REVIEW ARTICLE

Molecular insights into bacteroid development during *Rhizobium*–legume symbiosis

Andreas F. Haag[1], Markus F. F. Arnold[1], Kamila K. Myka[1], Bernhard Kerscher[1], Sergio Dall'Angelo[2], Matteo Zanda[2,3], Peter Mergaert[4] & Gail P. Ferguson[1,*]

[1]School of Medicine & Dentistry, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK; [2]School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK; [3]C.N.R.-Istituto di Chimica del Riconoscimento Molecolare, Milano, Italy; and [4]Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Gif-sur-Yvette Cedex, France

Correspondence: Andreas F. Haag, Novartis Vaccines & Diagnostics, Via Fiorentina 1, 53100 Siena (SI), Italy. Tel.: +39 0577 245343; fax: +39 0577 278600; e-mail: a.haag@live.co.uk

*Deceased.

Received 1 June 2012; revised 12 September 2012; accepted 14 September 2012. Final version published online 19 October 2012.

DOI: 10.1111/1574-6976.2012.12003

Editor: Hauke Hennecke

Keywords
Rhizobium–legume symbiosis; antimicrobial peptides; nodule-specific; cysteine-rich peptides; bacteroid differentiation; BacA; lipopolysaccharide.

Abstract

Rhizobial soil bacteria can form a symbiosis with legumes in which the bacteria fix atmospheric nitrogen into ammonia that can be utilized by the host. The plant, in turn, supplies the rhizobia with a carbon source. After infecting the host cell, the bacteria differentiate into a distinct bacteroid form, which is able to fix nitrogen. The bacterial BacA protein is essential for bacteroid differentiation in legumes producing nodule-specific cysteine-rich peptides (NCRs), which induce the terminal differentiation of the bacteria into bacteroids. NCRs are antimicrobial peptides similar to mammalian defensins, which are important for the eukaryotic response to invading pathogens. The BacA protein is essential for rhizobia to survive the NCR peptide challenge. Similarities in the lifestyle of intracellular pathogenic bacteria suggest that host factors might also be important for inducing chronic infections associated with *Brucella abortus* and *Mycobacterium tuberculosis*. Moreover, rhizobial lipopolysaccharide is modified with an unusual fatty acid, which plays an important role in protecting the bacteria from environmental stresses. Mutants defective in the biosynthesis of this fatty acid display bacteroid development defects within the nodule. In this review, we will focus on these key components, which affect rhizobial bacteroid development and survival.

Symbiotic nitrogen fixation by legumes

Nitrogen is essential for the growth of all organisms and although nitrogen gas (N_2) is the most abundant gas in Earth's atmosphere, this molecular form cannot be used by most organisms. Therefore, these organisms depend on the presence of fixed nitrogen for their nutritional needs. For example, nitrogen availability limits plant growth and crop yields and vast resources are spent on the production of nitrogen fertilizers, leading to a considerable carbon footprint of this industrial sector (Jensen *et al.*, 2012). Only few bacteria and archea are capable of fixing N_2 into ammonia that can be assimilated. Nevertheless, certain plants establish symbiotic interactions with soil-dwelling nitrogen-fixing bacteria and use N_2 for growth. One such class of plants are legumes, which interact with Gram-negative bacteria called rhizobia that belong either to the *Alphaproteobacteria* or *Betaproteobacteria* (α- and β-rhizobia, respectively). Based on this nitrogen-fixing symbiosis, legume crops require 35–60% less fossil-based energy than conventional, N-fertilized crops (Jensen *et al.*, 2012). On a global scale, biological nitrogen fixation in the legume–rhizobia symbiosis accounts for roughly 200 million tons of fixed nitrogen per year (Ferguson *et al.*, 2010). Therefore, legumes are agriculturally and ecologically very important and account for 25% of the world's primary crop production (Ferguson *et al.*, 2010). Peas and beans are important for human nutrition and soybean, clover and alfalfa provide valuable sources of animal feed, while agricultural practice also uses legumes in crop rotation to enrich the soil for bioavailable nitrogen and to ensure optimum growth of the following, non-leguminous crops.

Rhizobia trigger the formation of a new plant organ (the nodule) on roots and/or stems of leguminous

FEMS MICROBIOLOGY REVIEWS

plants by an intricate cross-communication between the two symbiotic partners (for comprehensive reviews see Jones *et al.*, 2007; Gibson *et al.*, 2008). Nodules are specialized to house intracellular nitrogen-fixing rhizobia and to make an efficient metabolic exchange possible between the symbiotic partners: in return for nitrogen provided to the plant, the symbiotic bacteria are protected from environmental stresses and are supplied with a carbon source within the plant cells (Halverson & Stacey, 1986; Poole *et al.*, 1994). Nitrogen fixation by legumes has been a major focus in research and our knowledge of how bacteria and plants can interact has advanced considerably. We focus here on the current advances in understanding the settlement of rhizobia within the nodule host cell.

Establishing the *Rhizobium*–legume symbiosis: formation and infection of nodules

Legumes secrete flavonoids (Fig. 1a), which are specifically recognized by a bacterial receptor, NodD, that in turn induces expression of the bacterial nodulation (*nod*) genes. These *nod* genes encode enzymes involved in the production of lipochito-oligosaccharides, the Nod factors (Fig. 1a), which are essential for the signalling of nodule development (Perret *et al.*, 2000; Jones *et al.*, 2007). The basic structure of Nod factors is conserved among rhizobia and consists of a chitin backbone with two to six β-1,4-linked *N*-acetyl-D-glucosamine residues to which an *N*-linked fatty acid is attached to the terminal nonreducing sugar (Cooper, 2007; Oldroyd & Downie, 2008). The substituent groups as well as the length and degree of saturation of the fatty acid moiety of Nod factors varies between rhizobia and is a key determinant of bacterial host specificity (Cooper, 2007; Oldroyd & Downie, 2008). Nod factor is in turn recognized by plant Nod factor receptor-like kinases of the LysM family in the epidermal root hair cells. Nod factor exposure triggers a calcium-dependent signal transduction pathway (Oldroyd & Downie, 2008). Very shortly after Nod factor recognition by the receptors, oscillations in the calcium concentrations in the nucleoplasm and nuclear-associated cytoplasm are induced, termed calcium spiking which in turn induces the expression of specific genes termed early nodulins (*ENODs*) (Oldroyd & Downie, 2008).

In parallel to the nuclear calcium spiking pathway, the Nod factors trigger a Ca^{2+} influx at the root hair tip causing an increase in Ca^{2+} levels in the cytosol (Felle *et al.*, 1999), a transient depolarization of the plasma membrane and the reorganization of actin and microtubule filaments in root hair cells (Sieberer & Emons, 2000; Sieberer *et al.*, 2002). This probably induces the developmental changes in root hair cells, resulting in the formation of root hair curls, the so-called Shepherd's crooks (Fig. 1b). The rhizobia are initially trapped within the curls and proliferate resulting in elevated Nod factor concentrations, which are thought to be required to reach a Nod factor threshold concentration. The Nod factors cause extensive remodelling of the root hair cells, which results in inward growth of the root hair tip and formation of a tubular structure known as an infection thread (Fig. 1b) (Brewin, 2004; Oldroyd & Downie, 2008). Infection threads display polar growth with new cell wall and membrane material being synthesized at their tip. The infection threads are filled with rhizobia which appear to be only actively dividing at the tip of the thread, as was shown with fluorescently labelled *Sinorhizobium meliloti* bacteria in alfalfa (Gage *et al.*, 1996; Gage, 2002). The formation and extension of the infection thread is dependent upon bacterial polysaccharides (Jones *et al.*, 2007). In *S. meliloti*, succinoglycan (EPS I) is the most important polysaccharide for infection thread initiation and elongation (Brewin, 2004). An *exoY* mutant, which is defective in the succinoglycan biosynthesis pathway, can induce root hair curling but is incapable of initiating infection threads (Cheng & Walker, 1998). Conversely, an *exoH* mutant, which produces succinoglycan missing a succinyl substituent, induces the formation of abortive and aberrant infection threads (Cheng & Walker, 1998). Like the succinoglycan *exoH* mutant, mutations in genes (*nodF* and *nodL*) that are necessary for the biosynthesis of the correctly decorated Nod factor result in aberrant infection threads (Ardourel *et al.*, 1994; Bisseling *et al.*, 2003). The formation of infection threads is not regulated by bacterial products alone. Several mutants in *Medicago truncatula* form abortive infection threads similar to *S. meliloti* mutants. The bacteria on an *M. truncatula lin* mutant are trapped in the root hair curls and, like the *S. meliloti exoY* mutant, do not form infection threads (Kuppusamy *et al.*, 2004). RNAi knockdown of the *M. truncatula* LysM Nod factor receptors also results in aberrant infection threads showing that the host's perception of rhizobia is important for correct formation of the infection threads (Brogden, 2005; Arrighi *et al.*, 2006).

Simultaneously with these root hair responses, mitotic activity is triggered in the underlying cortical cells, giving rise to a nodule primordium which will grow into a developing nodule (Perret *et al.*, 2000; Oldroyd & Downie, 2008). How the epidermal and cortical responses are coordinated remains largely unknown but it has been established that auxin and cytokinin phytohormone signalling is a central component in the mechanism (Oldroyd & Downie, 2008). Once the infection thread has passed through the root hair and has reached the cortical

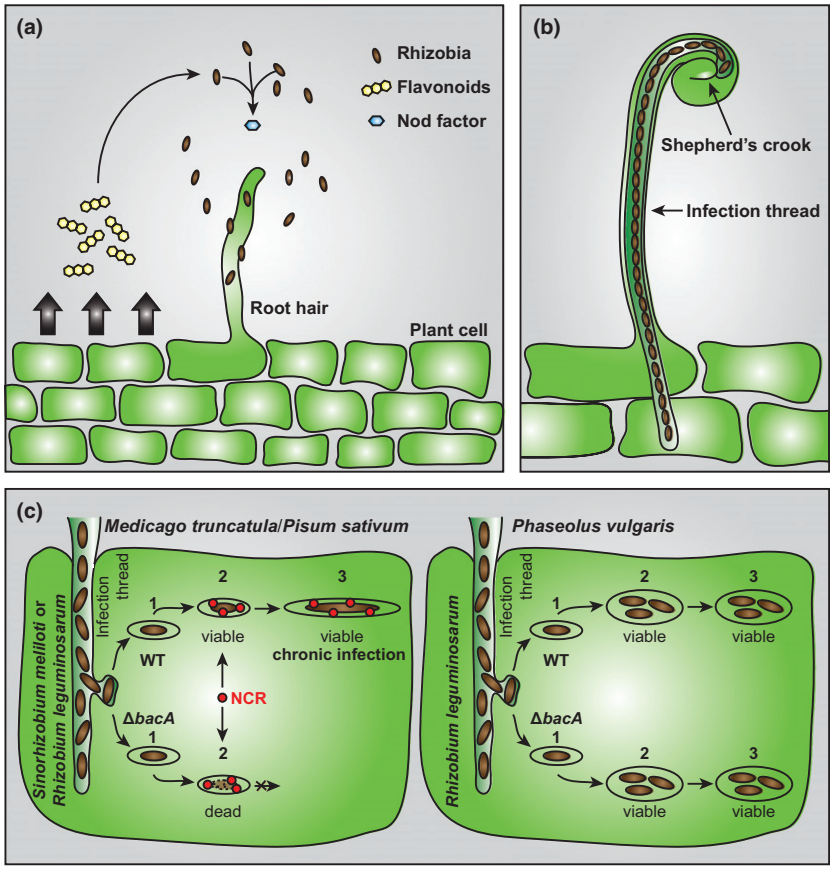

Fig. 1. Rhizobia interacting with legumes. (a) The legume secretes flavonoids which induce the rhizobia to produce Nod factors and attract them to the plant root hair cells. (b) Nod-factor signalling triggers a number of developmental changes, including root hair curling which traps the rhizobia in Shepherd's crooks. Inward growth of the root hair tip results in tubular structures called infection threads, which allow the rhizobia to enter the cortical cell layers of the plant root. (c) The rhizobia escape the infection thread and are taken into the host cell via an endocytosis-like process (1), which encompasses them in a host-derived membrane. These intracellular compartments are known as symbiosomes. In legumes of the IRLC clade such as *Medicago truncatula* and *Pisum sativum*, the rhizobia are challenged with NCR peptides (2) and differentiate into elongated bacteroids (3). The bacterial BacA protein is essential for protecting the rhizobia against the antimicrobial activity of NCR peptides (2). In contrast, BacA is dispensable for rhizobia infecting legumes of the phaseoloid clade that do not produce NCR peptides. In these host plants, rhizobia do not differentiate terminally and often multiple bacteroids can be found inside a single symbiosome membrane. WT, wild-type.

root cells, it ramifies and the resulting infection thread network grows through the cortical cells towards the growing nodule primordium (Perret *et al.*, 2000; Gage, 2004). For the initiation of infection threads in root hairs, the cell-to-cell passage of infection threads and the release of rhizobia into target host cells, plant cell walls have to be breached. This requires local degradation of these cell walls by pectin- and cellulose-degrading enzymes which can be produced by the infecting rhizobia (Mateos *et al.*, 2001; Robledo *et al.*, 2008, 2011) or by the host after local induction by rhizobium-produced Nod factors (Xie *et al.*, 2012).

When infection threads reach target host cells in the growing nodule primordium, bacteria are able to enter the host cell through structures termed infection droplets, which are unwalled outgrowths of the infection thread (Brewin, 2004). The bacteria are encompassed by the plasma membrane in an endocytosis-like process, giving rise to symbiosome compartments (Fig. 1c) (Brewin, 2004). The membrane separating the bacteria from the cytosol of the plant cell is termed the peribacteroid membrane (PBM) (Perret *et al.*, 2000). Although it retains aspects of the plasma membrane, the PBM undergoes a remodelling process through its interaction with the bacterium as it serves as the major player in the bacterial–plant interchange of nutrients (Robertson & Lyttleton, 1984; Udvardi & Day, 1997; Bolaños *et al.*, 2004; Limpens *et al.*, 2009). Within symbiosomes, bacteria differentiate into nitrogen-fixing bacteroids that are dependent on a constant energy and carbon supply from the host plant (Jones *et al.*, 2007; Gibson *et al.*, 2008).

Differences between free-living rhizobia and bacteroids

To fix nitrogen, the plant needs to provide a low-oxygen environment as bacterial nitrogenase is highly sensitive to oxygen, which irreversibly inactivates the enzyme. The legume host maintains a microaerophilic environment via a number of mechanisms. Firstly, the legume controls the permeability of the nodule cells to oxygen by an oxygen diffusion barrier, thereby allowing the plant to react to the available oxygen within the rhizosphere environment (Ott *et al.*, 2005; Wei & Layzell, 2006; Gibson *et al.*, 2008). Secondly, the microaerophilic environment necessary for nitrogen fixation is controlled by high-level expression of leghaemoglobin in the infected nodule cells, which reduces the free-oxygen concentration in the nitrogen-fixing zone of the plant nodule to 3–22 nM and is responsible for the pink colour of the plant root nodule (Ott *et al.*, 2005; Gibson *et al.*, 2008). Thus, the bacterial genes involved in nitrogen fixation are controlled by an oxygen-sensing two-component regulatory system, FixJ–FixL (Gong *et al.*, 2006), which regulates expression of the *nifA* gene encoding the master regulator of nitrogen fixation (Dixon & Kahn, 2004; Gong *et al.*, 2006).

The symbiosis between rhizobia and plants is a partnership resulting in the exchange of nutrients between the host cell and the contained bacteroids. To facilitate this process, the bacterial cell envelope and the PBM undergo massive remodelling (Robertson & Lyttleton, 1984; Whitehead & Day, 1997). Ammonia is delivered to the plant through ammonia channels in the plant membrane and to a smaller extent in the form of alanine (White *et al.*, 2007). In addition, many other metabolite transporters located on peribacteroid and bacterial membranes are required for the extensive nutrient exchange between the bacteroids and their host cells (White *et al.*, 2007). Interestingly, by examining the expression levels of branched-chain amino acid biosynthesis genes in bacteroids and cultures of *Rhizobium leguminosarum* bv. *viciae*, it was determined that the synthesis of branched chain amino acids was downregulated within the bacteroids to below the level required for efficient nitrogen fixation (Prell *et al.*, 2009). Thus, *R. leguminosarum* bacteroids become auxotrophic for the branched-chain amino acids leucine, isoleucine and valine (Prell *et al.*, 2009). This downregulation of branched-chain amino acid synthesis is conserved in *S. meliloti* (Barnett *et al.*, 2004; Capela *et al.*, 2006) and *Bradyrhizobium japonicum* bacteroids (Pessi *et al.*, 2007) in alfalfa and soybean, respectively. Three possible, non-exclusive explanations for the downregulation of branched-chain amino acid biosynthesis have been offered (Prell *et al.*, 2009): firstly, it might suggest that this is a conserved mechanism used by the plant to control the degree of bacterial infection, thereby preventing potentially detrimental, 'over-populated' nodule cells. Secondly, it might be energetically favourable for the plant to supply branched-chain amino acids to the bacteroids resulting in increased nitrogen fixation relative to carbon input. And thirdly, it was proposed that metabolic precursors of branched-chain amino acid biosynthesis could become limited due to bacteroid metabolism and therefore bacteroids have to be supplied with these amino acids by the plant host. However, metabolic changes are not only limited to the biosynthesis of branched-chain amino acids. In fact, many metabolic processes required for the conversion of higher carbon sources are downregulated and the bacteroids depend on the supply of dicarboxylic acids by the plant to obtain the energy required for nitrogen fixation (Lodwig *et al.*, 2003). Biological nitrogen fixation is a very energy-intensive process, which (dependent on the organism) consumes between 16 and 42 molecules of ATP for every two molecules of NH_3 synthesized (O'Brian, 1996). Downregulation of gene expression in bacteroids includes many bacterial genes required for cell growth and division, synthesis of ribosomal proteins, DNA repair and membrane protein biosynthesis (Barnett *et al.*, 2004; Becker *et al.*, 2004; Capela *et al.*, 2006; Karunakaran *et al.*, 2009). Moreover, under free-living conditions, bacteria regulate the amount of intracellular ammonia through a nitrogen-stress response regulatory system. However, this control is disabled in bacteroids to ensure the production of the high levels of ammonia required by the host plant (Yurgel & Kahn, 2008).

Depending on the host plant, changes in rhizobial metabolism can also be accompanied by changes in rhizobial cell size and shape. One can distinguish three bacteroid types (Vasse *et al.*, 1990; Mergaert *et al.*, 2006; Bonaldi *et al.*, 2011; Haag *et al.*, 2011b). The first bacteroid type develops in legumes of the Inverted Repeat-Lacking Clade (IRLC) (consisting of legumes such as *Medicago*, *Pisum*, *Vicia*, *Trifolium*, *Galega* and *Astragalus*). Infecting rhizobia undergo repeated rounds of genome amplification, increase their cell size by elongation and can even be branched (Fig. 1c) (Bisseling *et al.*, 1977; Paau *et al.*, 1979; Kobayashi *et al.*, 2001; Mergaert *et al.*, 2006). Bacteroids that undergo this type of metamorphosis are terminally differentiated and are no longer viable once extracted from the nodule (Mergaert *et al.*, 2006). The second type of bacteroids can be found in legumes such as those of the Dalbergoid clade (*Aeschynomene* and *Arachis* species). Bacteroids are enlarged by either elongation as for example in *Aeschynomene afraspera* or by forming large spheres as in *Arachis*, *Aeschynomene indica* or *Aeschynomene evenia* (Sen & Weaver, 1984; Bonaldi *et al.*, 2011). In contrast, the third type of bacteroids is

found in rhizobia that infect phaseoloid legumes (i.e. *Phaseolus*, *Vignia*, *Lotus* and *Glycine* species). In this case, rhizobia do not undergo terminal differentiation and retain a cell shape and size and DNA content similar to free-living bacteria (Fig. 1c) (Bisseling *et al.*, 1977; Paau *et al.*, 1979; Mergaert *et al.*, 2006). Because *Rhizobium* strains that can nodulate legumes of different clades adopt a bacteroid morphotype according to the host, it was concluded that bacteroid metamorphosis was induced by host factors rather than being encoded in the bacterial genome (Sen & Weaver, 1984; Mergaert *et al.*, 2006; Bonaldi *et al.*, 2011).

IRLC legume nodule-specific cysteine-rich peptides and bacteroid development

The availability of genome sequences and transcriptomes of legumes during the last decade has shed light onto the mechanisms of bacteroid differentiation during infection of *Medicago*. A class of nearly 600 genes encoding nodule-specific cysteine-rich peptides (NCRs) has been discovered and the expression of more than 300 NCRs confirmed (Mergaert *et al.*, 2003; Graham *et al.*, 2004; Young *et al.*, 2011). These NCRs have very little sequence conservation but share four or six conserved cysteine residues per peptide (Mergaert *et al.*, 2003). In their immature form, NCRs contain an N-terminal signalling peptide, which is critical for targeting NCRs to the symbiosome compartment in the host (Mergaert *et al.*, 2003; Van De Velde *et al.*, 2010). NCRs form a unique class of peptides which are characterized by the pattern of conserved cysteines and their distribution is restricted to the IRLC legumes (Scheres *et al.*, 1990; Frühling *et al.*, 2000; Jimenez-Zurdo *et al.*, 2000; Crockard *et al.*, 2002; Kaijalainen *et al.*, 2002; Kato *et al.*, 2002; Mergaert *et al.*, 2003; Graham *et al.*, 2004; Alunni *et al.*, 2007; Young *et al.*, 2011). Nevertheless, based on criteria of peptide structure, gene organization and family structure, NCRs clearly resemble defensins which are antimicrobial peptides that are part of the innate immune system in animals and plants (Maróti *et al.*, 2011). More generally, NCRs are part of the superfamily of cysteine-rich peptides (CRPs) found in all kingdoms of life. These are in general short peptides, usually no longer than 160 amino acids, contain an N-terminal secretion signal and are stabilized by disulphide bond formation between intrapeptide cysteine residues (Marshall *et al.*, 2011).

The NCR genes in *M. truncatula* as well as those of the other IRLC legumes are specifically and solely expressed in nodules (Scheres *et al.*, 1990; Frühling *et al.*, 2000; Jimenez-Zurdo *et al.*, 2000; Crockard *et al.*, 2002; Kaijalainen *et al.*, 2002; Kato *et al.*, 2002; Mergaert *et al.*, 2003; Graham *et al.*, 2004; Alunni *et al.*, 2007; Young *et al.*, 2011). Clustering NCR genes based on the distribution of expressed sequence tags and on microarray transcriptome analysis revealed that subsets of NCR genes have distinct spatiotemporal expression profiles (Mergaert *et al.*, 2003; Maunoury *et al.*, 2010). Those genes that were analysed *in situ*, with promoter–GUS fusions or *in situ* hybridization, were always found to be expressed in the infected, bacteroid-containing nodule cells with subsets of genes expressed in either young infected cells or in mature symbiotic cells (Mergaert *et al.*, 2003; Van De Velde *et al.*, 2010).

NCR peptides contain an N-terminal hydrophobic signal peptide and experiments in which a green fluorescent protein-tagged NCR peptide was expressed transiently in onion cells confirmed that NCR peptides were indeed targeted to the plant cell secretory pathway (Mergaert *et al.*, 2003). Purified nodule peptide fractions in combination with Western blotting and NCR-specific antibodies revealed that NCR peptides co-purified with bacteroids (Van De Velde *et al.*, 2010). Expression of NCRs in infected nodule cells and localization within the symbiosome compartments was further confirmed by immunolocalization and NCR peptide fusions with fluorescent proteins (Van De Velde *et al.*, 2010). This revealed that the NCR peptides were found within the bacteroid and did not solely associate with the symbiosome and bacteroid membranes. The *M. truncatula dnf1* mutant is defective in a nodule-specific signal peptidase complex involved in cleavage of the signal peptide in the endoplasmic reticulum (Wang *et al.*, 2010). In this mutant, trafficking of NCR peptides to the symbiosome compartments was blocked and this was correlated with the absence of bacteroid differentiation in these symbiosomes (Van De Velde *et al.*, 2010). It was further shown that expressing NCR genes in a legume such as *Lotus japonicus*, naturally lacking NCR genes and elongated bacteroid differentiation, resulted in the formation of elongated bacteroids. Moreover, treatment of *S. meliloti* cultures with NCR peptides *in vitro* could induce features of differentiated bacteroids such as increased DNA content and cell elongation relative to untreated control cultures (Van De Velde *et al.*, 2010). In agreement with this a fluorescein isothiocyanate (FITC)-labelled NCR peptide was found to accumulate at the bacterial division plane, suggesting that it may interfere with the bacterial division machinery. It was also shown that some but not all NCR peptides were inducing membrane damage and permeabilization, which could lead either directly to bacterial cell death or enable the NCR peptides to reach other periplasmic and/or intracellular targets (Van De Velde *et al.*, 2010). These data have added substantial evidence showing that NCR peptides are indeed a critical driving force of bacteroid differentiation.

Despite its widespread occurrence in the legume family and the considerable effort put into the process by the host plant, it remains unknown why the plant host induces terminal bacteroid differentiation. However, recent data suggest that terminally differentiated bacteroids are more efficient at fixing nitrogen than nonterminally differentiated bacteroids (Sen & Weaver, 1981; Oono & Denison, 2010; Oono *et al.*, 2010).

The rhizobial BacA protein is critical for bacteroid development in IRLC legumes

The *S. meliloti bacA* gene (bacteroid development factor A) was identified to be essential in the *S. meliloti–Medicago* symbiosis and was discovered from a Tn*phoA* transposon mutagenesis screen (Long *et al.*, 1988). An *S. meliloti bacA* mutant is unable to support nitrogen-fixing symbiosis although it is not impaired in nodule formation, infection thread development or host cell entry. Instead of differentiating into nitrogen-fixing bacteroids, the bacteria are rapidly killed after their release from infection droplets (Fig. 1c) (Glazebrook *et al.*, 1993). The BacA protein is predicted to be located in the inner membrane and is thought to form the transmembrane domain of an ATP-binding cassette (ABC) transporter system (LeVier & Walker, 2001). BacA proteins are conserved and widespread in bacteria and the homologues of *Brucella abortus* and *Escherichia coli* (named SbmA) were demonstrated to be functionally equivalent and able to complement the symbiotic defect of the *S. meliloti* mutant (Ichige & Walker, 1997; Wehmeier *et al.*, 2010).

Sinorhizobium meliloti bacA and *E. coli sbmA* mutants have an increased resistance towards several classes of antimicrobial peptides with intracellular targets. *E. coli* SbmA sensitises the bacterium to the prokaryotic antimicrobial peptides microcins B17 and J25 (Lavina *et al.*, 1986; Salomon & Farias, 1995) and both *Escherichia coli* SbmA and *S. meliloti* BacA sensitize the bacteria towards the glycopeptide antibiotic bleomycin and a bovine, proline- and arginine-rich peptide called Bac7 (Yorgey *et al.*, 1994; Ichige & Walker, 1997; Ferguson *et al.*, 2002; Benincasa *et al.*, 2004, 2009; Podda *et al.*, 2006; Mattiuzzo *et al.*, 2007; Scocchi *et al.*, 2008; Marlow *et al.*, 2009; Wehmeier *et al.*, 2010). These observations led to the hypothesis that BacA/SbmA might be involved in the uptake of these antimicrobial peptides, which was recently confirmed to be the case in both *S. meliloti* and *E. coli* (Mattiuzzo *et al.*, 2007; Wehmeier *et al.*, 2010). It was later confirmed that uptake of the Bac7 peptide in a BacA-dependent manner was also conserved in *R. leguminosarum* and *Rhizobium etli*, two rhizobia infecting *Pisum sativum* and *Phaseolus vulgaris*, respectively (Karunakaran *et al.*, 2010). However, it remains to be confirmed whether the BacA-dependent internalization of Bac7 involves a direct or indirect transport mechanism. While Bac7 is produced by bovine neutrophils (Scocchi *et al.*, 1994; Benincasa *et al.*, 2004) and therefore would not be encountered by *S. meliloti in planta*, these observations led to the proposal that BacA might be required for the uptake of the plant-derived NCR peptide(s) which control bacteroid differentiation and survival within the plant cell of the IRLC clade of legumes (Marlow *et al.*, 2009).

There is a range of experimental evidence that BacA proteins function as ABC transporters. The *S. meliloti* BacA protein was proposed to be the membrane spanning domain of an ABC transporter complex located in the inner membrane of the cell envelope (LeVier & Walker, 2001). ABC transporters require a functional ATPase domain and often use substrate binding proteins (SBPs) in the case of importers (Davidson & Chen, 2004). The required proteins to form complete ABC transporter complexes usually are transcribed together or are closely located together within the genome of bacteria. *Mycobacterium tuberculosis* BacA was not found to be cotranscribed with any other genes (Vallecillo & Espitia, 2009). This suggests that BacA is either functioning as an exporter which does not require a SBP or, if BacA is an importer, it functions without SBP or the SBP might be expressed somewhere else in the genome and associates with BacA to form a complete ABC transporter. Interestingly, the *S. meliloti bacA* gene does not contain an encoded ATPase domain, suggesting that BacA might associate with an orphan ATPase found in the *S. meliloti* genome.

In addition, over the last 2 years, increasing evidence has been presented that BacA was only required in rhizobia infecting legumes of the IRLC clade that make NCR peptides, but not in phaseoloid legumes that are devoid of NCR peptides (Table 1). These data are in agreement with BacA having an important role in the response of rhizobia towards NCR peptide challenge. We were able to investigate a potential role of BacA in the protection against NCR peptides, by synthesizing several specifically folded versions of NCR247 (Haag *et al.*, 2011b, \2012), a peptide that had been shown to have antimicrobial activity in previous studies (Van De Velde *et al.*, 2010). Using *in vitro* viability assays, an *S. meliloti bacA* mutant was identified to be hypersensitive towards the antimicrobial activity of the folded NCR247 relative to the wild-type strain (Haag *et al.*, 2011b). However, no difference was observed between the ability of sublethal doses of NCR247 to induce bacteroid-like features *in vitro* between the wild-type strain and its *bacA* mutant indicating that the BacA protein itself was not directly involved in the terminal differentiation of the bacteria but rather

Table 1. Correlation between NCR gene expression in nodules, requirement of BacA and bacteroid type

Legume species	Reference(s)	*Rhizobium* species	BacA*	Reference
Legumes without NCR peptides and reversibly differentiated bacteroids†				
Phaseolus vulgaris (bean)	http://compbio.dfci.harvard.edu/tgi/	*Rhizobium leguminosarum* bv. *phaseoli*	No	Karunakaran *et al.* (2010)
		Rhizobium etli	No	Karunakaran *et al.* (2010)
Vigna unguiculata (cowpea)	http://compbio.dfci.harvard.edu/tgi/	*Sinorhizobium* NGR234	No	Ardissone *et al.* (2011)
Lotus japonicus	Alunni *et al.* (2007)	*Mesorhizobium loti*	No	Maruya & Saeki (2010)
Glycine max (soybean)	Alunni *et al.* (2007)	*Bradyrhizobium japonicum*	ND	
Legumes with NCR peptides‡ and terminally differentiated bacteroid†				
Medicago truncatula	Mergaert *et al.* (2003), Graham *et al.* (2004)	*Sinorhizobium meliloti* Sm1021	Yes	Maunoury *et al.* (2010)
Medicago sativa (alfalfa)	Jimenez-Zurdo *et al.* (2000)	*Sinorhizobium meliloti* Sm1021	Yes	Glazebrook *et al.* (1993)
Pisum sativum (pea)	Scheres *et al.* (1990), Kato *et al.* (2002)	*Rhizobium leguminosarum* bv. *viciae*	Yes	Karunakaran *et al.* (2010)
Astragalus sinicus	Chou *et al.* (2006)	*Mesorhizobium huakuii*	Yes	Tan *et al.* (2009)
Trifolium repens (white clover)	Crockard *et al.* (2002)	*Rhizobium leguminosarum* bv. *trifolii*	ND	
Galega orientalis (goat's rue)	Kaijalainen *et al.* (2002)	*Rhizobium galegae*	ND	
Vicia faba (broad bean)	Frühling *et al.* (2000)	*Rhizobium leguminosarum* bv. *viciae*	ND	

ND, no data available.

*Requirement of a functional *bacA* gene for efficient, nitrogen-fixing symbiosis.

†Mergaert *et al.* (2006) and data not shown.

‡Demonstrated presence of NCR gene expression in nodules.

provided protection from NCR247-induced membrane permeabilization, which might be crucial in ensuring viable and metabolically functional bacteroids within the nodule (Haag *et al.*, 2011b). *In vivo*, an *S. meliloti bacA* mutant was shown to be challenged with NCR peptides although a significant amount of the total NCR peptide present was excreted from the nodule cell. This is thought to occur due to the lack of a sufficient number of *S. meliloti bacA* mutant bacteria being present in the infected cells relative to wild-type infected nodules. Excess NCR peptides would therefore not have a target within the nodule cell and as a result are excreted (Haag *et al.*, 2011b). In agreement with the hypersensitivity of the *S. meliloti bacA* mutant towards NCR247 *in vitro*, it was determined that *bacA* mutant bacteria are rapidly killed once they enter into the *M. truncatula* nodule cell, while the wild-type strain bacteria remained viable and differentiated into bacteroids (Fig. 1c) (Haag *et al.*, 2011b). Consistent with this, the *S. meliloti bacA* mutant was able to survive within the nodules of the nodule-specific signal peptidase *dnf1* mutant of *M. truncatula* (Wang *et al.*, 2010; Haag *et al.*, 2011b). *Sinorhizobium meliloti* is no longer challenged with NCR peptides in this *M. truncatula* mutant and thus survival of the *S. meliloti bacA* mutant in the *M. truncatula dnf1* mutant provides strong support for the role of BacA in protecting the rhizobia from being killed by the NCR peptide challenge even though it cannot be excluded that the transport of other peptides or proteins to the bacteroid-containing symbiosomes might be affected by the *dnf1* mutation and contribute to the *bacA* mutant phenotype.

In an effort to understand more about the function of NCR peptides and to investigate whether the formation of defined disulphide bridges and thus the presence of cysteine residues in the NCR peptide sequence was paramount to NCR function, the influence of the loss of the disulphide bridges and/or the loss of the cysteine residues was investigated (Haag *et al.*, 2012). This study showed that a properly folded cysteine-containing peptide was required to induce an optimal bacteroid differentiation response *in vitro* (see Supporting Information, Data S1 for information regarding NCR peptide synthesis). NCR peptides with an incorrect disulphide-bond formation, lacking cysteine residues or peptide truncations were either reduced in or had lost their ability to induce bacteroid features *in vitro* (Haag *et al.*, 2012). Truncated versions of NCR247 revealed that the antimicrobial activity of NCR247 was dependent on its positive charge. This was not entirely surprising as antimicrobial peptides such as defensins, known to act by membrane permeabilization,

are generally cationic peptides (Haag *et al.*, 2011b). This feature is also in agreement with the finding that only cationic NCR peptides appear to show antimicrobial activity against bacteria *in vitro* (Van De Velde *et al.*, 2010). However, it needs to be mentioned that the mode of action of NCR peptides on rhizobia has not yet been fully understood and there might well be other periplasmic and intracellular bacterial targets of NCR peptides. Interestingly, once the disulphide bonds were reduced by dithiothreitol, the antimicrobial potency of NCR247 was increased significantly (Haag *et al.*, 2012). This is similar to a phenotype observed for a mammalian defensin acting in the animal colon, which is thought to be stored in an inactive oxidized form and then is activated by reduction through thioredoxin proteins (Schroeder *et al.*, 2011). Remarkably, no difference in sensitivity against the reduced NCR247 peptide was observed between wild-type *S. meliloti* and the *bacA* mutant (Haag *et al.*, 2012).

The similarity of BacA to ABC transporters has long favoured a hypothesis in which the transport of (a) plant-derived molecule(s) might be important for the successful formation of the symbiosis and the loss of this transport would thus account for the inability of *bacA* mutants to differentiate (Glazebrook *et al.*, 1993; Ichige & Walker, 1997; Ferguson *et al.*, 2006; Domenech *et al.*, 2009; Marlow *et al.*, 2009; Karunakaran *et al.*, 2010; Wehmeier *et al.*, 2010). However, increasing evidence points to a role of BacA in specifically protecting the bacterium from the antimicrobial action of NCR peptides encountered in the legume nodule cells. The fact that the BacA protein only conferred protection against folded NCR peptides might indicate that it could be involved in the maintenance of the less toxic, folded peptide form, preventing reduction of the disulphide bonds *in planta*. It remains to be determined whether this occurs via a direct interaction of BacA with the NCR peptides or whether the peptide transport function of BacA could be involved in maintaining an oxidizing environment within the periplasm.

Role of unusual lipid A fatty acids in bacteroid development

Sinorhizobium meliloti is thought to encounter various other stresses such as low pH and high osmolarity within their intracellular compartments (Vedam *et al.*, 2003; Ferguson *et al.*, 2005). The bacteria need to adapt to these stresses to survive within the host. The main barrier protecting Gram-negative bacteria is their cell envelope (Fig. 2a). The majority of the outer leaflet of the outer membrane is composed of lipopolysaccharide (LPS), which consists of lipid A, an oligosaccharide core and the O-antigen polysaccharide (Raetz & Whitfield, 2002). Lipid A forms the hydrophobic anchor of LPS and consists of a sugar backbone that is modified with various fatty acids (Raetz & Whitfield, 2002). The lipid A molecules of *Rhizobiaceae* are modified with an unusual very-long-chain fatty acid (VLCFA) (Figs 2a and 3), which can be either a 27-OHC28:0, 27-OH(βOmeC4:0)C28:0 or 29-OHC30:0 VLCFA modification (Bhat *et al.*, 1991; Que *et al.*, 2000a, b; Ferguson *et al.*, 2002, 2004; De Castro *et al.*, 2008). The *S. meliloti bacA* mutant has a 50% reduction in the content of VLCFA-modified lipid A (Fig. 2a) (Ferguson *et al.*, 2004) and consistent with this displayed an increased sensitivity to ethanol and sodium dodecyl sulphate (SDS) indicating a compromised cell envelope (Ferguson *et al.*, 2002). This led to the hypothesis that lipid A VLCFA modifications might play an important role in establishing and/or maintaining a successful symbiosis (Ferguson *et al.*, 2004). It is therefore also possible that the increased sensitivity of the *S. meliloti bacA* mutant towards NCR peptides is related to the role of BacA in this cell envelope alteration. BacA has low sequence similarity with the human adrenoleukodystrophy protein (hALDP), which is a peroxisomal-membrane protein involved in the transport of long-chain fatty acids from the cytoplasm into the peroxisome, where they are degraded (Ferguson *et al.*, 2004). Site-directed mutations in conserved residues between the *S. meliloti* BacA protein and the hALDP resulted in a reduction of the LPS VLCFA content, further providing evidence that BacA might be involved in lipid transport (Fig. 2a) (Ferguson *et al.*, 2004). However, through the use of BacA site-directed mutants, it was possible to uncouple the peptide uptake function of BacA from its role in VLCFA transport. Of the four symbiotically defective site-directed BacA mutants with a confirmed reduction in their VLCFA content, two were still capable of taking up Bac7, suggesting that the reduced VLCFA content of LPS in the *bacA* mutant and the function of BacA in Bac7 peptide uptake are not related (Marlow *et al.*, 2009).

Biosynthesis and importance of unusual *S. meliloti* LPS lipids

Encoded within the rhizobial genome is a highly conserved gene cluster consisting of five to six genes involved in the biosynthesis of the VLCFA (Fig. 2b) (Sharypova *et al.*, 2003; Vanderlinde *et al.*, 2009; Haag *et al.*, 2011a). The VLCFA biosynthesis cluster is also present in *Brucella* species, but lacking the *fabZXL* gene (Fig. 2b). The absence of *fabZXL* does not coincide with the loss of the lipid A VLCFA modification, suggesting that core fatty acid biosynthesis genes can also be involved in VLCFA biosynthesis (Ferguson *et al.*, 2004).

Fatty acid chains are synthesized on small ($\leq$ 10 kDa), acidic proteins, known as acyl carrier proteins (ACPs)

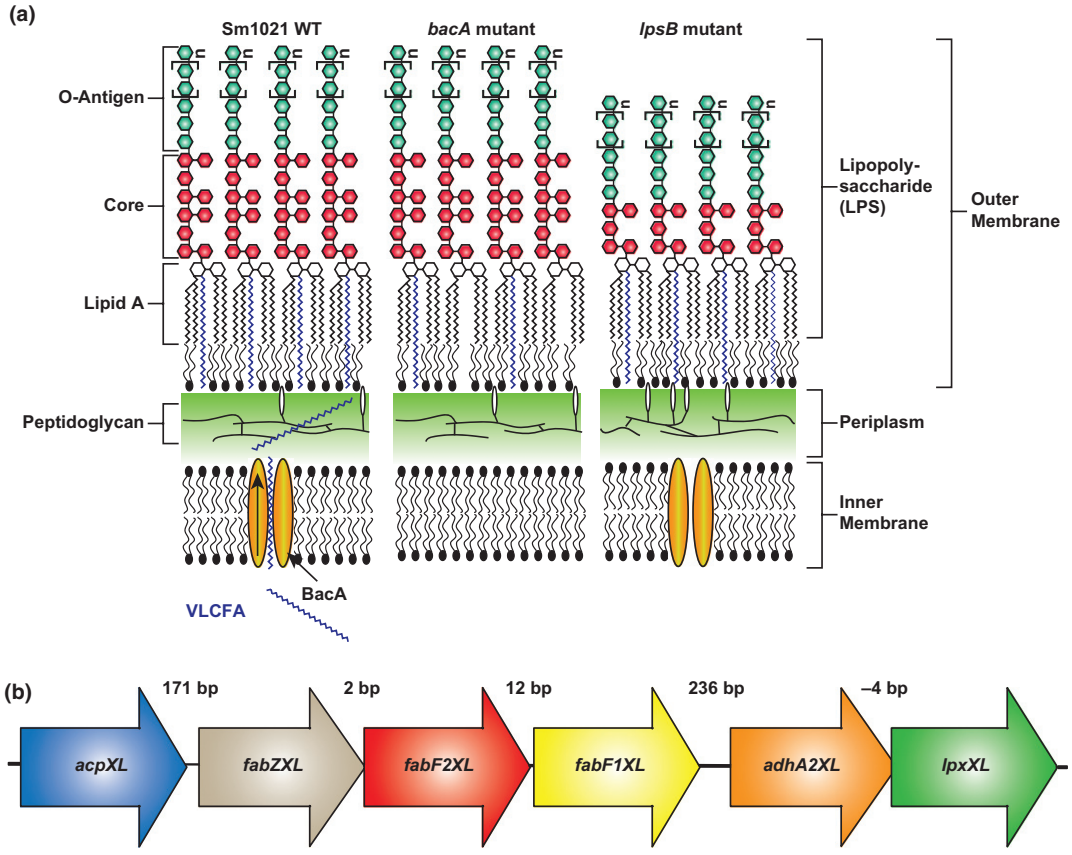

Fig. 2. The cell envelope of rhizobia. (a) Diagram of the composition of the envelope of the *Sinorhizobium meliloti* wild-type (Sm1021 WT), *bacA* and *lpsB* mutant strains. The inner membrane consists primarily of a phospholipid bilayer, in which membrane-bound proteins such as BacA are embedded. It is separated from the outer membrane by the periplasm and a layer of peptidoglycan. The outer membrane consists of layer of phospholipids on the periplasmic face and an outer layer of LPS. LPS comprises three major subunits: O-antigen, core and lipid A, which forms the hydrophobic anchor of LPS in the outer membrane. Every LPS molecule in the *S. meliloti* Sm1021 wild-type strain is modified with a VLCFA (indicated in blue), whereas only 50% of the LPS molecules in an *S. meliloti bacA* mutant are modified with a VLCFA. Therefore, the BacA protein has been hypothesized to be involved in the export of VLCFA from the cytoplasm to the periplasm. In contrast, the *S. meliloti lpsB* mutant has a truncated LPS sugar core. (b) The VLCFA biosynthesis in rhizobia is encoded in a six-gene cluster, which is involved in the different steps of cyclic fatty acid elongation. The following functions have been assigned to each of the genes: *acpXL*, VLCFA ACP; *fabZXL*, putative 3R-hydroxy-myristoyl [acyl carrier] dehydratase; *fabF2XL*, putative 3-oxo-acyl-carrier-protein synthase; *fabF1XL*, putative 3-oxo-acyl-carrier-protein synthase; *adhA2XL*, putative alcohol dehydrogenase; *lpxXL*, VLCFA acyl transferase.

(Cronan & Thomas, 2009). From these ACPs, the acyl chains are transferred to their target molecules by acyl transferases. ACPs play a crucial role in the assembly of the bacterial cell envelope and have been shown to play an important role in establishing a successful symbiosis (Geiger & López-Lara, 2002). Currently, six ACPs have been identified and their functions confirmed in *S. meliloti* (Geiger & López-Lara, 2002; Ramos-Vega *et al.*, 2009; Davila-Martinez *et al.*, 2010). A particular ACP is NodF, which is involved in the decoration of the Nod factor with a specific polyunsaturated fatty acid requiring also the NodE protein for its synthesis. A mutant in *nodF* lacks the Nod factor acyl decoration and is severely impaired in establishing infection threads (Ardourel *et al.*, 1994; Geiger & López-Lara, 2002; Davila-Martinez *et al.*, 2010).

Research has initially focused on two genes in the VLCFA biosynthesis cluster. AcpXL is the ACP onto which the VLCFA is synthesized (Brozek *et al.*, 1996; Sharypova *et al.*, 2003), while LpxXL transfers the VLCFA onto the rhizobial lipid A precursor (Basu *et al.*, 2002; Raetz *et al.*, 2007). Similar functions for AcpXL and LpxXL have been confirmed in various rhizobia (Sharypova *et al.*, 2003; Vedam *et al.*, 2003; Ferguson *et al.*, 2005; D'Haeze *et al.*, 2007; Ardissone *et al.*, 2011; Brown *et al.*, 2011). Mutants lacking AcpXL in *S. meliloti*, *R. leguminosarum* (bv. *viciae* and *phaseoli*) and *Rhizobium* sp. NGR234 have been shown to be devoid of the lipid A VLCFA modifications in their free-living states (Sharypova *et al.*, 2003; Vedam *et al.*, 2003; Ferguson *et al.*, 2005; Ardissone *et al.*, 2011; Brown *et al.*, 2011). The *S. meliloti*

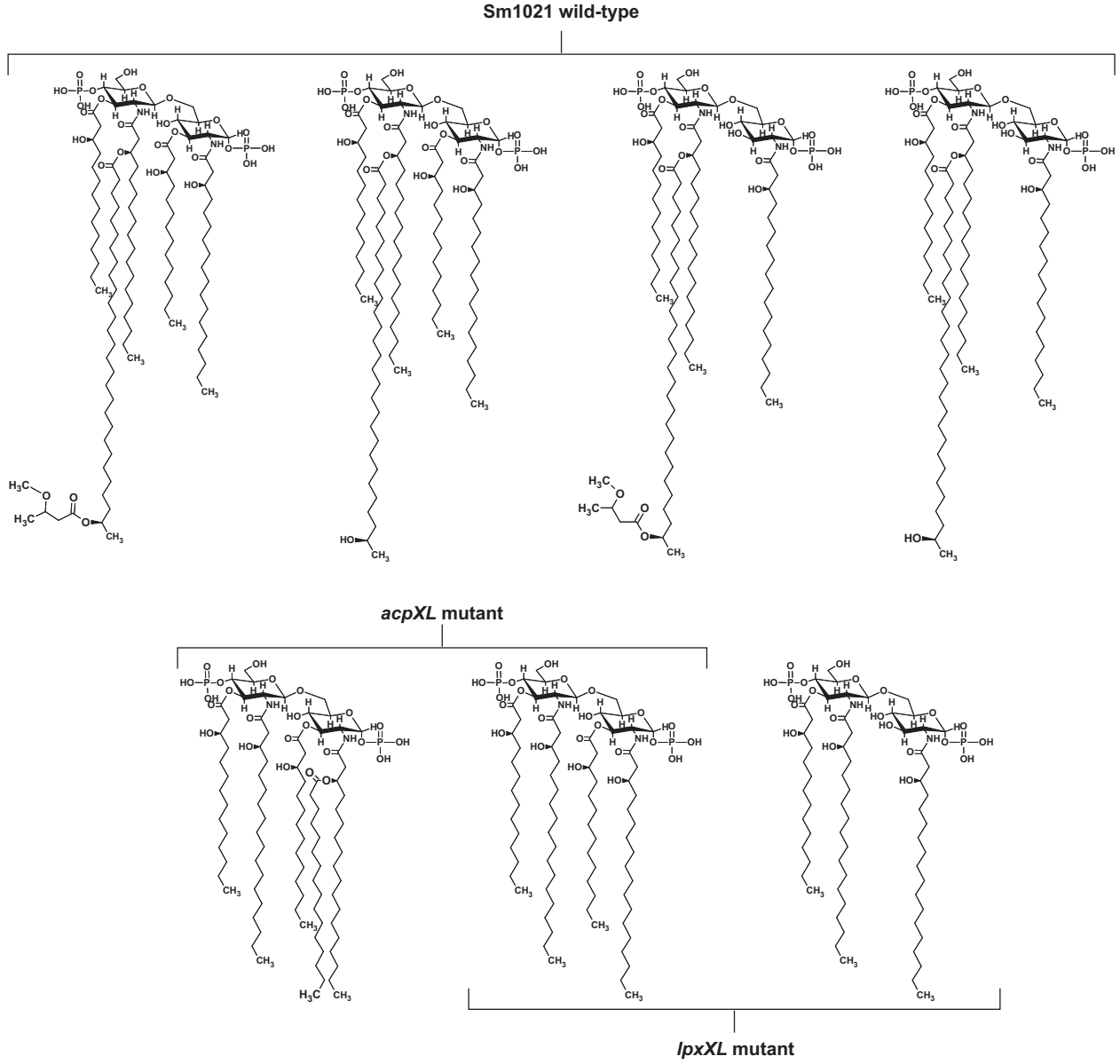

Fig. 3. Lipid A species found in the *Sinorhizobium meliloti* parent and VLCFA mutant strains. All Sm1021 wild-type strain lipid A molecules are modified with a VLCFA. The mutants in the *acpXL*/*lpxXL* gene cluster lack the VLCFA modification but differ in their acylation state. While the wild-type strain is penta- and tetra-acylated, in the mutants between *acpXL* and *adhA2XL*, the position of the VLCFA can be occupied by a shorter, unhydroxylated fatty acid with a chain length of either C16 or C18, whereas this position is unmodified in the *lpxXL* mutant. Furthermore, in the *lpxXL* mutant tri-acylated lipid A species are present.

and *R. leguminosarum acpXL* mutants are delayed in nodulation (Sharypova *et al.*, 2003; Vedam *et al.*, 2003) and the *R. leguminosarum acpXL* mutant also in the development of nitrogen fixation (Vedam *et al.*, 2004). Competition experiments with either the *S. meliloti* single mutants in the VLCFA biosynthesis cluster and the wild-type strain on alfalfa plants revealed that all mutant strains were less competitive than the parent strain even though they were able to support plant growth (Ferguson *et al.*, 2005; Haag *et al.*, 2011a). Transmission electron micrographs of *R. leguminosarum acpXL*-mutant-infected pea nodules showed that bacteroids were abnormally shaped and multiple bacteroids could be found within a single symbiosome (Vedam *et al.*, 2004). This was confirmed in an *S. meliloti acpXL* mutant and the bacteroids also appeared to be senescing prematurely (Haag *et al.*,

2009). *Sinorhizobium meliloti lpxXL* mutant bacteroids were found to have a considerably increased cell size and abnormal shape relative to the wild-type bacteroids (Haag *et al.*, 2009).

In the *S. meliloti acpXL* mutant, the lipid A VLCFA is replaced by a shorter (C16 or C18) unhydroxylated fatty acid (Fig. 3). The *S. meliloti lpxXL* mutant also lacks the lipid A VLCFA modifications but in contrast to the *acpXL* mutant, the VLCFA position on the lipid A was not further modified by a different-chain-length fatty acid (Fig. 3), suggesting that LpxXL in the absence of AcpXL is capable of transferring shorter unhydroxylated fatty acids onto the lipid A (Ferguson *et al.*, 2005). Interestingly, although both *S. meliloti* and *R. leguminosarum acpXL* mutants lack the lipid A VLCFA modification in their free-living state, host-extracted cells had a partial restoration of the lipid A VLCFA modification (Vedam *et al.*, 2006; Haag *et al.*, 2009). It is therefore possible that within the plant host, a different rhizobial ACP can be expressed and takes over the function of AcpXL.

Lipid A VLCFAs are thought to play an important part in stabilizing the membrane and protecting the bacterium from environmental stresses as the VLCFAs are long enough to span across the entire double layer of the outer membrane (Vedam *et al.*, 2003). In fact, mutants in the VLCFA biosynthesis gene cluster in either *R. leguminosarum* or *S. meliloti* have been found to show an increased sensitivity towards environmental factors such as osmotic stresses, pH and detergents relative to their parent strains (Vedam *et al.*, 2003, 2006; Ferguson *et al.*, 2005; Haag *et al.*, 2011a). Interestingly, the *S. meliloti lpxXL* mutant, lacking not only the VLCFA modification of its lipid A but also having only tetra-acylated lipid A (penta-acylated lipid A species were found in all the other VLCFA biosynthesis cluster mutants in *S. meliloti*) (Fig. 3), was found to be even more sensitive towards these stress conditions than the other VLCFA biosynthesis mutants (Ferguson *et al.*, 2005; Haag *et al.*, 2011a). The *S. meliloti lpxXL* mutant has the greatest salt requirement for growth of the cluster mutants, suggesting that the inability of this mutant to synthesize penta-acylated lipid A dramatically decreases the stability of its outer membrane relative to the other mutants. Therefore, the reduced competitiveness of the cluster mutants in legume colonization might just arise through an increased sensitivity to symbiosome-encountered stresses relative to the parent strain due to weakened outer membranes.

Role of *S. meliloti* LPS sugars in bacteroid development

The VLCFAs of the LPS molecule are not the only component of the *S. meliloti* LPS important for the development of symbiotic interaction with *Medicago* species. The *lpsB* mutant was also isolated from the Tn*phoA* transposon mutagenesis screen for non-nitrogen fixing *S. meliloti* (Long *et al.*, 1988). The LPS of this mutant migrates more than wild-type LPS using SDS-PAGE analysis and it was suggested that it was defective in a glycosyl transferase involved in the biosynthesis of the LPS core resulting in its truncation. However, this proposed model was difficult to reconcile with the observed changes in the sugar composition of the LPS core molecule and the presence of O-antigen. Therefore, it was proposed that the *lpsB* mutation might result in the enrichment of a different LPS species that is normally a minor component of the wild-type *S. meliloti* LPS (Campbell *et al.*, 2002). The *lpsB* mutant is capable of forming normal infection threads and of entering the host cell, yet is compromised in its ability to fix nitrogen, which ultimately leads to nitrogen starvation of the host (Campbell *et al.*, 2002). The LPS species of the *S. meliloti lpsB* mutant had an increased mobility on SDS and DOC LPS gels, resulting in an overall band shift relative to the parent strain but looked otherwise identical (Campbell *et al.*, 2003). This was attributed to the changes in the LPS sugar core (Campbell *et al.*, 2003). In its free-living state, the *lpsB* mutant showed an increased sensitivity towards cationic antimicrobial peptides such as polymyxin B, poly-L-lysine and melittin, which is thought to be the result of the O-antigen core truncation altering the charge of the bacterial LPS and thus permitting more cationic peptides to interact with the cell envelope (Campbell *et al.*, 2002).

The *S. meliloti lpsB* mutant has a striking symbiotic phenotype. Unlike in wild type infected nodules, where only a single bacteroid is found within a symbiosome compartment, the *lpsB* mutant-induced nodules contain multiple bacteroids within a single membrane-bound compartment (Campbell *et al.*, 2002). This is a feature more similar to bacteroids found in determinate nodules, where it is common for symbiosome compartments to contain multiple bacteroids (Mergaert *et al.*, 2006; Jones *et al.*, 2007). On the other hand, the *lpsB* mutant bacteroids are unable to persist within the symbiosomes and appear to be lysing (Campbell *et al.*, 2002). However, there are also some bacteroids that appear to be differentiated with elongated morphologies that show outer membrane damage (Campbell *et al.*, 2002). This could suggest that some bacteria are able to differentiate but cannot persist within the symbiosomes for extensive periods of time, which might also explain why plants infected with the *lpsB* mutant are able to grow and fix nitrogen for a short period but eventually die. The lysing bacteroids resemble the phenotype of the *S. meliloti bacA* mutant although this process seems to be delayed in the *lpsB* mutant.

Parallels between symbiosis and chronic mammalian pathogenesis

It has recently been shown that control of bacterial infection by antimicrobial peptides is a common phenomenon in diverse kingdoms of life. It was discovered that the control and maintenance of symbiotic bacteria within some insects (weevils) (Login *et al.*, 2011) is antimicrobial peptide-dependent and, similarly, animals ranging from *Hydra*, insects to mammals use antimicrobial peptides to control their gut microbiota (Tjepkema & Cartica, 1982; Soupene *et al.*, 1995; Wei & Layzell, 2006), indicating the necessity for the host to control bacteria in the symbiotic context.

Rhizobia reside within an intracellular, host-membrane-bound compartment that allows the bacteria to survive inside the plant cell and to establish the nitrogen fixing symbiosis with its host (Gibson *et al.*, 2008). In the IRLC legumes, bacteroid differentiation is mediated by hundreds of NCR peptides (see previous sections) inducing terminal differentiation of the bacteria (Van De Velde *et al.*, 2010; Haag *et al.*, 2011b). The rhizobial lifestyle within nodules cells is in many aspects similar to the lifestyle of intracellular microbial pathogens and can be considered a chronic infection (LeVier *et al.*, 2000). This parallel further extends to the NCR peptides expressed within legumes which show similarities to mammalian defensins of innate immunity (Fig. 4a) (Mergaert *et al.*, 2003). Antimicrobial peptides are key effectors of higher organisms to defend themselves against invading organisms and are also able to potentiate the immune system, i.e. guide immune cells to the site of infection (Izadpanah & Gallo, 2005). In the following section we first discuss the analogous lifestyle of two examples of diverse chronic mammalian pathogens compared with rhizobia and focus on similar mechanisms of survival under environmental conditions that these bacteria are exposed to.

Brucellosis

Brucella abortus is a non-motile, Gram-negative alpha-proteobacterium (Young, 1995) that is phylogenetically closely related to *S. meliloti* (LeVier *et al.*, 2000). *Brucella abortus* does not form a beneficial interaction with its mammalian host but establishes a chronic infection, which results in the disease known as brucellosis in humans (Young, 1995). With more than 500 000 new cases of human infections, it is the most prevalent zoonotic disease worldwide (Pappas *et al.*, 2006). Although brucellosis very rarely leads to the death of the patient, it is a seriously debilitating disease that presents with, among other symptoms, fever, fatigue, nausea and weight loss (Young, 1995). If not properly treated, brucellosis can become a chronic and asymptomatic disease that can re-emerge months after the initial infection (Young, 1995). Humans can be exposed to brucellae through contact with infected animals and material, such as blood or milk, or through the aerosol route (Fugier *et al.*, 2007). *Brucella* are highly infectious and doses as low as 10–100 bacteria are thought to be sufficient to cause the human disease (Fugier *et al.*, 2007). Brucellae are therefore considered to

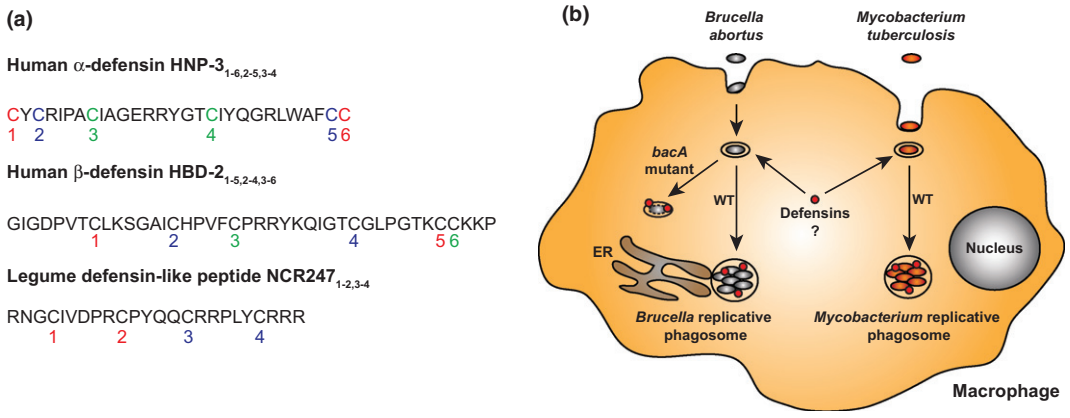

Fig. 4. Parallels between the *Rhizobium*–legume symbiosis and chronic mammalian infections. (a) Eukaryotic hosts are teeming with cysteine-rich peptides that form defined disulphide bonds. In addition to their role in the host's defence/immune response to bacterial challenges, these peptides could induce a chronic infection state as was found in the *S. meliloti*–legume symbiosis. (b) The intracellular mammalian pathogens *Brucella abortus* and *Mycobacterium tuberculosis* share a similar lifestyle with rhizobia. They are taken up into host-membrane-bound intracellular compartments, in which they can persist and are challenged with cysteine-rich peptides (defensins). Like the *S. meliloti bacA* mutant, the *B. abortus bacA* mutant is unable to survive in the host cell. Whether defensins are involved in the induction of a chronic infective state remains to be determined. ER, endoplasmic reticulum; WT, wild-type.

be targets for the development of biological weapons and several countries were suspected of trying to weaponize *Brucella* species during the Cold War (Guillemin, 2006; Fugier *et al.*, 2007).

The infection process of *Brucella* has been subject to intense research. The preferred cell types infected by brucellae are phagocytic cells such as macrophages (Fig. 4b) (Maria-Pilar *et al.*, 2005). While the symbiotic *Rhizobiaceae* are taken up through an endocytosis-like process from the infection threads (Jones *et al.*, 2007), *B. abortus* is taken up into a phagosome, which is then targeted to the endoplasmic reticulum, the replicative niche of *Brucella* within the host (Fig. 4b) (Maria-Pilar *et al.*, 2005; Fugier *et al.*, 2007). Like rhizobia, *Brucella* species persist within membrane-bound acidic compartments for extensive periods of time (LeVier *et al.*, 2000; Roop *et al.*, 2002, 2009). Evasion of the immune system of the host organism and targeting of the bacterium to its replicative niche are of key importance for the infection process in *B. abortus*. The bacterial cell envelope is the major point of interaction between brucellae and the host and, as such, molecules within the bacterial cell envelope play a significant part in the infection process. O-antigen and LPS structure are known to significantly affect the outcome of *Brucella* infections. For example, many mutants defective in the LPS O-antigen are attenuated in their virulence (Haag *et al.*, 2010). Smooth LPS (LPS containing the O-antigen) *Brucella* strains can interact with lipid rafts thereby acquiring lipid raft markers in their compartments, which facilitate the intracellular targeting of the bacteria (Haag *et al.*, 2010). The *S. meliloti* symbiosome also acquires certain endosomal marker proteins when taken up into the *M. truncatula* host cells, but is delayed in the acquisition of lytic vacuolar markers, which is thought to ensure the survival and maintenance of the bacteroids in the host cells (Limpens *et al.*, 2009). Rough LPS *Brucella* strains (without the O-antigen) are more likely to be targeted to lysosomes and are unable to establish a chronic infection (Haag *et al.*, 2010). Cyclic-β-1,2-linked glucans, which play a major role in the response of bacteria towards hypo-osmotic stresses (Miller *et al.*, 1986) play a role in determining the intracellular targeting of *Brucella* to either their replicative niche or a lytic vacuole (Haag *et al.*, 2010).

The *Brucella* BacA protein is highly similar to *S. meliloti* BacA and was found to be essential for *Brucella* to survive in macrophages *in vitro* (LeVier *et al.*, 2000). In addition, a *B. abortus* BacA-deficient mutant was found to have increased resistance to the glycopeptide antibiotic bleomycin (LeVier *et al.*, 2000). *Brucella abortus* contains, like *S. meliloti*, LPS that is modified with a VLCFA and loss of the BacA protein in *B. abortus* also results in a reduced VLCFA content in the *B. abortus* lipid A molecule (Ferguson *et al.*, 2004). Similar to an *S. meliloti bacA* mutant, a *B. abortus bacA* mutant is unable to persist within its host cell (LeVier *et al.*, 2000) and a plasmid-borne *B. abortus bacA* gene was able to restore a functional symbiosis, Bac7 peptide uptake and NCR peptide resistance in the *S. meliloti bacA* mutant background (Wehmeier *et al.*, 2010; Haag *et al.*, 2011b). These findings suggested that *B. abortus* BacA, just like *S. meliloti* BacA, is essential for the bacteria to persist within its host and that it helps the bacteria to withstand challenges of antimicrobial peptides within the host.

Mammalian host cells are teeming with peptides, some of which have similarities to plant peptides. For example, NCR peptides are similar to cysteine-rich defensins (Fig. 4a) (see following section) (Mergaert *et al.*, 2003). Research in *Rhizobium*/legume symbiosis and other symbiotic bacterial–host interactions (see above) have shown that host-derived antimicrobial peptides can alter bacterial development and behaviour. It remains to be determined whether the bacteria have adapted to use these peptides as a signal or whether the host uses them to control infection.

Tuberculosis

Mycobacterium tuberculosis is the causative agent of tuberculosis in humans, which is one of the most important infectious diseases in the world. Approximately one-third of the human population is latently infected with this pathogen and acute tuberculosis results in the death of about 2 million people each year (Harries & Dye, 2006; Barry *et al.*, 2009). *Mycobacterium tuberculosis* is an acid-fast bacterium with a unique cell wall composed of very long chain hydrophobic fatty acids, which make up approximately 60% of the bacterial cell mass (Anderson, 1943). *Mycobacteria* are normally only transmitted from human to human in the acute phase of the disease and enter via the aerosol route into the human lung. The bacteria then enter the terminal alveoli of the lungs and are able to multiply until they eventually spread into the blood circulation to infect vital parts of the human body (Harries & Dye, 2006). However, following infections the immune system often is able to contain the bacteria in lung tubercles and prevent spreading of the bacteria, resulting in a latent/chronic state of infection (Barry *et al.*, 2009).

Mycobacterium tuberculosis primarily resides inside macrophages and survives the killing action of the phagolysosome by inhibiting phagosome maturation (Cho & Harper, 1991). Lipoarabinomannan (LAM), a major mycobacterial cell wall component, is involved in this process. LAM, among others, is a lipoglycan, which is noncovalently attached to the cell plasma membrane and is able to modulate the host's immune system (Cho &

Harper, 1991). Then *M. tuberculosis* enters a dormant state with close to no physiological activity (Barry *et al.*, 2009). Only about 10% of these dormant infections result in active disease. Although following a mainly intracellular lifestyle, *M. tuberculosis* induces a range of antimicrobial peptides during its infection in the human host, including human neutrophil peptides (Tan *et al.*, 2006) and human beta-defensin 2 (Rivas-Santiago *et al.*, 2005, 2006).

Mycobacterium tuberculosis has a homologue to *S. meliloti* BacA, which is 639 amino acids in length and is therefore 219 amino acids longer than the *S. meliloti* BacA protein (Domenech *et al.*, 2009). This difference in length between the *S. meliloti* and the *M tuberculosis* BacA proteins is due to the presence of a putative ATPase domain in the *Mycobacterium* protein (LeVier *et al.*, 2000; Domenech *et al.*, 2009). Deletion of the *bacA* gene affected the ability of the *M. tuberculosis* strain H37Rv to form a latent infection within a murine model system (Domenech *et al.*, 2009). The *M. tuberculosis bacA* gene complements the functions of SbmA and BacA in sensitizing towards the glycopeptide antibiotic bleomycin and the truncated bovine antimicrobial peptide, $Bac7_{1–16}$, when expressed in the heterologous *sbmA* and *bacA* mutants in *E. coli* and *S. meliloti* (Domenech *et al.*, 2009). In *S. meliloti*, deletion of BacA results in a 50% reduction of the VLCFA content in the outer membrane (Ferguson *et al.*, 2004). However, the three most abundant fatty acids within the cell wall of *M. tuberculosis*, methoxy-, keto-, and α-mycolic acids, are not affected by deletion of *bacA* (Domenech *et al.*, 2009). Although the *M. tuberculosis* bacterium does not contain LPS, this suggested that unlike in *B. abortus* and *S. meliloti* (Ferguson *et al.*, 2004, 2005), BacA does not affect the composition of cell envelope fatty acids in *M. tuberculosis* (Domenech *et al.*, 2009) but might be involved in other conserved BacA functions such as peptide transport and antimicrobial peptide resistance. *Mycobactrium tuberculosis* BacA could sensitize *E. coli* and *S. meliloti* against the glycopeptide bleomycin, suggesting active peptide uptake as was shown for *S. meliloti* BacA (Domenech *et al.*, 2009; Wehmeier *et al.*, 2010). *Mycobactrium tuberculosis* BacA appears to play a key role during latent *M. tuberculosis* infections. The challenge of *M. tuberculosis* with defensins within the host environment and the BacA-mediated response to these molecules could play an important role in mediating a latent state of infection as is the case with NCR peptides in the legume symbiosis.

Concluding remarks

The recent developments in *Rhizobium*–legume research concerning bacteroid differentiation and maintenance promise to open new perspectives comparable to the identification of the Nod factor in the 1990s and the Nod factor signalling in the years since 2000. In case of IRLC legumes, the plant host appears to be controlling the fate of the bacteria via the expression of a very complex cysteine-rich peptide mix resulting in the terminal differentiation of rhizobia within these nodule cells. The plant deploys considerable resources to the expression of these peptides but probably also gains a significant competitive advantage over plants that do not induce terminal differentiation. NCR peptides are not homogenous mixtures with common properties for all peptides but they vary in charge and in expression time. Currently the targets of NCR peptides are unknown. Although some have been shown to associate with bacteria and bacteroids *in vitro* and *in vivo*, the sheer number of peptides expressed allows for other target molecules from the bacteria or the plant. Genetic screens will help to determine bacterial factors involved in the protection against antimicrobial NCR peptides. Other interactors could be identified using biochemical approaches such as pull-down assays with tagged NCR peptides or far-Western blotting on cell lysates. As NCR peptides will probably affect a whole range of metabolic processes within the bacterium, it will be worth looking for NCR peptide-induced changes on a more global level. Microarrays or RNAseq analysis of untreated and treated wild-type rhizobia and mutants affected in the response to NCR peptides could identify gene expression changes important for adaptation in the host environment. Proteomic analysis could determine global changes in protein expression induced by NCR peptides. With up to 600 NCR peptides expressed in *M. truncatula* alone, determining the precise roles of NCR peptides will be a challenging task for researchers over the next years.

BacA has been shown to be involved in establishing a chronic intracellular infection within a eukaryotic host by several bacterial species. Here, we have discussed that BacA is involved in protecting rhizobia against legume NCR peptides by reducing their antimicrobial activity. How BacA proteins achieve this protection is not yet fully understood. One possibility is that the loss of BacA causes a general loss of membrane stability and therefore an increased susceptibility of the bacteria towards NCR peptides. However, the fact that BacA specifically protects against folded NCR peptide versions seems to suggest that a more specific interaction is taking place and BacA could be directly or indirectly involved in conserving the oxidized state of the disulphide bridges in the NCR peptides. BacA has been shown to be essential for the uptake of proline-rich bovine peptides and it could be the transport function of BacA facilitating uptake or export of NCR and/or other peptides within the host. This could either alleviate the antimicrobial pressure from the cell envelope

by removing the peptide and transporting it into the cell or transport of peptides could help maintain a suitable oxidizing environment preventing the activation of the antimicrobial properties of NCRs. Whether the involvement of BacA in peptide transport is important for the symbiosis is unknown.

BacA also affects the modification of the rhizobial lipid A with a VLCFA and although the VLCFA *per se* has been shown not to be essential, successful infection might depend on the degree of VLCFA-lipid A present. Mutants lacking the VLCFA modified lipid A have developmental defects in the nodule cell and are less efficient at fixing nitrogen. From these observations it is clear that this unusual fatty acid is important for the symbiosis. As discussed in the above sections, bacterial pathogens of the order of *Rhizobiales* also have the capacity to modify lipid A with a VLCFA and this might be important for the pathogen to establish itself within a host cell. The *M. tuberculosis* cell envelope is modified with different types of unusual fatty acids, mycolic acids, which are involved in *M. tuberculosis* virulence and survival *in vivo* (Karakousis *et al.*, 2004). In particular, the wax-like compound phthiocerol dimycocerosate (PDIM) was shown to be important for full virulence of *M. tuberculosis* H37Rv in murine infection models (Domenech & Reed, 2009). However, the exact means by which PDIM supports virulence is very complex due to the many genes involved in its synthesis and transport and remains elusive (Domenech & Reed, 2009).

NCR peptides have the potential to be developed into a useful antimicrobial drug. It has been shown that positively charged NCR peptides possess potent antimicrobial activity. A key problem for developing a usable drug from these peptides will be to find the shortest possible version giving good antimicrobial properties. Furthermore, cysteine residues provide a challenge during the synthesis and can affect the quality and homogeneity of different peptide batches due to varying degrees of inter- and intra-peptide disulphide bridge formation. Various version of NCR247 were generated and have been shown to have altered antimicrobial properties. Substitution of the cysteine residues with serines reduced the antimicrobial potency of NCR247, while reduction of the cysteine bridges results in increased bacterial killing in *S. meliloti*. However, a truncated form of the peptide retained antimicrobial activity so long as they retained a cationic charge. Further characterization might be able to determine a core NCR peptide with the necessary properties.

Acknowledgements

This review is dedicated to the memory of Gail P. Ferguson who passed away on 27 December 2011. A.F.H. was and B.K. is funded by an Institute of Medical Sciences, University of Aberdeen PhD studentship, M.F.F.A. and K.K.M. are funded by SULSA PhD studentships, and P.M. is supported by the Agence Nationale de la Recherche, grant ANR-09-BLAN-0396-01.

References

Alunni B, Kevei Z, Redondo-Nieto M, Kondorosi A, Mergaert P & Kondorosi E (2007) Genomic organization and evolutionary insights on *GRP* and *NCR* genes, two large nodule-specific gene families in *Medicago truncatula*. *Mol Plant Microbe Interact* **20**: 1138–1148.

Anderson RJ (1943) The chemistry of the lipids of the tubercle bacillus. *Yale J Biol Med* **15**: 311–345.

Ardissone S, Kobayashi H, Kambara K, Rummel C, Noel KD, Walker GC, Broughton WJ, Deakin WJ (2011) Role of BacA in lipopolysaccharide synthesis, peptide transport and nodulation by *Rhizobium sp*. NGR234. *Journal of Bacteriology* **193**: 2218–2228.

Ardourel M, Demont N, Debelle FD *et al.* (1994) *Rhizobium meliloti* lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair-cells and induction of plant symbiotic developmental responses. *Plant Cell* **6**: 1357–1374.

Arrighi JF, Barre A, Ben Amor B *et al.* (2006) The *Medicago truncatula* lysin [corrected] motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. *Plant Physiol* **142**: 265–279.

Barnett MJ, Toman CJ, Fisher RF & Long SR (2004) A dual-genome Symbiosis Chip for coordinate study of signal exchange and development in a prokaryote-host interaction. *P Natl Acad Sci USA* **101**: 16636–16641.

Barry CE III, Boshoff HI, Dartois V *et al.* (2009) The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* **7**: 845–855.

Basu SS, Karbarz MJ & Raetz CR (2002) Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum*. *J Biol Chem* **277**: 28959–28971.

Becker A, Berge s H, Krol E *et al.* (2004) Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. *Mol Plant Microbe Interact* **17**: 292–303.

Benincasa M, Scocchi M, Podda E, Skerlavaj B, Dolzani L & Gennaro R (2004) Antimicrobial activity of Bac7 fragments against drug-resistant clinical isolates. *Peptides* **25**: 2055–2061.

Benincasa M, Mattiuzzo M, Herasimenka Y, Cescutti P, Rizzo R & Gennaro R (2009) Activity of antimicrobial peptides in the presence of polysaccharides produced by pulmonary pathogens. *J Pept Sci* **15**: 595–600.

Bhat UR, Carlson RW, Busch M & Mayer H (1991) Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipopolysaccharides from bacteria belonging to the alpha-2 subgroup of Proteobacteria. *Int J Syst Bacteriol* **41**: 213–217.

Bisseling T, Bos RCVD, Kammen AV, Ploeg MVD, Duijn PV & Houwers A (1977) Cytofluorometrical determination of the DNA contents of bacteroids and corresponding broth-cultured *Rhizobium* bacteria. *J Gen Microbiol* **101**: 79–84.

Bisseling T, Limpens E, Franken C, Smit P, Willemse J & Geurts R (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* **302**: 630–633.

Bolaños L, Redondo-Nieto M, Rivilla R, Brewin NJ & Bonilla I (2004) Cell surface interactions of *Rhizobium* bacteroids and other bacterial strains with symbiosomal and peribacteroid membrane components from pea nodules. *Mol Plant Microbe Interact* **17**: 216–223.

Bonaldi K, Gargani D, Prin Y *et al.* (2011) Nodulation of *Aeschynomene afraspera* and *A. indica* by photosynthetic *Bradyrhizobium* sp. strain ORS285: the nod-dependent versus the nod-independent symbiotic interaction. *Mol Plant Microbe Interact* **24**: 1359–1371.

Brewin NJ (2004) Plant cell wall remodelling in the *Rhizobium*-legume symbiosis. *Crit Rev Plant Sci* **23**: 293–316.

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**: 238–250.

Brown DB, Huang YC, Kannenberg EL, Sherrier DJ & Carlson RW (2011) An acpXL mutant of *Rhizobium leguminosarum* bv. *phaseoli* lacks 27-hydroxyoctacosanoic acid in its lipid A and is developmentally delayed during symbiotic infection of the determinate nodulating host plant *Phaseolus vulgaris*. *J Bacteriol* **193**: 4766–4778.

Brozek KA, Carlson RW & Raetz CRH (1996) A special acyl carrier protein for transferring long hydroxylated fatty acids to lipid A in *Rhizobium*. *J Biol Chem* **271**: 32126–32136.

Campbell GR, Reuhs BL & Walker GC (2002) Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. *P Natl Acad Sci USA* **99**: 3938–3943.

Campbell GR, Sharypova LA, Scheidle H, Jones KM, Niehaus K, Becker A & Walker GC (2003) Striking complexity of lipopolysaccharide defects in a collection of *Sinorhizobium meliloti* mutants. *J Bacteriol* **185**: 3853–3862.

Capela D, Filipe C, Bobik C, Batut J & Bruand C (2006) *Sinorhizobium meliloti* differentiation during symbiosis with alfalfa: a transcriptomic dissection. *Mol Plant Microbe Interact* **19**: 363–372.

Cheng HP & Walker GC (1998) Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J Bacteriol* **180**: 5183–5191.

Cho MJ & Harper JE (1991) Effect of inoculation and nitrogen on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. *Plant Physiol* **95**: 435–442.

Chou MX, Wei XY, Chen DS & Zhou JC (2006) Thirteen nodule-specific or nodule-enhanced genes encoding products homologous to cysteine cluster proteins or plant lipid transfer proteins are identified in *Astragalus sinicus* L. by suppressive subtractive hybridization. *J Exp Bot* **57**: 2673–2685.

Cooper JE (2007) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J Appl Microbiol* **103**: 1355–1365.

Crockard A, Bjourson J, Dazzo B & Cooper JE (2002) A white clover nodulin gene, *dd23b*, encoding a cysteine cluster protein, is expressed in roots during the very early stages of interaction with *Rhizobium leguminosarum* biovar *trifolii* and after treatment with chitolipooligosaccharide Nod factors. *J Plant Res* **115**: 439–447.

Cronan JE & Thomas J (2009) Bacterial fatty acid synthesis and its relationships with polyketide synthetic pathways. *Methods Enzymol*, **459**: 395–433.

Davidson AL & Chen J (2004) ATP-binding cassette transporters in bacteria. *Annu Rev Biochem* **73**: 241–268.

Davila-Martinez Y, Ramos-Vega AL, Contreras-Martinez S, Encarnacion S, Geiger O & Lopez-Lara IM (2010) SMc01553 is the sixth acyl carrier protein in *Sinorhizobium meliloti* 1021. *Microbiology* **156**: 230–239.

De Castro C, Molinaro A, Lanzetta R, Silipo A & Parrilli M (2008) Lipopolysaccharide structures from *Agrobacterium* and *Rhizobiaceae* species. *Carbohydr Res* **343**: 1924–1933.

D'Haeze W, Leoff C, Freshour G, Noel KD & Carlson RW (2007) *Rhizobium etli* CE3 bacteroid lipopolysaccharides are structurally similar but not identical to those produced by cultured CE3 bacteria. *J Biol Chem* **282**: 17101–17113.

Dixon R & Kahn D (2004) Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* **2**: 621–631.

Domenech P & Reed MB (2009) Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from *Mycobacterium tuberculosis* grown *in vitro*: implications for virulence studies. *Microbiology* **155**: 3532–3543.

Domenech P, Kobayashi H, Levier K, Walker GC & Barry CE III (2009) BacA, an ABC transporter involved in maintenance of chronic murine infections with *Mycobacterium tuberculosis*. *J Bacteriol* **191**: 477–485.

Felle HH, Kondorosi E, Kondorosi A & Schultze M (1999) Elevation of the cytosolic free $[Ca^{2+}]$ is indispensable for the transduction of the nod factor signal in alfalfa. *Plant Physiol* **121**: 273–279.

Ferguson GP, Roop RM II & Walker GC (2002) Deficiency of a *Sinorhizobium meliloti bacA* mutant in alfalfa symbiosis correlates with alteration of the cell envelope. *J Bacteriol* **184**: 5625–5632.

Ferguson GP, Datta A, Baumgartner J, Roop RM II, Carlson RW & Walker GC (2004) Similarity to peroxisomal-membrane protein family reveals that *Sinorhizobium* and *Brucella* BacA affect lipid-A fatty acids. *P Natl Acad Sci USA* **101**: 5012–5017.

Ferguson GP, Datta A, Carlson RW & Walker GC (2005) Importance of unusually modified lipid A in *Sinorhizobium* stress resistance and legume symbiosis. *Mol Microbiol* **56**: 68–80.

Ferguson GP, Jansen A, Marlow VL & Walker GC (2006) BacA-mediated bleomycin sensitivity in *Sinorhizobium*

meliloti is independent of the unusual lipid A modification. *J Bacteriol* **188**: 3143–3148.

Ferguson BJ, Indrasumunar A, Hayashi S, Lin MH, Lin YH, Reid DE & Gresshoff PM (2010) Molecular analysis of legume nodule development and autoregulation. *J Integr Plant Biol* **52**: 61–76.

Frühling M, Albus U, Hohnjec N, Geise G, Pühler A & Perlick AM (2000) A small gene family of broad bean codes for late nodulins containing conserved cysteine clusters. *Plant Sci* **152**: 67–77.

Fugier E, Pappas G & Gorvel JP (2007) Virulence factors in brucellosis: implications for aetiopathogenesis and treatment. *Expert Rev Mol Med* **9**: 1–10.

Gage DJ (2002) Analysis of infection thread development using Gfp- and DsRed-expressing *Sinorhizobium meliloti*. *J Bacteriol* **184**: 7042–7046.

Gage DJ (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev* **68**: 280–300.

Gage DJ, Bobo T & Long SR (1996) Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *J Bacteriol* **178**: 7159–7166.

Geiger O & López-Lara IM (2002) Rhizobial acyl carrier proteins and their roles in the formation of bacterial cell-surface components that are required for the development of nitrogen-fixing root nodules on legume hosts. *FEMS Microbiol Lett* **208**: 153–162.

Gibson KE, Kobayashi H & Walker GC (2008) Molecular determinants of a symbiotic chronic infection. *Annu Rev Genet* **42**: 413–441.

Glazebrook J, Ichige A & Walker GC (1993) A *Rhizobium meliloti* homolog of the *Escherichia coli* peptide-antibiotic transport protein SbmA is essential for bacteroid development. *Genes Dev* **7**: 1485–1497.

Gong ZY, He ZS, Zhu JB, Yu GQ & Zou HS (2006) *Sinorhizobium meliloti nifA* mutant induces different gene expression profile from wild type in Alfalfa nodules. *Cell Res* **16**: 818–829.

Graham MA, Silverstein KA, Cannon SB & VandenBosch KA (2004) Computational identification and characterization of novel genes from legumes. *Plant Physiol* **135**: 1179–1197.

Guillemin J (2006) Scientists and the history of biological weapons: a brief historical overview of the development of biological weapons in the twentieth century. *EMBO Rep* **7**: S45–S49.

Haag AF, Wehmeier S, Beck S, Marlow VL, Fletcher V, James EK & Ferguson GP (2009) The *Sinorhizobium meliloti* LpxXL and AcpXL proteins play important roles in bacteroid development within alfalfa. *J Bacteriol* **191**: 4681–4686.

Haag AF, Myka KK, Arnold MF, Caro-Hernandez P & Ferguson GP (2010) Importance of lipopolysaccharide and cyclic β-1,2-glucans in *Brucella*–mammalian infections. *Int J Microbiol* **2010**: 124509.

Haag AF, Wehmeier S, Muszynski A *et al.* (2011a) Biochemical characterization of *Sinorhizobium meliloti* mutants reveals gene products involved in the biosynthesis of the unusual lipid A very long-chain fatty acid. *J Biol Chem* **286**: 17455–17466.

Haag AF, Baloban M, Sani M *et al.* (2011b) Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biol* **9**: e1001169.

Haag AF, Kerscher B, Dall'angelo S *et al.* (2012) Role of cysteine residues and disulfide bonds on the activity of a legume root nodule-specific, cysteine-rich peptide. *J Biol Chem*, **287**: 10791–10798.

Halverson LJ & Stacey G (1986) Signal exchange in plant–microbe interactions. *Microbiol Rev* **50**: 193–225.

Harries AD & Dye C (2006) Tuberculosis. *Ann Trop Med Parasitol* **100**: 415–431.

Ichige A & Walker GC (1997) Genetic analysis of the *Rhizobium meliloti bacA* gene: functional interchangeability with the *Escherichia coli sbmA* gene and phenotypes of mutants. *J Bacteriol* **179**: 209–216.

Izadpanah A & Gallo RL (2005) Antimicrobial peptides. *J Am Acad Dermatol* **52**: 381–390; quiz 391-382.

Jensen E, Peoples M, Boddey R, Gresshoff P, Hauggaard-Nielsen H, Alves B & Morrison M (2012) Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agron Sustain Dev* **32**: 329–364.

Jimenez-Zurdo JI, Frugier F, Crespi MD & Kondorosi A (2000) Expression profiles of 22 novel molecular markers for organogenetic pathways acting in alfalfa nodule development. *Mol Plant Microbe Interact* **13**: 96–106.

Jones KM, Kobayashi H, Davies BW, Taga ME & Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium–Medicago* model. *Nat Rev Microbiol* **5**: 619–633.

Kaijalainen S, Schroda M & Lindstrom K (2002) Cloning of nodule-specific cDNAs of *Galega orientalis*. *Physiol Plant* **114**: 588–593.

Karakousis PC, Bishai WR & Dorman SE (2004) *Mycobacterium tuberculosis* cell envelope lipids and the host immune response. *Cell Microbiol* **6**: 105–116.

Karunakaran R, Ramachandran VK, Seaman JC *et al.* (2009) Transcriptomic analysis of *Rhizobium leguminosarum* biovar *viciae* in symbiosis with host plants *Pisum sativum* and *Vicia cracca*. *J Bacteriol* **191**: 4002–4014.

Karunakaran R, Haag AF, East AK *et al.* (2010) BacA is essential for bacteroid development in nodules of galegoid, but not phaseoloid, legumes. *J Bacteriol* **192**: 2920–2928.

Kato T, Kawashima K, Miwa M, Mimura Y, Tamaoki M, Kouchi H & Suganuma N (2002) Expression of genes encoding late nodulins characterized by a putative signal peptide and conserved cysteine residues is reduced in ineffective pea nodules. *Mol Plant Microbe Interact* **15**: 129–137.

Kobayashi H, Sunako M, Hayashi M & Murooka Y (2001) DNA synthesis and fragmentation in bacteroids during *Astragalus sinicus* root nodule development. *Biosci Biotechnol Biochem* **65**: 510–515.

Kuppusamy KT, Endre G, Prabhu R *et al.* (2004) *LIN*, a *Medicago truncatula* gene required for nodule differentiation

and persistence of rhizobial infections. *Plant Physiol* **136**: 3682–3691.

Lavina M, Pugsley AP & Moreno F (1986) Identification, mapping, cloning and characterization of a gene (*sbmA*) required for microcin B17 action on *Escherichia coli* K12. *J Gen Microbiol* **132**: 1685–1693.

LeVier K & Walker GC (2001) Genetic analysis of the *Sinorhizobium meliloti* BacA protein: differential effects of mutations on phenotypes. *J Bacteriol* **183**: 6444–6453.

LeVier K, Phillips RW, Grippe VK, Roop RM II & Walker GC (2000) Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. *Science* **287**: 2492–2493.

Limpens E, Ivanov S, van Esse W, Voets G, Fedorova E & Bisseling T (2009) *Medicago* N2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity. *Plant Cell* **21**: 2811–2828.

Lodwig EM, Hosie AH, Bourdes A *et al.* (2003) Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis. *Nature* **422**: 722–726.

Login FH, Balmand S, Vallier A *et al.* (2011) Antimicrobial peptides keep insect endosymbionts under control. *Science* **334**: 362–365.

Long S, McCune S & Walker GC (1988) Symbiotic loci of *Rhizobium meliloti* identified by random Tn*phoA* mutagenesis. *J Bacteriol* **170**: 4257–4265.

Maria-Pilar JDB, Dudal S, Dornand J & Gross A (2005) Cellular bioterrorism: how *Brucella* corrupts macrophage physiology to promote invasion and proliferation. *Clin Immunol* **114**: 227–238.

Marlow VL, Haag AF, Kobayashi H, Fletcher V, Scocchi M, Walker GC & Ferguson GP (2009) Essential role for the BacA protein in the uptake of a truncated eukaryotic peptide in *Sinorhizobium meliloti*. *J Bacteriol* **191**: 1519–1527.

Maróti G, Kereszt A, Kondorosi E & Mergaert P (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. *Res Microbiol* **162**: 363–374.

Marshall E, Costa LM & Gutierrez-Marcos J (2011) Cysteine-rich peptides (CRPs) mediate diverse aspects of cell-cell communication in plant reproduction and development. *J Exp Bot* **62**: 1677–1686.

Maruya J & Saeki K (2010) The *bacA* gene homolog, mlr7400, in *Mesorhizobium loti* MAFF303099 is dispensable for symbiosis with *Lotus japonicus* but partially capable of supporting the symbiotic function of *bacA* in *Sinorhizobium meliloti*. *Plant Cell Physiol* **51**: 1443–1452.

Mateos PF, Baker DL, Petersen M *et al.* (2001) Erosion of root epidermal cell walls by *Rhizobium* polysaccharide-degrading enzymes as related to primary host infection in the *Rhizobium*–legume symbiosis. *Can J Microbiol* **47**: 475–487.

Mattiuzzo M, Bandiera A, Gennaro R, Benincasa M, Pacor S, Antcheva N & Scocchi M (2007) Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. *Mol Microbiol* **66**: 151–163.

Maunoury N, Redondo-Nieto M, Bourcy M *et al.* (2010) Differentiation of symbiotic cells and endosymbionts in *Medicago truncatula* nodulation are coupled to two transcriptome-switches. *PLoS ONE* **5**: e9519.

Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A & Kondorosi E (2003) A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant Physiol* **132**: 161–173.

Mergaert P, Uchiumi T, Alunni B *et al.* (2006) Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*–legume symbiosis. *P Natl Acad Sci USA* **103**: 5230–5235.

Miller KJ, Kennedy EP & Reinhold VN (1986) Osmotic adaptation by gram-negative bacteria: possible role for periplasmic oligosaccharides. *Science* **231**: 48–51.

O'Brian MR (1996) Heme synthesis in the *Rhizobium*–legume symbiosis: a palette for bacterial and eukaryotic pigments. *J Bacteriol* **178**: 2471–2478.

Oldroyd GE & Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu Rev Plant Biol* **59**: 519–546.

Oono R & Denison RF (2010) Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant Physiol* **154**: 1541–1548.

Oono R, Schmitt I, Sprent JI & Denison RF (2010) Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. *New Phytol* **187**: 508–520.

Ott T, van Dongen JT, Gunther C *et al.* (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Curr Biol* **15**: 531–535.

Paau AS, Oro J & Cowles JR (1979) DNA content of free living rhizobia and bacteroids of various *Rhizobium*–legume associations. *Plant Physiol* **63**: 402–405.

Pappas G, Papadimitriou P, Akritidis N, Christou L & Tsianos EV (2006) The new global map of human brucellosis. *Lancet Infect Dis* **6**: 91–99.

Perret X, Staehelin C & Broughton WJ (2000) Molecular basis of symbiotic promiscuity. *Microbiol Mol Biol Rev* **64**: 180–201.

Pessi G, Ahrens CH, Rehrauer H, Lindemann A, Hauser F, Fischer HM & Hennecke H (2007) Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. *Mol Plant Microbe Interact* **20**: 1353–1363.

Podda E, Benincasa M, Pacor S, Micali F, Mattiuzzo M, Gennaro R & Scocchi M (2006) Dual mode of action of Bac7, a proline-rich antibacterial peptide. *Biochim Biophys Acta* **1760**: 1732–1740.

Poole PS, Schofield NA, Reid CJ, Drew EM & Walshaw DL (1994) Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology* **140**: 2797–2809.

Prell J, White JP, Bourdes A, Bunnewell S, Bongaerts RJ & Poole PS (2009) Legumes regulate *Rhizobium* bacteroid development and persistence by the supply of branched-chain amino acids. *P Natl Acad Sci USA* **106**: 12477–12482.

Que NL, Ribeiro AA & Raetz CR (2000a) Two-dimensional NMR spectroscopy and structures of six lipid A species from *Rhizobium etli* CE3. Detection of an acyloxyacyl residue in each component and origin of the aminogluconate moiety. *J Biol Chem* **275**: 28017–28027.

Que NL, Lin S, Cotter RJ & Raetz CR (2000b) Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*. Demonstration of a conserved distal unit and a variable proximal portion. *J Biol Chem* **275**: 28006–28016.

Raetz CRH & Whitfield C (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**: 635–700.

Raetz CR, Reynolds CM, Trent MS & Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* **76**: 295–329.

Ramos-Vega AL, Davila-Martinez Y, Sohlenkamp C, Contreras-Martinez S, Encarnacion S, Geiger O & Lopez-Lara IM (2009) SMb20651 is another acyl carrier protein from *Sinorhizobium meliloti*. *Microbiology* **155**: 257–267.

Rivas-Santiago B, Schwander SK, Sarabia C *et al.* (2005) Human b-defensin 2 is expressed and associated with *Mycobacterium tuberculosis* during infection of human alveolar epithelial cells. *Infect Immun* **73**: 4505–4511.

Rivas-Santiago B, Sada E, Tsutsumi V, Aguilar-Leon D, Contreras JL & Hernandez-Pando R (2006) beta-Defensin gene expression during the course of experimental tuberculosis infection. *J Infect Dis* **194**: 697–701.

Robertson JG & Lyttleton P (1984) Division of peribacteroid membranes in root nodules of white clover. *J Cell Sci* **69**: 147–157.

Robledo M, Jimenez-Zurdo JI, Velazquez E *et al.* (2008) *Rhizobium* cellulase CelC2 is essential for primary symbiotic infection of legume host roots. *P Natl Acad Sci USA* **105**: 7064–7069.

Robledo M, Jimenez-Zurdo JI, Soto MJ, Velazquez E, Dazzo F, Martinez-Molina E & Mateos PF (2011) Development of functional symbiotic white clover root hairs and nodules requires tightly regulated production of rhizobial cellulase CelC2. *Mol Plant Microbe Interact* **24**: 798–807.

Roop RM II, Robertson GT, Ferguson GP, Milford LE, Winkler ME & Walker GC (2002) Seeking a niche: putative contributions of the *hfq* and *bacA* gene products to the successful adaptation of the brucellae to their intracellular home. *Vet Microbiol* **90**: 349–363.

Roop RM II, Gaines JM, Anderson ES, Caswell CC & Martin DW (2009) Survival of the fittest: how *Brucella* strains adapt to their intracellular niche in the host. *Med Microbiol Immunol* **198**: 221–238.

Salomon RA & Farias RN (1995) The peptide antibiotic microcin 25 is imported through the *tonB* pathway and the SbmA protein. *J Bacteriol* **177**: 3323–3325.

Scheres B, van Engelen F, van der Knaap E, van de Wiel C, van Kammen A & Bisseling T (1990) Sequential induction of nodulin gene expression in the developing pea nodule. *Plant Cell* **2**: 687–700.

Schroeder BO, Wu Z, Nuding S *et al.* (2011) Reduction of disulphide bonds unmasks potent antimicrobial activity of human beta-defensin 1. *Nature* **469**: 419–423.

Scocchi M, Romeo D & Zanetti M (1994) Molecular cloning of Bac7, a proline- and arginine-rich antimicrobial peptide from bovine neutrophils. *FEBS Lett* **352**: 197–200.

Scocchi M, Mattiuzzo M, Benincasa M, Antcheva N, Tossi A & Gennaro R (2008) Investigating the mode of action of proline-rich antimicrobial peptides using a genetic approach: a tool to identify new bacterial targets amenable to the design of novel antibiotics. *Methods Mol Biol* **494**: 161–176.

Sen D & Weaver R (1981) A comparison of nitrogen-fixing ability of peanut, cowpea and siratro plants nodulated by different strains of *Rhizobium*. *Plant Soil* **60**: 317–319.

Sen D & Weaver RW (1984) A basis for different rates of N2-fixation by the same strains of *Rhizobium* in peanut and cowpea root nodules. *Plant Science Letters* **34**: 239–246.

Sharypova LA, Niehaus K, Scheidle H, Holst O & Becker A (2003) *Sinorhizobium meliloti acpXL* mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does not express a slow migrating form of lipopolysaccharide. *J Biol Chem* **278**: 12946–12954.

Sieberer B & Emons AMC (2000) Cytoarchitecture and pattern of cytoplasmic streaming in root hairs of *Medicago truncatula* during development and deformation by nodulation factors. *Protoplasma* **214**: 118–127.

Sieberer BJ, Timmers ACJ, Lhuissier FGP & Emons AMC (2002) Endoplasmic microtubules configure the subapical cytoplasm and are required for fast growth of *Medicago truncatula* root hairs. *Plant Physiol* **130**: 977–988.

Soupene E, Foussard M, Boistard P, Truchet G & Batut J (1995) Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. *P Natl Acad Sci USA* **92**: 3759–3763.

Tan BH, Meinken C, Bastian M *et al.* (2006) Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* **177**: 1864–1871.

Tan XJ, Cheng Y, Li YX, Li YG & Zhou JC (2009) BacA is indispensable for successful *Mesorhizobium-Astragalus* symbiosis. *Appl Microbiol Biotechnol* **84**: 519–526.

Tjepkema JD & Cartica RJ (1982) Diffusion limitation of oxygen uptake and nitrogenase activity in the root nodules of *Parasponia rigida* Merr. and Perry. *Plant Physiol* **69**: 728–733.

Udvardi MK & Day DA (1997) Metabolite transport across symbiotic membranes of legume nodules. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 493–523.

Vallecillo AJ & Espitia C (2009) Expression of *Mycobacterium tuberculosis* pe_pgrs33 is repressed during stationary phase and stress conditions, and its transcription is mediated by sigma factor A. *Microb Pathog* **46**: 119–127.

Van De Velde W, Zehirov G, Szatmari A *et al.* (2010) Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* **327**: 1122–1126.

Vanderlinde EM, Muszynski A, Harrison JJ *et al.* (2009) *Rhizobium leguminosarum* biovar *viciae* 3841, deficient in 27-hydroxyoctacosanoate-modified lipopolysaccharide, is impaired in desiccation tolerance, biofilm formation and motility. *Microbiology* **155**: 3055–3069.

Vasse J, de Billy F, Camut S & Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J Bacteriol* **172**: 4295–4306.

Vedam V, Kannenberg EL, Haynes JG, Sherrier DJ, Datta A & Carlson RW (2003) A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. *J Bacteriol* **185**: 1841–1850.

Vedam V, Haynes JG, Kannenberg EL, Carlson RW & Sherrier DJ (2004) A *Rhizobium leguminosarum* lipopolysaccharide lipid-A mutant induces nitrogen-fixing nodules with delayed and defective bacteroid formation. *Mol Plant Microbe Interact* **17**: 283–291.

Vedam V, Kannenberg E, Datta A, Brown D, Haynes-Gann JG, Sherrier DJ & Carlson RW (2006) The pea nodule environment restores the ability of a *Rhizobium leguminosarum* lipopolysaccharide *acpXL* mutant to add 27-hydroxyoctacosanoic acid to its lipid A. *J Bacteriol* **188**: 2126–2133.

Wang D, Griffitts J, Starker C *et al.* (2010) A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis. *Science* **327**: 1126–1129.

Wehmeier S, Arnold MF, Marlow VL *et al.* (2010) Internalization of a thiazole-modified peptide in *Sinorhizobium meliloti* occurs by BacA-dependent and -independent mechanisms. *Microbiology* **156**: 2702–2713.

Wei H & Layzell DB (2006) Adenylate-coupled ion movement. A mechanism for the control of nodule permeability to O2 diffusion. *Plant Physiol* **141**: 280–287.

White J, Prell J, James EK & Poole P (2007) Nutrient sharing between symbionts. *Plant Physiol* **144**: 604–614.

Whitehead LF & Day DA (1997) The peribacteroid membrane. *Physiol Plant* **100**: 30–44.

Xie F, Murray JD, Kim J, Heckmann AB, Edwards A, Oldroyd GE & Downie JA (2012) Legume pectate lyase required for root infection by rhizobia. *Proc Natl Acad Sci USA* **109**: 633–638.

Yorgey P, Lee J, Kordel J, Vivas E, Warner P, Jebaratnam D & Kolter R (1994) Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor. *P Natl Acad Sci USA* **91**: 4519–4523.

Young EJ (1995) An overview of human brucellosis. *Clin Infect Dis* **21**: 283–289.

Young ND, Debelle F, Oldroyd GE *et al.* (2011) The *Medicago* genome provides insight into the evolution of rhizobial symbioses. *Nature* **480**: 520–524.

Yurgel SN & Kahn ML (2008) A mutant GlnD nitrogen sensor protein leads to a nitrogen-fixing but ineffective *Sinorhizobium meliloti* symbiosis with alfalfa. *P Natl Acad Sci USA* **105**: 18958–18963.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Synthesis of NCR peptides.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.