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Rhizobium common *nod* genes are required for biofilm formation

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Summary

In legume nitrogen-fixing symbioses, rhizobial *nod* genes are obligatory for initiating infection thread formation and root nodule development. Here we show that the common *nod* genes, *nodD1ABC*, whose products synthesize core Nod factor, a chitin-like oligomer, are also required for the establishment of the three-dimensional architecture of the biofilm of *Sinorhizobium meliloti*. Common *nod* gene mutants form a biofilm that is a monolayer. Moreover, adding Nod Factor antibody to *S. meliloti* cells inhibits biofilm formation, while chitinase treatment disrupts pre-formed biofilms. These results attest to the involvement of core Nod factor in rhizobial biofilm establishment. However, luteolin, the plant-derived inducer of *S. meliloti*'s *nod* genes, is not required for mature biofilm formation, although biofilm establishment is enhanced in the presence of this flavonoid inducer. Because biofilm formation is plant-inducer-independent and because all nodulating rhizobia, both alpha- and beta-proteobacteria have common *nod* genes, the role of core Nod factor in biofilm formation is likely to be an ancestral and evolutionarily conserved function of these genes.

Introduction

For more than 20 years, *Rhizobium nod* genes, and their product, Nod Factor (NF), have been recognized as essential for the development of nitrogen-fixing nodules on legume roots (Lerouge *et al*., 1990). Mutations within or deletions of entire *nod* genes result in a loss of the ability of rhizobial bacteria to induce nodules on the host, and therefore fix atmospheric nitrogen. NF is a β-1, 4-linked *N*-acetylglucosamine oligomer, with a fatty acid chain attached to the terminal glucosamine and various substituents occurring on the chitin-like backbone. Although the precise mechanism whereby NF stimulates nodule formation remains uncertain, NF is known to trigger calcium spiking, cause root hair deformation and initiate nodule primordium formation (Geurts *et al*., 2005; Oldroyd and Downie, 2006).

The combination of the products from two different classes of *nod* genes, which are under the control of the *lysR*-like regulatory gene *nodD*, results in the synthesis of NF. NodD binds to the *nod* box region of the *nod* gene promoters; *nodD1*, one of three *nodD* genes in *Sinorhizobium meliloti*, is included among the common *nod* genes. The common nodulation genes (*nodDABC*) are found in all bacteria that nodulate legumes (with so far only one known exception; Giraud *et al*., 2007), including the beta-proteobacteria (beta-rhizobia), which also establish nodules on legume roots (Moulin *et al*., 2001). NodC is responsible for the biosynthesis of the *N*-acetylglucosamine trimeric to pentameric backbone, while NodB deacetylates a terminal glucosamine, leaving a free amino group, which is acylated by NodA. The second class of *nod* genes (in *S. meliloti*, *nodEF*, *nodG*, *nodH*, *nodPQ*, *nodL*) consists of the host-specific nodulation genes, whose products modify the *N*-acetylglucosamine backbone in ways that are unique to a particular *Rhizobium* species. Typically, the products of the host-specific *nod* genes are responsible for adding side group substituents and for determining the length and saturation of the fatty acid on the terminal glucosamine (Lerouge *et al*., 1990). These substituents confer specificity between a rhizobial species and the cognate host.

Sinorhizobium meliloti and *Rhizobium leguminosarum* bv. *viciae*, like many other bacteria, form biofilms on sterile inert substrates, including plastic, glass, sand and soil (Fujishige *et al*., 2006a). While screening symbiotic mutants for their effects on biofilm formation using microtiter plate and whole-root assays, we earlier found that *R. leguminosarum* bv. *viciae* deleted of pSym [an endogenous plasmid that carries both *nod* and *nif* (nitrogen fixation) genes] and *S. meliloti* pSymA-deletion mutants

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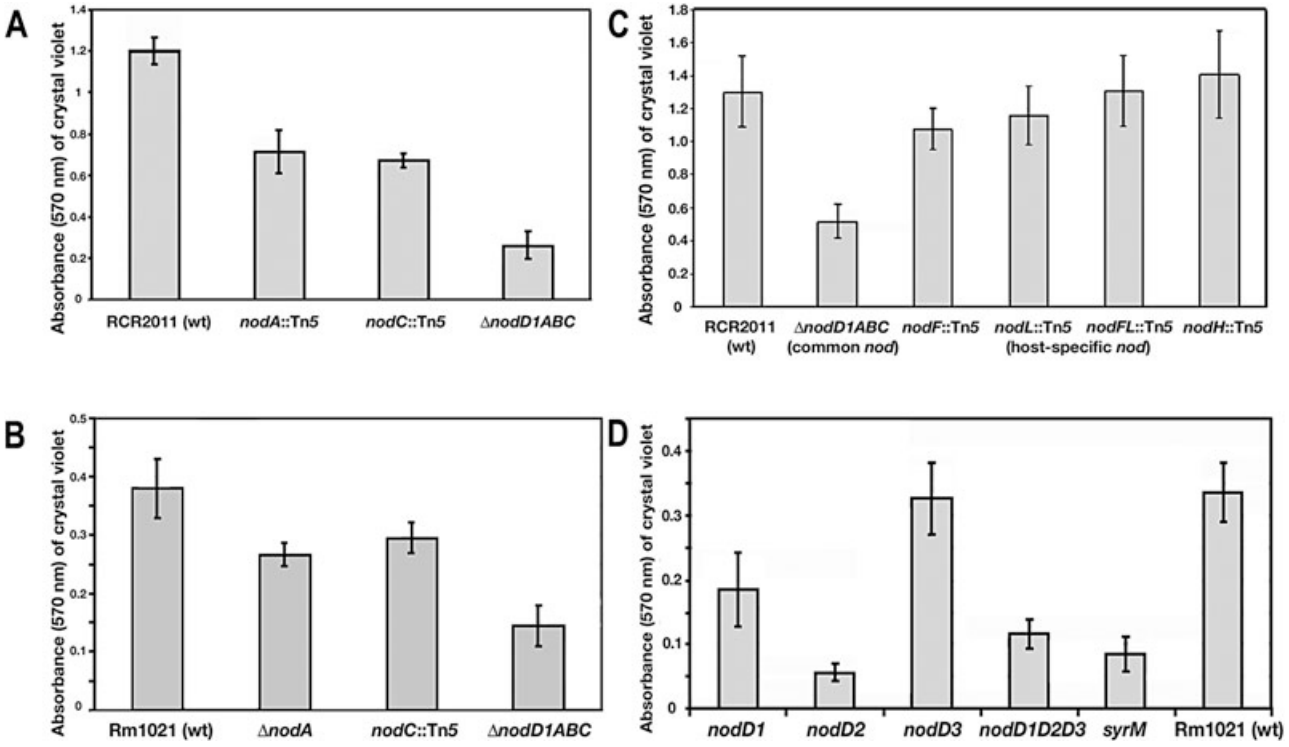

Fig. 1. Microtiter plate assay of biofilm formation by wild-type *S. meliloti* strain and *nod* mutants. The plates were assayed 24 h after inoculation; there was no difference in growth rate among the various strains tested.
A. RCR2011 *nod* gene mutants are reduced in biofilm formation compared with the wild type.
B. Rm1021 *nod* gene mutants are reduced in biofilm formation compared with the wild type.
C. Biofilms of RCR2011 (wild-type) and the host-specific *nod* (*nodF*, *nodL*, *nodFL*, *nodH*) mutants are not statistically different from the wild type. The Δ*nodD1ABC* mutant, which does not produce core NF, shows significantly reduced biofilm formation.
D. Except for the *nodD3* mutant, biofilms produced by the various Rm1021 *nodD* and *syrM* (a *nodD*-like gene) mutants are reduced compared with the wild-type biofilms.

exhibited significantly reduced biofilm formation (Fujishige *et al.*, 2006b). This led to testing the effect on biofilm formation of individual and multiple *nod* gene mutations. Here we report that the products of the common *nod* genes have a hitherto unrecognized function – holding a rhizobial biofilm together.

Results

Effect of nod *gene mutations on biofilm formation* in vitro

Individual common *nod* gene mutants, the class of *nod* genes common to nodulating rhizobia, including the β-rhizobia, as well as mutants in the second class of *nod* genes, the host-specific *nod* genes, were tested for biofilming ability. We determined that *S. meliloti* mutants deleted of any one of the common *nod* genes or *nodD1ABC* exhibited significantly reduced (50–70%) biofilm formation not only in microtiter plate assays (Fig. 1A and B), but also on environmentally relevant substrates, including roots (Fig. 2A). Mutations in individual genes, *nodA* or *nodC* (Table 1), either in *S. meliloti* RCR2011 (Fig. 1A) or in the sequenced strain Rm1021 (Galibert *et al.*, 2001; Fig. 1B), did not affect the growth of these bacteria compared with wild-type *S. meliloti* (data not shown), but did result in statistically significantly reduced biofilm formation. A similar result was observed for individual *nodB* mutants (data not shown). In contrast, host-specific *nod* mutants were not altered in their level of biofilm establishment compared with the wild-type strains in the RCR2011 (Fig. 1C) and Rm1021 genetic backgrounds (data not shown).

In *S. meliloti*, in addition to the three *nodD* genes, there is a *nodD*-like gene, *syrM*. SyrM and NodD3 together form a positive regulatory circuit (Swanson *et al.*, 1993; Dusha *et al.*, 1999a,b). In the microtiter plate assay, mutants of *nodD1*, *nodD2*, *nodD1D2D3* and *syrM* developed reduced biofilms, whereas the *nodD3* mutant was unaffected (Fig. 1D). The difference between *syrM* and *nodD3* may lie in SyrM's involvement in succinoglycan (EPSI) biosynthesis (Swanson *et al.*, 1993); we previously reported that *S. meliloti* EPSI mutants exhibit reduced biofilm formation (Fujishige *et al.*, 2006a). The *nodD1* gene is activated by plant-produced flavonoids, such as

Table 1. Strains and plasmids used in this study.

Strain	Relevant characteristics	Source or reference
RCR2011	Wild-type derivative of SU47	Jean Dénarié
Rm1021	Wild-type Sm^r derivative of 2011	Meade *et al.* (1982)
Rm1021 (pRm57)	Rm1021 *nodC*::*lacZ*	Mulligan and Long (1985)
GMI3253	Rm1021 Δ*nodA* null	Jean Dénarié
Rm5612	Rm1021 *nodC*::Tn*5*	Ethan R. Signer
SL44	Rm1021 Δ*nodD1ABC*	Sharon R. Long
GMI5383	RCR2011 *nodA*::Tn*5*	Jean Dénarié
GMI5389	RCR2011 *nodC*::Tn*5*	Jean Dénarié
GMI357	RCR2011 Δ*nodD1ABC*	Jean Dénarié
RCR2011-*gfp*	RCR2011 (pHC60)	This study
Rm1021 DsRed	Rm1021 (pDG77)	Bringhurst *et al.* (2001)
Rm1021-*gfp*	Rm1021 (pHC60)	Cheng *et al.* (1998)
Rm5612-*gfp*	Rm5612 (pHC60)	This study
GMI357-*gfp*	GMI357 (pHC60)	This study
TJ9B8	Rm1021 *nodD1*::Tn*5*	Sharon R. Long
RmD2	Rm1021 *nodD2*::tm	Honma and Ausubel (1987)
RmD3-1	Rm1021 *nodD3*::sp./g-1	Honma and Ausubel (1987)
Rm*D1D2D3*	Rm1021 *nodD1*::Tn*5*, *nodD2*::tm, *nodD3*:: sp./g-1	Honma and Ausubel (1987)
JAS105	Rm1021 *syrM*::Tn*5–233*	Swanson *et al.* (1993)
Rm*D1D2D3-gfp*	Rm*D1D2D3* (pHC60)	This study
GMI5378	RCR2011 Δ*nodF*	Jean Dénarié
GMI6436	RCR2011 *nodL*::Tn*5*	Jean Dénarié
GMI6628	RCR2011 Δ*nodF*, *nodL*::Tn*5*	Jean Dénarié
GMI2212	RCR2011 *nodH*::Tn*5*	Jean Dénarié
RCR2011 (p149)	RCR2011 carrying the common and host-specific *nod* genes	This study
RCR2011 (pRmJ30)	RCR2011 carrying the common *nod* genes	This study
GMI357 (pRmJ30)	GMI357 carrying the common *nod* genes	This study
A348	Wild-type *Agrobacterium tumefaciens*	Garfinkel *et al.* (1981)
A348 (pRmJ30)	A348 carrying the *S. meliloti* common *nod* genes	Hirsch *et al.* (1985)
Plasmids		
pPROBE-AT′	Broad host-range promoter-*gfp* vector	Miller *et al.* (2000)
pGMI149	*nodD1ABCIJQPGEFH*, *nodD3*, *syrM* on IncP plasmid	Lerouge *et al.* (1990)
pLAFR1	Broad host-range cosmid-cloning vector	Friedman *et al.* (1982)
pNF2	pPROBE-AT′ *nodA* promoter-*gfp*	This study
pRmJ30	8.7 kb EcoRI fragment carrying *nodD1ABCIJ*	Jacobs *et al.* (1985)

luteolin (Mulligan and Long, 1985), whereas *nodD2* is activated by plant-produced betaines (Phillips *et al.*, 1992). No change in biofilm formation was observed when betaine was added to the culture medium (data not shown), whereas luteolin addition enhanced biofilm establishment (see later section).

Confocal imaging of wild-type green fluorescent protein (GFP)-labelled rhizobia showed that mature biofilms consist of towers and ridges (Fig. 2D and E). This biofilm morphology contrasted with that produced by Nod$^-$ mutant bacteria, which only established a monolayer with few bacteria attached to one another (Fig. 2F and G). When viewed under transmission electron microscopy (TEM), wild-type *S. meliloti* biofilms revealed extensive cell-to-cell contacts (Fig. 3A), whereas the *nod* mutants remained as single cells or occasionally as doublets (Fig. 3B).

Effect of nod *gene mutations on biofilm formation* in vivo

We extended the findings from the microtiter plate assay by examining biofilm formation on roots of white sweet-clover (*Melilotus alba* Desr.). Confocal laser scanning micrographs of roots inoculated with Rm1021 showed distinct microcolonies along the root surface, which remained adherent after extensive washing (arrows, Fig. 2A). In contrast, few *nodC* mutant cells remained attached after washing (arrow, Fig. 2B). The number of colony-forming units (cfu) per gram of root tissue demonstrated a significant reduction (> 50%) in attachment ability of both *nodC* and *nodD1D2D3* mutants compared with the wild-type control Rm1021 (Fig. 2C).

Luteolin is not required for biofilm formation

We hypothesized that the common *nod* genes are expressed in the biofilm independently of plant-derived inducers because biofilms developed in the microtiter plate wells without adding luteolin (Fig. 1). To test this hypothesis specifically, a *nodA–gfp* transcription fusion was introduced into wild-type Rm1021 cells, which were inoculated onto sand particles. Using this transcriptional fusion, attached single cells and small microcolonies were visualized by their fluorescence 4 h after the initiation of

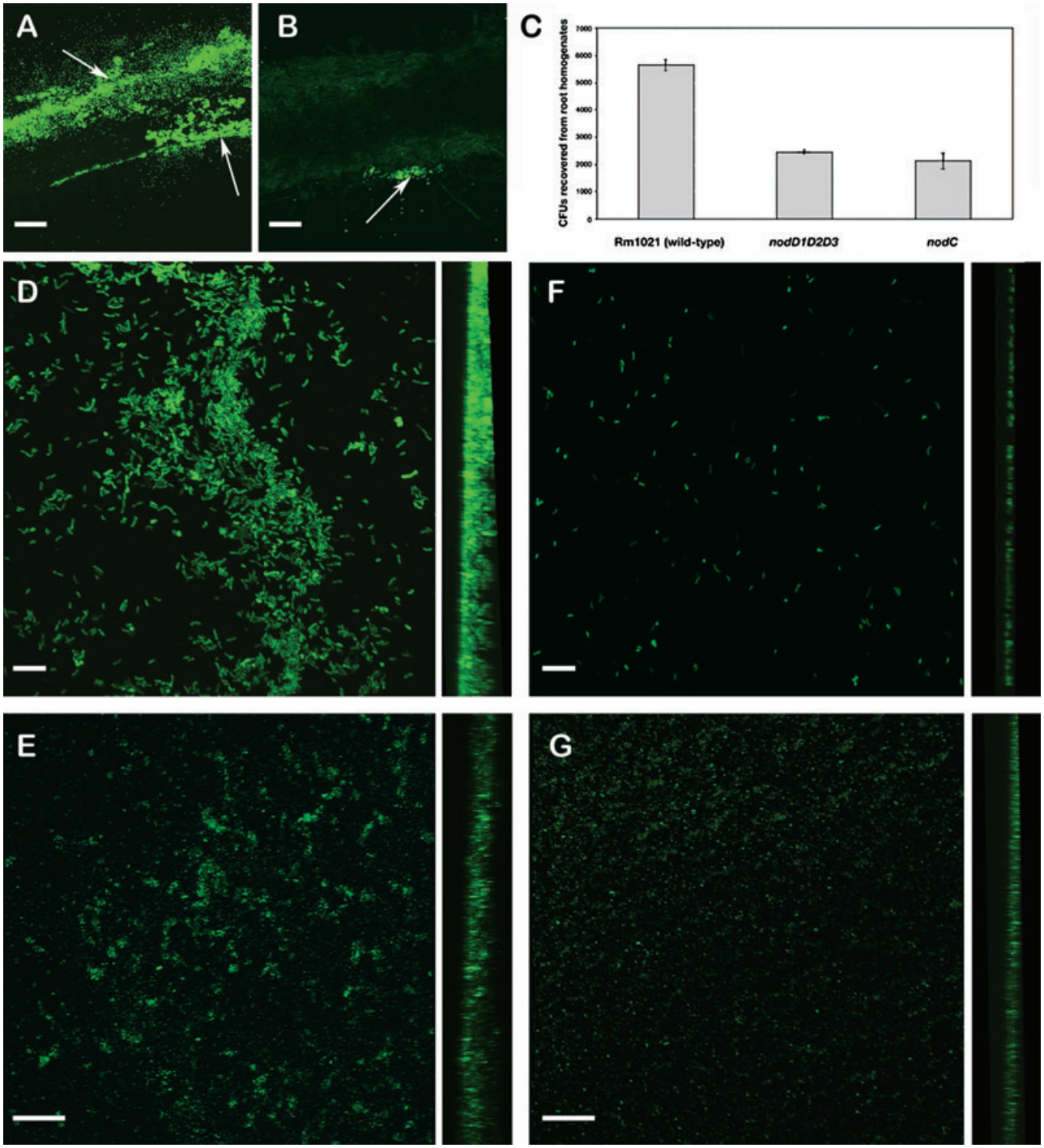

Fig. 2. Attachment *in vivo* and *in vitro*.
A and B. Confocal images showing the attachment of (A) wild-type *S. meliloti* (Rm1021) and (B) a *nodC* mutant (Rm5612) to roots of *Melilotus alba* Desr. 72 h post inoculation. Bar, 250 μm.
C. Colony-forming unit (cfu) counts from roots 48 h post inoculation with either wild-type (Rm1021) or *nod* mutant bacteria (*nodD1D2D3* and *nodC*).
D. Confocal imaging (top and side views) of a wild-type *gfp*-expressing RCR2011 strain 72 h post inoculation shows biofilms composed of ridges (arrows) and towers (arrowheads). Bar, 10 μm.
E. Confocal imaging (top and side views) of a wild-type *gfp*-expressing RCR2011 strain 5 days post inoculation. Bar, 100 μm.
F. Very few Δ*nodD1ABC* mutant cells (top and side views) remain attached to each other or to the glass coverslip 72 h post inoculation. Bar, 10 μm.
G. After 5 days, the Δ*nodD1ABC* mutant bacteria remain in a monolayer. Bar, 100 μm.

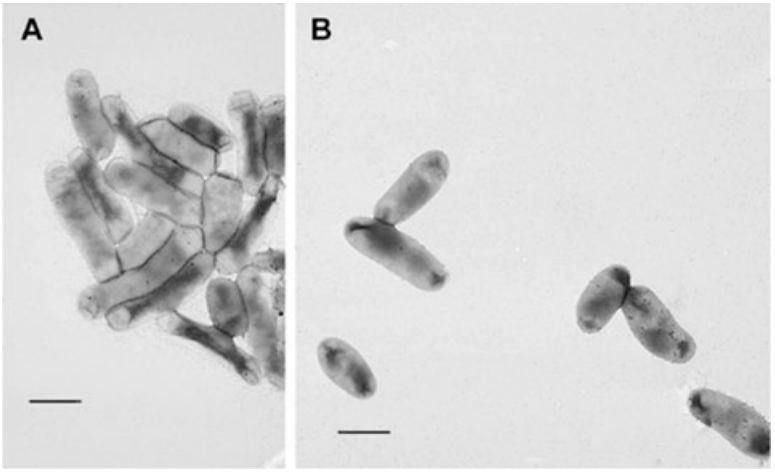

Fig. 3. Transmission electron micrographs of *S. meliloti* grown under biofilm conditions.
A. Wild-type RCR2011. The cells adhere to each other.
B. GMI357, the *ΔnodD1ABC* mutant in the RCR2011 genetic background, grown under biofilm conditions. Few cell aggregates are observed. Bar, 0.5 μm.

the experiment (Fig. 4A). By 24 h after the start of the experiment without the luteolin inducer, large GFP-positive colonies were observed on the sand particles (Fig. 4B).

Wild-type *S. meliloti* biofilm formation was enhanced by adding 1 μM luteolin to the culture medium, whereas the *nodC* mutant showed no difference in biofilm establishment in the presence or absence of luteolin (Fig. 4C). Luteolin induced an almost twofold increase in β-galactosidase activity in biofilmed *S. meliloti* carrying a *nodC–lacZ* transcriptional fusion (Mulligan and Long, 1985) over the control, which was treated with the solvent methanol (Fig. 4D). As expected, planktonic cells also showed increased β-galactosidase activity in the presence of luteolin.

Because biofilm formation correlated with augmented NF production brought about by luteolin addition, we examined the effect of NF overproduction by introducing the plasmid pRmJ30, which carries the common *nod* genes, or the plasmid pGMI149, which contains both common and host-specific *nod* genes, or pLAFR1, the vector control, into the RCR2011 genetic background (Table 1). Each *nod* gene containing plasmid enhanced biofilm formation in the absence of luteolin by approximately 25% (Fig. 5A). A similar result was found for the Rm1021 wild-type strain (data not shown). No enhancement was observed for strains containing the vector pLAFR1 (data not shown). Interestingly, we detected no statistical difference between strains carrying only the common *nod* genes versus those carrying a plasmid with the full complement of *nod* genes, indicating that the contribution of host-specific *nod* genes to biofilm formation is minimal (see also Fig. 1C). When the plasmid carrying *nodD1ABC* was introduced into a mutant deleted of these genes, biofilm formation was restored (Fig. 5B).

As a further gain-of-function test, we introduced a series of *nod* plasmids into another member of the Rhizobiaceae, *Agrobacterium tumefaciens* strain A348, which generates tumours on plant tissues (Garfinkel *et al*., 1981). In all cases, there was a clear enhancement of biofilm formation, even when only *nodD1ABC* was introduced (Fig. 5C). This finding shows that the minimalist or core NF contributes to the enhancement of biofilm formation in *A. tumefaciens* as it does in *S. meliloti*.

Core NF facilitates cell-to-cell adhesion

Based on the morphology of the wild-type versus *nod* mutant biofilms (Figs 2D–G and 3), we hypothesized that core NF causes the rhizobial cells to adhere to one another. Cell-to-cell adhesion would allow the rhizobial cells to remain closely attached to roots until an adequate population accumulated to produce a sufficient localized concentration of the host-specific signalling NF, which is required for root hair calcium spiking and deformation. We examined biofilms formed by mixing a GFP-labelled *nodC* mutant strain 1:1 with wild-type Rm1021 labelled with DsRed (Fig. 4E). Interestingly, the *nodC* mutant strain was excluded from the biofilm, suggesting that the lack of NF kept the mutant from integrating into the Rm1021 biofilm. A similar response was observed when a *nodC-gfp* mutant was mixed with a DsRed-labelled *exoY* mutant (see Fig. S1).

An *in silico* investigation revealed an overall similarity of 37% between *Staphylococcus epidermidis* IcaA and *S. meliloti* NodC on the protein level (data not shown). IcaA and other proteins encoded by the *ica* gene cluster synthesize a long chain of *N*-acetylglucosamines known as polysaccharide intercellular adhesin (PIA), which is essential for maintaining *S. epidermidis* biofilm adherence (Heilmann *et al*., 1996; Götz, 2002). PIA is detected in fibrous material surrounding the bacterial cells within the *S. epidermidis* biofilm (Vuong *et al*., 2004). However, we detected no cross reaction between PIA and *S. meliloti* or between PIA and NF (data not shown).

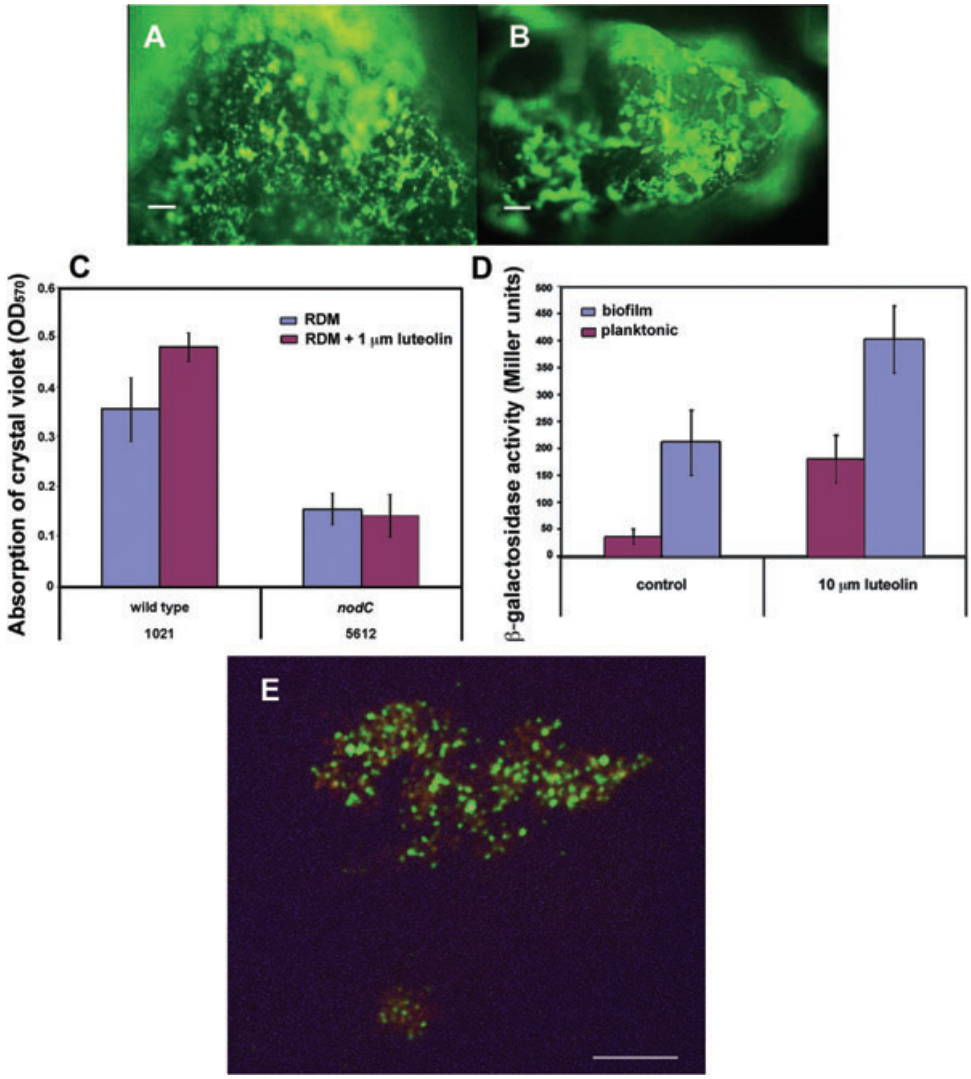

Fig. 4. Expression of *nod* genes in response to adherence to a surface and to the flavonoid inducer luteolin.
A. Rm1021 with a *nodA-gfp* transcriptional fusion fluoresced as single cells and as microcolonies attached to sand particles as early as 4 h after inoculation. Bar, 40 μm.
B. After 24 h, the microcolonies were significantly larger. Bar, 40 μm.
C. The microtiter plate assays show an increase in Rm1021 biofilms when 1 μM luteolin was added to RDM, but no change in the *nodC* mutant biofilms occurred between the luteolin-treated and untreated samples.
D. Luteolin induced an almost twofold increase in β-galactosidase activity over the control in biofilmed *S. meliloti* Rm1021 carrying a *nodC–lacZ* transcriptional fusion. The planktonic cells as expected also showed an increase in β-galactosidase activity.
E. Mixed biofilm of Rm5612-gfp (*nodC*::Tn*5*) and Rm1021-DsRed as viewed with epifluorescence. The green Nod$^-$ cells remained on the top of the biofilm. Bar, 20 μm.

We utilized an *S. meliloti* NF-specific antibody (Timmers *et al.*, 1998) conjugated to colloidal gold in TEM studies against biofilms (Lévesque *et al.*, 2004), but found no labelling of any definite structures on the bacterial cell surface. However, the gold-labelled antibody was detected in both the external milieu and on the cell membrane (Fig. S2). Because NF is only three to five *N*-acetylglucosamines long, detecting a fibrous component analogous to PIA, which is a long chain of 100–120 *N*-acetylglucosamine residues, is unlikely. On the other hand, we observed that wild-type *S. meliloti* biofilm formation was reduced in the microtiter plate assay with antibody dilutions ranging from 1:10 000 to 1:100 (Fig. 5D). The NF antibody at dilutions of 1:10 000 (Fig. 5E) and 1:1000 (data not shown) similarly reduced biofilm formation of *A. tumefaciens* A348 carrying pRmJ30, but no effect on A348 biofilms was detected at any concentration. This result strongly suggests that a molecule recognized by NF antibody is present on the surface of or within the biofilm matrix of agrobacteria expressing the common *nod* genes.

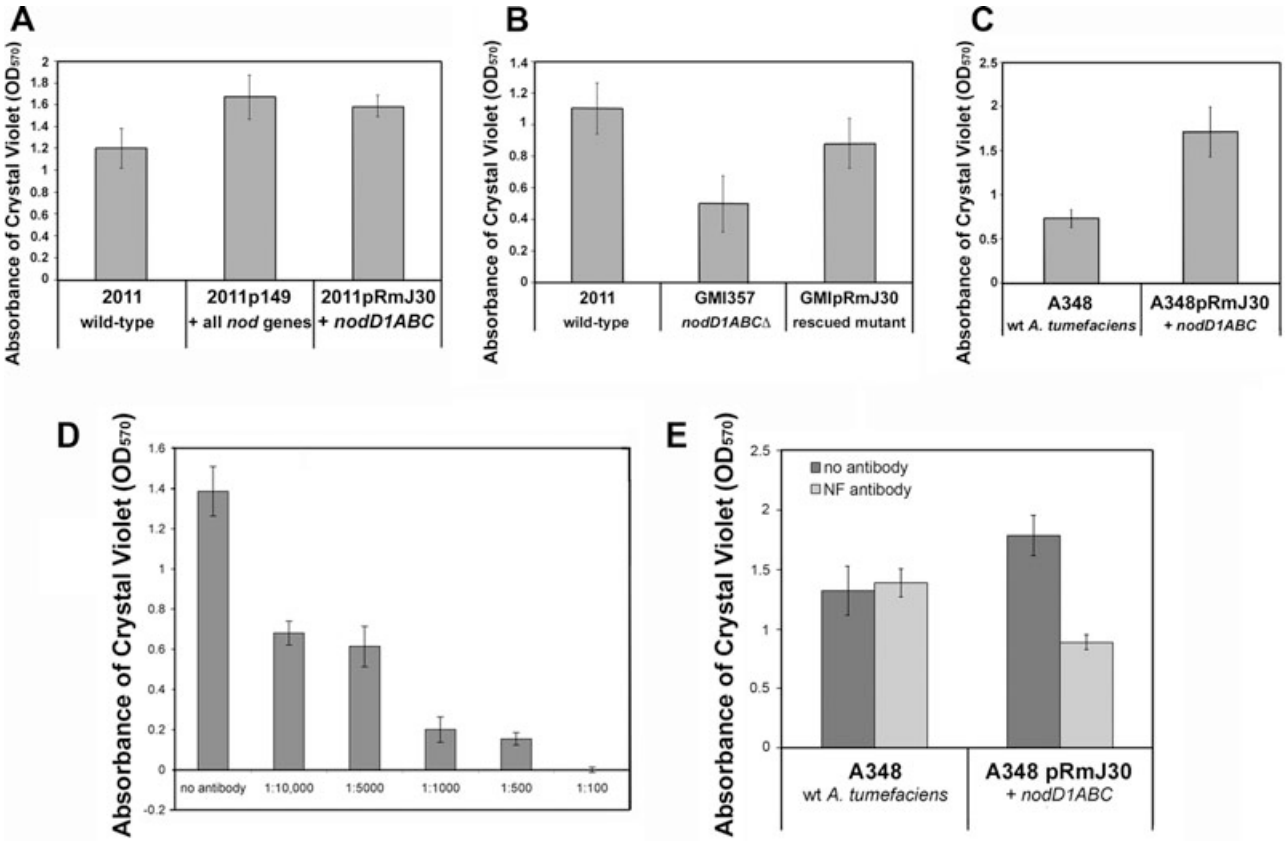

Fig. 5. Microtiter titer plate assays demonstrating the importance of core NF to biofilm formation.
A. Addition of *nod* genes, either the complete set or only the common *nod* genes, to RCR2011 enhanced biofilm formation over the wild-type control.
B. Addition of the core NF-encoding genes to the Δ*nodD1ABC* mutant restored biofilm formation to wild-type levels.
C. Adding the core NF-encoding genes to wild-type *A. tumefaciens* strain A348 enhanced its biofilm formation.
D. Adding NF antibody to wild-type *S. meliloti* cells interfered with biofilm formation even at the lowest dilutions.
E. NF antibody added at 1:10 000 decreased biofilm formation of an *A. tumefaciens* strain (A348) carrying the common *nod* genes, but had no effect on A348 biofilms. A similar result was found for a 1:1000 dilution (data not shown).

Sinorhizobium meliloti biofilms were next treated with chitinase, which caused the dispersal of a pre-formed 24-h-old Rm1021 biofilm, more than twofold over the control (Fig. 6A). Numerous cells were released from the biofilm after 90 min of chitinase treatment, resulting in large areas of the surface that were bacteria-free. After 180 min of chitinase treatment, the biofilm had completely dispersed. These data show that the structure of the biofilm is broken down by chitinase, which is consistent with NF composition.

Core NF is similar in structure to chitosan, which has been reported to promote *Escherichia coli* CSH57 adhesion by making the microbial surface more hydrophobic, thereby enhancing biofilm formation (Goldberg *et al*., 1990). Two different assays demonstrated that the wild-type strains are more hydrophobic than the Nod⁻ mutant strains. For example, in the salt aggregation test (Honda *et al*., 1983), both Rm1021 (Fig. 6B) and RCR2011 (Fig. 6C) aggregated at a lower concentration of ammonium sulphate than the Nod⁻ mutants. Treatment with 10 μM luteolin enhanced aggregation of the wild-type strains, but no increase in aggregation was observed for the nodulation-defective *S. meliloti* mutant (Fig. 6B and C).

Discussion

Taken together, the data presented herein indicate that NF is critical for establishing a mature rhizobial biofilm. This is a new function for NF, and is distinctly different from the established role as a morphogen for inducing legume nodule development. The involvement of core NF in biofilm establishment has been hitherto unrecognized in part because of the prior emphasis on the signalling functions of rhizobial NF (Ardourel *et al*., 1994). We propose that the biofilm function may reflect an earlier evolutionary development, as this property is encoded by genes common to all nodulating rhizobia, including *Burkholderia* and *Cupriavidus* strains, the so-called

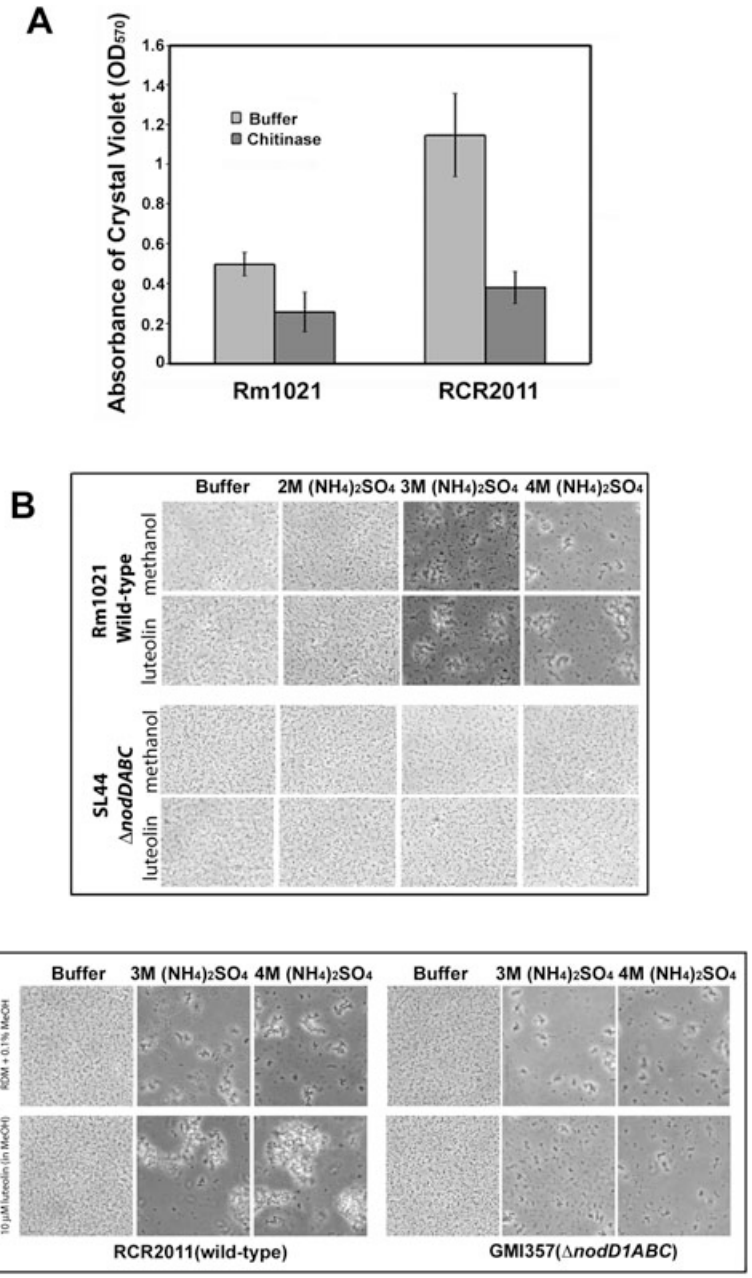

Fig. 6. Assays suggesting that core NF is either on the bacterial surface or in the biofilm matrix.
A. Chitinase disrupted pre-formed *S. meliloti* biofilms.
B. The salt aggregation test demonstrated that wild-type *S. meliloti* Rm1021 cells have a more hydrophobic surface than the Δ*nodD1ABC* mutant (SL44), because the wild-type rhizobia aggregated at a much lower salt concentration (2 M) than the mutant cells. Luteolin enhanced aggregation in Rm1021, but not in SL44.
C. RCR2011 bacteria aggregated more in the presence of 10 μm luteolin (dissolved in methanol) than the control bacteria (treated with methanol alone). GMI357 (Δ*nodD1ABC*) exhibited very little aggregation even in the presence of luteolin.

β-rhizobia (Moulin *et al.*, 2001), whereas the host-specific *nod* genes needed for the signalling function vary depending on the rhizobial species. In addition, plant-derived activators, such as luteolin, are not required for *S. meliloti* biofilm formation, further supporting the hypothesis that this function is more ancestral or primitive. Apparently, some change in rhizobial behaviour, brought about by contact to either abiotic surfaces or to roots, leads to the expression of the common *nod* genes. Supporting this is the fact that *S. meliloti* containing *nodC–lacZ* fusions, when tested in microtiter plates for β-galactosidase activity, turned blue even without luteolin addition (data not shown). Ongoing research may identify the factors important for manifesting this change.

Lending support to the idea that core NF may play a structural role, perhaps by associating with the bacterial cell surface, is the fact that we could not rescue the monolayer biofilms established by Nod⁻ rhizobia by adding purified NF (data not shown). Similarly, NF addition does not restore a wild-type phenotype to Nod⁻*S. meliloti* (Hirsch *et al.*, 1993), although it triggers the beginnings of nodule development on alfalfa, which is very sensitive to NF application (Truchet *et al.*, 1991). This lack of rescue may be characteristic of rhizobia that nodulate

indeterminate nodule-forming legumes, because purified NF does not rescue the aberrant phenotype of a *R. leguminosarum* bv. *viciae nodEnodO* double mutant either (Walker and Downie, 2000). On the other hand, adding NF rescues the Nod$^-$ phenotype of mutants of *Rhizobium* NGR234 and *Bradyrhizobium japonicum*, which nodulate determinate nodule-forming legumes (Relic *et al*., 1993). These results suggest that for rhizobia interacting with indeterminate nodule-forming plants, NF must be associated in some way with the rhizobial cell surface to complement both the biofilm and nodulation phenotypes of Nod$^-$ mutants. If NF is not localized to the cell surface, as is the case for exogenous NF, it is incompatible with cell-to-cell contact and subsequent rhizobial invasion.

Core NF's importance has been previously thought of only in terms of its being a backbone for host-specific determinants. However, core NF is critical for root colonization and biofilm formation in that it holds the rhizobia together until a threshold population density is achieved and sufficient host-specific signalling NF is synthesized to act as a morphogen. It may also protect non-spore forming prokaryotes, such as rhizobia from desiccation, especially in the absence of a host legume, by facilitating the adherence of cells together in a biofilm on soil particles or on non-host roots. Moreover, core NF's involvement in biofilm formation may shield attached rhizobia from host defence reactions, in a manner similar to PIA, which inhibits the defence mechanisms of human innate immunity (Vuong *et al*., 2004).

Interestingly, molecules similar to core NF are involved in non-signalling functions in other bacteria. For example, *N*-acetylglucosamines are reported to act as adhesins not only in *Staphylococcus* species (Heilmann *et al*., 1996; Götz, 2002), but also in *Caulobacter crescentus* (Merker and Smit, 1988; Ong *et al*., 1990). In the latter, *N*-acetylglucosamines are localized to the holdfast of the stalked cells, promoting cell-to-cell adhesion (rosette formation) and adherence to abiotic surfaces. Based on the fact that *N*-acetylglucosamines function as adhesins in a number of bacteria and on our results showing the importance of core NF for biofilm formation, we propose that core NF plays a similar role in *S. meliloti*.

The realization that core NF has dual functions, both as a structural component of the biofilm and independently as a precursor to host-specific morphogens, implies the likely existence of two different sets of control mechanisms, one luteolin-dependent and the other luteolin-independent, which regulate NF production. Also, based on our data, it is very likely that NodD1 probably regulates the expression of other genes, exclusive of *nod*, which are essential for biofilm formation. A fruitful area of future research will be to tease these systems apart, and thus arrive at an understanding of the factors that separately regulate production of the structural and morphogenic components.

Experimental procedures

Strains and plasmids

Bacterial strains and plasmids used for the biofilm analysis are listed in Table 1. Triparental matings were performed as described (Figurski and Helinski, 1979).

Construction of the nodA-gfp *transcriptional fusion*

A 400 bp EagI–SacI fragment of the *S. meliloti nodA* promoter was ligated into the multicloning site of the broad host-range promoter-GFP vector, pPROBE-AT′ (Miller *et al*., 2000). This fragment includes the *nod* box, to which the NodD transcriptional activators bind, and 81 bp of the *nodA* gene. The resulting plasmid, pNF2, was transformed into the chemically competent *E. coli* strain, DH5α (Sambrook and Russell, 2001). Plasmid pNF2 was conjugated into *S. meliloti* strain RCR2011 by triparental mating, using DH5α (pRK2013) as the helper plasmid (Figurski and Helinski, 1979).

Biofilm preparation

Biofilms were established as described (Fujishige *et al*., 2006a) and grown for 24 h before staining with crystal violet. Each data point is the average of at least 18 wells. Error bars indicate the standard deviation from the mean. Root biofilms were prepared (Fujishige *et al*., 2006c) and harvested 48–72 h post inoculation with either wild-type or *nod* mutant bacteria. The strains constitutively expressed GFP. Attached cells were quantified by counting cfus (Fujishige *et al*., 2006c). Error bars indicate the standard deviation from the mean.

β-Galactosidase activity

Biofilms were grown for 48 h in 96 well plates as previously described (Fujishige *et al*., 2006a). Biofilm and planktonic cells carrying a *nodC–lacZ* transcriptional fusion (Mulligan and Long, 1985) were grown in supplemented Rhizobium defined medium (RDM) culture medium (Fujishige *et al*., 2006a) with added solvent (methanol) or 10 μM luteolin dissolved in methanol. β-Galactosidase-specific activity of biofilm and planktonic cells was measured as described in Stanley *et al*. (2003). To reprise, 80 μl of planktonic bacteria were removed carefully from each well. The biofilms were gently rinsed three times with sterile RDM to remove the planktonic cells, and the biofilm cells were scraped from the wells into RDM. The wells were vigorously washed to remove all bacteria (wells were subsequently stained with crystal violet, as described above, to verify that bacteria were thoroughly removed). Biofilm cells were dispersed by extensive vortexing. To quantify cell number, the OD_{595} of the separate preparations of planktonic and biofilm cells was measured. The planktonic or biofilm cells were mixed with 100 μl of Z-buffer (60 mM Na_2HPO_4, 4 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol). To each of these samples, 1 μl of SDS (1% w/v) and 2 μl of chloroform were added. Reactions were pre-incubated at 28°C for 5 min, and then 20 μl of ONPG (4 mg ml^{-1}) were added to each sample. Reactions were stopped by the addition of 50 μl of Na_2CO_3. Samples were centrifuged for 2 min at 8000 *g*. The OD_{415} of the supernatants were measured, and Miller units were calculated as follows:

Miller units = $(1000 \times OD_{415})/(t \times v \times OD_{595})$; t = reaction time (min) and v = volume of culture (ml).

Microscopy

For confocal scanning laser microscopy, the bacteria were grown on flame-sterilized glass coverslips placed into 20 well microtiter plate wells for 72 h or 5 days. GFP-labelled biofilms were examined on a Zeiss LSM510 microscope, and the images obtained using a 10x/0.3 or 63x/1.4 oil-immersion objective with excitation at 488 nm in conjunction with the Zeiss LSM510 imaging software. Rm1021 *nodA-gfp* bacteria grown on sand particles (Fujishige *et al*., 2006a) were placed into the wells of depression slides, topped with a coverslip, and examined under epifluorescence using a Zeiss Axiophot microscope.

Chitinase assay

Rm1021 or RCR2011 biofilms were grown for 48 h in U-bottom polyvinyl chloride microtiter plate wells. The biofilms were rinsed once with chitinase buffer (200 mM potassium phosphate, 2 mM calcium chloride, pH 6.0) and then treated with chitinase (0.1 unit ml^{-1}), which was validated for purity by mass spectrometry analysis (see Fig. S3 and Table S1), or buffer alone for 1 h. The wells were rinsed once with buffer and processed for crystal violet staining.

Antibody interference assay

A polyclonal anti-NF antibody (Timmers *et al*., 1998) was diluted 1:10 in phosphate buffered saline (PBS), pH 7.2 (Sambrook and Russell, 2001). Liquid cultures of the Δ*nodD1ABC* mutant (GMI357) were grown to early stationary phase and centrifuged for 5 min at 7500 *g*. The cell pellets were resuspended in the antibody solution at a final concentration of 1×10^8 cfu ml^{-1}. The cell suspension was mixed on a rotary platform for 18 h at 4°C and then centrifuged for 5 min at 10 000 *g*. The supernatants containing the cleared antibody were filter-sterilized to remove residual bacteria. The cleared anti-NF antibody was diluted 1:100–1:10 000 in the culture medium (Fujishige *et al*., 2006a), which was filter-sterilized and used to resuspend *S. meliloti* RCR2011 cells, *A. tumefaciens* A348, or A348 (pRmJ30) to $OD_{600} = 0.2$. One hundred millilitres of cell suspension were added to individual wells of a 96 well PVC plate. Biofilm formation was assayed as described (Fujishige *et al*., 2006a).

Salt aggregation assay

Liquid cultures were supplemented with either 10 μM luteolin dissolved in 0.1% methanol or with 0.1% methanol alone. Cultures were grown to $OD_{600} = 1.5$, washed and resuspended in 2 mM sodium phosphate (pH 7.0) at a final concentration of 5×10^8 cfu ml^{-1}. On a microscope slide, 25 μl of the cell suspension was mixed with an equal volume of ammonium sulphate in 2 mM sodium phosphate (pH 7.0). Ammonium sulphate concentrations ranged from 0.5 M to 4.0 M. Bacterial aggregation was monitored by microscopy for at least 2 min.

Transmission electron microscopy

Twenty-four-hour-old biofilm cells were scraped off glass coverslips and resuspended in PBS, pH 7.4. A drop of each suspension was placed on a carbon-coated grid, and the bacteria were allowed to settle. The cells on the grid were negatively stained with 2% uranyl acetate for 35–60 s, and subsequently examined under a JEOL-100CX transmission electron microscope.

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Supplementary material

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