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The *Sinorhizobium meliloti* MsbA2 protein is essential for the legume symbiosis

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Sinorhizobium meliloti is a beneficial legume symbiont, closely related to *Brucella* species, which are chronic mammalian pathogens. We discovered that the *S. meliloti* MsbA2 protein is essential to ensure the symbiotic interaction with the host plant, alfalfa. *S. meliloti* invades plant cells via plant-derived structures known as infection threads. However, in the absence of MsbA2, *S. meliloti* remains trapped within abnormally thickened infection threads and induces a heightened plant defence response, characterized by a substantial thickening of the nodule endodermis layer and the accumulation of polyphenolic compounds. The *S. meliloti* MsbA2 protein is homologous to the *Escherichia coli* lipopolysaccharide/phospholipid trafficking protein MsbA. However, MsbA2 was not essential for the membrane transport of either lipopolysaccharide or phospholipids in *S. meliloti*. We determined that the *msbA2* gene is transcribed in free-living *S. meliloti* and that in the absence of MsbA2 the polysaccharide content of *S. meliloti* is altered. Consequently, we propose a model whereby the altered polysaccharide content of the *S. meliloti msbA2* mutant could be responsible for its symbiotic defect by inducing an inappropriate host response.

INTRODUCTION

The α-proteobacterium *Sinorhizobium meliloti* forms a beneficial symbiosis with leguminous plants such as alfalfa (*Medicago sativa*) (Niner & Hirsch, 1998). During this interaction, *S. meliloti* enters into the legume host via the root hairs and induces the formation of plant-derived structures known as infection threads. The bacteria multiply and traverse down the infection threads, where they are ultimately endocytosed into the plant cell within membrane-bound acidic compartments (Mellor, 1989). Within these compartments, *S. meliloti* differentiates into a nitrogen-fixing bacteroid, which persists for extensive periods. Although this interaction is beneficial for the legume host and is an important system in its own right, it has also been used to gain insights into the molecular basis of chronic mammalian pathogens such as *Brucella* species (Ferguson *et al.*, 2004; LeVier *et al.*, 2000). *S. meliloti* is closely related to *Brucella* species (Paulsen *et al.*, 2002), and commonalities exist in their infection processes despite the major differences in their eventual outcome on their respective hosts (LeVier *et al.*, 2000).

The BacA protein was found to be essential for the persistence of both *S. meliloti* and *Brucella abortus* within their hosts (Glazebrook *et al.*, 1993; LeVier *et al.*, 2000). Based on the distant sequence similarity between BacA and the adrenoleukodystrophy family of eukaryotic proteins, which are thought to be involved in the transport of very-long-chain fatty acids (VLCFA) out of the cytoplasm into peroxisomes, it was subsequently discovered that BacA affects the VLCFA modification of the lipid A in both *S. meliloti* and *B. abortus* (Ferguson *et al.*, 2004). Interestingly, in the absence of BacA, ~50 % of the lipid A molecules of *S. meliloti* and *B. abortus* lack the lipid A VLCFA modification. In contrast, every lipid A molecule of the parent strain possesses a VLCFA modification. The lipid A is a component of the lipopolysaccharide (LPS), which forms the outermost leaflet of the outer membrane of Gram-negative bacteria.

†These authors contributed equally to this work.

Abbreviations: DOC, deoxycholate; EPS, exopolysaccharide; GUS, β-glucuronidase; TEM, transmission electron microscopy; VLCFA, very-long-chain fatty acid.

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Consequently, these findings led to a model where BacA is involved in the transport of an activated VLCFA out of the cytoplasm that is used to modify the lipid A in the outer membrane (Ferguson *et al.*, 2004). If this model were correct, then the LPS would need to be transported across the inner membrane before it could be modified with the VLCFA. However, the mechanism by which lipid-containing macromolecules such as LPS are transported from their site of synthesis on the inner face of the inner membrane to the outer membrane is poorly understood (Doerrler, 2006; Ruiz *et al.*, 2006).

In *Escherichia coli*, the inner-membrane MsbA protein is essential (Doerrler *et al.*, 2001). However, since a temperature-sensitive *E. coli msbA* mutant accumulates LPS in the inner membrane at the non-permissive temperature, this provided evidence that MsbA is involved in the inner-membrane transport of rough LPS, which lacks O-antigen (Doerrler *et al.*, 2001, 2004). The *E. coli* MsbA protein is also thought to be involved in the transport of phospholipids across the inner membrane (Doerrler *et al.*, 2001). However, there is still some debate about the role of MsbA proteins in phospholipid transport since a *Neisseria meningitidis msbA* mutant was affected in LPS but not phospholipid transport (Tefsen *et al.*, 2005). Interestingly, the *S. meliloti* Rm1021 genome (Galibert *et al.*, 2001) encodes multiple proteins (Fig. 1a), which share between 26–34 % identity (47–58 % similarity) and 23–32 % identity (44–54 % similarity) over their entire length with the *E. coli* and *N. meningitidis* MsbA proteins, respectively. It was shown previously that the

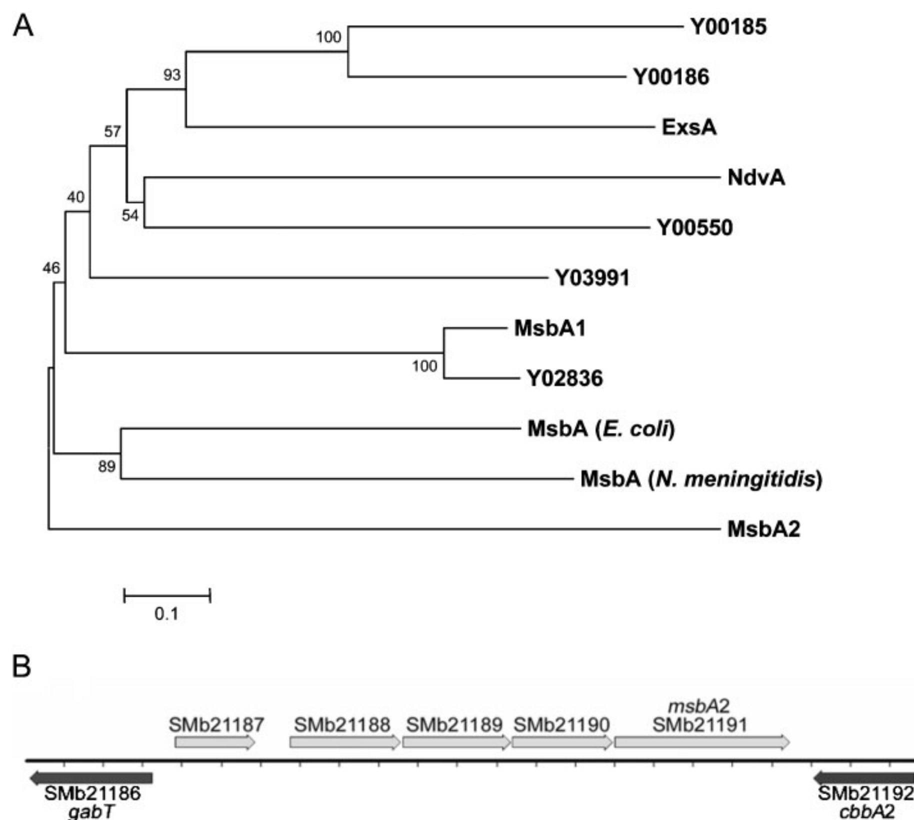

Fig. 1. *S. meliloti* MsbA2 is predicted to be an MsbA-like protein and is encoded in the genome immediately downstream of potential polysaccharide biosynthesis genes. (A) The *S. meliloti* Rm1021 genome was scanned for potential homologues of the *E. coli* MsbA protein using BLAST. Since *E. coli* MsbA is an ABC transporter, a large number of *S. meliloti* proteins showing similarity over the predicted ATP-binding site were identified. However, only *S. meliloti* proteins showing similarity across their entire length were aligned along with the *E. coli* and *N. meningitidis* MsbA proteins, using MEGA version 4 (Tamura *et al.*, 2007). A guide tree was then calculated using the neighbour-joining method (Saitou & Nei, 1987) and bootstrapped (500 replicates, random seeds). Previous studies have shown that NdvA is a transporter of β-(1,2)-glucans (Roset *et al.*, 2004; Stanfield *et al.*, 1988) and ExsA is predicted to be involved in succinoglycan transport (Becker *et al.*, 1995). Although predicted to be ABC transporters, the functions of the other MsbA-like proteins shown are unknown. (B) *msbA2* (*SMb21191*) is likely to be the last gene in a multi-gene operon with *SMb21188* (encoding a putative acyltransferase) and *SMb21189*/*SMb21190* (encoding putative glycosyltransferases). The predicted operon is surrounded by the upstream genes *gabT* (*SMb21186*, encoding a putative 4-aminobutyrate aminotransferase) and *SMb21187* (encoding a putative transcriptional regulator) and the downstream gene *cbbA2* (*SMb21192*, encoding a putative fructose-bisphosphate aldolase).

S. meliloti ExsA protein affects the molecular mass distribution of the exopolysaccharide (EPS) succinoglycan and has been proposed to play a role in the transport of succinoglycan across the inner membrane (Becker *et al.*, 1995). Although an *S. meliloti exsA* mutant forms a symbiosis with legumes, a *B. abortus exsA* deletion mutant has reduced survival in mice and induced protective immunity against the parent strain (Rosinha *et al.*, 2002). Additionally, the *S. meliloti* and *B. abortus* NdvA proteins (known as Cgt in *B. abortus*) are involved in the inner-membrane transport of a β-1,2-glucan and are essential for the host interaction (Dickstein *et al.*, 1988; Roset *et al.*, 2004; Stanfield *et al.*, 1988). Combined, these findings raised the possibility that the other *S. meliloti* MsbA-like proteins (Fig. 1A) could also be playing a role in the transport of a polysaccharide or lipid-containing polysaccharide such as LPS and these processes could play an important role in the host interaction.

To understand more about the roles of the potential MsbA-like proteins in *S. meliloti* (Fig. 1A), we constructed and characterized mutants defective in these proteins. In this paper, we report our findings for an *S. meliloti* Rm1021 *msbA2* insertional mutant. We initially focused our efforts on the *msbA2* gene since it is borne on the pSymB megaplasmid of *S. meliloti* Rm1021, which carries a large number of genes whose products are involved in the synthesis of cell-surface carbohydrates (Finan *et al.*, 2001), and is immediately downstream of genes whose products are predicted to be involved in the biosynthesis of a lipid-linked polysaccharide (Fig. 1B). Our characterization determined that the MsbA2 protein is essential for the legume symbiosis, and since the *S. meliloti msbA2* insertional mutant has a polysaccharide alteration, we propose that the MsbA2 protein could be involved in the transport of a novel polysaccharide.

METHODS

Bacterial growth. All bacterial strains and plasmids used in this study are shown in Table 1. All *S. meliloti* strains were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1982) or on LB agar supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC) and 500 μg streptomycin (Sm) ml^{-1}, unless defined otherwise. Where indicated, the LB medium or agar was also supplemented with 200 μg neomycin (Nm) ml^{-1}, 50 μg gentamicin (Gm) ml^{-1} or hygromycin (Hm) at either 100 (for LB) or 150 μg ml^{-1} (for LB/MC). The *E. coli* strains were grown in LB medium or on LB agar supplemented with 50 μg kanamycin (Km) ml^{-1} or 10 μg Gm ml^{-1} as defined.

Construction of the *S. meliloti msbA2* insertional mutant. The *S. meliloti msbA2* insertional mutant was constructed by amplifying an 853 bp internal fragment by PCR using the primers SmmsbA2+181F-*Xho*I (5′-ACGCTCGAGGCGGCATTTCCGCTTACTTTC-3′) and SmmsbA2+1015R-*Avr*II (5′-GACCCTAGGATTGCCCTGAGGAGC-CTTTG-3′). The *msbA2* PCR fragment was then purified (QIAquick, Qiagen), digested with *Xho*I and *Avr*II and then cloned into the *Sal*I and *Spe*I sites of pJH104 to create pJH104-*msbA2*in. After transformation into *E. coli* DH5α, pJH104-*msbA2*in was transferred into *S. meliloti* Rm1021 by tri-parental mating using *E. coli* MM294A with the helper plasmid pRK600 and transconjugants were selected on LB/MC Sm Nm agar after incubation at 30 °C for 4 days. To avoid contamination with the *E. coli* donor strains, a final concentration of 1 mg Sm ml^{-1} was used in the selection agar. The vector, pJH104, is able to replicate in *E. coli* but is unable to replicate in *S. meliloti*, hence the genomic *msbA2* gene is disrupted by homologous recombination upon transfer of pJH104-*msbA2*in into *S. meliloti*. The disrupted *msbA2* gene was transduced into Rm1021 and transductants were selected on LB Nm. All transductants were purified onto LB/MC agar containing the defined antibiotics and the disrupted *msbA2* gene was confirmed by PCR using the primers SmmsbA2+155F (5′-CGGAGATGGACAGGCCAAC-3′) and pJH104gusR (5′-GAGTTT-TTTGATTTCACGGGTT-3′). All transductants gave the expected PCR product of 0.9 kb with this primer pair.

Construction of the *bacA-uidA* BacA$^+$ fusion strain. A 0.94 kb fragment of the *S. meliloti bacA* gene, including upstream region, was amplified from pJG51A (Glazebrook *et al.*, 1993) by PCR using the primers SmBaUS1F-*Bam*HI (5′-TCGAGTTCCTTGCCTGGC-3′) and SmBaIN1R-*Xba*I (5′-TTGATCGCGACGCTGACC-3′) and cloned into TOPO using the pCR-2.1 TOPO cloning kit (Invitrogen). After sequencing, the *bacA* fragment was digested from TOPO using *Spe*I and *Xho*I and cloned into pJH104. The resulting pJH104-*bacA* was then transferred into *S. meliloti* by tri-parental mating, transconjugants were selected and then the insertion was transduced into Rm1021 exactly as described for the construction of the *S. meliloti msbA2*::pJH104 mutant. The insertion of pJH104-*bacA* was confirmed by PCR using the primers SmBaUS1F-*Bam*HI and pJH104gusR and all transductants gave the expected PCR product of 1 kb with this primer pair. Additionally, since BacA is essential for the alfalfa symbiosis, the presence of a wild-type copy of the *bacA* gene in this strain was also confirmed by the ability of the strain to form a successful symbiosis with alfalfa.

Cloning of the *S. meliloti msbA2G97A* gene. The *S. meliloti msbA2* gene was amplified by PCR using the primers SmmsbA-90F-*Xba*I (5′-CCGTCTAGATCGCGCGGCTTGCCATG-3′) and SmmsbA2+1832R-*Xba*I (5′-CCGTCTAGAATGGACAAGCGGCTGGGTCTT-3′) and digested with *Xba*I. The digested *msbA2* gene was ligated into the *Xba*I site of pJN105 (Newman & Fuqua, 1999), under control of the pBAD promoter, and transformants were selected on LB Gm supplemented with 0.1 % (w/v) glucose to repress transcription. After screening 60 transformants by digestion, we identified one clone containing an *msbA2* gene insert. Sequencing of the cloned *msbA2* gene revealed a point mutation (G97A) resulting in an asparagine residue instead of a serine at amino acid position 33 of the MsbA2 protein.

Polysaccharide isolation and analysis. Cultures of the defined strains were grown to late-exponential phase in LB/MC with the appropriate antibiotics and diluted into 5 l LB/MC to an OD_{600} ~0.1. The cultures were then grown for 48 h to late exponential phase (OD_{600} ~2), and the cells were harvested by centrifugation at 6000 r.p.m. for 10 min (Sorvall RC-3B). The crude polysaccharides were extracted from the cell pellets using a hot water/phenol procedure (Reuhs *et al.*, 1994) and were then treated with DNase (20 μg ml^{-1}), RNase (100 μg ml^{-1}) and proteinase K (100 μg ml^{-1}). After lyophilizing, the polysaccharide preparations were resuspended in distilled water to a concentration of 10 mg ml^{-1}. After centrifugation (4500 ***g*** for 15 min), the supernatant was ultracentrifuged (100 000 ***g***, 8 °C) for 3.5 h. The purified polysaccharide pellet was then washed twice with distilled water, freeze-dried and then 3 μg aliquots analysed by deoxycholate (DOC)-PAGE/silver staining as described previously using either periodate or alcian blue (0.005 % w/v) (Reuhs *et al.*, 1994).

Fluorescence microscopy and histochemical staining for polyphenolics. Polyphenolics were identified using previously published methods with modifications (Vasse *et al.*, 1993). In brief, for both methods, root nodules were sliced longitudinally and then fixed immediately in 2.5 % (w/v) glutaraldehyde and 10 mM PIPES

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
***S. meliloti* strains**		
Rm1021	Sm^r derivative of SU47	Meade *et al.* (1982)
SmSB1	*msbA2*::pJH104 (*uidA*) transduced into Rm1021 Nm^r	This study
SmGF3	*bacA*::pJH104 (*uidA*) $BacA^+$ transduced into Rm1021 Nm^r	This study
***E. coli* strains**		
DH5α	*supE44*Δ*lac*U169 (*ϕ*80*lacZ*ΔM15) *hsdR*17 *recA1 endA1 gyrA96 thi-1 relA1*	BRL
MT616	MM294A *recA*56 (pRK600) Cm^r	Finan *et al.* (1986)
Plasmids		
pJG51A	pRK404 carrying the *bacA* gene	Glazebook *et al.* (1993)
pJH104	*S. meliloti* suicide plasmid, Nm^r	Davis & Walker (2007)
pJN105	*araC*-P_{BAD} cassette cloned in pBBR1MCS5, Gm^r	Newman & Fuqua (1999)
pMS03	Broad-host-range plasmid pMB393 with constitutive *trp* promoter	Keating *et al.* (2002)
pJH104-*msbA2*in	pJH104 carrying 853 bp *msbA2* internal fragment	This study
p*msbA2*G97A	pJN105 carrying the entire *msbA2* gene containing a G97A mutation and 90 bp upstream	This study
pRK600	pRK2013 *npt*::Tn*9* Cm^r	Finan *et al.* (1986)

(pH 7.2) for 1 h. For the fluorescent microscopy, the nodule slices were then cleared by soaking in 50 % (v/v) bleach for 3 min, washed three times with sterile distilled water and then visualized for fluorescent polyphenolics by microscopy (10× magnification, Zeiss Axioskop) using UV excitation. For the histochemical staining, after fixing, the nodule slices were immersed in 0.04 % (w/v) potassium permanganate for 1 h, rinsed with 10 mM PIPES (pH 7.2), and then stained with 0.01 % (w/v) methylene blue for 2 min. The nodule slices were then immersed in 50 % (v/v) bleach for 3 min and visualized using bright-field optics (10× magnification, Zeiss Axioskop). Images were then processed by Axio Vision software.

***β*-Glucuronidase (GUS) assay.** Cultures of the defined strains were grown to late-exponential phase in LB/MC media with the appropriate antibiotics. An aliquot of culture (100 μl) was centrifuged, washed and resuspended in 0.75 ml lysis buffer [50 mM sodium phosphate buffer pH 7.0, 2.6 % (v/v) *β*-mercaptoethanol, 0.1 % (v/v) Triton X-100 and 1 mg lysozyme ml^{-1}]. One hundred microlitres of chloroform was added and the mixture was incubated at 37 °C for 10 min prior to the addition of 100 μl 10 mM *p*-nitrophenyl-*β*-D-glucuronide. The samples were incubated at 37 °C until the appearance of a yellow colour and then the reaction was stopped by the addition of 400 μl 2 M 2-amino-2-methylpropandiol. The cell debris was removed by centrifugation (microfuge, 13 000 r.p.m., 5 min) and then the GUS activity of the supernatant was determined by measuring the A_{415}. Units of GUS activity were then calculated using the Miller equation [A_{415}/time (min) × volume (ml) × OD_{600})] × 1000 (Miller, 1972).

***S. meliloti*-alfalfa interaction experiments.** To determine the ability of *S. meliloti* to form a successful symbiosis with alfalfa, 3-day-old seedlings were inoculated with 1 ml *S. meliloti* culture, resuspended to an OD_{600} 0.05 in sterile water, on Jensen's agar as described previously (Leigh *et al.*, 1985). The plates were incubated at 25 °C and then plant growth and nodule morphology were determined at different stages throughout a 4 week period. To confirm that the mutants were not reverting during the symbiosis, individual nodules were removed, surface sterilized with 50 % (v/v) bleach and crushed in 200 μl LB/MC supplemented with 5 % (w/v) glucose. The bacteria were then serially diluted and 10 μl aliquots from each dilution were spotted in triplicate onto LB/MC Sm agar (to determine total number of bacteria extracted) and LB/MC agar containing the appropriate antibiotic to select for the mutant strain. In all cases, we observed equivalent numbers of colonies on both selective and non-selective agar, indicating that the mutants were not reverting.

Light and transmission electron microscopy (TEM). The light microscopy and TEM of the nodules were performed as described previously (Krusell *et al.*, 2005). The nodules were halved and fixed in 2.5 % (w/v) glutaraldehyde in 0.1 M sodium cacodylate (pH 7.0) overnight at 4 °C. The fixed nodules were either dehydrated in an ethanol series and embedded in LR White acrylic resin (Agar Scientific) for light microscopy or post-fixed in 1 % osmium tetroxide (w/v), dehydrated in an ethanol series, and then embedded in Durcupan epoxy resin (Sigma-Aldrich) for conventional TEM. Semi-thin sections (1 mm) and ultrathin sections (70 nm) were taken from the resin-embedded samples (LR White and Durcupan) using a Reichert Ultracut E ultramicrotome. The semi-thin sections were collected on glass slides and stained with 1 % toluidine blue in borax (w/v) and viewed and photographed using a Zeiss Axioskop optical microscope fitted with an AxioCam digital camera (Carl Zeiss Imaging). The ultrathin sections for conventional TEM were collected on pioloform-coated copper grids and stained with uranyl acetate (10 min) and lead citrate (5 min) before being viewed with a JEOL 1200 EX transmission electron microscope.

Membrane localization of phosphate-containing lipids. *S. meliloti* strains were grown at 30 °C to an OD_{600} of ~0.5–0.6 in LB/MC containing appropriate antibiotics then labelled for 20 min with $^{32}P_i$ [2 μCi ml^{-1} (74 kBq ml^{-1})] before the cells were cooled on ice and recovered by centrifugation at 4 °C for 10 min at 4000 ***g***. Spheroplasts were prepared by treatment with EDTA/lysozyme and lysed by sonication. Washed membranes were isolated by centrifugation at 100 000 ***g*** and separated into inner- and outer-membrane fractions using a 30–60 % sucrose gradient (Doerrler *et al.*, 2004; Osborn & Munson, 1974). Portions of gradient fractions were subjected to mild acid hydrolysis in 0.4 ml 12.5 mM sodium acetate, pH 4.5, 1 % SDS to release the lipid A from the LPS core sugars, then extracted by the addition of 1 ml chloroform/methanol (1 : 1) to yield a two-phase solution. This treatment has no effect on phospholipids and allows the LPS and phospholipids to be analysed simultaneously (Zhou *et al.*, 1998). The aqueous upper phase was discarded and the lower phase was washed with fresh upper phase and dried. Alternatively, LPS was recovered free from phospholipids due to its insolubility (5 min, 20 000 ***g***) in a single-phase Bligh–Dyer solution, chloroform/methanol/H_2O (1 : 2 : 0.8) (Bligh & Dyer, 1959; Nishijima & Raetz, 1979) prior to mild acid treatment as described above. Lipid species were dissolved in chloroform/methanol (4 : 1), spotted onto silica gel 60 TLC plates (Merck) and resolved using the solvent chloroform/pyridine/88 % formic acid/H_2O (50 : 50 : 16 : 5). Plates were analysed using a Phosphorimager equipped with IQMac software.

RESULTS

An *S. meliloti msbA2* insertional mutant is defective in the legume symbiosis

To investigate the function of the MsbA2 protein in *S. meliloti*, we disrupted the *msbA2* gene in the sequenced strain Rm1021 (Galibert *et al.*, 2001) using an internal fragment of the *msbA2* gene cloned into the suicide vector, pJH104 (Davis & Walker, 2007). The *msbA2* gene is carried on one of the two megaplasmids, pSymB, in the *S. meliloti* genome (Galibert *et al.*, 2001), immediately downstream of two potential glycosyltransferases genes and an acyltransferase gene (Fig. 1B). Disruption of genes with pJH104 also creates a transcriptional fusion to the *uidA* gene, which encodes GUS. Once constructed, the *msbA2*::pJH104 disruption was then transduced into Rm1021 to create Rm1021 *msbA2*::pJH104t. To investigate whether the MsbA2 protein was playing a role in the *S. meliloti*–legume symbiosis, alfalfa seedlings were inoculated with cultures of either the parent strain, Rm1021, or the mutant strain on Jensen's agar, which lacks nitrogen and carbon sources. Plant growth and nodule development were monitored after 4 weeks (Fig. 2). In contrast to inoculation with the parent strain, which resulted in healthy, dark green alfalfa plants with a mean height of 14 cm (n=19), seedlings inoculated with the *S. meliloti msbA2*::pJH104t mutant were stunted [mean height 2 cm (n=21)] and the leaves were paler green/yellowish, indicative of an unsuccessful symbiosis (Fig. 2A and B, respectively). Additionally, compared to the pink, elongated, nitrogen-fixing root nodules induced by the parent strain, the root nodules induced by the Rm1021 *msbA2*::pJH104t mutant

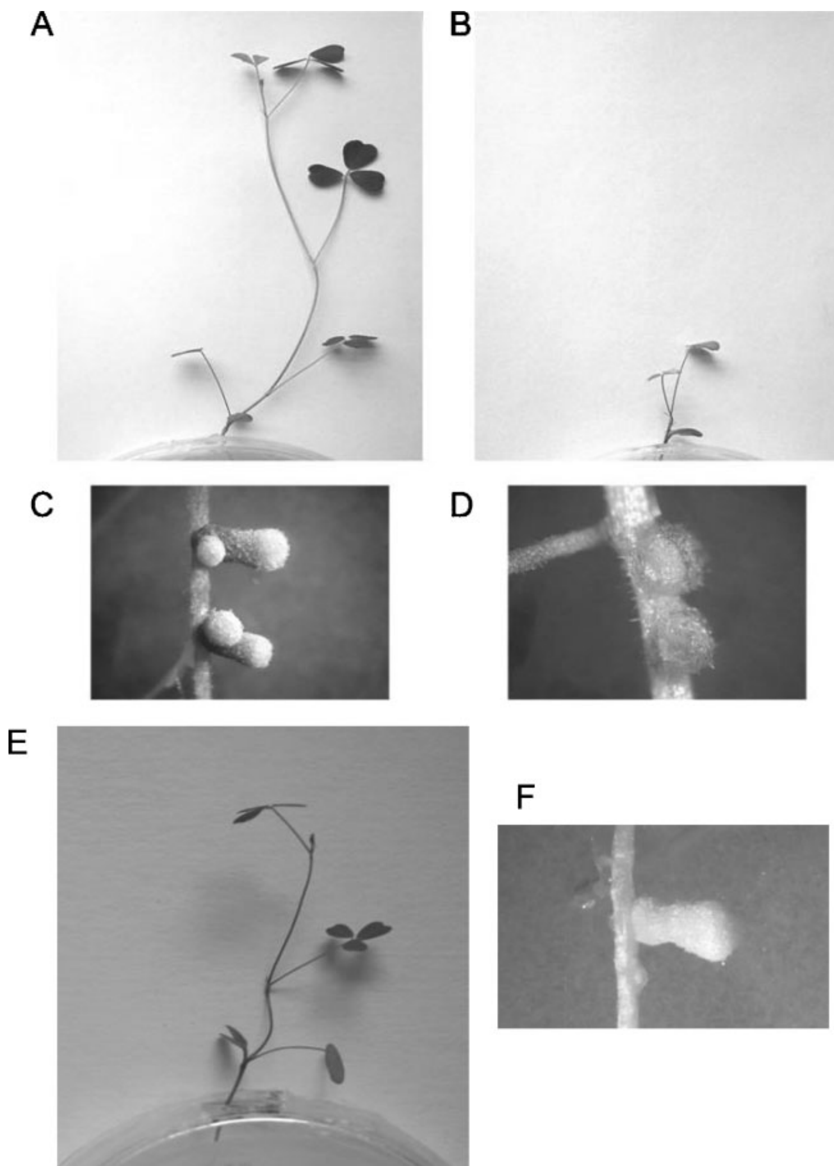

Fig. 2. *S. meliloti* MsbA2 is essential for the alfalfa symbiosis. Alfalfa seedlings were inoculated with either the parent strain *S. meliloti* Rm1021 (A and C), the *S. meliloti* Rm1021 *msbA2*::pJH104t mutant strain (B and D) or the Rm1021 *msbA2*::pJH104t mutant strain containing p*msbA2*G97A (E and F), and plant growth (A, B and E) and nodule development (C, D and F) were photographed 4 weeks post-infection.

were shorter and white, with brown tinges, indicative of a potential plant defence response (Fig. 2C and D, respectively). The same defective plant phenotypes were also observed for additional Rm1021 *msbA2*::pJH104 transductants and the original Rm1021 strain where the *msbA2*::pJH104 insertion was created (data not shown). Therefore, combined, these findings provide evidence that the MsbA2 protein is essential for *S. meliloti* to form a successful symbiosis with alfalfa.

Since the genes downstream of *msbA2* are encoded on the opposite strand in the Rm1021 genome (Fig. 1B) (Galibert *et al.*, 2001), it was unlikely that the insertion of pJH104 into the *msbA2* gene was exerting a polar effect on these genes. Additionally, another study had independently identified that disruption of the Rm1021 *msbA2* gene with a transposon insertion also prevented a successful legume symbiosis (J. Griffitts & S. Long, unpublished). Unfortunately, we were unsuccessful in our attempts to clone a wild-type copy of the *S. meliloti msbA2* gene into either TOPO (Invitrogen) or the broad-host-range vectors pMS03 (Keating *et al.*, 2002) and pJN105 (Newman & Fuqua, 1999) in *E. coli*. However, we were able to clone a mutated form of the *msbA2* gene (p*msbA2*G97A) into pJN105 (Newman & Fuqua, 1999), under control of an arabinose-inducible promoter in the presence of 0.1 % (w/v) glucose. The cloned *S. meliloti msbA2* gene contained a point mutation (G97A), which produced a mutated form of the MsbA2 protein with a substitution of an asparagine, instead of a serine residue, at amino acid position 33. We discovered that, although 7/15 plants inoculated with the *S. meliloti msbA2*::pJH104t mutant containing p*msbA2*G97A in the presence of 0.1 % (w/v) arabinose were light green/yellow with only brown nodules (7.0 ± 0.7) and white nodules (2 ± 3), 8/15 plants were dark green and had on average 2.6 ± 1.0 pink nodules per plant root (Fig. 2E and F, respectively). Since pJN105 had never been used previously to complement *in planta* defects of *S. meliloti* mutants, the variability in these results could be due to differences in the diffusion of arabinose into the root nodule to induce transcription of the *msbA2* gene. However, we determined that increasing the percentage of arabinose in the Jensen's agar had a detrimental effect on plant growth regardless of the strain used for inoculation (data not shown). In contrast, no pink nodules were observed on plants inoculated with the *S. meliloti msbA2*::pJH104t mutant containing the control plasmid, pJN105, in the presence of 0.1 % (w/v) arabinose ($n=9$, data not shown). Consequently, despite our inability to clone the wild-type *msbA2* gene, these data provide further support that the MsbA2 protein is important for the legume symbiosis and suggest that the serine at residue 33 is not critical for MsbA2 function.

The *S. meliloti msbA2* insertional mutant is defective in host cell entry

To investigate more precisely the host defect of the Rm1021 *msbA2*::pJH104t mutant, the root nodules induced by either the parent or the mutant strain were analysed by light microscopy and TEM, 2 and 4 weeks after inoculation (Figs 3 and 4, respectively). Unlike the nodules induced by the parent strain after 2 weeks, which were surrounded by an endodermis and were packed throughout the different developmental zones with *S. meliloti* bacteroids (Fig. 3A and B, respectively), the nodules induced by the Rm1021 *msbA2*::pJH104t mutant after 2 weeks had a significantly thickened endodermis and were devoid of bacteroids (Fig. 3C and D, respectively). The differences between the nodules induced by either the parent strain or the Rm1021 *msbA2*::pJH104t mutant strain were even more pronounced 4 weeks post-infection (Fig. 4A, B, C). Interestingly, we determined that, in the absence of MsbA2, *S. meliloti* appeared to be randomly colonizing the root hair cells (Fig. 4D). Additionally, within the Rm1021 *msbA2*::pJH104t mutant-induced nodules, we observed abnormal infection threads, which appeared to be dramatically thickened, were packed full of bacterial cells and had penetrated through the root hair (Fig. 4E and F). Randomly colonized root hair cells and swollen, abnormal infection threads were never visualized in the nodules induced by the parent strain (data not shown). Therefore, our observations show that the Rm1021 *msbA2*::pJH104t mutant is defective in host cell entry and that the interaction with the host is aborted whilst the bacterial cells are within the infection thread.

The *S. meliloti msbA2* insertional mutant induces a host defence response

Previous studies have revealed that *S. meliloti* mutants defective in infection thread development induce a plant defence-like response in the root nodules, characterized by the accumulation of polyphenolic compounds (Niehaus *et al.*, 1998). The production of these compounds in plant tissues can be detected either by their autofluorescence or by histochemical staining (Vasse *et al.*, 1993). To investigate whether the Rm1021 *msbA2*::pJH104t mutant induces a plant defence response in alfalfa, the nodules induced by the mutant strain were analysed by fluorescence microscopy and by bright-field microscopy after histochemical staining (Fig. 5A and B, respectively). As controls, nodules from alfalfa seedlings inoculated with the parent strain were also analysed under the same conditions (Fig. 5C and D, respectively). The nodules induced by the Rm1021 *msbA2*::pJH104t mutant had a dramatically increased autofluorescence and stained blue with potassium permanganate/methylene blue, indicating the accumulation of polyphenolics, whereas the nodules induced by the parent strain had less autofluorescence and were not stained. Combined with the microscopy analysis showing that the nodule endodermal layer is dramatically thickened in response to the Rm1021 *msbA2*::pJH104t mutant strain (Fig. 4C), these results provide strong evidence that the Rm1021 *msbA2*::pJH104t mutant induces a heightened defence response in alfalfa.

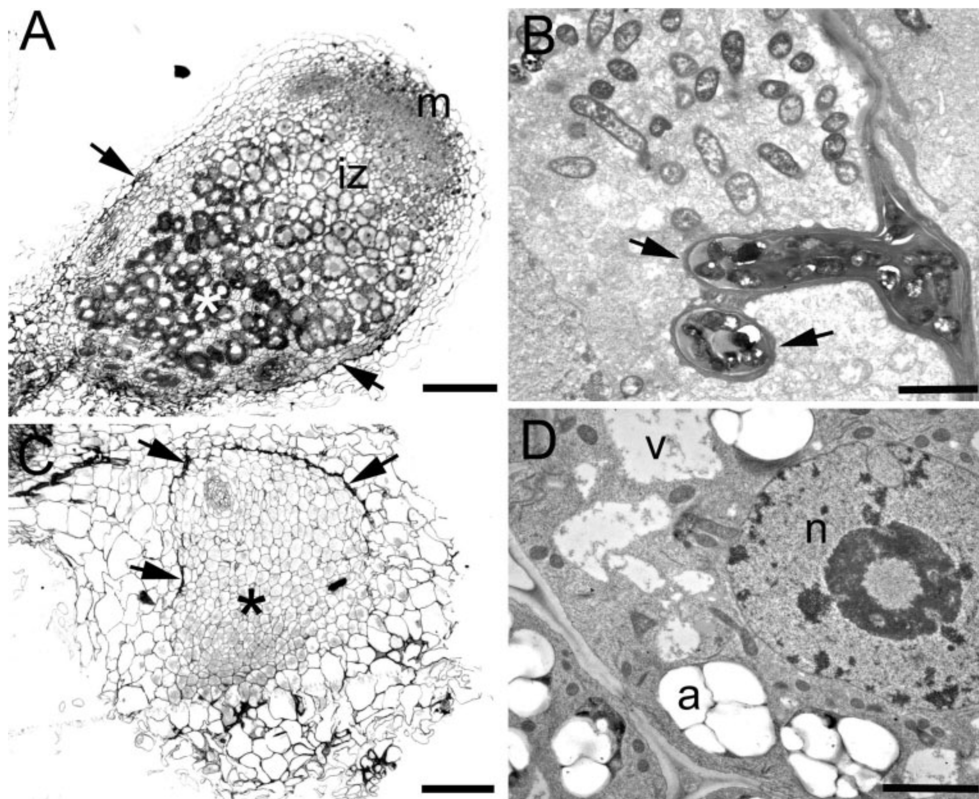

Fig. 3. MsbA2 is essential for *S. meliloti* to infect the nodule cells. Light (A and C) and transmission electron (B and D) microscopy images of alfalfa nodules after inoculation with either the *S. meliloti* Rm1021 parent strain (A and B) or the *S. meliloti* Rm1021 *msbA2*: : pJH104t mutant strain (C and D), 2 weeks post-inoculation. Arrows indicate the endodermis in (A) and (C). Nodules formed by the parent strain (A) are indeterminate, with a meristem (m), invasion zone (iz) and a central infected zone (*). The infection threads containing *S. meliloti* penetrate into the plant cell (arrows in B). The nodules induced by the *S. meliloti* Rm1021 *msbA2*: : pJH104t mutant (C) are much smaller, have no obvious zonation and the cells in the centre (*) are uninfected (D). Abbreviations: a, amyloplast; n, nucleus; v, vacuole. Bars, 200 μm (A), 2 μm (B and D) and 50 μm (C).

The *S. meliloti msbA2* insertional mutant has an altered polysaccharide content but does not affect the transport of phosphate-containing lipids

The MsbA2 protein shares 50 % similarity (26 % identity) and 44 % similarity (23 % identity) to the *E. coli* and *N. meningitidis* MsbA proteins, respectively, which are known to be involved in LPS transport (Doerrler *et al.*, 2001, 2004; Tefsen *et al.*, 2005), suggesting that MsbA2 could also be involved in the transport of a lipid-linked polysaccharide such as LPS. Additionally, the *msbA2* gene is located adjacent to two potential glycosyltransferease genes and an acyltransferase gene in the Rm1021 genome (Fig. 1B), providing further support that MsbA2 could be involved in the transport of a lipid-linked polysaccharide. Since previous studies have determined that the lipid A molecules of *S. meliloti* are modified with phosphate groups (Gudlavalleti & Forsberg, 2003; Sharypova *et al.*, 2003), we labelled the lipid A and phospholipids of the *S. meliloti* parent and *msbA2*: : pJH104t mutant by growth in the presence of $^{32}P_i$. However, analysis of the $^{32}P_i$-labelled lipids from either the inner- or outer-membrane fractions by TLC (data not shown), showed that disruption of the *msbA2* gene was not affecting the membrane localization of the either the lipid A or phospholipids. Additionally, using classical physiology tests, such as an increased sensitivity to detergents such as SDS and DOC, which have been successfully used to identify *S. meliloti* mutants with LPS alterations (Ferguson *et al.*, 2002, 2004), we observed no difference in the sensitivity of the parent strain and Rm1021 *msbA2*: : pJH104t mutant toward these agents on LB agar (data not shown). Additionally, small-scale analysis of the LPS by SDS-lysis followed by SDS-PAGE/periodate/silver staining revealed no LPS alterations between the parent strain and Rm1021 *msbA2*: : pJH104t mutant (data not shown), suggesting that if there were LPS alterations in this mutant strain then these could not be detected using this methodology. Therefore, these data suggest that the MsbA2 protein is either not expressed in free-living *S. meliloti* or is not essential for the transport of phosphate-containing lipids such as LPS across the inner membrane.

Since disruption of the *msbA2* gene with pJH104 created a transcriptional fusion to the *uidA* gene, encoding GUS, we

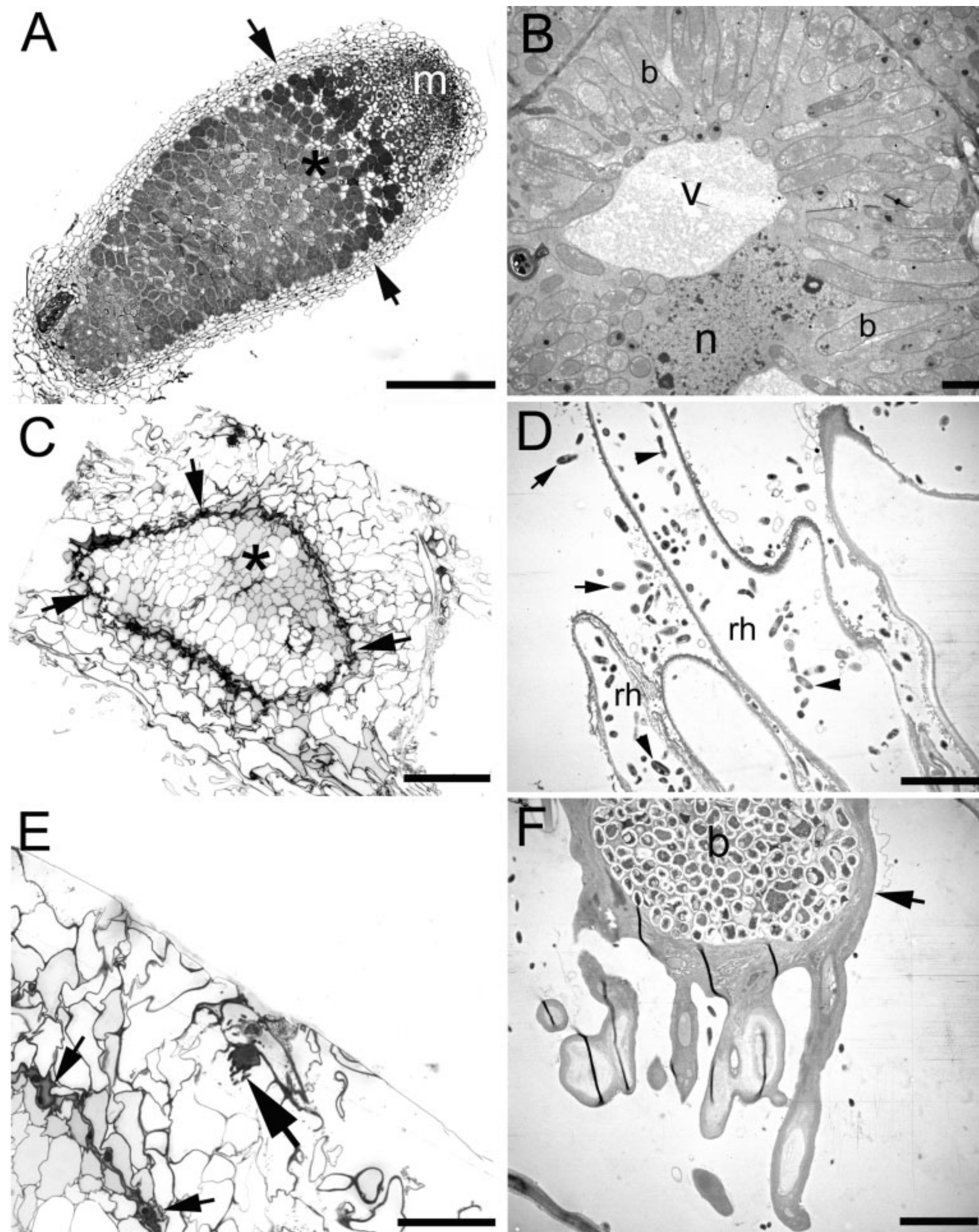

Fig. 4. The *S. meliloti msbA2* insertional mutant colonizes root hairs and induces abnormal infection threads. All images were taken 4 weeks post-infection. (A) Light microscopy of parent strain infected nodule [arrows indicate endodermis, meristem (m) and zone of *S. meliloti* nitrogen-fixing bacteroids (*)]. (B) TEM of parent strain infected nodule [*S. meliloti* bacteroids (b), plant nucleus (n) and plant vacuole (v)]. (C) Light microscopy of Rm1021 *msbA2* : : pJH104t mutant infected nodule [arrows indicate the dramatically thickened endodermis and the central zone lacks *S. meliloti* bacteroids (*)]. (D) TEM of the root hairs (rh) of alfalfa showing that the Rm1021 *msbA2* : : pJH104t mutant can colonize in a random fashion [arrows indicate the bacterial cells surrounding and within the root hair cells]. (E) TEM of Rm1021 *msbA2* : : pJH104t mutant-infected nodule (the small arrows show the thickened endodermis and the large arrow shows that a swollen infection thread has penetrated through the root hair. (F) TEM of swollen infection thread induced by the Rm1021 *msbA2* : : pJH104t mutant (arrow points to thickened plant cell wall material). Bars, 400 μm (A), 2 μm (B), 50 μm (C), 5 μm (D), 20 μm (E), and 3 μm (F).

investigated whether the *msbA2* gene was being transcribed in free-living *S. meliloti* by assaying for GUS activity (Fig. 6A). As controls we also determined the GUS activity of the free-living parent strain and an *S. meliloti* Rm1021 strain containing a pJH104 insertion in the *bacA* gene [Rm1021 *bacA* : : pJH104t (BacA^{+})] (Fig. 6A). Transcription of the *bacA* gene was used as a positive control since we have shown previously that deletion of the *bacA* gene in *S.*

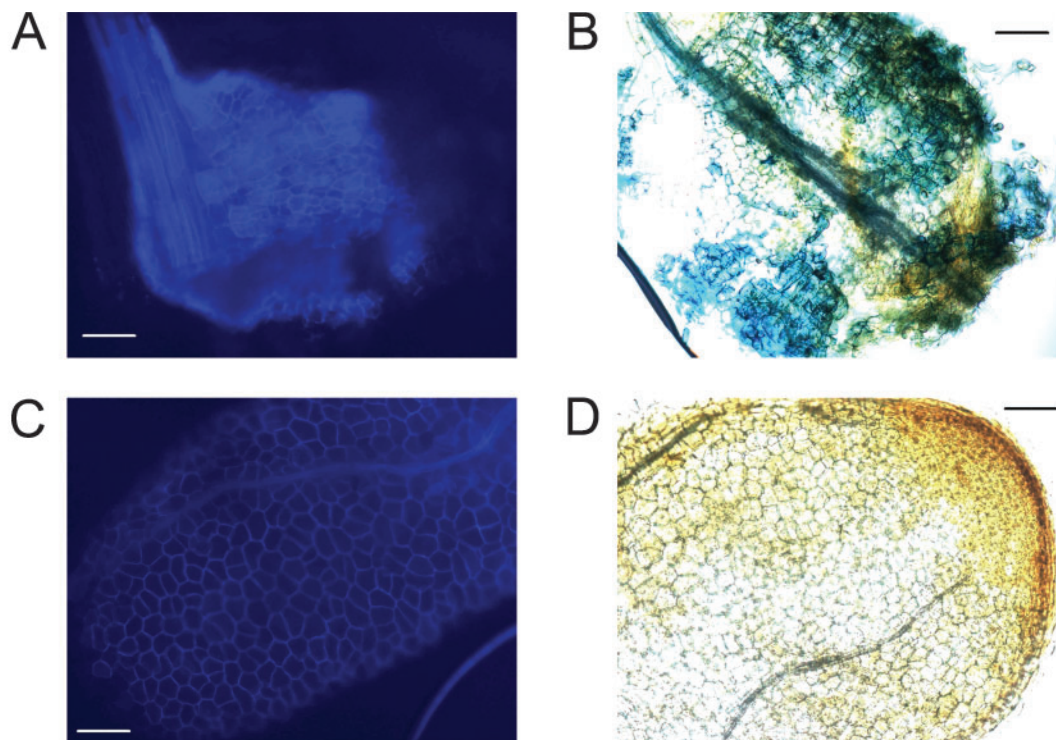

Fig. 5. The *S. meliloti msbA2* insertional mutant induces a heightened defence response within the nodules. Nodules were removed 3 weeks post-infection from alfalfa seedlings inoculated with either the *S. meliloti* Rm1021 *msbA2*::pJH104t mutant (A and B) or *S. meliloti* Rm1021 (C and D). The nodules were then thinly sliced and polyphenolic compounds were detected by either autofluorescence (A and C) or histochemical staining (B and D). Bars, 10 μm.

meliloti results in an LPS alteration in free-living *S. meliloti* (Ferguson *et al.*, 2002). Our GUS assay results provided evidence that the *msbA2* gene is being transcribed in free-living *S. meliloti*, although to a lower level than the *bacA* gene (Fig. 6A). Therefore, these findings suggest that MsbA2 is not essential for the transport of either LPS or phospholipids in *S. meliloti*.

To investigate whether disruption of the *msbA2* gene was having any effect upon the polysaccharide content of free-living *S. meliloti*, the polysaccharides from the parent and Rm1021 *msbA2*::pJH104t mutant strains were extracted using the hot water/phenol procedure, which separates polysaccharides according to their hydrophobicity. A previous study has shown that the ability to visualize polysaccharide alterations in bacterial mutants is often dependent upon the method used for extraction and analysis (Ridley *et al.*, 2000). The polysaccharides isolated into the aqueous and the phenol phases were then resolved by DOC-PAGE and treated with either periodate or alcian blue followed by silver staining. Using the periodate method, we determined that in the absence of MsbA2, a new high-molecular-mass polysaccharide band appeared in the phenol-phase-extracted material and there was also a reduction in the intensity of a lower molecular mass band (Fig. 6B). Differences were also observed in the phenol-phase-extracted material from the parent and *msbA2*::pJH104t mutant strain using the alcian blue method (Fig. 6C). In contrast, no reproducible differences were observed in the aqueous-phase-extracted material from the parent and *S. meliloti msbA2*::pJH104 mutant strains using either staining method (data not shown). However, these findings provide evidence that disruption of the *msbA2* gene in *S. meliloti* affects the polysaccharide content.

DISCUSSION

Our data showed that, despite the *S. meliloti* Rm1021 genome encoding multiple potential MsbA-like proteins (Fig. 1A), a sole disruption of the *msbA2* gene was sufficient to prevent *S. meliloti* Rm1021 from forming a successful symbiotic relationship with its plant host. We determined that in the absence of MsbA2, the *S. meliloti*–legume infection was aborted at the level of the infection threads and the bacterial cells were unable to be endocytosed into the host cells but instead remained within abnormally swollen infection threads. It appears that in the absence of MsbA2, *S. meliloti* induces a response in the plant host more characteristic of a pathogen, causing browning of the plant tissue, a substantial thickening of the plant endodermis, preventing bacterial entry into the plant cell, and a heightened production of polyphenolic defence compounds. Since similar heightened plant

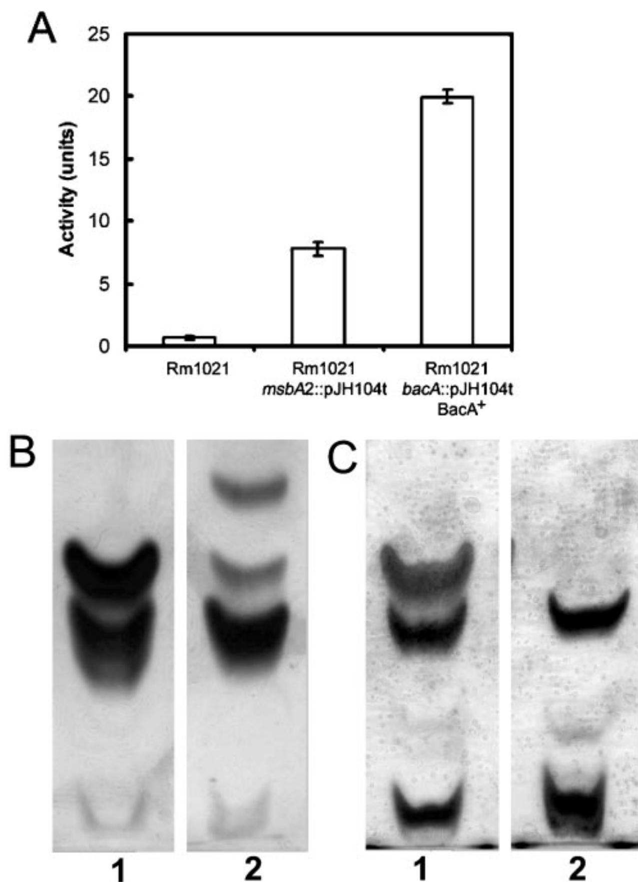

Fig. 6. The *msbA2* gene is transcribed in free-living *S. meliloti* and its disruption affects the polysaccharide profile. (A) The transcriptional activities of the *msbA2* and *bacA* genes were determined by measuring the GUS activity of the *S. meliloti* Rm1021 *msbA2*::pJH104t and *bacA*::pJH104t BacA$^+$ strains, respectively. (B) The phenol-phase polysaccharides from either *S. meliloti* Rm1021 parent strain (lane 1) or the *S. meliloti* Rm1021 *msbA2*::pJH104t mutant strain (lane 2) were resolved by DOC-PAGE and visualized by either periodate (B) or alcian blue (C) followed by silver staining. No differences were observed in the aqueous-phase polysaccharides (data not shown).

defence responses, accompanied by aborted infection threads, have also been observed during the infection of legumes with *S. meliloti* and *Rhizobium leguminosarum* mutants, which are known to have alterations in their polysaccharides (Niehaus *et al.*, 1993, 1998; Perotto *et al.*, 1994), our findings suggest that the polysaccharide alteration observed by PAGE analysis in the *S. meliloti msbA2* mutant could be responsible for at least some of the symbiotic defects of this mutant.

Since the *E. coli* MsbA protein affects the transport of phosphate-containing lipids such as LPS across the inner membrane, we initially investigated whether the *S. meliloti msbA2* mutant was also affected in the transport of phosphate-containing lipids. However, although the lipid A component of *S. meliloti* LPS is bisphosphorylated (Gudlavalleti & Forsberg, 2003; Sharypova *et al.*, 2003), we did not observe any differences in the membrane localization of $^{32}P_i$-containing lipids in the *S. meliloti msbA2* insertional mutant relative to the parent strain (data not shown), suggesting that MsbA2 is not essential for the transport of either phosphorylated LPS or phospholipids. Consistent with this, we did not observe any altered sensitivities of the *S. meliloti msbA2* mutant relative to the parent strain towards cell envelope disrupting agents usually associated with LPS mutants.

Although we determined that the MsbA2 protein is not essential for the transport of LPS and phospholipids in free-living *S. meliloti*, we cannot rule out at this stage that one or more of the other potential MsbA-like proteins in *S. meliloti* (Fig. 1A) could be masking a role for MsbA2 in the transport of LPS and/or phospholipids. With the exception of ExsA and NdvA, the functions of the other potential MsbA-like proteins in *S. meliloti* have not been extensively investigated. Our phylogenetic analysis showed that the MsbA1 and Y02836 proteins show the highest degree of similarity to the *E. coli* and *N. meningitidis* MsbA proteins (Fig. 1A). However, our preliminary analyses suggest that MsbA1 is not essential for the legume symbiosis and neither an *S. meliloti msbA1* nor an *msbA1*/*msbA2* double mutant was affected in the transport of phosphate-containing lipids (V. L. Marlow, A. Haag, S. Beck, W. T. Doerrler & G. P. Ferguson, unpublished data). However, the MsbA1 and Y02836 proteins are 91 % similar (84 % identical) to each other, suggesting that Y02836 may be able to compensate for loss of MsbA1. Consequently, future studies will focus on the creation and characterization of *S. meliloti* mutants with deletions in multiple *msbA*-like genes, so that their functions can be thoroughly elucidated. Additionally, since the MsbA1 and MsbA2 proteins are only 44 % similar (26 % identical), this suggests that the *msbA2* gene should be reannotated in future studies once the precise function of the MsbA2 protein is determined.

In *S. meliloti* Rm1021, succinoglycan is the symbiotically active form of EPS and is essential for infection thread formation (Niehaus *et al.*, 1998; Pellock *et al.*, 2000). Previous studies have shown that mutants affected in succinoglycan production often affect calcofluor fluorescence (Long *et al.*, 1988). However, we also did not observe any differences between the calcofluor fluorescence of the *S. meliloti* parent and *msbA2* insertional mutant strain, suggesting that succinoglycan could still be transported in the absence of MsbA2 (data not shown). Additionally, a previous study showed that the *S. meliloti exsA* gene forms part of the succinoglycan biosynthetic gene cluster and ExsA has been proposed to be involved in the transport of succinoglycan (Becker *et al.*, 1995). Consequently, further biochemical studies will be needed to determine the precise polysaccharide(s) affected in the *S. meliloti msbA2* mutant. However, since the compositional and structural analyses of *S. meliloti* polysaccharides to date

have focused on the aqueous-phase-extracted material (Ferguson *et al.*, 2002, 2004, 2005; Gudlavalleti & Forsberg, 2003; Sharypova *et al.*, 2003), extensive future studies will be necessary to enable the characterization of the phenol-phase polysaccharides from both the parent and the *msbA2* mutant strains. Since the *msbA2* gene appears to be closely associated with two glycosyltransferase genes and an acyltransferase gene (Fig. 1B), it is interesting to speculate that these genes form an operon and that the MsbA2 protein is involved in the transport of a novel as-yet-unidentified polysaccharide. Therefore, the polysaccharide alteration we observe in the absence of MsbA2 could be due to the intracellular accumulation of this species or to compensatory changes in the *S. meliloti* polysaccharide content resulting from the absence of this polysaccharide species. Interestingly, non-polar single deletion mutants in the upstream genes do not appear to affect the legume symbiosis (J. Griffitts & S. Long, unpublished), suggesting that either the novel polysaccharide is not essential for the legume symbiosis or another glycosyltransferase or acyltransferase encoded in the *S. meliloti* genome (Galibert *et al.*, 2001) can substitute for their function. Additionally, since the importance of the *msbA2* gene in the *S. meliloti* Rm1021–legume symbiosis was not identified in a previous study (Charles & Finan, 1991), which analysed large deletion mutants of pSymB (120–600 kb deletions), this suggests that the host defect observed in our *msbA2* insertional mutant could be due to the cytoplasmic accumulation of the novel polysaccharide, encoded by the upstream genes, which somehow interferes with the legume interaction. Thus, future studies investigating defined combinations of mutants in this region will be necessary to rule out this possibility. Additionally, since the Rm1021 strain background used in this study contains an insertion sequence in the *expR* gene, which is required for the production of symbiotically active EPS II (Pellock *et al.*, 2001), further studies will be necessary to determine whether disruption of the *msbA2* gene will result in the same symbiotic phenotypes in an *S. meliloti* Rm8530 strain, which has an intact *expR* gene.

If the altered polysaccharide observed in the *S. meliloti msbA2* mutant is responsible for the symbiotic defect, the question remains as to how this polysaccharide alteration could affect the host interaction. It has been suggested previously that *S. meliloti* polysaccharides are essential to suppress the plant defence response and enable proper infection thread development (Niehaus *et al.*, 1993, 1998; Perotto *et al.*, 1994). Consequently, since we observed that the *S. meliloti msbA2* mutant produced an elevated plant defence, the altered polysaccharides in this mutant may be less effective at suppressing the defence response and the plant would perceive this mutant as a pathogen rather than a symbiont. Interestingly, wild-type *S. meliloti* induce a heightened plant defence response and abnormal infection threads when inoculated onto a *nip* (numerous infections and polyphenolics) mutant of the legume *Medicago truncatula* (Veereshlingam *et al.*, 2004), suggesting that there could be some interplay between the *nip* locus in the host plant and bacterial-produced polysaccharides in suppressing the plant defence response. Polysaccharides are also thought to play a critical role in the interaction of *Brucella* species with their host. For example, cyclic glucans are proposed to be necessary for the intracellular trafficking of *Brucella* species within their hosts (Arellano-Reynoso *et al.*, 2005). Additionally, a *B. abortus bacA* mutant is defective in chronic infection (LeVier *et al.*, 2000), has an altered LPS (Ferguson *et al.*, 2004) and induces a greater amount of pro-inflammatory cytokines than the parent strain (Parent *et al.*, 2007), suggesting that suppression or avoidance of host immune responses by wild-type LPS is necessary for *B. abortus* to form a chronic infection.

In summary, these studies have shown that the MsbA2 protein is essential for *S. meliloti* to form a symbiosis with legumes and suggest that it may play a role in the transport of a lipid-linked polysaccharide.

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