Heterologous Complementation Reveals a Specialized Activity for BacA in the *Medicago*–*Sinorhizobium meliloti* Symbiosis

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The bacterium *Sinorhizobium meliloti* Rm2011 forms N₂-fixing root nodules on alfalfa and other leguminous plants. The pSymB chromid contains a 110-kb region (the ETR region) showing high synteny to a chromosomally located region in *Sinorhizobium fredii* NGR234 and related rhizobia. We recently introduced the ETR region from *S. fredii* NGR234 into the *S. meliloti* chromosome. Here, we report that, unexpectedly, the *S. fredii* NGR234 ETR region did not complement deletion of the *S. meliloti* ETR region in symbiosis with *Medicago sativa*. This phenotype was due to the *bacA* gene of NGR234 not being functionally interchangeable with the *S. meliloti bacA* gene during *M. sativa* symbiosis. Further analysis revealed that, whereas *bacA* genes from *S. fredii* or *Rhizobium leguminosarum* bv. *viciae* 3841 failed to complement the Fix[−] phenotype of a *S. meliloti bacA* mutant with *M. sativa*, they allowed for further developmental progression prior to a loss of viability. In contrast, with *Melilotus alba*, *bacA* from *S. fredii* and *R. leguminosarum* supported N₂ fixation by a *S. meliloti bacA* mutant. Additionally, the *S. meliloti bacA* gene can support N₂ fixation of a *R. leguminosarum bacA* mutant during symbiosis with *Pisum sativum*. A phylogeny of BacA proteins illustrated that *S. meliloti* BacA has rapidly diverged from most rhizobia and has converged toward the sequence of pathogenic genera *Brucella* and *Escherichia*. These data suggest that the *S. meliloti* BacA has evolved toward a specific interaction with *Medicago* and highlights the limitations of using a single model system for the study of complex biological topics.

The rhizobium-legume symbiosis is agriculturally and ecologically important. N₂-fixing root nodules are formed via an exchange of signaling molecules between the plant and bacteria, leading to the development of an infection thread and the eventual release of the rhizobia into cortical cells. The endocytosis-like release process results in the formation of a symbiosome, which is a rhizobial cell surrounded by a plant-derived membrane. Within symbiosomes, rhizobial cells differentiate into bacteroids and fix N₂ gas into ammonia that is provided to the plant. A major factor regulating the differentiation process are the nodule-specific

cysteine-rich (NCR) peptides that are found in legumes whose bacteroids are terminally differentiated (Alunni et al. 2007; Czernic et al. 2015; Kondorosi et al. 2013; Mergaert et al. 2006, 2003; Montiel et al. 2016; Van de Velde et al. 2010). NCR peptides are encoded by a large gene family with 700 members in *Medicago truncatula* and over 1,300 putative members in *Medicago sativa* (Guefrachi et al. 2014; Kondorosi et al. 2013; Maróti et al. 2015; O'Rourke et al. 2015; Young et al. 2011). NCR peptides are essential for bacteroid terminal differentiation as the inability of the plant to target NCR peptides to bacteroids or the expression of a rhizobial protein capable of cleaving NCR peptides results in loss of terminal differentiation (Price et al. 2015; Van de Velde et al. 2010). In vitro, NCR peptides display antimicrobial activity against a broad range of bacterial species; however, only a few NCR peptides have been studied in detail and the precise in planta activity of NCR peptides remains uncertain (Alunni and Gourion 2016; Farkas et al. 2014; Haag et al. 2011; Horváth et al. 2015; Kim et al. 2015; Kondorosi et al. 2013; Mikuláss et al. 2016; Penterman et al. 2014; Shabab et al. 2016; Tiricz et al. 2013; Van de Velde et al. 2010).

The ability of NCR peptides to promote terminal differentiation appears dependent on the rhizobial BacA or BacA-like proteins that are essential for symbiosis only with legumes expressing NCR peptides (Ardissone et al. 2011; Glazebrook et al. 1993; Guefrachi et al. 2015; Haag et al. 2011; Karunakaran et al. 2010; Maruya and Saeki 2010; Tan et al. 2009). BacA is a transmembrane protein capable of importing peptides and may function either as part of an ABC-type transport system or alone with transport driven by the electron motive force, as appears to be the case for the *Escherichia coli* BacA homolog (Glazebrook et al. 1993; Guefrachi et al. 2015; Haag et al. 2013; Marlow et al. 2009; Runti et al. 2013; Wehmeier et al. 2010). In free-living cells, *bacA* mutants have pleiotropic phenotypes that, depending on the species, can include increased sensitivity to NCR peptides and membrane-disturbing agents, increased resistance to certain antimicrobial compounds such as gentamicin, and altered lipopolysaccharide fatty acid composition (Ferguson et al. 2002, 2004; Glazebrook et al. 1993; Haag et al. 2011; Ichige and Walker 1997; LeVier and Walker 2001; Karunakaran et al. 2010). During symbiosis, *Sinorhizobium meliloti bacA* mutants rapidly die following release into *M. truncatula* or *M. sativa* nodule cells, except during symbiosis with plant mutants not able to target NCR peptides to the symbiosome (Glazebrook et al. 1993; Haag et al. 2011). The mechanisms by which BacA both protects the cell from the antimicrobial activity of NCR peptides and promotes symbiosis remain unclear (Alunni and Gourion 2016; Haag et al. 2011; Kondorosi et al. 2013).

BacA or BacA-like proteins are encoded by many nonrhizobial genomes, including *E. coli* (SbmA) and other pathogenic

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organisms, such as *Brucella abortus* and *Mycobacterium tuberculosis*, in which these proteins are required for persistent host infection (Domenech et al. 2009; LeVier et al. 2000). Heterologous expression of *bacA* across various species allows complementation of at least a subset of the free-living *bacA* mutant phenotypes (Kondorosi et al. 2013). *S. meliloti bacA* mutants form nodules that do not fix N_2 (Fix⁻) with *M. sativa* and the *bacA* orthologs from *B. abortus* and *E. coli* are able to fully complement this symbiotic defect (Ichige and Walker 1997; Wehmeier et al. 2010). In contrast, the *bacA* or *bacA*-like genes from *Mesorhizobium loti*, *Bradyrhizobium* species, or *Mycobacterium tuberculosis* only partially complemented the symbiotic phenotype (Arnold et al. 2013; Guefrachi et al. 2015; Maruya and Saeki 2010). Whereas *S. meliloti bacA* mutants induce small, white, and empty nodules, mutants expressing these three *bacA* genes induced small, transiently pinkish nodules filled with bacterial cells but that fixed little to no nitrogen. The *S. meliloti bacA* gene failed to complement both free-living and symbiotic phenotypes of a *Bradyrhizobium* species with a *bclA* (*bacA*-like) mutation; however, this may simply reflect difference in the energetics of transport as, unlike BacA, BclA is fused to an ATPase subunit (Guefrachi et al. 2015). Why all of these cross-species complementation attempts largely failed to restore N_2 fixation and what BacA features are required to ensure complementation remain to be determined (Kondorosi et al. 2013).

Our interest in BacA arises from our work to establish the necessary and sufficient rhizobial N_2 -fixing symbiotic genome using the model *S. meliloti*-*M. sativa* symbiosis (diCenzo et al. 2016). The 6.8-Mb genome of *S. meliloti* Rm1021/Rm2011 is split between a 3.7-Mb chromosome, a 1.7-Mb pSymB chromid, and a 1.4-Mb pSymA megaplasmid (Galibert et al. 2001). A recombination event transferred a 69-kb fragment, termed the ETR region, from the chromosome to pSymB in a *S. meliloti* ancestor (diCenzo et al. 2013, 2016). The ETR region contains two genes essential for cell viability and several genes implicated in N_2 -fixing symbiosis, the most notable being *bacA*. To identify the necessary and sufficient pSymA and pSymB symbiotic genes in *S. meliloti*, excluding the 'chromosomal' genes situated within the ETR region, the ETR region from *Sinorhizobium fredii* NGR234 was introduced into the *S. meliloti* chromosome and a derivative lacking pSymA and pSymB was constructed (diCenzo et al. 2016). *S. fredii* NGR234 is a broad-host range symbiont that can nodulate legumes from over 100 genera, although it generally displays poor symbiotic abilities with plants producing NCR peptides (Pueppke and Broughton 1999). The ETR region from *S. fredii* NGR234 was chosen for reintroduction into the *S. meliloti* chromosome as a phylogenetic analysis suggested that this version was more ancestral (diCenzo et al. 2013, 2016).

Here, we report that the ETR region from *S. fredii* NGR234 is not functionally equivalent in symbiosis to the *S. meliloti* ETR region and we present data to demonstrate that this functional difference resides in the *bacA* genes. The BacA protein of *S. meliloti* is shown to be phylogenetically distinct from that of most other rhizobia and, instead, groups together with the BacA of genera *Brucella* and *Escherichia*. Data are presented to support a specialized role of the *S. meliloti* BacA during symbiosis with plants of the *Medicago* genus that is not supported by other rhizobial BacA proteins, and we hypothesize that this is the driving force behind the divergence of *S. meliloti* BacA from the rest of the rhizobia.

RESULTS

The ETR region of *S. meliloti* and *S. fredii* NGR234 are not equivalent during symbiosis.

In previous genetic analyses of pSymB, various regions on pSymB, designated as, e.g., B109 and B122, were flanked

by flippase recognition target (*FRT*) sites and were deleted (Milunovic et al. 2014). Screening these deletion mutants for symbiotic phenotypes implicated the B109 and B122 regions as required for efficient N_2 fixation (diCenzo et al. 2016; Zamani et al. 2017). To extend our analysis of the symbiotic genes of the pSymB replicon and to look for potential redundancies, we deleted the 325-kb B202 region, located between B109 and B122 (Fig. 1A and B). Since this region included the ETR locus containing the *tRNA^{arg}* and *engA* genes that are essential for cell viability, we constructed the deletion in *S. meliloti* RmP3503, as it carried the ETR region of *S. fredii* NGR234 integrated into the *S. meliloti* chromosome (diCenzo et al. 2016). Surprisingly, the Δ B202 deletion in RmP3503 failed to fix nitrogen (Nod⁺Fix⁻) in

Fig. 1. Localization of the symbiotic locus within the *Sinorhizobium meliloti* B202 region. **A**, A schematic illustration, drawn to scale except for the right side of the B202 region and the deletion mutants within this region screened for symbiotic phenotypes. The thick black and gray line represents the B202 region of the *S. meliloti* pSymB replicon, with the gray indicating the location of the ETR region. The nucleotide borders are indicated and the positions of several loci of interest are marked. The above lines indicate the position of each deletion tested. **B**, An illustration of the entire pSymB replicon, indicating the relative position of the B202 region. **C**, The shoot dry weights of *Medicago sativa* plants inoculated with the deletions displayed in A are shown. Data points represent the mean of triplicate samples, with each sample consisting of five to eight plants, while the error bars indicate the standard error. Strains derived from the three *S. meliloti* backgrounds were tested on plants in independent experiments. RmP3503: Rm2011 containing the ETR region of *S. fredii* NGR234 integrated into the chromosome, RmP3951: RmP3503 with the *bacA* gene and promoter within the NGR234 ETR region deleted and replaced with the *bacA* gene and promoter of NGR234, and RmP3954: RmP3503 with the *bacA* gene and promoter within the NGR234 ETR region deleted and replaced with the *bacA* gene and promoter of Rm2011.

symbiosis with *M. sativa* (Fig. 1C). To investigate which genes were responsible for this symbiotic deficiency, we constructed and analyzed new smaller deletions corresponding to portions of the B202 segment. The symbiotic phenotype of these deletions on *M. sativa* localized the symbiotic deficiency to an approximately 30-kb region removed in Δ B110 but not in Δ B112 (Fig. 1). As this region contained the *bacA* gene known to be required for symbiosis (Glazebrook et al. 1993), the pSymB copy of *bacA* in RmP3503 was deleted and the resulting deletion was found to abolish symbiotic nitrogen fixation with *M. sativa* (Fig. 1C); this occurred despite the presence of the *bacA* gene of *S. fredii* NGR234 on the chromosome of *S. meliloti* strain RmP3503.

To determine whether loss of the pSymB *bacA* gene alone was responsible for the Fix^- phenotype of the Δ B202 deletion, strains were constructed in which the chromosomal *bacA* gene and upstream intergenic region (herein referred to as the promoter region, as transcriptional start site mapping and promoter prediction in *S. meliloti* [Schlüter et al. 2013] indicated that the *bacA* promoter was within the upstream intergenic region) within the *S. fredii* NGR234 ETR region was replaced with the *bacA* gene and promoter region of *S. meliloti* Rm2011 and, as a control, with the promoter region and *bacA* gene of *S. fredii* NGR234. Analysis of the symbiotic properties of the resulting strains revealed that replacing the chromosomal NRG234 *bacA* with the *S. meliloti* *bacA* complemented the Nod^+Fix^- phenotypes of both a pSymB Δ *bacA* mutant and the pSymB Δ B202 deletion mutant (Fig. 1C). These data established that the Fix^- phenotype of the Δ B202 deletion in the *S. meliloti* RmP3503 background was solely dependent on loss of the *S. meliloti* *bacA* gene.

Heterologous expression of rhizobial *bacA* genes in *S. meliloti*.

As *S. fredii* and *S. meliloti* are closely related species, it was expected that the *S. fredii* NGR234 *bacA* gene (*bacA_{Sf}*) would complement the Fix^- symbiotic phenotype of a *S. meliloti* *bacA* mutant. The lack of complementation prompted further investigation, and whether this might be due to improper transcription of the *bacA_{Sf}* gene in *S. meliloti* and whether expression of *bacA* from other rhizobia may function in place of the *S. meliloti* *bacA*. Accordingly, a *S. meliloti* Rm2011 Δ *bacA* deletion mutant was prepared. Constructs in which the *S. meliloti* Rm2011 *bacA* gene (*bacA_{Sm}*) expressed from its own promoter (*P_{Sm}*) as well as the *S. fredii* NGR234 *bacA* gene (*bacA_{Sf}*) or the *Rhizobium leguminosarum* bv. *viciae* 3841 *bacA* gene (*bacA_{RI}*) expressed from their own promoters (*P_{Sf}* and *P_{RI}*, respectively) or *P_{Sm}* were recombined into the Rm2011 genome in place of the wild-type *S. meliloti* *bacA* gene. The resulting *bacA* related strains are described in Table 1 and their construction is detailed below.

The gene region surrounding *bacA* in each species was visualized with progressiveMauve (Darling et al. 2010) and is shown in Supplementary Figure S1.

As free-living cells, *S. meliloti* *bacA* mutants show increased resistance to gentamicin, relative to wild type (Ichige and Walker 1997), and as an initial *bacA* complementation assay, we analyzed the Δ *bacA*::*bacA*⁺ constructs for their sensitivity to gentamicin. The results revealed that all strains grew equally well in LBmc (lysogeny broth with MgSO₄ and CaCl₂ supplementation) medium lacking gentamicin. In LBmc containing 20 μ g of gentamicin per milliliter, the Rm2011 Δ *bacA* mutant was clearly more resistant to gentamicin than the wild-type Rm2011 strain, and all of the Δ *bacA*::*bacA*⁺ strains showed increased sensitivity to gentamicin compared with the Δ *bacA* strain, although the level of sensitivity varied depending on the construct being examined (Supplementary Fig. S2). We therefore concluded that all *bacA* constructs were expressed in free-living cells and the *S. fredii* and *R. leguminosarum* *bacA* genes complemented the gentamicin-resistance phenotype of the *S. meliloti* Δ *bacA* mutant.

Heterologous *bacA* expression does not complement the *M. sativa* symbiotic phenotype of a *S. meliloti* *bacA* mutant.

M. sativa plants were inoculated with the various *bacA* strains and, as expected, the Δ *bacA* mutant nodules were small and failed to fix nitrogen (Fix^-), as measured by plant shoot dry weight and root acetylene reduction (Table 2; Fig. 2A). All of these phenotypes were fully complemented by expression of the wild-type *S. meliloti* *bacA* gene (Table 2; Fig. 2A). However, except for the *P_{Sm}*-*bacA_{Sf}* construct, expression of the *bacA_{Sf}* or *bacA_{RI}* genes failed to complement the phenotypes of the Δ *bacA* mutant, regardless of the promoter (Table 2; Fig. 2A). Plants inoculated with the *P_{Sm}*-*bacA_{Sf}* construct displayed very poor complementation, as there was not a statistically significant increase in shoot dry weight relative to the Δ *bacA* mutant, although some plants were slightly greener than those of the Δ *bacA* mutant and acetylene reduction activity was observed at an average rate approximately 14% of the wild type (Table 2; Fig. 2A).

The root nodules on *M. sativa* plants inoculated with the *P_{Sf}*-*bacA_{Sf}*, *P_{Sm}*-*bacA_{Sf}*, *P_{RI}*-*bacA_{RI}*, and *P_{Sm}*-*bacA_{RI}* strains were largely indistinguishable from the small, spherical, and white nodules induced by the *bacA* mutant, although in some cases there was a very slight pinkish color. However, confocal microscopy imaging of nodules showed that the *bacA_{Sf}* and the *bacA_{RI}* genes allowed the symbiosis to proceed to a developmentally more advanced stage than observed in Δ *bacA* nodules (Fig. 2B vs. D to G). Whereas the Δ *bacA* mutant nodules were devoid of bacterial cells aside from the first one or two plant cell layers near the nodule

Table 1. The primary *bacA* mutant strains used in this study

Strain	Genotype	Description
<i>Sinorhizobium meliloti</i>		
Rm2011	<i>bacA</i> ⁺	Wild type
RmP3898	Δ <i>bacA</i> :: <i>aacC4</i>	Rm2011 Δ <i>bacA</i> :: <i>aacC4</i>
RmP3903	Δ <i>bacA</i> :: <i>P_{Sm}</i> - <i>bacA_{Sm}</i>	RmP3898 with <i>aacC4</i> replaced by the <i>S. meliloti</i> Rm2011 <i>bacA</i> expressed from the Rm2011 <i>bacA</i> promoter
RmP3904	Δ <i>bacA</i> :: <i>P_{Sf}</i> - <i>bacA_{Sf}</i>	RmP3898 with <i>aacC4</i> replaced by the <i>S. fredii</i> NGR234 <i>bacA</i> expressed from the NGR234 <i>bacA</i> promoter
RmP3905	Δ <i>bacA</i> :: <i>P_{Sm}</i> - <i>bacA_{Sf}</i>	RmP3898 with <i>aacC4</i> replaced by the <i>S. fredii</i> NGR234 <i>bacA</i> expressed from the Rm2011 <i>bacA</i> promoter
RmP3906	Δ <i>bacA</i> :: <i>P_{RI}</i> - <i>bacA_{RI}</i>	RmP3898 with <i>aacC4</i> replaced by the <i>R. leguminosarum</i> bv. <i>viciae</i> 3841 <i>bacA</i> expressed from the 3841 <i>bacA</i> promoter
RmP3907	Δ <i>bacA</i> :: <i>P_{Sm}</i> - <i>bacA_{RI}</i>	RmP3898 with <i>aacC4</i> replaced by the <i>R. leguminosarum</i> bv. <i>viciae</i> 3841 <i>bacA</i> expressed from the Rm2011 <i>bacA</i> promoter
RmP3985	Δ <i>bacA</i>	RmP3898 with the <i>aacC4</i> gene removed
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>		
3841	<i>bacA</i> ⁺	Wild type
RU4040	<i>bacA</i>	3841 <i>bacA</i> ::pK19 (Karunakaran et al. 2010)
LMB247	<i>bacA</i>	RU4040 with the empty pRK404 vector (Karunakaran et al. 2010)
LMB248	<i>bacA</i> -/ <i>bacA_{Sm}</i> ⁺	RU4040 with <i>S. meliloti</i> Rm1021 <i>bacA</i> expressed in trans from pRK404 (Karunakaran et al. 2010)

meristem (Fig. 2B), nodules induced by any of the $P_{SF}\text{-}bacA_{SF}$, $P_{Sm}\text{-}bacA_{SF}$, $P_{RI}\text{-}bacA_{RI}$, or $P_{Sm}\text{-}bacA_{RI}$ constructs displayed two histologies; some appeared to be largely empty of bacterial cells (not shown), while many were either completely or partially full of bacterial cells (Fig. 2D to G). These bacterial cells appeared elongated, unlike those of the $\Delta bacA$ mutant, which was suggestive of at least partial bacteroid differentiation (Supplementary Fig. S3, including additional and higher magnification confocal microscopy images of *M. sativa* and *Melilotus alba* nodules).

The viability of bacterial cells within nodules was examined with the BacLight cell that differentially stains cells as viable or non-viable on the basis of membrane permeability. As expected, all nodule sections of nodules induced by the $P_{Sm}\text{-}bacA_{Sm}$ bacterial strain were dominated by viable cells, with all infected nodule cells containing viable bacteria (Fig. 2H). In contrast, a clear reduction in *S. meliloti* population viability was observed in nodules induced by the $P_{Sm}\text{-}bacA_{SF}$ or $P_{Sm}\text{-}bacA_{RI}$ strains (Fig. 2I and J). Although viable bacteria were detected in these nodules, many nodule cells had *S. meliloti* populations that were dominated by nonviable bacteria (Fig. 2I and J), a phenotype that we failed to detect in nodules induced by the $P_{Sm}\text{-}bacA_{Sm}$ strain. These observations illustrated that, compared with a *S. meliloti* $\Delta bacA$ mutant, *S. meliloti* strains expressing the *S. fredii* and *R. leguminosarum* *bacA* genes allowed the *S. meliloti*–*M. sativa* symbiosis to proceed to a developmentally more advanced stage, but an eventual loss of cell viability and nodule senescence was still observed.

Heterologous *bacA* expression complements the *Melilotus alba* symbiotic phenotype of a *S. meliloti* *bacA* mutant.

The ability of the *bacA_{SF}* and *bacA_{RI}* genes to allow the *S. meliloti*–*M. sativa* symbiosis to proceed to a more developmentally advanced stage compared with the $\Delta bacA$ allele prompted us to examine the symbiosis with an alternate host plant, *Melilotus alba* (Table 3). As for *M. sativa*, the $\Delta bacA$ mutant failed to fix nitrogen with *Melilotus alba* and this phenotype was fully complemented by the $P_{Sm}\text{-}bacA_{Sm}$ construct (Table 2; Fig. 3A). Strikingly, and in contrast to *M. sativa*, the $P_{Sm}\text{-}bacA_{SF}$ construct supported N_2 fixation at a rate 40 to 60% that of wild type, while the $P_{Sm}\text{-}bacA_{RI}$ construct supported N_2 fixation at a rate 80 to 90% that of wild type, as measured by shoot dry weights and acetylene reduction (Table 2; Fig. 3A). Moreover, the *bacA_{SF}* and *bacA_{RI}* complementation was dependent on expression from the *S. meliloti* *bacA* promoter, as this did not occur when *bacA_{SF}* or *bacA_{RI}* were expressed from their native promoter (Table 2; Fig. 3A).

On *Melilotus alba*, the $\Delta bacA$ nodules were small and white and essentially devoid of bacterial cells (Fig. 3B), whereas strains carrying the $P_{Sm}\text{-}bacA_{Sm}$, the $P_{Sm}\text{-}bacA_{SF}$, and the $P_{Sm}\text{-}bacA_{RI}$ constructs induced large, pink nodules filled with differentiated bacteroids (Fig. 3C, E, and G). Nodules induced by strains carrying the $P_{RI}\text{-}bacA_{RI}$ constructs were predominantly small and white, while $P_{SF}\text{-}bacA_{SF}$ nodules were small with a slight pink coloration. Resembling *M. sativa* nodules, the ineffective $P_{RI}\text{-}bacA_{RI}$ and $P_{SF}\text{-}bacA_{SF}$ nodules were developmentally more advanced than nodules induced by the $\Delta bacA$ mutant, when viewed with confocal microscopy, and presented two histologies, either completely or partially full of bacterial cells (Fig. 3D and F) or empty and likely senesced.

S. meliloti *bacA* supports nitrogen fixation by a *R. leguminosarum* bv. *viciae* 3841 *bacA* mutant with *Pisum sativum*.

As the *R. leguminosarum* *bacA* functionally replaced the symbiotic requirement of the *S. meliloti* *bacA* gene with *Melilotus alba* but not *M. sativa* (Table 2), it was of interest to determine if the *S. meliloti* *bacA* could function in place of the *R. leguminosarum*

bacA during the pea nodule symbiosis. Therefore, we examined whether the tetracycline-resistant broad-host range plasmid (pRK404-*bacA_{Sm}*), which contained the *S. meliloti* *bacA* gene, would complement the Fix^- symbiotic phenotype of a *R. leguminosarum* bv. *viciae* 3841 *bacA* mutant (Glazebrook et al. 1993; Karunakaran et al. 2010). The results from these experiments showed that the *S. meliloti* *bacA* gene supported nitrogen fixation by the *R. leguminosarum* *bacA* mutant (Table 3; Fig. 4A). However, the absolute level of complementation was uncertain, as the pRK404-*bacA_{Sm}* plasmid was lost during the symbiosis; roots of plants inoculated with the *bacA* mutant (pRK404-*bacA_{Sm}*) strain contained both white (65%) and pink (35%) nodules, and analysis of 11 nodules of each type revealed that approximately 60% of bacteria in the pink nodules carried the tetracycline resistance marker from pRK404-*bacA_{Sm}*, whereas all bacteria from the white nodules were tetracycline sensitive and, therefore, had lost pRK404-*bacA_{Sm}*. Microscopic analysis of nodules formed by the *bacA* mutant showed that the bacteria were released from the infection thread and appeared to have partially differentiated, followed by cell death (Fig. 4C, F, and G). The pink nodules induced by the *bacA* mutant carrying pRK404-*bacA_{Sm}* were similar to wild-type nodules and contained many plant cells filled with differentiated bacteroids (Fig. 4B and E vs. D and H). In contrast, the white nodules were equivalent to those induced by the *bacA* null mutant (not shown). Together, these data showed that expression of the *S. meliloti* *bacA* gene in a *R. leguminosarum* bv. *viciae* 3841 *bacA* mutant was sufficient to support nitrogen fixation with *P. sativum*.

The *S. meliloti* BacA protein is phylogenetically distinct from most rhizobia.

The 420-amino acid BacA proteins of *S. meliloti* Rm2011 and *S. fredii* NGR234 shared 59% amino acid identity. This low level of identity was unexpected, as all 2,022 putative orthologs, as determined via a Blast bidirectional best hit approach with an E-value cutoff of $1E-170$, shared an average of 88% identity. Similarly, whereas all 1,307 orthologs of *S. meliloti* Rm2011 and *R. leguminosarum* bv. *viciae* 3841 shared an average amino acid identity greater than 78%, their BacA amino acid sequences were just 58% identical (Supplementary Table S1; Supplementary Fig. S4). This was suggestive of rapid evolution of the BacA protein, which is under purifying selection in *S. meliloti* (Galardini et al. 2013), and therefore, we examined the phylogeny of a subset of BacA proteins.

As described below, the nonredundant set of BacA proteins from a subset of strains belonging to the order Rhizobiales and genera *Escherichia* and *Klebsiella* (82 proteins) were collected,

Table 2. Symbiotic phenotypes of *Sinorhizobium meliloti* strains with various *bacA* constructs^x

Genotype	<i>Medicago sativa</i>		<i>Melilotus alba</i>	
	SDW ^y	ARA ^z	SDW ^y	ARA ^z
Wild-type Rm2011	42.8 ± 6.8 a	400 ± 53 a	27.5 ± 1.4 a	439 ± 27 a
<i>dme tme</i>	7.8 ± 0.4 b	0 ± 0 b	4.8 ± 0.2 d	0 ± 0 c
$\Delta bacA::aacC4$	6.4 ± 0.2 b	0 ± 0 b	3.1 ± 0.7 d	0 ± 0 c
$\Delta bacA::P_{Sm}\text{-}bacA_{Sm}$	46.7 ± 6.7 a	343 ± 75 a	27.9 ± 3.7 a	398 ± 67 a
$\Delta bacA::P_{SF}\text{-}bacA_{SF}$	9.4 ± 0.6 b	0 ± 0 b	6.8 ± 1.0 d	18 ± 18 c
$\Delta bacA::P_{Sm}\text{-}bacA_{SF}$	12.1 ± 0.4 b	55 ± 8 b	13.3 ± 1.7 c	262 ± 23 b
$\Delta bacA::P_{RI}\text{-}bacA_{RI}$	7.1 ± 0.4 b	0 ± 0 b	5.0 ± 0.4 d	0 ± 0 b
$\Delta bacA::P_{Sm}\text{-}bacA_{RI}$	8.9 ± 0.6 b	13 ± 13 b	22.4 ± 1.0 b	401 ± 22 a

^x Data represent the mean ± standard error of triplicate samples, with each sample consisting of between five to eight plants. The *dme tme* strain represents a Nod⁺Fix[−] control. Different letters indicate statistically unique groups ($\alpha < 0.05$) and were determined via one-way analyses of variance, followed by Duncan's posthoc tests. Statistical analyses were performed separately for each plant and each variable.

^y SDS = shoot dry weight (milligrams per plant).

^z ARA = acetylene reduction activity (nanomoles per minute per plant).

and maximum likelihood phylogenies were built based on the BacA amino acid sequences (Fig. 5B, Supplementary Fig. S5A) or based on a multilocus sequence analysis (MLSA) of the corresponding genomes (Fig. 5A). It was immediately obvious that the topology of the BacA tree was incongruent with the topology of the MLSA tree. Notably, the BacA proteins from *S. meliloti* and a few other rhizobia formed a monophyletic group, together with the *Brucella*, *Ochrobactrum*, *Escherichia*, and *Klebsiella* BacA proteins, while the BacA proteins of the other *Sinorhizobium*, *Ensifer*, *Rhizobium*, *Neorhizobium*, *Agrobacterium*, and *Mesorhizobium* genera formed a second monophyletic group. No obvious domain was identified that drove the separation of these clades (Supplementary Dataset S1 includes a Newick formatted phylogeny, the BacA alignments, and the BacA logo.). The genes present immediately upstream (Smb21000) and downstream (Smb20998) of *bacA* in *S. meliloti* also flanked the *bacA* gene in 44 of the 53 *Rhizobiales* genomes examined in the above analysis. Phylogenies of the Smb21000

orthologs or the Smb20998 orthologs were congruent with the MLSA phylogeny (Fig. 5A). The synteny of the *smb20998-bacA-smb21000* region between *S. meliloti* and *Brucella* species and the observation that the phylogenetic pattern observed for BacA is not also observed for Smb20998 and Smb21000 strongly suggested that the observed phylogeny was not due to horizontal gene transfer of *bacA* but, instead, due to the *S. meliloti* BacA having undergone rapid, convergent evolution to that of the BacA proteins of the pathogenic genera *Brucella*, *Escherichia*, and *Klebsiella*.

DISCUSSION

Phylogenetic analyses showed that the BacA protein from *S. meliloti* was more similar to BacA from pathogenic organisms such as genera *Brucella*, *Ochrobactrum*, *Escherichia*, and *Klebsiella* than to the rest of the *Rhizobiaceae* family (Fig. 5). Data here and elsewhere suggested that this reflected some underlying difference in the activity of the BacA orthologs. The

Fig. 2. Symbiotic phenotypes of *Medicago sativa* plants inoculated with *Sinorhizobium meliloti*. **A**, *M. sativa* plants 28 days postinoculation with *S. meliloti*. From left to right, the plants are inoculated with wild-type Rm2011, RmP3898, RmP3903, RmP3904, RmP3905, RmP3906, and RmP3907. **B** to **G**, Confocal microscopy images of fixed nodules stained with the DNA binding dye Syto13. The scale bar represents 250 μm . **H** to **J**, Confocal microscopy images of fresh, nonfixed nodules stained with the BacLight Live/Dead stain (Invitrogen) that differentially stains living and dead cells. The scale bar represents 25 μm . The nodules were induced by the following strains: RmP3898 (**B**), RmP3903 (**C** and **H**), RmP3904 (**D**), RmP3905 (**E** and **I**), RmP3906 (**F**), and RmP3907 (**G** and **J**).

bacA orthologs of *B. abortus* and *E. coli* complemented the symbiotic N₂-fixation phenotype of a *S. meliloti bacA* mutant with *M. sativa* (Ichige and Walker 1997; Wehmeier et al. 2010), whereas the *bacA* orthologs of *Mesorhizobium loti* (Maruya and Saeki 2010), *S. fredii*, or *R. leguminosarum* did not (Fig. 2; Table 2). Thus, presumably, BacA proteins of the *Escherichia-Brucella* clade have a modified activity that the other BacA proteins lack, such as expanded substrate recognition or differing substrate affinity, perhaps allowing recognition and transport of a broader range or higher concentration of host peptides (NCR peptides during symbiosis). As the *S. fredii* and *R. leguminosarum bacA* genes were able to complement the symbiotic phenotype of a *S. meliloti bacA* mutant with *Melilotus alba* (Fig. 3; Table 2), we hypothesize that the symbiosis with plants of the *Medicago* genus was the driving force behind the divergence of the *S. meliloti* BacA from the other rhizobia. Additionally, these results highlight how the activity of BacA and potentially NCR peptides may differ greatly between symbioses

and the importance of studying multiple symbioses to understand what conclusions are generalizable and which are symbiosis dependent.

In addition to differences in the ability of the BacA proteins to functionally replace the *S. meliloti* BacA during symbiosis, a striking promoter effect was also observed, both in free-living and symbiotic cells. *S. meliloti* $\Delta bacA$ mutants that expressed the *R. leguminosarum bacA* under its native promoter were more sensitive to gentamicin than when it was expressed from the *S. meliloti bacA* promoter. During symbiosis with *Melilotus alba*, *S. meliloti* $\Delta bacA$ mutants that expressed either the *S. fredii* or the *R. leguminosarum bacA* genes from the *S. meliloti bacA* promoter formed effective nodules, whereas ineffective nodules were formed when these genes were expressed from their native promoters (Fig. 3; Table 2). However, the converse was not true; *S. meliloti bacA* complemented the symbiotic phenotype of a *S. meliloti* $\Delta bacA$ mutant regardless of whether it was expressed from the *S. meliloti* or the *S. fredii bacA* promoters (Supplementary Table S2). In previous work, the *E. coli bacA* homolog *smBA* was shown to complement the symbiotic phenotype of a *S. meliloti bacA* mutant when expressed from the *S. meliloti bacA* promoter but not from its native promoter (Ichige and Walker 1997), whereas the *S. meliloti bacA* can complement a *bacA* mutant regardless of whether it is expressed from its native promoter or the *P_{trp}* promoter (Wehmeier et al. 2010). Hence, it appears as though the *bacA* promoter, in addition to the coding sequence, has undergone rapid functional divergence and that the ability of a bacterial strain to fix nitrogen in symbiosis with a particular host is determined, in part, by an interplay between the *bacA* coding sequence and its cognate promoter.

Evidence is consistent with *S. meliloti bacA* mutants dying almost immediately upon release in the cytoplasm of the plant nodule cells (Figs. 2B and 3B) (Glazebrook et al. 1993) and that

Table 3. Fix phenotypes of *Pisum sativum* inoculated with *Rhizobium leguminosarum*^x

Genotype	SDW ^y	ARA ^z
Wild type	1,280 ± 67 a	27.1 ± 3.0 a
Uninoculated	385 ± 150 c	0 ± 0 b
<i>bacA::pK19</i>	330 ± 50 c	0 ± 0 b
<i>bacA::pK19, pRK404</i>	513 ± 86 c	0 ± 0 b
<i>bacA::pK19, pRK404(bacA_{Sm})</i>	827 ± 64 b	1.5 ± 0.2 b

^x Data represent the mean ± standard error of triplicate samples consisting of a single plant per replicate. Different letters indicate statistically unique groups ($\alpha < 0.05$) and were determined via one-way analyses of variance, followed by Duncan's posthoc tests that were performed independently for each column.

^y SDS = shoot dry weight (milligrams per plant).

^z ARA = acetylene reduction activity (nanomoles per minute per plant).

Fig. 3. Symbiotic phenotypes of *Medicago alba* plants inoculated with *Sinorhizobium meliloti*. **A**, *Melilotus alba* plants 28 days postinoculation with *S. meliloti*. From left to right, the plants are inoculated with wild-type Rm2011, RmP3898, RmP3903, RmP3904, RmP3905, RmP3906, and RmP3907. **B** to **G**, Confocal microscopy images of fixed nodules stained with the DNA binding dye Syto13. The scale bar represents 250 μ m. The nodules were induced by the following strains: RmP3898 (B), RmP3903 (C), RmP3904 (D), RmP3905 (E), RmP3906 (F), and RmP3907 (G).

this death is dependent on NCR peptides (Haag et al. 2011). Similarly, *R. leguminosarum* bv. *viciae* appeared to rapidly die upon release into the nodule cells of *P. sativum* cv. Avola (Karunakaran et al. 2010), as did *M. hakuui* upon release into *Astragalus sinicus* cells (Tan et al. 2009). Interestingly, this does not appear to be universally true, as *Bradyrhizobium bclA* (*bacA*-like) mutants are Fix^- but are capable of establishing a population in the nodule (Guefrachi et al. 2015), and a *R. leguminosarum* bv. *viciae* *bacA* mutant appeared to begin the differentiation process before undergoing cell death during symbiosis with *P. sativum* cv. Homesteader plants (Fig. 4C, F, and G). As shown here (Figs. 2 and 3; Table 2) and elsewhere (Guefrachi et al. 2015; Maruya and Saeki 2010), the initial cell death of *S. meliloti* *bacA* mutants upon release into the nodule can be complemented through heterologous expression of other rhizobial *bacA* genes; however, these bacterial strains remain either unable to fully differentiate into nitrogen-fixing bacteroids or support poor nitrogen fixation. Thus, BacA appears to play two roles during symbiosis, i.e., an early function in protecting the cell from lysis related to exposure to NCR peptides, which is not universally conserved, and a later role in promoting bacteroid differentiation. We wonder whether the initial cell death is a pleiotropic phenotype due to the overall reduced fitness and poor membrane stability of the *bacA* mutants

whereas the second role of BacA may be related to the transport of NCR peptides into the cell, thus allowing the NCR peptides to promote terminal differentiation. Previous studies have illustrated that the BacA proteins from *S. meliloti* Rm1021, *S. fredii* NGR234, and *R. leguminosarum* bv. *viciae* 3841 mediated transport of eukaryotic peptides (Ardissone et al. 2011; Karunakaran et al. 2010; Marlow et al. 2009), although the substrate specificity of each has not been compared. Given that NCR peptides appear to have rapidly evolved and diversified (Nallu et al. 2014; O'Rourke et al. 2015), there may have been a corresponding rapid evolution in the substrate specificity of the BacA proteins, potentially explaining the incomplete complementation following heterologous *bacA* expression in rhizobial *bacA* mutants.

We consistently observed that approximately 10 to 25% of *M. sativa* plants inoculated with *S. meliloti* $\Delta bacA$ mutants containing $P_{\text{Sm}}\text{-}bacA_{\text{sf}}$ (i.e., RmP3905) and, to a lesser extent, $P_{\text{SF}}\text{-}bacA_{\text{sf}}$ (e.g., RmP3904, RmP3556) formed several thin, elongated Fix^+ nodules on *M. sativa* that likely accounted for all the acetylene reduction activity of these plant root systems (Table 2). Bacteria isolated from these nodules maintained the original Fix phenotype when reinoculated onto *M. sativa* plants (not shown), indicating that the apparent ability of some of the

Fig. 4. Symbiotic phenotypes of *Pisum sativum* plants inoculated with *Rhizobium leguminosarum*. **A**, *P. sativum* plants 29 days postinoculation (dpi) with *R. leguminosarum* bv. *viciae* strains. From left to right, the plants are inoculated with: wild-type 3841, RU4040, LMB247, and LMB248. **B** to **H**, Confocal microscopy images of fixed nodules stained with the DNA binding dye Syto13. Nodules were collected either 29 dpi (**B**, **D**, **E**, and **H**) or 24 dpi (**C**, **F**, and **G**). The scale bar represents 250 μm (**B** to **D**) or 25 μm (**E** to **G**). The nodules were induced by the following strains: wild-type 3841 (**B** and **E**), RU4040 (**C**, **F**, and **G**), and LMB248 (**D** and **H**).

bacterial mutants to escape cell death in planta and fix nitrogen was likely due to variations in the *M. sativa* plant host, which has a tetraploid genome (O'Rourke et al. 2015). Given the relationship between BacA and NCR peptides (Haag et al. 2011; Kondorosi et al. 2013), it may be that certain *M. sativa*

plants produce lower amounts of NCR peptides or fail to produce one or more of the 1,300 putative NCR peptides (O'Rourke et al. 2015). Future study of the transcriptome of these Fix⁺ nodules may yield novel insights into the interplay between BacA and NCR peptides.

Fig. 5. Phylogenetic analysis of the BacA protein. **A**, An unrooted bacterial maximum likelihood phylogeny derived from the concatenated alignments of 25 proteins present in all 82 genomes analyzed. **B**, An unrooted BacA maximum likelihood phylogeny based on the amino acid sequences of the BacA proteins encoded by the same 82 genomes. Arrows indicate the position of the BacA proteins examined in this study. Numbers indicate bootstrap values from 100 replicas.

The analysis of the *S. meliloti* and *S. fredii* BacA proteins reported here was motivated by the goal of engineering a minimal symbiotic genome for *S. meliloti*. Thus, differences in the *bacA* genes falsified our assumption that the genes in the ETR region of *S. fredii* NGR234 would complement and allow deletion of the ETR region genes from the *S. meliloti* pSymB chromid. However, we now know that replacement of just the *bacA* gene within the *S. fredii* NGR234 ETR region in the *S. meliloti* chromosome was sufficient to complement the Fix⁻ phenotype of the Δ B202 deletion that removes the pSymB ETR region (Table 1). This new strain, RmP3954, will now serve as our primary background strain for identification of the necessary and sufficient symbiotic nitrogen-fixation genes of pSymA and pSymB. Additionally, the unexpected inability of the *S. fredii* NGR234 *bacA* gene to complement deletion of the *S. meliloti* *bacA* gene illustrates how the journey toward the minimal symbiotic genome is likely to reveal new insights into the functioning of the symbiosis.

MATERIALS AND METHODS

Construction of the *S. meliloti* pSymB Δ B202 deletion and subdeletions.

Primary strains and plasmids used in this study are described in Supplementary Table S3 (Blondelet-Rouault et al. 1997; Cowie et al. 2006; diCenzo and Finan 2015; diCenzo et al. 2013, 2014, 2016; Ditta et al. 1985; Finan et al. 1984, 1986; Glazebrook et al. 1993; Johnston and Beringer 1975; Karunakaran et al. 2010; Milunovic et al. 2014, 2016; Pérez-Mendoza et al. 2004; Zhang et al. 2012). The plasmid pTH1942, which contains a *FRT* site, was recombined via conjugation into the *S. meliloti* RmP2719 genome, which carries copies of the two essential pSymB genes on the chromosome. The Δ B179 deletion, which contains a *FRT* site, was recombined into the resulting strain via transduction from RmP2712, producing RmP3555. The region between the pTH1942 integrant and the Δ B179 deletion in RmP3555 was deleted by expression of the Fip recombinase from pTH2505, to produce RmP3556, containing the Δ B202 deletion. The presence of the Δ B202 deletion was confirmed by whole genome Illumina sequencing as described before (diCenzo et al. 2016). The Δ B202 deletion was recombined via transduction of the neomycin-resistant marker into RmP3503, producing RmP3587, for initial testing of the Δ B202 symbiotic phenotype.

Five subdeletions within the B202 region were constructed in the *S. meliloti* RmP3503 background. Δ B112 was recombined into RmP3503 via transduction from RmP823. Δ B179 was recombined into RmP3503 via transduction from RmP2712. Plasmid pTH1522 and pTH1937 integrants containing *FRT* sites and bordering the B110 region were transduced from RmP734 into RmP3503, and the Δ B110 deletion was made by expression of Fip from pTH2505. The Δ B116 and Δ B158 deletions were similarly made and the *FRT* sites were transduced from RmP800 and RmP896, respectively.

Construction of the *bacA* deletion and expression vectors.

The 3' end of the gene upstream of *bacA* (oligos: DF036 and DF037; Supplementary Table S4 lists the sequences of all oligonucleotides) and the region immediately downstream of *bacA* (oligos: DF038 and DF039) were polymerase chain reaction (PCR)-amplified and were simultaneously cloned into *Xba*I-digested pTH2919 (Tc^r, *sacB*). Transformants were pooled and digested with *Nsi*I, which cleaved in-between the two cloned PCR products, following which a *Nsi*I-digested *aacC4* cassette that was PCR-amplified (oligo DF040) from pHP45aac was ligated into the cut vector, producing pTH3127. The same procedure was followed to produce plasmids pTH3128 to pTH3130, except that the regions cloned into each vector differed. For construction of pTH3128, the region

immediately upstream (oligos: DF038 and DF041) and downstream (oligos: DF038 and DF039) of *bacA* were used. Plasmids pTH3129 (oligos: DF042 and DF043, DF044 and DF045) and pTH3130 (DF042 and DF046, DF044 and DF045) are equivalent to plasmids pTH3127 and pTH3128, respectively, except that the PCR-amplified fragments were amplified from *S. fredii* NGR234 and not from *S. meliloti*. Finally, plasmid pTH3159 refers to plasmid pTH3128 that has been digested with *Nsi*I to remove the *aacC4* gene and with the plasmid ends religated together.

To construct the *bacA* complementation vectors, plasmids pTH3127 and pTH3128 were digested with *Nsi*I to remove the *aacC4* gene. The *S. meliloti* *bacA* promoter region and coding sequence was PCR-amplified from *S. meliloti* Rm2011 (oligos: DF047 and DF048), and introduced into the *Nsi*I-digested pTH3127 via sequence- and ligation-independent cloning (Jeong et al. 2012), producing pTH3131; the primers were designed such that the entire *Nsi*I recognition sequence was removed following the cloning. The same procedure was performed using the *bacA* coding sequence and promoter region that was PCR-amplified from *S. fredii* NGR234 (oligos: DF049 and DF050) or *R. leguminosarum* bv. *viciae* 3841 (oligos: DF051 and DF052), producing plasmids pTH3132 and pTH3133, respectively. To express the *bacA* genes of *S. fredii* NGR234 (oligos: DF053 and DF050) and *R. leguminosarum* bv. *viciae* 3841 (oligos: DF054 and DF052) under control of the *S. meliloti* Rm2011 promoter, the *bacA* coding sequence from the respective genomes was PCR-amplified and was cloned into *Nsi*I-digested pTH3128 as described above, producing pTH3134 and pTH3135, respectively.

The same procedure was employed to construct the vectors for replacement of the *S. fredii* NGR234 *bacA* gene. The *bacA* promoter region and coding sequence of *S. meliloti* Rm2011 (oligos: DF055 and DF056) and *S. fredii* NGR234 (oligos: DF057 and DF058) were PCR-amplified and were cloned into *Nsi*I-digested pTH3129 to produce pTH3136 and pTH3137, respectively. For expression of the *bacA* gene of *S. meliloti* Rm2011 under the control of the *S. fredii* NGR234 promoter, the *bacA* coding sequence from *S. meliloti* Rm2011 was PCR-amplified (oligos: DF106 and DF056) and was cloned into *Nsi*I-digested pTH3130, producing pTH3138.

Allele replacement of the *S. meliloti* Rm2011 *bacA* gene.

In order to delete the *bacA_{sm}* promoter and coding sequence in the *S. meliloti* Rm2011 and RmP3503 backgrounds, double crossover recombinants of pTH3127 into the appropriate strains were recovered as described before (diCenzo and Finan 2015), producing RmP3898 and RmP3899, respectively. Deletion of just the *bacA_{sm}* coding sequence in the RmP3503 background was performed by isolating double crossover recombinants of pTH3128, producing RmP3900. A gentamycin-sensitive Δ *bacA* mutant was constructed through recovery of pTH3159 double crossover recombinants in RmP3898 (Δ *bacA::aacC4*), which would delete the *aacC4* gene. Expression of the five *bacA* constructs described above was accomplished by isolating double crossover recombinants of plasmids pTH3131 – pTH3135 into *S. meliloti* RmP3898 (Δ *bacA::aacC4*), producing RmP3903–RmP3907. Recombinants were recovered as described previously, and the correct genotype confirmed by gentamycin and tetracycline sensitivity and sucrose resistance and by PCR.

Allele replacement of the *bacA* gene in the NGR234 ETR region integrated into the *S. meliloti* chromosome.

The *bacA* gene of *S. fredii* NGR234 integrated into the *S. meliloti* chromosome as part of the ETR region was replaced with three constructs using the same procedure as described above for the allele swapping of *bacA* in *S. meliloti* Rm2011. First, the chromosomal *bacA_{sf}* gene in *S. meliloti* RmP3500 (contains the ETR region of *S. fredii* NGR234 and lacks the

pSymA and pSymB replicons) was replaced with the *aacC4* gene by isolation of pTH3129 double crossover recombinants as described above, producing RmP3901. The *aacC4* gene was then replaced with the desired *bacA* construct, by isolation of pTH3136, pTH3137, or pTH3138 double crossover recombinants in the RmP3901 background, as described above, producing RmP3909, RmP3910, and RmP3911, respectively.

The pSymA, pSymB, or pSymA plus pSymB replicons were transferred into these three strains from *S. meliloti* Rm5000 through overexpression of the RctB conjugal regulator protein, as described elsewhere (diCenzo et al. 2016; Nogales et al. 2013). The sole change was that transconjugants carrying pSymA, pSymB, or both from a single conjugation were isolated on a single medium, M9 minimal medium containing trigonelline, hydroxyproline, and thiamine. On this medium, transconjugants that obtained just pSymA form small colonies, those acquiring just pSymB form large white colonies, and those gaining both pSymA and pSymB form large pale colonies. The genotypes of the transconjugants were confirmed by patching on M9-trigonelline + thiamine, M9-hydroxyproline, and M9-trigonelline (diCenzo et al. 2016). Deletion of *bacA_{Sm}* or the B202 region in the resulting strains was performed through recombination via transduction from the appropriate donor strain.

Gentamicin sensitivity assays.

Overnight cultures of *S. meliloti* grown in LBmc were washed and resuspended to an optical density at 600 nm of 1.0. Cell suspensions (10 µl of each) were added to triplicate wells of a 96-well microtitre plate together with 180 µl of LBmc. The plate was then incubated at 30°C with shaking in a Biotek Cytation3 plate reader, as described previously (diCenzo et al. 2014). After 2 h, either 10 µl of double-distilled water (ddH₂O) (control) or 10 µl of 400 µg of gentamicin per microliter (Gm) (final concentration of 20 µg µl⁻¹) was added to each well, and the plate was again incubated in the Cytation3 plate reader. Data analysis was performed with a previously reported Perl script (diCenzo et al. 2014).

Plant materials and growth conditions.

Seeds of *M. sativa* cv. Iroquois and *Melilotus alba* (Parson's Seeds Limited) were surface-sterilized and germinated as described previously (diCenzo et al. 2015; Yarosh et al. 1989). *P. sativum* cv. Homesteader (Heritage Harvest Seed) seeds were sterilized based on the method of Bolaños et al. (2006); seeds were washed with 70% ethanol for 3 min, were rinsed with sterile ddH₂O, and were then washed with 1% hypochlorite for 20 min. Sterilized seeds were then washed with ddH₂O, were soaked in ddH₂O for 4 h, were spread on 1.5% agar, were incubated at 4°C overnight in the dark, and then, were germinated at room temperature in the dark overnight. Leonard assemblies were prepared as described before (diCenzo et al. 2015), and six to eight seedlings for *M. sativa* and *Melilotus alba* or either one or four seedlings for *P. sativum* were planted in each assembly. Plants were grown for up to 29 days, as indicated, in a Conviron growth chamber with a 18-h day (21°C) and 6-h night (17°C) cycle.

Symbiotic assays.

Shoot dry weight measurements, acetylene reduction assays, and isolation of rhizobia from surface-sterilized nodules were performed as detailed previously (diCenzo et al. 2015). Acetylene reduction activity was based on measurements every 6 min for 18 min for *M. sativa* and *Melilotus alba* or every 3 min for 9 to 12 min for *P. sativum*. The percentage of pink nodules on *P. sativum* was determined by counting approximately 800 nodules from a randomly chosen subset of the *P. sativum* root systems. The percentage of tetracycline-resistant rhizobia in the *P. sativum* nodules was determined by crushing surface-sterilized

nodules in 100 µl of tryptone yeast (TY) broth plus 300 mM sucrose and plating serial dilutions on TY medium with and without 1 µg of tetracycline per milliliter.

Confocal microscopy.

Examination of nodule and bacteroid morphology with confocal microscopy was performed as described previously (diCenzo et al. 2015; Zamani et al. 2017). Briefly, nodules were fixed with glutaraldehyde and were stored at 4°C for up to 2 weeks. Fixed nodules were sliced into 90-µm longitudinal cross-sections, were stained with the nucleic acid-binding dye Syto13, and were imaged (excitation and emission: 488/509) with a Lecia TCS SP2 confocal laser microscope equipped with an argon laser and a RSP 500 beam splitter.

Live and dead staining of nodule cross-sections was performed according to the method of Haag et al. (2011). Fresh, nonfixed nodules were sliced into 90-µm longitudinal nodule cross-sections, were stained with 7.5 µM Syto9 (excitation and emission: 488/500) and 20 µM propidium iodine (excitation and emission: 488/635), using the Invitrogen BacLight bacterial viability kit, and were imaged with a Lecia TCS SP2 confocal laser microscope equipped with an argon laser and a 488/543/633 beam splitter.

Blast bidirectional best hit analysis.

A custom pipeline consisting of shell and Perl scripts was assembled to perform the BLASTp bidirectional best hit analysis. The complete set of RefSeq (O'Leary et al. 2016) proteomes for the species of interest was downloaded through the National Center for Biotechnology Information (NCBI) ftp site (accessed April 10, 2016). The annotated BacA proteins of *S. fredii* NGR234 and *Agrobacterium fabrum* C58 were truncated on the N-terminal end, and this was fixed prior to the analysis. Blastp searches were performed using the BLAST 2.2.31+ suite from NCBI (Camacho et al. 2009). The complete *S. meliloti* Rm2011 proteome was searched against the complete proteome of each strain, and the top hit for each protein was recorded. The complete proteome of each genome of interest was then searched against the complete *S. meliloti* Rm2011 proteome, and the top hit for each protein was recorded. For each genome, the two BLAST lists were compared, and all bidirectional best hits were identified. Bidirectional best hits were defined as putative orthologs only if the E-value of the BLASTp hit in each direction was below the specified threshold.

BacA phylogenetic analysis.

The protein faa RefSeq (O'Leary et al. 2016) record of all the finished genomes publicly available through the NCBI ftp site (accessed March 21, 2016) were downloaded for the following genera: *Sinorhizobium*, *Ensifer*, *Rhizobium*, *Neorhizobium*, *Agrobacterium*, *Mesorhizobium*, *Brucella*, *Ochrobactrum*, *Bordetella*, *Bradyrhizobium*, *Azorhizobium*, *Nitrobacter*, *Escherichia*, *Klebsiella*, and *Mycobacterium*. This dataset was supplemented with all the *Sinorhizobium* and *Ensifer* strains present in scaffolds, to increase the sample size, as well as the *Mesorhizobium huakuii* 7653R and *Rhizobium leguminosarum* bv. *phaseoli* 4292 present in scaffolds, as the symbiotic phenotype of *bacA* mutants in these strains has been studied (Karunakaran et al. 2010; Tan et al. 2009). The annotated BacA proteins of *S. fredii* NGR234 and *Agrobacterium fabrum* C58 were truncated on the N-terminal end, and this was fixed prior to the analysis.

To analyze the phylogeny of the BacA protein, the SbmA_BacA hidden Markov model was downloaded from the Pfam database (Finn et al. 2016), all proteomes were searched using HMMER version 3.1b2 (Eddy 2009), and the best-scoring protein from each strain was collected. As no close BacA orthologs, excluding BclA orthologs, were identified in genera

Bordetella, *Bradyrhizobium*, *Azorhizobium*, *Nitrobacter*, and *Mycobacterium*, these genera were discarded from all further analyses. All BacA proteins were aligned with MAFFT-linsi version 7.294b (Katoh and Standley 2013), following which RAXML v8.2.8 (Stamatakis 2014) was used to identify identical proteins and to produce a fasta file containing only the unique sequences. The unique 82 proteins were aligned with MAFFT-linsi and were trimmed with trimAl version 1.2rev59, using the automated1 algorithm (Capella-Gutiérrez et al. 2009). Phylogenetic analysis of the trimmed alignment was performed with the RAXML BlackBox webserver (Stamatakis et al. 2008) and the maximum likelihood bootstrap best tree was produced following 100 bootstrap replicates. The phylogenetic reconstruction was performed using a gamma rate heterogeneity and the LG amino acid substitution model, as a preliminary RAXML analysis indicated the LG model was the best fit for the data.

MLSA.

A MLSA was performed with all genomes used in the final BacA phylogenetic analysis. The MarkerScanner Perl script of the AMPHORA2 pipeline (Wu and Scott 2012) was used to search all genomes for 31 proteins highly conserved in bacteria. Perl scripts were used to determine that 25 of the 31 proteins were identified in single copy in all the genomes and only these proteins were used further, i.e., Frr, InfC, NusA, RplA, RplB, RplC, RplE, RplF, RplK, RplL, RplM, RplN, RplP, RplT, RpoB, RpsB, RpsC, RpsE, RpsI, RpsJ, RpsK, RpsM, RpsS, SmpB, and Tsf. Each ortholog set was aligned with MAFFT-linsi, the resulting alignments were trimmed with trimAl, using the automated1 algorithm, and the 25 alignments were then concatenated, using a Perl script. A phylogeny of the concatenated alignment was produced using the RAXML BlackBox webserver, and the maximum likelihood bootstrap best tree was produced following 100 bootstrap replicates. The phylogenetic reconstruction was performed using a gamma rate heterogeneity and the LG amino acid substitution model, as a preliminary RAXML analysis indicated the LG model was the best fit.

Phylogenetic analysis of the genes upstream and downstream of BacA.

Our previously reported pipeline (Zamani et al. 2017) was used to identify the genes upstream and downstream of the *bacA* orthologs in all genomes analyzed and to determine whether they were orthologous to the genes upstream and downstream of *bacA* in *S. meliloti* Rm2011. Of the 53 *Rhizobiales* genomes included in the BacA analysis, both the upstream and downstream genes were conserved in 44 of the genomes. The BacA protein, the protein encoded upstream of *bacA* (Smb21000), and the protein encoded downstream of *bacA* (Smb20998) in these 44 genomes were aligned with MAFFT-linsi, were trimmed with trimAl, and maximum likelihood phylogenies were produced with the BlackBox RAXML webserver, as described above. For the RAXML phylogeny, the LG amino acid substitution model was used for BacA and Smb21000 while the JTTDCMUT model was used for Smb20998.

Amino acid sequence logo generation.

Following the MAFFT-linsi alignment of the complete set of BacA proteins, subsets of the aligned sequences were grouped separately. Amino acid sequence logos of the BacA subsets were generated with Skyline using the ‘weighted counts’ and ‘information content—above background’ options (Wheeler et al. 2014).

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