Supporting Information Corrected June 17, 2015

Supplementary Tables

Table S1. Bacterial strains and plasmids used in this study.

Strains	Relevant characteristic	Reference or source
Sinorhizobium meliloti		
1021	Sm^r derivative of 2011 (*expR*$^-$)	(1)
8530	1021 *expR*$^+$ derivative; Sm^r	(2)
Rm9020	8530 *exoY*::Tn*5*-132; Sm^r Otc^r	(3)
8530 ΔexpR	8530 derivative with *expR* deletion	(M.J. Soto)
8530 sinI::Km	8530 derivative with a Km resistant cassette inserted in *sinI*	(M.J. Soto)
2011mTn5STM.1.08.B12	2011 derivative with miniTn*5* in *SMb20391* gene; Nm^r	(4)
2011mTn5STM.4.13.E03	2011 derivative with miniTn*5* in *SMb20391* and oriented *gus* fusion; Nm^r	(4)
2011mTn5STM.4.03.E12	2011 derivative with miniTn*5* in *SMb20460* gene; Nm^r	(4)
SMb20391::Nm	8530 derivative with miniTn*5* *in SMb20391*; Str^r Nm^r	This work
SMb20391::Nm-gus	8530 derivative with miniTn*5* in *SMb20391* and oriented *gus* fusion; Sm^r Nm^r	This work
SMb20460::Nm	8530 derivative with miniTn*5* *in SMb20460*; Sm^r Nm^r	This work
Δ*SMb20390*	8530 derivative with *SMb20390* deletion; Sm^r	This work
Agrobacterium tumefaciens		
C58 LBA1010	C58 containing oncogenic pTiB6	(5)
Escherichia coli		
DH5α	*supE44*, Δ*lacU169*, Φ80, *lacZ*ΔM1, *recA1*, *endA1*, *gyrA96*, *thi1*, *relA1*, *5hsdR171*	(6)
One Shot® TOP10	(F−) *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ (araleu)7697 galU galK rpsL (Sm^r) endA1 nupG*	Invitrogene
β2163	(F−) RP4-2-Tc::Mu *dapA*::(*erm-pir*) [Km^r Em^r]	(7)
S17.1	Tmp^r, Sm^r, Sp^r*; thi, pro, recA, hsdR, hsdM, Rp4Tc::Mu, Km::Tn7*	(8)
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS$_B$*(r$_B^-$ m$_B^-$) λ(DE3 [*lacI lacUV5*-T7 gene 1 *ind1 sam7 nin5*])	Novagene
OmniMAX™	[*proAB lacIq lacZΔM15* Tn*10*(Tc^r) *Δ(ccdAB)*] *mcrA Δ(mrr hsdRMS-mcrBC) Φ 80(lacZ)ΔM15 Δ(lacZYA-argF)U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD*	Invitrogene
Plasmids		
pJB3Tc19	Ap^r, Tc^r; cloning vector, P$_{lac}$ promoter	(9)

pET28b+	Km^r expression vector, IPTG-inducible T7 promoter.	Novagene
pET28b::BgsA	Expression vector encoding BgsA with a His_6-tag in the N-terminus	This work
pQE80L	Medium-copy expression vector, IPTG-inducible T5 promoter, Ap^r for N-terminus His6-tag fusions	Qiagen
pQE80L::C-BgsA	Expression vector encoding the last 139 amino acids of BgsA with a His_6-tag in the N-terminus	This work
pET21::PA3353	Expression vector encoding a His_6-tag derivative of the PilZ domain protein PA3353 from *Pseudomonas aeruginosa*	(10)
pJBpleD*	Ap^r, Tc^r; pJB3Tc19 derivate bearing a 1423 bp XbaI/EcoRI fragment containing *pleD**	(11)
pJB9091	Ap^r, Tc^r; pJB3Tc19 derivate bearing a EcoRI fragment containing *SMb20390* and *SMb20391*	This work
pJBpleD*9091	Ap^r, Tc^r; pJB39091 derivate bearing a 1423 bp XbaI/EcoRI fragment containing *pleD**	This work
pCR-XL-TOPO	Km^r; cloning vector for PCR products	Invitrogen
pK18*mobsacB*	Km^r; mobilizable suicide plasmid	(12)
pK18Δ20390	Km^r; pCR-XL-TOPO carrying the deleted version of the *SMb20390* gene	This work

Ap^r, Gm^r, Km^r, Nm^r, Otc^r, Sm^r, Sp^r, Tc^r, Tmp^r, stand for resistance to ampicillin, gentamicin, kanamycin, neomycin, oxytetracycline, streptomycin, spectinomycin, tetracycline and trimethoprim, respectively.

Table S2. Primers used in this work

Primer	Product	Sequence (5' to 3')	Used in
op90-F	496 bp	5'GTCCCATTTCTATCTCCCTCG3'	Deletion of *SMb20390*
20390L-R		5'GACGCGGATCGCCAGGAGGAGGATGCC3'	
20390R-F	408 bp	5'TCCTGGCGATCCGCGTCACGCTCGCT3'	
SM_b20391-R		5'CGACGTAGCGGCTCATC3'	
op90-F	3720 bp	5'GTCCCATTTCTATCTCCCTCG3'	Amplification of *SMb20390-91 operon*
op91-R		5'CTCCCGCACGCTCTATGC3'	
op91-F	2068 bp	5'CATCGATAGGAGGTTGCTTGTGGTTCAG TCTCTTCTAGC3'	Overexpression of *SMb20391*
op91-R		5'CTCCCGCACGCTCTATGC3'	
C-BgsA-F	488bp	5'GGATCCGCCTCGCTTATCTGCTTC3'	Overexpression of C-terminus of *SMb20391*
C-BgsA-R		5'AAGCTTTCCCGCACGCTCTATGC3'	
SMb20390-F	144 bp	5'GGGATATTCCTCGTCGG3'	qRT-PCR
SMb20390-R		5'GGCTATCTGCTGGTCCG3'	
SMb20391-F	150 bp	5'TGCGTTACATGCTCTGGC3'	qRT-PCR
SMb20391-R		5'CGACGTAGCGGCTCATC3'	

Supplementary Figures

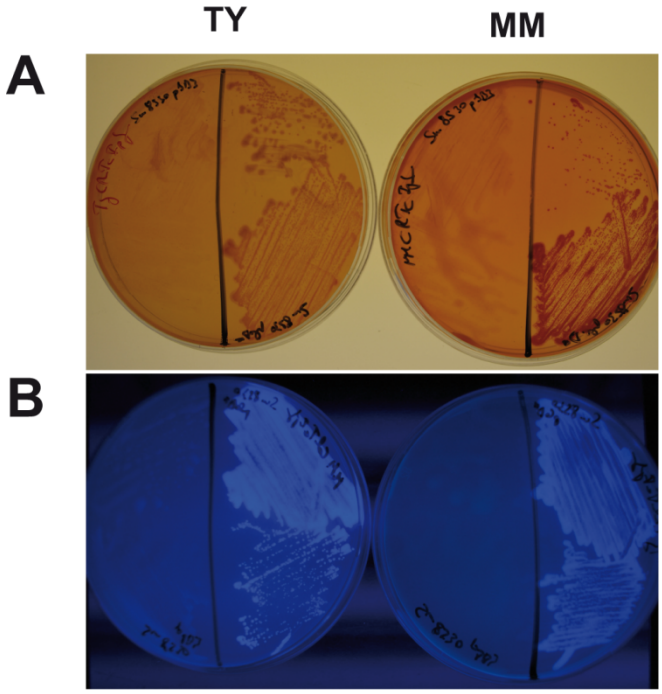

Figure S1. Influence of *pleD** on bacterial staining with different dyes.

S. meliloti 8530 carrying pJBpleD* plasmid (right side of plates) or the vector pJB3Tc19 (left side of plates), grown on rich TY or minimal (MM) medium. A, Media supplemented with Congo Red; B, Media supplemented with Calcofluor. Cultures were imaged under white (A) or UV light (B) after growth at 28°C for 3 days.

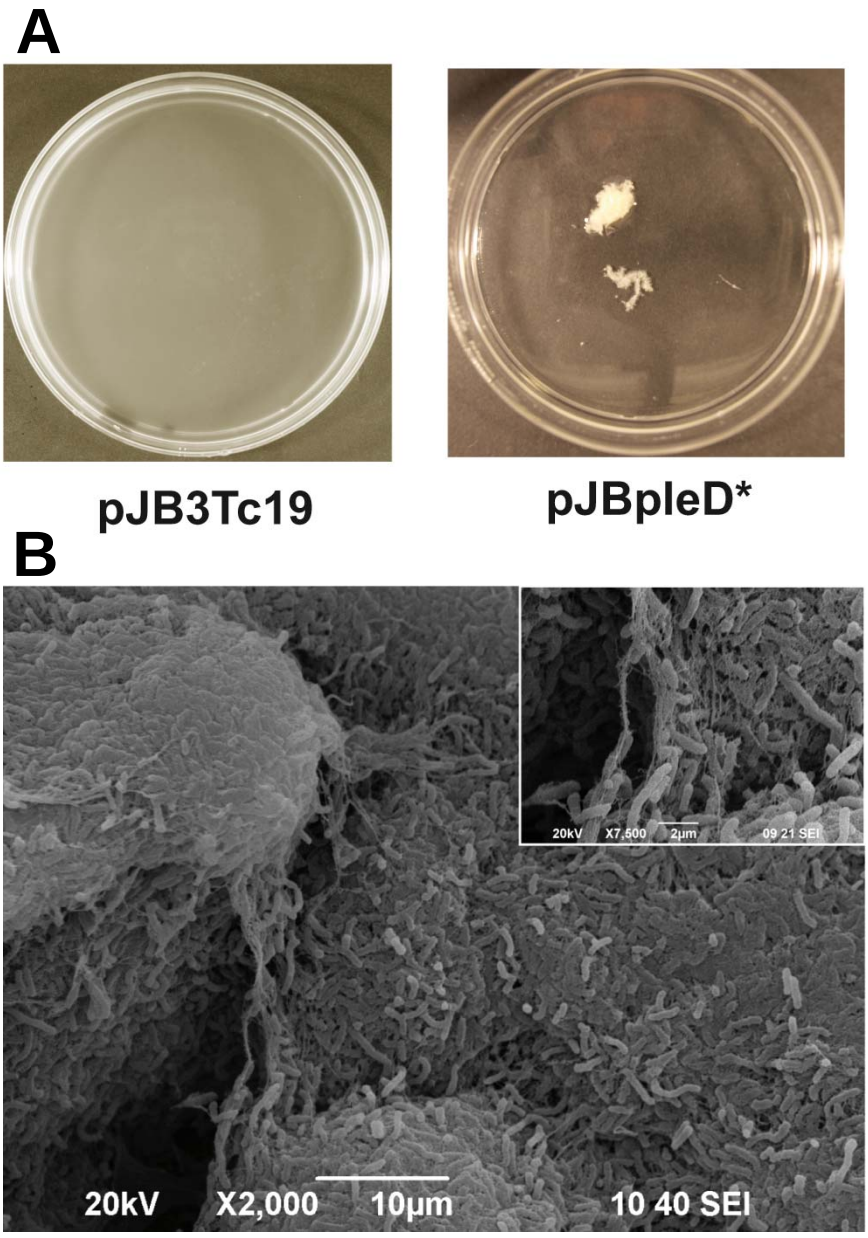

Figure S2. Elevated c-di-GMP levels in *Sme* promote floculation in liquid cultures. (A) Flocs produced by *Sinorhizobium meliloti* 8530 expressing *pleD** (pJBpleD*) after growth for 1 week at 28°C with shaking in liquid MM in plates, in contrast to the control strain (*Sme* pJB3Tc19). (B) Scanning electron microscopy (SEM) images at ×2,000 and ×7,500 magnification (inset) of a floc formed by *Sinorhizobium meliloti* 8530 expressing *pleD** (pJBpleD*). The images reveal details of the mesh of entangled filaments formed by *Sme* with high c-di-GMP levels.

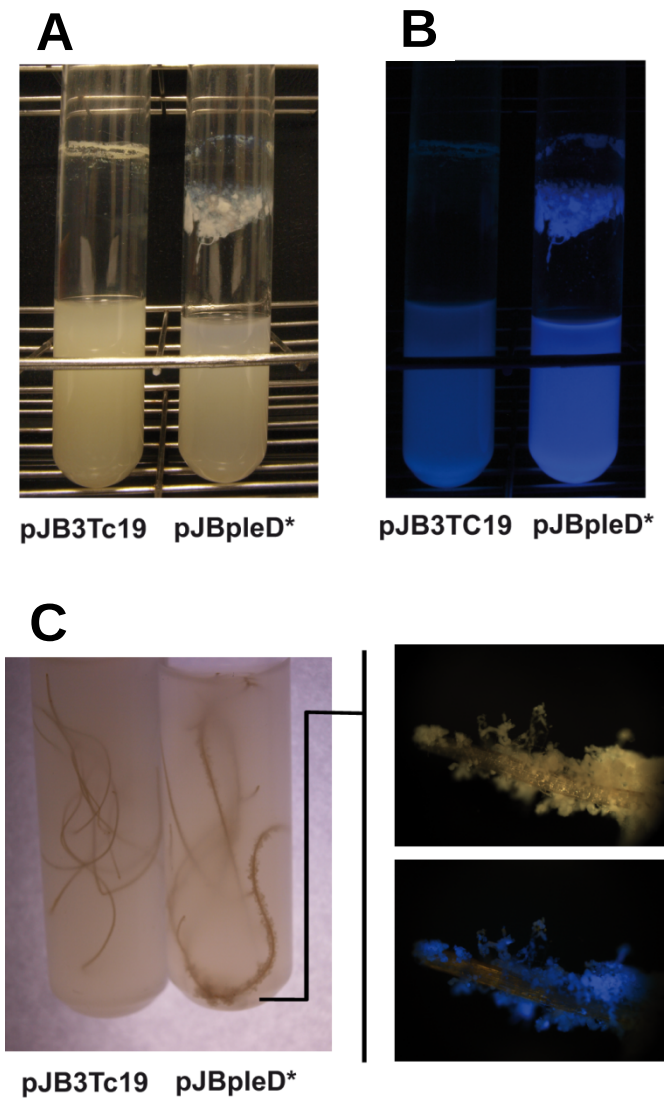

Figure S3. Elevated c-di-GMP levels in *Sme* promote biofilm formation and attachment to abiotic and biotic surfaces. (A and B) Biofilm formed by *Sinorhizobium meliloti* 8530 (pJBpleD*) after growth with shaking for 3 days at 28°C in glass tubes with liquid MM media supplemented with CF (100µM) under white (A) and UV illumination (B).

(C) Biofilm formation of *Sme* 8530 (pJBpleD*) on alfalfa root surface. Bacteria were grown at 28°C for 3 days in glass tubes with liquid MM media supplemented with CF (100 µM) in the presence of *Medicago sativa* roots. Images of the biofilm formed by *Sme* pJBpleD* over the roots were taken with a stereomicroscope under white- and UV-light.

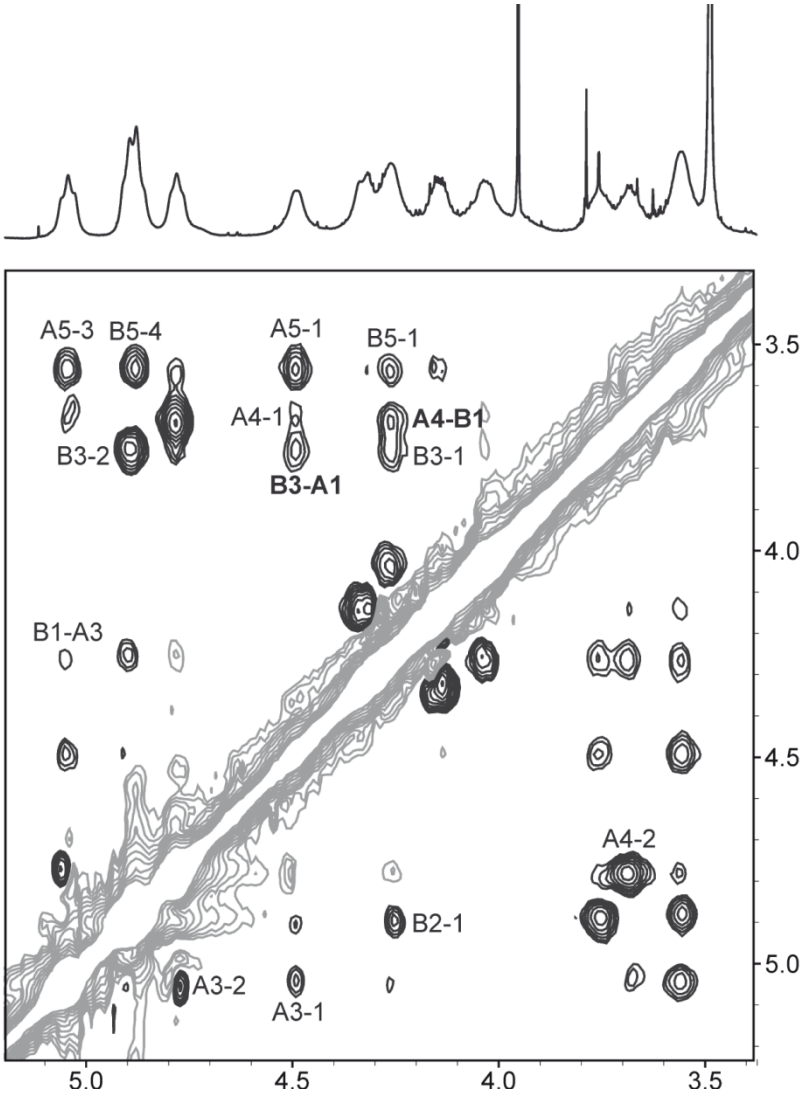

Figure S4. 500 MHz ROESY (mixing time 100 ms) of the acetylated mixed-linkage β-Glucan (MLG) purified from *Sinorhizobium meliloti* 8530 overexpressing *pleD** (*Sme* pJBpleD*). Inter-residue cross-peaks are indicated in bold.

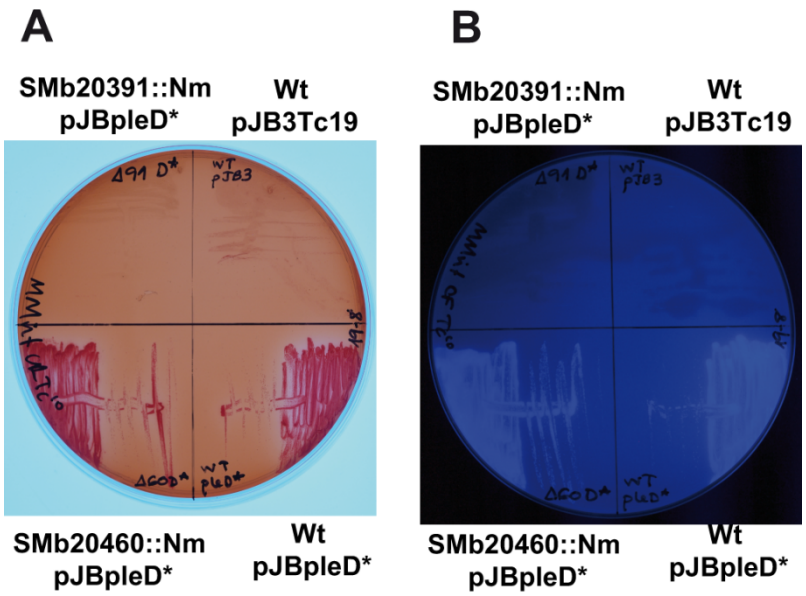

Figure S5. Influence of gene mutations on *S. meliloti* staining with different dyes. Appearances of *S. meliloti* 8530 (Wt) and *SMb20391* and *SMb20460* mutant strains expressing *pleD** (pJBpleD*). Cultures were imaged after growth at 28°C for 3 days in solid MM media supplemented with (A) Congo Red or (B) Calcofluor (UV light).

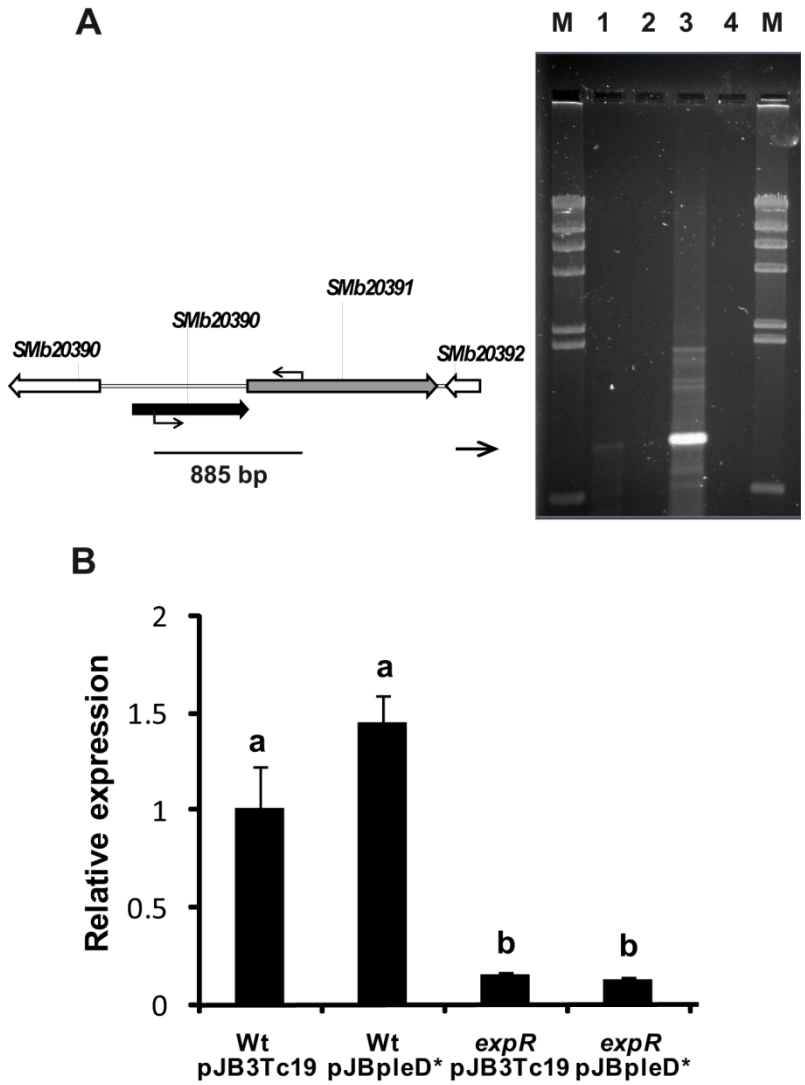

Figure S6. Transcription of the *Sinorhizobium meliloti SMb20390-91* operon.

(A) Cotranscription of *SMb20390* and *SMb20391* genes: M, Molecular ladder (λ HindIII); Reverse-Transcriptase (RT) PCR amplification of the depicted 885 bp fragment using *Sme* 8530 RNA carried out with (lane 1) or without (lane 2) the RT enzyme. Genomic DNA of *Sme* 8530 strain was used as positive control (lane 3) and MiliQ water as a blank (lane 4). (B) Relative transcript levels of *SMb20391* gene in *Sme* 8530 (wt) and a *expR* mutant derivative, in presence (pJBpleD*) or absence of *pleD** (pJB3Tc19). Error bars represent standard deviation; a and b denote analysis of variance categories for significant differences ($P < 0.05$).

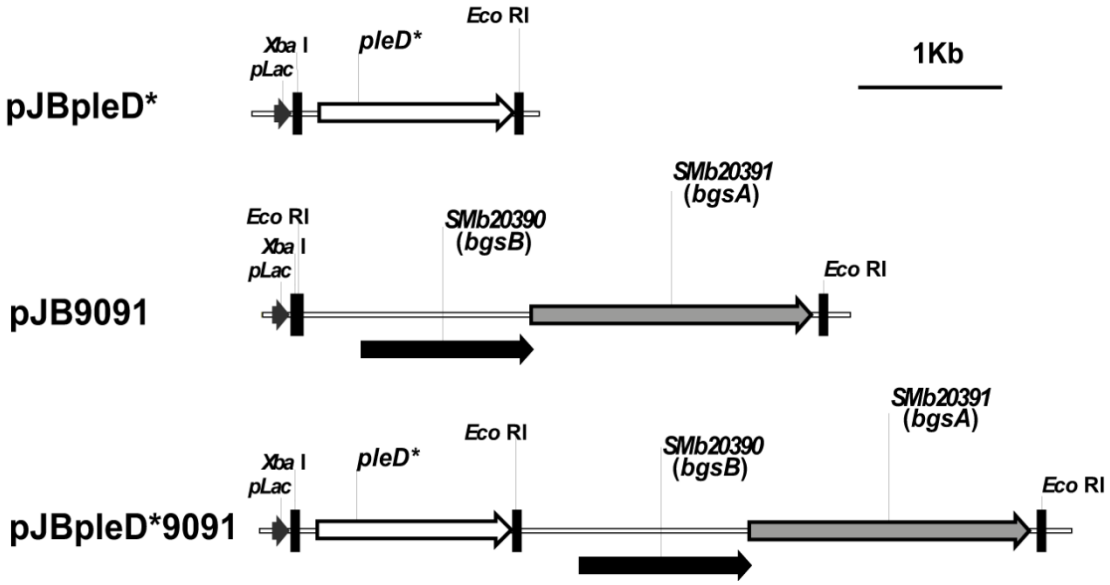

Figure S7. Diagram of various plasmid constructions.

Genes *pleD**, *SMb20390* and *SMb20391* are indicated as white, black and gray arrows, respectively. Relevant restriction sites and other plasmid features are depicted.

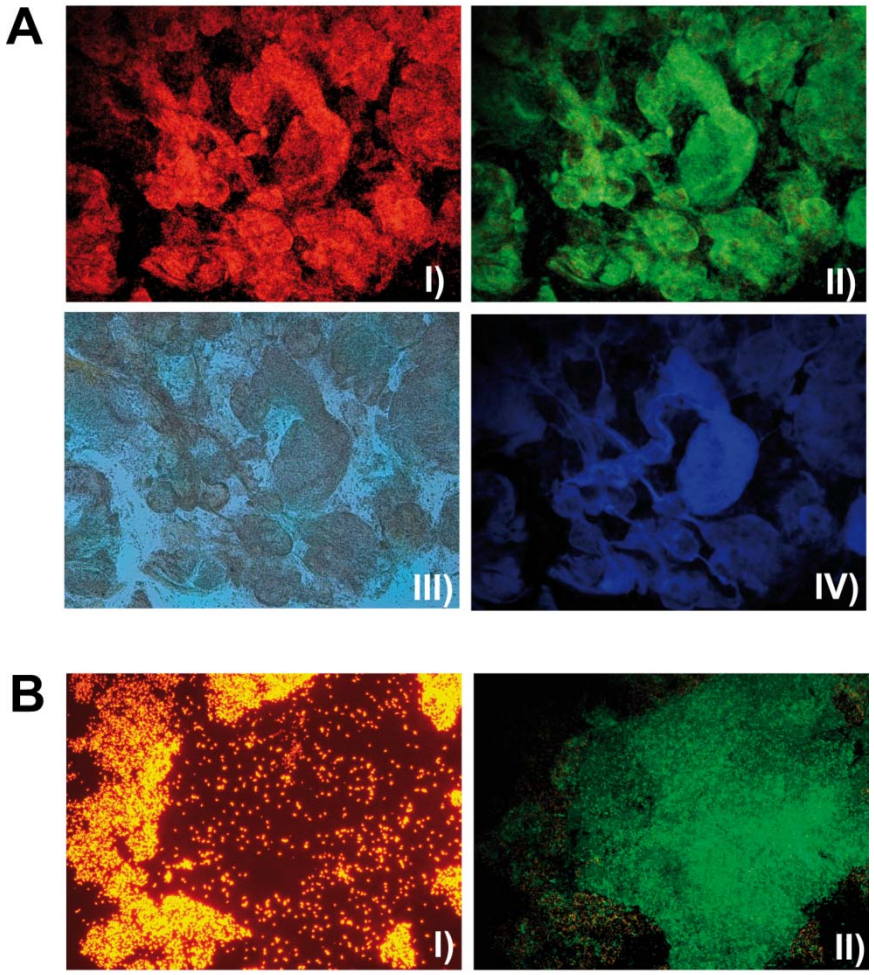

Figure S8. Epifluorescence micrographs at ×10 magnification of bacterial flocs. A, floc formed in flasks inoculated with 1:1 mixtures of 8530-GFP and 8530-dsRed, both overexpressing PleD*; B, floc formed in flasks inoculated with similar numbers of 8530-GFP and *SMb20391*-dsRed, both overexpressing PleD*; Filters: I, dsRed (G-2A); II, GFP (B-2A); IV, Calcofluor (CF; UV-2E/C); III, no filter.

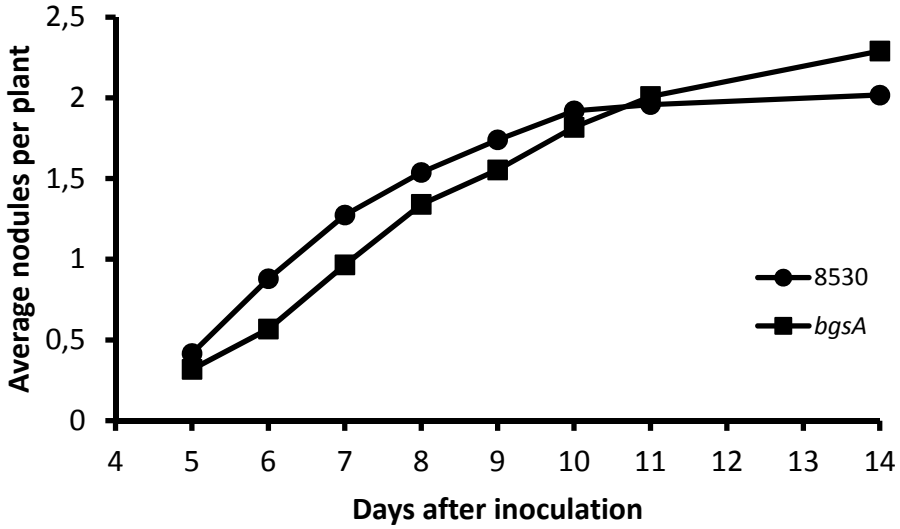

Figure S9. Nodulation kinetics of wild type 8530 and *SMb20391::Nm* mutant (*bgsA*), inoculated at 10^3 cells per root. Values are the means from 45 inoculated alfalfa plants and two independent experiments.

Supplementary Methods

Intracellular c-di-GMP measurements

c-di-GMP was extracted using a protocol based on a previous report (13). Three biological replicates of *Sinorhizobium meliloti* 8530 (*Sme*) expressing *pleD** (pJBpleD*) and its control *Sme* (pJB3Tc19), were grown in 10 ml of TY with shaking at 28ºC for 24h. Formaldehyde at a final concentration of 0.19 % was added and the cells harvested by centrifugation (10 min at 4000 rpm). The pellet was washed in 1 ml of iced deionised water, centrifuged for 3 min at 13000 rpm, resuspended in 0.5 ml of iced deionised water and heated to 95ºC for 5 min. A volume of 925 µl of iced absolute ethanol was added to reach a final concentration of 65%. Nucleotides were extracted by 30 sec of vortex followed by a centrifugation step (3 min at 13000 rpm). Supernatants containing extracted nucleotides were then evaporated to dryness at 50 °C in a speed-vacuum system. The pellet was resuspended by vigorous vortexing in 300 µl of $AcNH_4$ 10mM (pH 5,5). Samples were filtered through 0.45 µm GHP membranes (GHP Acrodisc, PALL). Samples were spiked at a concentration of 250 nM by mixing 100 µl of a solution of 750 nM of synthetic c-di-GMP (Axxora) disolved in $AcNH_4$ 10mM pH 5,5 with 200 µl of each sample. Samples were analysed by reversed phase-coupled HPLC-MS/MS. High performance liquid chromatography was performed on an Agilent 1100 coupled to a 3x125 mm column Waters Spherisorb 5 µm ODS2 (C-18). Running conditions were optimized using synthetic c-di-GMP as a reference. ESI-MS mass spectra were measured on an Esquire 6000 (Bruker Daltonics) and on a TSQ7000 (Finnigan) mass spectrometer. Matrix-assisted laser desorption ionization spectra were measured on a Reflex III spectrometer (Bruker Daltonics). To confirm the identity of the substance, relevant peaks were fragmented by ESI-MS using the positive ion mode. Three major ions were visible in the c-di-GMP fragmentation pattern, and m/z 152 and

540 corresponded to products from single bond fragmentation, and 248 originated from double bond fragmentations. The area of the ion m/z 540 peak was used to estimate the amount of c-di-GMP in each sample (similar values were obtained with the other ions). For quantification, a standard curve was established using synthetic c-di-GMP (Axxora) dissolved in ammonium acetate (10mM pH 5.5) at a range of concentrations (20 nM, 200 nM, 2 μM and 20 μM). After subtracting the basal 250nM spike, c-di-GMP concentrations in each strain culture were standardised with the total protein content, determined by Bradford assay (14).

Microscopy studies of flocs formed by Sinorhizobium meliloti

The flocs formed by *Sinorhizobium meliloti* strains expressing *pleD** in liquid MM were analyzed by epifluorescence and scanning microscopy. For the epifluorescence microscopy two different fluorescent constructions (pBBR-gfp and pBBR-dsRed) were introduced into *Sinorhizobium meliloti* strains. Equal number of cells (from starting cultures) of the two strains to be compared (WT-GFP versus WT-DsRed or WT-GFP versus *SMb20391*-DsRed) expressing *pleD** (pJBpleD*) were inoculated in flasks with 10 ml of liquid MM supplemented with Tc and CF and grown with shaking for 2 days at 28°C. The flocs formed were analysed using a epifluorescence microscope (Nikon eclipse 50i). Filters for Calcofluor (CF; UV-2E/C Ex/Em 358/461nm) and for fluorescent proteins GFP (B-2A Ex/Em 480/506nm) and DsRed (G-2A Ex/Em 558/583nm) were used.

For scanning microscopy flocs formed by untagged *Sinorhizobium meliloti* 8530 (*Sme*) expressing *pleD** (pJBpleD*) grown in MM with shaking for 2 days at 28°C were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH7.2, dehydrated in ethanol series and taken to the critical point (Baltec CPD030). Samples were then mounted on

stainless steel stubs, covered with gold in a Leica SCD050 and examined in a Jeol Scanning Electron Microscope (JSM-6490LV).

Flocculation and Biofilm assays

Starting cultures of rhizobial strains were prepared as above. Flocs were obtained by growing *Sinorhizobium meliloti* 8530 expressing *pleD** (pJBpleD*) for 2-7 days in Petri dishes at 28°C in liquid MM supplemented with tetracycline (10 µg/ml) with gentle shaking.

For biofilm assays, 6 ml of diluted cultures in MM supplemented with CF (100µM final concentration) were disposed in glass tubes and let grow with shaking at 28ºC for 72 h. For adhesion to biotic surfaces, bacteria were incubated in the presence of surface sterilized alfalfa roots. Roots were imaged using a fluorescent stereo microscope (Leica M165 FC). Biofilm quantification was done in 96-well plates by crystal violet staining as previously described (5).

Supplementary References

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conjugative machineries and their cognate *Escherichia coli* host strains. *Res.Microbiol.* 156(2):245-255.
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