A *Sinorhizobium meliloti* *minE* mutant has an altered morphology and exhibits defects in legume symbiosis

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Sinorhizobium meliloti differentiates from rod-shaped, free-living cells into pleomorphic, non-dividing, N₂-fixing bacteroids within alfalfa root nodules. Here, the role of the *minCDE* genes in bacteroid differentiation and in free-living cell division is examined. Disruption of the *minE* gene resulted in large, swollen and branched free-living cells, and in symbiosis a *minE* mutation resulted in a defect in nitrogen fixation with activity reduced by approximately 70 % compared to the wild-type. It has been demonstrated that the *minCDE* genes form an operon driven by a promoter located 173 bp upstream of *minC*. The *minCDE* genes were expressed in free-living cells and in both the infection zone and the symbiotic zone of alfalfa nodules; however, no changes in the free-living cell morphology, growth or symbiotic N₂ fixation were detected as a result of deletion of these genes. Induced production of individual or combinations of Min proteins in *S. meliloti* altered its rod-shaped cell morphology. Moreover, cell morphologies resulting from the overexpression of the *S. meliloti* Min proteins in *Escherichia coli* suggested similar functions for the *E. coli* and *S. meliloti* *min* genes. These data suggest that there is greater redundancy in the roles of cell division genes in *S. meliloti* compared with *E. coli*.

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INTRODUCTION

Division of a bacterial cell normally occurs at the midcell, and hence requires the cell to locate and position cell division machinery at this midcell site (Errington *et al.*, 2003). The first known step in the formation of the prokaryotic division machinery is the polymerization of the cytosolic tubulin homologue FtsZ into a ring-like structure (Z-ring) encircling the dividing cell at the position of a potential septum (Bi & Lutkenhaus, 1991). Proper localization of the Z-ring is determined by at least two proposed mechanisms: nucleoid occlusion and the Min system (Errington *et al.*, 2003). The nucleoid occlusion model states that all sites along the length of the cell are potential division sites competent for cell division; however, division is inhibited at positions occupied by nucleoids. Therefore, upon nucleoid segregation the midpoint becomes available for the placement of an FtsZ ring and the central division site.

In *Escherichia coli* the MinCDE proteins act together as a negative regulatory element, restricting the formation of septa at polar division sites, ensuring that the FtsZ ring is placed correctly at the midcell (Errington *et al.*, 2003). MinC acts as an inhibitor of septation and is given topological specificity by the action of MinE, whereas MinD is required

for the recruitment of MinC and MinE to the cytoplasmic membrane (Hale *et al.*, 2001). The MinCD proteins form a MinE-dependent spiral complex that oscillates from pole to pole and inhibits the formation of the Z-ring at polar locations while allowing proper localization of the Z-ring at the midcell (Hale *et al.*, 2001; Shih *et al.*, 2003).

The Min system is found in a diverse range of bacteria (Errington *et al.*, 2003). The Gram-positive organism *Bacillus subtilis* contains homologues to both MinC and MinD, but lacks a MinE counterpart (Cha & Stewart, 1997; Edwards & Errington, 1997). The MinC and MinD proteins do not oscillate from pole to pole and midcell selection is controlled by a protein, DivIVA, which lacks significant amino acid sequence similarity to MinE. The DivIVA protein stably associates with the *B. subtilis* cell poles and retains MinCD at the poles, thereby preventing division at those sites (Cha & Stewart, 1997; Edwards & Errington, 1997; Marston & Errington, 1999). Additionally, MinCDE homologues have been characterized in coccal *Neisseria gonorrhoeae* (Ramirez-Arcos *et al.*, 2001; Szeto *et al.*, 2001) and the spherical-celled cyanobacterium *Synechocystis* (Mazouni *et al.*, 2004).

Sinorhizobium meliloti is a Gram-negative, rod-shaped alphaproteobacterium that induces N₂-fixing root nodules on the legume alfalfa (*Medicago sativa*). The genome of this micro-organism consists of three replicons: a circular

Abbreviation: ESEM, environmental scanning electron microscopy.

chromosome (3.7 Mb) and two megaplasmids pSymA (1.3 Mb) and pSymB (1.7 Mb) (Galibert *et al.*, 2001). The *S. meliloti* genome sequence reveals a single copy of *minCDE* gene homologues on the pSymB megaplasmid (Finan *et al.*, 2001; Galibert *et al.*, 2001). During nodule formation, *S. meliloti* cells invade the host plant through root hairs and enter the plant cells where they differentiate from 1–2 µm long small rods into enlarged filaments and branched cells 5–10 µm in length (Mergaert *et al.*, 2006; Paau *et al.*, 1977). In a recent study Mergaert *et al.* (2006) examined the viability of *S. meliloti* differentiated bacteroids and concluded that they are non-dividing. They also reported that the genome content of the bacteroids is 24C (C being the haploid DNA content), while the content in free-living bacteria was 1–2C. The genes and factors involved in and controlling the morphological and genome content changes during bacteroid differentiation have not been well characterized. Here we report on experiments designed to investigate the requirement of *min* genes for cell division and symbiotic N₂ fixation in *S. meliloti*. We have constructed various *min* mutants and *min* reporter gene fusions, and overexpressed the *min* genes in *S. meliloti* and *E. coli*. Our findings demonstrate that while *minDE* genes are expressed in free-living cells and within nodules, they are not absolutely required for cell division and symbiotic N₂ fixation.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The strains and plasmids used in this work are listed in Table 1. The *E. coli* cultures were grown at 37 °C in Luria broth (LB), and *S. meliloti* strains were grown at 30 °C in LB containing 2.5 mM CaCl₂ and 2.5 mM MgSO₄ (LBmc). Defined M9 medium contained M9 salts (Miller, 1972) supplemented with 0.25 mM CaCl₂, 1 mM MgSO₄, 0.3 µg biotin ml⁻¹, 5 ng CoCl₂ ml⁻¹ (Watson *et al.*, 2001) and 15 mM succinate or glucose. When necessary, antibiotics were used as described previously (Finan *et al.*, 1986). For Min protein overexpression and electron microscopy, *E. coli* strains were grown to an OD₆₀₀ of 0.1 and the growth continued for 4 h after IPTG was added to a final concentration of 0.1 mM. *S. meliloti* cultures were grown to an OD₆₀₀ of 0.1 and then induced with IPTG (1 mM) for 16 h.

DNA manipulations and genetic techniques. Standard methods were used for DNA isolation, digestion, agarose gel electrophoresis, DNA ligation and transformation (Sambrook & Russell, 2001). Synthesis of oligonucleotides (Table 2) and sequencing of PCR products were performed at MOBIXLab (McMaster University, Hamilton, Ontario, Canada). Bacterial matings were done as described previously (Finan *et al.*, 1986).

To delete and replace the entire *minCDE* genes with the Ω Sp cassette from pHP45 Ω (Prentki & Krisch, 1984), the Ω Sp cassette was cloned between the 844 and 911 bp PCR-amplified DNA fragments (primer pairs AB32663/AB32664 & AB32661/AB32662, respectively) flanking the outside of *minC* and *minE*, serially cloned into pJQ200-mp18 (Quandt & Hynes, 1993). The resultant plasmid, pTH1426 (Fig. 1a), was transferred into *S. meliloti* wild-type strain Rm1021 and a single crossover recombinant was selected on an LB plate containing (µg ml⁻¹) streptomycin, (200), gentamicin (60) and spectinomycin

(200). The double crossover integrant SmK850 was obtained by plating the transconjugants on LB agar with 5 % sucrose and screening the colonies for gentamicin sensitivity and spectinomycin resistance.

To disrupt the *minC* gene, a 570 bp DNA fragment internal to *minC* was PCR-amplified using AB31700 and AB31698 primers, and cloned downstream of the *trp* terminator and upstream of *gusA* in a suicide vector, pVO155 (Oke & Long, 1999), to obtain plasmid pTH1218 (Fig. 1a). Integration of pTH1218 by homologous recombination into the *S. meliloti* genome resulted in a *minC* and *gusA* gene fusion, and promoterless *minC' DE* genes in SmK690 (Fig. 1b).

To make a polar *minD* mutation (i.e. *minC*⁺ *D*⁻ *E*⁻) a 773 bp DNA fragment carrying *minC* (without its translational start codon) was obtained by PCR using primers AB31700/AB31697 and cloned upstream of *gusA* and downstream of the *trp* terminator in pVO155 to yield pTH1219 (Fig. 1a). Homologous recombination of pTH1219 into *S. meliloti* genomic DNA produced a functional copy of *minC* and disrupted the *minCDE* operon in SmK691 (Fig. 1b). To disrupt the *minE* gene, a 1657 bp DNA fragment carrying the *minCD* gene region was PCR-amplified using primers AB31700/AB31699 and cloned downstream of the *trp* terminator and upstream of *gusA* in pVO155 to obtain pTH1220 (Fig. 1a). Strain SmK692, resulting from integration of pTH1220 into the *S. meliloti* genome, contained functional *minC* and *minD* genes with a *gusA* gene fusion at the 3' end of *minD* (Fig. 1b).

To overexpress *S. meliloti min* genes in both *E. coli* and *S. meliloti*, a broad-host-range expression vector, pTH1227, was constructed. A 1510 bp DNA fragment carrying the *lacI*⁻-*P*_{tac} cassette from pMAL-c2x (New England Biolabs) was PCR-amplified using primers AB32193/AB32194 and inserted upstream of *gusA* in a broad-host-range plasmid, pFUS1 (Reeve *et al.*, 1999), to obtain pTH1227. Overexpression of the *gusA* gene after induction with IPTG was determined by measuring GusA activities in *E. coli* M65 and *S. meliloti* SmK700, both of which contained the vector pTH1227. DNA fragments carrying *min* genes with a modified ribosome-binding site were PCR-amplified using primers AB31697/AB32186 (807 bp) for *minC*, AB31699/AB32187 (850 bp) for *minD*, AB32188/AB31979 (336 bp) for *minE*, AB31699/AB32186 (1694 bp) for *minCD*, AB32186/AB31979 (1995 bp) for *minCDE* and AB32187/AB31979 (1151 bp) for *minDE*. The resulting PCR products were cloned into pTH1227 to obtain pTH1369 (*P*_{tac}-*minC*), pTH1228 (*P*_{tac}-*minD*), pTH1229 (*P*_{tac}-*minE*), pTH1230 (*P*_{tac}-*minCD*), pTH1231 (*P*_{tac}-*minCDE*) and pTH1587 (*P*_{tac}-*minDE*). Transfer of these plasmids to *S. meliloti* Rm1021 resulted in strains SmK701 (pTH1228), SmK702 (pTH1229), SmK703 (pTH1230), SmK704 (pTH1231), SmK705 (pTH1369) and RmP321 (pTH1587), respectively.

To measure *min* gene expression and identify promoter(s) in the *minCDE* region, an 844 bp DNA fragment from 837 bp upstream of *minC* was PCR-amplified using primers AB32663/AB32664 and cloned into pUCP30T (Schweizer *et al.*, 1996). The *SphI*-*KpnI* fragment from the resulting plasmid (the promoter region) was then cloned into the reporter plasmid pFUS1 to give pTH1261 (Fig. 1a). Another 839 bp DNA fragment carrying the *minC* ORF and the *minC* and *minD* intergenic region was obtained by PCR using primers AB32919/AB32920 and then cloned upstream of *gusA* in pFUS1 to obtain pTH1273 (Fig. 1a). Following transfer of pTH1261 and pTH1273 into Rm1021, strains SmK712 and SmK710 were obtained, respectively. Plasmid pTH1445 was constructed for amplifying *minC* mRNA for primer extension. A DNA fragment carrying *minC* and the 1 kb region upstream of *minC* was PCR-amplified using primer AB32664/AB31697 and cloned into the *Apal/XbaI* sites in pBluescript SK (Stratagene) to obtain pTH1423. The *S. meliloti* DNA was then subcloned into the *KpnI/XbaI* sites in pFUS1 to obtain pTH1445. Strain SmK857 was obtained after transfer of pTH1445 into *S. meliloti* Rm1021.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristics	Reference or source
<i>E. coli</i>		
DH5 α	F ⁻ <i>endA1 hasdR17 supE44 thi-1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>)	Lab collection
MT616	MT607(pRK600), mobilizer, Cm ^r	Finan <i>et al.</i> (1986)
<i>S. meliloti</i>		
Rm1021	SU47, <i>str-21</i>	Finan <i>et al.</i> (1984)
Rm5000	SU47, <i>rif-5</i>	Finan <i>et al.</i> (1984)
RmP110	Rm1021 with changed wild-type <i>pstC</i> , Sm ^r	Yuan <i>et al.</i> (2006)
RmP319	Rm1021, <i>nifH::gusA</i> , Sm ^r Gm ^r	Lab collection
SmK690	Rm5000, <i>minC::gusA</i> , Rf ^r Nm ^r	This study
SmK691	Rm5000, 3' end of <i>minC::gusA</i> , Rf ^r Nm ^r	This study
SmK692	Rm5000, 3' end of <i>minD::gusA</i> , Rf ^r Nm ^r	This study
SmK700	Rm1021(pTH1227), P _{tac} - <i>gusA</i> , Sm ^r Tc ^r	This study
SmK701	Rm1021(pTH1228), P _{tac} - <i>minD</i> , Sm ^r Tc ^r	This study
SmK702	Rm1021(pTH1229), P _{tac} - <i>minE</i> , Sm ^r Tc ^r	This study
SmK703	Rm1021(pTH1230), P _{tac} - <i>minCD</i> , Sm ^r Tc ^r	This study
SmK704	Rm1021(pTH1231), P _{tac} - <i>minCDE</i> , Sm ^r Tc ^r	This study
SmK705	Rm1021(pTH1232), P _{tac} - <i>minC</i> , Sm ^r Tc ^r	This study
RmP321	Rm1021(pTH1232), P _{tac} - <i>minDE</i> , Sm ^r Tc ^r	This study
SmK709	Rm1021(pFUS1), Sm ^r Tc ^r	This study
SmK710	Rm1021(pTH1273), <i>minC</i> and <i>minD</i> intergenic region:: <i>gusA</i> , Sm ^r Tc ^r	This study
SmK712	Rm1021(pTH1261), P _{minC} :: <i>gusA</i> , Sm ^r Tc ^r	This study
SmK850	Rm1021, <i>minCDE::</i> Ω Sp, Sm ^r Sp ^r	This study
SmK857	Rm1021(pTH1445), Sm ^r Tc ^r	This study
SmFL5810	RmP110, <i>minD::gusA</i> , Gm ^r (pTH1522 library clone 5810)	Cowie <i>et al.</i> (2006)
Plasmids		
pBluescript-SK	Cloning vector	Lab collection
pFUS1	A broad-host-range plasmid carrying <i>gusA</i> , Tc ^r	Reeve <i>et al.</i> (1999)
pHP45 Ω	pBR322 derivative carrying Ω Sm ^r Sp ^r cassette, Ap ^r	Prentki & Krisch (1984)
pJQ200-mp18	Suicide vector carrying <i>sacB</i> , Gm ^r	Quandt & Hynes (1993)
pVO155	pUC119 derivative carrying <i>gusA</i> , Nm ^r Ap ^r	Oke & Long (1999)
pUCP30T	Cloning vector, Gm ^r	Schweizer <i>et al.</i> (1996)
pTH1218	pVO155 carrying 0.57 kb 5'- <i>minC</i> (AB31698/AB31700), Ap ^r Nm ^r	This study
pTH1219	pVO155 carrying <i>minC</i> (AB31697/AB31700), Ap ^r Nm ^r	This study
pTH1220	pVO155 carrying <i>minC</i> and <i>minD</i> (AB31699/AB31700), Ap ^r Nm ^r	This study
pTH1227	pFUS1 carrying <i>lacI</i> ^q -P _{tac} DNA from pMal-c2x (AB32193/AB32194), Tc ^r	This study
pTH1228	pTH1227 carrying <i>minD</i> (AB32187/AB31699), Tc ^r	This study
pTH1229	pTH1227 carrying <i>minE</i> (AB32188/AB31979), Tc ^r	This study
pTH1230	pTH1227 carrying <i>minC</i> and <i>minD</i> (AB32186/AB31699), Tc ^r	This study
pTH1231	pTH1227 carrying <i>minC</i> , <i>minD</i> and <i>minE</i> (AB32186/AB31979), Tc ^r	This study
pTH1369	pTH1227 carrying <i>minC</i> (AB32186/AB31697), Tc ^r	This study
pTH1261	pFUS1 carrying 0.84 kb DNA upstream of <i>minC</i> coding region, Tc ^r	This study
pTH1273	pFUS1 carrying <i>minC</i> and intergenic region of <i>minCD</i> (AB32919/AB32920), Tc ^r	This study
pTH1421	pJQ200-mp18 carrying 0.84 kb DNA upstream of <i>minC</i> (AB32663/AB32664), Gm ^r	This study
pTH1422	pTH1421 carrying 0.91 kb DNA downstream of <i>minE</i> (AB32661/AB32662), Gm ^r	This study
pTH1423	pBluescript-SK carrying <i>minC</i> promoter and <i>minC</i> (AB31697/AB32664), Ap ^r	This study
pTH1426	pTH1422 carrying Ω Sp cassette, Gm ^r Sp ^r Sm ^r	This study
pTH1445	pFUS1 carrying <i>minC</i> and 1 kb DNA upstream of <i>minC</i> (AB31697/AB32664), Tc ^r	This study
pTH1587	pTH1227 carrying <i>minDE</i> (AB32187/AB31979), Tc ^r	This study

Ap, Ampicillin; Gm, gentamicin; Nm, neomycin; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

RNA isolation and primer extension. *S. meliloti* SmK857 containing pTH1445 was grown in 20 ml LBmc and M9 glucose to an OD₆₀₀ between 0.4 and 0.6. After addition of 2 ml 5% (v/v) phenol

in ethanol (Bernstein *et al.*, 2002), cells were collected by centrifugation at 2500 g for 10 min at 4 °C. The cell pellet was resuspended in 325 μ l 10 mM sodium acetate (pH4.2) buffer containing 0.3 M

Table 2. Oligonucleotides used in this work

Restriction and ribosome-binding sites that were engineered into the primers are shown in bold or underlined respectively.

Oligonucleotide	Sequence (5'–3')
AB31697	CCCCTCTAGAAATCCGATAAATC
AB31698	CCCCTCTAGAAAGCGTCCCGTAGATATG
AB31699	CGGGTCTAGATTATGCTGCCCTCCGTCCGAATATCTTG
AB31700	CCCCGGATCCGCTAGCACCAAAGTGCTAACAGAC
AB31979	CGCCTCTAGACGGGGCTCGGTGATGCTG
AB32186	GGCAAGATCTAAGGAACGAAGATGACCAAAG
AB32187	CGCCAGATCTACAGGAGCGGGAAGAT
AB32188	GGGCAGATCTAAGGAGGCGAGCATGAGCATTTTC
AB32193	GCGCGT CGACAAGCTT GAAATGGTGCAAAAC
AB32194	CCCCTCGAGGTGAAGTGCTCGTGAAAAAC
AB32661	GCTCGAGCTCCACGAGAAAACGGTGAAACATC
AB32662	GGCGGGATCCGCAATGGCAACCGGATGGATAAC
AB32663	GCGCGGATCCTGGTCATCTTCGTTAATTAT
AB32664	TTATCTCGAGGGCCCGGATCACCTCATCCGCAATTCAAA
AB32919	TAACGAATTCGACCAAAGTGCTAACAGA
AB32920	GCAACTGCAGCTACTTTCCGCCATCTTC
ML2050	GAGCGGCCCTTGTGTGGATCGAGCGAGCGTCTGTTAGCA
ML9882	GGTCCGTTTCGCCTCCGCATGACGTG

sucrose and 50 mM EDTA. Following addition of 375 µl lysis buffer (2 %, w/v, SDS in 10 mM sodium acetate, pH 4.2), the mixture was incubated at 65 °C for 5 min. Pre-warmed acidic phenol (700 µl, 65 °C, pH 4.2) was added to the lysate and incubation was continued for a further 5 min with vortexing every 1 min. Total RNA was extracted with the acidic phenol twice, phenol/chloroform (50:50) twice and then chloroform, and the RNA was precipitated with ethanol. The RNA was treated with RNase-free DNase I at 37 °C for 30 min and then purified using a Qiagen RNeasy mini column, according to the manufacturer's instructions. The purity and quality of the RNA were checked by electrophoresis through a 1.2 % agarose TBE gel.

To identify the transcriptional start site through primer extension, primers ML2050 and ML9882 were end-labelled with γ-³²P-ATP (Amersham) by using T4 polynucleotide kinase (New England Biolabs) at 37 °C for 45 min. Unincorporated label was removed by passing the labelling mixture through Qiagen oligopurification columns. The primer extension reaction was carried out as described previously (Yuan *et al.*, 2006). The primer extension product was loaded onto a 6 % acrylamide/7 M urea sequencing gel and run alongside a sequencing ladder generated by using the same primer with plasmid template pTH1423 and the Sequenase Version 2 DNA Sequencing kit (USB).

β-Glucuronidase activity assay. To measure expression of the *gusA* gene encoding β-glucuronidase (GusA), *S. meliloti* cultures were grown up in 96 deep-well plates (ABgene) in 0.5 ml LBmc medium with antibiotics, and then subcultured into 150 µl LBmc or M9 media in 96-well plates (Costar). After 16 h growth in LBmc or 36 h growth in M9 media, 5 µl cultures were transferred to 95 µl assay buffer (50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.0125 %, w/v, SDS and 0.438 mg *p*-nitrophenyl-β-glucuronide ml⁻¹) in 96-well plates. The reaction was stopped with 100 µl 1 M Na₂CO₃ after 1 h. *A*₄₀₅ was measured using a Safire microplate reader (Tecan). GusA specific activities were calculated using the equation: specific activity = *A*₄₀₅ × 1000/60 × 0.005 × OD₆₀₀.

For nodule extracts, GusA specific activity was calculated using the equation: *A*₄₀₅ × 1000 × (time, min)⁻¹ × (volume, ml)⁻¹ × (mg protein)⁻¹.

Plant tests. Symbiotic N₂ fixation was assessed using alfalfa (*Medicago sativa*) grown under nitrogen-deficient conditions in Leonard assemblies as described previously (Finan *et al.*, 1986). Plants were harvested 28 days after inoculation and shoot dry weights were determined following drying in an oven. Measurement of GusA activity in nodules and histochemical staining for GusA were performed as described by Yuan *et al.* (2006). To determine the viable cell numbers in nodules, fresh nodules were surface-sterilized for 5 min in 2.5 % sodium hypochlorite, rinsed three times in 0.85 % (w/v) NaCl and then put into ice-cold MMS buffer (20 µl mg⁻¹) containing 40 mM MOPS (pH 7.0), 20 mM KOH, 2 mM MgSO₄ and 0.3 M sucrose. The nodules were crushed and then centrifuged for 2 min at 400 *g*. The supernatant was diluted serially in 0.85 % NaCl and then plated onto LB agar plates.

Environmental scanning electron microscopy (ESEM). ESEM was performed as described previously (Sibley *et al.*, 2006). Briefly, primary fixation of mid-exponential-phase LB-grown cells was done at room temperature for 30 min in 2 ml 0.2 M sodium cacodylate buffer (pH 7.4) containing 2.5 % (v/v) glutaraldehyde. Bacteroids were fixed in MMS buffer containing 2.5 % glutaraldehyde for 1 h. Fixed cells were washed five times in ddH₂O and then 1 µl was spotted onto a gold-coated cover glass (5 nm thick) that was mounted on an ESEM stub with conductive glue. Wet samples were viewed with an Electronscan 2020 Environmental Scanning Electron Microscope at 2.4–4.0 Torr with an accelerating voltage of 20–30 KeV.

Transmission electron microscopy. Primary fixed samples were washed twice in acetate/veronal buffer (59 mM sodium acetate, 35 mM sodium barbital, 145 mM NaCl and 12 mM CaCl₂, pH 6.1), resuspended in the same buffer containing 1 % (w/v) osmium tetroxide and then incubated at room temperature for 4 h. The cells were then washed twice in the acetate/veronal buffer and resuspended in

Fig. 1. Schematic diagram of the *minCDE* region on the megaplasmid pSymB of *S. meliloti*. (a) DNA fragments (solid line) were PCR-amplified and cloned to obtain the pVO155 derivatives pTH1218, pTH1219 and pTH1220, the pFUS1 derivatives pTH1261 and pTH1273, and the pJQ200-mp18 derivative pTH1426. The *minCDE* genes were replaced by a Ω Sp cassette in pTH1426. The +1 above *minC* refers to nt 1 of the translational start codon. (b) Transfer of the constructs into *S. meliloti*-generated *minCDE* mutants SmK690 and SmK850, *minDE* mutant SmK691 and *minE* mutant SmK692. The *trp* terminator (stem-loop) blocks transcription from the plasmid backbone. The *minC* promoter is indicated (P_{minC} , arrow). The structure of the *minD::gusA* gene fusion in SmFL5810 is also indicated.

2% (w/v) Nobel Agar (45°C) and cooled on a glass slide. Cells embedded in the agar cubes (1 mm³) were then stained with 0.5% (w/v) uranyl acetate in the acetate/veronal buffer at 4°C for 12 h. Samples were dehydrated at room temperature with sequential washes of increasing ethanol concentrations and finally with propylene oxide. Infiltrated samples were embedded in Spurr's resin (Spurr, 1969) and 60–90 nm sections were cut with an Ultracut Microtome. Thin sections were post-stained in a 1% (w/v) aqueous uranyl acetate solution for 20 min on carbon-coated copper EM grids (100 mesh) that had been previously covered with 0.3% (w/v) Formvar. Sections were then post-stained with Reynold's lead citrate for 6 min.

RESULTS

Structure and expression of the *minCDE* genes

The organization of the *minC*, *minD* and *minE* on the *S. meliloti* pSymB megaplasmid suggests that these genes are

transcribed as an operon, *minCDE*. There is a 101 bp region between *minC* and *minD* which contains a 43 bp partial RIME element (Østerås *et al.*, 1995) and there is a single nucleotide overlap between *minD* and *minE* (schematically outlined in Fig. 1). To identify promoters associated with these genes, an 844 bp DNA fragment encompassing the region upstream from the *minC* translational start codon and a 847 bp DNA fragment containing the *minC* ORF and the *minC*–*minD* intergenic region were cloned into the replicating *gusA* reporter plasmid pFUS1 to generate plasmids pTH1261 and pTH1273, respectively (Fig. 1). Analysis of GusA activity from *S. meliloti* strains carrying these plasmids (Fig. 2a) demonstrated strong promoter activity (20-fold above background) from upstream of *minC* (SmK712). The promoter activities from cells grown in both rich medium (LB) and minimal medium (M9-glucose) were comparable although the growth rate for *S. meliloti* was

Fig. 2. Expression of *minCDE* genes in *S. meliloti*. (a) Cultures were grown in LBmc (white bars) or M9-glucose media (black bars) in 96-well plates as described in Methods. GusA activity is represented as $A_{405} \times 1000/\text{time (min)} \times \text{sample volume (ml)} \times \text{OD}_{600}$. SmK709 contained pFUS1 as control, SmK710 contained pTH1273, i.e. pFUS1 with the *minC* gene and *minCD* intergenic region, and SmK712 contained pTH1261, i.e. pFUS1 with the *minC* promoter region. (b) Determination of transcriptional start sites of the *minC* promoter in *S. meliloti* through extension of ^{32}P -labelled ML9882 primer. Lanes: 1, RNA isolated from SmK857 cells grown in LBmc; 2, RNA isolated from SmK857 cells grown in M9 glucose. (c) Sequence of the *minC* promoter region, showing the transcriptional start (+1) site, and -35 and -10 regions (underlined). Predicted CtrA binding sites are boxed and underlined sequences show the locations of extension primers used for transcriptional start site mapping. Data for primer ML2050 are not shown.

about twofold slower in M9 minimal medium compared to LBmc. Consistent with an absence of promoter activity, only background GusA activity was observed from constructs carrying the *minC*-*minD* intergenic region (SmK710). These data demonstrated that, under the conditions examined, transcription emanated from upstream of *minC*. To map the transcriptional start site(s) upstream of *minC*, we used primer extension and two distinct primers (ML9882, Fig. 2b; ML2050, data not shown). Using mRNA extracted from both LBmc- and M9-glucose-grown cells, a

transcript initiating from 174 nt upstream from the *minC* start codon was identified in both extension reactions. The deduced -35 and -10 hexanucleotide promoter sequences, 5'-GCCTGC-N₁₇-CCATGC-3', showed little similarity to the recently derived *S. meliloti* promoter consensus sequences 5'-CTTGAC-N₁₇-CTATAT-3', particularly within the more conserved -35 region (MacLellan *et al.*, 2006). We note that the 174 nt untranslated region is relatively large for *S. meliloti* genes. This could reflect the presence of regulatory sequences and, in this respect, it is

interesting that two possible CtrA protein-binding sites (Barnett *et al.*, 2001) are positioned in this region (Fig. 2c), implying that the CtrA protein might be involved in regulation of *minCDE* expression in *S. meliloti*.

***S. meliloti minCDE* genes are dispensable for growth**

To investigate whether *minCDE* are required for cell viability, we sought to construct a strain where these genes were deleted and replaced with the Ω Sp cassette. We confirmed that the *min* genes were absent from the resulting strain SmK850 (Rm1021 $\Delta minCDE::\Omega$ Sp) by PCR, while a fragment from upstream of *minC* to the Ω Sp gene was readily amplified in four separate mutants. Additional *min* mutant derivatives were made via a single cross-over recombination between plasmids carrying fragments from the *min* gene region and the *min* genes in the genome. These co-integrates readily generated single cross-over polar mutations in the *minC* gene in strain SmK690 (*minC*[−] *minD*[−] *minE*[−]), in the *minD* gene in strain SmK691 (*minD*[−] *minE*[−]) and in the *minE* gene in strain SmK692 (*minE*[−]) (see Fig. 1b).

The growth of the resulting strains in LBmc and M9 media was examined and the viability of these cultures was

determined by plating dilutions onto LB agar (data not shown). In addition, the effects of the mutations on cell structure were determined by examining the cells by phase-contrast microscopy and by both scanning and transmission electron microscopy (Fig. 3).

With the exception of the *minE* mutant strain SmK692 which is described below, the various *min* mutants grew at similar rates (OD₆₀₀) and had similar cell viability to their parent strains. The morphologies of SmK850 ($\Delta minCDE::\Omega$ Sp) (Fig. 3b), SmK690 (*minC*[−] *minD*[−] *minE*[−]) (data not shown) and SmK691 (*minD*[−] *minE*[−]) (Fig. 3c) were indistinguishable from the rod-shaped, 1–2 μ m long wild-type cells (Fig. 3a). This differs from the minicell phenotype observed with *E. coli minCDE* and *minDE* mutants (de Boer *et al.*, 1989). Strikingly the cells from the *minE*[−] mutant strain SmK692 were swollen and branched with single or multiple asymmetric septa (Fig. 3d). This phenotype also contrasts with the cell filamentation phenotype seen with an *E. coli minE* mutant (de Boer *et al.*, 1989). Transmission electron micrographs showed that wild-type *S. meliloti* cells had normal mid-cell invaginations and an intact membrane surrounding the entire cell (Fig. 3e). The *minE* cells were very different from wild-type (Fig. 3f), as seen by the membrane connectivity of

Fig. 3. Cell morphology of *S. meliloti* strains. Environmental scanning electron micrographs of wild-type Rm5000 (a), *minCDE* mutant SmK690 (b), *minDE* mutant SmK691 (c) and *minE* mutant SmK692 (d); transmission electron micrographs of Rm5000 (e) and SmK692 (f). Cells were grown in LBmc medium to mid-exponential phase and processed for electron microscopy as described in Methods. Bars, 2 μ m (a, b, c, d) and 0.4 μ m (e, f).

the cytoplasm with shapes never seen in the wild-type sections. Thus, the branched cells appeared to represent individual bacteria with atypical morphology.

Symbiotic phenotype of *S. meliloti* *min* mutants

The symbiotic phenotype of the *S. meliloti* wild-type and *min* mutant strains was examined following their inoculation onto alfalfa seedlings. The *min* mutants formed wild-type-like pink nodules. With the exception of the *minE*[−] mutant SmK692 (discussed below), the dry weights of the alfalfa plants inoculated with the mutants were similar to those inoculated with the wild-type strains (Table 3). The dry weights of alfalfa inoculated with *minE*[−] mutant SmK692 were 30 % of the parent Rm5000, while the uninoculated plants were less than 5 % of the wild-type dry weight (Table 3). Thus the *minE* mutation resulted in a large decrease in symbiotic N₂ fixation.

To determine whether the *min* mutations reduced the viability of the bacterial cells in nodules, 4-week-old nodules were crushed and the resulting cell suspensions were plated onto LB agar. The viable cell numbers of SmK850 (Δ *minCDE*) and SmK691 (*minD*[−] *E*[−]) nodules were similar to that of the wild-type [9×10^7 (g nodule)^{−1}], while the number of viable cells in SmK692 (*minE*[−]) nodules was slightly reduced [3×10^7 (g nodule)^{−1}] relative to the wild-type. The morphology of the bacteroids was examined using ESEM. Wild-type strain Rm1021 bacteroids showed a heterogeneous morphology as described by Vasse *et al.* (1990) with enlarged filaments and Y shapes of 3–8 times the length of vegetative cells, and the various *min* mutant strains, SmK690, SmK850, SmK691 or SmK692, were indistinguishable from the wild-type (data not shown). We suggest that the reduced N₂-fixation phenotype of the *minE*[−] mutation is related to the aberrant cell division phenotype that results when *minC* and *minD* are expressed in the absence of *minE*. Our results demonstrate that the *min* genes are not essential for the development of N₂-fixing nodules.

Expression of *min* genes in nodules

The lack of a symbiotic phenotype for the *minCDE* deletion mutant coupled with the reduced fix phenotype of the *minE*[−] mutant prompted us to investigate the expression of these genes in nodules. Expression of the *minCDE* genes in nodules was measured following the inoculation of alfalfa seedlings with the *minD::gusA* reporter gene fusion in strain SmFL5810. The single cross-over that generated this strain also reconstituted a wild-type *minCDE* gene region (see Fig. 1b). As a control we employed strain RmP319 carrying a *nifH::gusA* gene fusion since *nifH* is highly expressed in bacteroids (Barnett *et al.*, 2004). Examination of nodules 4 weeks after inoculation revealed that the *nifH* gene was highly expressed with a GusA specific activity of 7739 ± 311 while the GusA specific activity from SmFL5810 nodule extracts was 1675 ± 26 or about 22 % that of *nifH*. These data demonstrate that the *minCDE* genes are expressed in nodules.

To investigate whether *min* gene expression was uniform throughout the nodule, longitudinal sections of nodules were examined following histochemical staining for GusA. The staining revealed that expression of the *nifH* gene occurred in the previously described interzone II to III (Vasse *et al.*, 1990) (data not shown), as reported by Labes *et al.* (1993) and Soupene *et al.* (1995). In contrast, the *minD* gene was expressed in all regions of the nodule containing the bacteria (SmFL5810, *minD::gusA*), including infection zone II (data not shown).

Overexpression of *min* genes in *S. meliloti* generated different phenotypes

With the exception of the aberrant cell division phenotype that resulted from the *minE*[−] mutation, the expression data and *min* mutant phenotypes were not informative as to a direct role for *min* genes in cell septation in *S. meliloti*. We therefore examined whether expressing the *min* genes from a strong plasmid-borne promoter would affect the cell morphology and viability of *S. meliloti*. Individual or

Table 3. Symbiotic phenotypes of *S. meliloti* strains

Alfalfa plants were harvested 28 days after inoculation. The dry weights are shoot dry weights (mean ± SEM). All viable nodule isolates obtained from plants inoculated with SmK690, SmK691 and SmK692 were Nm^r. All viable nodule isolates obtained from plants inoculated with RmP319, SmFL5810 were Gm^r, and all isolates from SmK850 inoculated plants were Sp^r.

Strain	Relevant genotype	Dry weight (mg per plant)	Percentage*
Control	No inoculation	4.7 ± 0.1	4.2
Rm1021	Sm ^r derivative of wild-type SU47	111.3 ± 7.3	100.0
Rm5000	Rf ^r derivative of wild-type SU47	106.0 ± 4.9	95.2
SmK690	Rm5000 <i>minC</i> [−] <i>minD</i> [−] <i>minE</i> [−]	97.2 ± 3.6	87.3
SmK691	Rm5000 <i>minD</i> [−] <i>minE</i> [−]	89.1 ± 2.2	80.0
SmK692	Rm5000 <i>minE</i> [−]	31.3 ± 2.2	28.1
SmK850	Rm1021 Δ <i>minCDE::</i> ΩSp	94.7 ± 1.7	85.1

*The percentages were calculated by comparison with the value for the wild-type strain Rm1021.

combinations of *min* genes with ribosome-binding sites were cloned downstream of the P_{tac} promoter in the expression vector pTH1227 which also carries the *lacI^q* gene upstream of the promoter. These cell structures of IPTG-induced *S. meliloti* cells carrying these plasmids were examined by ESEM, and cell viability was evaluated by diluting cultures of known OD₆₀₀ and plating onto LB agar. In the absence of added IPTG, alterations to the wild-type cell structures were not observed. As the *gusA* gene is located downstream of the P_{tac} promoter in pTH1227, we first examined *S. meliloti* cells overexpressing *gusA* alone. These cells resembled the short rods of wild-type Rm1021 (SmK700, Fig. 4a, b) and their viability was the same as wild-type cells (data not shown). When the *minC* gene was expressed in pTH1227, several cell phenotypes were observed, including wild-type-like short rods, filaments, and swollen and branched cells (SmK705, Fig. 4c). The viability of these cells was similar to the wild-type. Expression of the *minD* gene from pTH1227 generated multi-branched cells (Fig. 4d, e) and the cell viability of these cultures was over 10 000-fold lower than the wild-type. Hence overproduction of MinD had a much more inhibitory effect on cell growth and division than overproduction of MinC. A similar inhibitory effect and a filamentation phenotype was observed in *E. coli* upon increased synthesis of MinD (de Boer *et al.*, 1989). Neither cell division nor cell viability appeared compromised in cells overexpressing *minE* (SmK702, Fig. 4a, f). When *minE* was overexpressed with *minD* in RmP321, a mixture of wild-type and branching cells was generated (Fig. 4k, l) and the viability of these cells was about tenfold higher than the cells overexpressing *minD* alone. These data indicated that MinE could relieve the inhibitory effect of MinD on cell division and growth, although it is also possible that *minD* expression levels were reduced in these cells.

Expression of both *minC* and *minD* from pTH1227 (SmK703 Fig. 4g, h) generated a branching cell morphology with the reduced cell viability observed for cells in which *minD* alone was overexpressed. The overexpression of *minCDE* (SmK704, Fig. 4i, j) from pTH1227 also generated the cell morphology and cell viability phenotypes observed from when *minD* alone was overexpressed (Fig. 4g, h). We note that for these constructs the cell growth, cell viability and cell morphology were not affected under non-inducing conditions.

Overexpression of *S. meliloti* Min proteins in *E. coli*

The morphology of *E. coli* DH5 α cells carrying the various pTH1227 *min* derivatives was examined after IPTG-induced expression of *min* gene expression. Without IPTG induction all cells appeared like wild-type. IPTG-induced expression of the *gusA* gene from pTH1227 did not alter the rod-shaped morphology of *E. coli* (Fig. 5a). IPTG-induced overexpression of the entire *minCDE* operon resulted in a large number of short filaments and minicells (Fig. 5f), a classic minicell phenotype of *E. coli* (de Boer *et al.*, 1989). When viewed by ESEM, the same minicell phenotype was

also observed when the *S. meliloti minE* gene was expressed in *E. coli* (Fig. 5d). In addition, expression of the *minD* or *minCD* genes in *E. coli* caused formation of long, non-septate filaments (Fig. 5c, e), whereas *S. meliloti* MinC produced various sizes of filaments in *E. coli* (Fig. 5b). Filamentation resulting from overexpression of *S. meliloti* MinC and MinCD proteins was the same as that resulting from *E. coli* Min proteins (de Boer *et al.*, 1989). Taken together, the morphological changes in *E. coli* cells resulting from overexpression of the *S. meliloti* Min proteins imply that the *S. meliloti* and *E. coli* MinCDE proteins act similarly with respect to cell division.

DISCUSSION

We have demonstrated that the *minCDE* genes are not essential for cell viability or cell division in *S. meliloti*. Thus we readily obtained both *minCDE* deletion and single cross-over insertion mutants, whereas in a separate study, we were unable to recover single cross-over recombinants in the *parAB* genes encoding chromosome partitioning proteins ParA and ParB of *S. meliloti* (Cowie *et al.*, 2006). The *parAB* genes are essential in *Caulobacter crescentus* (Figge *et al.*, 2003; Mohl *et al.*, 2001). Our data clearly show that the *min* genes are expressed in free-living cells, and both the cell phenotype of the *minE* mutant and that of cells expressing *minD* from the P_{tac} promoter are consistent with these genes playing a role in cell septa location/formation. In the absence of MinE, it appears that the MinC and MinD proteins interfere with septum formation. This is consistent with the *E. coli* model where MinE prevents the MinCD division inhibitor from accumulating at the midcell, while permitting it to block division at other unwanted sites along the length of the cell (Shih *et al.*, 2003). In *E. coli*, the Min system prevents septation at potential division sites near cell poles without blocking septum formation at the desired midcell location. MinC interacts with the division protein FtsZ to prevent formation of stable FtsZ rings, a vital first step in assembly of the division machinery. MinE is responsible for giving site specificity to MinC, limiting its activity to the sites away from the desired midcell. MinD is responsible for the membrane association of MinC and MinE (Errington *et al.*, 2003). According to the *E. coli* Min model, deletion of *S. meliloti minCDE* genes, or a polar *minC* mutation or a polar *minD* mutation, should result in a minicell phenotype which was not observed in this study. Instead, the mutant cells were indistinguishable from wild-type. Alternatively, expression of the genomic copy of the *minC* and *minD* genes in the absence of the *minE* gene in *S. meliloti* would be predicated to produce long filaments, but cells actually showed a large, swollen and branched morphology (Fig. 4g, h).

Deletion of the *min* genes in *E. coli* and *B. subtilis* generates a minicell phenotype in which a large fraction of cells divide at polar positions of the cell instead of the middle (de Boer *et al.*, 1989; Cha & Stewart, 1997). In contrast, the *Synechocystis* Δ *minCDE* mutant displays spiralled, heart-shaped cells and minicells (Mazouni *et al.*, 2004). The

Fig. 4. The effect of *min* overexpression on the cell morphology of *S. meliloti* Rm1021. Environmental scanning electron micrographs of Rm1021 (a) overexpressing *gusA* (b), *minC* (c), *minD* (d, e), *minE* (f), *minCD* (g, h), *minCDE* (i, j) and *minDE* (k, l). Cells were grown in LBmc medium and then induced by IPTG as described in Methods. Bars, 1 µM (a, b, c, d, f, g, i, k, l), 5 µm (e, h, j).

Fig. 5. Effect of overexpressed *S. meliloti* Min proteins on the morphology of *E. coli* DH5 α . Environmental scanning electron micrographs of *E. coli* DH5 α (a) overexpressing *minC* (b), *minD* (c), *minE* (d), *minCD* (e) and *minCDE* (f). Cells were grown in LB medium and then induced with 0.1 mM IPTG as described in Methods. Bars, 4 μ m (a, b, d, f), 20 μ m (c, e).

minicells of *Synechocystis* are able to further divide, but those of *E. coli* and *B. subtilis* are anucleate. The *minC* and *minD* mutants of *Neisseria gonorrhoeae* do not show a minicell phenotype, but rather a gross enlargement of their round shape, causing cell lysis (Ramirez-Arcos *et al.*, 2001; Szeto *et al.*, 2001). Thus while the Min system is required for proper septum location in a wide range of bacteria, the phenotypes resulting from *min* gene mutations can be very different.

Both the free-living and nodule phenotypes of the Δ *minCDE* mutant demonstrate that in the absence of the Min proteins, other systems in *S. meliloti* may direct appropriate cell septation. Mutations in such systems may be lethal in the *min* mutant background, as in the case of the *slmA* mutants of *E. coli* (Bernhardt & de Boer, 2005). Many bacterial species lack Min homologues (*Caulobacter crescentus*, *Haemophilus influenzae* and *Staphylococcus aureus*) and thus must have other mechanisms to replace the role of the MinCDE proteins in determining Z-ring assembly (Margolin, 2003; Figge *et al.*, 2003). In this respect, we note that the megaplasmid location of the *min* genes in *S. meliloti* is consistent with a dispensable function. Moreover, the *minCDE* genes are located on the p42e plasmid of *Rhizobium etli* (Gonzalez *et al.*, 2006) and the pRL11 plasmid in *Rhizobium leguminosarum* (Young *et al.*, 2006).

Six of the nine genes required for cytokinesis in *E. coli* [*ftsA*, *ftsI*, *ftsK* (2), *ftsQ*, *ftsW* and *ftsZ* (2)] have been annotated in the *S. meliloti* genome (Galibert *et al.*, 2001). Recent transcriptome analysis of *S. meliloti* shows that the expression of these genes is down-regulated in bacteroids (Barnett *et al.*, 2004). Our mutant data show that while *minCDE* genes are not absolutely required for the bacteroid differentiation process, the *min* genes are expressed in nodules. Both the free-living and nodule phenotypes of the Δ *minCDE* mutant demonstrate that there is redundancy in the pathway(s) determining septa formation in *S. meliloti*.

The cellular concentrations of MinCDE proteins are maintained within certain levels to avoid disrupting cell division, as overexpression of *S. meliloti* *min* genes in wild-type or disruption of the *minE* gene resulted in aberrant morphology and growth. We identified a promoter upstream of the *minC* gene and identified two putative CtrA-binding sites (Fig. 2c). *S. meliloti* CtrA is essential and binds to the five CtrA-binding sites in the *ctrA* promoter region (Barnett *et al.*, 2001). CtrA is a DNA-binding response regulator and regulates expression of about 100 genes involved in cell cycle progression and morphogenesis in *Caulobacter crescentus* (Laub *et al.*, 2000). The presence of CtrA-binding sites upstream of the *minC* gene indicates that

CtrA may be involved in regulating *minCDE* expression, as implied for *Brucella abortus minC* expression (Bellefontaine *et al.*, 2002; Hallez *et al.*, 2004).

The complex cell morphological changes resulting from a *minE* mutation or overproduction of Min proteins are similar to those in *S. meliloti* overexpressing CcrM (Wright *et al.*, 1997), DnaA (Sibley *et al.*, 2006) and FtsZ (Latch & Margolin, 1997), or treatment with DNA-damaging agents (Latch & Margolin, 1997). This phenotype is correlated with blocked cell division (Latch & Margolin, 1997; Wright *et al.*, 1997; Sibley *et al.*, 2006) and loss of normal control of DNA replication initiation (Wright *et al.*, 1997). It remains to be established whether a blockage in cell division always leads to branching in *S. meliloti*; however, this possibility is of considerable interest given the cellular morphology of N₂-fixing bacteroids. We do not exclude the possibility that the MinCDE proteins may be involved in other cell process such as genome partitioning and segregation, as in *E. coli* (Åkerlund *et al.*, 2002).

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