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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, Bac71-16. 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 to 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 (SmBacA) 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 non-symbiotic 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 σE (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 (MtBacA) 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 host, the M. tuberculosis bacterium was found to be associated with human β-defensin 2 (HBD2) (17). During chronic murine infection, lung
alveolar epithelial cells harbor *M. tuberculosis* despite the produc-
tion of high levels of murine β-defensin 3, the mouse homolog to
HBD2 (18). Hence, like *S. melliloti* within the symbiosome com-
partment of *Medicago* species, *M. tuberculosis* must also defend
itself against the antimicrobial action of host cysteine-rich, cat-
tonic 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
protein has thus far been unsuccessful (13). The
functioning as ABC transporters (13, 16). Such ABC transporters
have a conserved region that was shown previously (11). However, whether this peptide uptake
function was determined. In contrast to this, the
*Mt* BacA or indirectly influenced by BacA remained to be determined.

**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. melliloti* strains are used as derivatives of the Sm1021 sequenced strain (21). For all exper-
iments, *S. melliloti* strains were grown in lysogeny broth (LB) (22) pre-
pared with 10 g liter⁻¹ NaCl and supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ (LB/MC) for 48 h at 37°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. melliloti*.** An *E. coli* and *S. melliloti* codon-
optimized *M. tuberculosis bacA* gene (Mt_bacA) was designed using the OptimumGene software, synthesized, and cloned into pUC57 to create
pUC57_Mt_bacA (GenScript Corporation, Piscataway, NJ). The plasmid was then isolated from *E. coli* GM272, digested with NsiI and XbaI, and
ligated into the pStl site of pRF771 (24) under the control of a trp pro-
moter. A C-terminally His-tagged version of the *Mt* BacA protein was

**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 pUC57_Mt_bacA
was amplified using the primers SDM*Mt_bacA_E576G_F and
SDM*Mt_bacA_E576G_R with the KAPA HiFi DNA polymerase system
(KAPA Biosystems). 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
DNA, the PCR product was then transformed into CaCl₂-com-
potent *E. coli* DH5α cells, and transformants were selected on LB Ap plates. The presence of the mutation was then verified by sequencing the
genome.

**Production of recombinant HBD2.** A recombinant human β-defen-
sin 2 (HBD2) was prepared in *E. coli* as follows (see supporting informa-
tion in the supplemental material for a detailed description). A codon-
optimized synthetic HBD2 gene was obtained from GenScript. The HBD2
genome 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 chromatog-
raphy-electron spray ionization/mass spectrometry (LC-ESI MS) on a
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Bruker Daltonics FT-ICR instrument (see Fig. S1 in the supplemental material). The data matched an HBBD2 peptide with three S-S bonds, and the behavior of this HBBD2 was identical to that of an HBBD2 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 HBBD2 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 (OD650) of 0.2. Cells were harvested and washed three times in an equal volume of 10 mM phosphate buffer supplemented with 0.05% (vol/vol) Tween 80 (pH 7.0) and diluted to a final OD650 of 0.1 in the assay. The bacteria were then treated with 10 μM recombinant HBBD2 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 (Bac71-16-BY and Bac71-16, respectively) and nonfluorescent fragment (residues 1 to 35) of Bac7 (Bac71-35) 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 OD650 of 0.05 in fresh LB growth medium. Then, Bac71-16-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 Bac71-16-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 the analysis of the cells, a Becton, Dickinson (BD) LSR II flow cytometer was used with a 488-nm laser. Bac71-16-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

MtbBacA 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 pMtBacA (a codon-optimized version of the MtBacA 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 pMtBacA 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 MtBacA protein in contrast to the white nodules of plants infected with the S. meliloti BacA-deficient mutant expressing the MtBacA protein, infection appeared to have progressed further than in nodules of 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 pMtBacA 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, MtbBacA could partially restore the ability of this mutant to infect nodules and form bacteroids. Although the MtbBacA 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.
mid-borne wild-type *S. meliloti* bacA gene (pSmbacA) 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.

**MtBacA 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 *M. tuberculosis* BacA protein was able to protect *S. meliloti* against the NCR AMP challenge.

We next aimed to investigate whether MtBacA 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 H9252-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 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-

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**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, N2 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.
by HBD2, and this sensitivity could be complemented by reintroducing the wild-type MtbacA 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 MtBacA to protect against antimicrobial peptides. We cloned a truncated version and a mutated version of the codon-optimized MtbacA gene into pRF771. The truncated *MtBacA* protein lacked the C-terminal ATPase domain, and the mutated *MtBacA* 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 pMtbacA<sub>trunc</sub> and pMtbacA<sub>E576G</sub>, respectively (see Fig. S2 in the supplemental material). To ensure that both modified *MtBacA* proteins were expressed in the *S. meliloti* BacA-deficient mutant, we constructed C-terminal His-tagged versions of all *MtBacA* proteins and determined the expression levels of all three His-tagged proteins by Western blotting. This showed that although the truncated *MtBacA* 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 *MtBacA* mutant version (E576G) was expressed at the same level as the wild-type *MtBacA* protein (see Fig. S2B and C, respectively, in the supplemental material).

To determine whether the ATPase domain was essential for the functions of the *MtBacA* protein during chronic plant infections, we initially analyzed the ability of pMtbacA<sub>E576G</sub> to complement the bacterioid development defect of the *S. meliloti* BacA-deficient mutant. Unlike pMtbacA<sub>trunc</sub>, pMtbacA<sub>E576G</sub> did not partially restore the ability of the *S. meliloti* BacA-deficient mutant to form bacteroids (Fig. 4A to C). Consistent with this, pMtbacA<sub>E576G</sub> 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 *MtBacA* protein being important for its *in vivo* function.

**MtBacA sensitizes an *S. meliloti* BacA-deficient mutant to Bac7<sub>1,16</sub> and mediates Bac7<sub>1,16</sub> uptake.** *S. meliloti* 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<sub>1,16</sub> (16). Therefore, we also investigated whether the *MtBacA* protein was able to sensitize the *S. meliloti* BacA-deficient mutant to two different truncated versions of Bac7, Bac7<sub>1,16</sub> and Bac7<sub>1,35</sub>. 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 *MtBacA* gene into the *S. meliloti* BacA-deficient mutant back-
The data obtained in this assay suggested that MtbBacA might have a role in transport of Bac71-16, and thus we used the fluorescent boron-dipyrromethene (BODIPY)-labeled Bac71-16-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 pMtbaca plasmids, respectively, with 1 μM Bac71-16-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 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 ≤ 0.05; **, P ≤ 0.01; *** P ≤ 0.001) were determined using ANOVA followed by Bonferroni’s posttest, and results are representative of at least two independent experiments.

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 pMtbacaESTAG. (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 ≤ 0.01; ***, P ≤ 0.001) were determined using ANOVA followed by Bonferroni’s posttest, and results are representative of at least two independent experiments.
where most cells of each peptide-treated population were detected. The analysis revealed that, in contrast to the Bac71-16-BY-treated BacA-deficient mutant, showing a pMFI of 192, the S. meliloti wild-type strain was able to take up Bac71-16-BY with a pMFI of 11,899 or more. As anticipated from the viability data, we found that the MtBacA protein (pMt bacA) was able to complement the loss of S. meliloti BacA, and it restored Bac71-16-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 MtBacA was involved in peptide uptake of Bac71-16-BY.

By treating an S. meliloti BacA-deficient mutant containing either pMt bacA trunc or pMt bacA E576G with Bac71-16, we were further able to show that neither the truncated nor the site-direct mutant MtBacA protein sensitized the S. meliloti BacA-deficient mutant to Bac71-16 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 MtBacA proteins to sensitize the S. meliloti BacA-deficient mutant to Bac71-16 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, Bac71-16-BY, by flow cytometry. We found that both the truncated (pMFI, 474) and the E576G (pMFI, 708) mutant MtBacA proteins were substantially (more than 10-fold) reduced in their capacity to mediate Bac71-16-BY peptide uptake into the S. meliloti BacA-deficient mu-
tant cells compared to the full-length MtBacA protein (pMFI, 8,473) (Fig. 5C). Therefore, these findings indicated that the putative ATPase domain of the MtBacA protein was important for the uptake of and bacterial sensitivity to Bac71_{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 MtbacA 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 MtbacA 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 MtBacA 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 MtBacA 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 MtBacA protein must be capable of at least partially overriding 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 SmBacA 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 MtbacA gene complemented the sensitivity phenotype of an S. meliloti BacA-deficient mutant to NCR247, suggesting that MtBacA 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 MtBacA 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 SmBacA protein was previously shown to be essential for the uptake of the AMPs Bac71_{1,16} and Bac71_{3,35}, 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 MtbacA gene only restored sensitivity of an S. meliloti BacA-deficient mutant to Bac71_{1,16} but not to Bac71_{3,35}. In contrast, the SmbacA 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 SmBacA has eight TMDs compared to six for MtBacA. 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 SmBacA 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 MtBacA protein to sensitize an S. meliloti BacA-deficient mutant to Bac71_{3,35}. 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 MtBacA and SmBacA proteins might be an additional reason for the inability of the MtBacA 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 MtBacA 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 Bac71_{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 MtBacA 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 MtBacA as an ABC transporter, these alterations prevented Bac71_{1,16}-BY from being taken up into the cells of the S. meliloti BacA-deficient mutant, suggesting also that Bac71_{1,16}-BY peptide uptake is an active and energy-dependent transport. Likewise, SmBacA-mediated bleomycin uptake by S. meliloti has previously been shown to be energy dependent (9). The ATPase-defective MtBacAE576G 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 MtBacA mediates peptide uptake and protection against different classes of peptides, indicating a broad spectrum of peptide specificity. Therefore, it is possible that MtBacA 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 MtBacA is critical for protection against HBD2 in vitro. Future studies will be necessary to understand how the putative ABC transport function of MtBacA 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 SmBacA 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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