Internalization of a thiazole-modified peptide in *Sinorhizobium meliloti* occurs by BacA-dependent and -independent mechanisms

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BacA proteins play key roles in the chronic intracellular infections of *Sinorhizobium meliloti, Brucella abortus* and *Mycobacterium tuberculosis* within their respective hosts. *S. meliloti, B. abortus* and *M. tuberculosis* BacA-deficient mutants have increased resistance to the thiazole-modified peptide bleomycin. BacA has been previously hypothesized, but not experimentally verified, to be involved in bleomycin uptake. In this paper, we show that a BacA-dependent mechanism is the major route of bleomycin internalization in *S. meliloti*. We also determined that the *B. abortus* and *S. meliloti* BacA proteins are functional homologues and that the *B. abortus* BacA protein is involved in the uptake of both bleomycin and proline-rich peptides. Our findings also provide evidence that there is a second, BacA-independent minor mechanism for bleomycin internalization in *S. meliloti*. We determined that the BacA-dependent and -independent mechanisms of bleomycin uptake are energy-dependent, consistent with both mechanisms of bleomycin uptake involving transport systems.

INTRODUCTION

*Sinorhizobium meliloti* can be found either free-living in the soil or in a symbiotic relationship with leguminous plants such as alfalfa (for recent reviews refer to Gibson et al., 2008; Jones et al., 2007). After entry into alfalfa cells, *S. meliloti* differentiates into a nitrogen-fixing bacteroid, which persists for extensive periods. Despite being beneficial for the host, this interaction can be viewed as a chronic intracellular infection (Gibson et al., 2008). The BacA protein is essential for the chronic intracellular infection of *S. meliloti* within alfalfa (Glazebrook et al., 1993), as in the absence of BacA, an *S. meliloti* mutant can enter into the plant cell but lyses and dies shortly after entry. The chronic mammalian pathogen *Brucella abortus* also has a BacA protein, which shares 68% identity with the *S. meliloti* BacA protein and is essential for chronic infections of BALB/c mice (LeVier et al., 2000). Recently, the *Mycobacterium tuberculosis* BacA protein (Rv1819c), which is 39% similar to the *B. abortus* BacA protein, was also determined to be involved in the maintenance of chronic B6D2/F1 mice infections (Domenech et al., 2009). Since the mechanism of chronic bacterial infections is poorly understood, elucidation of the function of different BacA proteins could reveal important insights into this process.

To understand more about the function of BacA proteins, *S. meliloti, B. abortus* and *M. tuberculosis* bacA mutants have also been characterized in their free-living states relative to their respective parent strains. The lipid A of *S. meliloti* and *B. abortus* is unusually modified with very-long-chain fatty acids (VLCFAs), and it was discovered that *S. meliloti* and *B. abortus* bacA null mutants have an approximately 50% reduction in their lipid A VLCFA content (Ferguson et al., 2004). Lipid A is a component of the lipopolysaccharide that forms the outer leaflet of the outer membrane of Gram-negative bacteria (Raetz & Whitfield, 2002). However, by constructing and characterizing *S. meliloti* acpXL and *lpxXL* mutants that are deficient in the biosynthesis of the lipid A VLCFA modification, it
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was found that the unusual modification of the lipid A is important but not essential for the chronic infection of *S. meliloti* in alfalfa (Ferguson et al., 2005; Haag et al., 2009; Sharpyova et al., 2003). Additionally, these studies demonstrated that the effect of BacA on the lipid A VLCFA modification is unlikely solely to account for its essential role in chronic infections. Consistent with this, the BacA protein of *M. tuberculosis* had no effect on the fatty acid content of membrane lipids (Domenech et al., 2009). Combined, these studies provide support for a model whereby BacA proteins also have another function or effect on bacterial cells that is important for their essential role in chronic bacterial infections.

The *S. meliloti* BacA protein also shares 64% identity with the *Escherichia coli* SbmA protein (Glazebrook et al., 1993; Ichige & Walker, 1997). The *sbmA* gene was first identified by its ability to confer sensitivity of *E. coli* to the antimicrobial peptide microcin B17 (Laviña et al., 1986; Yorgey et al., 1994). Microcin B17 is a glycine-rich, thiazole-modified peptide produced by *E. coli* strains carrying a naturally occurring plasmid and is an inhibitor of DNA gyrase (Yorgey et al., 1994). Since mutations in *sbmA* only protected *E. coli* against exogenous microcin B17 and conferred no growth advantage to the microcin B17-producing strain, this provided indirect evidence that SbmA is involved in peptide uptake (Laviña et al., 1986). It was subsequently shown, using fluorescently labelled peptides, that the SbmA protein is involved in the uptake of truncated Bac7 peptides into *E. coli* (Mattiuzzo et al., 2007). Full-length Bac7 is produced by bovine leukocytes and is a linear proline/arginine-rich peptide (Frank et al., 1990). At present, it is not known whether SbmA plays a direct or indirect role in peptide uptake in *E. coli* (Mattiuzzo et al., 2007). However, it has been proposed that SbmA and BacA proteins form the transmembrane domains of an ABC transporter and the *M. tuberculosis* BacA protein has a fused ATPase domain (Domenech et al., 2009; LeVier & Walker, 2001), suggesting that they could be directly involved in peptide uptake.

*S. meliloti* is highly resistant to killing by microcin B17 (Ichige & Walker, 1997). However, *S. meliloti* is sensitive to truncated Bac7 peptides, and the BacA protein has been shown to play an essential role in their uptake (Marlow et al., 2009). In contrast, there appear to have been no previous studies investigating the role of the *B. abortus* and *M. tuberculosis* BacA proteins in peptide uptake. Nevertheless, every *sbmA*/bacA mutant characterized to date shows increased resistance to the glycopeptide antibiotic bleomycin (Domenech et al., 2009; Ichige & Walker, 1997; LeVier et al., 2000). In *S. meliloti*, it was shown that reductions in the lipid A VLCFA do not confer resistance to bleomycin, providing evidence that the increased resistance of the *S. meliloti* bacA null mutant to bleomycin is independent of its lipid A alteration (Ferguson et al., 2006). Bleomycin is synthesized by the soil bacterium *Streptomyces verticillus* (Umezawa et al., 1966) and has been shown previously to damage DNA through a Fe(II)-mediated oxidative mechanism (Kane & Hecht, 1994). It had been proposed previously that BacA-mediated sensitivity to bleomycin could be due to BacA being involved in iron uptake (Ichige & Walker, 1997). However, an *M. tuberculosis* BacA-deficient mutant was unaffected in iron uptake, suggesting that this was not the case (Domenech et al., 2009). Interestingly, although the peptide sequences of bleomycin and microcin B17 differ, both peptides are modified with thiazole rings (Yorgey et al., 1994). Based on this finding and the increased resistance of bacA/sbmA mutants to bleomycin it was hypothesized that SbmA/BacA proteins could also be involved in the uptake of bleomycin into bacterial cells (Ichige & Walker, 1997; Yorgey et al., 1994). However, this hypothesis had never been verified experimentally. Bleomycin is also known to damage the cell wall and membrane of yeast cells (Lim et al., 1995). Therefore, we could not exclude the possibility that BacA/SbmA were protecting bacterial cells to bleomycin through another mechanism rather than uptake.

Since bleomycin is produced by a soil bacterium and plants produce thiazole-containing compounds (Umezawa et al., 1966; Yorgey et al., 1994), the effect of bleomycin on *S. meliloti* could have relevance to both its free-living and symbiotic states. Additionally, bleomycin is an antibiotic (Umezawa et al., 1966; Yorgey et al., 1994). Therefore, investigating the factors involved in sensitizing *B. abortus* to this peptide could provide information relevant to the treatment of chronic bacterial infections. In this study, we investigated the hypothesis that BacA proteins are involved in bleomycin internalization. We found that both the *S. meliloti* and *B. abortus* BacA proteins play important roles in bleomycin internalization in *S. meliloti*. However, we also discovered a second, BacA-independent, energy-dependent mechanism for bleomycin internalization in *S. meliloti*.

**METHODS**

**Bacterial strains and growth conditions.** All bacterial strains and plasmids used in this work are described in Table 1. The *S. meliloti* strains used are all derivatives of the Rm1021 sequenced strain (Galibert et al., 2001). For all experiments, *S. meliloti* strains were grown in either Luria–Bertani broth (LB) (Sambrook et al., 1982) prepared with 10 g NaCl L⁻¹ or LB supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ (LB/MC) for 48 h at 30 °C. Unless indicated otherwise, antibiotics were used at the following concentrations (µg ml⁻¹): gentamicin (Gm), 50; streptomycin (Sm), 500; spectinomycin (Sp), 100; neomycin (Nm) 200; and tetracycline (Tc), 5.

**Filter disc and liquid culture viability assays.** For the filter disc assays, late-exponential-phase cultures (OD₆₀₀ ~ 3.0) were washed in LB, resuspended to OD₆₀₀ ~ 0.2 and then the assays were performed exactly as described previously (Ferguson et al., 2002). The agar plates were incubated at 30 °C (72 h) and then the diameters of growth inhibition were recorded. The growth inhibition zone was measured from at least three plates for each strain and condition and the results were averaged. The liquid culture viability assays, unless stated otherwise, were also performed with late-exponential-phase cultures, which were harvested, washed and diluted to OD₆₀₀ ~ 0.1 in LB medium. After addition of the appropriate form of bleomycin, the
cultures were incubated at 30°C. At defined times, samples were removed, serially diluted in LB medium, then 10 μl aliquots were plated in triplicate on LB agar plates. C.f.u. were determined to OD600 ~0.1 in LB medium and the viability determined as described above. To determine whether DNP treatment affected sensitivity to bleomycin, the cultures were diluted to OD600 ~0.08 in dilution buffer (10 mM NaCl, 6.6 mM Na2SO4, 5 mM HEPES pH 7.0). The DAPI assay was performed following a previously described procedure (Johnson, 1994) except that DAPI was added to the cells at a final concentration of 0.1 μg ml⁻¹ in a final volume of 100 μl and the cells were incubated with DAPI overnight at 4°C. The fluorescence intensity was measured using a FLUOstar optima plate reader (BMG Labtech) spectrophotometer with excitation and emission at 350 and 450 nm, respectively.

### Cloning of the B. abortus and S. melloti bacA genes

The B. abortus bacA gene, including 150 bp upstream, was amplified by PCR using the primers BabacA +150-F (5’-GCTTGGCTGCAAGCCTAA- CACCCATTG GGGCGT-3’) and BabacA-R (5’-AGATGGATCCT- CGACGCCGCCCCTG-3’). The fragment was then digested with BamHI and PstI, and ligated into pRF771 (Wells & Long, 2002), under control of the trp promoter. The S. melloti bacA gene was amplified by PCR using the primers Smelloti_bacA_Nal-RBS_F (5’- CGTGAATGATGAAACGAGAGTGCCGTCCCCCTTGTTCCAA- TCCCTTCTCCC-3’) and Smelloti_bacA_XbaI-R (5’-TTATCGT- CTAGAATGAGGGGGATGTGCTGC-3’) and ligated into pRF771 under control of trp. In both cases, the ligated plasmids were transformed into E. coli DH5α and transformants were selected on LB Tc agar. The insertions were then confirmed by amplification of the inserts by PCR using the plasmid-specific primers pkX-US-F (5’-CGGCTCGTATGTTGTG-3’) and pkX-DS-R (5’-CGGAAGGGGATGTGCTGC-3’) followed by sequencing. The correct clones, pBabacA and pSmbacA, were then conjugated into the S. melloti Rm1021 bacA null mutant (Ferguson et al., 2002) using E. coli MT616 with the helper plasmid pRK600 (Finan et al., 1986), and selected on LB Sm Tc agar.

### S. melloti–alfalfa interaction experiments

To determine the ability of S. melloti to form a successful symbiosis with alfalfa, 3-day-old seedlings were inoculated with 1 ml S. melloti culture, resuspended to OD600 ~0.05 in sterile water, on Jensen’s agar as described previously (Leigh et al., 1985). The plates were incubated at 25°C, and plant growth and nodule morphology were determined after 4 weeks.

### Bac7 sensitivity and Bac7-16-BY uptake assays

The N-terminal fragments 1–16 and 1–35 of Bac7 were synthesized and prepared as described previously (Benincasa et al., 2004). The Bac7 sensitivity assays were performed using mid-exponential-phase cultures exactly as described previously (Marlow et al., 2009). A fluorescently labelled

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**Table 1. Bacterial strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<td><strong>S. melloti</strong></td>
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<td>Rm1021</td>
<td>Sm derivative of SU47</td>
<td>Meade et al. (1982)</td>
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<td>SmGf1</td>
<td>Rm1021, ΔbacA654::Spec'</td>
<td>Ferguson et al. (2002)</td>
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<td>DH5zx</td>
<td>supE44 AlacU169 (Δ80laczΔM15) hasdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>BRL</td>
</tr>
<tr>
<td>MT616</td>
<td>MM294A recA56 (pRK600) Cm'</td>
<td>Finan et al. (1986)</td>
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<td>Broad-host-range control plasmid, Tc'</td>
<td>Ditta et al. (1985)</td>
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<td>pJG51A</td>
<td>pRK404 carrying the S. melloti wild-type bacA gene with upstream region, Tc'</td>
<td>Glazebrook et al. (1993)</td>
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<td>pRF771</td>
<td>RK2 derivative Pthr expression vector, Tc'</td>
<td>Wells &amp; Long (2002)</td>
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<td>This study</td>
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<td>#M12</td>
<td>Generalized transducing phage for S. melloti Rm1021</td>
<td>T. Finan*</td>
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**4',6'-Diamidino-2-phenylindole (DAPI) fluorescence.** Cultures were treated with and without bleomycin as exactly as for the genomic DNA preparations except that following the centrifugation the cells were resuspended in 100 μl 1% (v/v) toluene, vortexed and stored at 4°C. When required, the cells were diluted to OD600 ~0.08 in dilution buffer (10 mM NaCl, 6.6 mM Na2SO4, 5 mM HEPES pH 7.0). The DAPI assay was performed following a previously described procedure (Johnson, 1994) except that DAPI was added to the cells at a final concentration of 0.1 μg ml⁻¹ in a final volume of 100 μl and the cells were incubated with DAPI overnight at 4°C. The fluorescence intensity was measured using a FLUOstar optima plate reader (BMG Labtech) spectrophotometer with excitation and emission at 350 and 450 nm, respectively.
version of Bac71–16, Bac71–16-BY, was prepared by linkage of the thiol-reactive dye BODIPY-FL N-(2-aminomethyl)maleimide (BY) (Invitrogen) to an additional C-terminal cysteine residue as reported previously (Scocchi et al., 2008). The uptake of Bac71–16-BY was performed as described previously (Marlow et al., 2009), with modifications. Mid-exponential-phase cultures of OD600 ~1.0 were harvested, washed and resuspended in fresh LB medium. After the addition of 0.5 μM Bac71–16-BY, the cultures were incubated at 30 °C for 1 h. To account for extracellular binding of Bac71–16-BY, the cultures were then treated with the extracellular quencher of fluorescence, Trypan Blue (TB; 1 mg ml−1) for 10 min at room temperature. The fluorescence of cells was determined on poly-L-lysine-coated slides using a Carl Zeiss Axioskop microscope (100 x objective magnification with UV light and FITC filter). Images were taken with a Canon Powershot camera and using the AxioVision version 2.0 software.

**Statistical analysis.** Where shown, the significance of differences among bacterial strains was assessed by the Student’s unpaired t-test using Microsoft Excel.

## RESULTS

**BacA sensitizes S. meliloti to different forms of bleomycin**

The R-group of bleomycin differs depending upon the form (Fig. 1a). Previous studies investigating the bleomycin-resistance phenotypes of S. meliloti Rm1021 bacA mutants have used the A2 form (Ferguson et al., 2006; Ichige & Walker, 1997; LeVier & Walker, 2001). To investigate whether the nature of the R-group affected BacA-mediated sensitivity of S. meliloti to bleomycin, we performed disc diffusion assays using different forms of bleomycin and compared the sensitivity of an S. meliloti Rm1021 bacA null mutant and the parent strain (Fig. 1b).

We found that the S. meliloti parent strain showed an increased sensitivity to all the forms of bleomycin tested relative to the S. meliloti bacA null mutant. We also determined that bleomycin sulphate and bleomycin A5 reduced the viability of S. meliloti Rm1021 in liquid culture and that deletion of bacA conferred protection against both forms (Fig. 1c, d). These findings show that BacA sensitizes S. meliloti to different forms of bleomycin, irrespective of the nature of the R-group.

**BacA is involved in the internalization of bleomycin in S. meliloti**

It has been shown previously by transmission electron microscopy (TEM) that bleomycin, in addition to damaging the DNA, also damages the cell wall and membrane of yeast cells (Lim et al., 1995). However, using TEM, we did not detect any bleomycin-induced lesions in the cell envelope components of S. meliloti Rm1021 or the bacA null mutant (data not shown). Additionally, unlike polymyxin B, which damages both the inner and outer membranes (Benincasa et al., 2009), bleomycin A5 treatment did not increase the amount of propidium iodide fluorescence of either the S. meliloti mutant lacking BacA or the parent strain (data not shown). To investigate a potential role for BacA in bleomycin uptake, we prepared fluorescently labelled bleomycin A5 (fluoro-BLM-A5) by conjugating it with FITC (Aouida et al., 2004). The resulting fluoro-BLM-A5 contained a 1:1 ratio of FITC and bleomycin A5 (data not shown). However, unlike with yeast cells (Aouida et al., 2004), we found that the conjugation of FITC to bleomycin A5 substantially reduced its toxicity and prevented uptake into S. meliloti (data not shown). For this reason, we used bleomycin-induced DNA degradation in the absence of membrane-damaging effects as an assay to monitor bleomycin A5 internalization in S. meliloti. We found that treatment of an S. meliloti Rm1021 culture with bleomycin A5 induced substantial degradation of the genomic DNA relative to the DNA from the untreated control culture (Fig. 2a), confirming the intracellular presence of bleomycin in S. meliloti. In contrast, we did not observe any significant loss of the genomic DNA after bleomycin A5 treatment of the S. meliloti Rm1021 bacA null mutant relative to the DNA from the untreated control culture (Fig. 2a). Additionally, the presence of pJG51A (encoding the S. meliloti wild-type bacA gene in pRK404) but not the control plasmid (pRK404) in the S. meliloti Rm1021 bacA null mutant strain increased the amount of bleomycin-induced DNA degradation relative to untreated control cultures (Fig. 2a). Taken together, these findings are consistent with a role for BacA in the internalization of bleomycin, which then leads to DNA damage and the subsequent degradation of the genomic DNA.

We also quantified the effect of bleomycin A5 treatment on the S. meliloti parent strain and bacA null mutant in situ by permeabilizing cells and measuring fluorescence of DAPI, a fluorescent dye that binds DNA (Johnson, 1994). We found a substantial decrease in the amount of DAPI fluorescence after treatment of either S. meliloti Rm1021 or the S. meliloti Rm1021 bacA null mutant with or without pRK404 (control vector) with bleomycin A5 resulted in a much smaller decrease in the amount of DAPI fluorescence relative to their untreated control cultures (Fig. 2b). These data confirm that BacA-dependent uptake is the major route of bleomycin internalization in S. meliloti. However, the fact that we observed a reduction in DAPI fluorescence after bleomycin treatment of the S. meliloti bacA null mutant shows that there is also a BacA-independent mechanism for bleomycin internalization in S. meliloti.

**BacA does not affect the sensitivity of S. meliloti to other DNA-damaging agents**

Although BacA is predicted to be in the inner membrane (Glazebrook et al., 1993), it has been demonstrated previously that the activation of inner-membrane transport
systems can alter the susceptibility of bacterial DNA to damaging agents by altering conditions in the cytoplasm (Ferguson et al., 2000). To rule this out, we analysed the sensitivity of the S. meliloti Rm1021 bacA null mutant and parent strain to methylglyoxal, mitomycin C and MMS, which are also known to induce DNA damage in bacteria (Ferguson et al., 2000; Grzesiuk & Janion, 1996; Otsuji & Murayama, 1972). We found that S. meliloti Rm1021 was sensitized to all three DNA-damaging agents, but in contrast to bleomycin A5, deletion of bacA did not increase the resistance of S. meliloti to these agents (data not shown). Additionally, although we found that treatment of S. meliloti Rm1021 with MMS resulted in DNA degradation, the presence of BacA did not affect the degree of MMS-induced DNA degradation (Fig. 2c). These findings show that BacA does not increase the susceptibility of S. meliloti to other DNA-damaging agents.

**B. abortus BacA complements the peptide uptake defects of an S. meliloti bacA null mutant**

The BacA protein is also responsible for conferring bleomycin sensitivity in B. abortus (LeVier et al., 2000). B. abortus is a highly infectious, containment level 3 pathogen (Franz et al., 1997). Therefore, to investigate the function of its BacA protein, we cloned the B. abortus bacA gene into the broad-host-range vector pRF771 (Wells & Long, 2002), under control of a constitutively active trp promoter, to create pBabacA. We found that the B. abortus BacA protein sensitized the S. meliloti bacA null mutant to bleomycin A5 relative to the mutant strain with the control plasmid (Fig. 3a). Additionally, the B. abortus BacA protein substantially reduced the amount of DAPI fluorescence in the S. meliloti bacA null mutant relative to the mutant strain with the control plasmid (Fig. 3b), demonstrating that the BacA protein is functionally homologous to S. meliloti in the internalization of bleomycin.

To determine whether B. abortus BacA is also involved in the uptake of truncated Bac7 peptides, we investigated the viability of the S. meliloti bacA null mutant with either pBabacA or pRF771 (control plasmid) in the presence of truncated Bac7 peptides, relative to the parent strain...
We determined that the *B. abortus* BacA protein greatly sensitized the *S. meliloti* bacA null mutant to Bac71–16 (RRIRPRPPRLPRPRP) and Bac71–35 (RRIRPRPPRLPRPRPRPPRLPFP) (Fig. 3c), consistent with a role for the *B. abortus* BacA protein in their uptake. To confirm this, cultures of *S. meliloti* Rm1021 bacA null mutant with either pBabacA or the control vector were incubated with Bac71–16 labelled with the fluorescent dye BODIPY (Bac71–16-BY) (Marlow et al., 2009; Mattiuzzo et al., 2007) and then analysed by fluorescence microscopy. To prevent the fluorescence of extracellular Bac71–16-BY, cultures were also treated with Trypan Blue, which quenches extracellular fluorescence (Benincasa et al., 2009). We found that cells of the *S. meliloti* Rm1021 bacA null mutant with pBabacA were highly fluorescent whereas, with the exception of an occasional cell, the cells of the mutant with control vector were non-fluorescent (Fig. 3d, i and ii, respectively). These results show that the *B. abortus* BacA protein is also involved in the internalization of truncated Bac7 peptides into *S. meliloti*.

**B. abortus** and *S. meliloti* *bacA* genes complement the chronic infection defect of the *S. meliloti* *bacA* null mutant in alfalfa when expressed under control of the *trp* promoter

It was demonstrated previously that the *E. coli* *sbmA* gene complemented the alfalfa chronic infection defect of the *S. meliloti* *bacA* null mutant, but only when cloned under control of the *S. meliloti* *bacA* gene promoter and 168 bp upstream of the *bacA* coding sequence (Ichige & Walker, 1997). This finding led to the hypothesis that expression of the *S. meliloti* *bacA* gene is regulated during the legume infection and that the correct expression is key to the essential role of BacA in the plant infection. However, we found that the *B. abortus* *bacA* gene (pBabacA) complemented the chronic infection defect of the *S. meliloti* Rm1021 bacA null mutant within alfalfa, when expressed from the *trp* promoter in pFL771 (Table 2). Since we included ~150 bp upstream of the *B. abortus bacA* coding sequence in pBabacA, we could not rule out the possibility that this region was playing a regulatory role during legume symbiosis. To determine whether the upstream region and promoter of the *S. meliloti* *bacA* gene are needed during legume symbiosis, we cloned the *S. meliloti* *bacA* gene with 19 bp upstream under control of *ptrp*...
We found that the \textit{S. meliloti bacA} gene cloned under control of \textit{p}_{\text{trp}} was able to complement the symbiotic defect of the \textit{S. meliloti bacA} null mutant to a similar extent to \textit{S. meliloti bacA} cloned under control of its own promoter with an extended upstream region (pJG51A) (Table 2). These findings show that the regulated expression of \textit{bacA} is not essential for \textit{S. meliloti} to form the legume symbiosis.

\textbf{Fig. 3.} BacA of \textit{B. abortus} is also involved in peptide uptake. (a) Cultures, containing approximately $1 \times 10^9$ c.f.u. ml$^{-1}$, were treated with bleomycin $A_2$ (20 \text{\mu}g \text{ml}^{-1})$ for 2 h and then the viability was determined. (b) Same as (a) except that cultures were treated or not with bleomycin and then the DNA was quantified by DAPI fluorescence. The fluorescence intensity for each bleomycin-treated strain is shown as a percentage relative to that of the untreated control. (c) Cultures of the indicated strains were incubated with and without Bac7$_{1-16}$ and Bac7$_{1-35}$ (1 \text{\mu}M) in LB medium for 1 h and then the viability was assessed. The arrows indicate that no viable cells were detected. The datasets shown are representative of the trends observed in two independent experiments and the error bars represent SD ($n=3$) for one experiment. The significance values (***$P<0.001$) represent comparison of the Rm1021 \textit{\Delta bacA} mutant carrying pRF771 (control) compared with pBabacA. (d) Cultures of the indicated strains were incubated with Bac7$_{1-16}$ labelled with the fluorescent dye BODIPY (Bac7$_{1-16}$-BY), treated with Trypan Blue and then analysed by microscopy as indicated. The images shown are representative fields of view from at least two independent experiments (bars, 20 \text{\mu}m).
Spermine protects S. meliloti against different forms of bleomycin

Our earlier findings showed that there is also a BacA-independent mechanism for bleomycin A\textsubscript{5} internalization in S. meliloti. Since the R-group of bleomycin A\textsubscript{5} has structural similarities to polyamines, it was proposed that a polyamine uptake system(s) could be involved in its uptake (Aouïda et al., 2004, 2005). Consistent with this, pre-treatment of yeast cells with the polyamines spermine or spermidine substantially reduced the uptake of fluorescently labelled bleomycin A\textsubscript{5} (fluoro-BLM-A5) (Aouïda et al., 2004). To investigate whether there was any relationship between polyamines and bleomycin in S. meliloti, we assessed the sensitivity of the parental strain S. meliloti Rm1021 to different forms of bleomycin (Fig. 4b), showing that spermine-mediated protection is independent of the BacA protein. Bleomycin damages DNA in the presence of iron and oxygen, through the generation of reactive oxygen species (Kane & Hecht, 1994). Although spermine has been shown in vitro to protect DNA against damage induced by reactive oxygen species generated by transition metals such as iron and copper (Pedreno et al., 2005), we found that spermine did not protect S. meliloti Rm1021 against H\textsubscript{2}O\textsubscript{2} and mitomycin C (Fig. 4c). Since H\textsubscript{2}O\textsubscript{2} and mitomycin also damage DNA through redox reactions with transition metals (Inlay & Linn, 1988), these results show that spermine does not have a generalized effect in protection of S. meliloti DNA against damaging agents.

Internalization of bleomycin in S. meliloti is energy-dependent

The BacA protein is predicted to form the transmembrane domain of an ABC transporter, and polyamine uptake is mediated by ABC transport systems in bacteria (Igarashi & Kashiwagi, 1999; LeVier & Walker, 2001). To gain further insights into the BacA-dependent and -independent mechanisms of bleomycin uptake in S. meliloti, we examined the effect of DNP pre-incubation on the sensitivity of S. meliloti Rm1021 parent strain and bacA null mutant to bleomycin A\textsubscript{5}. DNP is an uncoupler, which dissipates the proton-motive force and prevents ATP synthesis (McLaughlin, 1972). We found that DNP exposure reduced the viability of both S. meliloti Rm1021 and the bacA null mutant to a similar extent (Fig. 5a). However, pre-incubation of Rm1021 and the bacA null mutant with DNP increased their resistance on subsequent exposure to bleomycin A\textsubscript{5} (Fig. 5b, c). These findings are consistent with both the BacA-dependent and -independent mechanisms of bleomycin internalization in S. meliloti involving energy-dependent transport mechanisms.

Table 2. B. abortus and S. meliloti bacA genes complement the symbiotic defect of an S. meliloti BacA-deficient mutant

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Alfalfa colour</th>
<th>Alfalfa height (cm)*</th>
<th>No. of pink nodules</th>
<th>No. of white nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm1021/pRF771</td>
<td>Dark green</td>
<td>8.5 ± 2.5 (n=10)</td>
<td>14 ± 9</td>
<td>1 ± 3</td>
</tr>
<tr>
<td>S. meliloti ΔbacA mutant:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+pRF771</td>
<td>Light green</td>
<td>5.0 ± 1.2 (n=9)</td>
<td>0</td>
<td>18 ± 13</td>
</tr>
<tr>
<td>+pBabacA</td>
<td>Dark green</td>
<td>7.3 ± 2.2 (n=13)</td>
<td>8 ± 3</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>+pG51A</td>
<td>Dark green</td>
<td>8 ± 2.3 (n=5)</td>
<td>8 ± 6</td>
<td>15 ± 13</td>
</tr>
<tr>
<td>+pSmbacA</td>
<td>Dark green</td>
<td>8.3 ± 2.4 (n=10)</td>
<td>8 ± 4</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

n, Number of plants analysed.
Combining these results with those of our previous study (Marlow et al., 2009), we found that the *S. meliloti* BacA protein is involved in the uptake of both proline-rich and...
thiazole-modified peptides. Therefore, within the soil and during legume symbiosis, BacA-mediated uptake of different classes of peptides and/or thiazole-containing compounds into \textit{S. meliloti} could be important for environmental survival and/or bacteroid development. Interestingly, plants produce thiazole-containing compounds in response to invading micro-organisms (Yorgey et al., 1994). Therefore, the BacA-mediated uptake of one or more of these compounds could be important during the legume symbiosis. Additionally, hundreds of nodule-specific cysteine-rich (NCR) peptides have been identified in the legume host of \textit{S. meliloti} (Alunni et al., 2007; Mergaert et al., 2003, 2006). The NCR peptides have recently been shown to play a key role in \textit{S. meliloti} bacteroid differentiation (Van de Velde et al., 2010). At present, \textit{S. meliloti} factors involved in the NCR-mediated response have not been identified. However, since it has been shown that NCR peptides can enter \textit{S. meliloti} (Van de Velde et al., 2010) and since we found that BacA is involved in the uptake of structurally diverse peptides, BacA may also be playing a key role in the uptake of one or more of these NCR peptides.

We found by functional complementation experiments that the \textit{B. abortus} BacA protein is also involved in uptake of bleomycin and truncated Bac7 peptides in \textit{S. meliloti}. These results suggest that BacA-mediated peptide uptake could also be important for \textit{B. abortus} during host infections. \textit{B. abortus} causes abortions in cattle, and in humans it results in brucellosis, a severe debilitating disease (Franz et al., 1997). During mammalian infections, \textit{B. abortus} is expected to encounter a number of different classes of peptides such as the proline-rich Bac7 peptides used in this study, as they were derived from a full-length peptide produced by bovine leukocytes (Frank et al., 1990). Other mammalian hosts are also known to produce proline-rich peptides (Gennaro et al., 2001). During mammalian infections, BacA may also be playing a key role in the uptake of one or more of these NCR peptides.

In summary, our findings show the importance of BacA proteins in the uptake of structurally diverse peptides. Since the BacA protein also sensitizes \textit{M. tuberculosis} to bleomycin (Domenech et al., 2009), it will be interesting to determine whether this protein is also involved in peptide uptake in this pathogen, and whether BacA-mediated peptide uptake is important during the host infection.

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