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Partial Complementation of *Sinorhizobium meliloti bacA* Mutant Phenotypes by the *Mycobacterium tuberculosis* BacA Protein

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The *Sinorhizobium meliloti* BacA ABC transporter protein plays an important role in its nodulating symbiosis with the legume alfalfa (*Medicago sativa*). The *Mycobacterium tuberculosis* BacA homolog was found to be important for the maintenance of chronic murine infections, yet its *in vivo* function is unknown. In the legume plant as well as in the mammalian host, bacteria encounter host antimicrobial peptides (AMPs). We found that the *M. tuberculosis* BacA protein was able to partially complement the symbiotic defect of an *S. meliloti* BacA-deficient mutant on alfalfa plants and to protect this mutant *in vitro* from the antimicrobial activity of a synthetic legume peptide, NCR247, and a recombinant human β-defensin 2 (HBD2). This finding was also confirmed using an *M. tuberculosis* insertion mutant. Furthermore, *M. tuberculosis* BacA-mediated protection of the legume symbiont *S. meliloti* against legume defensins as well as HBD2 is dependent on its attached ATPase domain. In addition, we show that *M. tuberculosis* BacA mediates peptide uptake of the truncated bovine AMP, $Bac7_{1-16}$. This process required a functional ATPase domain. We therefore suggest that *M. tuberculosis* BacA is important for the transport of peptides across the cytoplasmic membrane and is part of a complete ABC transporter. Hence, BacA-mediated protection against host AMPs might be important for the maintenance of latent infections.

For more than 20 years, the *Sinorhizobium meliloti* BacA protein has been known to be essential for the differentiation and persistence of *S. meliloti* within root nodules on the leguminous plant alfalfa (*Medicago sativa*) (1). *S. meliloti* establishes a symbiotic interaction with *Medicago* species whereby it enters into the symbiosome compartment within the legume root nodules and differentiates into persisting, nitrogen-fixing bacteroids (for recent reviews, see references 2 and 3). Bacteroid differentiation is mediated by nodule-specific, cysteine-rich antimicrobial peptides (known as NCR AMPs) produced by the plant, which are trafficked to the symbiosome compartments (4). NCR AMPs are similar to the defensins of eukaryotic innate immunity, as they are cationic and have conserved cysteine residues, which form defined disulfide (S-S) bridges and exhibit antimicrobial activity (5). In the absence of BacA, *S. meliloti* is hypersensitive to the action of NCR AMPs and is killed shortly after entering the host cell instead of differentiating into bacteroids (6). Moreover, an *S. meliloti* BacA-deficient mutant has alterations in its outer membrane lipid A structure and is, therefore, hypersensitive to detergents *in vitro* (7, 8). The *S. meliloti* BacA protein (*Sm*BacA) and its *Escherichia coli* homolog, SbmA, were also found to be involved in the uptake of diverse peptides, which suggested that this function might be necessary for the survival of *S. meliloti* within the host environment (9–11). Thus, it is unknown whether BacA-mediated protection against *Medicago* NCR AMPs is due to an indirect effect on the bacterial cell envelope or to a direct peptide transport function of BacA or to both (6).

BacA homologs are found in many bacteria, including nonsymbiotic or nonpathogenic ones, thus suggesting that the function of the BacA protein is not specific to bacterium-eukaryote interactions and that BacA also has a housekeeping function. In agreement with this, we have demonstrated many phenotypes of an *S. meliloti* BacA-deficient mutant grown in culture in the absence of a host (6, 7, 8, 12, 13). However, the precise physiological housekeeping role of BacA remains to be determined. One interesting indication is that *E. coli sbmA* is under the control of the transcriptional regulator σ^E (RpoE), suggesting that the SbmA protein has a role in the response to envelope stresses (14).

Homologs to BacA were identified in the chronic pathogens *Brucella abortus* (15) and *Mycobacterium tuberculosis* (16). The *M. tuberculosis* BacA protein (*Mt*BacA) was found to be important for the maintenance of the chronic infection in a murine infection model (16). *M. tuberculosis* is a respiratory pathogen, and upon uptake into its mammalian hosts, it encounters and induces the production of β-defensins by lung alveolar epithelial cells (17). Once it had entered these cells within the human host, the *M. tuberculosis* bacterium was found to be associated with human β-defensin 2 (HBD2) (17). During chronic murine infection, lung

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alveolar epithelial cells harbor *M. tuberculosis* despite the production of high levels of murine β-defensin 3, the mouse homolog to HBD2 (18). Hence, like *S. meliloti* within the symbiosome compartment of *Medicago* species, *M. tuberculosis* must also defend itself against the antimicrobial action of host cysteine-rich, cationic peptides in order to form persistent infections.

BacA proteins are predicted to be integral membrane proteins functioning as ABC transporters (13, 16). Such ABC transporters transport substrates into and/or out of the bacterial cell at the expense of ATP hydrolysis (19, 20). As the *S. meliloti* BacA-deficient mutant displayed resistance to certain types of antimicrobial peptides, a function in the uptake of these peptides was proposed and shown previously (11). However, whether this peptide uptake was facilitated through a direct transport mechanism involving BacA or indirectly influenced by BacA remained to be determined. In contrast to this, the *S. meliloti* BacA-deficient mutant showed hypersensitivity to NCR AMPs, indicating that BacA might be functionally diverse depending on the type of peptide present. A particular difficulty for researchers was that no ATPase domain was associated with the rhizobial BacA proteins, and identifying potential orphan ATPase proteins to associate with the BacA protein has thus far been unsuccessful (13). The *Mt*BacA protein is 39% similar (22% identical) to *Sm*BacA and contains a fused putative ATPase domain at the C terminus of the protein (13, 16).

In this paper, the heterologous expression of the *Mt*BacA protein in an *S. meliloti* BacA-deficient strain allowed us to address several questions related to the function of BacA-like proteins. We thus investigated the role of the *Mt*BacA protein in the uptake of and protection against host-derived peptides. We show that conserved mechanisms are involved in establishing latent bacterial infections within a eukaryotic host cell and that the function of the BacA protein in peptide uptake and protection against cysteine-rich host peptides, at least in *M. tuberculosis*, is dependent on the presence of a functional ATPase domain.

MATERIALS AND METHODS

Bacterial growth. All bacterial strains and plasmids used in this study are described in Table S1 in the supplemental material. The *S. meliloti* strains used are all derivatives of the *Sm*1021 sequenced strain (21). For all experiments, *S. meliloti* strains were grown in lysogeny broth (LB) (22) prepared with 10 g liter^{-1} NaCl and supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (LB/MC) for 48 h at 30°C. All *E. coli* strains were grown in LB (10 g liter^{-1} NaCl) at 37°C. *M. tuberculosis* strains were grown in GAST medium, which consists of glycerol alanine salts (GAS) without added iron and with Tween 80 (23). Growth media were supplemented with the appropriate antibiotics. Unless indicated otherwise, antibiotics were used at the following concentrations (in μg ml^{-1}): streptomycin (Sm), 500; chloramphenicol (Cm), 12.5; ampicillin (Ap), 100; tetracycline (Tc), 10; hygromycin (Hy), 50; and kanamycin (Km), 25.

Synthesis of a codon-optimized *M. tuberculosis bacA* gene for expression in *E. coli* and *S. meliloti*. An *E. coli* and *S. meliloti* codon-optimized *M. tuberculosis bacA* gene (Mt*bacA*) was designed using the OptimumGene software, synthesized, and cloned into pUC57 to create pUC57Mt*bacA* (GenScript Corporation, Piscataway, NJ). The plasmid was then isolated from *E. coli* GM272, digested with NsiI and XbaI, and ligated into the PstI site of pRF771 (24) under the control of a *trp* promoter. A C-terminally His-tagged version of the *Mt*BacA protein was generated by amplification of the Mt*bacA* gene from pUC57Mt*bacA* using the primers *Mt*_NsiI_*bacA*_F and *Mt_bacA*_6his_XbaI_R. Primer sequences used in this study are provided in Table S2 in the supplemental material. The reverse primer contained sequences coding for 6 histidine residues at their 5′ ends. The His-tagged version of the Mt*bacA* gene with the site-directed mutation E576G in the ATPase site (see below) was generated by amplification of the codon-optimized Mt*bacA* gene from pUC57Mt$bacA_{E576G}$ using the primers *Mt*_NsiI_*bacA*_F and *Mt_bacA*_6his_XbaI_R. All PCR fragments were then digested with NsiI and XbaI and ligated into the PstI site of pRF771 under the control of the *trp* promoter. In all cases, the ligated plasmids were transformed into *E. coli* DH5α and transformants were selected on LB Tc agar plates. The correct inserts were then confirmed by PCR using the plasmid-specific primers, pKX-US-F and pKX-DS-R, purified, and then sequenced. The correct clones were then conjugated into the *S. meliloti* *Sm*1021 BacA-deficient mutant (7) using *E. coli* MT616 with a helper plasmid, pRK600 (25), and selected on LB Sm Tc agar.

Site-directed mutagenesis of the *M. tuberculosis* BacA protein. The site-directed mutation E576G was introduced into the *Mt*BacA protein using a PCR-based mutagenesis method. Plasmid pUC57Mt$bacA_{E576G}$ was amplified using the primers SDM_Mt*bacA*_E576G_F and SDM_Mt*bacA*_E576G_R with the KAPA HiFi DNA polymerase system (KAPAbiosystems). PCR was performed using an initial denaturation step of 98°C for 3 min followed by 30 cycles of denaturation at 98°C for 30 s, annealing at 50°C for 30 s, and an extension at 68°C for 6 min. A final extension step of 10 min was then performed, followed by the addition of 10 units DpnI. After incubation at 37°C for 3 h to digest methylated template DNA, the PCR product was then transformed into $CaCl_2$-competent *E. coli* DH5α cells, and transformants were selected on LB Ap plates. The presence of the mutation was then verified by sequencing the gene.

Production of a truncated codon-optimized *M. tuberculosis bacA* gene and His-tagged construct. A truncated Mt*bacA* gene encoding only the membrane-spanning part of the protein was generated by amplifying the Mt*bacA* gene from pUC57MTbacA using the primers *Mt*_NsiI_*bacA*_F and *Mt_bacA*_trunc_XbaI_R (see Table S2 in the supplemental material). A C-terminal His-tagged version of the truncated *Mt*BacA protein was generated by amplification of the Mt*bacA* gene from pUC57MTbacA using the primers *Mt*_NsiI_*bacA*_F and *Mt_bacA*_trunc_6his_XbaI_R (see Table S2 in the supplemental material), cloned into pRF771, and mated into the *S. meliloti* BacA-deficient mutant as described above.

Western blotting. Whole-cell extracts were prepared from defined *S. meliloti* strains by French pressing in phosphate-buffered saline (PBS) (pH 7.0) twice with a 500-psi gauge. Cell debris was removed by centrifugation, and the total protein concentration in the supernatant was determined using the Bradford assay (Bio-Rad protein assay) according to the manufacturer's instructions. Western blotting of the obtained extracts was performed using an anti-penta-His antibody conjugated to horseradish peroxidase (HRP) (Sigma). HRP activity on the blots was detected using an Amersham ECL Plus Western blotting detection kit (GE Healthcare).

Production of recombinant HBD2. A recombinant human β-defensin 2 (HBD2) was prepared in *E. coli* as follows (see supporting information in the supplemental material for a detailed description). A codon-optimized synthetic HBD2 gene was obtained from GenScript. The HBD2 gene was then fused to the C terminus of *E. coli* thioredoxin (Trx) in a pET-32b-derived plasmid with a tobacco etch virus (TEV) protease site engineered between the Trx and HBD2 proteins. The N-terminally His-tagged Trx-HBD2 fusion was expressed under standard conditions (1 mM isopropyl-β-D-thiogalactopyranoside [IPTG], 30°C, 3 h) in a BL21(DE3) host. An extract containing the soluble Trx-HBD2 fusion was obtained by sonication and the His-tagged protein purified using Ni-NTA resin (Qiagen). The purified fusion was digested overnight with recombinant TEV protease at 4°C. His-tagged Trx and uncut Trx-HBD2 were removed by passage over Ni-NTA, and the HBD2 protein solution was desalted and then freeze-dried. TEV cleavage did not leave any unwanted amino acid additions on the recombinant peptide. The purity and oxidation status of the mature HBD2 were confirmed by high-resolution liquid chromatography-electron spray ionization/mass spectrometry (LC ESI MS) on a

Bruker Daltonics FT-ICR instrument (see Fig. S1 in the supplemental material). The data matched an HBD2 peptide with three S-S bonds, and the behavior of this HBD2 was identical to that of an HBD2 published previously (26).

***S. meliloti*-alfalfa interaction.** To determine the ability of *S. meliloti* strains to form a successful symbiosis with alfalfa, 3-day-old alfalfa seedlings were inoculated with *S. meliloti* on Jensen's agar exactly as described previously (27). Plant growth and nodule morphology were monitored over a 4-week incubation period. Root nodules from 3-week-old seedlings were removed, fixed, and analyzed by light microscopy and transmission electron microscopy (TEM) exactly as described previously (27, 28).

DOC sensitivity assay. Deoxycholate (DOC) sensitivity assays were performed as described previously using gradient plates (12). Growth was recorded after 72 h, and the results from four independent measurements were averaged. The error bars represent the standard deviations (SD) from the means.

***S. meliloti* and *M. tuberculosis* viability experiments.** The synthetic NCR247 peptide was prepared exactly as described previously with defined disulfide bridges between cysteines 1 and 2 and between cysteines 3 and 4 (6). The effect of NCR247 and HBD2 on *S. meliloti* cell viability was determined as described before using early-exponential-phase cultures in 10 mM sodium phosphate buffer, pH 7.0 (6). *M. tuberculosis* strains were grown in GAST medium to an optical density at 650 nm (OD_{650}) of 0.2. Cells were harvested and washed three times in an equal volume of 10 mM sodium phosphate buffer supplemented with 0.05% (vol/vol) Tween 80 (pH 7.0) and diluted to a final OD_{650} of 0.1 in the assay. The bacteria were then treated with 10 μM recombinant HBD2 peptide in triplicate wells for 24 h at 37°C. After the treatment, the cells were immediately diluted in 7H9 Middlebrook medium supplemented with 5 g liter^{-1} bovine serum albumin (BSA) fraction V, 2 g liter^{-1} glucose, 0.02% (vol/vol) glycerol, and 0.05% (vol/vol) Tween 80 and plated on 7H11 Middlebrook/oleic acid-albumin-dextrose-catalase (OADC) agar plates for colony enumeration (16).

Bac7 sensitivity assays. Fluorescent and nonfluorescent N-terminal fragments (residues 1 to 16) of Bac7 ($Bac7_{1-16}$-BY and $Bac7_{1-16}$, respectively) and nonfluorescent fragment (residues 1 to 35) of Bac7 ($Bac7_{1-35}$) were synthesized and prepared as described previously (29). The Bac7 sensitivity assays were performed using mid-exponential-phase cultures exactly as described previously (11).

Flow cytometry analysis. Uptake assays were performed as described previously (11). Mid-exponential-phase cultures of the indicated strains were washed and resuspended to an OD_{600} of 0.05 in fresh LB growth medium. Then, $Bac7_{1-16}$-BY was added, and the reaction mixtures were incubated at 30°C for 1 h followed by one wash in fresh LB medium to remove any $Bac7_{1-16}$-BY not taken up into the cells. Then, the cells were resuspended in 50 mM sodium phosphate buffer (pH 7.0). The cultures were then treated with 1 mg ml^{-1} of the extracellular fluorescence quencher trypan blue (TB) for 10 min at room temperature before analysis. For analysis of the cells, a Becton, Dickinson (BD) LSR II flow cytometer was used with a 488-nm laser. $Bac7_{1-16}$-BY fluorescence was measured using a 530/30-nm band-pass filter. Ten thousand events were acquired and recorded using BD Diva software (TreeStar Inc.) for each sample. Data analysis was performed using FlowJo (Tree Star Inc.).

Statistical analysis. Where shown, the significance of differences among bacterial strains was assessed using GraphPad Prism using analysis of variance (ANOVA) analysis followed by Bonferroni's multiple-comparison test for multiple comparisons.

RESULTS

***Mt*BacA partially rescues the bacteroid defect of an *S. meliloti* BacA-deficient mutant.** *S. meliloti* induces the formation of pink root nodules (due to the presence of leghemoglobin [Lb]) on the roots of *Medicago* species such as alfalfa, in which the plant cells are filled with persisting and nitrogen-fixing bacteroids (Fig. 1A to C) that enable the plant to grow in the absence of exogenous ammonia (30). In contrast, an *S. meliloti* BacA-deficient mutant is defective in forming a persistent infection within the plant cells and induces the formation of stunted and white nodules (without Lb) that are devoid of nitrogen-fixing bacteroids (Fig. 1D to F) (1). We found that neither the *S. meliloti* BacA-deficient mutant with the control plasmid pRF771 nor that with the plasmid pMt*bacA* (a codon-optimized version of the Mt*bacA* gene cloned in the plasmid pRF771) supported the growth of alfalfa in the absence of an exogenous nitrogen source (data not shown). The *S. meliloti* BacA-deficient mutant with pMt*bacA* resulted in transiently pale pink nodules, which ultimately turned white, or in white nodules (Fig. 1G and J, respectively). Transiently pink nodules were never found on alfalfa infected with the *S. meliloti* BacA-deficient mutant. We found that in contrast to the white nodules of plants infected with the *S. meliloti* BacA-deficient mutant, the transiently pink nodules induced by the *S. meliloti* BacA-deficient mutant expressing the *Mt*BacA protein were filled with bacteroids throughout the whole nodule (Fig. 1G to I). Even in the white nodules of plants infected with the *S. meliloti* BacA-deficient mutant expressing the *M. tuberculosis* BacA protein, infection appeared to have progressed further than in nodules of plants infected by the *S. meliloti* BacA-deficient mutant with pRF771 (compare Fig. 1H and K to E and B). This was confirmed by analyzing infected nodule cells using transmission electron microscopy (Fig. 1C, F, I, and L). While bacteroids in plants infected with the wild-type *S. meliloti* strain had differentiated and were shaped normally, only undifferentiated bacteria exiting the infection threat were found in nodule cells infected with the *S. meliloti* BacA-deficient mutant (Fig. 1C and F, respectively). The *S. meliloti* BacA-deficient mutant containing pMt*bacA* was able to form bacteroids within the nodule cells, but these were aberrantly shaped and the white nodules contained poly-hydroxy-butyrate granules, thus indicating that these bacteroids might be arrested early in development. Therefore, *Mt*BacA could partially restore the ability of this mutant to infect nodules and form bacteroids. Although the *Mt*BacA protein was unable to restore growth of alfalfa infected with the *S. meliloti* BacA-deficient mutant, its ability to enable the partial differentiation of bacteria into bacteroids and even the formation of slightly pink nodules suggests that the BacA proteins of *M. tuberculosis* and *S. meliloti* might have analogous functions that could be specific to their natural host environment.

The *Mt*BacA protein does not restore the cell envelope defect of an *S. meliloti* BacA-deficient mutant. Abnormally shaped bacteroids have been previously observed in alfalfa nodules infected with *S. meliloti* mutants that were defective in the biosynthesis of the lipopolysaccharide very-long-chain fatty acid (LPS VLCFA) (39). The *S. meliloti* BacA-deficient mutant also has a 50% decrease in its LPS VLCFA content, which results in a reduction of cell envelope integrity, making this mutant more susceptible to detergent stress (7, 8). To determine whether the ability of the *Mt*BacA protein to partially support bacteroid development of the *S. meliloti* BacA-deficient mutant was due to lack of VLCFA, we tested whether the *M. tuberculosis* BacA protein would also restore resistance of the *S. meliloti* BacA-deficient mutant to the detergent deoxycholate (DOC). Consistent with previous results (7), we determined that an *S. meliloti* BacA-deficient mutant with the control plasmid pRF771 had an increased sensitivity to increasing DOC concentrations relative to the wild-type strain with the control plasmid (Fig. 2A). However, while the introduction of a plas-

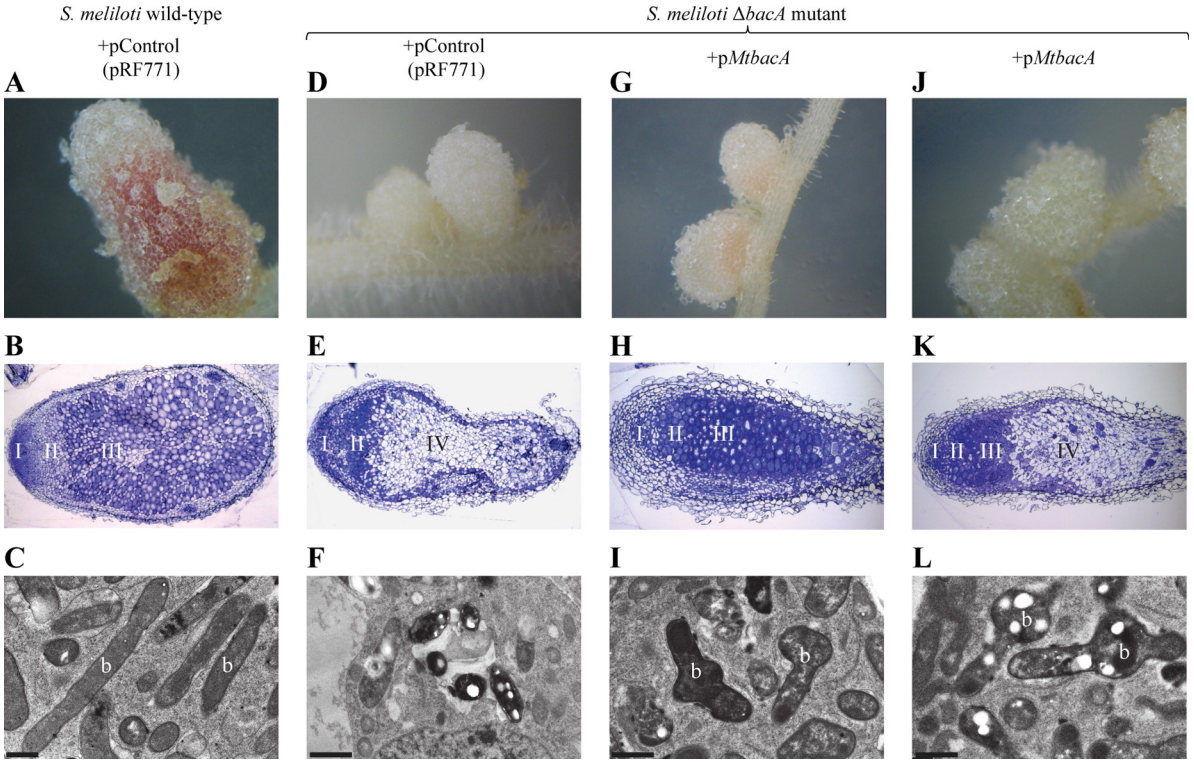

FIG 1 *M. tuberculosis* BacA partially complements the *S. meliloti* BacA-deficient mutant bacteroid defect *in planta*. (A, D, G, and J) Three-week-old *Medicago sativa* (alfalfa) root nodules formed after infection with the defined *S. meliloti* strains. The *S. meliloti* BacA-deficient mutant with pMt*bacA* formed nodules that were either transiently pink (G) or white (J). (B, E, H, and K) Light microscopy images of whole-nodule sections stained with toluidine blue. The different infection zones are as follows: I, meristem; II, invasion zone; III, N_2 fixation zone; IV, senescent zone. (C, I, F, and L) Transmission electron microscopy images of nodule sections showing typical bacteroid morphologies (C, I, L) and bacteria released from the infection threads (F), which did not differentiate into bacteroids. Scale bars, 1 μm; b, bacteroids.

mid-borne wild-type *S. meliloti bacA* gene (pSm*bacA*) fully restored DOC resistance of the *S. meliloti* BacA-deficient mutant, we found that a plasmid-borne Mt*bacA* gene had no effect on the sensitivity of the *S. meliloti* BacA-deficient mutant to DOC (Fig. 2A). Consequently, these findings showed that, unlike the *S. meliloti bacA* gene, the cloned Mt*bacA* gene was unable to complement the detergent sensitivity phenotype of the *S. meliloti* BacA-deficient mutant and was, therefore, unlikely to complement the defect in the LPS of this mutant strain.

***Mt*BacA complements the hypersensitivity of the *S. meliloti* BacA-deficient mutant to NCR AMPs and HBD2.** Recently, we have shown that the inability of the *S. meliloti* BacA-deficient mutant to survive within hosts in the legume genus *Medicago* was the result of a hypersensitivity of this mutant to challenge with nodule-specific cysteine-rich antimicrobial peptides (6). We therefore investigated whether the *Mt*BacA protein was able to protect the *S. meliloti* BacA-deficient mutant against the *in vitro* challenge with one such NCR AMP, NCR247 (4, 6) (Fig. 2B). In agreement with previous results (6, 31), the *S. meliloti* BacA-deficient mutant with a control plasmid was hypersensitive to NCR247 *in vitro* compared to the wild-type strain with the control plasmid. Introduction of the plasmid-borne Mt*bacA* gene into the *S. meliloti* BacA-deficient mutant background restored the resistance of the *S. meliloti* BacA-deficient mutant to NCR247 to the level of the wild-type strain with the control plasmid pRF771 (Fig. 2C). Therefore, despite its inability to complement the detergent sensitivity phenotype of the *S. meliloti* BacA-deficient mutant, the *M. tuberculosis* BacA protein was able to protect *S. meliloti* against the NCR AMP challenge.

We next aimed to investigate whether *Mt*BacA also protected an *S. meliloti* BacA-deficient mutant against HBD2, a defensin relevant for *M. tuberculosis* infections in the human host. The chemical synthesis of mammalian β-defensins is complex due to the presence of 6 cysteine residues (32), and hence there is a need to form three different S-S bridges. For this study, HBD2 was produced as a recombinant peptide (Fig. 3A). We employed an *E. coli* expression system for thioredoxin-tagged HBD2, cleaved the tag, purified the liberated HBD2 peptide, and verified its purity and oxidation status by mass spectrometry (see Fig. S1 in the supplemental material). The recombinant HBD2 peptide was dissolved in 0.05% (vol/vol) acetic acid in order to prevent reduction of the peptide. To test whether any observed antimicrobial effects were due to HBD2 and not to the acetic acid, we treated the *S. meliloti* strains with acetic acid only and under our assay conditions. We found no effect of 0.05% acetic acid on the viability of *S. meliloti* (data not shown). When treating *S. meliloti* strains with HBD2 for 3 h, we found that the *S. meliloti* wild-type strain was sensitive to the recombinant HBD2 AMP and over 99% of the cells were killed after treatment with a 10 μM concentration of the peptide (Fig. 3B). This demonstrated that the recombinant HBD2 peptide had antimicrobial activity. The *S. meliloti* BacA-deficient mutant with the control plasmid was hypersensitive to the anti-

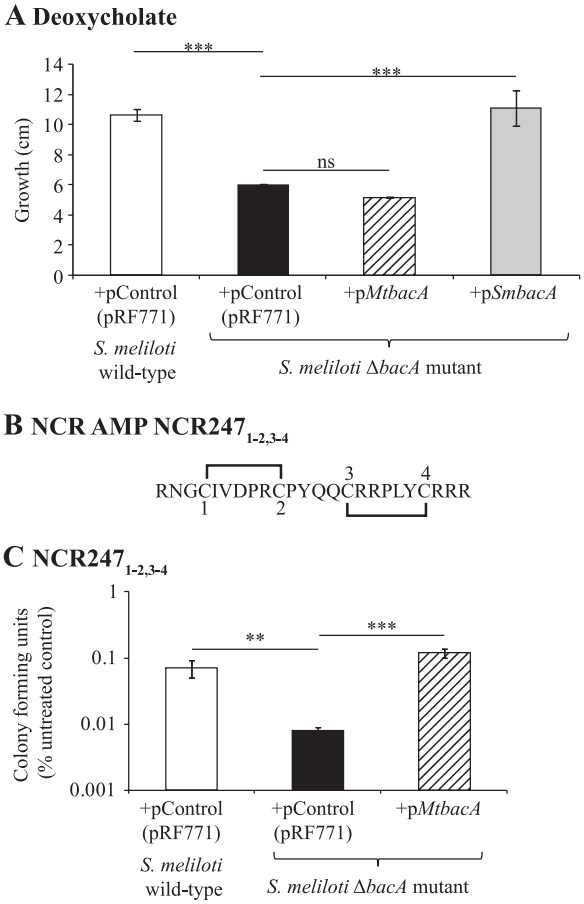

FIG 2 *M. tuberculosis* BacA protects *S. meliloti* against an NCR AMP but not deoxycholate. (A) Cultures were streaked onto LB agar with a gradient of sodium deoxycholate (0 to 25 mM), and growth was determined after 72 h. (B) Synthetic NCR247 peptide sequence showing the two S-S bridges between cysteine residues 1 and 2 and between residues 3 and 4. (C) Colony-forming ability of the *S. meliloti* strains was assessed after exposure to 20 μM NCR247 for 3 h. Bars represent means ± SD. Significant values (**, $P \leq 0.01$; ***, $P \leq 0.001$) were determined using ANOVA followed by Bonferroni's posttest, and the results are representative of at least two independent experiments.

microbial action of recombinant HBD2 relative to the wild-type strain with the control plasmid (Fig. 3B). Introduction of plasmid-borne versions of both the *S. meliloti* (pSm*bacA*) and *M. tuberculosis* BacA (pMt*bacA*) proteins restored the resistance of the mutant strain to HBD2 to wild-type-levels (Fig. 3B). Therefore, this showed that both the *M. tuberculosis* and *S. meliloti* BacA proteins provided *S. meliloti* with protection against various cysteine-containing peptides relevant to their respective host environments.

An *M. tuberculosis* BacA-deficient mutant exhibits a sensitivity phenotype to HBD2. We next aimed to determine whether an *M. tuberculosis* BacA-deficient mutant was also sensitive to the antimicrobial action of HBD2 relative to its parental strain. Therefore, the *M. tuberculosis* H37Rv strain, its *bacA*::*hyg* mutant, and the chromosomally complemented *bacA*::*hyg* mutant referred to as pKLMt5 were treated with 10 μM HBD2 for 24 h. In contrast to our findings with *S. meliloti*, we found that *M. tuberculosis* H37Rv was completely resistant to the antimicrobial action of the recombinant defensin HBD2. However, in the absence of BacA, the *M. tuberculosis bacA*::*hyg* mutant strain was susceptible to being killed by HBD2, and this sensitivity could be complemented by reintroducing the wild-type Mt*bacA* gene into the mutant strain (Fig. 3C). Hence, the BacA protein was also essential for the protection of *M. tuberculosis* against being killed by HBD2.

The putative ATPase domain is critical for *Mt*BacA to protect against antimicrobial peptides. We cloned a truncated version and a mutated version of the codon-optimized Mt*bacA* gene into pRF771. The truncated *Mt*BacA protein lacked the C-terminal ATPase domain, and the mutated *Mt*BacA protein had a site-directed mutation of a conserved glutamate residue at position 576, which in other ABC transporters has been shown to be important for the protein transport activity (19). These plasmids were named pMt$bacA_{trunc}$ and pMt$bacA_{E576G}$, respectively (see Fig. S2 in the supplemental material). To ensure that both modified *Mt*BacA proteins were expressed in the *S. meliloti* BacA-deficient mutant, we constructed C-terminal His-tagged versions of all *Mt*BacA proteins and determined the expression levels of all three His-tagged proteins by Western blotting. This showed that although the truncated *Mt*BacA protein was expressed within the *S. meliloti* BacA-deficient mutant, its expression levels were significantly lower than those of the wild-type BacA protein. In contrast, the site-directed *Mt*BacA mutant version (E576G) was expressed at the same level as the wild-type *Mt*BacA protein (see Fig. S2B and C, respectively, in the supplemental material).

To determine whether the ATPase domain was essential for the functions of the *Mt*BacA protein during chronic plant infections, we initially analyzed the ability of pMt$bacA_{E576G}$ to complement the bacteroid development defect of the *S. meliloti* BacA-deficient mutant. Unlike pMt*bacA*, pMt$bacA_{E576G}$ did not partially restore the ability of the *S. meliloti* BacA-deficient mutant to form bacteroids (Fig. 4A to C). Consistent with this, pMt$bacA_{E576G}$ did not confer protection to the *S. meliloti* BacA-deficient mutant against either NCR247 or HBD2 (Fig. 4D and E). Taken together, these findings are consistent with the ATPase domain of the *Mt*BacA protein being important for its *in vivo* function.

***Mt*BacA sensitizes an *S. meliloti* BacA-deficient mutant to Bac7$_{1-16}$ and mediates Bac7$_{1-16}$ uptake.** *Sm*BacA and its *E. coli* homolog SbmA have been shown to be essential for the sensitivity to and uptake of the bovine neutrophil peptide Bac7, suggesting a role of these proteins in peptide transport (10, 11). The *M. tuberculosis* BacA protein was previously shown to sensitize a spontaneous *E. coli sbmA* mutant to Bac7$_{1-16}$ (16). Therefore, we also investigated whether the *Mt*BacA protein was able to sensitize the *S. meliloti* BacA-deficient mutant to two different truncated versions of Bac7, Bac7$_{1-16}$ and Bac7$_{1-35}$. We treated the *S. meliloti* wild-type and the BacA-deficient mutant strains with either the control plasmid or a plasmid-borne version of the Mt*bacA* gene with a 1 μM concentration of the defined Bac7 peptides and then determined the numbers of viable cells relative to the untreated controls. We found, in agreement with previous studies (11, 33), that the *S. meliloti* BacA-deficient mutant with the control plasmid was completely resistant and that the wild-type strain with the control plasmid was sensitive to being killed by any of the Bac7 peptides (Fig. 5A and B). Introduction of the plasmid-borne Mt*bacA* gene into the *S. meliloti* BacA-deficient mutant background restored the sensitivity of the strains to Bac7$_{1-16}$ (Fig. 5A) but did not restore their sensitivity to Bac7$_{1-35}$ (Fig. 5B). Therefore, the *Mt*BacA protein appeared to display an overlapping but distinct specificity for Bac7-derived peptides compared to the *S. meliloti* BacA protein.

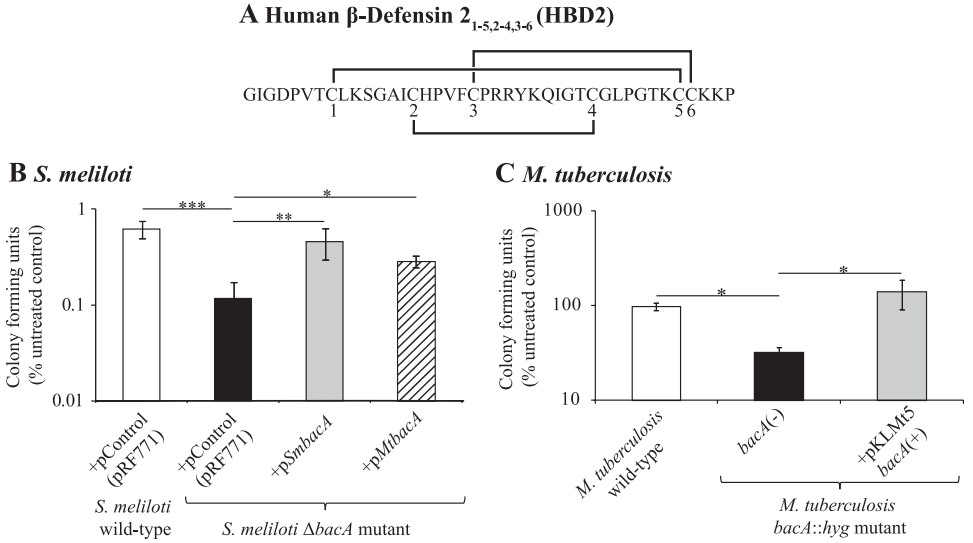

FIG 3 The *M. tuberculosis* BacA protein protects against a recombinant human beta defensin. (A) Recombinant HBD2 with the indicated S-S bridges between cysteine residues 1 and 5, 2 and 4, and 3 and 6. (B) The colony-forming ability of the *S. meliloti* strains was assessed after exposure to 10 μM recombinant HBD2 for 3 h. (C) The colony-forming ability of the *M. tuberculosis* strains was assessed after exposure to 10 μM recombinant HBD2 for 24 h. The results shown are the averages of three independent cultures for each strain. The results shown in panels A and B are representative of at least two independent experiments. Bars represent means ± SD. Significant values (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) were determined using ANOVA followed by Bonferroni's posttest, and results are representative of at least two independent experiments.

The data obtained in this assay suggested that *Mt*BacA might have a role in transport of $Bac7_{1-16}$, and thus we used the fluorescent boron-dipyrromethene (BODIPY)-labeled $Bac7_{1-16}$-BY to determine whether the peptide was taken up into the bacterial cell (11). We treated the *S. meliloti* wild-type and BacA-deficient mutant strains containing the control and pMt*bacA* plasmids, respectively, with 1 μM $Bac7_{1-16}$-BY and analyzed treated and untreated cell populations for BY fluorescence using flow cytometry. To quantify peptide uptake the peak mean fluorescent intensity (pMFI) was then determined, indicating the fluorescence peak

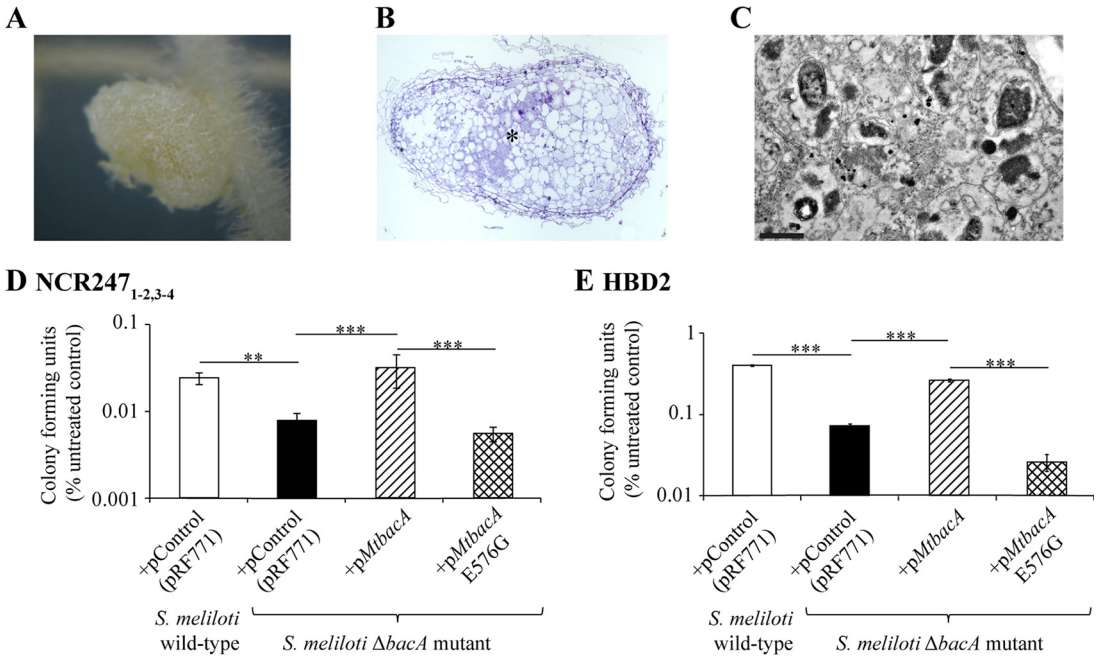

FIG 4 The ATPase domain of *M. tuberculosis* BacA is essential for its function. (A, B, and C) Alfalfa roots were infected with an *S. meliloti* BacA-deficient mutant harboring pMt$bacA_{E576G}$. (A) Photograph of a root nodule; (B) light microscopy image of a nodule section stained with toluidine blue; (C) transmission electron microscopy image of a nodule section (scale bar = 1 μm). (D and E) The colony-forming ability of the *S. meliloti* strains was assessed after exposure to 20 μM NCR247 for 3 h (D) and to 10 μM recombinant HBD2 for 3 h (E). Bars represent means ± SD. Significant values (**, $P \leq 0.01$; ***, $P \leq 0.001$) were determined using ANOVA followed by Bonferroni's posttest, and results are representative of at least two independent experiments.

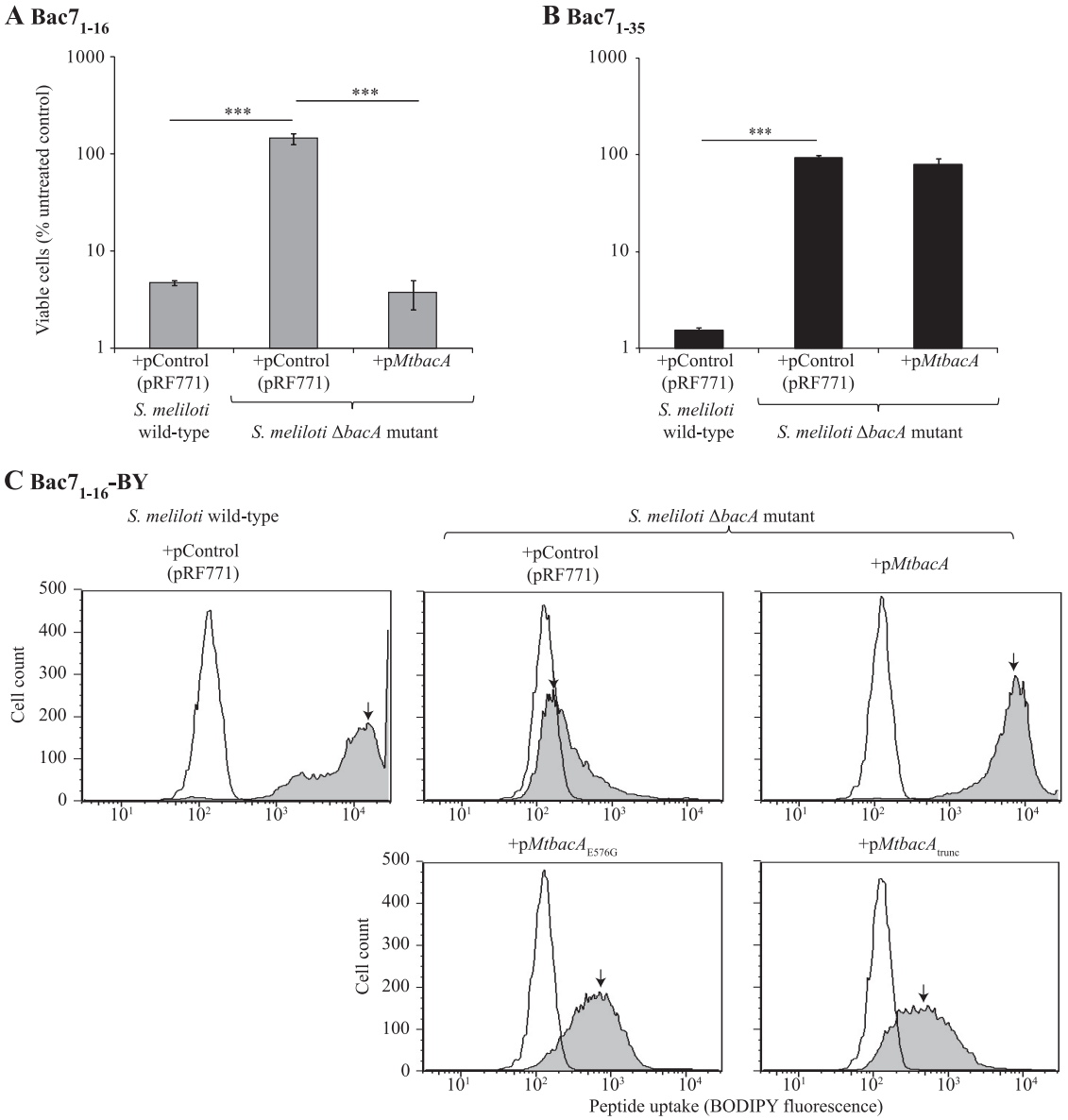

FIG 5 *M. tuberculosis* BacA is involved in sensitizing an *S. meliloti* BacA-deficient mutant to $Bac7_{1-16}$. Defined strains were treated with either 1 μM $Bac7_{1-16}$ (A) or 1 μM $Bac7_{1-35}$ (B) for 1 h, and the populations (CFU ml^{-1}) were assessed relative to an untreated control. (C) Flow cytometry analysis of *S. meliloti* strains untreated (empty histograms) and treated with 2 μM $Bac7_{1-16}$-BY (shaded histograms) of the indicated strains. All data sets are representing trends observed in at least two independent experiments. Bars represent means ± SD. Significant values (***, $P \leq 0.001$) were determined using ANOVA followed by a Bonferroni posttest and represent comparisons of the indicated results.

where most cells of each peptide-treated population were detected. The analysis revealed that, in contrast to the $Bac7_{1-16}$-BY-treated BacA-deficient mutant, showing a pMFI of 192, the *S. meliloti* wild-type strain was able to take up $Bac7_{1-16}$-BY with a pMFI of 11,899 or more. As anticipated from the viability data, we found that the *Mt*BacA protein (pMt*bacA*) was able to complement the loss of *S. meliloti* BacA, and it restored $Bac7_{1-16}$-BY uptake of the *S. meliloti* BacA-deficient mutant compared to the control plasmid by increasing the pMFI more than 40-fold, from 192 to 8,473 (Fig. 5C, arrows). These findings strongly suggested that *Mt*BacA was involved in peptide uptake of $Bac7_{1-16}$-BY.

By treating an *S. meliloti* BacA-deficient mutant containing either pMt$bacA_{trunc}$ or pMt$bacA_{E576G}$ with $Bac7_{1-16}$, we were further able to show that neither the truncated nor the site-direct mutant *Mt*BacA protein sensitized the *S. meliloti* BacA-deficient mutant to $Bac7_{1-16}$ compared to an *S. meliloti* BacA-deficient mutant with pMt*bacA* (see Fig. S3 in the supplemental material). In order to confirm that the inability of the truncated and mutated *Mt*BacA proteins to sensitize the *S. meliloti* BacA-deficient mutant to $Bac7_{1-16}$ was due to the lack of peptide uptake, we tested whether the *S. meliloti* BacA-deficient mutant strains expressing these proteins were able to take up the fluorescently labeled Bac7 peptide, $Bac7_{1-16}$-BY, by flow cytometry. We found that both the truncated (pMFI, 474) and the E576G (pMFI, 708) mutant *Mt*BacA proteins were substantially (more than 10-fold) reduced in their capacity to mediate $Bac7_{1-16}$-BY peptide uptake into the *S. meliloti* BacA-deficient mu-

tant cells compared to the full-length *Mt*BacA protein (pMFI, 8,473) (Fig. 5C). Therefore, these findings indicated that the putative ATPase domain of the *Mt*BacA protein was important for the uptake of and bacterial sensitivity to $Bac7_{1-16}$-BY.

DISCUSSION

In this study, we investigated whether the *M. tuberculosis* BacA protein has *in vitro* and *in vivo* functions similar to those of its well-characterized *S. meliloti* counterpart. We found that the Mt*bacA* gene partially restored the ability of an *S. meliloti* BacA-deficient mutant to develop into bacteroids. However, these were unable to support a functional symbiosis. This symbiotic defect may be resulting from the inability of the Mt*bacA* gene to restore the cell envelope integrity defect of the *S. meliloti* BacA-deficient mutant, as determined by the DOC gradient assay. In addition, the *Mt*BacA protein is only 39% similar (22% identical) to the *S. meliloti* BacA protein and, in addition, contains a fused ATPase domain (see Fig. S4 in the supplemental material). Therefore, this protein is sufficiently different from the *S. meliloti* BacA protein to account for this inability. Indeed, it is not surprising that the *Mt*BacA protein does not complement the LPS defect of an *S. meliloti* BacA-deficient mutant, as *M. tuberculosis* does not itself contain LPS (34). However, our findings indicated that the *Mt*BacA protein must be capable of at least partially overcoming the inability of the *S. meliloti* BacA-deficient mutant to survive within the root nodule, which suggests that it might have an analogous function to the *Sm*BacA protein.

During symbiosis, the root nodules of the legume genus *Medicago*, in which *S. meliloti* resides, produce over 600 different NCR peptides that show a pattern of conserved cysteine residues, similar to mammalian defensin peptides (5, 35). Within the host compartment of *Medicago* root nodules, BacA-mediated protection of *S. meliloti* against these NCR peptides is critical for the survival of the bacteria and for enabling them to differentiate into persisting bacteroids (4) (see Fig. S5 in the supplemental material). We observed that the Mt*bacA* gene complemented the sensitivity phenotype of an *S. meliloti* BacA-deficient mutant to NCR247, suggesting that *Mt*BacA might also be involved in the protection of *M. tuberculosis* against host peptides during chronic infection. Like *S. meliloti*, *M. tuberculosis* encounters cysteine-rich mammalian peptides throughout its host infection. In particular, *M. tuberculosis* is exposed to β-defensins within the respiratory tract and in lung alveolar epithelial cells both in the chronic murine infection model and in human infections (17, 18, 36). Lung alveolar epithelial cells from mice chronically infected with *M. tuberculosis* produce high levels of murine β-defensin-3, the mouse homolog to HBD2 (18). In support of our hypothesis, we were able to show that *Mt*BacA protected the *S. meliloti* BacA-deficient strain and *M. tuberculosis* against the antimicrobial activity of HBD2, suggesting that BacA might be important for the protection of *M. tuberculosis* against defensins *in vivo*. BacA-mediated protection against mammalian β-defensins might be critical for the maintenance of *M. tuberculosis* during chronic infection (see Fig. S5 in the supplemental material). Although the sensitivity of an *M. tuberculosis* BacA-deficient mutant to HBD2 was rather modest, the low growth rate of *M. tuberculosis in vitro* might account for this phenotype and impose an increased basal level of resistance to defensin-like peptides on the bacterium. Thus, *in vivo* the *M. tuberculosis* BacA-deficient mutant might display a sensitivity phenotype to mammalian defensins. This result could explain the increased survival of mice infected with the *M. tuberculosis bacA* mutant as opposed to wild-type *M. tuberculosis*-infected mice (16).

The *Sm*BacA protein was previously shown to be essential for the uptake of the AMPs $Bac7_{1-16}$ and $Bac7_{1-35}$, and thus loss of BacA resulted in protection (9, 11). This indicated a role of BacA in the transport of certain peptide classes into the bacterial cell. Here, we found that the plasmid-carried Mt*bacA* gene only restored sensitivity of an *S. meliloti* BacA-deficient mutant to $Bac7_{1-16}$ but not to $Bac7_{1-35}$. In contrast, the Sm*bacA* gene cloned into the same plasmid sensitized the mutant strain to both truncated Bac7 peptides (9). These findings could indicate different peptide specificities for the two BacA proteins, which might be due to the pore size of the ABC transporters' transmembrane domains (TMDs). Predicted TMDs of the *S. meliloti* and *M. tuberculosis* BacA proteins with the TMHMM algorithm (37) revealed that *Sm*BacA has eight TMDs compared to six for *Mt*BacA. Aligning the two BacA protein sequences to highlight the predicted TMDs shows that four TMDs appear to be similar. However, the first and the last two TMDs of *Sm*BacA each appear to be aligned with a single *M. tuberculosis* BacA TMD (see Fig. S4 in the supplemental material). This structural difference could account for the inability of the *Mt*BacA protein to sensitize an *S. meliloti* BacA-deficient mutant to $Bac7_{1-35}$. Moreover, it is also known that the specificity of the *S. meliloti* BacA protein for certain truncated forms of Bac7 is dependent on the growth conditions (i.e., the growth medium) used to culture the strains (33). Furthermore, the distinct specificities for different peptides displayed by the *Mt*BacA and *Sm*BacA proteins might be an additional reason for the inability of the *Mt*BacA protein to fully complement the symbiotic defect of a BacA-deficient *S. meliloti* strain since host plants produce a very large panel of different peptides, some of which may be transported by *Mt*BacA but others not.

The BacA protein of *M. tuberculosis*, in contrast to the *S. meliloti* BacA protein, is fused to a putative ATPase domain, indicating its functional role as an ABC transporter. We initially confirmed that the *M. tuberculosis* BacA protein was facilitating the uptake of fluorescently labeled $Bac7_{1-16}$-BY, which is in agreement with the viability data. To investigate the potential role of the fused ATPase domain in this process, we created a truncated *Mt*BacA version or mutated a conserved glutamate residue of the ATPase domain, which was shown to be important for the ATPase activity of other ABC transporters (19, 38). In agreement with the proposed function of *Mt*BacA as an ABC transporter, these alterations prevented $Bac7_{1-16}$-BY from being taken up into the cells of the *S. meliloti* BacA-deficient mutant, suggesting also that $Bac7_{1-16}$-BY peptide uptake is an active and energy-dependent transport. Likewise, *Sm*BacA-mediated bleomycin uptake by *S. meliloti* has previously been shown to be energy dependent (9). The ATPase-defective $MtBacA_{E576G}$ protein was also unable to protect the *S. meliloti* BacA-deficient mutant against NCR247 and HBD2 AMPs, thus suggesting that an active transport is also required to protect the bacterial cells from the antimicrobial activity of cysteine-rich host peptides. *S. meliloti* and *B. abortus* BacA proteins lack a fused ATPase domain (13), yet our findings suggest that these too might function as ABC transporters, associating with unknown orphan ATPase proteins, encoded elsewhere in their bacterial genomes (21). Nevertheless, no evidence has been put forward so far to prove that the BacA proteins directly transport Bac7 and bleomycin or that the protection of the bacteria from cysteine rich-AMPs is a direct interaction. In addition, it still remains to be determined

whether it is the transport of the cysteine-rich AMPs that is providing the BacA-mediated protection. We suggest that *Mt*BacA mediates peptide uptake and protection against different classes of peptides, indicating a broad spectrum of peptide specificity. Therefore, it is possible that *Mt*BacA might also be involved in the interaction with/transport of other peptide substrates.

In summary, we provide conclusive experimental evidence that BacA proteins function as ABC transporters with a functional ATPase domain. In addition, we were able to show that *Mt*BacA is critical for protection against HBD2 *in vitro*. Future studies will be necessary to understand how the putative ABC transport function of *Mt*BacA confers host AMP protection and to determine if the protection against HBD2 or murine β-defensin 3 is actually responsible for the *in vivo* phenotypes of the *M. tuberculosis bacA*-deficient strain. One possible way to address this issue might be silencing of defensin genes in experimental mouse models to prevent defensin exposure of the bacteria in the host. Preventing the targeting of NCR AMPs in *Medicago truncatula* has already provided valuable insight into the role of the *Sm*BacA protein in the legume symbiosis (6). We have successfully exploited the *S. meliloti*-legume interaction to gain insights into the *M. tuberculosis* chronic mammalian infection, thus suggesting that this interaction might provide a valuable model system to facilitate investigations into the difficult-to-study *M. tuberculosis* infection.

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