Sinorhizobium meliloti functions required for resistance to antimicrobial NCR peptides and bacteroid differentiation

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Running title: NCR resistance in *Sinorhizobium meliloti*

Abstract

Legumes of the *Medicago* genus form symbiosis with the bacterium *Sinorhizobium meliloti* and develop root nodules housing large numbers of the intracellular symbionts. Members of the Nodule-specific Cysteine Rich peptide (NCRs) family induce the endosymbionts into a terminal differentiated state. Individual cationic NCRs are antimicrobial peptides that have the capacity to kill the symbiont but the nodule cell environment prevents killing. Moreover, the bacterial broad-specificity peptide uptake transporter BacA and exopolysaccharides contribute to protect the endosymbionts against the toxic activity of NCRs. Here, we show that other *S. meliloti* functions participate in the protection of the endosymbionts, including an additional broad-specificity peptide uptake transporter encoded by the *yejABEF* genes, lipopolysaccharide modifications mediated by *lpsB* and *lpxXL* as well as *rpoH1*, encoding a stress sigma factor. Mutants in these genes show *in vitro* a strain-specific increased sensitivity profile against a panel of NCRs and form nodules in which bacteroid differentiation is affected. The *lpsB* mutant nodule bacteria do not differentiate, the *lpxXL* and *rpoH1* mutants form some seemingly fully differentiated bacteroids although most of the nodule bacteria are undifferentiated, while the *yejABEF* mutants form hypertrophied but nitrogen-fixing bacteroids. The nodule bacteria of all the mutants have a strongly enhanced membrane permeability, which is dependent on the transport of NCRs to the endosymbionts. Our results suggest that *S. meliloti* relies on a suite of functions including peptide transporters, the bacterial envelope structures and stress response regulators to resist the stressful assault of NCR peptides in the nodule cells.

Importance

The nitrogen fixing symbiosis of legumes with rhizobium bacteria has a predominant ecological role in the nitrogen cycle and has the potential to provide the nitrogen required for plant growth in agriculture. The host plants allow the rhizobia to colonize specific symbiotic organs, the nodules, in large numbers in order to produce sufficient reduced nitrogen for the plant needs. Some legumes, including *Medicago* spp., produce massively antimicrobial peptides to keep this large bacterial population in check. These peptides, known as NCRs, have the potential to kill the rhizobia but in nodules, they only inhibit the division of the bacteria, which maintain a high nitrogen fixing activity. In this study, we show that the tempering of the antimicrobial activity of the NCR peptides in the *Medicago* symbiont *Sinorhizobium meliloti* is multifactorial and requires the YejABEF peptide transporter, the lipopolysaccharide outer membrane and the stress regulator RpoH1.

Introduction

Antimicrobial peptides (AMPs) are a diverse group of defense peptides, which are essential mediators of innate immunity in eukaryotes. Their function is to attack and kill unwanted and potentially harmful invading microbes. Animals deficient in a specific AMP or in the regulation of AMP expression are more sensitive to bacterial or fungal infections, while the ectopic expression of AMPs in animals and plants can enhance resistance (1–3). AMPs are also widely used as essential regulators of bacteria in symbiotic associations (2). In symbiosis, host organisms intentionally maintain bacterial partners and the role of "symbiotic" AMPs is therefore not to eradicate the symbiotic microbes but rather to police them or to optimize their metabolic integration with the hosts (2, 4).

An extreme case of deployment of AMPs for controlling endosymbiont populations, involving hundreds of peptides, is described in the rhizobium-legume symbiosis (2, 5–9). Legumes form a symbiosis with phylogenetically diverse nitrogen-fixing soil bacteria, collectively called rhizobia. This nutritional symbiosis provides reduced nitrogen to the plants, enabling them to grow in nitrogen-poor soils that are normally limiting growth. The symbiosis implies the formation of nodules, specific symbiotic organs, on the roots of the plants. These nodules house the nitrogen-fixing rhizobia, which transfer their produced ammonia to the plant in return for the exclusive niche that the plant creates for them in the nodules where they multiply massively from a single or very few infecting bacteria to a population of millions.

After endocytic uptake by the symbiotic nodule cells, the multiplied bacteria reside intracellularly in vesicles called symbiosomes. The nodule cells and symbiosomes establish the optimal conditions for nitrogen fixation and metabolic exchange with the endosymbionts and at the same time, keep them in check. The low oxygen levels prevailing in the symbiotic nodule cells transform the rhizobia into a differentiated physiological state, called bacteroid, which is adapted for nitrogen fixation. Moreover, in certain legume clades like the Inverted Repeat Lacking Clade (IRLC) and the Dalbergioids, the physiological transition of the bacteroids is accompanied with a remarkable differentiation process that is manifested in an irreversible loss of the capacity of bacteroids to divide (10, 11). These terminally differentiated bacteroids have a partially permeabilized cell membrane. They are giant bacterial cells, being either long, wide rods, resulting from polar cell elongation, or large spheres, resulting from an isodiametric growth. A switch in the bacterial cell cycle, from a regular cycle of replication and division to a cycle of repeated genome replications without divisions, drives this cell enlargement, resulting in polyploid bacteroids.

The terminal differentiation is triggered by a family of Nodule-specific Cysteine-Rich (NCR) peptides, produced by the symbiotic nodule cells (11–13). In *Medicago truncatula*, over 600 *NCR* genes were identified in the genome, and from a few to several hundreds of them are present also in other species of the IRLC and the Dalbergoids (11, 12, 14, 18). The 600 *NCR* genes in *M. truncatula* are specifically

expressed in the symbiotic cells (15). However, they are activated in different waves during the

differentiation of the symbiotic cells and bacteroids, including sets of *NCR* genes activated at the onset

and others at the intermediate or final stages of the differentiation. These temporal profiles indicate

that the different *NCR* genes have specific functions during the bacteroid formation process.

The NCR peptides have structural features shared with AMPs and at least some NCRs, in particular the

cationic ones, can *in vitro* kill or inhibit the growth of not only the rhizobium symbionts but also many

other bacteria and even fungi (16). Their major antibacterial activity results from their capacity to

disturb the integrity of the inner and outer membranes of bacteria (13, 17), although some NCRs have

also inhibiting activities on essential intracellular machineries (18). However, bacteroids remain

metabolically active for very long, despite the high burden of NCRs. Possibly, the environment of the

symbiotic nodule cells and symbiosomes contribute to temper the antimicrobial activity of the

peptides. Importantly, also specific functions of the bacteria themselves are NCR-resistance

determinants in the bacteroids.

Sinorhizobium meliloti, the symbiont of *Medicago* plants, requires the peptide transporter BacA to

counter the NCR peptides inside the symbiotic nodule cells and establish a chronic infection (19). *S.*

meliloti bacA mutants are hypersensitive to the antimicrobial NCRs. They induce nodules and infect

their symbiotic cells seemingly normally but the mutants die as soon as they are released in the

symbiotic cells. This death can be avoided by blocking NCR transport to the infecting rhizobia in the *M.*

truncatula dnf1 mutant (19). BacA proteins are broad-specificity peptide uptake transporters (20–22).

They can promote the uptake of NCR peptides, suggesting that BacA provides resistance by redirecting

the peptides away from the bacterial membrane, thereby limiting membrane damage.

Exopolysaccharide (EPS) is another known factor of *S. meliloti* that helps the endosymbionts to

withstand the NCRs (14, 23). This negatively charged extracellular polysaccharide traps the cationic

AMPs, reducing their effective concentration in the membrane vicinity.

Bacterial resistance to AMPs is usually multifactorial (24), suggesting that besides BacA and EPS,

additional functions of *S. meliloti* bacteroids contribute to resist the NCRs in the symbiotic nodule cells.

The literature on *S. meliloti* is rich in the description of bacterial genes that are required for symbiosis.

However, often the reporting on these mutants lacks precise information on their bacteroid phenotype

and/or on their sensitivity to NCRs. Moreover, transcriptome and Tn-seq (transposon sequencing)

analyses of NCR-treated cells and NCR-protein interaction studies identified a whole suite of additional

candidate NCR-responsive functions in *S. meliloti* (18, 25–27). Together with BacA and EPS, some of

these *S. meliloti* functions may contribute to alleviate the NCR stress on the bacteroids. To test this

hypothesis, we have selected in the present study three candidate functions and analyzed the

phenotype of the corresponding mutants in NCR resistance and bacteroid formation.

Results

Sinorhizobium meliloti mutants with enhanced sensitivity to NCR peptides

The *S. meliloti* functions selected in this study include a broad specificity peptide uptake transporter encoded by the *yejABEF* genes (SMc02829-SMc02832), lipopolysaccharide (LPS) modifications mediated by *lpsB* (SMc01219) and *lpxXL* (SMc04268) as well as *rpoH1* (SMc00646), encoding a stress sigma factor. The YejABEF ABC transporter was selected on the basis of a genetic screen by Tn-seq in *S. meliloti* revealing that the mutant has an increased sensitivity to the peptide NCR247 (25). LPS structure is one of the major determinants of AMP resistance and sensitivity in Gram-negative bacteria (24, 28). The selected genes *lpsB* and *lpxXL* encode respectively a glycosyltransferase involved in the synthesis of the LPS core and a very-long-chain fatty acid acyl transferase involved in the biosynthesis of lipid A. Mutants in these genes are affected in resistance to AMPs and in symbiosis (29, 30). Finally, the *rpoH1* gene is a global stress regulator in *S. meliloti* (31). The gene as well as its target genes are upregulated in NCR247 treated cells (26, 27). An *rpoH1* mutant is also affected in symbiosis (32). These selected genes are expressed in nodules, with peak expression in different regions of the nodule where bacteria infect plant cells, undergo the differentiation process or fix nitrogen (15, 33)(Fig. S1).

Mutants in these candidate genes were constructed (*yejA*, *yejE*, *yejF*, *rpoH1*) or were obtained from previous studies (*lpsB*, *lpxXL*). Log-phase bacteria were treated with a small panel of NCR peptides that were previously shown to have antimicrobial activity (14). These tested peptides were NCR169, NCR183, NCR247 and NCR280, which displayed three different expression patterns in the nodule tissues (15). The *NCR280* gene is expressed in the younger nodule cells, the *NCR169* gene in the older cells while *NCR183* and *NCR247* have an intermediate expression pattern (Fig. S1). The bacterial survival after treatment was determined by counting colony-forming units. The selected mutants were tested along with the wild-type strain and the *bacA* mutant, which was previously shown to be hypersensitive to NCRs (19). The four tested NCR peptides had a strong antimicrobial activity against the wild-type strain, which displayed a survival rate ranging from 8% to 0.03% depending on the tested peptide (Table S1). In agreement with previous results, the *bacA* mutant was hypersensitive to the four peptides (Table S1; Fig. 1). Interestingly, the newly analyzed mutants all displayed a higher sensitivity to at least one of the peptides compared to the wild type (Table S1; Fig. 1). The *lpxXL* mutant was more sensitive to the four peptides, the *lpsB*, *yejE* and *yejF* mutants were more sensitive to NCR183, NCR247 and NCR280. The *yejA* mutant was more sensitive to NCR280 and the *rpoH1* mutant was more sensitive to NCR247. The differential response towards peptide NCR247 of the *yejA* mutant on the one hand and the *yejE* and *yejF* mutants on the other hand, corresponds to the previously described Tn-seq screen with this peptide (25). Taken together, this analysis indicated that each mutant displayed a specific sensitivity profile to the panel of tested peptides.

Nodule formation by NCR-sensitive *Sinorhizobium meliloti* mutants

Next, the phenotype of these mutants in symbiosis with *M. truncatula* was compared with the wild-

type strain and the *bacA* mutant. Measurement of the nitrogen fixation activity and macroscopic

inspection of the root system of plants inoculated with the wild type and seven mutants (Fig. S2)

revealed that, besides the wild type, mutants in the *yejA*, *yejE*, *yejF* and *lpxXL* genes formed functional

nodules (Fix$^+$), although the *yejE*, *yejF* and *lpxXL* mutants had a reduced nitrogen-fixation activity. On

the other hand, the *bacA*, *lpsB* and *rpoH* mutants formed non-functional (Fix$^-$) and abnormal looking

nodules that were small and white, in agreement with previous descriptions (19, 29, 32).

The histological organization of the nodules formed by the mutants and the formation of infected

symbiotic cells and the viability of the bacteria they contain, were analysed using confocal microscopy

(Fig. 2A). The used staining procedure highlights the nodule bacteria with a green fluorescence signal

(SYTO^{TM}9) when their membranes are well preserved and with a red fluorescence (propidium iodide)

when their membranes are highly permeable. As previously reported, wild-type nodules formed

symbiotic cells infected with green-labelled elongated bacteroids, while the nodules infected with the

bacA mutant contained symbiotic cells carrying small undifferentiated bacteria staining red (19).

On the other hand, contrary to what we expected from the macroscopic inspection of the nodules and

their ability to fix nitrogen, the *yejA*, *yejE* and *yejF* mutant bacteroids were substantially altered

compared to wild-type bacteroids. A high proportion of them were red-stained indicating that their

membranes were strongly permeabilized. Nevertheless, other host cells contained bacteroids stained

in green by the SYTO^{TM}9 dye. LpxXL is known to be important, but not essential to *S. meliloti* during

symbiosis with alfalfa (*Medicago sativa*) (34). The mutant forms hypertrophied bacteroids (30). In our

experiments, the *lpxXL* mutant displayed elongated bacteroids but mostly permeable to propidium

iodide (stained in red). This observation confirmed that *S. meliloti* LpxXL is also essential for the normal

bacteroid differentiation process in *M. truncatula* but that it is not crucial for infection and nitrogen

fixation. The *rpoH1* mutant formed elongated bacteroids that were, however, strongly stained by

propidium iodide, in agreement with the Fix$^-$ phenotype of the nodules and the described phenotype

of the mutant in alfalfa (32). Inside the small bumps elicited by the *lpsB* mutant, no cell seems to be

colonised by bacteria as revealed by confocal microscopy. Thus, the *S. meliloti lpsB* mutant failed to

colonize the host cells and is defective at an earlier stage in infection than the *bacA* mutant, which

does form infected plant cells. It was previously reported that the *lpsB* mutant colonizes nodules cells

in *M. sativa* suggesting a more severe defect in *M. truncatula* (35, 36). Therefore, we tested the

phenotype of the selected mutants also on *M. sativa* (Fig. S3). The *lpsB*, *rpoH1* and the *yejA* mutants

had indeed milder phenotypes on this host while the other mutants had similar phenotypes on the

two hosts.

Can the increased membrane permeability of the NCR-sensitive *Sinorhizobium meliloti* mutants be
attributed to the NCRs in nodules?
The high membrane permeability of the nodule bacteria of the *bacA* mutant is the result of the action
of the NCR peptides in the nodule cells on this NCR-hypersensitive mutant (19). Possibly, the same is
true for the other mutants. To test this hypothesis, we made use of the *M. truncatula dnf1* mutant,
which is defective in a nodule-specific subunit of the signal peptidase complex (37). This mutant cannot
transport NCR peptides to the symbiosomes (13). As a result, the wild-type *S. meliloti* nodule bacteria
in this mutant are not differentiated. The *bacA* mutant, which is strongly permeabilized and stained by
propidium iodide in nodules of wild-type *M. truncatula* plants, is not so in the *dnf1* mutant nodule cells
because the bacteria are not challenged anymore with the NCRs (Fig. 2B)(19). Similarly to the *bacA*
mutant, the *yejA*, *yejE*, *yejF*, *lpxXL* and *rpoH1* mutants did not display membrane permeability, as
revealed by the absence of propidium iodide staining, in the infected nodule cells of the *dnf1* mutant
(Fig. 2B). This suggests thus that these mutants become membrane-permeabilized by the action of the
NCRs and that their symbiotic defects are at least in part due to their hypersensitivity to the NCRs (Fig.
1). The *lpsB* mutant did not form detectable nodule-like structures on the *dnf1* roots. We can therefore
not conclude on the involvement of the NCR peptides in the symbiotic phenotype of this mutant.
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Bacteroid differentiation in the NCR-sensitive *Sinorhizobium meliloti*
A very strong cell enlargement and an increase in the ploidy level of the bacteria characterize the
differentiated bacteroids in *Medicago* nodules. These parameters are readily measured by DAPI
staining and flow cytometry (10). The wild-type bacteroids from *M. truncatula* nodules had a high DNA
content, over twenty fold higher than the DNA content in free-living *S. meliloti* (Fig. 3) and increased
scatter parameters reflecting the cell enlargement (Fig. S4). As previously reported, the nodules
infected with the *bacA* mutant did not contain differentiated bacteria (Fig. 3, Fig. S4)(21). The bacteria
in nodules induced by the *lpsB* mutant, probably located in infection threads, had a profile that
confirmed the complete absence of differentiated bacteria (Fig. 3, Fig. S4). Nodules infected with the
rpoH1 mutant had mostly undifferentiated bacteria although a small amount of fully differentiated
cells was detected, as well as cells in an intermediate stage. Also the nodules of the *lpxXL* mutant
contained many undifferentiated bacteria as well as fully differentiated ones (Fig. 3, Fig. S4). In
contrast, the *yejA*, *yejE* and *yejF* mutant nodules contained large numbers of fully differentiated
bacteria (Fig. 3, Fig. S4).
It was reported previously that the *lpxXL* mutant formed nodules containing misshapen hypertrophied
and larger bacteria than the wild-type bacteroids (30). In our study, this difference was detectable in
the flow cytometry measurements showing a higher DAPI fluorescence and scatter for the small
portion of differentiated bacteria in these nodules (Fig. 3, Fig. S4). Moreover, we noticed that the

bacteroids in the nodules infected with the *yejA*, *yejE* and *yejF* mutants displayed similar higher DNA

fluorescence and scatter (Fig. 3, Fig. S4), suggesting that also these bacteroids have abnormal

morphologies.

These patterns, described here for *M. truncatula* nodules were overall similar in the *M. sativa* host

plant although the *rpoH1* mutant showed a higher number of intermediate and fully differentiated

bacteroids and the *yejA* mutant had a profile similar to the wild type in nodules at 21 dpi while larger

than normal bacteroids were only detected at 32 dpi (Fig. S5). These differences corresponded well

with the macroscopic and microscopic differences in the nodules between the two host plants (Fig. 2,

Fig. S2, Fig. S3).

Defective bacteroid differentiation in *yejE* and *yejF* mutants

To confirm the altered bacteroid morphologies in the *yej* mutants, suggested by the cytometry

analysis, the *yejE* and *yejF* mutants were observed at high magnification by microscopy. Confocal and

transmission electron microscopy of *M. sativa* nodule sections showed that the nodule cells infected

with the *yejE* or *yejF* mutant contained a very heterogeneous population of abnormal bacteroid

morphs, including elongated, spherical, pear-shaped or irregular blob-like cells (Fig. 4A, Fig. S6A). These

cells contrasted strongly with the narrow, elongated wild-type bacteroids. The difference was also

obvious by fluorescence microscopy of preparations of purified nodule bacteria (Fig. 4B). The

quantification of cell morphology parameters by image analysis with MicrobeJ of free-living bacteria

and the purified nodule bacteria confirmed the irregular forms of *yejE* and *yejF* bacteroids, notably

showing that these bacteroids are broader and more spherical than the elongated wild-type bacteroids

(Fig. 4C; Fig. S6B). Transmission electron microscopy further showed that the cytoplasm and inner

membrane of many of the *yejF* nodule cells was retracted, leaving very large intermembrane spaces,

which in some cases even developed into vacuoles, entirely surrounded by cytoplasm. Some cells had

multiple vacuoles, small and large (Fig. S6A).

The strongly abnormal bacteroids in the *yej* mutants is *a priori* contradictory with the nitrogen fixation

activity of these nodules. Expression of the *nifH* gene is a marker for nitrogen-fixing bacteroids (38). A

GFP gene under the control of the *nifH* promoter was introduced into the wild-type strain and the *bacA*

(as a negative control), *yejE* and *yejF* mutants. Analysis of the GFP fluorescence in nodule bacteria of

M. truncatula (Fig. 5) and *M. sativa* (Fig. S7) by confocal microscopy and flow cytometry showed that

despite their aberrant morphology, the *yejE* and *yejF* bacteroids are functional.

In contrast to the bacteroids, in the cultured bacteria, no differences were observed in the

ultrastructure of the wild-type and *yejF* mutant bacteria (Fig. S8). Moreover, the mutants behaved like

wild type in unstressed growth in culture or in the presence of hydrogen peroxide, sodium chloride

and acid stress (Fig. S8). On the other hand, the *yej* mutants were more sensitive than wild type to the

exposure with the detergent sodium dodecyl sulfate (Fig. S8), indicating that these mutants have a

reduced ability to cope with membrane stress. Together, these phenotypes are in agreement with the

bacteroid defects resulting from a nodule-specific stress such as one derived from the NCR peptides.

NCR247 uptake by the YejABEF transporter

In *E. coli*, both YejABEF and the BacA homolog SbmA mediate the transport of microcin C peptides.

Therefore, we tested whether the overlap in substrates of YejABEF and BacA can be extended to NCR

peptides which are known substrates of BacA (20, 21). We tested the impact of the mutants in the

YejABEF transporter on NCR247 uptake and compared it with a mutation in *bacA*. With a flow

cytometry based assay and a fluorescent derivative of the NCR247 peptide, we found that while the

NCR247 uptake is completely abolished in the *bacA* mutant, as expected, its uptake is also reduced but

not completely abolished in the three *yej* mutants (Fig. 6). This suggests that the YejABEF transporter

contributes to NCR uptake.

Discussion

Multiple functions of *Sinorhizobium meliloti* contribute to NCR resistance and are required for

bacteroid formation and persistence

The NCR peptides are a two-edged sword. On the one hand, they maneuver the rhizobial

endosymbionts into a terminally differentiated state via a multitude of activities on the bacteria from

which the membrane permeabilization, cell cycle perturbation (polyploidization) and cell enlargement

are the most visible ones. On the other hand, NCRs have antimicrobial activity, potentially killing the

endosymbionts. Therefore, rhizobia have to defend themselves to be able to establish a chronic

infection in the NCR-producing symbiotic cells of the nodules. Previous work has identified the BacA

peptide transporter and the EPS barrier in the bacterial envelope as defenses of *Sinorhizobium* strains

against the NCRs of their *Medicago* hosts (14, 19, 23).

Here, we defined three new functions in *S. meliloti*, the LPS, the sigma factor RpoH1, and the YejABEF

peptide transporter as additional determinants in bacteroids, required to cope with the NCR peptides.

We show that knockout mutants in these genes are more sensitive to a panel of antimicrobial NCRs

and that this hypersensitivity is correlated with a strongly enhanced membrane permeability of the

nodule bacteria and abnormalities in their morphology and ploidy levels. It is striking, however, that

the different mutants have markedly different bacteroid phenotypes, ranging from undifferentiated

to hyper-differentiated. This can be attributed to at least three factors. Each mutant has a specific NCR-

sensitivity profile when tested against a small panel of peptides and the "NCR landscape" present in

the developing symbiotic cells is continuously changing because of the expression of the hundreds of

NCR genes in different waves during symbiotic cell differentiation (15). Accordingly, each mutant could

accumulate NCR-induced damage at a different rate and reach the breaking point at different stages

over the course of the bacteroid differentiation process (Fig. S1). Moreover, the expression pattern of

the bacterial genes in the nodule zones is different, suggesting that their principal impact is situated

at different stages of the symbiotic cell development and bacteroid differentiation. In addition, we

cannot exclude that these genes provide supplementary contributions to the survival of the nodule

bacteria, other than the response to the NCR peptides. Nevertheless, blocking the targeting of the NCR

peptides to the nodule bacteria by the use of the *M. truncatula dnf1* mutant prevents the membrane

permeabilization of the nodule bacteria in the mutants. This observation places the symbiotic role of

these bacterial genes downstream of the peptide targeting to the symbiosomes (except for *lpsB*, see

below) and links them, at least in part, to the NCRs.

The functions of *S. meliloti* described here and before probably picture only part of the full toolkit of

this symbiont to survive the NCR challenge. Many additional *S. meliloti* mutants are described with

symbiotic phenotypes that are suggestive for a similar contribution to NCR resistance. (26, 39–50). In

addition, other genes that could be important for symbiosis, were discovered in a Tn-seq screen with

the peptide NCR247 (25). This screen identified here-described functions like the BacA and YejABEF

transporters, the LPS and EPS biosynthesis, but also never analyzed functions. It would be of great

interest to explore the function of these genes in relation to the NCR response of bacteroids. Note that

some of these genes might have escaped identification in previous genetic screens for symbiosis

mutants because mutations might provoke only subtle phenotypes with a weak overall effect on

nitrogen fixation in standard laboratory conditions but with bacteroid alterations in morphology and

persistence as illustrated here with the *yejABEF* mutants.

Response to NCR-induced stress regulated by the alternative sigma factor RpoH1

Bacteria deal with different types of stress conditions by global transcriptional responses mediated by

alternative sigma factors. As a general classification, the RpoH sigma factors are believed to control

cytoplasmic stress responses, while the RpoE sigma factors are known to respond to periplasmic and

membrane stress (51). Given the membrane damage provoked by NCRs on the one hand, and the

general function in bacteria of RpoE sigma factors in periplasmic and membrane stress response,

including in response to AMPs (52), a role of the RpoE regulators of *S. meliloti* in bacteroid

differentiation and NCR response could *a priori* have been expected. *S. meliloti* has 11 RpoE-like sigma

factors. Remarkably, despite the fact that some RpoEs have a considerable effect on gene

transcription, all single mutants, all possible double mutants and even a mutant lacking all 11 genes

showed no detectable phenotypic difference with wild type in symbiosis or during many tested free-

living growth conditions, including growth in the presence of membrane stresses (53, 54). Thus, the 11

S. meliloti RpoEs do not have the expected role in regulating the envelope stress response.

This role seems to be taken up in part by RpoH in *S. meliloti*, in agreement with its role in symbiosis

and NCR resistance and the observation that growth of the *rpoH1* mutant is affected in the presence

of various membrane-disrupting agents (32). The latter suggests that RpoH in *S. meliloti* is not

respecting the canonical division of labor between alternative sigma factors and that its function is not

restricted to cytoplasmic stress but also encompasses membrane stresses. We propose that the here

uncovered role of RpoH1 in NCR resistance and in bacteroid differentiation is connected to its

regulation of membrane stress.

The lipopolysaccharide barrier against NCR membrane damage

In Gram-negative bacteria like rhizobia, LPS constitutes the first point of attack of cationic AMPs. In a

two-stage process, AMPs make first electrostatic interactions with the negatively charged LPS, allowing

the AMPs to approach the membrane lipids and subsequently to insert into the lipid bilayer, perforate

it and translocate into the periplasm (55). The chemical composition of LPS can influence the efficiency

of the AMP attack and pathogens have evolved mechanisms to recognize the presence of AMPs and

to modify in response the composition of the LPS to lower the potency of the AMP attack (56).

S. meliloti lpsB encodes a glycosyl transferase that participates in the biosynthesis of the LPS core (57)

and the mutation of the gene affects both the core structure and O-antigen polymerization (38, 39).

The O-antigen is thought to be a camouflage, masking the membrane and the charges in the

membrane vicinity. In the *lpsB* mutant, the shorter O-antigen could be a less efficient shield against

the NCRs, offering to the peptides an easier access to membrane-proximal charges resulting in the

increased sensitivity of the *lpsB* mutant to the NCRs (this work) as well as to other AMPs (29, 36).

The *lpsB* mutant forms nodules on *M. sativa*, which contain infected cells and has a phenotype that is

similar to the *bacA* mutant. However, this *lpsB* mutant forms uninfected nodules on *M. truncatula*

roots. Therefore, it was not possible to test the implication of NCRs in this symbiotic phenotype with

the use of the *dnf1* mutant, since the *DNF1* gene is expressed only in infected nodule cells (37). Since

also the *NCR* genes are nearly exclusively expressed in the infected symbiotic cells (15), the blockage

of the mutant before release of bacteria into nodule cells suggests that the LPS could provide

protection against another stressor produced very early on in the infection process in *M. truncatula*.

On the other hand, it was recently reported that some *NCR* genes are expressed in infected root hairs

or Nod factor-stimulated root epidermal cells (58, 59). Thus, it is possible that the challenge with these

early NCR peptides is already detrimental to the mutant, blocking any further progress in the infection

process.

LpxXL is a specific acyl transferase that introduces in the lipid A moiety of LPS the very long chain fatty

acid 27-OHC28:0 (34). This LpxXL-dependent acylation is expected to make the lipid A hydrophobic,

forming the biophysical basis for LpxXL-dependent NCR resistance. Hydrophobic lipid A increases the

thickness of the outer layer of the outer membrane and reduces the membrane fluidity, which on its

turn prevents or delays AMP insertion and membrane damage. Increasing lipid A hydrophobicity by

introducing additional acyl chains is a well-known mechanism used by *Salmonella* to enhance its

resistance against host AMPs during infection (56).

The YejABEF peptide transporter provides resistance to membrane-damaging peptides and

sensitizes bacteria to AMPs with intracellular targets

The YejABEF ABC transporter has never been analyzed before in the context of the rhizobium-legume

symbiosis. Furthermore, even though this transporter is highly conserved among proteobacteria, its

physiological role in whichever bacterium has been characterized only in a few instances. One of them

is the uptake of microcin C in *E. coli*. This translation-inhibiting AMP of bacterial origin has no action

on the bacterial membrane but has an intracellular target. A mutant in the YejABEF transporter cannot

uptake microcin C and is resistant to the AMP (60, 61). Conversely, the *Salmonella* and *Brucella yejABEF*

mutants are more sensitive to peptides with membrane-damaging activity such as defensins,

polymyxin B, protamine and melittin. Consequently, these mutants have reduced pathogenicity

because their capacity to survive in macrophages and in animals is reduced (62, 63). Thus, the

increased sensitivity of the *yejA*, *yejE* and *yejF* mutants of *S. meliloti* towards NCRs, which was

suggested by Tn-seq (25) and demonstrated here, is coherent with these previous findings.

The characteristics of the YejABEF peptide transporter towards membrane-damaging peptides versus

peptides with intracellular action are intriguingly parallel to the features of the BacA peptide

transporter (SbmA in *E. coli* and *Salmonella*). These latter transporters are required for the import of

diverse peptides with intracellular targets (microcins B and J, Bac7, Bac5 and bleomycin) and mutants

are therefore resistant to them while the same mutants are hypersensitive to membrane-active

peptides (defensins, NCRs) (19, 64, 65). Moreover, the range of peptides that can be imported overlap

between these two types of transporters. Both, YejABEF and SbmA can uptake derivatives of microcin

C (32). Furthermore, we show that both BacA and YejABEF contribute to NCR247 uptake. Intriguingly,

our genetic analysis suggests that both transporters cooperate to import this peptide, since

inactivation of one or the other abolishes or strongly reduces uptake. How these two transporters can

physically interact and cooperate is an issue of interest for future research. Nevertheless, BacA can

function (partially) in the absence of YejABEF while the inverse is not the case. This could be the basis

for the markedly different symbiotic phenotype of the *bacA* and *yej* mutants. While the *bacA* mutation

is detrimental from the earliest contact with NCRs and bacteria die as soon as they are released from

infection threads in the symbiotic cells, the *yej* mutants cope with the NCR peptides much longer and

show abnormalities only at the end of the bacteroid differentiation process and bacteroid lifespan. The

yejA gene encodes the periplasmic binding protein of the transporter. The *yejA* mutant had a different

NCR sensitivity profile than the *yejE* and *yejF* mutant and this was correlated with a different symbiotic

phenotype in the *M. sativa* host. This suggest that YejA contributes to the interaction of only a subset

of the NCR peptides.

How does the YejABEF and BacA transporters contribute to resistance to NCRs and other membrane-

damaging peptides? The most straightforward model that has been proposed before for BacA (19–21)

as well as for the unrelated SapABCDF peptide uptake transporter (66), is the reduction below a critical

threshold of the AMP concentration in the vicinity of the inner membrane. Alternatively, the presence

or activity of the transporters might indirectly affect the bacterial envelope structure, rendering it

more robust against AMPs. The higher sensitivity of the *yej* mutants towards SDS is in agreement with

this possibility. A similar detergent sensitivity and membrane alterations were reported in the *bacA*

mutant of *S. meliloti* (67).

Conclusions

The multifaceted NCR resistance required for symbiosis and chronic infection of the nodule cells

mirrors the multitude of AMP resistance mechanisms in animal pathogens, which collectively

contribute to the pathogenicity of these bacteria (2, 3, 68). However, one dimension of this strategy in

pathogens is not known in *S. meliloti*, and consists of the direct recognition of host AMPs by receptors

triggering an adaptive response. Probably the best studied AMP receptor is the two-component

regulator PhoPQ in *Salmonella*, which adjusts the LPS composition in response to the presence of host

peptides (56). Perhaps the *S. meliloti* ExoS-ChvI or FeuP-FeuQ two component regulators, which are

upregulated by NCR treatment and essential for symbiosis (26, 39, 40), form such a regulatory module,

recognizing NCRs and controlling an appropriate response in the symbiotic nodule cells. In this respect,

it is of interest to note that the rhizobial LPS structure changes strongly in bacteroids of NCR-producing

nodules (69, 70).

As shown here, rhizobia have to defend themselves to be able to establish a chronic infection in the

NCR-producing symbiotic cells of the nodules. On the other hand, the profile of NCR peptides produced

in the nodule cells is also determinant for the outcome of the symbiosis and some *M. truncatula*

mutants in individual *NCR* genes or *M. truncatula* accessions, expressing specific *NCR* alleles, display

incompatibility with *S. meliloti* strains (71–74). Thus, a fine balance must be established in the

symbiotic nodule cells between the NCR landscapes and matching multifactorial bacterial

countermeasures. Perturbations in the host or in the endosymbiont, as the ones described here,

affecting this equilibrium leads to a breakdown of the symbiosis.

Materials and Methods

Bacterial strains, plant growth and nodulation assays and analysis

The procedures for the growth of the *S. meliloti* Sm1021 strain and its derivatives, plant culture of the *M. sativa* cultivar Gabès, the *M. truncatula* accession Jemalong A17, and the A17 *dnf1* mutant, nodulation assays, acetylene reduction assays, bacteroid isolation, flow cytometry measurements and confocal microscopy were performed as described before (75). Bacterial mutants in *rpoH1*, *yejA*, *yejE* and *yejF* were obtained by plasmid insertion and gene deletion (Table S2) as described before (76). Quantitative analysis of the shape of free-living bacteria and bacteroids was performed with the MicrobeJ plugin of ImageJ (77). Bacteroid extracts and exponential phase cultures were stained with 2.5 nM SYTO™9 for 10 minutes at 37°C and mounted between slide and coverslip. Bacteria imaging was performed on a SP8 laser scanning confocal microscope (Leica microsystems) equipped with hybrid detectors and a 63x oil immersion objective (Plan Apo, NA: 1.4, Leica). For each condition, multiple z-stacks (2.7μm width, 0.7 μm step) were automatically acquired (excitation: 488 nm; collection of fluorescence: 520-580 nm). Stacks were transformed as maximum intensity projections using ImageJ software (78). Bacteria in the image stacks were automatically detected with MicrobeJ using an intensity based threshold method with a combination of morphological filters. To ensure high data quality every image was manually checked to remove false positive objects (mainly plant residues). Morphological parameters were directly extracted from MicrobeJ and figures were created with Excel or ggplot2 in R.

In vitro **sensitivity and peptide uptake assays**

NCR sensitivity assays were carried out essentially as described (20). To measure the resistance of strains to sodium dodecyl sulfate (SDS), H_2O_2, HCl and NaCl stress, overnight cultures of the wild type and mutants were diluted to OD_{600}=0.2. A total of 100 μL of these suspensions was added to 3 ml soft agar (0.7% agar) and poured onto standard 1.5% agar plates. After solidification of the soft agar, filter paper disks (5 mm diameter) were placed on the center of the plate, and 5 μL of 10% (w/v) SDS, 2 M HCl or 30% H_2O_2 was added to the disks. Plates were incubated at 30°C for 3 days, and the diameter of the clearing zone was measured. Peptide uptake assays were performed as described (20, 79).

TEM

Bacterial suspensions at OD_{600}=6 or 21 days old nodule samples were incubated in fixative (3% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate, pH 6.8) for one to three hours, and washed with 0.1 M cacodylate buffer, pH 6.8. Samples were then incubated one hour in 1% osmium tetroxide, 1.5% potassium ferrocyanide in water. After washing, bacterial suspensions were pelleted in 2% low

melting point agarose to facilitate their manipulation. Samples were dehydrated by incubation in
increasing concentrations of ethanol (nodule samples for a total of 4 hours in 10-20-30-50-70-90-
100%-absolute ethanol-propylene oxide; bacterial pellets for a total of 2 hours in 10-30-50-70-90-
100%-absolute ethanol), followed by infiltration with epoxy resin (low viscosity Premix Kit medium,
Agar) (for nodule samples, 3 days of infiltration; for bacterial pellets, 24h of infiltration) and
polymerization for 24h at 60°C. Ultrathin sections (80-70 nm) were obtained with an ultramicrotome
EM UC6 (Leica Microsystems) and collected on formvar carbon-coated copper grids (Agar). Sections
were stained with 2% uranyl acetate (Merck) and lead citrate (Agar) before observation with a JEOL
JEM-1400 transmission electron microscope operating at 120kV. Images were acquired using a
postcolumn high-resolution (11 megapixels) high-speed camera (SC1000 Orius; Gatan) and processed
with Digital Micrograph (Gatan).

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Author contributions
QN, QB and NB have initiated jointly this work. QN, QB, NB, ML, RL, CB and TT constructed strains and
performed nodulation assays. QN, QB, RL, CB and TT performed microscopy and flow cytometry. QB,
SJ, AK and EK performed the *in vitro* experiments and provided reagents. QN, QB, NB, TT, BA and PM
conceived the study and analyzed the data. PM wrote the manuscript with input from all authors.

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 Figure legends

 Figure 1. Sensitivity profile of *Sinorhizobium meliloti* strains to a panel of NCR peptides. The heatmap
 shows the relative survival, expressed in % of the mutant strains compared to the wild type (WT)
 survival set at 100% for each peptide treatment.

 **Figure 2. Symbiotic phenotype of *Sinorhizobium meliloti* Sm1021 mutants during symbiosis with
 Medicago truncatula wild type and *dnf1* mutant. A.** Membrane permeability in bacteroids of *S.
 meliloti* Sm1021 wild type (WT) and mutants in wild-type *M. truncatula* nodules, determined by live-
 dead staining of nodule sections and confocal microscopy. **B.** Membrane permeability of *S. meliloti*
 Sm1021 WT and mutants in nodules of the *M. truncatula dnf1* mutant, determined by live-dead
 staining of nodule sections and confocal microscopy. Nodule phenotype at 21 days post inoculation.
 Top row images, full nodule sections; Bottom row images, enlarged images of symbiotic cells. Scale
 bars are indicated in each panel. The staining procedure of nodule sections with a mixture of the dyes
 propidium iodide, SYTO™9 and calcofluor-white highlights the bacteroids and nodule bacteria with a
 green fluorescence signal (SYTO™9) when their membranes are well preserved and with a red
 fluorescence (propidium iodide) when their membranes are highly permeable. Plant cell walls are
 stained blue (calcofluor-white).

 Figure 3. DNA content of nodule bacteria in *Medicago truncatula*. Flow cytometry analysis of the DNA
 content of bacteria in culture or isolated from nodules infected with the indicated strains and stained
 with 4',6-diamidino-2-phenylindole (DAPI). The cell counts (y-axes) are represented in function of the
 DAPI fluorescence intensity (x-axes). The arrow in each graph indicates the mean DNA content of wild-
 type (WT) bacteroids as in the upper left panel.

 Figure 4. Bacteroid morphology of *yejE* and *yejF* mutants in *Medicago sativa* nodules. A. Sections of
 nodules infected with the wild type (WT) and the *yejE* or *yejF* mutants were stained with a mixture of
 the dyes propidium iodide, SYTO™9 and calcofluor-white and observed by confocal microscopy. Scale
 bars (10 μm) are indicated in each panel. **B.** Preparations of cultured bacteria or purified *M. sativa*
 nodule bacteria of the wild type and the *yejF* mutant were observed by fluorescence microscopy. The
 panels are composite images and the shown individual cells were cut from original images and
 recombined in a single panel. Each panel is at the same magnification and the scale bar (10 μm) is
 indicated in the left panel. **C.** Cell shape parameters of free-living bacteria and bacteroids determined
 by MicrobeJ. Dot plots show the mean width and length of cells of the indicated strains. Each point
 represents the values of one bacterium. Numbers of analysed cells (n) are indicated.

Figure 5. Nitrogenase expression in the *yejE* and *yejF* mutant bacteroids in *Medicago truncatula*
nodules. A. Confocal microscopy of sections of nodules infected with *S. meliloti* Sm1021.p*pnifH-GFP*
(WT), *bacA*.p*pnifH-GFP* (*bacA*), *yejE*.p*pnifH-GFP* (*yejE*) or *yejF*.p*pnifH-GFP* (*yejF*) and stained with
propidium iodide. **B.** Flow cytometry of GFP levels in differentiated nodule bacteria (upper panels) and
heat-killed nodule bacteria (lower panels). The green square shows the position of the GFP-positive
bacteroids.

Figure 6. Uptake of NCR247 mediated by YejABEF. Flow cytometry assay of the NCR247-FITC peptide.
In the WT, a subpopulation of the bacteria import the NCR247-FITC peptide and in the *bacA* mutant,
this population is absent. In the *yejA*, *yejE* and *yejF* mutants, the FITC-positive subpopulation is strongly
reduced compared to wild type.

Figure S1. Expression pattern in nodules of bacterial and *NCR* genes. A. Schematic drawing of a
Medicago nodule organized in tissues. The meristem is bacteria-free and contains dividing cells
allowing the organ to grow. In the early infection zone, bacteria proliferate in infection threads (green
lines) and are released by endocytosis inside cells derived from the meristem. In the late infection zone
and the so-called interzone, the bacteria differentiate into bacteroids. The fixation zone contains the
fully differentiated, nitrogen-fixing bacteroids. The pictures show bacteria inside the nodule cells and
are presented at the same scale (bar is 1 μm) allowing to appreciate the transformation of the bacteria.
B. Against the backdrop of a changing landscape of NCR peptides (rainbow colors representing
peptides appearing and disappearing at different stages of symbiotic cell differentiation), the bacterial
functions described in this study are critical at distinct stages of the bacteroid differentiation process.
It should be noted that the functions that are essential in early stages, such as LpsB and BacA, can also
be important in later stages of the bacteroid differentiation. However, the phenotypic analysis of the
corresponding mutants cannot reveal these putative late roles. **C,D.** The relative expression profile (%
of total) of the studied bacterial genes *lpsB*, *lpxXL*, *rpoH1*, *yejF*, *yejE*, *yejB*, *yejA* and *bacA* (**C**) and of
NCR280, *NCR247*, *NCR183* and *NCR169* (**D**) in the meristem, early infection zone, late infection zone,
interzone and fixation zone are displayed. Data was extracted from (15) and was obtained by RNA-seq
analysis on laser-microdissected nodule tissues (33).

Figure S2. Symbiotic phenotypes of NCR sensitivity mutants in *Medicago truncatula*. A. Nitrogen
fixation activity determined by the acetylene reduction assay on whole roots of nodulated plants
infected with the indicated bacterial mutants at 21 days post inoculation. NI, non-inoculated control
plants; WT, plants nodulated by the wild-type strain Sm1021. Boxplots were generated from 15 plants

each. Letters associated with each condition represent statistically different classes determined by a

non-parametric Dunn test, with a α threshold equal to 0.05. **B.** Nodule phenotypes at 21 days post

inoculation. Arrowheads indicate small nodules elicited by the *lpsB* mutant. Scale bar (2 mm) applies

to all panels.

Figure S3. Symbiotic phenotype of *Sinorhizobium meliloti* Sm1021 mutants during symbiosis with

***Medicago sativa*. A.** Leaves of plants inoculated with the indicated strains. Scale bar = 2 cm. **B.** Nodule

phenotype at 21 days post inoculation. Scale bar = 1 mm. **C.** Bacteroid viability determined by live-

dead staining of nodule sections and confocal microscopy. Top row images, full nodule sections;

Bottom row images, enlarged images of symbiotic cells. Scale bars are indicated in each panel. **D.**

Composite image of a *yejF*-infected nodule section showing the rapid permeabilization of the

membranes of the internalized bacteria (red staining). Note the bacteria in infection threads (white

arrows) are not permeabilized (green staining). The images of the composition are separated by

dashed lines. **E.** Nitrogen fixation activity determined by the acetylene reduction assay on whole roots

of nodulated plants infected with the indicated bacterial mutants at 21 days post inoculation. NI, non-

inoculated control plants; WT, plants nodulated by the wild-type strain Sm1021. Boxplots were

generated from 15 plants each. Letters associated with each condition represent statistically different

classes determined by a non-parametric Dunn test, with a α threshold equal to 0.05.

Figure S4. Morphological parameters in nodule bacteria of *Medicago truncatula* nodules. Flow

cytometry analysis of the morphology of bacteria isolated from nodules infected with the indicated

strains. The forward scatter (FSC, y-axes) is represented in function of the side scatter (SSC, x-axes).

The "X" indicates the peak values for each sample. The position of the peak value in the cultured

bacteria and in the wild-type bacteroids are indicated in each panel by the hatched lines.

Figure S5. DNA content and morphological parameters in nodule bacteria in *Medicago sativa*. A.

Flow cytometry analysis of the DNA content of bacteria in culture or isolated from nodules infected

with the indicated strains and stained with 4',6-diamidino-2-phenylindole (DAPI). The cell counts (y-

axes) are represented in function of the DAPI fluorescence (x-axes). The arrow indicates the mean DNA

content of wild-type bacteroids. The rows represent three sets of independent experiments and the

values of DAPI fluorescence cannot be compared between experiments because of differences in

instrument settings. The first two rows are samples prepared from 21 dpi nodules; the third row is

from 32 dpi nodules. The latter shows that the phenotype of the *yejA* mutant evolves in older nodules.

B. Flow cytometry analysis of the morphology of bacteria isolated from nodules infected with the

indicated strains. The forward scatter (FSC, y-axes) is represented in function of the side scatter (SSC,

x-axes).

Figure S6. Cellular defects in the *yejF* mutant bacteroids in *Medicago sativa* nodules. A. Transmission

electron microscopy of wild-type and *yejF* mutant bacteroids. The arrows indicate retracted

innermbranes in the yejF bacteroids, "v" indicate vacuoles, "a" indicate amyloplasts and "*" indicate

an enlarged peribacteroid space found in the angle of a branched bacteroid. **B.** Boxplots of morphology

parameters of free-living bacteria and bacteroids of the indicated strains, measured by MicrobeJ. Area

is the measure of the surface; circularity is calculated by $4\pi * area$ / perimeter^2, and describes how close

an object is to a perfect circle (value of 1); aspectRatio is the ratio of small and large diameters of the

objects' fitted ellipses; width.stdev is the standard deviation calculated from all the measured widths

along an object's medial axis. On each graph, panels group measurements made on one strain, either

in culture (left box) or extracted from mature nodules (right box). Numbers of analysed cells (n) are

identical as in Fig. 4C.

Figure S7. Nitrogenase expression in the *yejE* and *yejF* mutant bacteroids in *Medicago sativa*

nodules. A. Confocal microscopy of sections of nodules infected with *S. meliloti* Sm1021.p*pnifH-GFP*,

bacA.p*pnifH-GFP*, *yejE*.p*pnifH-GFP* or *yejF*.p*pnifH-GFP* and stained with propidium iodide. **B.** Flow

cytometry of GFP levels in differentiated nodule bacteria (upper panels) and heat killed nodule bacteria

(lower panels). The square shows the position of the GFP-positive bacteroids.

Figure S8. Phenotypes of wild-type and *yej* mutant bacteria in culture. A. Ultrastructure by

transmission electron microscopy. Scale bars are 1 μm. **B.** Growth in liquid medium. **C.** Growth

inhibition in the presence of SDS. **D.** Growth inhibition in the presence of H_2O_2. **E.** Growth inhibition in

the presence of HCl. **F.** Growth inhibition in the presence of NaCl. Growth inhibitions in C. to F. were

determined by the size of a halo formed around a round paper patch soaked in the stress compound.

Table S1. Survival after NCR treatment of wild-type and mutant *Sinorhizobium meliloti*.

Table S2. Primers used for mutant construction.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure S1

A

meristem – bacteria free

early infection zone
bactrial release

late infection zone
bactrial differentiation

interzone
bactrial differentiation

fixation zone
differentiated bacteroids

B

LpsB
BacA

RpoH1

LpxXL

YejABEF

C bacterial gene expression in nodule zones

relative gene expression (% of total)

lpsB *lpxXL* *rpoH1* *yejF* *yejE* *yejB* *yejA* *bacA*

■ meristem ■ early infection zone ■ late infection zone ■ interzone ■ fixation zone

D *NCR* gene expression in nodule zones

relative gene expression (% of total)

NCR280 *NCR247* *NCR183* *NCR169*

■ meristem ■ early infection zone ■ late infection zone ■ interzone ■ fixation zone

Figure S2

A

B

Figure S3

| | WT | *bacA* | *yejA* | *yejE* | *yejF* | *lpxXL* | *lpsB* | *rpoH1* |

A 2 cm

B 1 mm

C 100 µm 100 µm 100 µm 100 µm 100 µm 100 µm 100 µm 100 µm

10 µm 10 µm 30 µm 30 µm 20 µm 50 µm 30 µm 30 µm

D *yejF* 25 µm

E ethylene (nmol C₂H₄/h/plant)

af bd a cf bc bde cf de e

NI WT *bacA* *lpsB* *rpoH1* *lpxXL* *yejA* *yejE* *yejF*

Figure S4

Figure S5

Figure S6

Figure S7

peptide (concentration)	survival (%) relative to untreated bacteria (n=3)	Standard deviation	survival relative to WT (%)
NCR169 (10 µM)			
WT	8,72727273	3,40708416	100
bacA	4,8	4,42920941	55
yejA	10,9090909	8,51771041	125
yejE	9,45454545	1,70354208	108,333333
yejF	13,6727273	8,85841882	156,666667
lpxXL	1,01818182	0,34070842	11,6666667
lpsB	6,54545455	1,70354208	75
rpoH	7,56363636	0,68141683	86,6666667
NCR183 (10 µM)			
WT	0,04654545	0,02725667	100
bacA	0,00581818	0,01362834	12,5
yejA	0,128	0,10902669	275
yejE	0	0	0
yejF	0	0	0
lpxXL	0	0	0
lpsB	0,00232727	0,00545133	5
rpoH	0,22690909	0,1499117	487,5
NCR247 (25 µM)			
WT	0,03025455	0,016354	100
bacA	0,00116364	0,00272567	3,84615385
yejA	0,04654545	0	153,846154
yejE	0,00116364	0,00272567	3,84615385
yejF	0	0	0
lpxXL	0	0	0
lpsB	0	0	0
rpoH	0	0	0
NCR280 (25 µM)			
WT	0,05352727	0,01090267	100
bacA	0,0128	0,02453101	23,9130435
yejA	0,00116364	0,00272567	2,17391304
yejE	0	0	0
yejF	0,00581818	0,01362834	10,8695652
lpxXL	0	0	0
lpsB	0	0	0
rpoH	0,07214545	0,02180534	134,782609

	Gene	Primer name	Sequence	Description
Insertion mutants	SMc02829 (*yejF*)	2829forward	CAAATCGGTGTCGACCAACTCGATCCTGAAGCTC	Primers used to amplify a region of *SMc02829* CDS, flanked with SalI & XbaI sites
		2829reverse	GTTGGCTCTAGACATGGCTATCATTAC	
		2829out-f	CATCTCCTTCGACATCAAG	Primers used for verification
		2829out-r	CTCGTCGGCGATCAGGAGCTC	
	SMc02830 (*yejE*)	2830forward	CTTCGGCCTGTCGACGTTTGCCGAGTTCATC	Primers used to amplify a region of *SMc02830* CDS, flanked with SalI & XbaI sites
		2830reverse	CAGCGCTCTAGAGAAAAGGACCGAGATG	
		2830out-f	CTGGCTCTTCCTCCTCCTCTTC	Primers used for verification
		2830out-r	GATGATGAGCAGGATGTAGAG	
	SMc02832 (*yejA*)	2832forward	GCTACGTCGACGATCGGAGAACCCAAATAC	Primers used to amplify a region of *SMc02832* CDS, flanked with SalI & XbaI sites
		2832reverse	GTGACTCTAGACTCGCCTGTCTTTTC	
		2832out-f	CAATGCCGAGGAACAACC	Primers used for verification
		2832out-r	GAGAATGTTCGGCAGTTC	
	SMc00646 (*rpoH1*)	RpoH1-Sal1-forward	CGCACAGTCGACTAACGAGCCATCTTCGCCTC	Primers used to amplify a region of *SMc00646* CDS, flanked with SalI & XbaI sites
		RpoH1-Xba1-Reverse	CGAAGATCTAGAGCTCACGATCGTTCAGAACC	
		RpoH1-up	AAGCGGTATCAGGAG	Primers used for verification
		RpoH1-down	CCTCCTGCACCTTTT	
Deletion mutants	SMc02829 (*yejF*)	2829fup	GCAACGGCACCATCATGGATCCGCACC	Up fragment used for deletion of *SMc02829*
		2829rup	GGAGAAACTCGGAACGTCTTTCTCG	
		2829fdown	CGTTCCGAGTTTCTCCCATGC	Down fragment used for deletion of *SMc02829*
		2829rdown	CACCCACTCGCTCATCCAAGCTTTCAGG	
	SMc02830 (*yejE*)	2830fup	GGACACGTGTTCCGCAACGGATCCCTGATC	Up fragment used for deletion of *SMc02830*
		2830rup	CGTCGCGCCAGACGTCCCTCCG	
		2830fdown	GGACGTCTGGCGCGACGATGACAG	Down fragment used for deletion of *SMc02830*
		2830rdown	GCGAACATGCCTGAAAGCTTCGTCG	
	SMc02832 (*yejA*)	2832fup	GGTCGAACCGCAGGGATCCAGCGCAGCACG	Up fragment used for deletion of *SMc02832*
		2832rup	GCAAGAGCTCCGGCCGGTACCCC	
		2832fdown	CGGCCGGAGCTCTTGCGCTCAGG	Down fragment used for deletion of *SMc02832*
		2832rdown	CATGATGCCGACGATCGTCAAGCTTATCAGC	