A *Mesorhizobium loti* mutant with reduced glucan content shows defective invasion of its host plant *Lotus japonicus*

Yasuyuki Kawaharada, Shima Eda, Kiwamu Minamisawa and Hisayuki Mitsui

Random transposon mutagenesis led to the isolation of a novel *Mesorhizobium loti* mutant that is defective in nitrogen fixation during symbiosis with *Lotus japonicus*. The mutated locus, designated *cep*, encodes a putative cell-envelope protein displaying no significant sequence similarity to proteins with known functions. This mutant elicits the formation of nodule-like bumps and root-hair curling, but not the elongation of infection threads, on *L. japonicus* roots. This is reminiscent of the phenotypes of rhizobial mutants impaired in cyclic β-glucan biosynthesis. The *cep* mutant exhibits partially reduced content of cell-associated glucans and intermediate deficiency of motility under hypo-osmotic conditions as compared to a glucan-deficient mutant. Second-site pseudorevertants of the *cep* mutant were isolated by selecting for restoration of symbiotic nitrogen fixation. A subset of pseudorevertants restored both symbiotic capability and glucan content to levels comparable to that of the wild-type. These results suggest that the Cep product acts on a successful symbiosis by affecting cell-associated glucan content.

**INTRODUCTION**

*Mesorhizobium loti* is a symbiotic partner of *Lotus japonicus*, a model legume widely used for molecular genetic studies. It dwells in the soil and, on its host plant, elicits the formation of root nodules through a complex interaction between the two partners. Specific lipochito-oligosaccharides produced by rhizobia, called Nod factors, have been well documented for many rhizobial species, including *M. loti*; these compounds trigger root-hair curling and nodule organogenesis in a host-specific manner (Hadri & Bisseling, 1998; Niwa et al., 2001). Rhizobia colonize the curled root hairs and invade the developing nodules via infection threads (ITs), which are formed by invagination of the root-hair cell membrane. Rhizobial cells are finally released into the host nodule cells, where they carry out symbiotic nitrogen fixation. The specificity between rhizobia and host legumes is also exhibited at stages later than those at which Nod factors act. This is exemplified in the symbiotic phenotype of *Rhizobium etli* on *L. japonicus*, in which early nodule senescence occurs despite the fact that the Nod factors from *M. loti* and *R. etli* have the same structure (Banba et al., 2001). To fully understand the molecular basis for the interaction between the model symbiotic pair *M. loti* and *L. japonicus*, additional symbiotic genes need to be identified extensively in the *M. loti* genome.

In rhizobia, the cell envelope should first come to the bacterium–plant interface and mediate communication with the host through attachment or signal exchange. Also, the cell envelope contains essential machinery for nutrient uptake and electron transport, some components of which play specific roles in symbiosis (Delgado et al., 1998; Lodwig & Poole, 2003). As constituents of the cell envelope, cell-surface carbohydrates such as lipopolysaccharides, extracellular polysaccharides, capsular polysaccharides and cyclic β-glucans are known to be critical for establishing a successful symbiosis (reviewed by Fraysse et al., 2003). Therefore, the cell-surface components have been important targets for which novel symbiotic mutants are screened.

Because the whole-genome sequence data are available for *M. loti* strain MAFF303099 (Kaneko et al., 2000), a genetic approach has become more efficient to identify novel symbiotic genes. The transposon Tn5 derivative Tn*phoA* (Tn5 *IS50*::*phoA*) is a useful tool for obtaining mutants where genes encoding secretory or transmembrane proteins are mutated (Manoil & Beckwith, 1985), and in fact it has been used successfully to identify a number of symbiotic genes in *Sinorhizobium meliloti* and *Bradyrhizobium*...
M. loti (Long et al., 1988; Müller et al., 1995). In this work, we attempted to use TnphoA to isolate novel symbiotic mutants of M. loti. By characterizing one of the isolates, we demonstrate that the mutated gene plays a critical role in symbiosis by affecting cell-associated glucan content.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The strains and plasmids used in this study are listed in Table 1. M. loti strains were grown at 30 °C in tryptone–yeast (TY) medium, which contains (per litre) tryptone (5 g), yeast extract (3 g), CaCl₂·H₂O (0.87 g) and agar (15 g, if needed) (pH 7.2), unless otherwise indicated. Glutamic acid–d-mannitol–salts (GMS) medium, which was used for glucan analysis, contains (per litre) glutamic acid (1.0 g), d-mannitol (5.0 g), K₂HPO₄ (1.0 g), MgSO₄·7H₂O (0.2 g), CaCl₂·2H₂O (0.04 g), FeCl₃·6H₂O (2.5 mg), H₃BO₃ (0.01 mg), ZnSO₄·7H₂O (0.01 mg), CoCl₂·6H₂O (0.01 mg), CuSO₄·5H₂O (0.01 mg), MnCl₂ (1 mg) and Na₂MoO₄·2H₂O (0.01 mg) (pH 7.0), as described by Zevenhuizen & Van Neerven (1983) except that biotin and thiamine were omitted. The osmolarity of TY medium was measured with a vapour pressure osmometer (Vapro 5520, Wescor). Antibiotics were added at the following concentrations: streptomycin (Sm), 200 μg ml⁻¹; neomycin (Nm), 200 μg ml⁻¹; tetracycline (Tc), 10 μg ml⁻¹; gentamicin (Gm), 50 μg ml⁻¹. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani medium (Miller, 1992).

**Screening of TnphoA-generated M. loti mutants.** Mutagenesis with TnphoA was carried out by conjugal transfer of pRK609 from MM294A into ML001, as described by Glazebrook & Walker (1991). The M. loti transconjugants were selected on TY plates supplemented with Sm, Nm and 3-bromo-4-chloro-3-indolyl phosphate (XP; Sigma-Aldrich; 40 μg ml⁻¹) after incubation at 30 °C for 4–6 days. Each of the blue colonies was used for nodulation testing.

**Nodulation test.** Seeds of *L. japonicus* B-129 cv. Gifu (Handberg & Stougaard, 1992) were scarified with sandpaper, surface-sterilized in 0.8% (v/v) sodium hypochlorite solution, rinsed and immersed for 2 days in sterilized water. They were transferred into sterile 180 ml plastic jars containing vermiculite and nitrogen-free plant growth medium (B&D medium; Broughton & Dilworth, 1971) or onto B&D plates solidified with 1% (w/v) agar. The jars or plates were incubated at 25 °C for 3–4 days. The germinated seeds were then inoculated with M. loti cells that had been grown to late exponential phase in TY medium, washed and finally resuspended in sterilized water. The jars or plates containing the inoculated plants were placed in a growth chamber held at 25 °C with a photoperiod regime of 16 h illumination and 8 h darkness. The photosynthetically active radiation on the rack in the growth chamber was 80 μmol m⁻² s⁻¹. The appearance of plants was examined 4 weeks after inoculation.

**Identification of the TnphoA-insertion locus.** DNA manipulations were performed according to standard protocols (Sambrook & Russell, 2001). For Southern hybridization analysis of MYa179, its total DNA was digested with PstI and probed by the 1558 bp internal fragment of TnphoA. To identify the mutated locus, a DNA region flanking the TnphoA insertion was amplified by inverse PCR with its total DNA as a template; the DNA had been digested with PstI and then self-ligated. The primer set 5'-AGAGAATTCACCGAGCAGG-CAGTCT-3' and 5'-GTCTGTGTTGTCGCGGGTTCTGTGTGT-3', designed for the internal sequence of TnphoA, and AccuPrime Taq DNA polymerase (Invitrogen) were used for the PCR, the cycling conditions of which comprised an initial denaturation step at 94 °C for 2 min followed by 25 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min.

Table 1. Strains and plasmids

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<tr>
<th>Strain or plasmid</th>
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<td>MAFF303099</td>
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<td>ML001</td>
<td>MAFF303099 Sm'</td>
<td>H. Mitsui</td>
</tr>
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<tr>
<td>YML1011</td>
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<td>YML1012</td>
<td>ML001 ΔndvB::aadA</td>
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<td>Tc', broad-host-range cloning vector</td>
<td>Dombrechet et al. (2001)</td>
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<td>pFAl1702 containing 2.1 kb cep' fragment</td>
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<td>pFAl1702 containing lacZ fused to groES1 promoter</td>
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<td>Becker et al. (1995)</td>
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<td>pK18mob</td>
<td>Suicide vector, Nm'</td>
<td>Schäfer et al. (1994)</td>
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Completion analysis. The 2105 bp DNA fragment containing the mlr6538 (cep) ORF and flanking sequences (beginning at nucleotide no. 5343738 and ending at no. 5341634 of the MAFF303099 chromosome, in reference to RhizoBase) was amplified by PCR using cosmid DNA (clone no. 261) as a template; this clone was derived from the ordered genomic library of MAFF303099 (Hattori et al., 2002). Primers used for the PCR were 5'-GGGGGATATTTCGCAACATATGGGCTC-3' and 5'-CCTGGGATATTTCGCAACATATGGGCTC-3'. (BamHI sites added for convenience of cloning are underlined). The PCR product was digested with BamHI and cloned into pFAJ1702, yielding pYK33. This plasmid was conjugated into MYa179, and the resulting Tc resistance transconjugant was used for the nodulation test and glucan analysis.

Construction of a cep mutant. The BamHI fragment in pYK33 was cloned into pUC19. The resulting plasmid was digested with NspV, blunted with Klenow fragment, and ligated to the fragment that contains the adaA gene encoding Sm/Sp resistance (pHP450; Prentki & Krisch, 1984), creating a cep::adaA insertion at nucleotide no. 925 of the cep ORF. This construct was cut out as a BamHI fragment and inserted into PK18mob (Schafer et al., 1994), yielding pYK56. To generate a strain harbouring the cep::adaA mutation, pYK56 was conjugated into ML001 by triparental mating using pRK600 (Finan et al., 1986), and a double-crossover event was selected by screening for Sp resistance and Nm sensitivity. The resulting strain YML1011 was confirmed to have correct gene replacement by PCR.

Observation of infection processes. We fused the E. coli lacZ ORF to the sequence upstream of the S. meliloti groES1 gene, which is constitutively expressed in both cultured cells and bacteroids of S. meliloti (Bittner et al., 2007; Mitsui et al., 2004). This was inserted into the HindIII–KpnI site of pFAJ1702. The resulting plasmid pAK37 was mobilized to M. loti strains to express β-galactosidase (LacZ). L. japonicus was inoculated with the LacZ-tagged strains and grown for 21 days, as described above. Nodule-like bumps and effective nodules were each counted under a stereomicroscope. ITS were examined by LacZ assay to visualize nodulation processes. The samples were then rinsed three times with 200 mM sodium phosphate (pH 7.0) containing 0.8 mg X-Gal ml\(^{-1}\) and then incubated three times with 200 mM sodium phosphate (pH 7.0), and then incubated at 30 °C. The samples were then rinsed three times with 200 mM sodium phosphate (pH 7.0), and finally observed for ITS under a light microscope.

Analyses of cell-associated glucans. Glucans were isolated and quantified as described by Breedveld et al. (1995) with some modifications. M. loti cultures were grown in 11 or 150 ml of TY medium, 250 ml of TY medium supplemented with sucrose to 0.15 M, or 250 ml of GMS medium to an OD\(_{560}\) of 0.7. Cell pellets were washed once with water and extracted with 30 ml (or 10 ml for pellets from 250 ml or 150 ml culture) of 70% (v/v) ethanol at 70 °C for 1 h. After centrifugation, the supernatants were concentrated to dryness under vacuum and redissolved in water (3 ml). The sample was centrifuged at 100,000 g for 1 h, and the supernatant was chromatographed on a HiPrep 16/60 Sephacryl S-100 HR column (1.6 × 60 cm; GE Healthcare), which was mounted on a BioLog chromatography system (Bio-Rad). It was eluted at room temperature at a rate of 0.3 ml min\(^{-1}\) (i.e. 9 cm h\(^{-1}\)) with 0.15 M ammonium acetate (pH 7.0) containing 7% (v/v) 1-propanol. Each of the fractions (1.5 ml) was assayed for total carbohydrate content as glucose equivalents by the anthrone/sulfuric acid method (Koehler, 1952), and fractions containing glucans were pooled. For separation of neutral and anionic glucans, the pooled glucan fraction was concentrated and desalted on a Sephadex G-10 column (1.5 × 74 cm; GE Healthcare) with 7% (v/v) 1-propanol as the eluant. The sample (5 ml) was applied to a HiLoad 26/10 Q-Sepharose HR column (2.6 × 10 cm; GE Healthcare) mounted on a BioLog system. The column was first eluted at a rate of 2.0 ml min\(^{-1}\) with 85 ml of 10 mM Tris/HCl (pH 7.4) containing 7% (v/v) 1-propanol, and then a 150 ml linear gradient was applied at a rate of 2.0 ml min\(^{-1}\), beginning with 0 mM NaCl and ending with 400 mM NaCl in the same buffer. Fractions (2.0 ml) were collected and assayed for total carbohydrate. Reducing sugars were measured as described by Park & Johnson (1949).

Alkaline phosphatase assay. M. loti cells grown in TY medium (1 ml) were harvested, suspended in 1 M Tris/HCl (pH 8.0) and their OD\(_{600}\) determined. To start the reaction, 100 μl of 4-nitrophenyl phosphate (4 mg ml\(^{-1}\)) was added and incubated at 30 °C. After cells were pelleted, the A\(_{420}\) of the supernatant was measured. Units of alkaline phosphatase were calculated as described by Charles et al. (1991) as follows: 1000 × A\(_{420}\)/time (min) × OD\(_{600}\).

Motility assay. M. loti strains were grown to late exponential phase in TY medium and spotted in 0.5 μl portions onto TY soft-agar (0.25% agar) plates. The plates were incubated at 30 °C for 5 days or 18 °C for 12 days.

Construction of ndvA and ndvB mutants. To disrupt the ndvA gene (mlr8326), a 2363 bp DNA fragment containing ndvA ORF and its flanking sequences was amplified with PCR and cloned in pK18mob. With the resulting plasmid, we created a ΔndvA::aacC1 construct in which a 633 bp EcoRI fragment within the ORF is replaced with the Gm resistance cassette consisting of the aacC1 gene (pMS266; Becker et al., 1995). The resulting plasmid, pYK40, was conjugated into ML001, and a double-crossover event was selected, yielding strain YML1006. To disrupt the ndvB gene (mlr8325), a 1934 bp DNA fragment (nucleotides 281–2214 of the ndvB ORF) and an 1891 bp DNA fragment (nucleotides 6555–8445 of the ndvB ORF) were amplified from the M. loti total DNA by PCR, and both the fragments were cloned in pK18mob in the proper orientation. Then the fragment was inserted between these fragments in this plasmid. The resulting plasmid pYK59, which is to replace a 4340 bp region within the ORF, was conjugated into ML001, and a double-crossover event was selected, yielding strain YML1012. Correct gene replacements were confirmed by PCR.

Analysis of TnphoA insertion in the revertants. PCR was carried out with the total DNA of MYa179 or its revertants as a template. The primer set used for the PCR, 5'-TAGTCGCTATCGCTGTGGT-3' and 5'-AGAGAATCCAGCGAGCAGCCT-3', was designed to amplify a 496 bp DNA fragment stretching over the cep::TnphoA fusion junction present in the MYa179 genome.

RESULTS

Isolation of a novel symbiotic mutant of M. loti

TnphoA can randomly generate fusion between a target gene and 'phoA, the gene for E. coli alkaline phosphatase lacking its signal peptide. The resulting chimeric protein exhibits phosphatase activity only when the protein fused in-frame to phosphatase provides a signal peptide that
promotes export to the periplasm or external milieu. Whereas the parental M. loti strain ML001 expressed low levels of endogenous alkaline phosphatase activity (less than 2 U) after growth in TY medium, it formed white colonies on TY agar in the presence of the chromogenic phosphatase substrate XP. This allowed us to distinguish phosphatase-positive mutants from the background. By mobilizing TnphoA into ML001, we found a small number of blue colonies in a mass of white colonies on XP-containing plates. We collected 699 such phosphatase-positive mutants and then inoculated each of them onto L. japonicus seedlings in nitrogen-free medium. Inoculated plants were examined visually for the symbiotic phenotype in comparison with ML001-inoculated and uninoculated plants. In this screen, six of the 699 strains were ineffective in symbiotic nitrogen fixation (Fix− phenotype), as judged by the stunted and chlorotic appearance of the plants. Southern hybridization analysis was carried out for each of the Fix− mutants by using the internal fragment of TnphoA as a probe, confirming that each strain contained a single copy of TnphoA within its genome (data not shown). By conducting DNA sequence analysis of the TnphoA-flanking regions in reference to RhizoBase and subsequent complementation analysis using cosmids that were chosen to cover the mutated loci from the library (Hattori et al., 2002), we established that the four mutants had the symbiotic deficiency due to TnphoA insertions within the ORFs of the following genes: mll3723 (pstS; a gene for a component of high-affinity phosphate transport system), which was identified in the two strains; mll2707 (a gene for Rieske iron–sulfur protein); and mll6538 (a gene of unknown function). The other two mutants examined were not complemented with such cosmids. In this study we decided to further characterize strain MYa179, which harbours the TnphoA insertion in mll6538, to search for a novel function required for an effective symbiosis.

Mll6538 is predicted to encode a 617 aa protein with a molecular mass of 64.1 kDa. In MYa179, the TnphoA insertion results in production of an Mll6538–PhoA fusion protein containing the 132 amino-terminal residues encoded by mll6538. We cloned the wild-type mll6538 ORF with its upstream 210 bp and downstream 41 bp regions into cloning vector pFAJ1702 at the site protected from read-through transcriptional activity of the vector sequence (Dombrech et al., 2001), yielding plasmid pYK33. We conjugated pYK33 into MYa179 and examined the resulting transconjugant for its symbiotic phenotype. We observed the complementation of the MYa179 phenotype into effective symbiosis (Fix+) on L. japonicus, thus confirming again that the Fix− phenotype was due to the insertion mutation of mll6538. This result also suggests that the DNA region cloned in pYK33 involves sequences required for the gene expression. MYa179 and its derivative carrying pYK33 showed similar levels of alkaline phosphatase activity (8–10 U) with TY-grown cells, suggesting little effect of the gene product on its expression.

Mll6538 displays no significant sequence similarity to proteins with known functions in currently available databases. The amino-terminal sequence had the features of the signal peptides typically found in bacterial secretory proteins (Pugsley, 1993). By using the SignalP program (available online from the Center for Biological Sequence Analysis (CBS), Technical University of Denmark: http://www.cbs.dtu.dk/services/SignalP/), we predicted that the 31 amino-terminal residues composed the signal peptide (data not shown). On the other hand, the remaining carboxy-terminal portion (residues 32–617) was predicted by the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) to contain no transmembrane helix (data not shown). These analyses suggest that mll6538 encodes a periplasmic protein or a peripheral membrane protein facing the periplasm. This is consistent with the phosphatase activity exhibited by MYa179 cells grown in TY medium. Thus we designated this gene cep for the cell-envelope-related protein whose loss causes a pleiotropic phenotype, as a consequence of our analysis described below. BLASTP search revealed that cep homologues are conserved in some members of the family Rhizobiacae, although their function is as yet unknown: BruAb1_1595 of Brucella abortus 9–941 (Halling et al., 2005), Smc00838 of S. meliloti 1021 (Galibert et al., 2001), RL0767 of Rhizobium leguminosarum biovar viciae 3841 (Young et al., 2006) and RHE_CH00718 of R. etli CFN42 (González et al., 2006) are 34%, 29%, 27% and 27% identical, respectively, to Cep in their deduced amino acid sequences. In addition, the preserved order of genes is found in the genomic regions surrounding the cep homologues among these bacteria; M. loti genes making up the synteny are mll6527 (aceA), mllr6529, mllr6533 (potF), mllr6534 (potG), mllr6536 (potH), mllr6537 (potI), cep, mllr6539 (acsA), mllr6540 (a gene for sensor histidine kinase), mllr6541 and trnH, all of which are located within the 18 kb genomic region. In reference to the Sinorhizobium meliloti Fusion Library Database (available online from McMaster University, Canada: http://www.sinorhizobium.org/), it has been shown that Smc00838 and acsA2 are certainly transcribed divergently from each other in free-living S. meliloti cells.

### Symbiotic properties of the cep mutant

MYa179 showed a Fix− phenotype on L. japonicus (Fig. 1a); numerous nodule-like bumps (Fig. 1c) and few, if any, small white nodules were formed on the roots. This is in contrast to the case in the wild-type ML001, which induced the formation of smaller numbers of round pink nodules (Fig. 1a, b). To visualize the infection process, we introduced the groES1–lacZ (constitutively expressed; see Methods) gene fusion into MYa179 and ML001 and inoculated the resulting strains onto L. japonicus seedlings. Under a light microscope we observed elongated ITs (Fig. 1d) at a rate of 36±5 (mean ±s.e.) per plant inoculated with ML001 (data derived from 10 plants). In contrast, elongated ITs were very rare on plants inoculated with MYa179; among the 18 plants examined we found no
ITs on 14 plants, one IT on three plants, and three ITs on one plant. Root-hair deformation and curling, however, were observed on both the roots inoculated with ML001 and those with MYa179 (Fig. 1d, e). To confirm the symbiotic phenotype with an additional cep allele, we generated another mutant in the cep gene, strain YML1011 (see Methods), and inoculated it onto L. japonicus. We found that YML1011 was Fix− and elicited the formation of nodule-like bumps on roots as did MYa179 (data not shown).

These results suggest that mutants in the cep gene are blocked for the invasion of host plants, but not for the induction of root-hair curling and cortical-cell division in host plants. The root-hair curling and cortical-cell division are observed on L. japonicus roots to which purified Nod factors have been added (Niwa et al., 2001). The invasion defect of the cep mutant resembles those of the ndvB/cgs mutants (defective in cyclic β-1,2-glucan biosynthesis) of S. meliloti (Dylan et al., 1986, 1990b) and M. loti, which was assayed with Lotus tenuis (D’Antuono et al., 2005).

**Analysis of cell-associated glucans**

Cyclic β-glucans, counterparts of E. coli membrane-derived oligosaccharides (MDOs) as periplasmic glucans, are present widely among members of the family Rhizobiaceae, including M. loti. These molecules consist of an average of about 20 β-1,2-linked glucose residues, and are synthesized and exported, respectively, through the function of the chvB/ndvB/cgs and chvA/ndvA/cgt genes (Breedveld & Miller, 1994; D’Antuono et al., 2005; Lepek et al., 1990; Roset et al., 2004). Some residues are modified by non-glycosidic anionic substituents; sn-1-phosphoglycerol and O-succinyl ester have been identified in S. meliloti (Miller et al., 1988). Notably, mutants impaired in cyclic β-1,2-glucan synthesis generally show a variety of defects in both free-living states and bacterium–plant interactions. The free-living defects, which are indicative of an altered cell surface, are most pronounced under low-osmolarity conditions; such defects include impaired growth and loss of motility in hypo-osmotic media. In addition, biosynthesis of cyclic β-glucans is subjected to osmotic regulation in many bacterial species: growth in a low-osmolarity medium causes a large accumulation of glucans (Breedveld & Miller, 1994; Cangelosi et al., 1990; Dylan et al., 1990a). The genome of M. loti MAFF303099 (the parent strain of ML001) has been annotated to contain the ndvB (mlr8325) and ndvA (mlr8326) genes in RhizoBase.

The symbiotic phenotype prompted us to examine the cep mutant for alterations in cyclic β-glucans, whereas the glucan-minus mutants of M. loti had not been characterized in terms of symbiosis with L. japonicus. ML001 and the ndvB mutant YML1012, which was generated (see Methods) and included as a negative control, were grown in TY medium and harvested. The cell pellets were extracted with hot ethanol, and the extracts were subjected to gel-filtration chromatography. The size profiles of carbohydrates present in these extracts are shown in Fig. 2(a, b). A small peak is observed near the void volume in each of the chromatograms; material in this peak has not been further characterized, but its size suggests polysaccharides of high molecular mass. Next, one large peak...
column. Kav cell extracts were chromatographed on a Sephacryl S-100 medium, and the cell pellets were extracted with 70% ethanol. The cep mutant (b) and MYa179 (c) were grown in TY medium, and the cell pellets were extracted with 70% ethanol. The size profile occurred at a $K_{av}$ of 0.45–0.7 for ML001, although its right-shoulder peak was not always resolved in each experiment (Fig. 2a). In contrast, just a small peak appeared at this $K_{av}$ for YML1012 (Fig. 2b), suggesting that the major peak consists mainly of cyclic $\beta$-glucans in ML001. Thus, fractions covered by this peak were pooled. We analysed total acid hydrolysate of the pooled carbohydrates by gas chromatography, showing that glucose accounted for the majority of material present in the sample (see Supplementary Fig. S1, available with the online version of this paper). In addition, reducing sugar accounted for 0.20 % (in moles) of the total glucose units present in the sample; this indicates that approximately 96% of the molecules do not have reducing end-groups, assuming an average size of 20 glucose residues per glucan molecule.

By using the same method, we analysed the MYa179 cell extracts by gel-filtration chromatography. The size profile of carbohydrates was similar to that for ML001, but a peak at a $K_{av}$ of 0.45–0.7 was partially reduced in amount compared to that obtained for ML001 (Fig. 2c, Table 2). The major peak for MYa179 appears to shift slightly to the right relative to that for ML001 in the chromatogram; this might be partly due to smaller molecular masses of glucans in MYa179, which are mostly unsubstiated, in contrast to glucans with anionic substituents in ML001 (see below). A reduced amount of carbohydrates in a peak at the same $K_{av}$ was also observed for MYa179 cells grown under different conditions. Growth in a medium of elevated osmolarity by addition of sucrose (0.15 M) lowered the carbohydrates to about a half of that with TY medium for each of the strains, but still retained the difference between ML001 and MYa179 (19 mg and 10 mg, respectively, per g cell dry weight; result from one experiment); this is also the case with GMS medium (29 mg and 21 mg for ML001 and MYa179, respectively; result from one experiment). Moreover, another cep mutant, strain YML1011, also showed a reduced amount of carbohydrates in a peak at the $K_{av}$ although glucan content in YML1011 is somewhat higher than that in MYa179 (Table 2); we have not investigated whether this is due to the different cep alleles or the presence of an additional mutation in either of the strains. The complementing plasmid pYK33 (cep+) increased the amount of carbohydrates in MYa179 (Table 2). These results collectively suggest that mutation in the cep gene reduces cell-associated glucan content in M. loti.

Then we analysed the carbohydrates in a major peak from the gel filtration by anion-exchange chromatography. For both ML001 and MYa179, carbohydrates were separated into one fraction not adsorbed on anion-exchange resin (peak N) and three fractions eluting with salt-gradient buffer (peaks A1, A2 and A3), indicating one neutral fraction and three anionic fractions, respectively (Fig. 3a, b). Similar separation profiles of glucans were reported previously for Agrobacterium tumefaciens, S. meliloti and Brucella abortus (de Iannino et al., 2000; Miller et al., 1987, 1988). In the case of MYa179, however, anionic fractions were greatly reduced in amount compared to those in ML001 (Fig. 3b). If we normalize the amount per dry weight of cells, in contrast, the neutral fraction was not so much affected or rather increased in MYa179 (1.5 times the amount in ML001 according to the data shown in Fig. 3). We observed a similar profile of neutral and anionic glucans for YML1011 to that for MYa179 using TLC (see Supplementary Fig. S2, available with the online version of this paper). We confirmed that the complementing plasmid pYK33 restored the profile to the wild-type one in MYa179 (Fig. S2). Thus, mutation in the cep gene appears to have a greater influence on anionic glucans than on neutral glucans.

**Pleiotropic effects of the mutation in the cep gene**

We examined the cep mutant for an additional property related to alteration in cyclic $\beta$-glucans. On a TY soft-agar
plate, MYa179 was partially less motile at 30 °C as compared to ML001 and glucan-deficient strain YML1006 (ndvA mutant; see Methods) (Fig. 4). The difference between MYa179 and ML001 was less obvious at 18 °C (data not shown), which is a similar tendency to that observed for the M. loti cgs mutant (D’Antuono et al., 2005). Such intermediate swarming at 30 °C was also observed for another cep mutant, YML1011, and pYK33 (cep+) restored the motility to the wild-type levels in MYa179 (data not shown). The osmolarity of TY medium was determined to be approximately 60 mosM, which is hypo-osmotic to the bacterial cell interior (about 300 mosM; Stock et al., 1977). Therefore, the intermediate motility defect of the cep mutants would be attributable to their altered glucan content.

Symbiotic pseudorevertants of the cep mutant

When we inoculated MYa179 onto 106 seedlings of L. japonicus, we found four plants growing better than the others in nitrogen-free medium. Each of the four plants had a single pink nodule on its roots, as well as a number of bumps. We isolated bacteria from each of the pink nodules and checked that all the isolates retained the Nm resistance associated with the presence of TnphoA. PCR amplified a 0.5 kb fragment containing the cep::TnphoA fusion junction (see Methods) from the genomic DNAs of all the four strains that were recovered from the respective nodules, as well as the MYa179 DNA, confirming that the TnphoA insertion was still located at the original site for all the strains. Then we reinoculated L. japonicus with these strains. All of them turned out to be Fix+; whereas the two strains SRM-9 and SRM-11 supported plant growth at levels comparable to that of ML001, the others (SRM-10 and SRM-12) induced slower plant growth than ML001 did (Fig. 1a; data not shown for SRM-11 and SRM-12). These results indicate that the occurrence of effective nodules after inoculation with MYa179 was not due to a leaky phenotype of the cep mutant but due to the presence of suppressor mutations. Restoration of symbiotic capability was different in degree between suppressor mutations. SRM-9 and SRM-10 expressed alkaline phosphatase activities at similar levels (9 U) to that for MYa179, indicating that the restoration is not due to overexpression of truncated Cep protein derived from the cep::TnphoA fusion.

We examined whether the pseudorevertants simultaneously reversed other phenotypic properties exhibited by

![Fig. 3. Anion-exchange chromatography profiles of cell-associated glucans from M. loti strains. Carbohydrate fractions derived from the major peak on the gel filtration (see text) were pooled, concentrated and desalted. A 5 ml sample containing a fixed amount (2.5 mg) in glucose equivalents was applied to a Q-Sepharose column (2.6×10 cm). It was eluted first with 85 ml of 10 mM Tris/HCl (pH 7.4) containing 7 % (v/v) 1-propanol and then with a 150 ml linear gradient beginning with 0 mM NaCl and ending with 400 mM NaCl in the same buffer. Fractions (2.0 ml) were collected and assayed for total carbohydrate. The neutral glucan peak is marked N, and anionic glucan peaks are marked A1, A2 and A3. (a) ML001 (wild-type); (b) MYa179 (cep mutant); (c) SRM-9 (symbiotic pseudorevertant of MYa179).](http://mic.sgmjournals.org)

### Table 2. Quantification of carbohydrates in a peak migrating with cyclic β-glucans on gel-filtration chromatography

Each measurement was performed at least in duplicate, in which values varied from the mean by not more than 7 % except for the cases of MYa179 and YML1012. Two values from the measurement of YML1012 differed from the mean value by 23 %.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbohydrate [mg (g cell dry weight)^-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML001 (wild-type)</td>
<td>35±0.2*</td>
</tr>
<tr>
<td>MYa179 (cep::TnphoA)</td>
<td>19±3.8*</td>
</tr>
<tr>
<td>MYa179 complemented with pYK33 (cep+)</td>
<td>48†</td>
</tr>
<tr>
<td>YML1011 (cep::aadA)</td>
<td>24±1.5†</td>
</tr>
<tr>
<td>YML1012 (ΔndvB::aadA)</td>
<td>1.8†</td>
</tr>
<tr>
<td>SRM-9 (symbiotic pseudorevertant)</td>
<td>31†</td>
</tr>
<tr>
<td>SRM-10 (symbiotic pseudorevertant)</td>
<td>27†</td>
</tr>
</tbody>
</table>

*Mean ± s.d. of three biological replicates.
†Mean of two biological replicates.
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MYa179. SRM-9 showed total glucan content at levels comparable to that observed for ML001 (Table 2). Moreover, profiles of neutral and anionic glucans were essentially the same between ML001 and SRM-9 (Table 2). In contrast, SRM-10 showed total glucan content at intermediate levels between those of SRM-9 and MYa179 (Table 2), and a similar level of glucans to that of SRM-10 was also observed for SRM-12 by TLC analysis (data not shown). These results suggest a close relationship between the symbiotic defect and the reduced glucan content in the cep mutant. In addition, SRM-9 and SRM-10 had substantially enhanced motility relative to MYa179 (Fig. 4), which is consistent with their restored glucan content.

**DISCUSSION**

In this study, we isolated an *M. loti* mutant in the cep gene, which encodes a putative cell-envelope protein with unknown function. This mutant exhibits pleiotropic phenotype in terms of both symbiotic and free-living properties; it is Fix- on the host plant *L. japonicus* (Fig. 1) and altered in cell-associated glucans (Figs 2 and 3; Table 2). The mutation reduces total glucan content and has a more striking influence on the anionic fraction than the neutral fraction in *M. loti*. It is not clear whether the alteration in glucan is the primary cause of the symbiotic defect, or whether both result separately from some other primary defect derived from a loss of the Cep protein. We isolated several second-site pseudorevertants of the cep mutant that restored an effective symbiosis. A subset of pseudorevertants that restored full symbiotic capability (strains SRM-9 and SRM-11) also regained glucan content to levels comparable to that of the wild-type, in terms of both total and anionic glucans (Table 2, Fig. 3). Therefore, we consider it more likely that the cep mutation causes symbiotic defects through the alteration in cell-associated glucans. At this time, we do not have convincing evidence to determine what amount of total or anionic glucans is critical to a successful symbiosis, but it is possible that anionic glucans are more effective than neutral glucans in promoting symbiosis.

Despite the structural diversity of glucan backbones in various species of Gram-negative bacteria, it is accepted that the periplasmic glucans commonly act as osmoprotectants or play a structural role in the envelope organization (Bohin, 2000). In addition, the glucans have a specific role in symbiosis between rhizobia and legumes, although the action of the molecules during plant–microbe interactions remains elusive. Most of our knowledge regarding the symbiotic role of glucans comes from studies done with strains of *Sinorhizobium/Rhizobium* on legume hosts forming indeterminate-type nodules (e.g. alfalfa) or strains of *Bradyrhizobium* on legume hosts forming determinate-type nodules (e.g. soybean). *B. japonicum* has a distinct class of glucans, cyclic β-1,3-β-1,6-glucans, the synthesis of which requires the function of the ndvA, ndvB and ndvC genes (Bhagwat et al., 1993; Miller et al., 1990; Tully et al., 1990). It is possible that the glucans have distinct functions during symbiosis between different rhizobium–legume pairs. Whereas glucans with predominantly β-1,3 linkages, which are produced by the ndvC mutant of *B. japonicum*, do not function effectively in symbiosis with soybean, glucans with identical glycosyl-linkage composition are effective in an ndvB mutant of *S. meliloti* to promote symbiosis with alfalfa (Bhagwat et al., 1993, 1996; Dunlap et al., 1996). Moreover, it was reported that cyclic β-1,3-β-1,6-glucans act as suppressors of the host defence response in soybean, but cyclic β-1,2-glucans do not (Bhagwat et al., 1999; Mithöfer et al., 1996). In contrast, cyclic β-1,2-glucans have a critical role in *Sinorhizobium fredii* for an effective symbiosis with soybean (Bhagwat et al., 1992). Thus, the *M. loti*–*L. japonicus* pair, which is also the combination of a rhizobium possessing ndvAB genes homologous to those of *Sinorhizobium/Rhizobium* and a legume forming determinate nodules, should provide important material for studying the symbiotic functions of glucans.

The *M. loti* mutant in the cep gene is severely impaired for invasion of its host plant. In contrast, it shows intermediate motility between those of the wild-type and ndvA mutant (Fig. 4); the motility appears to correlate with glucan content. This suggests that the cell-associated glucans are required more strictly to be abundant in symbiosis than in free-living function. Alternatively, it may support the notion that the symbiotic deficiency can be separated from the other free-living properties in glucan-deficient mutants, which was previously demonstrated (Bhagwat et al., 1996; Dylan et al., 1999b).

Reversion of phenotypic properties is not unusual in glucan-deficient mutants; pseudorevertants of ndvA or...
ndvB mutants were obtained by selecting for restoration of symbiosis, motility or hypo-osmotic tolerance. However, these revertants still lacked glucans (Chen et al., 2003; Dylan et al., 1990b; Quandt et al., 1992). In view of the fact that a subset of pseudorevertants restores glucans to wild-type levels, we infer that the cept gene may not be directly involved in glucan biosynthesis. Instead, a loss of Cep might cause some physiological or structural perturbation in the cell envelope to affect glucan content. One possibility is that such structural damage would affect the capacity for glucans in the periplasmic space, releasing slightly more glucans into the external environment than in normal cells. Another possibility is that such a physiological change would act to downregulate the glucan synthesis. The synthesis of cyclic (1,2)-β-glucans is repressed under high-osmolarity conditions (Breedveld et al., 1990; Miller et al., 1986). This osmotic regulation was shown to occur principally at the level of modulation of enzyme activity, rather than at the level of gene expression; this was reported with the enzymes associated with cyclic (1,2)-β-glucan biosynthesis in A. tumefaciens, MDO biosynthesis in E. coli, and transfer of phosphoglycerol moieties to the cyclic (1,2)-β-glucans in S. meliloti (Breedveld & Miller, 1995; Rumley et al., 1992; Zorreguieta et al., 1990). A loss of Cep might result in an increase in intracellular ionic strength, which was suggested to be the cause of inhibition of the enzymic activity in A. tumefaciens (Zorreguieta et al., 1990); alternatively, a loss of Cep might result in a false signal to invoke the feedback inhibition of glucan biosynthesis, which was found to operate in response to the level of MDO in the periplasm in E. coli (Rumley et al., 1992). Our analysis showed that the cept mutation had a greater influence on the anionic glucans than on the neutral glucans. Because neutral glucans are the biosynthetic precursors of anionic glucans (Geiger et al., 1991), this might be caused by a cumulative effect of the inhibition of the transfer of anionic substitutents to the glucan backbone in addition to the inhibition of the backbone synthesis.

We cannot yet completely rule out the possibility that cell-surface perturbation caused by a loss of the Cep protein affects the symbiotic capability directly, not through altered glucan content. Whereas further study is necessary to elucidate the primary effect of the mutation in the cept gene, this gene might define a new class of genes that affect cyclic (1,2)-β-glucan content in rhizobia. Mapping of the suppressor mutations obtained in this study will greatly contribute to the resolution of this issue.

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