The *Sinorhizobium meliloti* chromosomal origin of replication

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The predicted chromosomal origin of replication (*oriC*) from the alfalfa symbiont *Sinorhizobium meliloti* is shown to allow autonomous replication of a normally non-replicating plasmid within *S. meliloti* cells. This is the first chromosomal replication origin to be experimentally localized in the *Rhizobiaceae* and its location, adjacent to *hemE*, is the same as for *oriC* in *Caulobacter crescentus*, the only experimentally characterized alphaproteobacterial *oriC*. Using an electrophoretic mobility shift assay and purified *S. meliloti* DnaA replication initiation protein, binding sites for DnaA were mapped in the *S. meliloti oriC* region. Mutations in these sites eliminated autonomous replication. *S. meliloti* that expressed DnaA from a plasmid *lac* promoter was observed to form pleomorphic filamentous cells, suggesting that cell division was perturbed. Interestingly, this cell phenotype is reminiscent of differentiated bacteroids found inside plant cells in alfalfa root nodules.

INTRODUCTION

Bidirectional replication of the bacterial chromosome requires the specific interaction of the ATPase DnaA at a precise region on the replicon, termed the chromosomal origin of replication (*oriC*) (Fuller *et al.*, 1981, 1984). *oriC* is the location of the initial strand opening event that precedes replication and the site of replisome assembly (Bramhill & Kornberg, 1988b). Many of the molecular events that occur at the *Escherichia coli oriC* have been well characterized, providing a general model of the essential biochemical process which results in the formation of two replication forks and duplication of *oriC* (Bramhill & Kornberg, 1988a). While *E. coli oriC* is the most extensively characterized chromosome origin, sequences capable of autonomous replication have been isolated from many bacterial chromosomes (Harding *et al.*, 1982; Zyskind *et al.*, 1983; Yee & Smith, 1990; Zakrzewska-Czerwinska & Schrepf, 1992; Fujita *et al.*, 1990, 1992; Calcutt & Schmidt, 1992; Schaper *et al.*, 2000; Moriya *et al.*, 1992; Salazar *et al.*, 1996; Jakimowicz *et al.*, 1998). In most sequenced bacterial chromosomes, *oriC* has been predicted on the basis of sequence analysis where a switch in the asymmetry of the two strands of DNA coincides with the origin and terminus of the chromosome (Lobry, 1996). The architecture of the bacterial chromosome origin is nevertheless diverse with respect to the physical sequence as well as the number and organization of binding sites for proteins that control the timing of origin firing. Binding sites for the replication initiator DnaA (DnaA boxes) appear to be one of the hallmark features of bacterial chromosome origins and because of this the location of DnaA boxes in the bacterial genome has been

used as a method for predicting *oriCs* (Mackiewicz *et al.*, 2004). Another feature of many functional origins is that adjacent to the DnaA boxes is an exceptionally AT-rich region which is the site of initial strand separation. Bacterial chromosome origins are commonly located in close vicinity to the *dnaA* gene and attempts have been made to exploit this linkage in the cloning of *oriCs* (Smith *et al.*, 1991; Fujita *et al.*, 1990, 1992; Schaper *et al.*, 2000; Moriya *et al.*, 1992; Salazar *et al.*, 1996; Jakimowicz *et al.*, 1998). Examples do exist in which hallmarks of *oriCs* have not been found to be linked to the *dnaA* replication initiation gene, such as the chromosomes of *Synechocystis* spp. (Richter & Messer 1995), *Prochlorococcus marinus* (Richter *et al.*, 1998) and *Sinorhizobium meliloti* (Margolin *et al.*, 1995).

S. meliloti is a Gram-negative bacterium that forms root nodules on alfalfa. The colonization of alfalfa roots by *S. meliloti* provokes a complex differentiation program resulting in morphological changes in both organisms. Nitrogen-fixing bacteroids have been reported to contain more nucleic acid per cell than the free-living form and thus may undergo a process of endoreduplication within alfalfa (Paau *et al.*, 1977). It is unclear what role DNA replication of the *S. meliloti* genome has during differentiation; however, the origins of replication in the *S. meliloti* genome may serve as important elements that coordinate this process within plant cells. The tripartite genome of *S. meliloti* (Galibert *et al.*, 2001) is composed of a circular chromosome (3.6 Mb) and two megaplasmids, pSymA (1.35 Mb) and pSymB (1.68 Mb). Previously, the chromosomal origin was predicted to be located adjacent to the *hemE* gene (encoding uroporphyrinogen decarboxylase) on the basis of DNA

strand asymmetry (Capela *et al.*, 2001). Here we show that a DNA fragment encompassing this region can confer autonomous replication to a non-replicating plasmid and that these minichromosomes are maintained in *S. meliloti* at copy numbers of less than one per host chromosome. Using a combined bioinformatic, genetic and biochemical approach we have mapped essential DnaA-binding sites within *oriC*. We previously characterized the replication origins from both megaplasmids carried by *S. meliloti* (Chain *et al.*, 2000; MacLellan *et al.*, 2005) and this work

completes our initial characterization of the third origin of replication carried by this bacterium.

METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *E. coli* was grown at 37 °C in LB broth and *S. meliloti* was grown at 30 °C in LB supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc) or on LB solidified with agar (16 g l⁻¹).

Table 1. Bacterial strains and plasmids

Plasmid	Genotype or characteristics	Source or reference
pET431a	Ap ^R , NusAHis6 tag expression vector, inducible with IPTG	Novagen
pRK600	pRK2013 <i>npt::Tn9</i> ; Cm ^R	Finan <i>et al.</i> (1986)
pBBR1MCS-5	Gm ^R , broad host-range cloning vector, LacZα peptide	Kovach <i>et al.</i> (1995)
pUCP30T	Gm ^R , ColEI <i>oriV</i> cloning vector, <i>oriT</i>	Schweizer <i>et al.</i> (1996)
pTH838	pUCP30T with 3 kb <i>oriC</i> (AB24853–AB24854) cloned via <i>EcoRI</i> , <i>hemE</i> same orientation as <i>Plac</i>	This work
pTH879	710 bp <i>HindIII</i> – <i>Sall</i> <i>oriC</i> fragment cloned into pUCP30T	This work
pTH880	875 bp <i>BamHI</i> – <i>Sall</i> <i>oriC</i> fragment cloned into pUCP30T	This work
pTH881	861 bp <i>SacI</i> <i>oriC</i> fragment cloned into pMB439 suicide plasmid	This work
pTH1081	pET431a with <i>dnaA</i> (AB26340–AB26341) cloned via <i>BamHI</i> / <i>EcoRI</i>	This work
pTH1091	pBBR1MCS-5 with <i>dnaA</i> and ribosome-binding site (AB29744–AB29675) cloned via <i>XbaI</i> / <i>SacI</i> , same orientation as <i>Plac</i>	This work
pTH1205	pTH838 with 3 bp deletion in DnaA box #1 TCATCCGCC→TCCGCC, created via site-directed mutagenesis (AB31447–AB31448)	This work
pTH1206	pTH838 with 4 bp deletion in DnaA box #2 TCATCCACA→TCACA, created via site-directed mutagenesis (AB31449–AB31450)	This work
pTH1207	pTH838 with 4 bp deletion in DnaA box #3 TTGTCCACA→TTGTA, created via site-directed mutagenesis (AB31451–AB31452)	This work
pTH1245	pUCP30T with 483 bp <i>hemE</i> – <i>Y02793</i> intergenic region (AB32323–AB32324) cloned via <i>EcoRI</i>	This work
pTH1253	pTH838 with 4 bp deletion in DnaA box #4 TTATCAACA→CAACA, created via site-directed mutagenesis (AB32750–AB32751)	This work
pTH1416	pUCP30T with 2289 bp <i>oriC</i> (ML1817–ML1818) cloned via <i>EcoRI</i>	This work
pTH1442	pUCP30T with 2472 bp <i>oriC</i> (ML1817–AB24853) cloned via <i>EcoRI</i>	This work
pTH1443	pUCP30T with 2823 bp <i>oriC</i> (ML1818–AB24854) cloned via <i>EcoRI</i>	This work
pTH1451	pUCP30T with 2402 bp <i>oriC</i> (ML2444–ML1818) cloned via <i>EcoRI</i>	This work
pTH1452	pUCP30T with 2421 bp <i>oriC</i> (ML2443–ML1818) cloned via <i>EcoRI</i>	This work
pTH1453	pUCP30T with 1783 bp <i>oriC</i> (ML2444–ML2445) cloned via <i>EcoRI</i>	This work
pTH1454	pUCP30T with 1802 bp <i>oriC</i> (ML-2443–ML2445) cloned via <i>EcoRI</i>	This work
Strain		
<i>E. coli</i> DH5α	F [−] <i>endA1 hsdR17</i> (r _K [−] , m _K [−]) <i>supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA)</i> U169 Φ80d ^{lacZ} ΔM15	BRL
<i>E. coli</i> MT616	MM294A <i>recA</i> -56 (pRK600)	Finan <i>et al.</i> (1986)
<i>E. coli</i> MT620	MM294A <i>recA</i> -56 Rf ^R	Strain collection
<i>E. coli</i> BL21 STAR	F [−] <i>ompT hsdSB</i> (r _B [−] , m _B [−]) <i>gal dcm rne131</i> (DE3)	Stratagene
<i>S. meliloti</i> Rm1021	SU47 str-27	Meade <i>et al.</i> (1982)
<i>S. meliloti</i> Rm5004	Rm1021 <i>recA::Tn5</i>	Strain collection
<i>S. meliloti</i> K569	G212 Φ pTH1044	Strain collection
<i>S. meliloti</i> K1012	Rm5004(pTH838) small colony	This work
<i>S. meliloti</i> K1013	Rm5004(pTH838) large colony	This work

Bacterial matings. Plasmids were transferred from *E. coli* (donor) cells to *S. meliloti* (recipients) in triparental matings using MT616, a helper strain that carries transfer functions on pRK600. Strains used in the triparental mating were grown overnight and then washed twice with saline. The *E. coli* donor and recipient cells were adjusted to have twice the cell density as the *S. meliloti* recipient. Equal volumes of cells (20 μ l) were spotted onto an LB agar plate and incubated overnight at 30 °C. The mating spot was resuspended in 0.85% NaCl, serially diluted and 100 μ l volumes were plated on LB with antibiotic to select for the *S. meliloti* recipient cells (200 μ g streptomycin ml⁻¹) and on LB plates containing antibiotics used to select for the recipient and the transferred test plasmid (200 μ g streptomycin ml⁻¹ and 60 μ g gentamicin ml⁻¹). *E. coli* MT620 recipients were selected for on LB plates containing 20 μ g rifampicin ml⁻¹ and transconjugants on LB plates containing 20 μ g rifampicin ml⁻¹ and 10 μ g gentamicin ml⁻¹.

General molecular biology. Plasmid DNA isolation, genomic DNA isolation, restriction analysis, PCR and site-directed mutagenesis were all performed according to standard protocols. PCR products were purified using QIAquick spin columns (Qiagen). Sequencing and primer synthesis was performed at the Mobix Central Facility (McMaster University, Hamilton, Ontario, Canada) using the ABI PRISM 3100 Genetic Analyser using the BigDye terminator chemistry.

Copy number determination. Total DNA was prepared from cultures grown to an OD₆₀₀ of 0.5–0.6, according to a standard protocol. Genomic DNA (10 μ g) was digested in a 30 μ l reaction volume for 4 h. Digested genomic DNA was loaded onto a 0.8% agarose gel and electrophoresed at 15 V overnight. Southern hybridization was done using either linearized plasmid DNA or purified PCR products as probes which were radioactively labelled with [α -³²P]dATP using the Roche Random Primed DNA Labelling Kit. Following hybridization, the membrane was exposed to a Storage Phosphor Screen (Amersham Biosciences) for 1 h. After exposure, the screen was scanned on a Storm 820 Phosphorimager (Molecular Dynamics) at a pixel size of 50 μ m. Band intensities were calculated using the Image Quant 5.2 program (Molecular Dynamics). Plasmid copy number was calculated as a ratio of plasmid signal to chromosome signal. Copy number was determined for triplicate samples and a mean copy number is reported.

Protein purification. The *dnaA* gene was PCR-amplified from genomic DNA using the AB26340 and AB26341 primers (Table 2) and cloned via engineered *Bam*HI and *Eco*RI restriction sites into the pET43a NusAHis₆ tag expression vector (Novagen) to create pTH1081. The pTH1081 plasmid was transformed into *E. coli* BL21 STAR (Stratagene) to create the protein overexpression strain J1027. This strain was grown at 37 °C in 5 ml LB broth containing 50 μ g ampicillin ml⁻¹ and subcultured into 100 ml LB broth with antibiotic. The culture was grown to an OD₆₀₀ of 0.6 at 37 °C and then IPTG was added to a final concentration of 0.3 mM. Following incubation for an additional 2.5 h at 30 °C, the cells were collected and resuspended in 10 ml ice-cold resuspension buffer (50 mM NaH₂PO₄, pH 8.0, 500 mM NaCl, 10 mM imidazole). Lysozyme was added to a 150 μ g ml⁻¹ final concentration and incubated on ice for approximately 30 min. The cell suspension was then sonicated twice with a Branson Sonifier Cell Disruptor with a 1/8" (2.7 mm) tapered probe for 20 s at a power level of 3.5. Lysates were kept on ice between sonications. The crude lysate was centrifuged at 15 000 r.p.m. to remove insoluble material.

His-tagged protein was purified from the soluble cell lysate as a batch purification. Soluble lysate was incubated with 1 ml equilibrated Ni-NTA resin (Qiagen) at 4 °C on a rotator for 1 h. The sample was then spun in a swinging bucket rotor at 2000 r.p.m. for 3 min in a Clay Adams Dynac 1 centrifuge to pellet the resin and most of the

supernatant was removed. The collected resin was added to a Bio-Rad Poly-Prep chromatography column and the flow-through was collected. The resin was then washed with 2 ml ice-cold resuspension buffer and then 2 ml ice-cold wash buffer #1 (50 mM NaH₂PO₄, pH 8.0, 500 mM NaCl, 20 mM imidazole). The resin was then washed with 4 ml ice-cold wash buffer #2 (50 mM NaH₂PO₄, pH 8.0, 500 mM NaCl, 40 mM imidazole). The fusion protein was eluted from the column with 1 ml ice-cold elution buffer (50 mM Na₂PO₄, pH 8.0, 500 mM NaCl, 250 mM imidazole). The eluate (1 ml) was added to a pre-soaked Pierce 3.5K Slide-A-Lyser with a cold syringe. Dialysis was carried out overnight at 4 °C in 4 l buffer containing 50 mM NaH₂PO₄ (pH 8.0) and 300 mM NaCl. The protein sample was removed from the dialysis cartridge and 0.5 μ l thrombin (1.5 U μ l⁻¹; Novagen) was added. Following incubation at room temperature for 2.5 h, the reaction was added to a Bio-Rad Poly-Prep column, the flow-through was collected and the resin was washed with 0.5 ml resuspension buffer, 0.5 ml wash buffer #1, 0.5 ml wash buffer #2; finally the NusA tag was eluted with 0.5 ml elution buffer. Collected samples were analysed by SDS-PAGE.

Electrophoretic mobility shift assay. Probe DNA (~100–200 bp purified PCR product) was quantified visually on a 1.8% agarose gel. One picomole of 5' termini was then end-labelled with [γ -³²P]ATP using T4 polynucleotide kinase (New England Biolabs). Following the labelling reaction, probes were purified with a QIAquick PCR Purification Kit (Qiagen). Specific activity of the labelled probe was calculated using a liquid scintillation counter.

The binding reaction was set up as described by Schaper & Messer (1995). In a microfuge tube on ice the following were mixed (in this order): 4 μ l 5 $\times$ binding buffer (100 mM HEPES-KOH, pH 8.0, 25 mM magnesium acetate, 5 mM Na₂EDTA, 20 mM DTT, 25 mg BSA ml⁻¹, 1% Triton X-100, 25% glycerol), ddH₂O, 0.4 μ l ATP (50 mM), probe DNA (50 nM), 1 μ l poly dI:dC (100 ng μ l⁻¹) and purified DnaA (100–500 nM) in a total reaction volume of 20 μ l. The reaction was incubated on ice for 10 min and then at room temperature for 20 min. The reactions were loaded onto a 4% polyacrylamide gel and electrophoresed at 14 V cm⁻¹ (252 V) for 10 min and then 9 V cm⁻¹ (162 V) for 2.5 h at room temperature. The gel was dried and exposed to Kodak Scientific Imaging Film and a Storage Phosphor Screen for quantification.

Environmental scanning electron microscopy. For glutaraldehyde fixation, several colonies were used to inoculate 2 ml LBmc with 30 μ g gentamicin ml⁻¹ and the culture was grown to an OD₆₀₀ of 0.5. The culture was pelleted in a microfuge tube and the supernatant was removed. The pellet was then resuspended in 1 ml 0.2 M sodium cacodylate buffer (pH 7.4). One millilitre of 0.2 M sodium cacodylate buffer containing 5% glutaraldehyde (pH 7.4) was added, thus changing the effective concentration of glutaraldehyde to 2.5%, and the tubes were inverted a couple of times and left for 1 h at room temperature.

For slide preparation, a cover glass was mounted on an aluminium ESEM stub with conductive glue (equal parts white Elmers glue and colloidal graphite) making sure that a line of conductive glue was made from the edge of the glue spot to the edge of the cover glass and just around to the sample side of the glass. The mounted cover glass was air-dried for 30 min and then coated with a 5 nm layer of gold using a Sputter Coater. The glutaraldehyde fixed sample was washed six times in 2 ml ddH₂O to remove all traces of salt. Then, 1 μ l resuspended sample was spotted onto the gold-coated glass slide and the spot was allowed time to air dry. For visualization and image capturing the stub was placed into an Electroscan 2020 Environmental Scanning Electron Microscope and set to Wet mode. Samples were viewed at 2.4–4.0 Torr with an accelerating voltage of 20–30 keV. Various magnifications were used and images were saved as TIFF files.

Table 2. Oligonucleotides used in this study

Restriction sites that were engineered into the primers are shown in bold.

Oligonucleotide name	Sequence (5'→3')
AB24853	AAGAATTCCAGCCGCGCAAGCGCCAG
AB24854	CCGAATTCACGACAAGGATGACGATGG
AB26340	CGCGGATCCATGCGGATGAATTTGGCG
AB26341	CGCGAATTCCTATTCGTTGATCAGTCGC
AB27527	GCCAAGCTTCTGCAGCACAACCGAGC
AB27528	GGCGAATTCTTAGGTGTCGCGTCATCG
AB27526	GCCGAATTCCTGCAACATTCCTACTCTCC
dnaA32AS	AATGAATTCCTCCAGGTGCCGTCGCC
AB29675	GAGCTCTATTCGTTGATCAGTCGCTTC
AB29744	AATCTAGATAACTGAAATTGGAAGGCGCAAGATGCG
AB30423	GTAGAATTCCTCAGGCGTTTCCAGGTAGTCGC
AB31447	GCGGACGGATGCGATCCGCCGCGGACCGGCTCG
AB31448	CGAGCCGGTCCGGCGGATCGCATCCGTCCGC
AB31449	CGCAGATCGACTCCTCACAGGAACCGCAAATCCCG
AB31450	CGGGAGTTTGGCGTTCCTGTGAGGAGTCGATCTGCG
AB31451	GGCCATGAGTCTTGTATGCCCGGGCAAGAGATTTCCGG
AB31452	CCGGAAATCTCTTGCCCGGGCATAACAAGACTCATGGCC
AB32750	GCTGTTTTTGTCCCGCCCAACAGACCGCGGAGAATTGCG
AB32751	CGCAATTCCTCCGCGGTCTGTTGGGCGGGACAAAAACAGC
AB32323	TAGAATTCATCAGCCAGATGGGGG
AB32324	ATGAATTCACGCAATTCTCCGCGG
ML700	GTTTGCGGTTCTGTGGATGAGG
ML701	CTCCGCGGTCTGTTGATAAGGC
ML702	GATGCGTTTCGCTCACTTGCCG
ML703	GCAAATCCCGATCGCCGGC
ML1182	GTTTGCGGTTCTGTGAGGAGTC
ML1183	CTCCGCGGTCTGTTGGGCG
ML1817	TAGAATTCCTTTTCATCGCGATCCTCGAACG
ML1818	ATGAATTCGCGGTTTCGCTCTATCTAAGCC
ML2444	ATGAATTCGAGGTCGTCCGGTTGCCG
ML2443	ATGAATTCGCGCCTTGATCCACAGATAGAGG
ML2445	TAGAATTCGTCGACGATCGTGTAGAGTACG
ML2796	CCGCCATCCAGGAGATCACCGC
ML2797	CCATCGCGGTCGCTGCCTTTGC
ML3257	CCTTGCCACGCTCGTGTAATCC
ML3258	AACTCGAGTGCCGTCGCCAAATTCATCCG
ML3852	GCAATGATGTGGAGCGCCTTGACAGATAGAGGTCGTCCGG
ML3853	CCGGACGACCTCTATCTGTCAAGGCGCTCCACATCATTGC

RESULTS AND DISCUSSION

Sequence analysis of predicted *oriC* reveals hallmark features of chromosomal replication origins

The origin of replication on the *S. meliloti* chromosome has been predicted to be approximately 400 kb from *dnaA* and adjacent to *hemE* on the basis of GC skew (Capela *et al.*, 2001) and this corresponds to the same location as the well studied chromosome origin from *C. crescentus* (Marczynski & Shapiro, 1992; Marczynski *et al.*, 1995; Brassinga & Marczynski, 2001). The *C. crescentus* chromosome origin is

located ~ 5 kb from the *dnaA* gene region, a sequence with a similar genetic arrangement to the *S. meliloti* *dnaA* region (Brassinga *et al.*, 2001). This *fadB1-rpsT-dnaA* region is unusual in that it deviates from the *rnpA-rmpH-dnaA-dnaN-recF-gyrB-rnpA* structure linked to many bacterial chromosome origins (Yoshikawa & Ogasawara, 1991; Ogasawara & Yoshikawa, 1992). Interestingly, the *parB-parA-gidA-gidB-thdF* gene cluster located ~ 70 kb from the *C. crescentus* *oriC* is within 8 kb of the predicted *S. meliloti* *oriC*. We examined the predicted *S. meliloti* *oriC* region for features characteristic of bacterial chromosome origins. The most obvious feature found was an exceptionally AT-rich (~ 30 mol% G + C over 80 nt) region contrasting with the

Fig. 1. A G+C content graph of a 3 kb region encompassing the *S. meliloti* *oriC* region (a), and the location of putative DnaA-binding sites that match the expanded *E. coli* DnaA box consensus sequence [(T/C)(T/C)(A/T/C)(T/A/C)(A/G)(A/C/T)(A/C) (Schaefer & Messer, 1991)] in the *S. meliloti* origins of replication for the chromosome (b) (the sequence of the *hemE*-Y02793 intergenic region is provided with predicted DnaA shown in bold), (c) pSymA and (d) pSymB. Closed triangles represent 9/9 matches and open triangles represent 8/9 matches to the consensus sequence. The sequences of the putative DnaA boxes are provided in (e). The numbers below the DnaA boxes are how the binding sites are referred to in this study and are the numbers to the left of the sequence in (e).

flanking DNA and the rest of the chromosome which has a G+C content of 62 mol% (Fig. 1a). In *E. coli*, Bramhill & Kornberg (1988b) showed that duplex opening occurred in the AT-rich region of *oriC* and presumably the lower double-stranded thermodynamic stability in this region promotes strand dissociation.

DnaA-binding sites are a consistent feature of bacterial *oriCs*. Sequences recognized by DnaA have been most extensively studied in *E. coli* and these 9 bp sequences seem relatively conserved amongst bacteria. We scanned the predicted *S. meliloti* *oriC* region for matches based upon the expanded *E. coli* consensus sequence (T/C)(T/C)(A/T/C)(T/A/C)(A/G)(A/C/T)(A/C) (Schaefer & Messer 1991). As shown in Fig. 1(b), five putative DnaA boxes were identified in the 3 kb *oriC* region. An additional box was also found in the *dnaA* gene region. This DnaA box (box 5, Fig. 1b) is an 8/9 bp match to the *E. coli* consensus sequence and is an exact match to an experimentally identified DnaA box found in the *C. crescentus* chromosome origin (Marczynski & Shapiro, 1992). DnaA box 3 (also an 8/9 bp match to the consensus) is identical to the sequences of DnaA boxes found in chromosome origins from *Streptomyces* spp. (Majka *et al.*, 2001) and *Micrococcus luteus* (Fujita *et al.*, 1990).

Since the initiation of replication of the megaplasmid origins may also be subject to regulation by DnaA, the *repABC* replicator regions for the pSymA and pSymB megaplasmids were also scanned for DnaA-binding sites. As shown in Fig. 1(c), five putative DnaA boxes were identified

in the pSymA *repABC* region. Interestingly, DnaA box 2, found in the *hemE*-Y02793 intergenic region, is found twice in the pSymA replicator region, once 158 bp upstream of the translational start site of *repA2* and again inside the *repA2* ORF. Eleven DnaA-binding sites were predicted in the pSymB *repABC* locus (Fig. 1d). It has been documented that only the *repC* ORF and sequence downstream of *repC* is necessary for *repABC* plasmid replication (Ramirez-Romero *et al.*, 2000) and thus the origin of replication must be encoded either within *repC* or just downstream of the gene. This work predicts a single DnaA box in the sequence downstream of *repC* in both megaplasmid replicator regions and this box may therefore serve a role in recruiting the cellular replication machinery to the megaplasmid origins. However, the distribution of these putative DnaA boxes appears to be biased to upstream of or within the *repA1* gene (8 of 11 predicted sites in the region). DnaA may possibly be involved in regulating *repABC* gene expression. There is no experimental evidence for the involvement of DnaA in *repABC* plasmid replication, but this work might point to such a role.

Autonomous replication of the DNA region encompassing the predicted *oriC*

Exploiting the ability of a cloned sequence to support autonomous replication of a normally non-replicating plasmid in the host cell has been a successful strategy for isolating the chromosome origins from *Enterobacter aerogenes*, *Klebsiella pneumoniae* (Harding *et al.*, 1982), *Erwinia carotovora* (Takeda *et al.*, 1982), *Vibrio harveyi* (Zyskind

et al., 1983), *Pseudomonas aeruginosa*, *Pseudomonas putida* (Yee & Smith, 1990) and *Streptomyces lividans* (Zakrzewska-Czerwinska & Schrempf, 1992). We used this strategy to examine whether the previously predicted *S. meliloti* *oriC* region could support autonomous replication. For this purpose a 3 kb region encompassing the AT-rich region, the *hemE* and *Y02793* genes, and the five putative DnaA boxes was amplified by PCR from *S. meliloti* genomic DNA using primers AB24853 and AB24854. This DNA was cloned into pUCP30T (a plasmid that cannot replicate in *S. meliloti*) to form plasmid pTH838 (see Fig. 3b). pTH838 was transferred from *E. coli* donor cells into a *recA*⁻ derivative of *S. meliloti* (Rm5004) via conjugation. The ability of pTH838 to promote the formation of transconjugant colonies on medium containing an antibiotic (60 µg gentamicin ml⁻¹) to select for its presence is indicative of autonomous replication. The pTH838 plasmid transferred into both Rm5004 and wild-type Rm1021 cells and transconjugants were obtained at a frequency of 10⁻¹ per recipient cell. The high transfer frequency into both recipient strains reflects an ability of pTH838 to autonomously replicate because the *recA* mutation in Rm5004 prevents homologous recombination of the plasmid with the *hemE* locus on the chromosome. To our surprise, every time this mating experiment was performed, both small and large *S. meliloti* transconjugant colonies arose on selective medium after incubation. Both small and large Rm5004(pTH838) transconjugants colonies were purified three times on selective medium, then tested for the presence of an autonomously replicating plasmid by Southern blot hybridization. A single small colony and a single large colony (done in triplicate) was used to inoculate LB and total DNA was prepared following growth for 36 h. As a control, total DNA was prepared from a pUCP30T cointegrant strain RmK569 (a wild-type

Rm1021 derivative in which a single copy of pUCP30T has integrated at the *pstS* locus). Total transconjugant genomic DNA was restricted and probed in a Southern blot with labelled pUCP30T. pTH838 DNA was detected as a single restriction fragment and this is indicative of the plasmid being maintained as a closed circular molecule in the transconjugant cells. Two restriction fragments hybridized with the pUCP30T probe in the control strain RmK569. Similarly, integration of pTH838 into the chromosomal *hemE* locus would result in two restriction fragments, whereas only a single fragment was observed (data not shown), revealing that pTH838 is an autonomously replicating plasmid.

The conjugal transfer of pTH838 into Rm5004 resulted in both small and large transconjugants with approximately 50 times more small than large colonies. The rate and extent to which colonies form upon selection medium is at least in part dependent on the phenotypic level of resistance conferred by the antibiotic resistance gene, and the resistance gene dosage (i.e. copy number of plasmid carrying the gene) might influence this parameter. To investigate whether the difference in colony size was due to differences in the copy number of the pTH838 plasmid, we carried out a Southern blot hybridization to assess the relative amounts of the autonomously replicating pTH838 plasmid in both the small and large transconjugants. Large and small colonies were streak-purified to single colonies three times and total DNA was prepared in triplicate from cultures inoculated with the purified strains, designated SmK1012 and SmK1013 for small and or large colonies, respectively. The DNA was restricted with *Hind*III, transferred onto nitrocellulose and probed with the 477 bp PCR fragment (amplified with primers AB32323 and AB32324) of the

Fig. 2. Southern blot used to assess the copy number of the pTH838 *oriC* plasmid in *S. meliloti*. *Hind*III-restricted total DNA prepared from cultures inoculated with either a single small Rm5004(pTH838) transconjugant colony or a single large Rm5004(pTH838) transconjugant colony. Lanes: 1, small #1; 2, small #2; 3, small #3; 4, large #1; 5, large #2; 6, large #3; 7, RmK569(ΦpUCP30T *pstS*); 8, Rm1021 (wild-type); 9, Rm5004; 10, *Hind*III-restricted pTH838 plasmid DNA. A PCR product of the 477 bp *hemE*–*Y02793* intergenic region (using primers AB32323 and AB32324) was randomly prime-labelled and used as a probe. The 5.8 kb band is probe-hybridized with plasmid DNA and the 3.4 kb band corresponds to the chromosomal signal. Intensities of the bands were determined and relative copy number was calculated as a ratio of plasmid to chromosome signal. The mean copy number was calculated from the three independent colonies tested.

hemE-Y02793 intergenic region (Fig. 2). The intensities of the plasmid signal (5.8 kb) and the chromosome signal (3.5 kb) were quantified with a phosphoimager. A ratio of plasmid signal : chromosome signal was determined for each transconjugant sample and then the three ratios for each of the small and large strains were used to calculate a mean number of plasmid molecules per chromosome. The mean copy number of the three small transconjugants was determined to be 0.053 plasmids per chromosome and the mean copy number of the three large transconjugants was 0.135 plasmids per chromosome. Thus the large colonies contained approximately twice as much autonomously replicating pTH838 plasmid DNA as the small colonies.

It is not uncommon to observe altered colony morphologies in bacterial cells containing plasmid copies of the host chromosome origin. Colony morphologies consistent with the delayed colony development phenotype reported for *S. meliloti* in this work have been observed in *C. crescentus* cells harbouring minichromosomes (Marczynski *et al.*, 1995). Mutations that impair the replication of *C. crescentus* minichromosomes do so in a way that leads to a reduction of the copy number of the autonomously replicating plasmid and results in a delayed growth phenotype on selective medium. This parallels our observation that Rm5004(pTH838) transconjugant colony size directly correlates with the copy number of the pTH838 minichromosomes.

To validate the calculated pTH838 copy number of less than one, K1012 and K1013 and the control strain RmK569 (Rm1021 with a single copy of pUCP30T that has integrated at the *pstS* locus) were grown in LB with antibiotic selection to mid-exponential phase and plated onto LB plates without added antibiotic. Two hundred colonies were then patched back onto selective medium and incubated at 30 °C for

4 days. A total of 20 of the 200 (0.1) patches and 13 of the 200 (0.065) patches were gentamicin-resistant from the K1013 and K1012 cultures, respectively. As expected, all 200 of the RmK569 patches maintained gentamicin resistance because of the stability of the chromosomally integrated copy of pUCP30T. These values are very close to the mean copy number calculated from the Southern blot (Fig. 2), 0.053 vs 0.065 for the small transconjugants and 0.135 vs 0.1 for the large transconjugants. These ratios represent the fraction of cells in the cell population growing in the presence of gentamicin that actually contain a pTH838 plasmid molecule or the gentamicin 3'-acetyltransferase protein encoded by the pTH838 plasmid.

We employed a second indirect method to establish that pTH838 was autonomously replicating in Rm5004. The method is based on the premise that autonomously replicating plasmids, but not integrated plasmids, can be readily transferred from *S. meliloti* to *E. coli*. Thus we tested strains K1012 [Rm5004(pTH838) small transconjugant], K1013 [Rm5004(pTH838) large transconjugant] and strain RmK569 (as a control) for their ability to transfer pTH838 (as monitored by gentamicin resistance) to *E. coli*. The results from these experiments (Table 3) clearly showed a high frequency of transfer of gentamicin resistance from the Rm5004(pTH838) transconjugants K1012 and K1013 to *E. coli* ($>10^{-1}$ per donor). In contrast no transfer of the integrated copy of pUCP30T in RmK569 to *E. coli* was detected ($<10^{-8}$ per donor). Restriction analysis of plasmid DNA prepared from gentamicin-resistant *E. coli* transconjugant cells confirmed that the transferred plasmid was pTH838 (data not shown). These data demonstrate that pTH838 is capable of autonomous replication and therefore represents a mini-derivative of the chromosomal basic replicon.

Table 3. Transconjugation frequencies per recipient and donor resulting from the transfer of pTH838 from *S. meliloti* into *E. coli*

Three *S. meliloti* strains were used as donors; *E. coli* MT620 was used as the recipient and MT616 was used as the mobilizing strain in the triparental mating. The mating spot was resuspended, serial-diluted and plated onto selective medium. Transconjugants were selected for on LB agar with 20 µg rifampicin ml⁻¹ and 10 µg gentamicin ml⁻¹, recipients were selected for on LB agar with 20 µg rifampicin ml⁻¹ and donors were selected for on LB agar with 200 µg streptomycin ml⁻¹ and 60 µg gentamicin ml⁻¹. The transconjugation frequency for *S. meliloti* K569 and *E. coli* MT616 was 0 ($<10^{-8}$ per donor).

Donor	Transconjugation frequency	
	Transconjugants/ <i>E. coli</i> recipient	Transconjugants/ <i>S. meliloti</i> donor
<i>S. meliloti</i> K1013	0.23	0.13
<i>S. meliloti</i> K1012	0.28	0.13

Delineation of the minimal sequence that supports autonomous replication

As described above, the nucleotide sequence coincident with the predicted *oriC* encodes hallmark features of chromosomal origins, including an AT-rich sequence and five putative DnaA boxes, and a 3 kb region encompassing these features is able to autonomously replicate in *S. meliloti* cells. To delineate the *oriC* region required to allow the suicide plasmid pUCP30T to replicate in *S. meliloti*, various subfragments from the insert in pTH838 were subcloned into pUCP30T. The resulting plasmids were examined for their ability to replicate in Rm5004 (Fig. 3b). The sequence encompassing the AT-rich region and DnaA boxes 2, 3 and 4 (pTH1245) was not sufficient for autonomous replication (transconjugant colony formation). The 5' limit of the sequence required for autonomous replication was mapped to within 19 bp (the difference between pTH1451 and pTH1452, and the difference between pTH1453 and pTH1454) and within this essential 19 bp is the predicted DnaA box 5. The 3' limits required for transconjugant colony formation (pTH1454) extends 250 bp into the Y02793 ORF and thus the plasmid copy of Y02793 is not

Fig. 3. (a) A genetic map of the *S. meliloti* chromosomal origin of replication showing the *hemE* and *Y02793* loci, and the *dnaA* gene region of the *S. meliloti* chromosome which is located 400 kb away from *oriC*. A G+C content graph indicates the location of an exceptionally AT-rich region in the *hemE*–*Y02793* intergenic region. DnaA boxes that match the expanded *E. coli* consensus sequence (T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C) (Schaefer & Messer, 1991) are shown with dark triangles and boxes that match the consensus 8/9 are represented with open triangles. (b) Subclones of the 3 kb origin in pTH838 are scored for their ability to generate transconjugant colonies when cloned into a suicide plasmid (pUCP30T) and transferred into an *S. meliloti* *recA*[–] strain (Rm5004). (c) Location of site-directed mutations in the *S. meliloti* *oriC*. The locations of the site-directed deletions are indicated with an inverted triangle. Transconjugation frequencies are provided in the accompanying table for transfer into Rm1021 and Rm5004.

required for autonomous replication. The minimal size of the *S. meliloti* *oriC* is 1802 bp and this is much larger than the 437 bp *C. crescentus* minimal chromosome origin (Marczynski & Shapiro, 1992). It may be that the *hemE* ORF itself is not required for replication, but only the DnaA box located downstream of *hemE* and thus deletion of the intervening 956 bp *hemE* gene would reduce the size of the *S. meliloti* *oriC* to 846 bp. The minimal nucleotide sequence required to support autonomous replication of the suicide plasmid pUCP30T suggests that the functional *S. meliloti* *oriC* sequence may be defined by the distribution of DnaA boxes that flank the AT-rich region.

We therefore examined the importance of the predicted DnaA boxes to replication by using a mutagenesis approach. The five DnaA boxes identified in pTH838 were mutated by oligonucleotide site-directed mutagenesis. The mutations in DnaA boxes 5, 2, 3 and 4 were 4 bp deletions and the mutation in DnaA box 1 was a 3 bp deletion (Fig. 3c). Plasmids carrying mutations in boxes 2, 3 and 4 lost the ability to autonomously replicate in *S. meliloti*, suggesting that these sequences are required for replication of plasmid-borne *oriC* (Fig. 3c). As expected, transconjugant colonies formed at a low frequency when mutant *oriC* plasmids were transferred into Rm1021, probably due to homologous

recombination at the *oriC* genomic locus. Rm1021 was used as a control recipient in the replication assay to ensure that the *oriC* plasmids were still mobilizable after mutagenesis. The mutation in DnaA box 1 did not influence pTH838 replication and, surprisingly, the mutation carried on pTH1518 (mutation in DnaA box 5) also did not abolish replication even though our deletion analysis (Fig. 3b) demonstrated that this sequence was essential for replication. It is possible that the removal of the 4 bp in DnaA box 5 generated a sequence still capable of interacting with the replication initiator. DnaA box 5 (5'-TGATCCACA-3') is an 8/9 bp match to the expanded *E. coli* DnaA box consensus (Fig. 1.); the site-directed mutation (deletion of GATC) in this predicted binding site generates 5'-TCA-CAGATA-3' and this sequence only deviates from the consensus sequence at positions 4 and 6. In other words, the 4 bp deletion in box 5 may result in the reconstitution of a sequence that can still bind DnaA.

DnaA binds to predicted binding sites in the *hemE*-Y02793 intergenic region

The ability of DNA fragments carrying the various putative DnaA-binding sites to bind purified DnaA protein was examined in electrophoretic mobility shift assays. We tested mutations in putative DnaA-binding sites that abolish replication of plasmid-borne *oriC* for their influence on DnaA binding. The six DNA sequences that were used in DnaA-binding experiments included a 190 bp probe (ML700/ML701) that contained all three predicted DnaA boxes in the *hemE*-Y02793 intergenic region (DnaA boxes 2, 3 and 4), a 178 bp probe (ML1182/ML1183) with the same sequence as the 190 bp probe except with 4 bp deletions in all three predicted DnaA boxes, a 117 bp probe (ML2796/ML2797) containing DnaA box 5 downstream of *hemE*, a 197 bp fragment (ML3257/ML3258) from the *dnaA* promoter that contains a predicted DnaA box overlapping the translational start site of the *dnaA* gene, a 233 bp *repA2* promoter probe (ML702/ML703) which includes a DnaA box with an exact match to DnaA box 2 in *oriC* and a 125 bp

repA1 promoter probe (AB27527/AB27526) containing two DnaA boxes.

Two complexes were resolved with the 190 bp *oriC* probe as seen in Fig. 4; however, in some cases using this probe resulted in the formation of three complexes (data not shown), consistent with the number of binding sites in the probe. Three rounds of site-directed mutagenesis of pTH838 were required to introduce the three mutations needed to generate a template that could be used for PCR amplification of the 178 bp probe. DnaA did not interact with this target as it did with the wild-type sequence and thus it appears that the mutations created in the *hemE*-Y02793 intergenic region represent bona fide deletions in DnaA boxes (Fig. 3c). Only a weak interaction was observed with the 117 bp DnaA box 5 probe. Complexes were not detected with the *dnaA* promoter probe or the *repA1* promoter probe. DnaA did complex with the *repA2* promoter probe, probably at the predicted DnaA box 158 bp upstream of the *repA2* translational start site. DnaA has been implicated as a transcription factor as it autoregulates *dnaA* gene expression (Atlung *et al.*, 1985) and either represses or activates expression from several other genes (Messer & Weigel, 1997). Thus the DnaA-binding site upstream of the *repA2B2C2* operon may be biologically relevant such that expression of the pSymA genes encoded in the replicator region are transcriptionally coordinated with chromosome replication.

Expression of *S. meliloti* DnaA in *E. coli* and *S. meliloti* results in a block in cell division

DnaA seems to be conserved in bacteria as a regulator of replication initiation events at the chromosome origin and it has been reported that overexpression of DnaA in *E. coli* stimulates the initiation reaction at *oriC* (Atlung *et al.*, 1987; Skarstad *et al.*, 1989). This results in a loss of the correct timing in the cycle of replication initiation and leads to a block in cell division, thus generating filamentous cells (Pierucci *et al.*, 1989). This block in cell division as a result of

Fig. 4. Electrophoretic mobility shift assay with target DNA containing predicted DnaA boxes and purified *S. meliloti* DnaA. Several target DNA fragments were used in a binding reaction with purified DnaA. Three concentrations of protein were added to the binding reaction (100, 300 and 500 nM) and the DNA fragments were also loaded without added protein. The size and description of each labelled DNA fragment is indicated above the lanes. The first left-hand lane for each DNA target is DNA without protein added and the next three lanes (left to right) are the DNA target incubated with 100, 300 and 500 nM purified DnaA, respectively.

increased DnaA levels has also been reported for *Mycobacterium smegmatis* (Greendyke *et al.*, 2002). We examined whether DnaA would have similar consequences on *S. meliloti* cell morphology if overexpressed. To this end the *dnaA* gene and 20 bp upstream of the predicted translational start (including a predicted ribosome-binding site) was PCR-amplified using primers AB29744 and AB29675. Primer AB29744 was designed with three engineered stop codons in all three reading frames to prevent a fusion to the LacZ peptide encoded in pBBR1MCS-5. The PCR product was cloned into pBBR1MCS-5 such that the *dnaA* gene was under the transcriptional control of the *E. coli lac* promoter, generating plasmid pTH1091. The pTH1091 DnaA expression plasmid was transferred into wild-type *S. meliloti* Rm1021 and *E. coli* DH5 α and transconjugant cells were visualized with an environmental scanning electron microscope. Many more of the *E. coli* cells expressing *S. meliloti* DnaA appeared filamentous compared to the parent DH5 α (compare Fig. 5a and b), suggesting that *S. meliloti* DnaA

has some form of cross-functionality and can perturb cell division in other *Proteobacteria*. *S. meliloti* cells expressing DnaA from the plasmid promoter also displayed an apparent block in cell division, with many of the cells growing up to 15 μ m in length compared to the 1 μ m length of Rm1021 wild-type cells (compare Fig. 5c and d). The *S. meliloti* filaments are complex and in most cases the cells display many branches with swollen areas flanked by regions that appear partially septated, as shown under higher magnification (Fig. 5e and f).

We cannot deduce the molecular mechanism underlying this phenotype; however, the morphological changes resulting from the plasmid-mediated expression of *S. meliloti* DnaA are very similar to the pleomorphic cell shape phenotype reported for *S. meliloti* cells overexpressing FtsZ or when treated with DNA-damaging agents (Latch & Margolin, 1997). It would seem that the default pathway for a block in cell division that arises from perturbations in

Fig. 5. Environmental scanning electron micrographs of *E. coli* and *S. meliloti* cells expressing *S. meliloti* DnaA from the *E. coli lac* promoter in the pBBR1MCS5 plasmid derivative, pTH1091. (a) shows *E. coli* DH5 α harbouring pBBR1MCS5 and (b) shows *E. coli* DH5 α carrying pTH1091. (c) shows wild-type Rm1021 harbouring the pBBR1MCS5 plasmid and (d), (e) and (f) are wild-type Rm1021 cells expressing DnaA from pTH1091. Bars, 25 μ m (a, b), 30 μ m (c, d) and 15 μ m (e, f).

chromosome replication in this endosymbiont is a complex change in cell shape. Interestingly, many of the cells assume branched Y-shaped architectures that look very similar to the fully differentiated bacteroids found inside plant cells in root nodules.

Here we report the identification of a replication origin from the *S. meliloti* circular chromosome which represents the first such origin to be localized in the *Rhizobiaceae* and the second chromosome origin to be experimentally defined in the *Alphaproteobacteria*. This is the third autonomously replicating sequence to be identified in the *S. meliloti* genome. The other two sequences were isolated from the pSymB megaplasmid (Margolin & Long, 1993; Chain *et al.*, 2000). The *S. meliloti oriC* possesses the hallmark features of a bacterial chromosome origin, such as sequences that interact specifically with the replication initiator DnaA (DnaA boxes) flanking an exceptionally AT-rich region. Interestingly, the *S. meliloti* chromosome origin is located greater than 400 kb from the *dnaA* gene which, in other bacteria, is often closely linked to the replication origin. This explains why an autonomously replicating sequence was not detected in the vicinity of *dnaA* in previous attempts to localize this replication origin (Margolin *et al.*, 1995). *S. meliloti* transconjugant cells harbouring *oriC* plasmids display a delayed growth phenotype, perhaps explaining why a screen for autonomously replicating sequences encoded in the *S. meliloti* genome conducted by Margolin & Long (1993) did not detect the chromosome origin.

Previous reports have demonstrated that requirements for minichromosome replication may be very different than those for the chromosomal origin. The necessity of certain DnaA-binding sites and DNA-bending proteins HU and IHF for *oriC* plasmid replication, but not for chromosome replication (Weigel *et al.*, 2001; Asai *et al.*, 1998), suggests that the plasmid-borne *oriC* may adopt a very different DNA topology than the chromosomal origin. It is possible that constraints on DNA topology in *S. meliloti* minichromosomes may cause a severe reduction in the number of replication initiation events occurring at *oriC* in pTH838 and thus result in copy numbers of much less than one per chromosome.

The copy numbers of plasmids encoding bacterial *oriC* sequences is quite diverse. The best characterized bacterial minichromosome from *E. coli* has a copy number of approximately 38 per cell (Lobner-Olesen *et al.*, 1987), but this does not represent the typical situation. Plasmids replicating from the cloned *Pseudomonas* chromosome origin have been reported to be present at as low as 0.7 copies per cell (Yee & Smith, 1990). The ColE1 replication origin has also been shown to exert a strong effect on the copy number of *Mycobacterium tuberculosis* minichromosomes and can reduce the copy number from approximately 17 to 0.6 plasmids per chromosome (Qin *et al.*, 1999). The cause of this instability is unknown, but we do not rule out the possibility that the ColE1 replication origin present in

pUCP30T may be the cause of the low *S. meliloti* minichromosome copy number.

While we refer to the region identified in this report as the *oriC*, we note that direct experimental proof that replication of the *S. meliloti* chromosome initiates from this region has yet to be obtained. Similarly, the presumed replication origins for the pSymA and pSymB megaplasmids have yet to be experimentally verified. Such verification would seem to be very worthwhile and since this would require synchronization of cell division, these experiments could also address whether replication of the chromosome and two megaplasmids is coordinately regulated.

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