, 1991). Thus, additional mutants should be studied to clarify the role of proline biosynthesis in rhizobium-legume symbiosis.

During our recent studies of an *S. meliloti* strain with a significantly reduced genome (diCenzo *et al.*, 2014), we identified redundancy in the genes encoding the ProC enzyme (diCenzo & Finan, 2015). By disrupting both the *proC* and *smb20003* genes, we constructed a *S. meliloti* proline auxotroph that was unable to synthesize L-proline *de novo* from L-glutamate (diCenzo & Finan, 2015). In the current study, we examined the symbiotic characteristics of this *S. meliloti* proline auxotroph. Our results indicated that L-proline biosynthesis is required for a fully effective symbiosis, with the proline auxotroph showing a plant-specific Fix^- phenotype.

METHODS

Media, growth conditions and genetic manipulations. All media (LB, LBmc, TY and M9), antibiotic concentrations (streptomycin, spectinomycin, neomycin, kanamycin, tetracycline, gentamicin and chloramphenicol) and growth conditions were as described previously (diCenzo *et al.*, 2014). All carbon sources were added to a final concentration of 10 mM. Where noted, M9 media were supplemented with 1 mM L-proline, 1 mM L-ornithine or 1 mM stachydrine. Bacterial matings and ϕ M12 transductions were performed as described previously (Cowie *et al.*, 2006; Finan *et al.*, 1984). Growth curves were established and analysed as described previously (diCenzo *et al.*, 2014).

Strain construction. All bacterial strains and plasmids used in this study are listed in Table 1. The Rm2011 proline auxotroph was complemented by the introduction of a cosmid library clone that carries *smb20003*. To construct a proline auxotroph in which the *ocd* gene was also disrupted, an unmarked deletion of the pSymB region from nt 1 679 723 to 49 523 (Δ B161; includes *smb20003*) was made via the expression of *flp* in RmP1054 from pTH2505, after which pTH2505 was cured from the cell. The *proC-1::Tn5-B20* allele from RmP3103 (Rm2011, *proC-1::Tn5-B20*) was transduced into the resulting strain, producing proline auxotrophy. Finally, the *ocd::pTH1522* allele from RmFL3575 was recombined into this proline auxotroph, creating a *proC smb20003 ocd* genotype.

Symbiotic assays. Plant growth experiments were performed largely as described previously (Yarosh *et al.*, 1989). Magenta jars were prepared with a 1 : 1 (w/w) mixture of quartz sand/vermiculite and 250 ml of Jensen's medium (Jensen, 1942), and then autoclaved. All seeds (alfalfa, *Medicago sativa* cv. Iroquois; white sweet clover, *Melilotus alba* cv. Polara) were sterilized with 95% ethanol for 5 min followed by 2.5% hypochlorite for 20 min. Seeds were then rinsed and soaked in double-distilled water for 1–3 h at room temperature and optionally soaked overnight at 4 °C. Seeds were germinated for 48–60 h on 1.5% agar at room temperature in the dark. Between six and eight germinating seedlings were potted in each Leonard assembly and grown for two nights, at which point $\sim 10^8$ c.f.u. of *S. meliloti* was added to each jar. Plants were grown for 28 nights in a Convicon

Table 1. Strains and plasmids used

Strain or plasmid	Characteristics	Reference
<i>S. meliloti</i>		
Rm2011	WT SU47 <i>str-3</i> ; Sm ^R	Laboratory collection
RmFL3575	RmP110, <i>ocd</i> : : pTH1522; Sm ^R Gm ^R	Cowie <i>et al.</i> (2006)
RmG994	Rm1021, <i>dme-3</i> : : Tn5, <i>tme-4</i> : : ΩSp ^R ; Sm ^R Sp ^R Nm ^R	Driscoll & Finan (1996)
RmP1054	RmP110, FRT sites at pSymB nt 1 679 723 – 49 523; Sm ^R Nm ^R Gm ^R	Milunovic <i>et al.</i> (2014)
RmP110	WT Rm1021 with fixed <i>pstC</i> ; Sm ^R	Yuan <i>et al.</i> (2006)
RmP3103	Rm2011, <i>proC-1</i> : : Tn5-B20; Sm ^R Nm ^R	diCenzo & Finan (2015)
RmP3371	Rm2011, Δ <i>smb20003</i> : : <i>aacC4</i> ; Sm ^R Gm ^R	diCenzo & Finan (2015)
RmP3372	Rm2011, <i>proC-1</i> : : Tn5-B20, Δ <i>smb20003</i> : : <i>aacC4</i> ; Sm ^R Nm ^R Gm ^R	diCenzo & Finan (2015)
RmP3479	RmP3372 (pTH2877); Sm ^R Nm ^R Gm ^R Tc ^R	This study
RmP3514	RmP110, ΔB161 (pSymB nt 1 679 723 – 49 523, Δ <i>smb20003</i>); Sm ^R	This study
RmP3515	RmP3514, <i>proC-1</i> : : Tn5-B20 via φRmP3103; Sm ^R Nm ^R	This study
RmP3516	RmP3515, <i>ocd</i> : : pTH1522 via φRmFL3575; Sm ^R Nm ^R Gm ^R	This study
<i>Escherichia coli</i>		
MT616	MM294A <i>recA-56</i> (pRK600), mobilizer; Cm ^R	Finan <i>et al.</i> (1986)
Plasmids		
pTH2505	<i>flp</i> gene under protocatechuate-inducible promoter in pRK7813; Tc ^R	Zhang <i>et al.</i> (2012)
pTH2877	pLAFR1 library clone carrying <i>smb20003</i> ; Tc ^R	diCenzo & Finan (2015)

growth chamber with a day (18 h, 21 °C) and night (6 h, 17 °C) cycle. At the end of the growth period, shoots were cut off and dried at 50 °C for 1–2 weeks prior to weighing.

Acetylene reduction experiments were performed as described previously (Zhang *et al.*, 2012). All root systems per pot were placed in a 50 ml test tube, to which acetylene was added to a final concentration of 10 %. Ethylene production was determined at 0, 5, 10 and 15 min post-addition of acetylene and measured with an HP6890 gas chromatograph system (Agilent Technologies) as described previously (Zhang *et al.*, 2012). The rate of ethylene production was determined by fitting a linear line of best fit to the data and comparison to a standard curve.

Nodule wet weight was determined by measuring all nodules per pot. After 1 week of incubation at 50 °C, nodules were reweighed to determine dry weight. The c.f.u. of *S. meliloti* per mg nodule wet weight was determined by collecting all nodules from one or two root systems per pot. Nodules were weighed, washed with double-distilled water, surface sterilized with 1 % hypochlorite for 15 min, washed twice with TY + 300 mM sucrose, squashed in 0.25 ml TY + 300 mM sucrose and dilutions plated on TY agar. Phenotypes of *S. meliloti* were screened on appropriate media following their isolation.

Nodulation kinetics experiments were performed in 18 × 150 mm test tubes containing slants of Jensen's medium (Jensen, 1942), pH 7, solidified with 1 % agar. Tubes were closed with translucent polypropylene Kim-Kap caps. A single, sterile, 2-day-old white sweet clover seedling was transferred to each slant and inoculated with ~10⁶ c.f.u. of *S. meliloti* 3 days later. Plants were grown for 30 nights in a Conviron growth chamber with a day (18 h, 21 °C) and night (6 h, 17 °C) cycle. The root systems of the plants were examined every few days to identify the presence of nodules. Each *S. meliloti* strain was inoculated on 20 plants.

Microscopy. For confocal microscopy, nodules were harvested from plants 25 days post-inoculation. Nodules were fixed in 2.5 % (v/v) glutaraldehyde in 100 mM PIPES buffer at pH 7.0, vacuum infiltrated for 15 min and then incubated for 1 h at room conditions. Fixed nodules were washed extensively with fresh 100 mM PIPES buffer, mounted upon a specimen plate using crazy glue (Instant Crazy Glue;

Elmer's Products) and sliced using a vibrating blade microtome (VT1000; Leica) into 50 μm longitudinal sections. Nodule sections were stained in 80 mM PIPES buffer with SYTO 13 at 1 μl ml⁻¹ (Life Technologies) for 13 min and then washed twice with 80 mM PIPES for several minutes. Confocal images were acquired using a Leica SP5 microscope.

For transmission electron microscopy, nodules were harvested from plants 25 days post-inoculation. Nodules were fixed in 2 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, vacuum infiltrated for 15 min and then incubated for 1 h at room conditions. The samples were rinsed twice in 0.1 M sodium cacodylate buffer, post-fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h and then dehydrated through a graded ethanol series (50, 70, 70, 95, 95, 100, 100 %). Final dehydration was done in two changes of 100 % propylene oxide. The tissue pieces were slowly infiltrated with Spurr's resin through a graded series (2 : 1 propylene oxide/Spurr's, 1 : 1 propylene oxide/Spurr's, 1 : 2 propylene oxide/Spurr's, 100 % Spurr's, 100 % Spurr's, 100 % Spurr's), with rotation of the samples in between solution changes. The samples were then transferred to embedding moulds filled with fresh 100 % Spurr's resin and polymerized overnight at 60 °C. Thin sections cut on a Leica UCT ultramicrotome were post-stained with uranyl acetate and lead citrate, and sections viewed in a JEM 1200 EX TEMSCAN transmission electron microscope (JEOL) operating at an accelerating voltage of 80 kV.

RESULTS

Proline auxotrophy results in an impaired symbiosis with alfalfa

To examine the necessity of *S. meliloti* L-proline biosynthesis during the *S. meliloti*–legume symbiosis, we first examined the symbiotic phenotype of *S. meliloti* inoculated on alfalfa. Following 4 weeks of growth, alfalfa plants inoculated with the proline auxotroph (RmP3372; *proC*

smb20003) were green (Figs 2a and S1, available in the online Supplementary Material), indicative of a Fix^+ symbiosis; however, the shoot dry weights were $\sim 50\text{--}60\%$ of plants inoculated with the WT, or the *proC* or the *smb20003* single mutants (Fig. 2b). The shoot dry weight phenotype of the *proC smb20003* double mutant was fully complemented when *smb20003* was expressed *in trans* (Fig. S1 and data not shown). Acetylene reduction experiments corroborated the results of the shoot dry weights; the rate of ethylene production by plants inoculated with RmP3372 was decreased $\sim 60\%$ per plant compared with the WT (Fig. 2c). This decrease in nitrogenase activity appeared to be due to a decrease in total nodule mass per plant (Fig. 2e), as the rate of ethylene production per mg nodule weight was equal across all treatments (Fig. 2c). Although proline auxotrophy had an effect on the rate of nodule mass formation (Fig. 2e), proline auxotrophy did not appear to impact nodule initiation as the number of nodules per plant was not different (Fig. 2e) nor was the size of the viable *S. meliloti* population per mg nodule (Fig. 2d).

Proline auxotrophy results in a Fix^- phenotype in symbiosis with white sweet clover

To examine whether the requirement for *S. meliloti* proline biosynthesis during symbiosis was plant specific, the phenotype of RmP3372 was examined with white sweet clover. Following 4 weeks of growth, the shoot dry weight of white sweet clover inoculated with the proline auxotroph was statistically indistinguishable from that of a Fix^- control (*dme tme*) (Table 2). Most plants were yellow; however, a couple of plants per pot were green (Figs 3 and S2). Examination of the root systems of these plants indicated that nodules were formed, although the majority were spherical or slightly elongated white nodules and only a few were pink in coloration (Table 2, Fig. 3). Nodulation kinetics experiments confirmed that the total number of nodules per plant was equal between the WT and proline auxotroph, although a slight delay in the rate of nodule appearance was observed (Fig. 4). The apparent Fix^- nature of the majority of nodules was confirmed by acetylene reduction experiments (Table 2). Analysis of bacteria isolated from the few pink nodules formed on plants inoculated with the auxotroph showed that these were proline prototrophs and true revertants of the proline auxotroph (data not shown).

S. meliloti proline auxotroph induces largely empty nodules on white sweet clover

Confocal microscopy and transmission electron microscopy were used to examine the structure of nodules from white sweet clover plants inoculated with either WT *S. meliloti* or the proline auxotroph (Fig. 5). Nodules induced by WT *S. meliloti* contained many host cells filled with nitrogen-fixing bacteroid cells (Fig. 5a, c, e). In contrast, for the most part, the plant cells in the nodules induced

by the *S. meliloti* proline auxotroph were empty and of the infected host cells, only relatively few bacterial cells were observed (Fig. 5b, d, f). The cells of the proline auxotroph appeared bacteroid-like under transmission electron microscopy; however, unlike the WT, clear accumulation of poly-3-hydroxybutyrate (PHB)-like granules was observed (Fig. 5e, f). Undifferentiated *S. meliloti* in the infection thread and nodule can accumulate PHB, but these PHB stores are mobilized during the process of differentiation and the mature, nitrogen-fixing bacteroids do not accumulate PHB (Hirsch *et al.*, 1983; Ratcliff *et al.*, 2008). Thus, the presence of many PHB granules within the bacteroids of the *S. meliloti* auxotroph is consistent with these bacteroids not performing nitrogen fixation. The detection of bacterial cells within at least some of the host cells, as well as the presence of infection threads (not shown), suggested that the *S. meliloti* proline auxotroph was able to grow in, and be released from, the infection thread. Instead, the absence of a large population of differentiated, nitrogen-fixing bacteroids appeared to be the result of an inability to extensively multiply and fully differentiate once within the host cell.

ocd (*smb21494*) encodes an ornithine cyclodeaminase

Previous work has shown that *S. meliloti* is able to convert exogenously provided L-ornithine to L-proline through the activity of an ornithine cyclodeaminase (Fig. 1) (diCenzo & Finan, 2015; Soto *et al.*, 1994). However, the genetic determinant(s) for this activity have not been elucidated experimentally. *S. meliloti* strains lacking pSymB are unable to grow with L-ornithine as a sole carbon source (diCenzo *et al.*, 2014), indicating the necessary genes are located on this replicon. Two genes on pSymB, *eutC* and *ocd*, are putatively annotated as encoding ornithine cyclodeaminases (Finan *et al.*, 2001). A single cross-over plasmid integrant that disrupts *ocd* resulted in an inability to grow using L-ornithine as a sole carbon source (Fig. 6a). Transfer of the *ocd* plasmid integrant into RmP3515 [proline auxotroph; RmP110 *proC*: : Tn5-B20 Δ B161 (which includes *smb20003*)] prevented growth in M9-sucrose supplemented with L-ornithine, but not in M9-sucrose supplemented with L-proline (Fig. 6b). Thus, the *ocd* product is required for the conversion of L-ornithine to L-proline, presumably through the activity of an ornithine cyclodeaminase (Soto *et al.*, 1994).

Ornithine cyclodeaminase activity is not required for symbiosis of the *S. meliloti* proline auxotroph with alfalfa

To determine whether the Fix^+ phenotype of RmP3372 on alfalfa was due to residual L-proline synthesis via L-ornithine, the symbiotic phenotype of *S. meliloti* RmP3516 [RmP110, *proC* Δ B161(*smb20003*) *ocd*] was examined on alfalfa. The shoot dry weights of alfalfa plants inoculated with RmP3372 or RmP3516 were

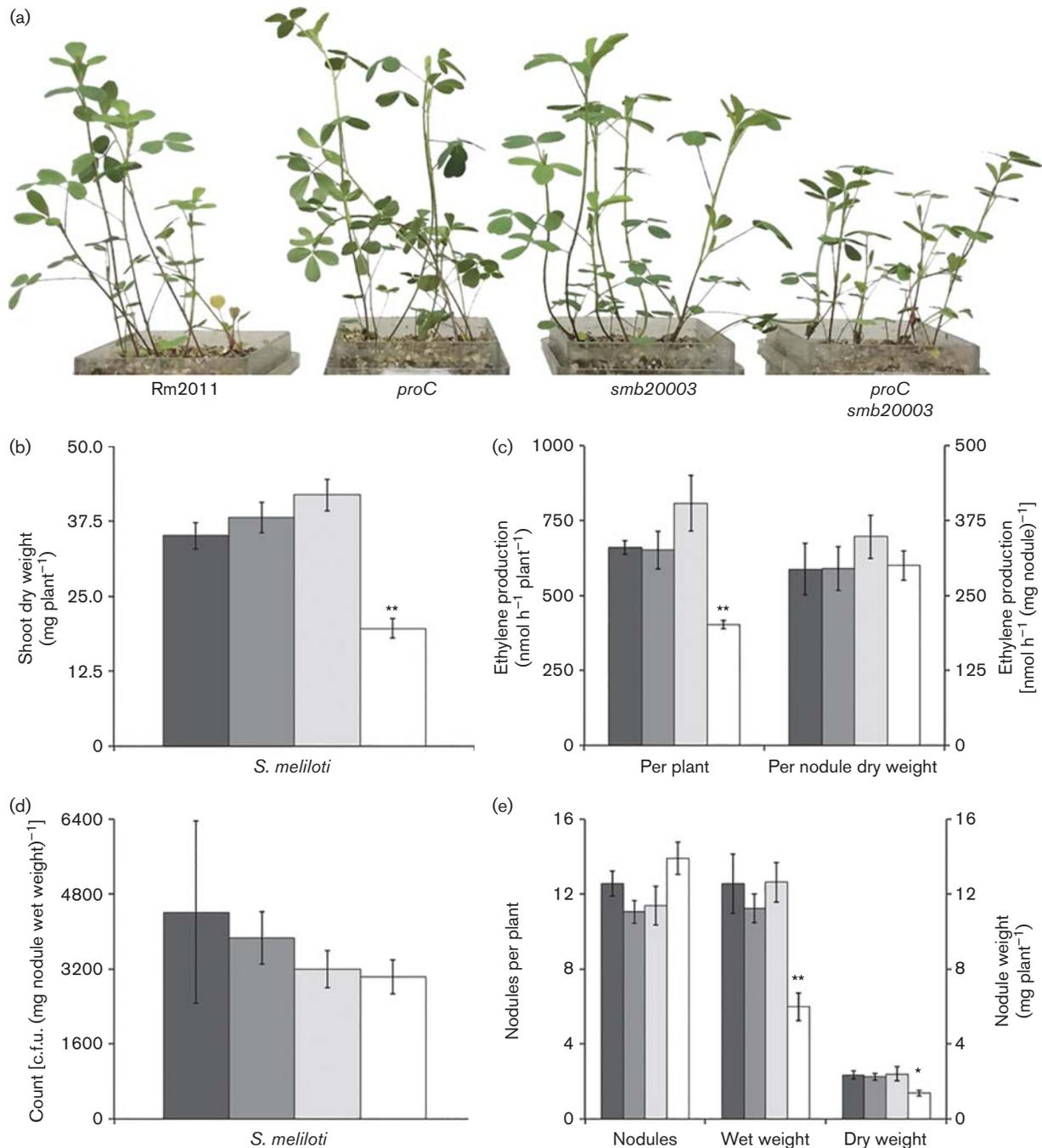


Fig. 2. Symbiotic phenotype of *S. meliloti* strains with alfalfa. (a) A picture of alfalfa plants 28 days post-inoculation with *S. meliloti* strains with the indicated genotype. (b) Shoot dry weights. (c) Rate of acetylene reduction of alfalfa root systems. (d) The c.f.u. of *S. meliloti* isolated from crushed nodules. (e) Nodule number and nodule mass. Data represent mean \pm SE from four Magenta jars. Statistically significant differences were identified using one-way ANOVAs, followed by a post-hoc test. **Treatment was statistically different ($P < 0.05$) as determined with a Tukey honestly significant difference post-hoc test. *Treatment was statistically different ($\alpha < 0.05$) as determined with a Student–Newman–Keuls post-hoc test, but not with a Tukey honestly significant difference post-hoc test. Black, WT Rm2011; dark grey, *proC*; light grey, *smb20003*; white, *proC smb20003*.

statistically equal (mean \pm SE: 12.5 ± 1.8 versus 11.0 ± 1.7 mg plant⁻¹; $P > 0.5$, Student's *t*-test), and plants inoculated with either had green shoots and pink nodules

(Fig. S1 and data not shown). Whilst we note that RmP3372 and RmP3516 are not in isogenic backgrounds (Rm2011 versus RmP110 and Δ *smb20003* :: *aacC4* versus

Table 2. Symbiotic phenotype of *S. meliloti* strains with white sweet clover

Values represent the mean (SE) of triplicate samples. The lower-case letters indicate the statistically unique groups in each row as determined by a Tukey honestly significant difference post-hoc test ($P < 0.05$) following one-way ANOVAs.

	WT	<i>proC</i>	<i>smb20003</i>	<i>proC smb20003</i>	<i>proC smb20003 smb20003</i> ⁺	<i>dme tme</i>
Shoot dry weight (mg plant ⁻¹)	46.7 (8.8) ^a	32.2 (1.5) ^a	32.0 (2.0) ^a	4.8 (0.2) ^b	30.5 (4.0) ^a	4.6 (0.6) ^b
Fix ⁺ nodules per plant	22.5 (3.7) ^a	18.4 (3.0) ^a	17.6 (2.1) ^a	1.5 (0.5) ^b	28.0 (4.5) ^a	0 ^b
Ethylene production (nmol h ⁻¹ plant ⁻¹)	883 (165) ^a	820 (85) ^a	868 (107) ^a	13.7 (13.7) ^b	888 (41) ^a	0 ^b

ΔB161), any difference between these strains is likely to exaggerate the phenotype of RmP3516 relative to RmP3372 (Table S1). Thus, the lack of a statistically significant difference between the shoot dry weight of alfalfa plants inoculated with RmP3372 versus RmP3516 indicates that *ocd* is not required for nitrogen fixation in the absence of *proC* and *smb20003*.

DISCUSSION

The study of rhizobial amino acid auxotrophs has revealed that biosynthesis of many, but not all, amino acids is

required during at least one step of symbiosis and that the requirement for the synthesis of a particular amino acid is not necessarily general to all rhizobia (Dunn, 2014). Whilst amino acid auxotrophy generally impacts the Fix, but not Nod, phenotype of the rhizobium, branched-chain amino acid auxotrophy results in a severe nodulation defect (de las Nieves Peltzer *et al.*, 2008). It has previously been observed that L-proline biosynthesis is required for *B. japonicum*–*Glycine max* (soybean) symbiosis (King *et al.*, 2000), whereas an *R. leguminosarum* proline auxotroph was able to establish a fully Fix⁺ symbiosis with *Pisum sativum* (pea) (Chien *et al.*, 1991).

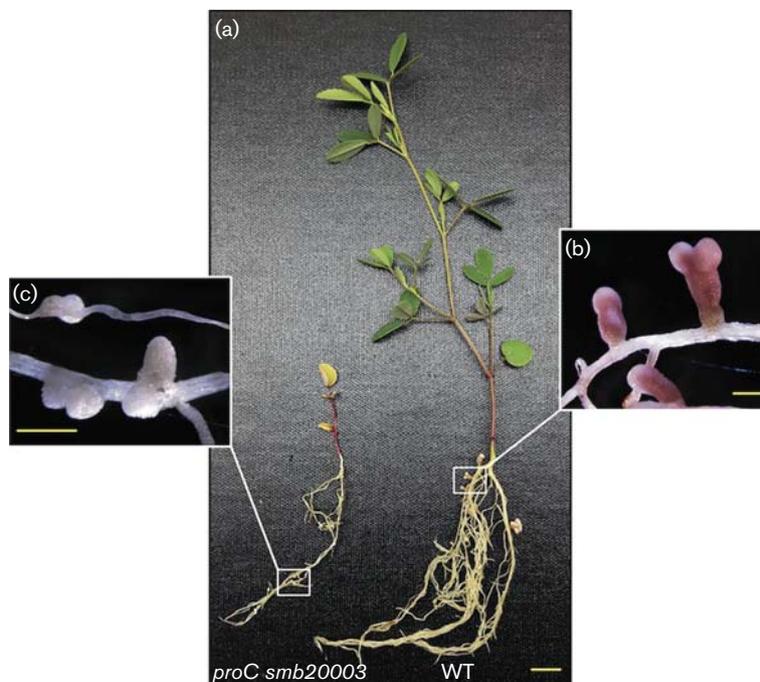


Fig. 3. Phenotypes of white sweet clover plants inoculated with *S. meliloti*. The shoot and nodule phenotypes of white sweet clover inoculated with *S. meliloti* Rm2011 (WT) and the proline auxotroph *S. meliloti* RmP3371 (Rm2011 *proC smb20003*) are shown. (a) The overall plant phenotype of white sweet clover inoculated with the WT (right) and the proline auxotroph (left). (b, c) The nodule morphology for plants inoculated with the WT (b) or the proline auxotroph (c). Bar: (a) 1 cm and (b, c) 1 mm.

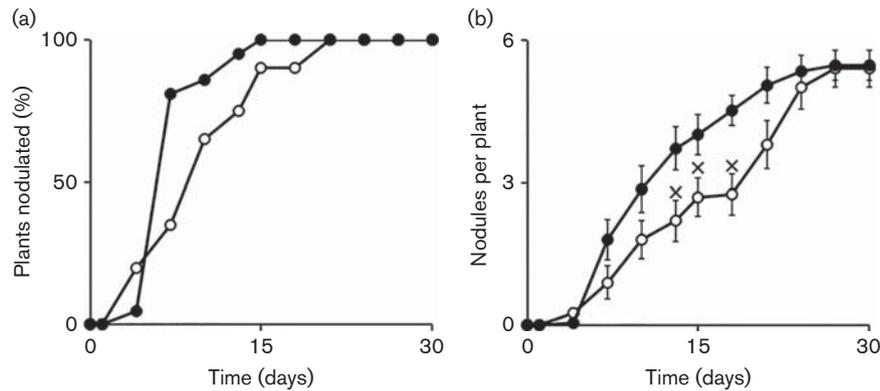


Fig. 4. The effect of proline auxotrophy on *S. meliloti* nodule kinetics. Twenty white sweet clover plants per strain were monitored for a 30 day period and (a) the percentage of plants with visible nodules and (b) the number of visible nodules per plant were recorded every few days. Closed circles, WT *S. meliloti* Rm2011; open circles, proline auxotroph *S. meliloti* RmP3372 (Rm2011 *proC smb20003*). The crosses indicate statistically significant differences (Student's *t*-test, $P < 0.05$).

However, the *R. leguminosarum* auxotroph could be complemented by exogenous L-glutamate, opening up the possibility that the Fix^+ phenotype was due to plant-derived L-glutamate and not L-proline. To help clarify the role of L-proline biosynthesis in rhizobium–legume symbioses, the symbiotic phenotype of a *S. meliloti* L-proline auxotroph was examined with alfalfa and white sweet clover.

The symbiotic phenotypes of *S. meliloti* RmP3372 (*proC smb20003*) on both white sweet clover and alfalfa (Figs 2 and 3, Table 2) suggest that L-proline biosynthesis is a general requirement for rhizobium–legume symbiosis to be fully effective, but the extent of the phenotype is influenced by the host. The ability of the *S. meliloti* proline auxotroph to form Fix^+ nodules on alfalfa was not dependent upon L-proline synthesis from L-ornithine, as knocking out the *ocd* gene did not result in a Fix^- phenotype. This suggests that throughout all stages of symbiosis with alfalfa, but not with white sweet clover, *S. meliloti* has access to a plant-derived source of L-proline, although it remains possible that *S. meliloti* indirectly obtains L-proline from the plant through the metabolism of stachydrine (Phillips *et al.*, 1998). However, it is unclear whether *S. meliloti* obtains stachydrine from alfalfa as the symbiotic phenotypes of stachydrine catabolic mutants may be independent of stachydrine metabolism (Burnet *et al.*, 2000; Goldmann *et al.*, 1994) and only one of the two stachydrine inducible catabolic loci is induced in alfalfa nodules (Barnett *et al.*, 2004). Nevertheless, this possibility is intriguing as it could explain why the symbiotic phenotype of the *S. meliloti* proline auxotroph was host dependent, as *Medicago* species produce stachydrine whereas other legumes do not (Phillips *et al.*, 1995, 1998; Wyn Jones & Storey, 1981); indeed, stachydrine complemented the proline auxotrophy of the mutants examined in this study (Fig. S3).

Given that L-proline biosynthesis was clearly required for a fully effective *S. meliloti*–legume symbiosis, we were interested in which stage of the symbiosis it was important. In conceptualizing rhizobium–legume symbiosis, we envisaged the biosynthetic demand for amino acids to be greatest in the actively dividing and differentiating bacteroids in zone II and the interzone II–III, and not in the non-growing, nitrogen-fixing bacteroids of zone III (Vasse *et al.*, 1990). Whilst the rhizobia are also actively dividing in the infection thread, we reasoned the requirement for amino acids by the infecting bacteria is relatively minor in comparison with the amount of amino acids that would be required to support the multiplication and enlargement of the bacteria as they differentiate and fill up the plant cells. If this were true, it would be expected that amino acid biosynthetic genes are expressed higher in the nodule zone II and interzone II–III than in the nitrogen-fixing zone III. To test this, we examined transcriptomic data for all *S. meliloti* genes annotated as being involved in amino acid biosynthesis, regardless of whether the annotation is experimentally supported, from the RNA sequencing dataset of Roux *et al.* (2014). By combining RNA sequencing with laser-capture microdissection of *Medicago truncatula* nodules, the authors determined the transcriptome of both the plant and infecting *S. meliloti* in five unique nodule zones. Based on their data, the majority (~62%; 65/105) of putative amino acid biosynthetic genes are downregulated in the nitrogen-fixing zone III (Table 3). Of the 40 that were not downregulated in zone III, only 10 (25%; ~10% overall) were expressed primarily in zone III (Table 3). Thus, these data support the notion that the biosynthetic demand for amino acids by *S. meliloti* in the nodule is much higher in the growing and differentiating bacteria than in the differentiated and nitrogen-fixing bacteroids. However, one must be cautious in interpreting the changes in expression across the nodule

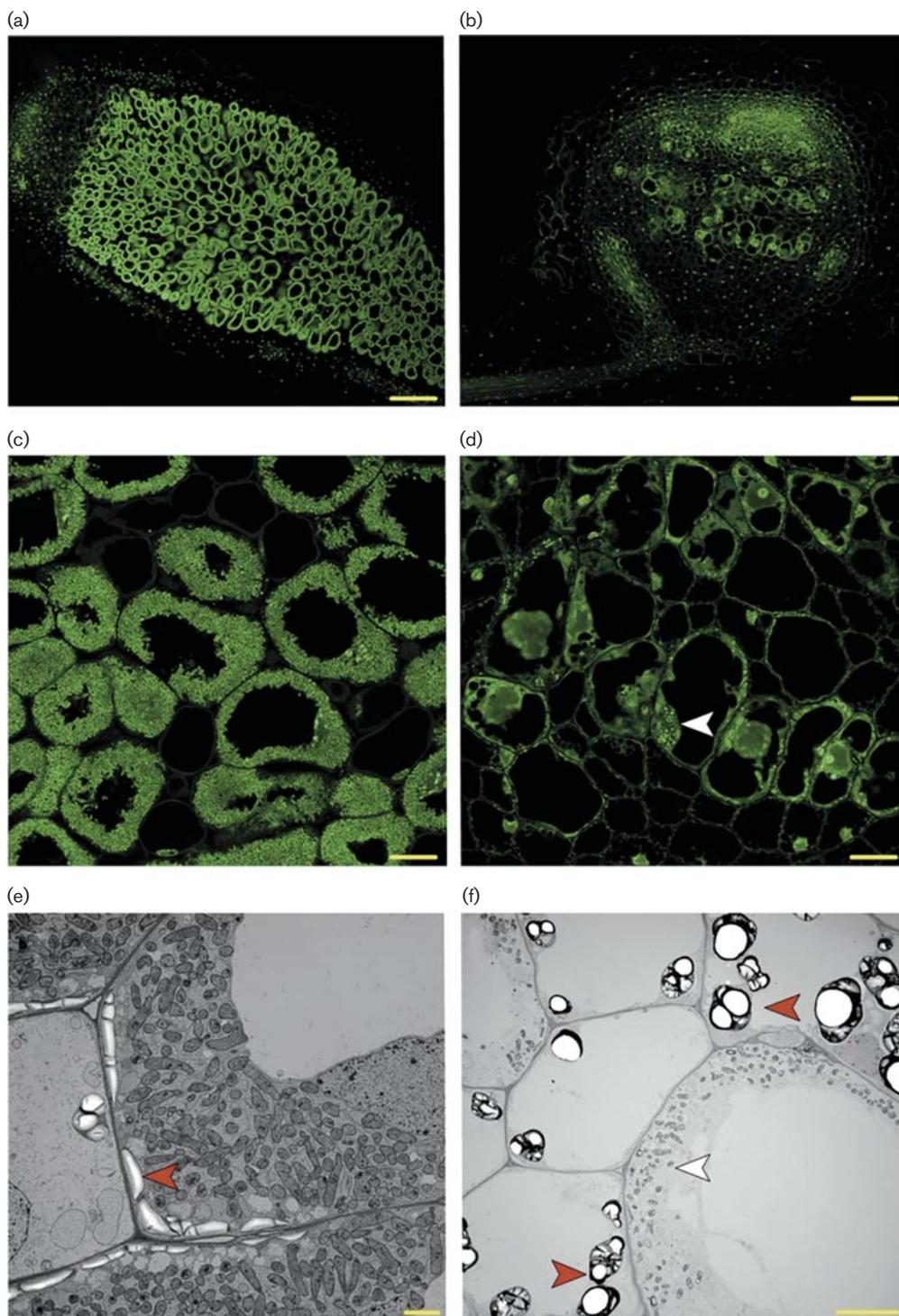


Fig. 5. White sweet clover nodules induced by the *S. meliloti* auxotroph contain few bacteroids. (a–d) Confocal and (e, f) transmission electron microscopy images of white sweet clover nodules from plants 25 days post-inoculation. (a, b) Confocal microscopy images showing the overall structure of nodules induced by WT *S. meliloti* (a) or the proline auxotroph RmP3372 (b). (c, d) Higher magnification view of cells from the central portion of nodules induced by the WT (c) or RmP3372 (d); the white arrow in (d) highlights some of the observed RmP3372 cells. (e, f) Transmission electron micrographs of nodules induced by the WT (e) or RmP3372 (f); the red arrows point to starch granules and the white arrows point to bacterial cells. Bar: (a, b) 250, (c, d) 25, (e) 2 and (f) 10 μm .

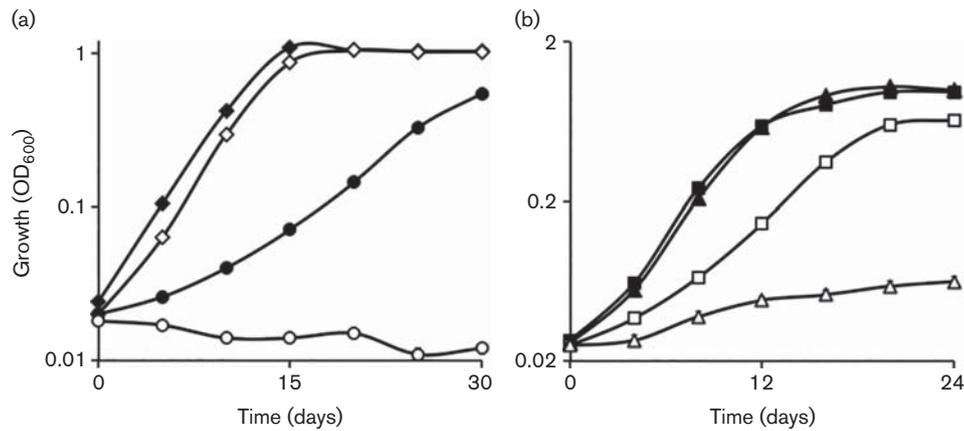


Fig. 6. The *ocd* gene encodes a functional ornithine cyclodeaminase. (a) Growth of *S. meliloti* RmP110 (solid symbols) and *S. meliloti* RmFL3575 (RmP110, *ocd*) (open symbols) in M9-sucrose (diamonds) and M9-L-ornithine (circles). (b) Growth of RmP3515 [RmP110, *proC* ΔB161(*smb20003*)] (solid symbols) and RmP3516 (RmP110, *proC* ΔB161 *ocd*) (open symbols) in M9-sucrose + L-proline (squares) and M9-sucrose + L-ornithine (triangles). Data represent mean ± SD of triplicate samples.

as representative of changes in amino acid availability, as most amino acid biosynthetic genes do not appear to be regulated by the corresponding amino acid. As shown in Table S2, a selection of reporter gene fusions to genes annotated as involved in amino acid, purine and pyrimidine biosynthesis showed little difference in expression whether they were grown in the presence (LB) or absence (M9) of these compounds. Nevertheless, a previous microarray analysis found a global transcriptional downregulation following bacteroid differentiation (Capela *et al.*, 2006), which is presumably correlated with a decrease in protein production and hence a reduced need for *de novo* amino acid biosynthesis.

This model is consistent with the symbiotic observations reported here. Following a slight delay, the proline auxotroph was able to induce WT numbers of nodules on white sweet clover (Fig. 4). The presence of the *S. meliloti* proline auxotroph within at least some of the legume cells of the root nodules indicated that it was able to grow in the infection thread and infect the host cells (Fig. 5). Thus, whilst perhaps not as efficient as the WT, the proline auxotroph was able to complete the infection process and thus the Fix⁻ phenotype is not due to the strain being unable to infect the plant. However, the majority of the plant cells were empty, and the cells that were infected contained few bacteria and these cells did not appear fully differentiated, consistent with the proline auxotroph failing to multiply and differentiate within the host cell. This is further supported by the phenotype of the mutant with alfalfa. The proline auxotroph was able to elicit Fix⁺ nodules on alfalfa, presumably due to a less severe proline limitation, but the size of the nodules was smaller (Fig. 2). We argue this was due to proline limitation restricting the rate of bacterial multiplication and differentiation within the host cell. However, proline biosynthesis appeared to be dispensable once the cells stopped

Table 3. Spatial expression of *S. meliloti* amino acid biosynthetic genes within *Medicago truncatula* nodules

Summary of the expression profile across the *Medicago truncatula* nodule of 105 *S. meliloti* genes putatively involved in amino acid biosynthesis. These data were extracted from the dataset of Roux *et al.* (2014). The clusters (first column) were defined by Roux *et al.* (2014) according to the expression pattern of the entire *S. meliloti* and *Medicago truncatula* gene set irrespective of gene function. Thus, different steps of the same biosynthetic pathway may be represented by separate clusters. The number of putative amino acid biosynthetic genes in each cluster is indicated in the final column. For each of the 105 genes, the expression in each nodule zone is presented as a ratio relative to zone ZIII, and values for genes belonging to the same cluster are presented as means. Approximately 62% of the genes showed decreased expression in the nitrogen-fixing zone (ZIII): clusters 3, 4, 5, 9 and 11. Notably, *proC* belongs to cluster 5, whilst *smb20003* belongs to cluster 8. The complete list of genes included in this table can be found in Table S3. ZI, zone I (meristematic region); ZII_d, zone II (infection zone) distal to zone III; ZII_p, zone II proximal to zone III; IZ, interzone II–III; ZIII, zone III (nitrogen-fixing zone).

Cluster	Nodule zone					No. of genes
	ZI	ZII _d	ZII _p	IZ	ZIII	
3	4.24	6.09	3.55	1.37	1.00	5
4	21.8	16.1	7.23	1.27	1.00	1
5	6.13	5.04	4.03	2.49	1.00	11
6	0.07	0.04	0.09	0.86	1.00	1
7	0.03	0.07	0.09	0.15	1.00	2
8	0.18	0.19	0.19	0.21	1.00	8
9	0.33	0.41	1.59	7.35	1.00	1
11	3.80	4.11	6.92	7.07	1.00	47
12	0.28	0.26	0.61	1.00	1.00	3
13	0.57	0.50	0.51	0.64	1.00	11
Not clustered	1.32	1.18	1.01	1.13	1.00	15
Total						105

multiplying and differentiating as the measured rate of acetylene reduction per mg nodule mass was not impacted; the availability of proline did not limit the rate at which the differentiated bacteroids fixed nitrogen. Thus, when considered together, these observations support the notion that *S. meliloti* L-proline biosynthesis, and potentially amino acid biosynthesis in general, is primarily a transient requirement for symbiosis due to the high biosynthetic demand encountered when the cells are attempting to differentiate and multiply within the infected host cell. Given that the requirement for rhizobial L-proline biosynthesis is conserved with the *B. japonicum*–soybean symbiosis (King *et al.*, 2000), and that *B. japonicum* bacteroids receive a physiologically relevant amount of L-proline from its legume host (Kohl *et al.*, 1988), we suggest that this is a conserved characteristic of rhizobium–legume symbioses.

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