Novel mixed-linkage β-glucan activated by c-di-GMP in Sinorhizobium meliloti

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An artificial increase of cyclic diguanylate (c-di-GMP) levels in Sinorhizobium meliloti 8530, a bacterium that does not carry known cellulose synthesis genes, leads to overproduction of a substance that binds the dyes Congo red and calcofluor. Sugar composition and methylation analyses and NMR studies identified this compound as a linear mixed-linkage (1→3)(1→4)-β-D-glucan (ML β-glucan), not previously described in bacteria but resembling ML β-glucans found in plants and lichens. This unique polymer is hydrolyzed by the specific endoglucanase lichenase, but, unlike lichenan and barley glucan, it generates a disaccharidic β-(1→3)→(1→4)β-D-glucan-β-glucan repeating unit. A two-gene operon bgsBA required for production of this ML β-glucan is conserved among several genera within the order Rhizobiales, where bgsA encodes a glycosyl transferase with domain resemblance and phylogenetic relationship to curdlan synthases and to bacterial cellulose synthases. Its expression is regulated by c-di-GMP, although only the allosteric activation of CSs through c-di-GMP binding to its C-terminal domain. To our knowledge, this is the first report on a linear mixed-linkage (1→3)(1→4)-β-glucan produced by a bacterium. The S. meliloti ML β-glucan participates in bacterial aggregation and biofilm formation and is required for efficient attachment to the roots of a host plant, resembling the biological role of cellulose in other bacteria.

In bacteria the second messenger cyclic diguanylate (c-di-GMP) regulates the transition from a motile to a sedentary lifestyle, so that high concentrations enhance the production of extracellular matrix components, exopolysaccharides (EPSs), and proteins (1–5). The c-di-GMP is synthesized from two molecules of GTP by the action of diguanylate cyclases (DGCs) and is hydrolyzed to 5′-phosphoguananyl-(3′-5′)-guanosine by specific phosphodiesterases. It has been determined that artificial increments of the intracellular levels of c-di-GMP by the overproduction of specific EPSs is required for production of this ML β-glucan in Sinorhizobium meliloti, an endosymbiont of alfalfa (Medicago sativa), produces two structurally distinct EPSs: EPS-I, or succinoglucon, and EPS-II, or galactoglucon; both are regulated by the ExpR/Sin quorum-sensing system and are important for nodulation of legume hosts (13, 15, 16).

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Significance

We report a novel linear mixed-linkage (1→3)(1→4)-β-glucan produced by the bacterium Sinorhizobium meliloti upon raising cyclic diguanylate (c-di-GMP) intracellular levels. This unique bacterial polysaccharide resembles (1→3)(1→4)-β-glucans found in cereals and certain lichens but has a distinctive primary structure. Genes, proteins, and regulatory pathways for producing this new polymer are described. Our findings open the possibility of using bacteria to produce (1→3)(1→4)-β-glucans, which are receiving increasing interest as bioactive compounds, and provide new elements to disclose molecular mechanisms of c-di-GMP regulation as well as for investigating the evolution, activity, and specificity of glycosyl transferases.

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of BcsA/CelA proteins, has been demonstrated (21–23). A PilZ domain apparently is absent in curdlan synthases (24); nonetheless c-di-GMP activation could occur by a different mechanism, because this second messenger is able to regulate the production of other EPSs by interacting with proteins involved in EPS extrusion (25, 26). Here we have exploited c-di-GMP activation of bacterial EPSs to unveil a undisclosed linear mixed-linkage (ML) (1→3)(1→4)-β-glucan in a cellulose-nonproducing bacterium, *S. melliloti* 8530.

**Results**

**Effects of Increased c-di-GMP Levels on the Production of EPSs by *S. melliloti* 8530.** In cellulose-producing (Cet+) bacteria, raising c-di-GMP intracellular levels (i.e., by overexpression of a DGC) triggers production of cellulose and other extracellular molecules (5, 6, 27). *S. melliloti* 8530 does not bear any of the known CS operons in its genome; however, overexpression of the DGC PleD* generated wrinkled and red-stained colonies in medium with Congo red (CR), in deep contrast to the wild-type colonies (*SI Appendix*, Fig. S1A). Furthermore, the overexpression of pleD* (pJBpleD*) also increased the calcifluor (CF)-derived fluorescence in both solid and liquid cultures (Fig. 1 and *SI Appendix*, Fig. S1B).

In liquid media and particularly in minimal medium (MM), *Sme* overexpressing pleD* (*Sme* pJBpleD*) displayed a strong aggregative behavior with most bacteria forming flocs (*SI Appendix*, Fig. S2A). Scanning electron microscopy of flocs showed that bacteria were encased in a dense matrix of filaments (*SI Appendix*, Fig. S2B). Adherence to and biofilm formation on test tube walls also were enhanced (*SI Appendix*, Fig. S3A); moreover, those biofilms fluoresced in the presence of CF under UV light (*SI Appendix*, Fig. S3B), indicating that the CF+ substance was part of the biofilm matrix. However, biofilm formation could not be quantified because it detached easily from the abiotic surfaces and was removed during the Cristal Violet staining process (Materials and Methods). The PleD*-dependent EPS matrix produced by *Sme* also enhanced its adhesion to alfalfa roots and the formation of a thick CF+ biofilm coating the root surface (*SI Appendix*, Fig. S3C). All these phenotypes correlated with a strong increase in the c-di-GMP intracellular levels provoked by PleD*. Extracts from *Sme* harboring the plasmid pJBpleD* contained 596.86 pmol of c-di-GMP/mg total protein, whereas the amounts of c-di-GMP from *Sme* carrying the pJB3Tc19 empty vector were below the HPLC-MS limit of detection (*SI Appendix, Supplementary Methods*).

**Characterization of a Novel β-Glucan Produced by *S. melliloti*.** CR fluorescence in both solid and liquid cultures (Fig. 1 and *SI Appendix*). The PleD*-dependent EPS matrix produced by *Sme* also enhanced its adhesion to alfalfa roots and the formation of a thick CF+ biofilm coating the root surface (*SI Appendix*, Fig. S3C). All these phenotypes correlated with a strong increase in the c-di-GMP intracellular levels provoked by PleD*. Extracts from *Sme* harboring the plasmid pJBpleD* contained 596.86 pmol of c-di-GMP/mg total protein, whereas the amounts of c-di-GMP from *Sme* carrying the pJB3Tc19 empty vector were below the HPLC-MS limit of detection (*SI Appendix, Supplementary Methods*).

**Fig. 1.** Quantification of CF-derived fluorescence of *S. melliloti* 8530 and its derivative Smb20391::Nm mutant harboring different plasmid constructions. Results are expressed in arbitrary units standardized by the OD600nm ± SE from three independent cultures.

**Fig. 2.** CF-derived fluorescence of *S. melliloti* 8530 (Wt) and mutant derivatives carrying the plasmid pJBpleD* or the empty vector pJB3Tc19. (Right) Bacterial cultures were imaged under UV light after growth at 28 °C in solid TY medium supplemented with CF (200 µg/mL). (Left) The diagram depicts the positions of the strains.

Whereas CF is more specific for polymers containing β-(1→3)- or β-(1→4)-β-glucopyranosyl units (28, 29), *Sme* produces at least one EPS, known as “EPS-I” or “succinoglycan,” which binds CF but not CR (30, 31). Indeed, *Sme* shows a greenish CF-derived fluorescence; however, pJBpleD* conferred a blue CF fluorescence (Fig. 2) which was retained in an exoY mutant lacking EPS-I (32). This finding suggested that high c-di-GMP levels generated by PleD* stimulated the production of a CF-binding polymer different from EPS-I.

A purification approach was designed to isolate and identify this substance. The *Sme* pJBpleD* strain was grown in flasks with liquid MM, and the flocs generated were recovered after 3 d using a sieve (Materials and Methods). Like curdlan and cellulose, the *Sme* substance was water insoluble, but, unlike cellulose, it could be dissolved in diluted NaOH solutions (0.5 M). Curdlan is soluble in dilute alkali (0.25 M NaOH) and, after aqueous suspensions are heated above 80 °C and subsequently cooled, produces high-set thermo-reversible gels (33). This gelling behavior was not observed with the *Sme* substance, so several rounds of boiling/cooling/centrifugation cycles were followed by a lyophilization step to obtain a substance with a cottony appearance. Monosaccharide analysis showed that d-glucose was the only component, and the methylation analysis indicated that these glucopyranoses were substituted at positions →3 and →4 in a 1:1 ratio. The polysaccharide was insoluble in water or in DMSO; therefore a few milligrams were treated with a mixture of acetic anhydride and pyridine to acetylate its hydroxyl groups and improve its solubility in organic solvents. After purification by size-exclusion chromatography (SEC), a fraction of the polysaccharide was solubilized in CDCl3 and was studied by NMR. Assignments were made using bidimensional experiments [DQF-COSY, total correlated spectroscopy (TOCSY), rotating-frame Overhauser spectroscopy (ROESY), and heteronuclear single-quartum coherence (HSQC)] and are listed in Table 1. NMR spectra showed two different units of glucose, labeled as “unit A” and “unit B,” with a ratio close to 1:1. Chemical shifts (1H and 13C) indicated their β configuration and that they were present in the pyranose form (Fig. 3A). Moreover, unit A was substituted at position →4, whereas unit B was substituted at position →3. The results were in good agreement with the chemical shifts of acetylated cellulose (34) and (1→3)-β-d-glucopentose peracetate (35). Finally, the ROESY spectrum (*SI Appendix*, Fig. S4) allowed the sequence of residues in the polysaccharide to be deduced, because intense cross-peaks were found between signals H-1 in unit A and H-3 in unit B and between H-1 in unit B and H-4 in unit A. Considering all these data, we propose that the acetylated polysaccharide has the repeating unit →3)-β-D-GlcP-(1→4)-β-D-GlcP-(1→x (Fig. 3B).

This β-glucan structure resembles ML β-glucans such as lichenan and barley β-glucan, which can be hydrolyzed specifically by lichenase, an endohydrolase whose target site is widely assumed to be all (1→4) bonds immediately following (1→3).
bonds (36). It does not hydrolyze pure (1→3)-β-D-glucans (i.e., curdlan) nor (1→4)-β-D-glucans (cellulose). Incubation of lichenan and barley β-glucan with lichenase produced distinctive mixtures of tri-, tetra-, and larger cello-oligosaccharides which could be separated by TLC (Fig. 4), in agreement with previous reports (37). Lichenase also was able to hydrolyze the S. meliloti β-glucan, although in this case the only product of hydrolysis was a disaccharide (Fig. 4). This result agreed with the NMR data and further supported the notion that the repeating unit of the β-glucan contains alternating β(1→3) and β(1→4) bonds (Fig. 3B).

The bgsBA Operon Is Required for ML β-Glucan Production. The S. meliloti 8530 genome encodes at least two glycosyl transferases (GTs) of unknown function, SMb20391 and SMb202460, annotated as hypothetical CSs (38). The PleD*-dependent phenotypes were tested with transposon-induced mutants in both genes (SI Appendix, Table S1). The SMb20460 mutant behaved like the wild type; however, the SMb20391 mutant had lost the PleD*-dependent CR* and CF* phenotypes and the aggregative behavior observed in the wild type.

The SMb20391 ORF overlaps by 19 bp the upstream ORF SMb20390, which encodes a putative membrane fusion protein (MFP) belonging to the AcrA/EmrA/HlyD family of transporters. RT-PCR experiments indicated that SMb20390 and SMb20391 are cotranscribed (SI Appendix, Fig. S6A). An in-frame deletion of SMb20390 (∆SMb20390) was constructed, and, like the SMb20391 mutant, the ∆SMb20390 pJBpleD* mutant showed a CR* and CF* phenotype and did not aggregate or form biofilm at high c-di-GMP levels. This result indicated that both the SMb20390 and SMb20391 genes are responsible for the c-di-GMP–induced phenotypes observed in Sme. This notion was confirmed further after genetic complementation. To avoid possible functional problems resulting from an imbalance between SMb20390 and SMb20391 protein levels, two new plasmids were constructed, one harboring the complete operon SMb20390-91 cloned in pJB3Tc19 (pJB9091) and a second one carrying SMb20390-91 and pleD* (pJBpleD*9091) (SI Appendix, Fig. S7). Both plasmid constructions were able to restore the CR* and CF* phenotypes in the SMb20391::Nm mutant (Fig. 1). Although the CF-derived fluorescence was much higher when pleD* was present, the two genes in multicopy were able to provide CR* and CF* phenotypes even under physiological c-di-GMP conditions. We also measured the relative yield of ML β-glucan in cultures, which represented 47.8% of the dry weight of the wild-type Sme (pJBpleD*) cultures. As expected, no ML β-glucan was produced by the SMb20391::Nm mutant overexpressing PleD*. The SMb20391::Nm mutant complemented with pJB9091 displayed a low (2.1% of culture dry weight) but nevertheless measurable yield of ML β-glucan. The ML β-glucan yield was highest in cultures of the mutant carrying both pleD* and SMb20390-20391 genes in multicopy (pJBpleD*9091), accounting for 77.5% of the culture dry weight. Therefore, two conditions seem to be required for high production of the ML β-glucan by Sme: (i) the presence of a functional copy of the SMb20390-91 operon and (ii) high intracellular levels of c-di-GMP. Given their requirement for ML β-glucan production, we propose that SMb20391 be renamed bgsA (with “bgs” standing for “β-glucan synthesis”) and that SMb20390 be renamed “bgsB.”

c-di-GMP Binding to BgsA. The bgsB gene product contains an HlyD-like domain found in diverse proteins involved in the export of a broad variety of compounds (39) and shows homology to MFPs, accessory proteins that cooperate with a variety of transporters. MFPs span the periplasm, linking the inner and outer membranes and facilitating the formation of a channel to expel the substrate from the cell (40).

Hydropathy analysis of the 664-aa BgsA protein sequence using the TMHMM 2.0 prediction program (41) revealed seven potential transmembrane (TM) helices (Fig. 5), in contrast to the
using a previously reported fluorescence-based dot blot assay with (46). We nevertheless tested the ability of BgsA to bind c-di-GMP, although BgsA expression probably was poor, because these cells expressing full-length BgsA gave weak positive results, whereas BgsA cells were dispersed (Fig. 7). The impact of c-di-GMP on the transcription of the bgsBA operon was assayed by quantitative RT-PCR (RT-qPCR) in the absence and presence of PleD*. Although bgsA transcription was slightly higher with PleD*, this variation was not statistically significant and suggested that c-di-GMP does not affect the transcription of bgsBA (SI Appendix, Fig. S6B). This suggestion was verified in a strain carrying a transcriptional bgsA::gus fusion. β-Glucuronidase activity in strain SMb20391::Nm-gus carrying pJBpleD* was 158.4 ± 13.85, similar to 171.7 ± 22.04 in the strain with the empty vector pJB3Te19.

bgsA (SMb20391) was found previously among Sme genes whose expression was dependent on expR and sinI genes (48) and recently has been confirmed to be a member of the Sin/ExpR regulon (49). We confirmed the ExpR dependence of bgsA transcription (SI Appendix, Fig. S6B) and extended it to bgsB, which showed a 50-fold transcript reduction in the expR mutant. Moreover, expR and sinI mutants overexpressing PleD* were impaired in the emission of the c-di-GMP-induced blue CF fluorescence (Fig. 2), showed a CR− phenotype, and did not exhibit the PleD2-dependent flocculation in liquid media. Furthermore, the ExpR dependence of ML β-glucan production was confirmed in the expR mutant Rm1021 (38), which did not display any of the c-di-GMP–associated phenotypes described for Sme.

ML β-Glucan Is Involved in the Attachment of S. melloti to Alfalfa Roots. We evaluated the impact of the new ML β-glucan on bacterial colonization and attachment to alfalfa roots. For microscopy studies, variants of wild-type Sme and bgsA (SMb20391::Nm) mutant strains expressing two different fluorescent proteins, GFP and DsRed, were used. As a control for plasmid stability and to rule out any possible interference of the fluorescent proteins with bacterial physiology or ML β-glucan production, a flocculation assay was carried out first with mixtures of the Sme (pJBpleD*) strain tagged with either of the fluorescent proteins, and flocs formed after 3 d were observed under the epifluorescence microscope. These flocs were constituted by similar numbers of each tagged variant (SI Appendix, Fig. S8A). In contrast, when bacterial mixtures contained the wild-type (pJBpleD*, pBBR-gfp) and the bgsA mutant (pJBpleD*, pBBR-dsRed) cells, the wild-type cells were found almost exclusively in the flocs. Most mutant cells were in the surrounding medium, and few were located within the flocs (SI Appendix, Fig. S8B), suggesting that cells not producing the ML β-glucan were excluded from the cell network.

Confocal laser scanning microscopy (CLSM) was used to study competitive root attachment and colonization under high c-di-GMP conditions. Twenty-four hours after inoculation Bgs* cells firmly attached to roots exceeded the bgsA mutant cells by more than 200-fold and formed a strong biofilm with large cell aggregates covering most of the root surface, whereas Bgs− cells were dispersed (Fig. 7).

ExpR Activates Transcription of the bgsBA Operon. The impact of c-di-GMP on the transcription of the bgsBA operon was assayed by quantitative RT-PCR (RT-qPCR) in the absence and presence of PleD*. Although bgsA transcription was slightly higher with PleD*, this variation was not statistically significant and suggested that c-di-GMP does not affect the transcription of bgsBA (SI Appendix, Fig. S6B). This suggestion was verified in a strain carrying a transcriptional bgsA::gus fusion. β-Glucuronidase activity in strain SMb20391::Nm-gus carrying pJBpleD* was 158.4 ± 13.85, similar to 171.7 ± 22.04 in the strain with the empty vector pJB3Te19.

Fig. 4. TLC of β-glucans hydrolyzed with lichenase. Lane 1: untreated S. melloti ML β-glucan; lane 2: S. melloti ML β-glucan incubated with lichenase; lane 3: untreated lichenan; lane 4: lichenan + lichenase; lane 5: untreated barley β-glucan; lane 6: barley β-glucan + lichenase; lane 7: mixture of glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose.}

Fig. 5. Topology model of BgsA (SMb20391) in the inner cell membrane (IM) predicted by TMHMM 2.0 (41) and presented by TOPOZ (www.sacs.ucsf.edu/cgi-bin/open-topo2.py). The putative catalytic residues of the D,D,D35QxxRW motif are shown in yellow, and the predicted DXD motif is enclosed in a box. The 139-aa C-BgsA peptide used in c-di-GMP–binding experiments is shown as gray diamonds.

Eight TMs present in CSs (42, 43). The large hydrophilic region between TM III and IV (amino acid residues 90–375) contains residues and motifs conserved in the GT-2 glycosyl transferases family (D,D,D35QxxRW), including the DXD signature involved in the addition of the UDP-sugar to the nascent polysaccharide (44) and the QXXRW sequence motif likely required for holding the growing glucan chain in the active site (Fig. 5) (45). After TM VII a C-terminal cytoplasmic segment was predicted (residues 532–664) which contains no known functional domains. This protein organization strongly resembles the structure predicted for the A. tunefaciens curdulan synthase (CrdS) (24) and CS; the latter contains a PilZ signature in its C-terminal cytoplasmic domain involved in c-di-GMP binding (22). However, neither CrdS nor BgsA seems to bear a PilZ domain nor the recently described c-di-GMP–binding GIL domain that is present in BecE-like proteins (46). We nevertheless tested the ability of BgsA to bind c-di-GMP, using a previously reported fluorescence-based dot blot assay with 2-Fluo-AHC-c-di-GMP (47). Crude extracts of Escherichia coli cells expressing full-length BgsA gave weak positive results, although BgsA expression probably was poor, because these cells had strongly delayed growth, and the protein could not be detected in SDS/PAGE gels. However, extracts of E. coli cells expressing the cytoplasmic C-terminal segment of BgsA (BgsA-C; the last 139 residues beginning with Ala526) (Fig. 5) showed significant binding to 2-Fluo-AHC-c-di-GMP compared with cell extracts expressing the empty vector (Fig. 6A). The signal from the BgsA-C extract was lower than with the PilZ domain protein PA3353 (46), but this difference could be caused by different protein-expression levels and/or affinities for the ligand. Moreover, the binding of the BgsA-C extract to 2-Fluo-AHC-c-di-GMP could be outcompeted by growing concentrations of pure, unlabeled c-di-GMP (Fig. 6B). These results provide strong evidence that BgsA can bind c-di-GMP through its C-terminal cytoplasmic domain, suggesting allosteric activation of this GT.
Bacterial attachment to roots also was studied at physiological levels of c-di-GMP. Plant roots were inoculated with 1:1 mixtures of the wild type and the \textit{bgsA} SMb20391::Nm mutant at two different inoculum cell densities, 10^5 or 10^3 total cells per root. In both instances each strain occupied nearly 50% of the colonizing roots with 1:1 mixtures of the wild type and the \textit{bgsA} SMb20391::Nm mutant. At physiological c-di-GMP levels the \textit{BgsA} operon is required for the synthesis and/or secretion of the novel ML \(\beta\)-glucan uncovered in \textit{Sme}. Given the features predicted for the encoded proteins, it is most likely that \textit{BgsA} is the synthetase and that \textit{BgsB} may participate in the export of the glucan chain. Similar to what occurs in cellulose or curdlan production (18, 20), it is likely that additional yet unknown components are necessary for full activity of the ML \(\beta\)-glucan synthesis system. We could identify \textit{bgsBA}-like operons in genomes of the genera \textit{Sinorhizobium}, \textit{Rhizobium}, and \textit{Agrobacterium} in the Rhizobiaceae and \textit{Methyllobacterium} in the Methylbacteriaceae, all within order Rhizobiales (Fig. 9). For instance, all available sinorhizobial (\textit{S. meliloti} and \textit{S. fredii}) and \textit{Methyllobacterium} genomes bear a \textit{bgsBA}-like operon, although only some strains of \textit{Rhizobium} carry these genes. Among agrobacteria, only \textit{A. radiobacter} K84 (a nonpathogenic biocontrol strain) carries a \textit{bgsBA}-like operon. A phylogenetic analysis of \textit{BgsA} (\(\beta\)-glucan), CrdS (curdlan), and CelA/BgsA/AcsA (cellulose) synthases showed two monophyletic clades separating Cel synthases from Crd and Bgs synthases (Fig. 9), with the two latter forming clearly diverging clusters. Unlike \textit{S. meliloti} 8530, which carries no CS genes, a Cel system coexists with either a Bgs or a Crd system in most available Rhizobiales genomes. Coexistence of Crd and Bgs systems is far less frequent, although distinctive Cel, Crd, and Bgs synthases are borne by some strains of methylbacteria (Fig. 9). Taken together, the data suggest that CrdS and BgsA share a common ancestor and that divergent evolution may have shaped their specific activities toward synthesis of either (1,3)-\(\beta\)-glucan or (1,3) (1,4)-\(\beta\)-glucan, respectively.

Influence of ML \(\beta\)-Glucan on Nodulation. Alfalfa nodulation with bacteria at high c-di-GMP levels could not be tested properly because the \textit{PleD} plasmid was lost rapidly under nonselective conditions. At physiological c-di-GMP levels the \textit{BgsA} mutant formed pink wild-type lacking the ML \(\beta\)-glucan. Taken together, the results support the notion that production of the ML \(\beta\)-glucan is required for efficient attachment of \textit{S. meliloti} to the alfalfa root surface and consequently for root colonization.

**Discussion**

c-di-GMP is a common activator of several bacterial EPSs, such as cellulose in many bacteria, poly-\(\beta\)-1,6-N-acetylglucosamine in enterobacteria, or alginate, Psl, and Pel in Pseudomonads (50, 51). In this work, we have exploited this feature to uncover an otherwise cryptic EPS produced by the \(\alpha\)-proteobacterium \textit{S. meliloti} 8530 when its intracellular c-di-GMP levels are artificially increased. This new bacterial polymer is a linear ML (1→3)(1→4)-\(\beta\)-\(\beta\)-glucan. It resembles the ML \(\beta\)-glucans found in cereals and lichens, but the bacterial ML \(\beta\)-glucan presents a unique primary structure. Whereas the plant ML \(\beta\)-glucans contain distinctive ratios of cello-oligosaccharides of 3, 4, or 5, and higher degrees of polymerization, linked by (1→3) bonds (37), the bacterial ML \(\beta\)-glucan contains a (→3)-\(\beta\)-\(\beta\)-Glcp-(1→4)-\(\beta\)-\(\beta\)-Glcp-(1→3)-\(\beta\)-\(\beta\)-Glcp-(1→4) repeating unit. ML \(\beta\)-glucans are attracting increasing industrial interest as bioactive ingredients and food additives (52). The structural features of ML \(\beta\)-glucans, including the ratio of (1→4)/(1→3) linkages, the abundance of long cellulosic fragments, and molecular size, determine their physical and physiological properties (53). The \textit{S. meliloti} ML \(\beta\)-glucan shares some properties with other bacterial \(\beta\)-glucans such as its insolubility in water (i.e., cellulose and curdlan) and solubility in dilute alkali (curdlan); however, the precise description of its
physical features and rheological properties shall be the subject of future specific studies.

At least two genes, bgsA and bgsB, likely encoding a GT-2 and a membrane fusion protein, respectively, are required for the production of this extracellular ML β-glucan. BgsA is a strong candidate to be the ML β-glucan synthase, because it shares sequence, domain conservation, and predicted secondary structure with other bacterial GT-2 proteins, particularly curdian (CrdS) and Cel synthases. In Cel synthases the C-terminal region encloses the PilZ domain involved in c-di-GMP binding and required for enzyme activation (22). Although BgsA does not seem to contain a PilZ signature, we provide strong evidence that it binds c-di-GMP through its C-terminal region, which could represent a new c-di-GMP-binding domain that awaits characterization. Therefore, it is likely that induction of ML β-glucan by c-di-GMP takes place through allosteric activation of the BgsA GT. It also will be of interest to identify the DGC(s) involved in the provision of c-di-GMP to BgsA and the physiological conditions that promote its activation. Synthesis of the ML β-glucan also is dependent on the ExpR/SinI system which regulates bgsBA transcription, thereby linking ML β-glucan to the production of other S. meliloti EPSs (7). Thus, ML β-glucan biosynthesis seems to be regulated at multiple levels, a situation that also has been proposed for cellulose biosynthesis in A. tumefaciens (54).

We observed that high production of ML β-glucan facilitates aggregation and biofilm formation by S. meliloti 8530 under free-living conditions and also on the alfalfa root surface. ML β-glucan overproduction greatly enhances bacterial attachment to the host plant root and the formation of a dense biofilm on the root surface, from which mutants unable to produce ML β-glucan seem to be excluded. Moreover, under physiological c-di-GMP levels bgsA mutants show reduced attachment to alfalfa roots. The involvement of ML β-glucan in S. meliloti’s firm attachment to roots and its aggregation and biofilm formation on abiotic and biotic surfaces suggest that it can be of particular importance for bacterial persistence and multiplication in the soil and rhizosphere under field conditions. This evokes the biological role of related glucans such as cellulose, an important adherence factor in very diverse bacteria (55), including rhizobia. Like the S. meliloti ML β-glucan, cellulose also provides Rhizobium leguminosarum with a root attachment advantage; however, both cellulose-deficient and -overproducing strains show wild-type nodulation properties (56, 57) similar to those of the S. meliloti bgsA mutants. With resemblance, A. tumefaciens cellulose-deficient mutants also show reduced attachment to plant cells but remain fully virulent (54) except under very particular experimental conditions [e.g., when the infection sites are washed shortly after inoculation], suggesting that cellulose may be important for bacterial attachment to plant wound sites under adverse field conditions (58). Phylogenetic analyses suggest that, like S. meliloti, other Rhizobiales, including nonnodulating plant-interacting bacteria such as Agrobacterium and Methylobacterium, also are able to produce ML (1→3)(1→4)-β-D-glucan. This novel polymer adds to the plethora of surface polysaccharides that help rhizobia and other plant-associated bacteria thrive in the changing soil and rhizosphere environments.

**Materials and Methods**

**Bacterial and Culture Conditions.** Bacterial strains and plasmids used in this work are listed in SI Appendix, Table S1. Starting cultures of S. meliloti strains were grown overnight at 28 °C in a one-time-use broth and then diluted 1:100 in TY (59) or MM medium (60). When required, antibiotics and other compounds were added at the following final concentrations: tetracycline, 10 μg/mL; CR, 50 μg/mL; CF, 200 μg/mL (in solid medium) or 100 μg/mL (in liquid medium). The plasmids pJBpleD*, pJB3Tc19, pJB9091, and pJBpleD9091 were introduced in rhizobia by conjugation using the E. coli p2163 donor strain (61) in matings as previously described (62).

**Construction of S. meliloti Mutants and Complementing Plasmids.** Molecular biology techniques were performed according to standard protocols and manufacturers’ instructions. For the construction of Sme Smb20391::Nm and Smb20460::Nm mutants, the phage Φ11 (63) was used to transduce mutations from the donors S. meliloti 2011mTn5STM.1.08.B12, 2011mTn5STM.4.13. E03, and 2011mTn5STM.4.03.E12 to S. meliloti, as previously described (64). Mutants were verified by PCR and Southern hybridization with specific probes. For the construction of Sme SΔSmb20390 with an unmarked in-frame deletion, two fragments flanking the ORF were amplified separately, using primer pairs op90-F/20390L-R (496 bp) and 20390R-FSM_b20391-R (408 bp) (SI Appendix, Table S2). These two fragments were purified and used as template DNA for an overlapping PCR with primers op90-F and SM_b20391-R. The final fragment was cloned into pCR-SacB (65), generating the pK18ΔSmb20390. The pK18ΔSmb20390 plasmid was introduced into S. meliloti by biparental conjugation with an E. coli S17.1 donor, and the Smb20390 deletion of 1,035 bp (402490–403524) was generated by homologous recombination following the procedures described in ref. 65. The SΔSmb20390 mutant was corroborated by PCR and Southern hybridization with specific probes. Diagrams of the plasmid constructions used in transconjugation experiments are depicted in SI Appendix, Fig. 57. For the construction of Sme SMb20391::Nm mutants, two primers flanking the Smb2039-91 operon were used (op90-F and op91-R) in a PCR with Sme genomic DNA as a template. The 3,720-bp fragment containing both genes was cloned in pCR-XL-TOPO (Invitrogen) and sequenced. A correct EcoRI DNA insert was isolated and subcloned into pJB3TC19 and pJBpleD* to generate pJB9091 and pJBpleD9091, respectively. The correct orientation of both constructions was verified with appropriate restriction enzymes.

**Gene-Expression Assays.** For RT-PCR experiments, RNA extractions were carried out using the Qiagen RNeasy RNA purification kit (Qiagen). Total RNA (1 μg) treated with RNase-free Dnase I Set (Roche) was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and random hexamers (Roche) as primers. RT-qPCR was performed on an iCycler IQ5 (Bio-Rad). Each 25-μL reaction contained 1 μL of cDNA, 200 nM of each primer, and 1X SyBrGreen Supermix (Bio-Rad). Control PCRs of the RNA samples also were performed to confirm the absence of contaminating DNA. Samples were denatured initially by heating at 95 °C for 3 min, followed by a 35-cycle amplification and quantification program (95 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s). Melting-curve analysis was conducted to ensure amplification of a single product. The efficiency of each primer pair (E) was determined by running 10-fold serial dilutions of Sme genomic DNA as a template and generating a standard curve by plotting the log of the dilution factor against the cycle threshold (Ct) value during amplification of each dilution. Amplification efficiency was calculated using the formula:

\[ E = \left( \frac{10^{(-1/Ct)} - 1}{2} \right) \times 100 \]
Relative expression of the SMb20390 and SMb20391 genes was normalized to that of 16S rRNA gene. A transcriptional reporter gene fusion also was used to study the effect PleD* on the expression bgsBA operon. A strain with an oriented miniTn5-gus inserted in bgsA was used (SMb20391::Nm-gus) (SI Appendix, Table S1), and β-glucuronidase was determined as previously described (62).

**CF-Binding Assays.** Starting cultures of rhizobial strains were prepared as detailed above. After washing with MM, cultures were diluted 1:100 into 10-mL flasks containing MM supplemented with CF (100 μM final concentration) at 28 °C under agitation for 48 h. Then cultures of the different strains were centrifuged for 10 min at 3,220 × g. Supernatant containing broth with unbound CF of each biological replicate was removed. The pellet was suspended in 2 mL of distilled water and placed in the wells of 24-well plates. Similar growth of all strains was confirmed, and CF-binding measurements for three biological replicates of each strain were performed in a PTI fluorimeter (Photon Technology International); the results were expressed in arbitrary units ± SE.

**Bacterial Attachment to Plant Roots.** Alfalfa (Medicago sativa L.) seeds were surface-sterilized and germinated as described by Olivares et al. (66). Seedlings were placed on 12 × 12 cm plates (12 seedlings per plate) containing nitrogen-free medium (66) solidified with 1.15% (wt/vol) purified agar and were incubated for 3 d in a plant-growth chamber (16-h light/8-h dark photoperiod, 24 °C/16 °C day/night temperature, 75% relative humidity) before inoculation. For root attachment of bacteria at high c-di-GMP levels, seedlings were transferred into a flask containing 25 mL of nitrogen-free plant nutrient solution with a mixture of the wild type and the SMb20391::Nm mutant (109 cells each), both expressing PleD* and tagged with GFP or dRed, and were agitated gently at 28 °C for 24 h. After this time, roots were separated from shoots, and four groups of three roots each were prepared and washed vigorously four times with 1 mL of sterile deionized water in Eppendorf tubes to remove bacteria unbound or weakly bound to roots. After these washings, one group of three roots was used for CLSM with a Nikon Eclipse TE2000-U microscope at the technical service of Estación Experimental del Zaidín Consejo Superior de Investigaciones Científicas. One milliliter of MM with 2 mM EDTA was added to each of the remaining three groups of roots, and bacteria firmly bound to roots were released by two cycles of vortexing for 1 min and sonication for 1 min. Serial dilutions were spread onto TY with and without Nm and were incubated at 28 °C for 3–4 d; then cfus of each strain were counted. For root attachment of bacteria at physiological c-di-GMP levels, a modification of the protocol described by Anollés and Favelukes was followed (67). Seedlings prepared as above were kept in plates, and each root was inoculated with 30 μL of nitrogen-free plant nutrient solution containing mixtures of Sme and SMb20391::Nm mutant in a 1:1 ratio. Two different inoculum densities were tested, so 109 or 107 cells were applied to each root. Plants were kept in the plant-growth chamber, and bacteria firmly attached to roots were recovered and counted as above, at 1 and 3 d after inoculation.

**Nodulation Experiments.** Alfalfa seedlings were prepared as above, except that nine plants per plate were used. For nodulation kinetics each strain was inoculated onto the roots of 45 plants by spreading 30 μL of a bacterial suspension (prepared in a nitrogen-free plant nutrient solution) along each root surface. Two inoculum densities were tested, so 105 or 103 cells were loaded on each root. Plants were maintained in the growth chamber, and the number of visible nodules was recorded daily. For competitive nodulation, plants were prepared as above, but bacterial suspensions contained...
mashes of the wild type and the SMb20391::Nm mutant in a 1:1 ratio. Two inoculum densities were tested, so 10^5 or 10^7 total cells were loaded on each root. Numbers of nodule was determined 15 d after inoculation. At least 100 nodules were collected, surface sterilized, and individually crushed. The contents of each nodule were spread on TY-agar plates with and without Nm and were incubated at 28 °C for 3–4 d.

**ML β-Glucan Isolation and Chemical Characterization.** Starting cultures of Sme pBpI38* were diluted 1:100 in 5-L flasks containing 0.5 L of liquid MM supplemented with tetracycline and were grown with slow shaking (80 rpm) for 3 d at 28 °C. The floccs formed were recovered using a sieve with a 0.25-μm cutoff and then placed in a 50-mL Falcon tube (Fisher Scientific) and washed with 30 mL of boiling Milli-Q water (Millipore Corporation). After boiling for 5 min, the material was cooled at room temperature and centrifuged for 20 min at 3,220 × g. The supernatant was discarded, and the washing process was repeated four times. The β-glucan obtained was frozen and lyophilized overnight. For quantification of β-glucan yield, the different strains were grown in 35 mL of MM in 250-mL flasks and were incubated and processed as described above, except that the sieve runoffes were recovered and centrifuged, and bacterial pellets were lyophilized and weighed to calculate the amount of the β-glucan dry weight relative to the total dry weight of the culture. Monosaccharides of the β-glucan were identified upon gas liquid chromatography (GLC-MS separation of per-O-trimethylsilylated methyl glycosides (68). The absolute configuration of monosaccharides was assigned following GLC-MS analysis of their per-O-trimethylsilylated (S)- and (R)-2-buty1 glycosides as described by Gerwig et al. (69). For methylation analysis, a further purification step on the β-glucan purification was applied to increase its final purity. The β-glucan was obtained as described above, and the floccs trapped on the sieve were dissolved in 15 mL of 1% NaOH for 1 h with shaking and then were dialyzed in water using molecular porous-membrane tubing (Spectrum Labs) until neutral pH was obtain in the sample, which then was frozen and lyophilized overnight. Lyophilized samples were methylated using the method of Ciancu and Costello (70). The methylated polysaccharide was purified by SEC on Sephadex LH-20, using CH32Cl2:MeOH (1:1 by vol) as the eluent. Hydrolysis was carried out first with 88% HCOOH (100 °C, 1 h) and then, after evaporation of the acid, with 2 M trifluoroacetic acid (120 °C, 4 h). Reduced with NaBD4 and acetylation were performed according to the methods of Kim and coworkers (71), yielding the corresponding partially methylated and acetylated alditois, which were solubilized in CH3CN and analyzed by GLC-MS. For acetylation of polysaccharide, 6 mg of polysaccharide were resuspended in 1 mL of pyridine:acetic anhydride (1:1 ratio) for 24 h at room temperature. The solution was poured into 5 mL of ice water, and the precipitate was recovered by centrifugation, rinsed with cold water, and freeze dried. This procedure was repeated once. The sample was resuspended in CH32CN:MeOH (1:1 ratio, vol/vol) and purified by SEC on Sephadex LH-20 with the same eluent. Fractions containing carbohydrates were vacuum-evaporated, dissolved in 300 μL of CDCl3, and studied by NMR. Spectra were recorded at 300 K on a Bruker P10-CVI-5800 and 201440E026; L.R.-J. was supported by a JAE-Pre fellowship and later by Grants a Junta de Ampliación de Estudios (JAE)-doc CSIC contract and later by Grants.

**Phylogenetic Analyses.** Protein sequence similarity searches were carried out with the BLASTP program from the National Center for Biotechnology Information (74). Alignment was performed with CLUSTALW (75). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 5.2 (76), using the following phylogenetic parameters: analysis, phylogeny reconstruction; statistical method, neighbor-joining; number of bootstrap replications, 1,000; substitutions type, amino acid; model/method, Poisson model; rates among sites, uniform rates; pattern among lineages, same (homogeneous); gaps as missing data; and pairwise deletion.

**Dot Blot c-di-GMP–Binding Assays.** Plasmids pET2Bb::BgsA and pQE80L::C-BgsA were constructed to express the entire or the last 139 amino acids of BgsA, respectively. A 2,068-bp DNA fragment containing full-length bgsA was amplified with primers opplF-1 and opplR-1, cloned into pCR-XL-TOPO, and subsequently subcloned as an EcoRI fragment into pET2Bb. The correct orientation of the construction was checked using Xhol. Primers C-BgsA-F and C-BgsA-R were used to amplify a 488-bp DNA fragment encoding the last 139 amino acids of BgsA. The amplon was cloned into pCR-XL-TOPO and subsequently was subcloned as a HindIII/BamHI fragment into pQE80L. E. coli strains carrying pET2Bb::BgsA, pQE80L::C-BgsA (SI Appendix, Table S1), respective empty vectors (as negative controls), and pET21.PA3353 over-expressing the Pseudomonas aeruginosa PilZ domain protein PA3353 (used as a positive control (77)) were grown on OMD medium 0.5–0.7 and induced with 1 mM of 3-f-AMP (Sigma) for 3 h at 37 °C. After centrifugation, pellets from 8 mL of culture were resuspended in 500 μL of lysis buffer [50 mM Tris (pH 7.5), 10 mM NaCl, 1 mM DTT; and 5% glycerol] and were lysed by sonication (six cycles of 15 s on ice; Branson Sonifier 200). Protein expression was evaluated by SDS-PAGE, and all lysates were standardized with the total protein content determined by Bradford assay (77). For the c-di-GMP dot blotting assays, we modified a previously described protocol (47). Briefly, 3 μg of each lysate sample was spotted on 0.45-μm nitrocellulose membrane discs (Millipore) and left to dry. The membranes were blocked in Tris buffer saline with 0.2% Tween 20 (TBS-T) and 5% skim milk for 1 h at room temperature. Blocked membranes were incubated with 1 μM of 2’-O-(fluoresceinyl)amino-n-hexyl(2-carboxyamido)-c-di-GMP (2’-Fluor-AHC-c-di-GMP; Biológ) in TBS-T containing 5% skim milk for 1 h at room temperature in a dark room. After washing with TBS-T for 10 min, the dot blots were scanned with a Phars FX Plus fluorescence imager (Bio-Rad) using the SYBR Green I settings and were quantified by densitometry with the Quantity One software (Bio-Rad). Competitive assays were performed as indicated above, but membranes were incubated with 2’-O-(fluoresceinyl)amido-c-di-GMP and increasing quantities (0.1–30 μM) of unlabeled c-di-GMP.

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**Supplementary Materials**

**SI Appendix.** Table S1, SI Methods

**References**


