THE *S. MELILOTI* CHROMOSOMAL ORIGIN OF REPLICATION

IDENTIFICATION AND CHARACTERIZATION OF THE *SINORHIZOBIUM MELILOTI* CHROMOSOMAL ORIGIN OF REPLICATION AND THE REPLICATION INITIATOR DnaA

By

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ABSTRACT

DNA replication initiates at a precise location on the bacterial chromosome, the origin of replication (*oriC*). This work has localized the origin of DNA replication on the *Sinorhizobium meliloti* chromosome to a region spanning the *hemE* gene. A genetic dissection of the locus revealed that a much larger fragment of DNA (1802 bp) is required for a functional *oriC* than that of the other characterized alpha-proteobacterial chromosome origin from *Caulobacter crescentus*. Site-directed mutations of predicted DnaA binding sites has identified several essential elements for replication of the plasmid borne *oriC*. Mutations in these DnaA boxes also reduce transcription of *hemE* and thus it is likely that transcription of *hemE* and replication of the *S. meliloti* chromosome are coupled. The ColE1 plasmid pUCP30T can autonomously replicate when the *S. meliloti oriC* is cloned into the suicide vector (pTH838) and can be efficiently mobilized out of *S. meliloti* into *E. coli*. The pTH838 *oriC* plasmid when transferred into *S. meliloti* results in both small and large colonies and both of these

transconjugant classes take longer to form than the *S. meliloti recA*::Tn5 recipient. We attributed this phenotype to the very low copy number of the pTH838 plasmid which was determined to be 0.053 - 0.135 copies per chromosome.

The DnaA protein responsible for replication initiation in many bacteria has been purified and used in electrophoretic mobility shift assays. The DnaA protein interacts specifically with sequences in the *hemE - Y02793* intergenic region and upstream of the *repA2* gene on the pSymA megaplasmid. The DnaA protein has also been implicated as a link between DNA replication and cell division in *S. meliloti* as overexpression of DnaA in both *E. coli* and *S. meliloti* results in filamentation.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ARS	autonomously replicating sequence
ATP	adenonsine triphosphate
bp	base pairs of DNA
BSA	bovine serum albumin
Cori	*C. crescentus* chromosomal origin of replication
Cm	chloramphenicol
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
Gm	gentamicin
IPTG	isopropylthio-β-D-galactoside
kb	kilobase pairs of DNA
kDa	kilodalton
LB	Luria-Bertani complex media
LBmc	Luria-Bertani complex media with calcium and magnesium
OD	optical density
oriC	chromosomal origin of replication
PCR	polymerase chain reaction
Rf	rifampicin
Sm	streptomycin
Tc	tetracycline
V	volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1. LITERATURE REVIEW

Historical Perspective

All life is committed to the replication and faithful segregation of daughter chromosomes during cellular division. Bidirectional DNA replication initiates at a particular time in the cell cycle (at a precise location) on the chromosome. Research on how genetic material is replicated in all domains of life has revealed both the vast diversity and common principles in the control of replication initiation. Experiments characterizing the mechanism and factors involved in *Escherichia coli* chromosome replication provides us with a model to conceptualize the molecular events occurring at a bacterial chromosome replication origin.

The classical view of regulatory processes controlling protein synthesis in bacteria could not be used to explain DNA synthesis. Rather in the early 1960's all the information regarding DNA replication could be described by a system of positive regulation in which a cytoplasmic factor was predicted to have an active function in the

replication process. A model was proposed stating that a unit capable of replication, a replicon, would carry two specific determinants: a structural gene controlling the synthesis of a specific initiator and a replicator or operator of replication, the site at which the initiator acts. An implication of this model was that it should be possible to isolate mutants that have lost the ability to multiply idependently. This was first accomplished by Jacob *et al* (Francois Jacob, Sydney Brenner, and Francois Cuzin, 1963) by the identification of temperature sensitive mutations in the *E. coli* F plasmid that were unable to replicate at 42°C and could only be maintained in Hfr cells (cells in which the F plasmid is integrated into the *E. coli* chromosome) or if a wildtype copy of F was also present. Many years later a similar situation was described by Nishimura *et al* (Nishimura *et al.*, 1971), in which isolation of revertants of a temperature sensitive mutation defective in chromosome replication initiation identified a novel mechanism termed integrative suppression. This type of suppression required the integration of F at a variety of sites on the chromosome and utilization of the F*oriV* in the Hfr strain. Nishimura

et al (1971) proposed that this suppression was in fact due to a take-over of the F replication system of the replication of the entire *E. coli* chromosome, thus the chromosome was an inert piece of DNA replicating as part of the F episome.

The replicator of the *E. coli* chromosome was initially isolated by the detection of F′ plasmids that could replicate in Hfr strains and were thus insensitive to the incombatibility mechanism of the inc gene product. This suggested that F replication was initiating from what was now referred to in the literature with the symbol oriC, the origin of replication of the chromosome (Hiraga, 1976). The chromosome origin was more precisely mapped by ligating restricted genomic DNA to an ampicillin resistance gene (*bla*) and selection of ampicillin resistant transformants. Autonomously replicating plasmid DNA was isolated from *recA*- strains and an integrated copy of ampicillin resistance was detected in *recA*+ strains. The integrated copy of the *bla* gene was used to transductionally map the location of *oriC* at 83 minutes on the genetic map of the *E. coli* chromosome (Yasuda and Hirota, 1977). The position of *oriC* was confirmed by using the specialized transducing

phage λ (Miki *et al.*, 1978;K.Von Meyenburg *et al.*, 1978). When *oriC* was present on λ*asn* it allowed these phages to establish themselves as plasmids or minichromosomes with copy numbers greater than 6 and as high as 15 to 30 per cell (K.Von Meyenburg *et al.*, 1978).

Temperature sensitive mutants defective in DNA replication fall into two classes: mutants that stop DNA synthesis immediately when shifted to a non permissive temperature (elongation defective mutants) or others that appear to finish rounds of replication already in progress but fail to initiate new rounds, termed initiation defective mutants (Tomizawa and Selzer, 1979;Y.Hirota, A.Ryter, and F.Jacob, 1968). The genetic locus of the first characterized mutant from this second class was designated *dnaA* (Wechsler and Gross, 1971). The cloning, sequencing (Y.Hirota *et al.*, 1978;Buhk and Messer, 1983;Sugimoto *et al.*, 1979) and a definition of the minimal sequence (245 base pairs) required for autonomous replication of *E. coli oriC* (Oka *et al.*, 1980) as well as the cloning and sequencing of *dnaA* (Hansen and von Meyenburg, 1979;Hansen, Hansen, and von Meyenburg, 1982) now allowed for an *in vitro* biochemical dissection of the

specific initiation reaction occurring at the origin with the DnaA initiator protein and was not hindered by the large bulk of chromosomal DNA.

Replication Initiation of the *Escherichia coli oriC*

It became clear that a soluble enzyme system specifically recognizes the *E. coli* sequence for initiation of replication and that this initiation event is absolutely dependent on the DnaA protein, RNA polymerase and numerous replication proteins including DnaB and the single-stranded DNA binding protein (Fuller, Kaguni, and Kornberg, 1981). Using this soluble enzyme system it was shown with an electron microscopic study that replication fork movement away from *oriC* preceeds bidirectionally (Kaguni, Fuller, and Kornberg, 1982). Fuller and Kornberg were the first to demonstrate an interaction of DnaA with *oriC* (Fuller and Kornberg, 1983).

The sequence 5'TTAT(C/A)CA(C/A)A is highly conserved within the minimal *oriC* sequence of *E. coli* (appearing four times in the right boundary) and in other chromosome origins from Enterobacteriaceae (*Salmonella typhimurium*,

Enterobacter aerogenes, Klebsiella pneumoniae and *Erwinia carotovora*)(Zyskind *et al.*, 1983). This 9-mer sequence appears in the origin of replication of P1 phage and plasmids ColE1, pSC101, pBR322, CloDF13, R1, R100 and F (Fuller, Funnell, and Kornberg, 1984) and is also found at the outer end of the IRL segment of the transposon Tn5 in a region required for transposition of Tn5 (Johnson and Reznikoff, 1983).

Binding of DnaA to these conserved regions (now called DnaA boxes) was shown with a fragment retention assay and DNase I Footprinting (Fuller, Funnell, and Kornberg, 1984). It has been suggested that 20 to 40 DnaA protein monomers are required for an optimal reaction with one *oriC* plasmid molecule and a cooperative interaction between protein monomers generates a massive DnaA protein-*oriC* complex (Fuller, Funnell, and Kornberg, 1984). Bramhill and Kornberg (Bramhill and Kornberg, 1988b) used purified enzymes to replicate supercoiled *oriC* templates and were able to divide the sequence of events that proceed DNA synthesis into four stages: first, the cooperative binding of DnaA to *oriC;* second, incorporation of DnaB and DnaC into a prepriming complex; third, the generation of two forks by the bi-directional unwinding by DnaB; and fourth,

priming by DnaG primase for chain elongation by DNA polymerase III holoenzyme (Kaguni and Kornberg, 1984). Due to their sensitivity to the single strand-specific nuclease P1, three tandemly repeated A/T rich 13-mers (Bramhill and Kornberg, 1988a) in the 60 base pair left boundary of *oriC* were implicated as the sites of duplex opening after binding of DnaA (Bramhill and Kornberg, 1988b). These 13-mers are also the sites to which IciA, the inhibitor of chromosome replication initiation binds (Hwang and Kornberg, 1990;Thony *et al.*, 1991). The model of replication initiation proposed by Bramhill and Kornberg (Bramhill and Kornberg, 1988a) postulates that DnaA performs three crucial roles: 1) it binds tightly to 9-mer repeats to form an initial complex, 2) it successively melts three AT-rich 13-mer repeats to form an open complex and 3) it guides the DnaB-DnaC complex into this melted region to form a prepriming complex marking the future forks of bidirectional replication.

The precise organization of DnaA boxes has to be maintained on the correct side of the helix for *oriC* to function because insertion mutations that change the distance between DnaA boxes (except for insertions or deletions between DnaA boxes R3 and R4 of exactly one

helical turn) inactivate the origin (Woelker and Messer, 1993). The organization of DnaA boxes in the nucleoprotein structure has been hypothesized to be such that all DnaA boxes are oriented in the same direction. In this nucleoprotein structure (*oriC* wrapped around many DnaA monomers) binding sites for Integration Host Factor (IHF) and FIS (factor for inversion stimulation) are positioned in areas requiring DNA bending (Gille *et al.*, 1991;Hwang and Kornberg, 1992). FIS interacts with *oriC* with the highest affinity site lying between DnaA boxes R2 and R3 (Gille and Messer, 1991;Filutowicz *et al.*, 1992) Stable maintenance of *oriC* plasmids and duplex opening requires negatively supercoiled DNA and either the HU or IHF proteins (Kano *et al.*, 1991;Bramhill and Kornberg, 1988b). It has been proposed that appropriate levels of FIS, IHF and DnaA are required for the timely assembly of the pre-replication complex and thus ultimately the timing of origin unwinding (Ryan *et al.*, 2004).

OriC contains another noteworthy feature, 11 GATC sites are present surrounding *oriC* and these are sites methylated by the *E. coli* DNA adenine methylase (Dam). These sites are required for the appropriate timing of replication initiation. The synchrony of initiation on

multiple chromosomes present in rapidly growing cells is altered in *dam-* cells (Bakker and Smith, 1989;Boye and Lobner-Olesen, 1990;Smith *et al.*, 1985). Hemimethylated DNA exists in the wake of the replication forks and is quickly sequestered to the membrane immediately following duplication (Landoulsi *et al.*, 1990) by the SeqA protein (Lu *et al.*, 1994;von Freiesleben, Rasmussen, and Schaechter, 1994;Shakibai *et al.*, 1998). However, sequesteration of *oriC* by Dam methylation is restricted to Enterobacteriaceae.

Principles of replication initiation deduced from studying the *E. coli oriC* and *dnaA* have shed light on all eubacterial replication systems. However, it is still worthwhile to compare and contrast chromosome replication initiation in other well characterized replication systems such as those from *Bacillus subtilis*, and *Caulobacter crescentus* because the models of replication initiation at these chromosome origins are very different from *E. coli*.

The *Bacillus subtilis* Chromosomal Origin of Replication

Attempts to isolate the origin of replication from the Gram positive *B. subtilis* chromosome was ongoing well

after the *E. coli oriC* had been cloned and partially characterized. All attempts to isolate an autonomously replicating sequence from the chromosome via a shot-gun approach had failed. However, a sequence was found on the chromosome that inhibits replication of plasmids in *B. subtilis* by acting in a cis-configuration (Seiki, Ogasawara, and Yoshikawa, 1981). The sequence and the limits of the segment required for this inhibition were mapped to within 500 base pairs (Seiki, Ogasawara, and Yoshikawa, 1982). The site of replication initiation was determined by localizing the first-replicating DNA strands on the chromosome using molecular hybridization techniques (Ogasawara, Mizumoto, and Yoshikawa, 1984). Approximately 10 kb in the *oriC* region was sequenced and it was found to contain the *dnaA* gene with predicted DnaA boxes both upstream and downstream of the putative replication initiation gene (Moriya, Ogasawara, and Yoshikawa, 1985).

At this time there was no direct evidence for involvement of either the *dnaA* gene or the DnaA boxes in initiation of chromosomal replication in *B. subtilis* due to the lack of *dnaA* mutations and the failure to isolate autonomously replicating *oriC* minichromosomes such as in *E. coli*. Moriya *et al* (Moriya *et al.*, 1988) provided the

first genetic evidence that DnaA boxes were responsible for the strong incompatibility detected when the regions flanking *dnaA* were cloned onto replicating plasmids by isolating mutations in DnaA boxes that alleviated this growth inhibition. The increased copy number of DnaA boxes was presumed to influence replication initiation of the chromosome by competing for DnaA. The DnaA boxes were further divided into three regions: six DnaA boxes are present upstream of the *dnaA* promoter, eight DnaA boxes are located between the promoter and the *dnaA* coding region, and seven binding sites are present downstream of *dnaA*. These regions were termed *incA*, *incB* and *incC* respectively.

The *B. subtilis* DnaA protein was purified and used to confirm that the replication initiator was interacting with all three of the incompatibility clusters and that there was a good correlation between the degree of growth inhibition exerted by the inc regions with the strength that DnaA bound to the various DnaA boxes (Fukuoka *et al.*, 1990). Two approaches were used to obtain an autonomously replicating sequence from *B. subtilis*: one used vectors that contained an antibiotic resistance marker which was selective at one copy per cell (Cm), and the second was to clone large fragments with all three inc regions (Moriya *et*

al., 1992). These approaches were successful and after a decade of trying an autonomously replicating plasmid was finally isolated from *B. subtilis*. The *oriC* minichromosome required DnaA boxes both upstream and downstream of the *dnaA* gene (most of which could be removed), was unstable, and had a copy number of approximately one per cell. Characterization of the unique structure of the *B. subtilis oriC* now made it possible to construct an in vitro replication system using soluble cell extracts (Moriya *et al.*, 1994) and these assays revealed that replication initiated in the downstream DnaA box region. This result was corroborated with the use of a two-dimensional gel method confirming that replication initiation occurs in the downstream DnaA box cluster on the *B. subtilis* chromosome *in vivo* (Moriya and Ogasawara, 1996).

The Caulobacter crecentus Origin of Replication (*Cori*)

The Gram negative alpha-proteobacterium *Caulobacter crescentus* exhibits asymmetric control of DNA replication in its progeny cells. Each asymmetric cell division yields a motile swarmer cell and a stalked cell,

however, replication only occurs in the stalked cell. Direct isolation of the *C. crescentus* chromosome origin was accomplished by preparative-scale pulse field gel electrophoresis. The cloned origin has a minimal size of 430 base pairs and was shown to support autonomous replication in stalked cells but not in swarmer cells. Thus the plasmid borne origin responds to the same cell cycle signals as the chromosome (Marczynski and Shapiro, 1992). *Cori* was later validated as the chromosome replication origin with a two-dimensional DNA neutral/neutral gel electrophoresis technique which mapped the origin to a 1.6 kb BamHI fragment spanning the *hemE* - *RP001* intergenic region (Brassinga and Marczynski, 2001) and confirmed that *Cori* is a bidirectionally replicating bacterial origin. Interestingly, a cluster of binding sites for the essential replication initiator DnaA (Gorbatyuk and Marczynski, 2001) does not exist, rather only a single DnaA box (TGATCCACA) appears to be essential for *Cori* replication (Marczynski and Shapiro, 1992).

Cori contains other significant features: a weak promoter directing most of the HemE protein synthesis, a strong promoter that is a regulatory replication element (Marczynski, Lentine, and Shapiro, 1995), novel 8-mer

elements, an exceptionally A/T rich region, an integration host factor (IHF) binding site and five binding sites for the global cell cycle regulator CtrA. The genetic organization around the *C. crescentus* origin (Brassinga, Siam, and Marczynski, 2001) and the binding sites for both IHF and CtrA are also present in the *Rickettsia prowazekii* chromosomal origin of replication (Brassinga *et al.*, 2002).

CtrA is classified as a global cell-cycle regulator because it controls 26% of the transcripts that vary during the cell-cycle in *C. crescentus* (Laub *et al.*, 2000). CtrA is a link between replication and cell division because it represses the strong *Cori* promoter (required for replication) in swarmer cells and controlls expression of essential cell division genes such as *ftsZ* (Kelly *et al.*, 1998). *Brucella abortus*, another alpha-proteobacterium, also encodes a CtrA homologue that has been shown to bind promoter elements in the *minCDE* cell division operon (Bellefontaine *et al.*, 2002). *C. crescentus* CtrA autoregulates its own expression (Domian, Reisenauer, and Shapiro, 1999) and also binds the *S. meliloti ctrA* promoter in vitro (Barnett *et al.*, 2001a). CtrA is subject to phosphorylation (like all response regulators) and this increases the affinity of the protein for its DNA target

10-50 fold (Siam and Marczynski, 2000). It has been proposed that the origin of replication on the circular *C. crescentus* chromosome is the first place to bind CtrA in the entire genome and is likely the last place to release CtrA (Marczynski and Shapiro, 2002).

The Initiator of Chromosome Replication DnaA

The literature regarding the *E. coli* replication initiator DnaA (52 kDa) is vast and has been reviewed on several occasions (Skarstad and Boye, 1994;Messer, 2002;Kaguni, 1997;Messer *et al.*, 2001). The DnaA protein has been functionally divided into four domains. The N-terminus of the protein (domain 1) is required for DnaA-DnaA interactions and for the interaction with the replicative helicase DnaB (Weigel *et al.*, 1999;Seitz, Weigel, and Messer, 2000). Domain 2 appears to be relatively variable in size amongst DnaA proteins and it has been suggested that these residues function as a flexible linker (Messer, 2002). Domain 3 contains a characteristic P loop (Walker A motif) which is found in AAA+ ATPase type proteins (Saraste, Sibbald, and

Wittinghofer, 1990), and a second region that interacts with DnaB (Marszalek *et al.*, 1996). The DNA binding activity of DnaA is encoded in the C-terminus of the protein (domain 4) and mutations in this domain have been isolated that prevent DNA binding (Blaesing *et al.*, 2000). Recently the crystal structure of domain four of *E. coli* DnaA has been solved (Fujikawa *et al.*, 2003).

DnaA binds ATP and ADP with high affinity (K_D of 30 nm and 1 μM respectively)(Sekimizu, Bramhill, and Kornberg, 1987) however only the ATP nucleotide bound form is active in replication (Yung, Crooke, and Kornberg, 1990). It is the oscillations in the various forms of DnaA that are speculated to coordinate the precise timing of DNA replication initiation (Kurokawa *et al.*, 1999). The intrinsic DNA dependent ATPase activity of DnaA is activated following replication initiation and this activity is stimulated by the sliding clamp, a ring shaped dimmer of the β subunit of DNA polymerase III (Katayama *et al.*, 1998) and requires the Hda protein (Kato and Katayama, 2001). Hda has been shown to directly interact with the β subunit of DNA polymerase III (Su'etsugu *et al.*, 2004). This regulation of the nucleotide bound forms of DnaA has

been termed RIDA for regulatory inactivation of DnaA (Katayama *et al.*, 1998).

The abundance of the DnaA protein in the cell is also tightly regulated and *dnaA* gene expression is correlated with the initiation mass which is defined as the cell mass at the time of initiation divided by the number of origins to be initiated (Lobner-Olesen *et al.*, 1989). The initiation mass is also affected by the introduction of plasmids with high affinity DnaA binding sites (Christensen, Atlung, and Hansen, 1999). Several mechanisms are exploited to regulate the availability of DnaA to participate in an initiation reaction (Katayama, Fujimitsu, and Ogawa, 2001). The *dnaA* gene is autoregulated (Atlung, Clausen, and Hansen, 1985;Braun, O'Day, and Wright, 1985) by binding of the protein to a DnaA box located between the two *dnaA* promoters. The *dnaA* promoter is also controlled through an interaction with the membrane because along with *oriC,* the hemimethylated *dnaA* promoter remains sequestered at the membrane for an average of four fold longer than any other region on the *E. coli* chromosome (Campbell and Kleckner, 1990). There are three hundred predicted DnaA boxes in the *E. coli* genome but one sequence called *datA* that encodes five high affinity DnaA

boxes is capable of cooperatively binding approximately 370 DnaA molecules (Kitagawa *et al.*, 1996). Mutations in this region alter the control of initiation (Ogawa *et al.*, 2002) and it has been proposed that this is a mechanism to prevent binding of DnaA to the weakest affinity binding site in the origin (R3), the only DnaA box that is not protected by DnaA throughout the cell-cycle until the replication initiation event (Samitt *et al.*, 1989).

Protein localization in the bacterial cell through out the cell cycle is of great interest. It is estimated that there is approximately 800 - 2000 DnaA molecules per *E. coli* cell and DnaA can be recovered in the particulate (membrane) fractions of non-overproducing strains (Sekimizu, Yung, and Kornberg, 1988). Experiments have implicated acidic phospholipids in a fluid bilayer in the presence of ATP, *oriC* DNA and the replication enzymes in promoting an exchange of ADP-DnaA to ATP-DnaA bound to DnaA (Sekimizu and Kornberg, 1988;Crooke, Castuma, and Kornberg, 1992). Mutations have been made in DnaA that prevent the functional interaction with phospolipids and map to domain three of the protein (Hase *et al.*, 1998). In whole cells, immunogold electron microscopy and immunofluoresecence microscopy eventually localized DnaA at the membrane and it

has been speculated that by being localized to the membrane the DnaA protein itself may play a role in directing proper chromosome inheritance (Newman and Crooke, 2000).

A comprehensive mutational analysis of DnaA boxes in *oriC* revealed the essential sequence and sequence context for effective replication initiation at the origin (Langer *et al.*, 1996). The most stringent definition of a DnaA box has been proposed by Schaper and Messer (Schaper and Messer, 1995) by determining binding constraints: (5′ T T A/T N C A C A 3′). It was noted that DnaA induces a 40° bend in the DNA upon binding and that the primary sequence is not sufficient to predict binding sites because the sequence context of DnaA boxes appears to be important for interaction with the replication initiator. Thus experimental evidence must be provided as proof that predicted DnaA binding sites are biologically relevant (Schaper and Messer, 1995). In vitro DNase I footprinting experiments using DnaA and *oriC* DNA grants a more relaxed consensus binding site (5′ T T/C A/T T A/C C A C/A A 3′)(Fuller, Funnell, and Kornberg, 1984). This consensus was further expanded based on an *in vivo* transcriptional termination assay where bound DnaA was used to block

transcribing RNA polymerase (5′ T/C T/C T/A/C T A/C C A/G A/C/T A/C 3′)(Schaefer and Messer, 1991). Recently, two DnaA boxes have been identified in *oriC* that preferentially bind DnaA-ATP and are required for *in vivo* strand opening that do not match either of these consensus sequences (McGarry *et al.*, 2004).

As well as the role of DnaA boxes in replication initiation at *oriC,* DnaA boxes have been found in promoter elements other than the *dnaA* promoter and these sites have been proven biologically relevant. Thus DnaA acts as a transcription factor in addition to its role in replication initiation. Transcriptional repression of various genes (*dnaA, mioC, rpoH, uvrB* and *proS*) and transcriptioanl activation of *nrd, glpD* and *fliC* has been reported (Messer and Weigel, 1997). The relevance of putative DnaA boxes found upstream of a number of open reading frames requires analysis and the mechanism by which DnaA influences gene expression remains to be elucidated.

Chromosome Segregation

The physical separation of daughter chromosomes must be efficiently cooridinated with cell division to ensure

that new born cells receive the full complement of genetic material. *E. coli oriC* plasmids have an average copy number of 38 copies per cell and have a loss frequency of 2 x 10^{-2} to 4 x 10^{-2}. This high loss frequency may be because of competetion of the minichromosomes for host replication factors. However, these minichromosomes can be stabilized by the *sop* genes of the F plasmid (required for efficient partitioning of F plasmids during cell division) to a loss frequency of 10^{-3} and thus it is speculated that the instability of *oriC* plasmids is due to occasional segregational failure rather than an incompatibility mechanism (Lobner-Olesen, Atlung, and Rasmussen, 1987).

In new born *E. coli* cells the *oriC* segment is localized at a nucleoid border and the replication terminus at an opposite nucleoid border (Niki and Hiraga, 1998). However, minichromosomes are localized randomly throughout the cytoplasm and when the *sopABC* segment of the F plasmid is cloned onto the *oriC* plasmids these molecules are localized at mid-cell or both quarter positions (Niki and Hiraga, 1999). Using a fluorescent *in situ* hybridization approach Niki and Hiraga (1999) were able to show that *E. coli oriC* plasmids were partitioned towards daughter cells randomly resulting in uneven segregation. They concluded

that *E. coli* minichromosomes do not include a cis-acting site essential for bipolar migration of the *E. coli oriC* region and that because *oriC* plasmids carrying the *sopABC* segment were always localized at specific cellular sites they are actively partitioned into daughter cells.

A possible mechanism that determines chromosome polarity comes from an observation in *B. subtilis* in which proteins such as PolC are localized to the middle of the bacterial cell suggesting a factory model of DNA replication in which there is a stationary replisome (Lemon and Grossman, 1998). This model predicts that the physical movement of DNA through this replication factory is responsible for the migration of newly replicated origins to the cell poles. However, the *E. coli oriC* is still localized to the cell poles when DNA replication initiates at other sites on the chromosome (in Hfr strains) and the core origin sequence is not sufficient for this localization (Gordon, Shivers, and Wright, 2002). To date, no specific sequences nor interacting proteins have been identified to explain this phenomenon. This observation does suggest that there is an origin-localizing factor that is independent of the directional force derived from DNA replication (Gordon, Shivers, and Wright, 2002).

The phenomenon of *oriC* partitioning to the cell poles is not restricted to *E. coli*, this effect has also been detected in *B. subtilis* and *C. crescentus* (Glaser *et al.*, 1997;Jensen and Shapiro, 1999). The SpoOJ protein colocalizes with the *B. subtilis oriC* sequence, binds to multiple sequences adjacent to *oriC* however normal migration of *oriC* was detected in *spoOJ* mutants (Lin and Grossman, 1998). Two sequences within the *oriC* have been implicated for accurate segregation of the *B. subtilis* chromosome: the 3′ end of the *dnaA* gene and the A/T rich sequence in the *dnaA* - *dnaN* intergenic region (site of strand opening at *oriC*)(Kadoya *et al.*, 2002). Proteins that interact with these sequences have yet to be identified.

In *C. crescentus* the operon encoding the chromosome partition proteins *parAB* is located within 80 kb of the origin of replication. Immediately downstream of *parB* is an A/T rich segment that serves as a binding site for the ParB protein, termed *parS* (Mohl and Gober, 1997). The spatial distribution of ParB is coupled to cell cycle events and approximates the movement of newly replicated chromosomes towards the cell poles. Increased levels of ParA and ParB in *C. crescentus* cells increase the number of

anucleate cells and therefore is likely partly responsible for effective chromosome segreation during cellular division. Strains with *parB* null mutations undergo a block in cell division by blocking the assembly of the FtsZ ring and thus the accumulation of filamentous cells (Mohl, Easter, Jr., and Gober, 2001). Therefore it has been proposed that *parAB* may not only function in chromosome partitioning but their major role may be regulators of cell division. The *parAB* genes of *S. meliloti* are situated approximately 6 kb from the chromosomal origin of replication reported in this work and the location of the the ParB binding site (*parS*) remains to be determined.

This Work

Several strategies have been utilized to detect ARS from a bacterial genome. Many ARS have been detected for their ability to confer autonomous replication of a ColE1 plasmid in an *E. coli polA* mutant such as the chromosome origins from *Enterobacter aerogenes, Klebsiella pneumoniae* (Harding *et al.*, 1982), *Vibrio harveyi* (Zyskind *et al.*, 1983) and *Erwinia carotovora* (Takeda *et al.*, 1982). An autonomously replicating sequence from *Coxiella burnetii*

was also isolated in this fashion, however, 2D-gel electrophoresis did not confirm that it was the chromosome origin and is thus speculated to be an alternative origin under cetain growth conditions (Suhan *et al.*, 1994). This strategy was unsuccessful in the isolation of chromosome origins from *Pseudonmonas aeruginosa* and *Pseudomonas putida* because these chromosome origins do not function in *E. coli* rather they were isolated by selecting for DNA that conferred autonomous replication of a ColE1 plasmid in the host strain (Yee and Smith, 1990). Screening genomic libraries for autonomously replicating sequences localized the chromosome origins discussed above as well as from *Streptomyces lividans* (Zakrzewska-Czerwinska and Schrempf, 1992). Chromosome origins have been defined by identifying conservation of DnaA binding sites in the *dnaA* region from *Mycoplasma capricolum* (Fujita, Yoshikawa, and Ogasawara, 1992), *Micrococcus luteus* (Fujita, Yoshikawa, and Ogasawara, 1990) and *Streptomyces coelicolor* (Calcutt and Schmidt, 1992). Experimental evidence that DnaA interacts with DnaA binding sites in the *dnaA* region has been enough to support the identification of chromosome origins from *Thermus thermophilus* (Schaper *et al.*, 2000) and *Helicobacter pylori* (Zawilak *et al.*, 2001)

The location of the *dnaA* gene in the bacterial genome has aided in the identification of many chromosome origins of replication such as: *B. subtilis* (Moriya *et al.*, 1992), *Micrococcus luteus* (Fujita, Yoshikawa, and Ogasawara, 1990), *Mycobacterium* spp. (Salazar *et al.*, 1996), *Mycoplasma capricolum* (Fujita, Yoshikawa, and Ogasawara, 1992), *Pseudomonas putida* (Smith *et al.*, 1991), *Thermus thermophilus* (Schaper *et al.*, 2000) and *Streptomyces* spp. (Jakimowicz *et al.*, 1998). However, initial attempts to localize the chromosomes origins from *Synechocystis* spp. (Richter and Messer, 1995), *Prochlorococcus marinus* (Richter *et al.*, 1998) and *S. meliloti* (Margolin, Bramhill, and Long, 1995) did not detect autonomously replicating sequences in the vicinity of the *dnaA* gene.

S. meliloti is a Gram negative alpha-proteobacterium that is found free living in the soil or engaged in a symbiotic relationship with alfalfa (*Medicago sativa*). The infection of the root hairs and colonization of the host plant is characterized by the formation of nitrogen fixing nodules in which differentiated bacteroids are able to reduce atmospheric nitrogen to ammonia. The biology of this microorganism as a natural fertilizer has guided most

of the *S. meliloti* research. However, *S. meliloti* is also an interesting organism for the study of DNA replication (MacLellan, Sibley, and Finan, 2004). The genetic material of *S. meliloti* is distributed over three large replicons, a primary chromosome (3.6 Mb), and two megaplasmids; pSymA and pSymB (1.35 Mb and 1.68 Mb respectively)(Galibert *et al.*, 2001). The megaplasmids encode plasmid-like *repABC* replicator regions (Chain *et al.*, 2000) and the chromosome origin has been only predicted (Capela *et al.*, 2001) despite its use in a localization study (Kahng and Shapiro, 2003) without any experimental evidence to support its identification.

This work has identified an autonomously replicating sequence from *S. meliloti* and thus provides the needed experimental evidence to support the site of replication initiation on the *S. meliloti* chromosome. The *oriC* is located in the *hemE-Y02793* region (approximately 400 kb away from *dnaA*) and has been genetically dissected to determine the minimal sequence required for replication of plasmid borne *oriC*. This has revealed that a much larger segment of DNA is required for a functional *oriC* as opposed to that from the other characterized alpha-proteobacterial origin from *C. crescentus*. However commonalities exist

between the two origins such as the presence of 8-mers and the transcriptional coupling of *hemE* (Uroporphyrinogen decarboxlase) with replication of the chromosome.

The copy number of the plasmid autonomously replicating from the cloned *oriC* has been determined to be variable and less than one copy per cell but does not appear to exert a strong incompatibility against the *S. meliloti* genome.

Site-directed muatgenesis on predicted DnaA boxes has determined several essential binding sites in the *hemE-Y02793* intergenic region for the replication of *oriC*. The replication initiator DnaA has been partially purified and used in electrophoretic mobility shift assay to confirm binding of the protein to the predicted DnaA boxes. DnaA boxes have also been mapped in the other two replication origins from the megaplasmids but their role in replication of these large extrachromosomal elements remains unknown. However, it appears that DnaA does bind to the *repA2* promoter on pSymA and may modestly influence its activity. A direct link between DNA replication and cell division has also been shown with an *S. meliloti* strain that grows as complex filaments resulting from constitutive overexpression of DnaA. This work has extended our

knowledge of the bacterial chromosome replication origin and confirms common principles that govern prokaryotic replication initiation.

CHAPTER 2. MATERIALS AND METHODS

MATERIALS

Bacterial strains and growth conditions

S. meliltoi and *Agrobacterium tumefaciens* were grown at 30°C and *E. coli* was grown at 37°C or 30°C. Cultures were inoculated with single colonies that had been streak purified three times on selective media. Usually, 2 ml liquid cultures were grown in 18 mm glass test tubes on a rotating wheel. When larger volumes of culture was required, overnight cultures were subcultured (0.2% vol/vol) into 100 ml of the appropriate broth in a 250 ml Erlenmeyer flask and agitated at 200 rpm in a chest incubator until the desired optical density (OD_{600}) was achieved. Plasmids and the bacterial strains used in this study are listed in Tables 2 and 3.

The complex media Luria-Bertani (LB) contains 10 g tryptone (Difco), 5 g Yeast extract (Difco), and 5 g NaCl per liter of nanopure water. LB was used for growth of *E. coli*, LB supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBmc) was used for growth of *S. meliloti*. Solid media was prepared by the addition of 15 g agar (Difco) to 1 L of LB

before sterilization. All media was sterilized by autoclaving at 15 pounds/square inch at 121°C for 20 min. When required, solid growth media was supplemented with antibiotics to the concentrations listed in Table 1.

Table 1. Concentrations of antibiotics used in solid media

Antibiotic	Concentration in LB agar for *E. coli* (μg/ml)	Concentration in LB agar for *S. meliloti* (μg/ml)	Stock Concentration
Na-Ampicillin (Ap)	100		100 mg/ml in ddH_2O
Chloramphenicol (Cm)	10		20 mg/ml in 50% ethanol
Gentamicin sulphate (Gm)	10	40 - 60	10 mg/ml in ddH_2O
Neomycin sulphate (Nm)		200	100 mg/ml in ddH_2O
Rifampicin (Rf)	10	20	20 mg/ml 100% ethanol
Streptomycin sulphate (Sm)		200	200 mg/ml in ddH_2O
Spectinomycin dihydrochloride (Sp)		200	20 mg/ml in ddH_2O
Tetracycline hydrochloride (Tc)	10	5	5 mg/ml in 50% ethanol

All antibiotic concentrations were halved for liquid media. Antibiotics were filter sterilized through a 0.2 μm filter if prepared in ddH_2O and were stored at -20°C. To screen for loss of β-galactosidase activity upon ligation of insert DNA into the *lacZα* gene of plasmids pUC119, pUCP30T and pBBR1MCS-5 solid media was supplemented with 20 μg/ml 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-Gal).

Table 2. Plasmids used in this study

Plasmid	Genotype or Characteristics	Source or Reference
pBADHisA	Ap^R, His_6 tag expression vector, inducible with L-arabinose	Invitrogen
pGEX-5x-1	Ap^R, GST tag expression vector, inducible with IPTG	Pharmacia
pET431a	Ap^R, NusAHis6 tag expression vector, inducible with IPTG	Novagen
pRK600	pRK2013 npt::Tn9; Cm^R	(Finan *et al.*, 1986)
pBBR1MCS-5	Gm^R broad host range cloning vector, *LacZα peptide*	(Kovach *et al.*, 1995)
pOT1	Gm^R promoterless *gfp* reporter plasmid, pBBR1MCS5 derivative	(Allaway *et al.*, 2001)
pUC119	Ap^R ColE1 *oriV* cloning vector, *LacZα* peptide	(Vieira and Messing, 1987)
pFUS1	Tc^R promoterless *gusA* reporter plasmid, pMP220 derivative	(Reeve *et al.*, 1999)
pUCP30T	Gm^R ColEI *oriV* cloning vector, oriT	(Schweizer, T.R.Klassen, and T.Hoang, 1996)
pMB439	Ap^R, Sp^R, cloning vector, oriT, pBluescript derivative	(Barnett, Oke, and Long, 2000)
pTH760	pBADHisA with *dnaA* (AB23165-AB23166) cloned via *SacI/HindIII*	This work
pTH798	pGEX-5X-1 with dnaA (AB24859-AB24860) cloned via *EcoRI/BamHI*	This work
pTH838	pUCP30T with 3 kb *oriC* (AB24853-AB24854) cloned via	This work

	EcoRI, *hemE* same orientation as P*lac*	
pTH879	710 bp *HindIII/SalI oriC* fragment cloned into pUCP30T	This work
pTH880	875 bp *BamHI/SalI oriC* fragment cloned into pUCP30T	This work
pTH881	861 bp *SacII oriC* fragment cloned into pMB439, P*hemE* same orientation as T3 promoter	This work
pTH882	861 bp *SacII oriC* fragment cloned into pMB439, P*hemE* same orientation as T7 promoter	This work
pTH1044	0.5 kb internal *pstS EcoRI* fragment in pUCP30T	Y. Zechun
pTH1081	pET431a with *dnaA* (AB26340-AB26341)cloned via *BamHI/EcoRI*	This work
pTH1091	PBBR1MCS-5 with *dnaA* and ribosome binding site (AB29744-AB29675) cloned via *XbaI/SacI*, same orientation as P*lac*	This work
pTH1145	pUCP30T with *SmaI* to *HindIII* sites removed in the MCS, white on Xgal	This work
pTH1146	pFUS1 with 147 bp *dnaA* promoter fragment (dnaA-147S - dnaA32AS) cloned via *HindIII/EcoRI*	This work
pTH1147	pFUS1 with 200 bp *repA2* promoter fragment cloned via *HindIII/EcoRI* (pSymA-200S - AB27528)	This work
pTH1167	pUCP30T with 2.9 kb *oriC* (AB24854-AB30425) cloned via *EcoRI*, *hemE* same orientation as P*lac*	This work
pTH1168	pUC119 with 416 bp *HindIII/SmaI* fragment from pTH1167 with 4bp deletion in XmaIII site	This work
pTH1173	pTH1145 with 2.9 kb *oriC* (AB24854-AB30425) cloned via *EcoRI*	This work
pTH1174	pET431a with *ctrA* (AB30798-AB30423) cloned via *SmaI/EcoRI*, maintained thrombin recognition sequence	This work
pTH1177	pTH1173 with 416 bp *HindIII/SmaI* fragment replaced with 412bp *HindIII/SmaI* fragment from pTH1168	This work
pTH1178	pET431a with *ctrA* (AB30799-AB30800) cloned via *EcoRI/XhoI*	This work
pTH1179	pUCP30T with 2.9 kb (AB24854-AB30425) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1186	pUCP30T with 2.9 kb *oriC* from pTH1177 cloned via *EcoRI*,	This work

	hemE same orientation as P*lac*	
pTH1187	pUCP30T with 2.9 kb *oriC* from pTH1177 cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	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 *hemE-Y02793* intergenic reigon (AB32323-AB32324) cloned via *EcoRI*	This work
pTH1253	pTH838 with 4 bp deletion in DnaA box #4 TTATCAACA → CAACA, created via site-directed mutagenesis (AB32750/AB32751)	This work
pTH1265	pBBR1MCS5 with intergenic PCR product (AB32323-AB32324) from pTH838 template cloned via *EcoRI*	This work
pTH1266	pBBR1MCS5 with intergenic PCR product (AB32323-AB32324) from pTH1186 template cloned via *EcoRI*	This work
pTH1267	pBBR1MCS5 with intergenic PCR product (AB32323-AB32324) from pTH1206 template cloned via *EcoRI*	This work
pTH1268	pBBR1MCS5 with intergenic PCR product (AB32323-AB32324) from pTH1207 template cloned via *EcoRI*	This work
pTH1270	pBBR1MCS5 with intergenic PCR product (AB32323-AB32324) from pTH1253 template cloned via *EcoRI*	This work
pTH1281	pFUS1 with 500 bp *BamHI/KpnI* fragment from pTH1265 cloned into BglII/KpnI, *Y02793* promoter upstream of *gusA*	This work
pTH1282	pFUS1 with 500 bp *HindIII/BamHI* fragment from pTH1265	This work

	cloned into *BglII/HindIII*, *hemE* promoter upstream of *gusA*	
pTH1283	pFUS1 with 500 bp *BamHI/KpnI* fragment from pTH1266 cloned into *BglII/KpnI*, *hemE* promoter upstream of *gusA*	This work
pTH1284	pFUS1 with 500 bp *HindIII/BamHI* fragment from pTH1266 cloned into *BglII/HindIII*, *Y02793* promoter upstream of *gusA*	This work
pTH1285	pFUS1 with 500 bp *BamHI/KpnI* fragment from pTH1267 cloned into *BglII/KpnI*, *Y02793* promoter upstream of *gusA*	This work
pTH1286	pFUS1 with 500 bp *HindIII/BamHI* fragment from pTH1267 cloned into *BglII/HindIII*, *hemE* promoter upstream of *gusA*	This work
pTH1287	pFUS1 with 500 bp *BamHI/KpnI* fragment from pTH1268 cloned into *BglII/KpnI*, *hemE* promoter upstream of *gusA*	This work
pTH1288	pFUS1 with 500 bp *HindIII/BamHI* fragment from pTH1268 cloned into *BglII/HindIII*, *Y02793* promoter upstream of *gusA*	This work
pTH1289	pFUS1 with 500 bp *BamHI/KpnI* fragment from pTH1270 cloned into *BglII/KpnI*, *Y02793* promoter upstream of *gusA*	This work
pTH1290	pFUS1 with 500 bp *HindIII/BamHI* fragment from pTH1270 cloned into *BglII/HindIII*, *hemE* promoter upstream of *gusA*	This work
pTH1342	pTH1207 with 4 bp deletion in DnaA box #2, created via site-directed mutagenesis (AB31449/AB31450)	This work
pTH1347	pTH1342 with 4 bp deletion in DnaA box #4, created via site-directed mutagenesis (AB32750/AB32751)	This work
pTH1416	pUCP30T with 2289 bp *oriC* (ML1817-ML1818) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1442	pUCP30T with 2472 bp *oriC* (ML1817-AB24853) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1443	pUCP30T with 2823 bp *oriC* (ML1818-AB24854) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1444	pUCP30T with 2823 bp *oriC* (ML1818-AB24854) cloned via *EcoRI*, *hemE* same orientation to P*lac*	This work

pTH1451	pUCP30T with 2402 bp *oriC* (ML2444-ML1818) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1452	pUCP30T with 2421 bp *oriC* (ML2443-ML1818) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1453	pUCP30T with 1783 bp *oriC* (ML2444-ML2445) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1454	pUCP30T with 1802 bp *oriC* (ML-2443-ML2445) cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1465	pOT1 with 518 bp *repA2* promoter (ML2877-ML2879) driving *gfp* expression cloned via *XbaI/HindIII*	S. MacLellan
pTH1472	pBBR1MCS5 with 3 kb *oriC* from pTH1186 cloned via *EcoRI*, *hemE* opposite orientation to Plac	This work
pTH1474	pBBR1MCS5 with 3 kb *oriC* from pTH1206 cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1475	pBBR1MCS5 with 3 kb *oriC* from pTH1207 cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1476	pBBR1MCS5 with 3 kb *oriC* from pTH1253 cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1514	pOT1 with 800 bp *dnaA* promoter (ML3257-ML3258) cloned as a *HindIII/SalI* fragment into *HindIII/XhoI*	This work
pTH1518	pTH838 with 4 bp deletion in DnaA box #5 TGATCCACA → TCACA, created via site-directed mutagenesis (ML3852/ML3853)	This work
pTH1527	pTH1465 with 4 bp deletion in DnaA box in *repA2* promoter TCATCCACA → TCACA, created via site-directed mutagenesis (ML3854/ML3855)	This work
pTH1562	pBBR1MCS5 with 3 kb *oriC* from pTH838 cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work
pTH1563	pBBR1MCS5 with 3 kb *oriC* from pTH1205 cloned via *EcoRI*, *hemE* opposite orientation to P*lac*	This work

Table 3. Bacterial strains used in this study

Strain	Genotype	Reference
DH5α	F^-, *end*A1, *hsd*R17 (r_K^-, m_K^-), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*ZYA) U169, Φ80d*lac*Z, ΔM15	BRL Inc.
MT616	MM294A recA-56 (pRK600)	(Finan *et al.*, 1986)
MT620	MM294A recA-56 Rf^R	T. M Finan
TOP10	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G	Invitrogen
BL21 Codon Plus RIL	F^- *omp*T *hsd*S($r_B^-m_B^-$) *dcm*+ TetR *gal* *end*A Hte [*arg*U *ile*Y *leu*W CmR]	Stratagene
BL21 STAR	F^- *omp*T *hsd*S B ($r_B^-m_B^-$) *gal* *dcm* *rne*131 (DE3)	Stratagene
J243	DH5α (pBBR1MCS5)	Strain collection
J252	DH5α (pUCP30T)	Strain collection
J525	DH5α (pFUS1)	Strain collection
J668	TOP 10 (pTH760)	This work
J710	BL21 Codon Plus RIL (pGEX-5X-1)	This work
J763	DH5α (pTH838)	This work
J803	DH5α (pTH879)	This work
J804	DH5α (pTH880)	This work
J805	DH5α (pTH881)	This work

J806	DH5α (pTH882)	This work
J819	BL21 Codon Plus RIL (pTH798)	This work
J1026	BL21 STAR (pET431a)	This work
J1027	BL21 STAR (pTH1081)	This work
J1037	DH5α (pTH1091)	This work
J1090	DH5α (pTH1145)	This work
J1091	DH5α (pTH1146)	This work
J1092	DH5α (pTH1147)	This work
K20	DH5α (pTH1167)	This work
M1	DH5α (pTH1168)	This work
M6	DH5α (pTH1173)	This work
M10	DH5α (pTH1177)	This work
M11	BL21 STAR (pTH1174)	This work
M12	BL21 STAR (pTH1178)	This work
M13	DH5α (pTH1179)	This work
M24	DH5α (pTH1186)	This work
M25	DH5α (pTH1187)	This work
M41	DH5α (pTH1205)	This work
M42	DH5α (pTH1206)	This work
M43	DH5α (pTH1207)	This work
M92	DH5α (pTH1245)	This work
M103	DH5α (pTH1253)	This work
M116	DH5α (pTH1265)	This work
M117	DH5α (pTH1266)	This work
M118	DH5α (pTH1267)	This work
M119	DH5α (pTH1268)	This work
M121	DH5α (pTH1270)	This work
M135	DH5α (pTH1281)	This work

M136	DH5α (pTH1282)	This work
M137	DH5α (pTH1283)	This work
M138	DH5α (pTH1284)	This work
M139	DH5α (pTH1285)	This work
M140	DH5α (pTH1286)	This work
M141	DH5α (pTH1287)	This work
M142	DH5α (pTH1288)	This work
M143	DH5α (pTH1289)	This work
M144	DH5α (pTH1290)	This work
M195	DH5α (pTH1342)	This work
M202	DH5α (pTH1347)	This work
M288	DH5α (pTH1416)	This work
M325	DH5α (pTH1442)	This work
M326	DH5α (pTH1443)	This work
M327	DH5α (pTH1444)	This work
M334	DH5α (pTH1451)	This work
M335	DH5α (pTH1452)	This work
M336	DH5α (pTH1453)	This work
M337	DH5α (pTH1454)	This work
M349	DH5α (pTH1465)	This work
M356	DH5α (pTH1472)	This work
M358	DH5α (pTH1474)	This work
M359	DH5α (pTH1475)	This work
M360	DH5α (pTH1476)	This work
M401	DH5α (pTH1514)	This work
M405	DH5α (pTH1518)	This work
M415	DH5α (pTH1527)	This work

M448	DH5α (pTH1562)	This work
M449	DH5α (pTH1563)	This work
Rm1021	SU47 str-27	(Meade *et al.*, 1982)
Rm5004	Rm1021 *recA*::Tn5	T. M Finan
K476	Rm1021 (pBBR1MCS5)	This work
K569	G212 Φ pTH1044	Y. Zechun
K726	Rm1021 (pTH1281)	This work
K727	Rm1021 (pTH1282)	This work
K728	Rm1021 (pTH1283)	This work
K729	Rm1021 (pTH1284)	This work
K730	Rm1021 (pTH1285)	This work
K731	Rm1021 (pTH1286)	This work
K732	Rm1021 (pTH1287)	This work
K733	Rm1021 (pTH1288)	This work
K734	Rm1021 (pTH1289)	This work
K735	Rm1021 (pTH1290)	This work
K762	Rm1021 (pTH1091)	This work
K801	Rm5004 (pFUS1)	This work
K943	Rm5004 (pTH1472)	This work
K945	Rm5004 (pTH1474)	This work
K946	Rm5004 (pTH1475)	This work
K947	Rm5004 (pTH1476)	This work
K962	Rm5004 (pTH1281)	This work
K963	Rm5004 (pTH1282)	This work
K964	Rm5004 (pTH1283)	This work
K965	Rm5004 (pTH1284)	This work
K966	Rm5004 (pTH1285)	This work
K967	Rm5004 (pTH1286)	This work
K968	Rm5004 (pTH1287)	This work
K969	Rm5004 (pTH1288)	This work

K970	Rm5004 (pTH1289)	This work
K971	Rm5004 (pTH1290)	This work
K972	Rm5004 (pTH1265)	This work
K973	Rm5004 (pTH1266)	This work
K974	Rm5004 (pTH1267)	This work
K975	Rm5004 (pTH1268)	This work
K976	Rm5004 (pTH1270)	This work
K1009	Rm5004 (pBBR1MCS5)	This work
	Rm1021 (pFUS1)	This work
K1010	Rm5004 (pTH1562)	This work
K1011	Rm5004 (pTH1563)	This work
K1012	Rm5004 (pTH838) small colony	This work
K1013	Rm5004 (pTH838) large colony	This work

Preparation of frozen permanents were made by mixing 600µl of overnight culture with 600µl of LB containing 14% dimethylsulfoxide (DMSO) in a glass vial resulting in a final concentration of 7% DMSO. Frozen permanents were stored at -70°C.

Table 4. Oligonucleotides used in this study

Primer name	Sequence (5′→ 3′)	Description	Length (bp)	Tm (°C)	% GC
AB23165	AATGAGCTCATGCGGATGAATTTGGC G	Sense primer for *dnaA*	27	60	48
AB23166	CGCAAGCTTCTATTCGTTGATCAGTC GC	Antisense primer for *dnaA*	28	61	50
AB24859	TTAGGATCCCAATGCGGATGAATTTG GCGACGGC	Sense primer for *dnaA*	34	67	53
AB24860	CCGAATTCCTATTCGTTGATCAGTCG CTTCAAAAGC	Antisense primer for *dnaA*	36	64	44
AB24853	AAGAATTCCAGCCGGCGAAGCGCCAG	Antisense primer for *oriC*	26	64	62
AB24854	CCGAATTCCCACGACAAGGATGACGA TGG	Sense primer for *oriC*	29	54	55
AB26340	CGCGGATCCATGCGGATGAATTTGGC G	Sense primer for *dnaA*	27	63	58
AB26341	CGCGAATTCCTATTCGTTGATCAGTC GC	Antisense primer for *dnaA*	28	63	56
AB27527	GCCAAGCTTCTGCAGCACAACCGAGC	Sense primer for amplification of 125 bp *repA1* promoter for EMSA target	26	64	62
AB27528	GGCGAATTCTTAGGTGTCGCGTCATC G	Antisense primer for amplification of 200 bp *repA2* promoter for EMSA target	27	63	56
AB27526	GCCGAATTCCTGCAACATTCCTACTC TCC	Antisense primer for amplification of 125 bp *repA1* promoter for	29	63	52

		EMSA target			
AB28779	GTC**AAGCTT**AGAACCATCCGGTGCAG TCG	Antisense primer for 518 bp *repA2* promoter	29	64	55
dnaA-147S	GCG**AAGCTT**CGTGGCGGGATATGAGG	Sense primer for *dnaA* promoter	26	64	62
dnaA32AS	AAT**GAATTC**CCTCCAGGTGCCGTCGC C	Antisense primer for *dnaA* promoter	27	64	59
pSymA-200S	TCA**AAGCTT**AGAACCGGCATGCACTG GCG	Sense primer for *repA2* promoter	29	64	55
AB30425	GT**GAATTC**TCGAAGGCCAGTCCTGCA TTGG	Antisense primer to occlude the 3′ SmaI site in *oriC*	30	64	53
AB29675	**GAGCTC**TATTCGTTGATCAGTCGCTT C	Antisense primer for *dnaA*	27	60	48
AB29744	AA**TCTAGA**TAACTGAAATTGGAAGGC GGCAAGATGCG	Sense primer for dnaA, contains stop codons in all three frames and ribosome binding site	37	64	43
AB30423	GTA**GAATTC**TCAGGCGGTTTCCAGGT AGTCGC	Antisense primer for *ctrA*	32	66	53
AB30798	TTA**CCCGGG**GCTCTATGCGGGTTCTA CTGATCG	Sense primer for *ctrA*, designed to maintain the thrombin recognition sequence in pET431a	33	68	58
AB30799	TGC**GAATTC**ATGCGGGTTCTACTGAT CGAAGACG	Sense primer for *ctrA*	34	66	50
AB30800	AAA**CTCGAG**TCAGGCGGTTTCCAGGT AGTCGC	Antisense primer for *ctrA*	32	67	56
AB30802	TCAGCGCTACGGTTTCGATAGTTTCG	Internal *dnaA*	26	60	50

		sequencing primer			
AB29679	GAACGCCAGCACATGGAC	Forward pET431a sequencing primer	18	53	61
AB29680	TTCACTTCTGAGTTCGGCATG	Reverse pET431a sequencing primer	21	52	48
AB31447	GCGGACGGATGCGATCCGCCGCCGGACCGGCTCG	Site-directed mutagenesis primer to create a 3 bp deletion in DnaA box #1	31	78	79
AB31448	CGAGCCGGTCCGGCGGATCGCATCCGTCCGC	Reverse complement of AB31447	31	78	79
AB31449	CGCAGATCGACTCCTCACAGGAACCGCAAACTCCCG	Site-directed mutagenesis primer to create a 4 bp deletion in DnaA box #2	36	77	61
AB31450	CGGGAGTTTGCGGTTCCTGTGAGGAGTCGATCTGCG	Reverse complement of AB31449	36	77	61
AB31451	GGCCATGAGTCTTGTATGCCCGGGCAAGAGATTTCCGG	Site-directed mutagenesis primer to create a 4 bp deletion in DnaA box #3	38	77	58
AB31452	CCGGAAATCTCTTGCCCGGGCATACAAGACTCATGGCC	Reverse complement of AB31451	38	77	58
oriC780S	TTAGAATTCCCGATCGCATCCGCACCG	*oriC* sequencing primer used to sequence pTH1205	27	63	56
oriC1483S	GTAGAATTCGTCCCGCTTGTCGCATCAGC	*oriC* sequencing primer used to sequence pTH1206 and pTH1207	29	64	55
AB32750	GCTGTTTTTGTCCCGCCCAACAGACCGCGGAGAATTGCG	Site-directed mutagenesis primer to	39	78	59

		create a 4 bp deletion in DnaA box #5			
AB32751	CGCAATTCTCCGCGGTCTGTTGGGCG GGACAAAAACAGC	Reverse complement of AB32751	39	78	59
AB32323	TA**GAATTC**CATCAGCCAGATGGGGG	Sense primer for *oriC* intergenic region	25	59	52
AB32324	AT**GAATTC**CACGCAATTCTCCGCGG	Antisense primer for oriC intergenic region	25	59	52
ML700	GTTTGCGGTTCCTGTGGATGAGG	Sense primer for amplification of 190 bp EMSA target	23	59	57
ML701	CTCCGCGGTCTGTTGATAAGGC	Antisense primer for amplification of 190 bp EMSA target	22	59	59
ML702	GATGCGTTTCGCTCACTTGCCG	Sense primer for amplification of 231 bp EMSA target	22	59	59
ML703	GCAAACTCCCGATCGCCGGC	Antisense primer for amplification of 231 bp EMSA target	20	60	70
ML1182	GTTTGCGGTTCCTGTGAGGAGTC	Sense primer for amplification of 178 bp EMSA target	23	59	57
ML1183	CTCCGCGGTCTGTTGGGCG	Antisense primer for amplification of 178 bp EMSA target	19	60	74
ML1817	TA**GAATTC**CCTTTCATCGCGATCCTC GAACG	Sense primer for *oriC*	31	63	48
ML1818	AT**GAATTC**CGCGGTTCGCTCTATCTA AGCC	Antisense primer for *oriC*	30	63	50
ML1888	CGGTTTACAAGCATAAAGC	pOT-1 forward	19	47	42

		sequencing primer			
ML2444	AT**GAATTC**GAGGTCGTCCGGTTGCCCG	Sense primer for *oriC*	27	64	59
ML2443	AT**GAATTC**GCGCCTTGATCCACAGATAGAGG	Sense primer for *oriC*	31	63	48
ML2445	TA**GAATTC**GGTCGACGATCGTGTAGAGTACG	Antisense primer for *oriC*	31	63	48
ML2796	CCGCCATCCAGGAGATCACCGC	Sense primer for amplification of 117 bp EMSA target containing DnaA box #5	22	62	68
ML2797	CCATCGCGGTCGCTGCCTTTGC	Antisense primer for amplification of 117 bp EMSA target containing DnaA box #5	22	62	68
ML2877	CAC**TCTAGA**TTAGGTGTCGCGTCATCG	Sense primer for 518 bp *repA2* promoter	27	61	52
ML3162	TACTGGTCCGGAATTAGCTTGC	pFUS1 reverse sequencing primer	22	55	50
ML3257	CCTTGCCACGCTCGTGTCAATCC	Sense primer for *dnaA* promoter	23	61	61
ML3258	AA**CTCGAG**TGCCGTCGCCAAATTCATCCG	Antisense primer for *dnaA* promoter	29	64	55
ML3852	GCAATGATGTGGAGCGCCTTGACAGATAGAGGTCGTCCGG	Site-directed mutagenesis primer to create a 4 bp deletion in DnaA box #5	40	78	58
ML3853	CCGGACGACCTCTATCTGTCAAGGCGCTCCACATCATTGC	Reverse complement of ML2852	40	78	58
ML3854	CCCGGCTCTGTGCGTCACAGCGCGCATTGATGGCC	Site-directed mutagenesis primer to	35	79	69

		create a 4 bp in DnaA box upstream of repA2			
ML3855	GGCCATCAATGCGCGCTGTGACGCAC AGAGCCGGG	Reverse complement of ML3854	35	79	69
M13f	GTAAAACGACGGCCAGT	Sequencing primer	17	47	53
M13r	AACAGCTATGACCATG	Sequencing primer	16	41	44

The melting temperature of site-directed mutagenesis primers were calculated using the equation Tm = 81.5 + 0.41 (%GC) - 675/N - % mismatch. For all the other primers the melting temperatures were calculated using the Oligocalculator program (http://www.pitt.edu/~rsup/OligoCalc.html) and primer sequences were analyzed for the presence of secondary structures using the Generunner program. Primers were always resuspended to a final concentration of 100 pmol/µl and stored at -20°C. Sequencing primers were diluted from the 100 pmol/µl stock to 1 pmol/µl. Engineered restriction sites that were used in cloning of the PCR products are shown in bold, extra base pairs that were added to the 5′ end of the primer to ensure effective restriction of the PCR product were selected based on the recommendation provided in the Cleavage Close to the Ends of DNA Fragments Table in the 2002-3 New England Biolabs catalogue (page 242).

METHODS

Genetic techniques

Bacterial Matings

Plasmids were transferred from an *E. coli* donor to an *S. meliloti* recipient via conjugation involving the mobilizing strain MT616 that contains the plasmid pRK600. The pK600 plasmid encodes the *tra* genes required for the transfer functions *in trans*. Recipient, donor and mobilizing strains were grown overnight in liquid broth in the presence of the appropriate antibiotic to maintain plasmids. The cultures (1.5 ml) were pelleted and then resuspended in 0.5 ml of 0.85% NaCl. Mating spots were created on an LB plate by spotting 20 µl of the mobilizer, donor and recipient strains (in this order) making sure to avoid air bubble formation. The mating spot was then incubated at 30°C overnight. Following the overnight incubation the mating spot was scrapped off the LB plate with an sterile stick and resuspended in 1 ml of 0.85% NaCl. In order to calculate transconjugation frequency in a mating experiment, 10-fold serial dilution series were made and 100 µl of the appropriate dilutions were plated onto selective media. In some cases, transconjugant

strains were isolated by streaking the mating spot directly onto selective media.

DNA techniques

Plasmid DNA Isolation

Alkaline Lysis

Plasmid DNA was isolated from *E. coli* using the protocol outlined by Sambrook and Russell(Sambrook and D.W.Russell, 2001). 1.5 mL of overnight culture was centrifuged for 30 seconds and the supernatant removed. The bacterial pellet was completely resuspended in 100 µL ice-cold solution #1 (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). Solution #2 (1% SDS in 0.2 N NaOH)(200 µL) was then added and mixed rapidly by inverting the tubes five times. The tubes were stored on ice for 3-5 minutes. Ice-cold solution #3 (60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL ddH20)(150 µL) was added and vortexed for several seconds. The tubes were stored on ice for 3-5 minutes, then centrifuged at 12,000 g for 5 minutes. The supernatant was removed into a new tube. A phenol:chloroform mixture (1:1) (100 µL) was added to the supernatant, mixed well by vortexing, and centrifuged for 3

minutes. The upper aqueous layer containing the plasmid DNA was transferred to a new tube without disturbing the interface. Chloroform was added, mixed by vortexing, and centrifuged for 2 minutes. The upper aqueous layer was then transferred to a new tube. Plasmid DNA was ethanol precipitated, washed with 70% ethanol and residual ethanol was removed by storing the pellet at 37°C for approximately 20 minutes. The DNA was resuspended in 50 µl $T_{10}E_1$ (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) containing DNAse-free pancreatic RNAse (20 µg/mL). Plasmid DNA was stored at -20°C.

Plasmid DNA was also prepared with the QIAquick miniprep kit (Qiagen) and the Gene Elute miniprep kit (Sigma) by following the manufactures directions.

Total Genomic DNA Isolation

Total genomic DNA was prepared from 6 ml of overnight culture or 100 ml of culture at an OD_{600} = 0.1. Cells were pelleted and washed with 5 ml of 0.85% NaCl in a 15 ml Falcon tube. Cells were resuspended in 1 ml T_{10} E_{25} (10 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0) then supplemented with 50 µl 25% SDS, 25 µl Proteinase K (10 mg/ml) and 125 µl 5 M

NaCl. The suspension was mixed by gently swirling and incubated at 65°C for 30 minutes. The lysate was cooled to room temperature, extracted three times with equal volumes of phenol:chloroform (1:1) and once with a large volume of chloroform in 15 ml Falcon tubes. The aqueous phase was transferred to a new 15 ml Falcon tube, ammonium acetate was added from a 10M stock to a final concentration of 2M and two volumes of 100% ethanol was added. Precipitated DNA was pelleted, supernatant removed and the DNA pellet was washed with 2 ml of 70% ethanol. The 15 ml tubes were spun again in a Beckman centrifuge and supernantant was removed. The pellet was incubated at 37°C to remove residual ethanol. The DNA was resuspended by the addition of 50 - 200 µl of T_{10} E_1 with 20 µg/ml Rnase A and incubated at 65°C for 30 minutes. Genomic DNA was the loaded (5 µl) onto a 0.8% agarose gel and run overnight at 15 V.

Polymerase Chain Reaction

Standard Reactions Conditions

Primers were synthesized (Mobix Central Facility) and were resuspended to 100 pmol/µl in ddH_2O. The primer sequences used in this study can be found in Table 4. A

dNTP mix was made prior to the PCR reaction in bulk and used for many reactions: 6.25 µL of each dNTP (100 mM) was added to 475 µL of ddH_20 creating a solution that has a final concentration of 1.25 mM for each dNTP. When required as template plasmids were diluted 1:250 and 1:500 and 5 µl was used in each reaction. When genomic DNA was required for template, 10 ng was used. A master mix was prepared containing 1X buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), ddH_20, 20 µM each dNTP, 1 pmol of each primer and $MgCl_2$ (1 to 5 mM). The master mix was placed on ice. Template DNA was added to PCR tubes, Platinum Taq DNA Polymerase (5 U/µl) was added to master mix (2.5 U/reaction), vortexed and master mix was then added to the PCR tubes. Reactions were always set up in triplicate. The 50 µl reactions were placed in a Perkin Elmer Gene Amp PCR system 2400 thermocycler. Reactions were heated to 94°C for 5 minutes and then 25 - 35 cycles of denaturing at 94°C for 30 seconds, annealing at 54 - 59°C (5°C lower than the lowest Tm of the primer pair) for 30 - 40 seconds, and extension at 72°C for 1 minute per kb of the predicted PCR product size. After the 25 - 35 cycles the reaction was extended for an additional 7 minutes and then cooled to

4°C. PCR products were examined by electrophoresing 5 µl of a reaction on an agarose gel. The remaining reaction was purified using the QIAquick PCR purification kit.

Site-directed mutagenesis

Two primers that are complementary to each other were designed to contain the desired deletion in the middle of the oligonucleotide with at least 15 bp on each side of the mutation. Several concentrations of plasmid template were prepared in a volume of 29 µl (from 1 µl plasmid DNA in 28 µl ddH_20 to 10 µl plasmid DNA in 19 µl of ddH_20), approximately 5 - 50 ng, and added to PCR tubes. A master mix was prepared with 1X Pfu buffer (20 mM Tris-HCl (pH8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 1 mg/ml nuclease-free BSA), 12.5 pmols of each primer, 125 µM of each dNTP and 2.5 U units of Pfu turbo (Stratagene). The master mix (21 µl) was added to the plasmid templates and tubes were placed in the Perkin Elmer Gene Amp PCR system 2400 thermocycler. Reactions were heated to 95°C for 2 minutes and then 18 cycles of denaturing at 95°C for 40 seconds, annealing at 55°C for 1 minute and extension at 68°C for 1.5 minutes per kb of plasmid template. The reactions were checked by electrophoresing 15 µl on an agarose gel. The remaining 35

µl of the successful reactions were incubated with 1 µl *DpnI* (20 U/µl) at 37°C for 1.5 hours. The digested PCR products were then purified using a QIAquick PCR purification kit and eluted from the column with 30 µl 10 mM Tris-HCl (pH 8.0). The 30 µl samples were then transformed into freshly prepared DH5α competent cells. Plasmid DNA was prepared from three transformant colonies and sent for sequencing at the Mobix Central Facility.

Colony screening by PCR

PCR was used to screen recombinant plasmids for insert DNA instead of restriction analysis in some cases. A fresh colony was picked from the plate with a toothpick, patched onto a master plate and the toothpick was placed into a microfuge tube containing 50 µl ddH_2O. The tubes were left open and vortexed to resuspend the cells, the toothpick was removed and the tubes were sealed then placed in a boiling water bath for 5 minutes. The tubes were then spun in a microfuge for 2 minutes at high speed. 10 µl of the supernatant was added to a PCR tube and this was used as template DNA in the reaction. A standard master mix was prepared and aliquots (40 µl) were added to the template DNA. Standard PCR conditions were used on the 50 µl

reaction. The reactions were checked by electrophoresing 5 µl of the reaction on a agarose gel. The putative positives were then inoculated into liquid broth, plasmid DNA was prepared and restriction analysis was always conducted to confirm the composition of the recombinant plasmid.

DNA modifications

Restriction digests, removal of 5′ overhangs with Mung Bean Nuclease and 5′ fill-in reactions with the Klenow fragment were carried out according to manufacture's recommendations. Restriction digests were always carried out using Roche buffers.

Agarose Gel Electrophoresis

Agarose gels were prepared from 0.8% to 1.8% in 100 ml of 0.5X TBE (45 mM Tris Borate, 1 mM EDTA). DNA Samples were mixed with 6X loading buffer (0.42% bromophenol blue and 25% ficoll type 400) before loading into the gel. Samples were electrophoresed at 50 - 100 V for 45 - 120 minutes. In some cases gels were run overnight at 15 V.

Following electrophoresis the gel was stained for 20 - 30 minutes in ddH_20 with ethidium bromide (~ 1 µg/ml) and destained in ddH_20 for 20 minutes before photo-documentation on a transilluminator.

Ligation Reactions

Plasmid DNA was restricted with the appropriate restriction enzymes. Insert DNA was prepared by restricting purified PCR product or by extracting a band from an agarose gel using the Qiagen Gel Extraction kit. Approximately three times excess amount of insert DNA (~1 µg) was added to restricted plasmid DNA. The mixture was then passed through a QIAquick PCR Purification Kit and eluted with 50 µl 10 mM Tris HCl (pH 8.0). Buffer was then removed from the sample by spinning in an Eppendorf Vacufuge at 45°C for 20 minutes. The pellet was resuspended in 1X ligation buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin), ligase (200 U) in a 10 µl final volume. The reaction was incubated at 16°C overnight upon which the entire ligation was transformed into *E. coli*.

Competent Cell Preparation

A single colony of *E. coli* DH5α was used to inoculate 5 ml LB. The following morning the cells were subcultured into 100 ml of LB and grown to an OD_{600} = 0.4. The culture was placed on ice for ~ 30 minutes and then spun down in two 50 ml Falcon tubes in a pre-chilled rotor. The supernatant was removed by pouring off into the used flask and by wicking with paper towel. The two pellets were each resuspended in 10 ml ice-cold 100 mM $CaCl_2$ by swirling not vortexing and stored on ice for at least 30 minutes. The tubes were spun again in a cold rotor and resuspended in 2 ml ice-cold 100 mM $CaCl_2$. At this point the cells were either used directly in a transformation reaction or stored for up to 18 hours in the 100 mM $CaCl_2$ on ice in the cold room to increase the transformation efficiency. After the 18 hour incubation the cells were used in transformation reactions or frozen. The cells were prepared for freezing by the addition of 70 µl DMSO to each 2ml volume followed by incubation on ice for 10 minutes. Another 70 µl of DMSO was added to the cell suspension and mixed. Microfuge tubes were pre-chilled in liquid nitrogen and 100 µl aliquots were added to the cold tubes, capped and submerged in the

liquid nitrogen. The frozen competent cells were transferred to a pre-chilled freezer box and stored at -70°C.

Transformation

Ligation reactions or plasmid DNA (~10 ng) was placed on ice to cool the tube. 100 µl of competent cells were added to the DNA and the mixture was incubated on ice for 30 minutes. The cells were subjected to heat shock treatment at 42°C for 1 minute and then returned to ice for 2 minutes. 500 µl of LB was then added and the tube was taped to the wheel that was rotating at 37°C. The cells were incubated at 37°C for at least 45 minutes before plating the transformation onto selective media.

Southern Blotting

Restriction of genomic DNA

Genomic DNA was quantified on the Varian CARY UV-Visible Spectrophotometer by measuring absorbance at 260 nm (A_{260} = 1.0 = 50 µg/ml). 10 µg of genomic DNA was digested in a 30 µl reaction volume for 4 hours. Digested genomic

DNA was loaded onto a 0.8% agarose gel and electrophoresed at 15 V overnight. The gel was stained with ethidium bromide, destained in ddH_2O and photographed with a fluorescent ruler adjacent to the lane with the 1 kb molecular weight ladder (MBI Fermentas).

Transfer to positively charged membrane

The gel was trimmed to remove the lanes with molecular weight ladder as well as the area above the wells. The gel was then agitated in alkaline transfer buffer (0.4 M NaOH, 1 M NaCl) for 30 minutes, changing the buffer mid-way. Whatman filter paper (3 mm) was cut into eight pieces (same size as the trimmed gel), four of which were soaked in alkaline transfer buffer. The transfer apparatus was assembled by stacking many layers of paper towel (approximately 8 cm high), four dry filter papers, two wet filter papers, the Zeta Probe Blotting Membrane (BIO RAD), the gel, two wet filter papers and finally a pre-soaked wick that had the ends submerged in alkaline transfer buffer in two elevated reservoirs. The DNA was transferred to the membrane in a downward direction for approximately six hours. After the transfer the apparatus was disassembled and the membrane was soaked for 15 min in 0.5

M Tris-HCl (pH 7.5), 1 M NaCl. The membrane was allowed time to air dry was wrapped in saran wrap and exposed to UV light on the transilluminator for 2 minutes.

Random Prime Labeling of Probe

Linearized plasmid DNA and purified PCR products were radioactively labeled using the Roche Random Primed DNA Labeling Kit (cat no. 1 004 760). Approximately 200 - 400 ng of DNA was added to ddH_20 to a total volume of 9 µl. The sample was incubated in a boiling water bath for 10 minutes and the tube was then plunged into an ice/water bath. 2 µl of the hexanucleotide nucleotide mixture in a 10X buffer, 3 µl of dNTPs (0.5 mM dCTP, 0.5 mM dGTP and 0.5 mM dTTP), 50 µCi [α-^{32}P]dATP (3000 Ci/mmol) and 1 µl Klenow enzyme (2 U/µl) were mixed in a final volume of 20 µl. The reaction was then incubated at 37°C in a water bath for 50 minutes. The ^{32}P- labeled probe was purified away from unicorporated nucleotides using a QIAquick PCR Purification Kit.

Hybridization

Standard stock solutions of 20X SSC (3 M NaCl, 0.3 M Na citrate (pH 7.0)), 50X Denhardt's Reagent (1% Ficoll 400, 1% polyvinylpyrollidone, 1% BSA (Sigma Fraction V)),

20% SDS, sheared Herring sperm DNA (10 mg/ml), and 1% Na pyrophosphate were made. The stock solutions were used to make 20 ml the prehybridization solution (5X SSC, 5X Denhardt's Reagent, 0.5% SDS, 100 µg/ml sheared Herring sperm DNA, 50% formamide, 0.1% Na pyrophosphate). The membrane was transferred into a glass bottle with 10 ml of the prehybridization solution and was incubated in a hybridization oven (Amersham Biosciences) at 42°C for at least 2 hours. The ^{32}P-labeled probe was denatured in a boiling water bath for 10 minutes and then rapidly cooled in an ice/water bath. The probe was added to the prehybrization solution at 42°C and hybridization occurred overnight.

Detection

After overnight hybridization the hybridization solution was removed from the bottle and the membrane was washed with the remaining 10 ml of prehybridization solution at room temperature for 15 minutes. The membrane was then washed for 15 minutes in wash buffer #1 (2X SSC, 0.5%SDS) for 15 minutes at room temperature. Wash buffer #2 (2X SSC, 0.1% SDS) was then used to wash the membrane for 15 minutes at room temperature. A high stringency wash

at 65°C for 3 hours was then done in wash buffer #3 (0.1X SSC, 0.1%SDS). The membrane was removed from the glass bottle and transferred to a plastic dish and washed briefly in wash buffer #4 (0.1X SSC). The membrane was air dried, wrapped in saran wrap and exposed to Kodak Scientific Imaging Film (Cat no. 165 1454) in a cassette with an intensifying screen. The film was developed in a Kodak X-OMAT 2000A Processor and the molecular weights of the bands were calculated by referring to the photograph of the agarose gel that was taken with the ruler along side the 1 kb ladder.

Calculation of Band Intensities

The membrane was also exposed to a Storage Phosphor Screen (Amersham Biosciences) for many different times (15 minutes - 18 hours). After the exposure the screen was scanned on a Storm 820 Phosphoimager (Molecular Dynamics) at a pixel size of 50 µm. Band intensities were calculated using the Image Quant 5.2 program (Molecular Dynamics) using the volume report function. The size of the calculated area (number of pixels) encompassing each signal was kept constant for each band. Intensities were

calculated for all the different exposure times and were used to generate a standard deviation.

DNA Sequencing and Analysis

In all cases cloned PCR products were sequenced before plasmids were entered into the strain collection. Sequencing was performed at the Mobix Central Facility using the ABIPRISM 3100 Genetic Analyzer using the BigDye terminator chemistry. Nucleotide sequences were aligned using the Lalign program (http://fasta.bioch.virginia.edu/fasta/lalign2.htm) and amino acid sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/).

Biochemical Techniques

β-glucuronidase Enzyme Assays

The procedure for the β-glucuronidase assay was taken from Reeve *et al.* 1998. Strains were grown overnight in LBmc with antibiotic selection and subcultured into 5 ml to

an OD_{600} of 0.05 and allowed time to grow to an OD_{600} of 0.4 - 0.6. Cells were centrifuged in a 15 ml falcon tube, supernatant removed and cells were washed with 5 ml 0.85% NaCl. Cells were spun again in the Beckman centrifuge and resuspended in 5 ml of resuspension buffer (50 mM Na_2HPO_4, 50 mM DTT, 1 mM EDTA (pH 7.0)). The OD_{600} was recorded and cells were kept on ice. 200 µl of cells were transferred into 790 µl resuspension buffer in a microfuge tube. One drop of toluene was added to each tube with a disposable transfer pipette, vortexed and tubes were incubated at 37°C for 40 minutes with the caps open. Tubes were removed from the incubator and 10 µl of p-nitrophenyl β-glucuronide (35 mg/ml) was added to tubes at 15 second intervals. The reactions were left until a yellow colour developed or for 60 minutes. Reaction time was recorded when the reactions were terminated by the addition of 700 µl 0.46 M Na_2CO_3 to 200 µl of the reaction. The microfuge tubes were spun at 13 000 rpm for 1 minute to pellet cellular debris. The 900 µl stopped reaction was added to a disposable cuvette and absorbance was read in the Varian CARY UV-Visible Spectrophotometer at 405 nm that had been zeroed with a blank containing 700 µl Na_2CO_3 and 200 µl resuspension buffer. β-glucuronidase activity was calculated and

expressed in Miller Units (Miller, 1972). Each strain was tested in triplicate and these were used to calculate a mean and standard deviation.

Green Flourescent Protein Assay

For each strain, three 2 ml volumes inoculated from single colonies were grown overnight at 30°C. 200 to 300 µl of each culture was subcultured into 5 ml LB broth to achieve an OD_{600} of approximately 0.1 to 0.2, and incubated until an OD_{600} of approximately 0.6 to 0.7 was reached. 1 ml of cells was centrifuged at 13 000 rpm and washed with 1 ml of 0.85% NaCl and resuspended in 1 ml of 0.85% NaCl. 200 µl aliquots were transferred to both clear and black 96-well microtiter plate for absorbance (600 nm) and GFP readings, respectively. For GFP fluorescence readings, the excitation wavelength was set to 405 nm and the emission wavelength was set to 505 nm. The absorbance and fluorescence readings were conducted using a Tecan Safire Fluorimeter/Spectrophotometer. The triplicate readings were used to generate mean and standard deviation values.

Overexpression of Fusion Proteins, Cell Extracts and Protein Purification

E. coli protein overexpression strains were grown at 37°C in 5 ml LB broth containing 50 µg/ml Amp and subcultured into 100 ml LB broth with antibiotic. The 100 ml culture was grown to an OD_{600} of 0.6 at 37°C and then isopropylthio-β-D-galactoside (IPTG) was added from a 100 mM stock to a final concentration of 0.3 mM. Cells were grown for an additional 2.5 hours at 30°C, spun down and resuspended in 10 ml ice-cold resuspension buffer (50 mM Na_2PO_4, 500 mM NaCl, 10 mM Imidazole (pH 8.0)). Lysozyme was added from a 10 mg/ml stock to a final concentration of 150 µg/ml and incubated on ice for approximately 30 minutes. The cells were then sonicated twice with the Branson Sonifier Cell Disruptor with a 1/8" tapered probe for 20 seconds at a power level of 3.5. Lysates was kept on ice between sonications. The crude lysate was poured into a pre-chilled 50 ml centrifuge bottle and spun in a Beckman Avanti J-25 Centrifuge using a JA-20 rotor at 15,000 rpm to remove insoluble material.

The soluble cell lysate was added to 1 ml Ni-NTA resin (Qiagen) that had been equiliberated with 0.5 ml

resuspension buffer (50 mM Na_2PO_4, 500 mM NaCl, 10 mM Imidazole (pH 8.0)) and was incubated in a 15 ml Falcon tube at 4°C on a rotator. The sample was then spun in a swing bucket rotor (2,000 rpm for 3 minutes) to pellet the resin, and most of the supernatant was removed. The collected resin was added to a BIO RAD disposable column and the flow through was collected. The resin was then washed with 2 ml of ice-cold resuspension buffer and then 2 ml of ice-cold wash buffer #1 (50 mM Na_2PO_4, 500 mM NaCl, 20 mM Imidazole (pH 8.0)). The resin was then washed with 4 ml of ice-cold wash buffer #2 (50 mM Na_2PO_4, 500 mM NaCl, 40 mM Imidazole (pH 8.0)). The fusion protein was eluted from the column with 1 ml of ice-cold elution buffer (50 mM Na_2PO_4, 500 mM NaCl, 250 mM Imidazole (pH 8.0)). The eluted protein fraction was collected in a microfuge tube on ice. The 1 ml elution was added to a pre-soaked Pierce 3.5K Slide-A-Lyzer with a cold syringe. Dialysis was carried out overnight at 4°C in 4 L of buffer containing 50 mM Na_2PO_4, 300 mM NaCl (pH 8.0). Protein sample was removed from the dialysis cartridge and 0.5 µl Thrombin (Novagen, 1.5 U/µl) was added and sample was incubated at room temperature for 2.5 hours. After the protease digestion the reaction was added to a BIO RAD disposable column and

the flow through was collected, the resin was washed with 0.5 ml resuspension buffer, 0.5 ml wash buffer #1, 0.5 ml wash buffer #2 and the finally the NusA tag was eluted with 0.5 ml elution buffer. Collected samples were analysed by SDS polyacrylamide gel electrophoresis. Protein samples were stored on ice in a sealed styrofoam box at 4°C.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was conducted according to the method of Laemmli (Laemmli, 1970) using 12% or 10% polyacrylamide. Gels were cast with 1.5 mm spacers in a BIO RAD Protean II Minigel. Prior to loading protein samples onto the gel they were mixed with 4X loading buffer (4% SDS, 0.1 M Tris-HCl (pH 8.9), 2 mM EDTA, 0.1% bromophenol blue, 20% glycerol, 0.25 DTT). Gels were run at 170 V for ~ 45 minutes and stained with for 30 minutes in a staining solution (0.1% coomassie blue, 40% methanol, 10% acetic acid) and destained for 2 hours in 40% methanol, 10% acetic acid, 50% ddH_2O.

Protein Determination

The concentration of purified protein samples was determined with using the Coomassie blue BIO RAD Protein Assay Dye Reagent (Bradford 1976). Samples were added to 1 ml of diluted reagent and incubated for 5 minutes at room temperature and the A_{595} was measured. A standard curve was made using 1 µg to 10 µg of bovine serum albumin. The concentration of purified protein was determined by converting A_{595} to µg protein and factoring in the amount of protein being measured.

Electrophoretic Mobility Shift Assays

Probe DNA (~100 - 200 bp purified PCR product) was quantified visually on an a 1.8% agarose gel by loading different amounts and comparison with known quantities of bands in the 100 bp molecular weight ladder (MBI fermentas). After the concentration of target DNA had been determined the amount of DNA required to contribute 1 pmol of 5′ termini to the labeling reaction was calculated according to Appendix A4.36 in Molecular Cloning(Sambrook and D.W.Russell, 2001), in all cases this was less than one

µl of PCR product. The (γ-^{32}P) ATP stock 150 mCi/ml (6000 Ci/mmol) was diluted 1:24 in ddH_20 to give a concentration of 1 pmol/ul. Less than 1 µl probe DNA, 2 µl 10X buffer (700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM DTT), ddH_20, T4 Polynucleotide Kinase (New England Biolabs), and 5 µl (γ-^{32}P) ATP (1 pmol/ul) were mixed in a 20 µl final volume. The reaction was incubated at 37°C for 50 minutes and then PCR purified with a QIAquick PCR Purification Kit (eluted with 50 µl). 1 µl of labeled probe was spotted onto a small square of filter paper, which was then added to scintillation fluid in a vial and counts per minute were read in a liquid scintillation counter.

A 60 ml 4% polyacrylamide solution was prepared by mixing 8 ml 30% Acrylamide (29:1 acrylamide:bis), 6 ml 5X TBE, 46 ml ddH_20, 400 µl 10% ammonium persulphate and 40 µl N,N,N'N'-tetramethylethylenediamine (TEMED). The glass plates were set in the BIO RAD Protean II xi Gel Caster and the edges of the gel were sealed with a 1% agarose solution in 0.5X TBE. The 60 ml solution was then poured between the glass plates and polymerized for at least 4 hours, in some cases the gel polymerized overnight wrapped in saran wrap at 4°C. The polymerized gel was then placed in the BIO RAD Protean II xi Cell and the upper reservoir and the

entire buffer chamber was filled with 0.5X TBE. The wells of the gel were rinsed with buffer and the gel was pre-run at 8 V/cm for 1 hour at room temperature.

The binding reaction was set up as described by Schaper and Messer 1995. In a microfuge on ice the following were mixed (in this order); 4 µl 5X Binding buffer (100 mM HEPES KOH (pH 8.0), 25 mM Mg acetate, 5 mM Na_2EDTA, 20 mM DTT, 25 mg/ml BSA, 1% Triton X-100, 25% glycerol), ddH_20, 0.4 µl ATP (50 mM), Target DNA (20,000 cpm), 1 µl poly dI:C (100 ng/µl), and purified DnaA (100 ng - 500 ng) in a total reaction volume of 20 µl. The reaction was incubated on ice for 10 minutes and then 20 minutes at room temperature. The reaction was loaded onto the 4% polyacrylamide gel and electrophoresed at 14 V/cm (252 V) for 10 minutes and then 9 V/cm (162 V) for 2.5 hours at room temperature. The gel was removed from the gel plates with whatman filter paper and covered in saran wrap. The gel was dried in a BIO RAD Gel Dryer Model 583 for 2 hours on cycle 1. The dried gel was exposed to Kodak Scientific Imaging Film and the Storage Phosphor Screen.

Environmental Scanning Electron Microscopy (ESEM)

For glutaraldehyde fixation several colonies were used to inoculate 2 ml LBmc with gentamicin (30 µg/ml) and the culture was grown until an OD_{600} = 0.5. The culture was pelleted in a microfuge tube and supernatant was removed. The pellet was then resuspended in 1 ml 0.2 M sodium cacodylate buffer pH (7.4). 1 ml of 0.2 M sodium cacodylate buffer containing 5% glutaraldehyde (pH 7.4) was added therefore changing the effective concentration of glutaraldehyde to 2.5%, tubes were inverted a couple of times and left for 1 hour at room temperature. Glutaraldehyde fixed samples were stored at 4°C.

For slide preparation a cover glass was mounted on an aluminum ESEM stub with conductive glue (equal parts white Elmers glue and collodial graphite). The glue was added to the stub, glass placed onto the glue spot and 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 minutes and then coated with a 5 nm layer of gold using the Sputter Coater.

The glutaraldehyde fixed sample was washed six times in 2 ml ddH_2O to remove all traces of salt and 1 µl of resuspended sample was spotted on the gold coated glass slide and the spot was allowed time to air dry. The orientation of multiple spots was achieved by gluing the end of a toothpick to one corner of the slide, spots were separated by a known number of scratches made in the gold with a scalpel.

For visualization and image capturing the stub was placed into the 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 1 Mb .tif files.

CHAPTER 3. MOLECULAR GENETIC CHARACTERIZATION OF THE SINORHIZOBIUM MELILOTI CHROMOSOMAL ORIGIN OF REPLICATION

Isolation of the *Sinorhizobium meliloti* chromosomal origin of replication

The origin of replication on the *S. meliloti* chromosome (*oriC*) has been predicted on the basis of G/C skew to be located in a region close to the *hemE* open reading frame (Capela *et al.*, 2001). An origin is also predicted in the homologous region of the *A. tumefaciens* circular chromosome. Figure 1 depicts these regions from both replicons, several genes that are commonly found in close proximity to bacterial origins are found adjacent to *hemE* such as *parAB* and *gidAB*. Experimental evidence from another alpha proteobacterium, *C. crescentus*, has localized the chromosome origin to the *hemE* - *RP001* intergenic region(Marczynski and Shapiro, 1992;Brassinga and Marczynski, 2001).

To examine the *hemE* locus from the *S. meliloti* chromosome for the presence of an origin of replication, a 3 kb exceptionally A/T rich region flanked by the *hemE* and *Y02793* genes by was PCR amplified from *S. meliloti* genomic

DNA and cloned via engineered restriction sites (*EcoRI*) into the gentamicin resistant suicide plasmid pUCP30T. This recombinant plasmid, pTH838, was transferred into the *S. meliloti recA*::Tn5 mutant (Rm5004) via conjugation with selection on Sm (200 µg/ml) Gm (60 µg/ml) plates. The pTH838 plasmid transferred into Rm5004 and wildtype Rm1021 at a frequency of 10^{-1} transconjugants per recipient cell. We assumed that this high transfer frequency into both Rm1021 and Rm5004 relected an ability of pTH838 to autonomously replicate since the *recA* mutation in Rm5004 would prevent homologous recombination into the genome. Every time this mating experiment was done both small and large tranconjugants arose on selective media after 6 days of incubation at 30°C as seen in Figure 2. No colonies were ever observed when the parent plasmid pUCP30T was transferred into either Rm5004 or Rm1021.

Minimal sequence required for transconjugant colony formation in the replication assay

We used the mating of recombinant plasmids into Rm5004 as a replication assay to map the sequence requirements of the *S. meliloti* origin of replication. A characteristic

feature of bacterial origins of replication is an A/T rich region and the presence of 9 bp DnaA binding sites (DnaA boxes) required for the replication initiation. A plot of the GC content of the 3 kb region cloned in pTH838 revealed the presence of an A/T rich sequence approximately 100 base pairs upstream of the translational start site of the *hemE* gene as seen in Figure 1. Moreover scanning of this sequence using the expanded *E. coli* DnaA box consensus (Schaefer and Messer, 1991) (T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C) for 9/9 and 8/9 matches revealed five putative DnaA boxes.

Subclones of pTH838 that contained the A/T rich region but did not contain all predicted DnaA boxes were initially made utilizing restriction sites (pTH879, pTH880 and pTH881) that flanked the *hemE - Y02793* intergenic region. These constructs did not result in any transconjugant colony formation when mated into Rm5004 suggesting that these subclones did not contain all the necessary sequence required for autonomous replication. To further confirm that the *hemE - Y02793* intergenic region alone could not support replication, the 477 bp region was amplified from pTH838 and cloned into pUCP30T. The resulting plasmid pTH1245 when transferred into Rm5004 did not yeild

transconjugant colonies (Figure 3). The 5′ and 3′ limits of the chromosome origin were estabilished using cloned PCR products. The 5′ limits of sequence required for transconjugant colony formation was mapped to within 19 base pairs (the difference between pTH1451 and pTH1452 and the difference between pTH1453 and pTH1454 as seen in Figure 3). A predicted DnaA box (TGATCCACA) maps within this 19 base pair sequence. The 3′ limits of the region required for transconjugant colony formation (pTH1454) extended approximately 250 base pairs into *Y02793*, Figure 3.

DnaA boxes are required for replication of cloned *oriC*

The 5 predicted DnaA boxes in pTH838 were mutated by oligonucleotide site-directed mutagenesis. The mutations in DnaA boxes 5, 2, 3 and 4 were 4 base pair deletions and the mutation in DnaA box 1 was a 3 bp deletion. Conjugation experiments revealed that the plasmids with mutations in DnaA boxes 2, 3, and 4 yielded no Rm5004 transconjugants suggesting that these mutations abolish replication of plasmid borne *oriC* (Figure 4). As expected, transconjugant colonies formed when these plasmids were

transferred into Rm1021 likely due to recombination into the genome ensuring that the plasmids are still mobilizable after the mutagenesis. Mutations in DnaA boxes 5 and 1 did not change the transconjugation frequency as compared to the transfer frequency of the wildtype *oriC* plasmid suggesting that these binding sites are not required for replication or that these mutations do not prevent DnaA binding (Figure 4).

Identification of a site in the *hemE - Y02793* intergenic region required for replication

A 4 base pair deletion was created at a unique XmaIII site in the *hemE - Y02793* intergenic region. This was done by first subcloning a 416 base pair HindIII/SmaI fragment (containing the intergenic region) into pUC119. Digestion of this recombinant plasmid with XmaIII (XmaIII recognition sites are absent from pUC119) then treatment with Mung Bean Nuclease to remove the 5′ overhangs followed by religation created the 4 base pair deletion. The 416 base pair HindIII/SmaI fragment in the wildtype *oriC* was then replaced with the 412bp HindIII/SmaI fragment from pUC119. The resulting plasmid (pTH1186) was unable to replicate in Rm5004 (Figure 4). Therefore the CCGG nucleotides in the

XmaIII recognition site are required for autonomous replication of the cloned *oriC*.

DnaA boxes in the three *Sinorhizobium meliloti* origins of replication

The *repABC* replicator regions from the *S. meliloti* pSymA and pSymB megaplasmids were scanned for the presence of binding sites that matched the expanded *E. coli* DnaA box consensus sequence (Schaefer and Messer, 1991). Figure 5 depicts the locations of the predicted DnaA boxes in the three *S. meliloti* origins of replication. DnaA box #5 in *oriC* has an 8/9 match to the consensus sequence however it shows an exact match to a DnaA box found in the *C. crescentus* chromosome origin (*Cori*). DnaA box #3 is also an 8/9 match to the consensus sequence but exactly matches DnaA boxes found in *Streptomyces* (Majka, Zakrzewska-Czerwinska, and Messer, 2001) and *Micrococcus luteus* (Fujita, Yoshikawa, and Ogasawara, 1990) where DnaA has been shown to bind. Interestingly DnaA box#2 found in the *hemE - Y02793* intergenic region is found twice in the pSymA replicator region, once 158 base pairs upstream of the *repA2* translational start site and once inside the *repA2* openreading frame. In addition, the 3′ end of the *repA2*

gene, contains a predicted binding site that matches a putative DnaA box that overlaps with the translational start site of the *dnaA* gene. The latter is gene known to be autoregulated in other organisms (Atlung, Clausen, and Hansen, 1985;Braun, O'Day, and Wright, 1985;Ogura *et al.*, 2001). There are 11 putative DnaA boxes in the pSymB replicator region none of which are duplicated in either of the other two origins. There is a single binding site in both megaplasmids downstream of the *repC* gene. Sequence upstream or *repC*, the *repC* open reading frame and sequence downstream of the *repC* gene is required for a minimal pSymB origin of replication (unpublished data). The DnaA box downstream of *repC1* is found in this sequence required for autonomous replication of the cloned pSymB origin in *A. tumefaciens*, and is located 32 base pairs downstream of the translation stop codon of *repC*.

Motifs in the *Sinorhizobium meliloti oriC*

Localization of the *S. meliloti oriC* was facilitated by the characterization of the *C. crescentus* origin of replication therefore we decided to look for motifs that are shared between the *Cori of C. crescentus* and the *oriC*

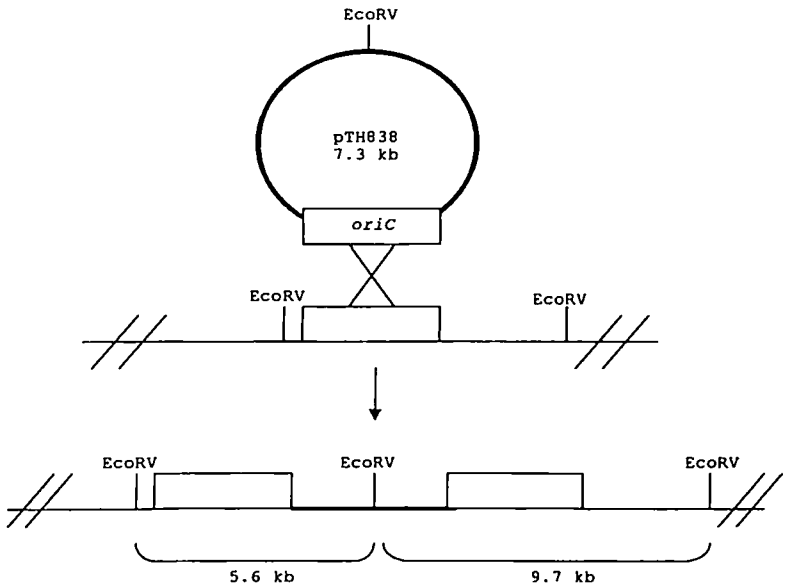
of *S. meiloti*. Table 5 shows 2 sets of 8-mers that are common to both origins. Repetitive motifs that are unique to the *S. meliloti oriC* are shown in Table 6, 4 11-mers and 14 8-mers were found in the 3 kb *oriC* cloned in pTH838. The biological function of these motifs remains unknown however it is common in the initial identification of a bacterial origin to report such elements.

Plasmid borne *oriC* is autonomously replicating and not integrating into the genome

Small and large Rm5004 (pTH838) transconjugants were streak purified 3 times on selective media (LB Sm Gm). Streak purification of these colonies resulted in both types of colony sizes if either a small or large colony was purified. A single small colony and a single large colony (done in triplicate) was used to inoculate LBmc Sm Gm and total DNA was prepared from these cultures. As a control total genomic DNA was also isolated from RmK569 which contains a copy of pUCP30T integrated at the *pstS* locus of Rm1021.

To determine whether pTH838 was replicating autonomously or had integrated into the Rm5004 genome genomic DNA (10 µg) was restricted with EcoRV and a

southern blot was preformed using labeled pUCP30T as probe. If pTH838 was replicating autonomously digestion with EcoRV would result in a single band of 7.3 kb, while integration of pTH838 into the chromosome (at *oriC*) would result in 2 bands of 5.6 kb and 9.7 kb (see Figure below). Integration of pTH838 at other sites in the genome would also result in two restriction fragments.



A digestion of RmK569 DNA with EcoRV should result in two bands of 2.7 kb and 2.2 kb (data not shown). Rm5004 and Rm1021 were both used as negative controls because neither of these strains should contain sequences that hybridize with the pUCP30T probe. All transconjugant colonies contained a single 7.3 kb band (Figure 6). Thus

pTH838 had not integrated into the genome and was autonomously replicating within Rm5004 cells.

Further evidence that pTH838 was replicating autonomously was obtained from experiments in which we tested for conjugal transfer of pTH838 from Rm5004 transconjugant colonies. A mating spot was created on an LB plate using either Rm5004 (pTH838) small (K1012), Rm5004 (pTH838) large (K1013) or RmK569 as donor strains. The recipient, *E. coli* MT620 was rifampicin resistant and *E. coli* MT616 (pRK600) was used as the mobilizing strain. The mating spot was incubated overnight at 30°C, resupended in 0.85% NaCl, serially diluted and then plated onto LB Sm Gm, LB Rf Gm, and LB Rf. Transconjugation frequencies were calculated as the # transconjugants / recipient cell and # transconjugants / donor cell these values are provided in Table 7.

As shown in Table 7 the integrated copy of pUCP30T in RmK569 was not mobilized into *E. coli* whereas pTH838 was very efficiently mobilized from K1012 and K1013 into MT620. An EcoRI and HindIII restriction analysis of the transferred plasmid prepared from the *E. coli* tranconjugants confirmed that the transferred plasmid yielded the same fragment sizes as pTH838 (Figure 7).

EcoRI was used to excise the 3 kb *oriC* from the 4.3 kb pUCP30T and HindIII was used to confirm the same orientation of the *hemE* gene with respect to the *lac* promoter (Figure 7). The transferred plasmids were also run uncut on an agarose gel (Figure 8) and 2 of the 16 plasmids tested appeared to be of a much higher molecular weight although the restriction analysis was indistinguishable from that of pTH838.

Rm5004 (pTH838) transconjugant colony size is a consequence of pTH838 copy number

The conjugative transfer of pTH838 into Rm5004 resulted in small and large transconjugant colonies with approximately fifty times more small than large colonies. To investigate whether this was due to differences in the copy number of the pTH838 plasmid, total DNA was prepared from liquid cultures inoculated with small and large colonies (each done in triplicate). The resulting DNA was restricted with HindIII, blotted onto nitrocellulose and probed with the 477 base pair *hemE* - *Y02793* intergenic region (Figure 9). The intensities of the plasmid signal (5.8 kb) and the chromosome signal (3.5 kb) were quantified with a phosphorimager. A ratio of plasmid signal :

chromosome signal was determined for each transconjugant and then the three ratios for each of the small and large strains tested were used to calculate an average relative copy number. The average copy number of the three small transconjugants was 0.053 plasmids / chromosome and the average copy number of the three large transconjugants was 0.135 plasmids / chromosome. Thus the large colonies contained approximately twice as much pTH838 plasmid DNA as the small colonies.

To further support the calculated copy numbers of less than one, K1012 and K1013 were grown in LBmc Sm Gm to mid-exponential phase and plated onto LB without added antibiotic. Plates were incubated for four days at 30°C, colonies developed normally. Two hundred colonies were then patched back onto selective media (Sm Gm), the patch plates were incubated at 30°C for four days. The number of Gm resistant colonies that grew are reported in Table 8. 20 / 200 (0.1) patches grew for the K1013 culture and 13 / 200 (0.065) patches grew for the K1012 culture, both values are very close to the average copy number values calculated from the southern blot, 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 selecting for pTH838 that actually contain a plasmid molecule. The RmK569 strain with an integrated copy of pUCP30T in the *pstS* gene was used as a control strain, all two hundred patches grew when patched back onto selective media suggesting that the integrated copy of pUCP30T is stably maintained in the *S. meliloti* genome. The results of these experiments suggests that transconjugant colony formation is much slower in the presence of gentamicin than without selection because 1 / 10 to 1 / 20 cells in the population actually contain the plasmid encoding gentamicin resistance which is replicating from the *S. meliloti* chromosomal origin of replication.

Mutations in the *hemE-Y02793* intergenic region that abolish replication of cloned *oriC* reduce transcription of *hemE*

Four mutations in the *hemE-Y02793* intergenic region abolished replication from the cloned *S. meliloti* chromosomal origin of replication. Three of these mutations were four base pair deletions in putative DnaA binding sites and one was a four base pair deletion at the XmaIII recognition site. The entire intergenic region including the ATG of *hemE* and the GTG of *Y02793* was PCR

amplified from wildtype and mutant templates and cloned via engineered EcoRI sites into the broad host range plasmid pBBR1MCS5. These recombinant plasmids were sequenced to determine the orientation of the cloned insert DNA and to confirm that all the intergenic sequences contained the proper mutations. The intergenic inserts were then excised from the pBBR1MCS5 plasmid and directionally cloned in both orientations into the pFUS1 reporter plasmid. The pFUS1 plasmid encodes a promoterless *gusA* and therefore β-glucuronidase activity is used as an assay for promoter strength. The pFUS1 plasmids were transferred into Rm5004, β-glucuronidase assays were done on permeabilized cells and GusA activity was calculated in Miller Units (Figure 10). All mutations appeared to alter the *hemE* promoter activity with little or no effect on the *Y02793* promoter. To investigate the possibility that the various cloned intergenic regions were altering the copy number of the reporter plasmid, total DNA was prepared from the same cultures used for the GusA enzyme assays. Total DNA was also prepared from Rm5004 strains with the wiltype and mutant intergenic regions cloned into pBBR1MCS5. The genomic DNA was restricted with EcoRI which excised the 477 base pair intergenic region from the plasmids and generated

a 3.5 kb fragment containing the chromosomal copy of the *hemE -Y02793* intergenic region. The wildtype 477 bp intergenic region was random prime labeled and used as a probe in a southern blot (Figure 11). The relative copy number of the pFUS1 and pBBR1MCS5 plasmids were calculated as a ratio of plasmid signal to chromosome signal, standard deviations of instrument variability were determined using several exposure times on the phosphor storage screen. These values are represented graphically in Figure 12. The pFUS1 reporter plasmid was in fact increasing in copy number when any mutant intergenic sequence was cloned into the pFUS1 plasmid as compared to the copy number of pFUS1 with the wildtype intergenic region. The same trend was observed when mutant intergenic regions were cloned into pBBR1MCS5. Miller Units were corrected by dividing the Miller Units by the copy number calculated from the southern blot. Figure 13 illustrates the differences between Miller Units and the corrected Miller Units. All four mutations reduce *hemE* transcription by 2 - 3 fold suggesting that *hemE* transcription and replication of the *S. meliloti* chromosome are coupled.

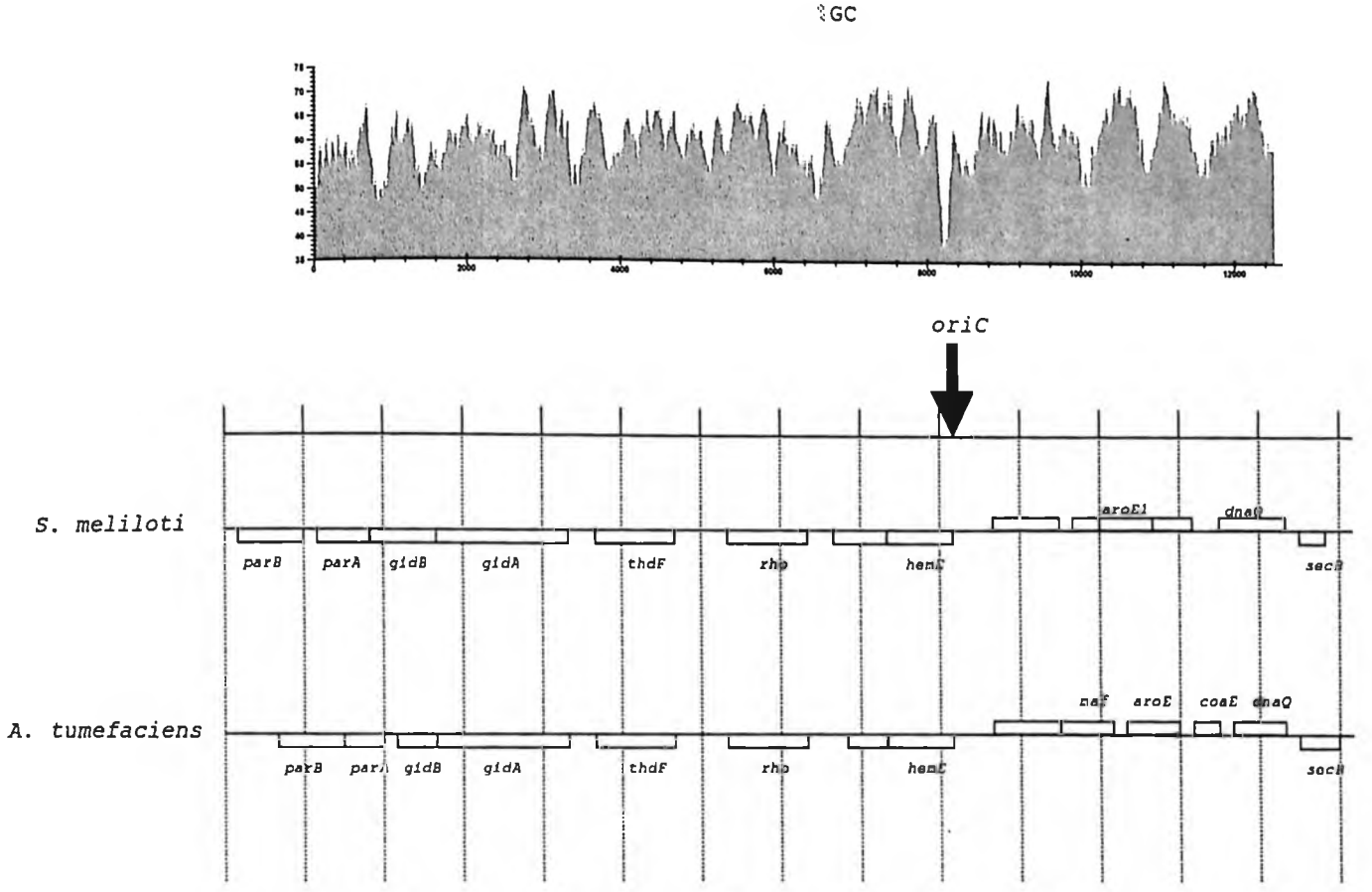
Wildtype and mutant *oriC* sequences reduce the copy number of the broad host range plasmid pBBR1MCS5 modestly in exponential phase

We speculated that perhaps the reason for such a low copy number of the autonomously replicating minichromosome pTH838 (less than one) was due to an incompatibility of the cloned chromosome origin against the *S. meliloti* genome and thus the effects on copy number of the broad host plasmid pBBR1MCS5 as a result of cloning wildtype and mutant 3 kb *oriC* sequences has been determined. Total DNA was prepared from Rm5004 strains harbouring pBBR1MCS5 and pBBR1MCS5 derivatives with 3 kb *oriC* sequences cloned via EcoRI. The DNA was prepared from cultures at early exponential phase (OD600 = 0.1) and late stationary phase (OD600 = 7.0).

Mutant origins with deletions in DnaA boxes 1, 2, 3, 4 and the deletion in the intergenic XmaIII recognition site were used. Total DNA was restricted with EcoRI and probed in a southern blot with the random prime labeled 800 base pair *dnaA* promoter and linearized pBBR1MCS5 (Figure 14). The relative copy number of each plasmid was calculated in both exponential and stationary phase as the ratio of plasmid signal (4.7 kb) to chromosome signal (5.9 kb). The calculated copy numbers are represented graphically in

Figure 15. The copy number of plasmids with the cloned wildtype or mutant origins was slightly reduced as compared to the copy number of the pBBR1MCS5 plasmid in exponentially growing cells. These changes in copy number were not detected in the stationary phase samples. In all cases the relative copy number of the pBBR1MCS5 plasmid in exponential phase was greater than twice that of cells that had entered stationary phase.

Figure 1. Genetic organization surrounding the chromosomal origin of replication from *S. meliloti* and the putative origin of replication of the circular chromosome from *A. tumefaciens*. A %GC graph of the corresponding 12 kb region from the *S. meliloti* chromosome is shown above the genetic maps. The % protein identity between homologous *S. meliloti* and A. tumefaciens proteins is scored below the genetic map.



	parB	parA	gidB	gidA	thdF	rho	hemE	aroE	dnaQ	secB
% Protein Identity	77	81.1	62.1	70.3	46.3	96.4	70.3	68.3	64.9	73.2

Figure 2. *S. meliloti* (pTH838) colony morphologies of A, Rm5004; B, Rm1021; C, Rm5004 (pTH838); and D, Rm1021 (pTH838) transconjugants. The pTH838 plasmid was transferred into Rm5004 and Rm1021 by conjugation. Transconjugants were selected on LB Sm Gm. All colonies were photographed following incubation at 30°C for 6 days.

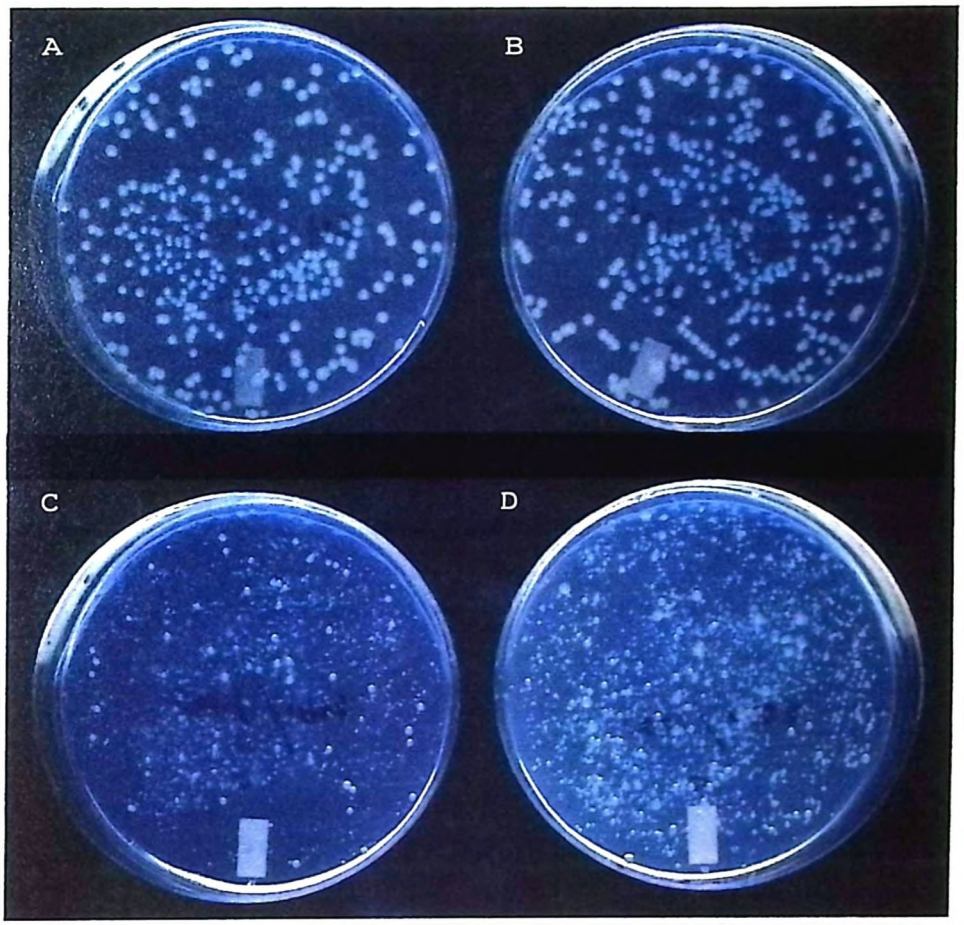
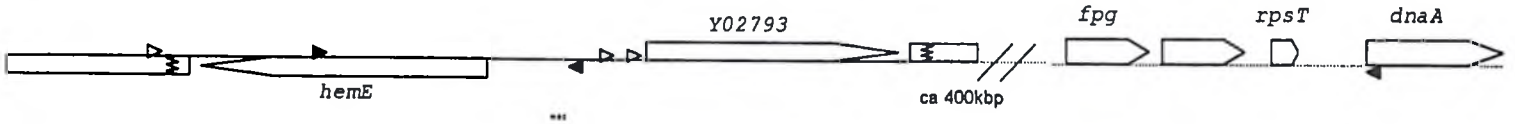
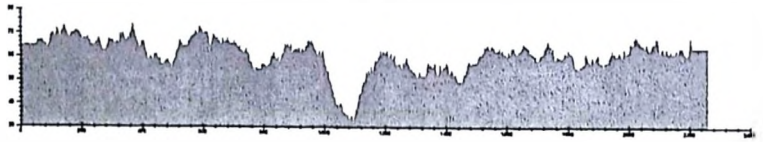
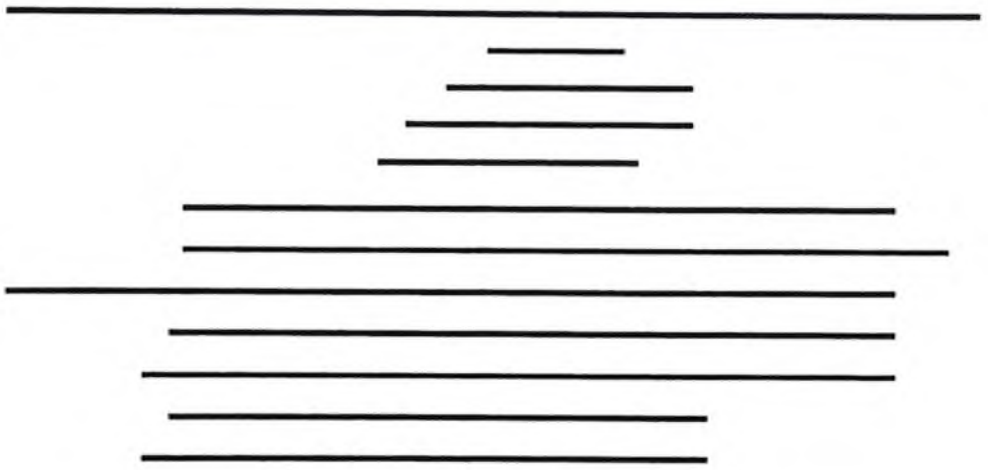

A
B
C
D

Figure 3. Genetic map of the *S. meliloti* chromosomal origin of replication. The organization around the origin is shown and the distance from the *dnaA* gene. A %GC graph indicates the location of an exceptionally A/T rich region in the 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) are shown with dark triangles and boxes that match the consensus 8/9 are represented with open triangles. Subclones of the 3 kb origin in pTH838 are scored for there ability to generate transconjugant colonies when cloned into a suicide plasmid (pUCP30T) and transferred into an *S. meliloti recA-* strain (Rm5004).

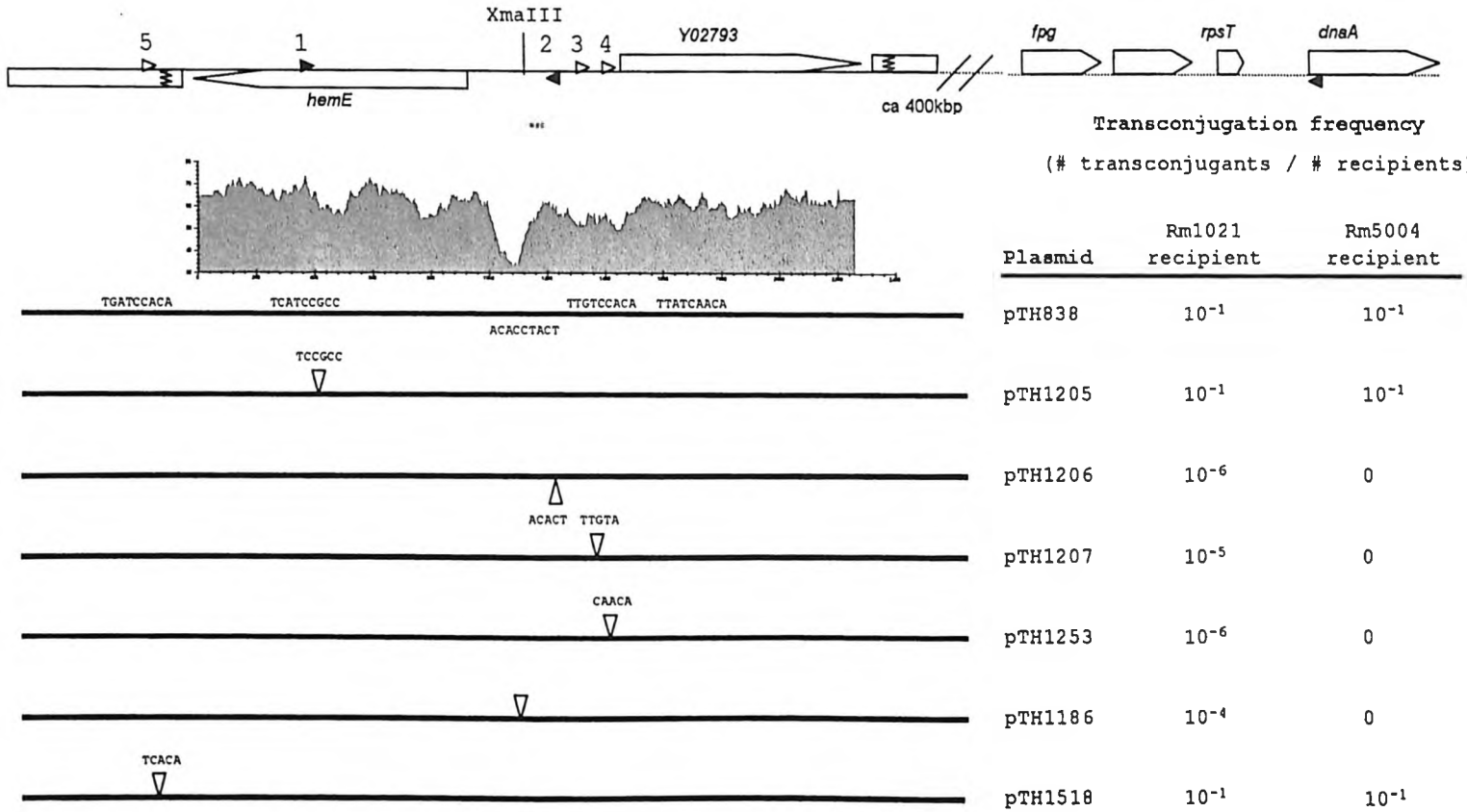






Plasmid	Transconjugation frequency (# transconjugants / # Rm5004 recipients)
pTH838	10^{-1}
pTH1245	0
pTH879	0
pTH880	0
pTH881	0
pTH1416	10^{-7}
pTH1442	10^{-6}
pTH1443	10^{-1}
pTH1451	10^{-7}
pTH1452	10^{-1}
pTH1453	10^{-7}
pTH1454	10^{-1}

Figure 4. Location of site-directed mutations in the *S. meliloti oriC*. The wildtype sequences of the putative 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)(►) and boxes that match the consensus 8/9 (▻) are provided on the line representing the wildtype *oriC* (pTH838). The sequence that results from the site-directed mutagenesis on pTH838 is provided on the mutant plasmid lines at the site of the deletion (∇). Transconjugation frequencies into Rm1021 and Rm5004 for the wildtype and mutant origins are indicated in the accompanying Table.



Transconjugation frequency

(# transconjugants / # recipients)

Plasmid	Rm1021 recipient	Rm5004 recipient
pTH838	10^{-1}	10^{-1}
pTH1205	10^{-1}	10^{-1}
pTH1206	10^{-6}	0
pTH1207	10^{-5}	0
pTH1253	10^{-6}	0
pTH1186	10^{-4}	0
pTH1518	10^{-1}	10^{-1}

Figure 5. Organization and the sequences of putative DnaA binding sites in the origins of replication for the (A) chromosome, (B) pSymA and (C) pSymB from *S. meliloti.* The schematic of each origin includes the location of the binding sites (dark triangles) and the sequence of the sites. The sites in left to right order are given in each Table in a corresponding descending order.

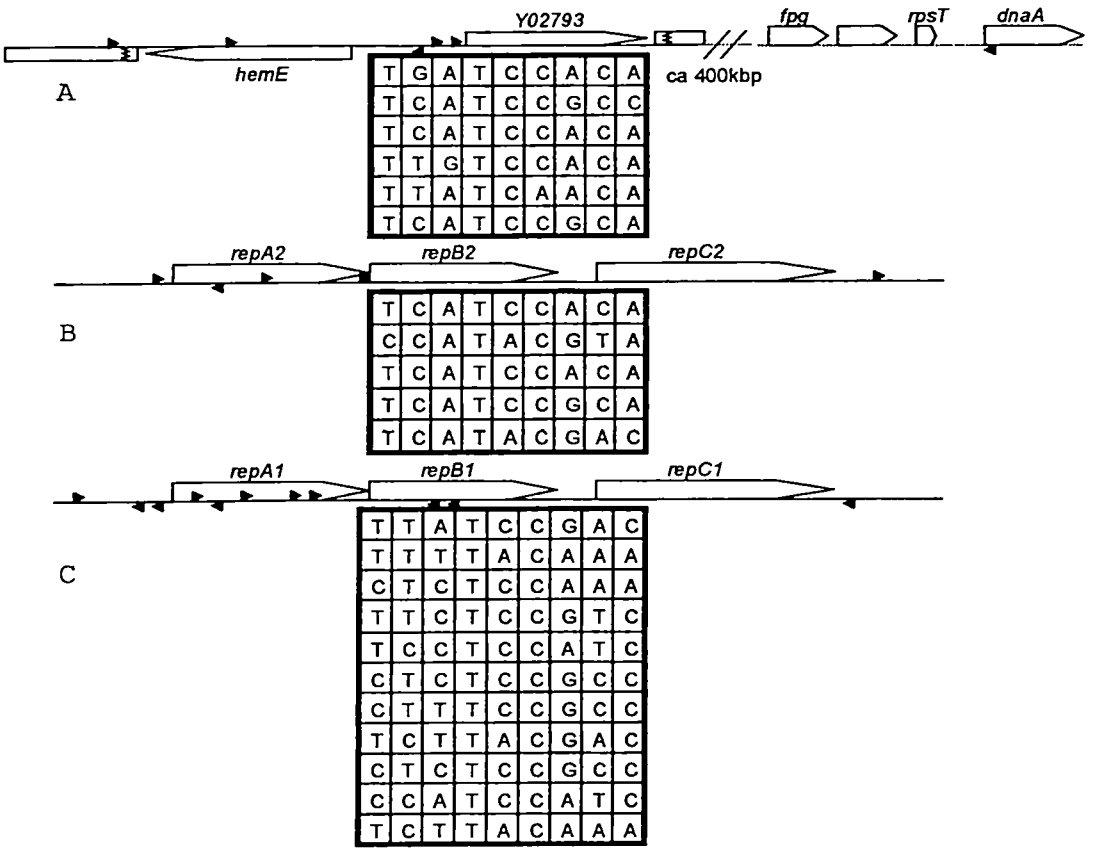

Y02793
fpg
rpsT
dnaA
hemE
ca 400kbp
A
T G A T C C A C A
T C A T C C G C C
T C A T C C A C A
T T G T C C A C A
T T A T C A A C A
T C A T C C G C A
repA2
repB2
repC2
B
T C A T C C A C A
C C A T A C G T A
T C A T C C A C A
T C A T C C G C A
T C A T A C G A C
repA1
repB1
repC1
C
T T A T C C G A C
T T T T A C A A A
C T C T C C A A A
T T C T C C G T C
T C C T C C A T C
C T C T C C G C C
C T T T C C G C C
T C T T A C G A C
C T C T C C G C C
C C A T C C A T C
T C T T A C A A A

Table 5. Motifs present in both the *C. crescentus* and *S. meliloti* chromosomal origins of replication. The positions of the sequences (in base pairs) in the 3 kb *oriC* are provided as starting numbers to the left of the sequence and ending numbers to the right of the sequence.

8-mer	GGCCTTCC	
101	A-------	94
514	---G----	521
1096	---G----	1103
1446	-------G	1453
2914	-------G	2921
8-mer	AAGCCCGG	
1451	---G----	1444
1734	--A-----	1741
1849	-T------	1856
1916	-----A--	1923
2521	-C------	2514
2573	-------A	2566
2922	-G------	2929

Table 6. Repetitive motifs that are found in the *S. meliloti* chromosomal origin of replication. The positions of the sequences in the 3 kb *oriC* (in base pairs) are provided as starting numbers to the left of the sequence and ending to the right of the sequence.

11-mer	CATCGTCATCC	
20	----------	10
541	-----CG----	551
1067	T----------	1057
1654	A---C------	1664

8-mer	CGCCGCCG	
353	-------A	360
683	----A---	690
753	--------	746
872	-------C	865
977	--G-----	984
984	--G-----	977
1103	---G----	1110
1125	----A---	1132
1924	--------	1931
2042	--------	2049
2301	--------	2308
2669	-------C	2662
2788	---G----	2781
2929	G-------	2936

Figure 6. Southern blot of *EcoRV* restricted genomic DNA prepared from cultures inoculated with either a single small Rm5004 (pTH838) transconjugant colony or a single large Rm5004 (pTH838) transconjugant colony and control strains. Lanes: 1, small #1; 2, small #2; 3, small #3; 4, large #1; 5, large #2; 6, large #3; 7, K569; 8, Rm1021; 9, Rm5004; 10, EcoRV restricted pTH838 plasmid DNA. The suicide plasmid pUCP30T (backbone of pTH838) was randomly prime labeled and used as a probe.

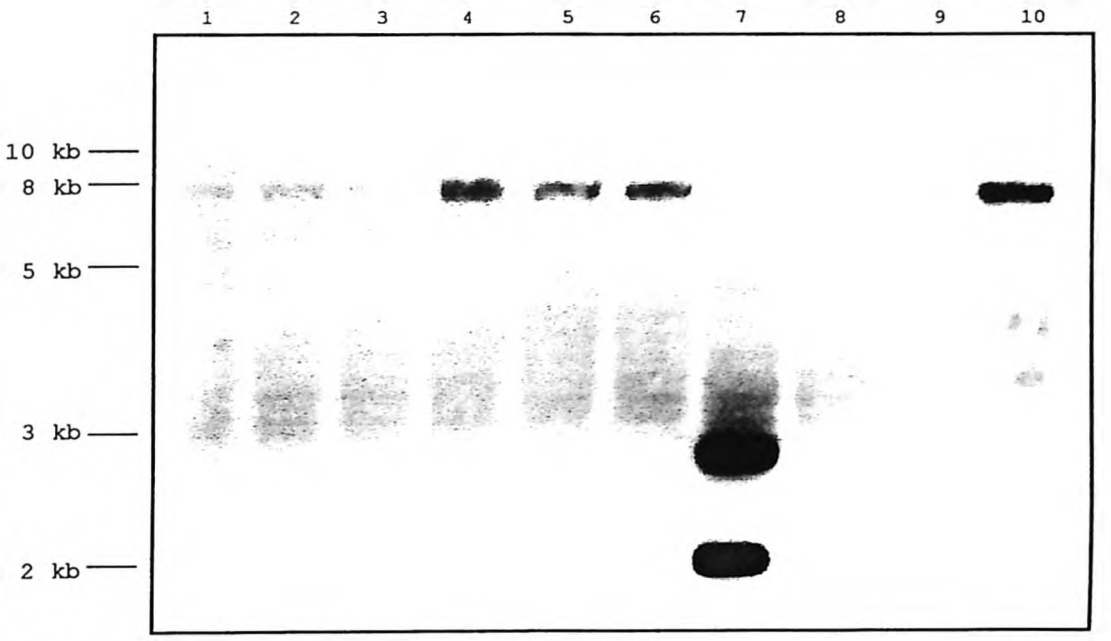

1
2
3
4
5
6
7
8
9
10
10 kb
8 kb
5 kb
3 kb
2 kb

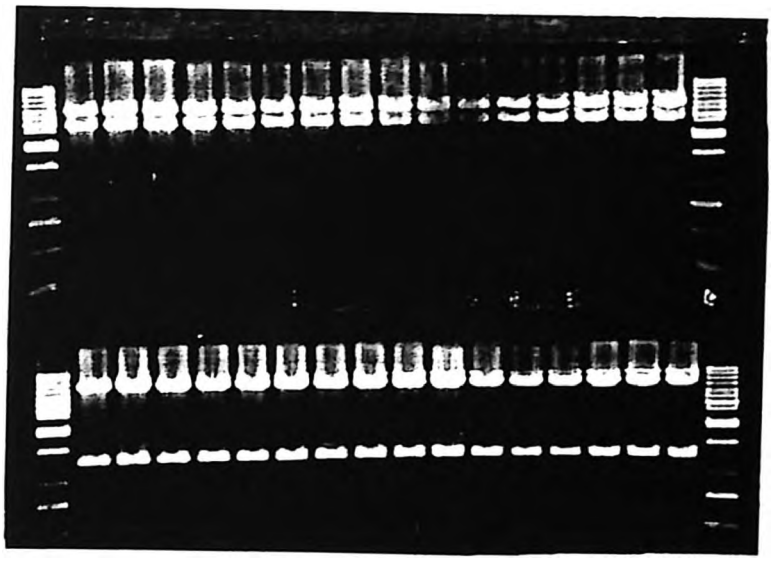
Table 7. Transconjugation frequencies per recipient and donor resulting from the transfer of pTH838 from *S. meliloti* into *E. coli*. Three *S. meliloti* strains were used as donors, *E. coli* MT620 was used as the recipient and MT616 was used as the mobilizing strain in the triparental mating. The mating spot was resuspended, serial diluted and plated onto selective media. Transconjugants were selected for on Rf(20 µg/ml)Gm(10 µg/ml), recipients were selected for on Rf(20 µg/ml) and donors were selected for on Sm(200 µg/ml)Gm(60 µg/ml).

Donor	Transconjugation frequency (# transconjugants / # *E. coli* recipients)	Transconjugation frequency (# transconjugants / # *S. meliloti* donors)
Rm5004(pTH838)large	0.23	0.13
Rm5004(pTH838)small	0.28	0.13
K569	0	0
MT616	0	0

Figure 7. Restriction digest of 8 plasmids prepared from MT620 (pTH838) transconjugants. From left to right; 1 kb ladder, 8 plasmids that were transferred from K1012, 8 plasmids that were transferred from K1013, 1kb ladder. (A) *EcoRI* digest (B) *HindIII* digest.

A

B



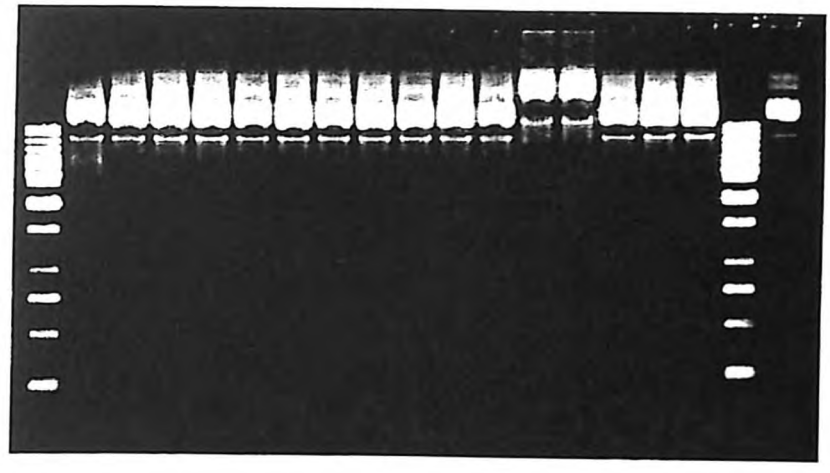
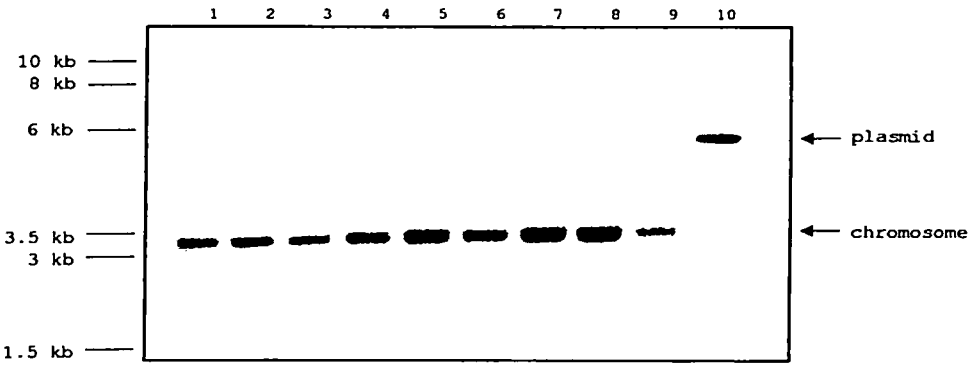


Figure 9. Southern Blot of *HindIII* restricted genomic DNA prepared from cultures inoculated with either a single small Rm5004 (pTH838) transconjugant colonies or a single large Rm5004 (pTH838) transconjugant colonies. Lanes: 1, small #1; 2, small #2; 3, small #3; 4, large #1; 5, large #2; 6, large #3; 7, RmK569 (ΦpUCP30T *pstS*); 8, Rm1021 (wildtype); 9, Rm5004; 10, *HindIII* restricted pTH838 plasmid DNA. A PCR product of the 477 bp *hemE - Y02793* intergenic region was randomly prime labeled 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 average copy number was calculated as an average of the three independent colonies tested.



Relative Copy Number (Plasmid signal / chromosome signal)	0.05	0.059	0.049	0.13	0.11	0.166				

Average copy # for small colony = 0.053

Average copy # for large colony = 0.135

Table 8. Rm5004(pTH838) small and large purified transconjugants were grown in LBmc Sm(100 µg/ml)Gm(30 µg/ml) and then plated onto LB without antibiotic. Plates were incubated at 30°C for 6 days and then 200 colonies were patched back onto selective media LB Sm(200 µg/ml)Gm(60 µg/ml) and plates were incubated for 4 days.

Strain	Number of colonies that grew on LB Sm(200 µg/ml)Gm(60 µg/ml)
Rm5004 (pTH838)large	20 / 200
Rm5004 (pTH838)small	13 / 200
Rm5004	0 / 200
RmK569	200 / 200

Figure 10. Promoter strengths from the wildtype and mutant *hemE - Y02793* intergenic region. The wildtype and mutant *hemE - Y02793* intergenic regions were cloned into the transcriptional reporter plasmid pFUS1 in both orientations and β-glucuronidase assays were preformed on Rm5004 transconjugant strains. Transcriptional activity is expressed in Miller Units and standard deviations were calculated from triplicate samples.

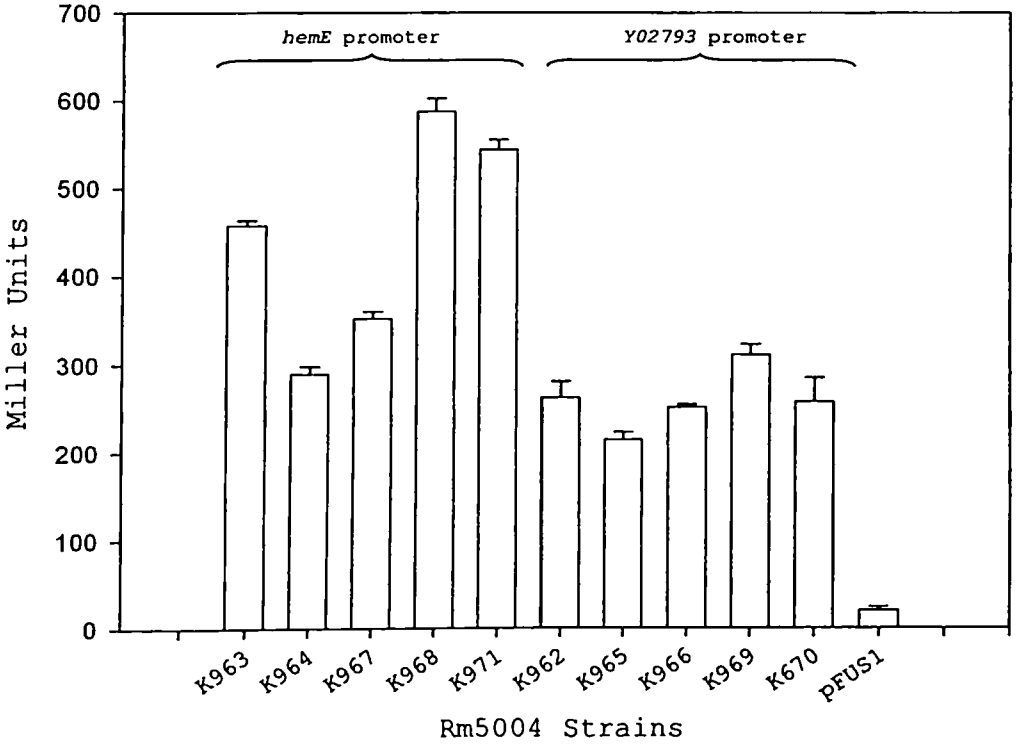

700
600
500
400
300
200
100
0
Miller Units
hemE promoter
Y02793 promoter
K963
K964
K967
K968
K971
K962
K965
K966
K969
K670
pFUS1
Rm5004 Strains

Figure 11. Southern blot of *EcoRI* restricted genomic DNA prepared from Rm5004 strains harboring pFUS1 and pBBR1MCS5 plasmids with the cloned *hemE - Y02793* intergenic region. Genomic DNA was prepared from the same cultures used in the GusA enzyme assays (Figure 10). The 477 bp intergenic region was random prime labeled and used as a probe. Relative copy number of the plasmids was determined by calculating the ratio plasmid signal to chromosome signal. Lanes: 1, Rm1021; 2, Rm5004; 3, K963; 4, K964; 5, K967; 6, K968; 7, K971; 8, K972; 9, K973; 10, K974; 11, K975; 12, K976.

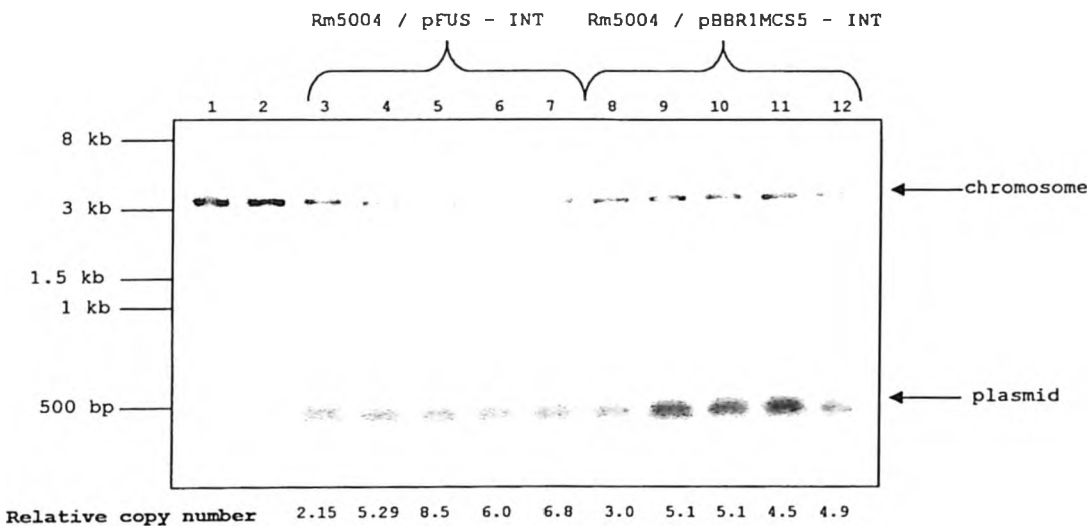

Rm5004 / pFUS - INT
Rm5004 / pBBR1MCS5 - INT
1 2 3 4 5 6 7 8 9 10 11 12
8 kb
3 kb
1.5 kb
1 kb
500 bp
chromosome
plasmid
Relative copy number
2.15 5.29 8.5 6.0 6.8 3.0 5.1 5.1 4.5 4.9

Figure 12. Relative copy numbers of pFUS1 and pBBR1MCS5 plasmids with the cloned wildtype and mutant *hemE - Y02793* intergenic regions. The intergenic regions from the wildtype pTH838 *oriC* plasmid and the intergenic regions from pTH1186, pTH1206, pTH1207 and pTH1253 corresponding to 4 base pair deletions in the XmaIII site, DnaA box 2, DnaA box 3 and DnaA box 4 respectively, were cloned into both the transcriptional reporter plasmid pFUS1 and the broad host range plasmid pBBR1MCS5. The relative copy numbers were calculated by quantification of band intensities from a southern blot (Figure 11) and are represented graphically. The relative copy numbers of the pFUS1 reporter plasmids containing the wildtype and mutant *hemE* promoters upstream of the *gusA* gene were calculated. The copy number calculations of reporter plasmids with the intergenic region in the opposite orientation were not done.

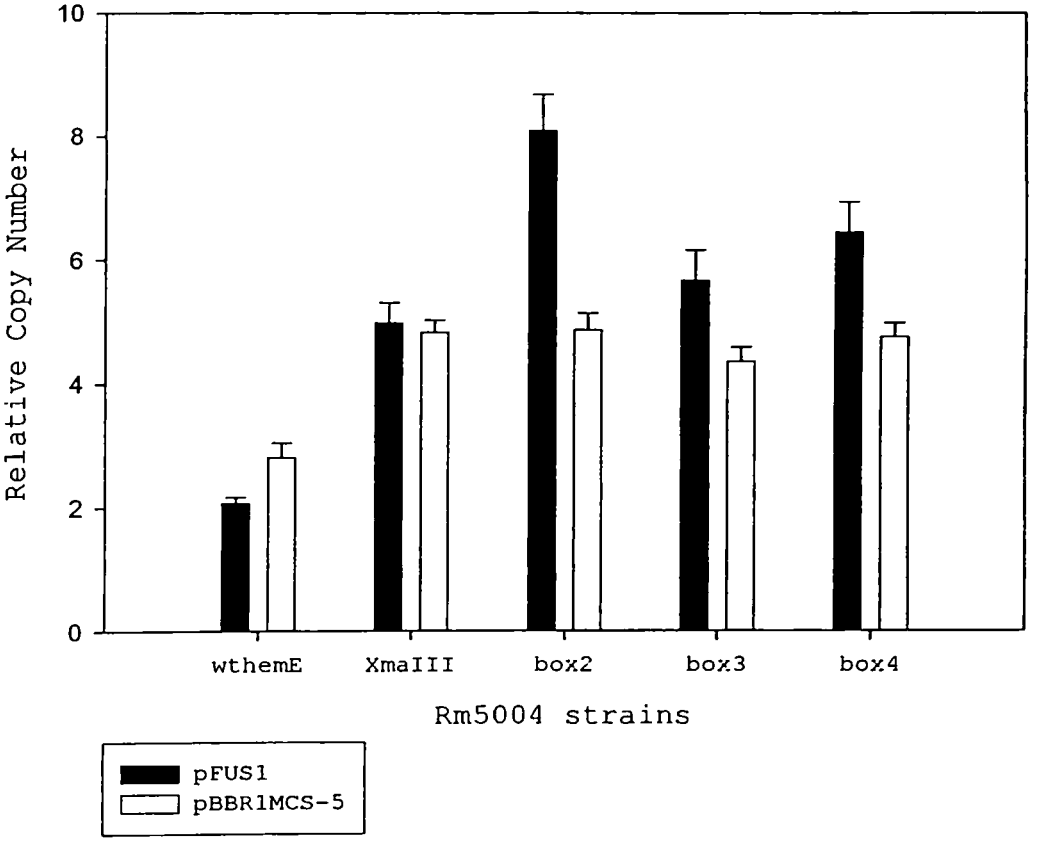

10
8
6
4
2
0
Relative Copy Number
wthemE
XmaIII
box2
box3
box4
Rm5004 strains
pFUS1
pBBR1MCS-5

Figure 13. Activity from wildtype and mutant *hemE* promoters. The mutant promoters tested contain four base pair deletions in the intergenic XmaIII site, DnaA box 2, DnaA box 3 and DnaA box 4. The Miller units calculated from the β-glucuronidase assays and the corrected Miller units (which were calculated by dividing Miller units by the relative copy number of the reporter plasmid) are compared graphically.

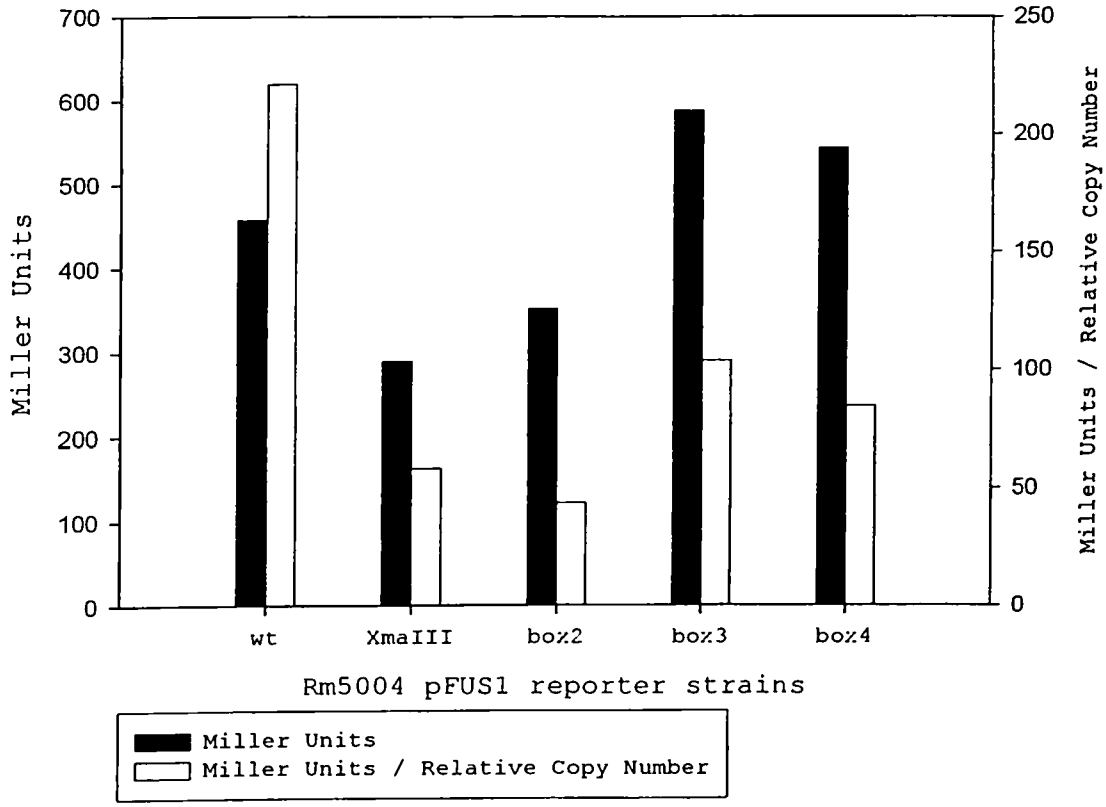
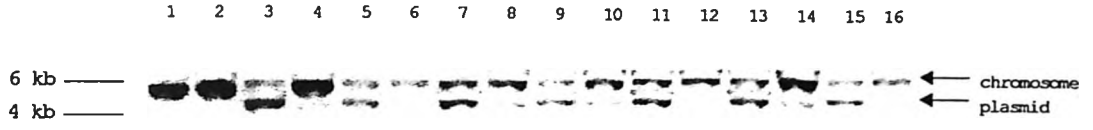
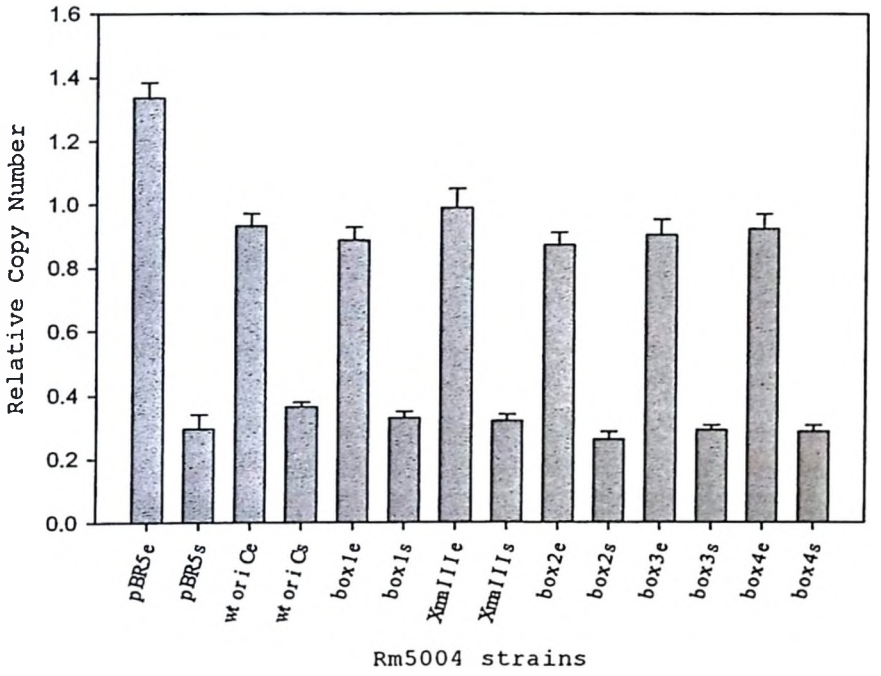

700
600
500
400
300
200
100
0
Miller Units
250
200
150
100
50
0
Miller Units / Relative Copy Number
wt
XmaIII
box2
box3
box4
Rm5004 pFUS1 reporter strains
Miller Units
Miller Units / Relative Copy Number

Figure 14. Southern blot of *EcoRI* restricted genomic DNA prepared from Rm5004 strains harboring pBBR1MCS5 plasmids with cloned 3 kb wildtype and mutant origins from exponential and stationary phase cultures. The random prime labeled 800 base pair *dnaA* promoter and linearized pBBR1MCS5 were used as probes. Lanes: 1, Rm1021; 2, Rm5004; 3, Rm5004(pBBR1MCS5) E; 4, Rm5004 (pBBR1MCS5) S; 5, K1010 E; 6, K1010 S; 7, K1011 E; 8, K1011 S; 9, K943 E; 10, K943 S; 11, K945 E; 12, K945 S; 13, K946 E; 14 K946 S; 15, K947 E; 16, K947 S. E = exponential phase culture (OD600 = 0.1) and S = stationary phase culture (OD600 = 7.0). Relative copy numbers are reported below each lane as a ratio of plasmid signal (4.7 kb) to chromosome signal (5.9 kb).



Relative copy number	1.3	0.3	0.93	0.37	0.89	0.33	0.99	0.32	0.88	0.26	0.90	0.29	0.92	0.29

Figure 15. Graphical representation of the relative copy number of pBBR1MCS5 and pBBR1MCS5 derivative plasmids with 3 kb cloned wildtype and mutant *oriC* sequences. The mutant origins tested included a three base pair deletion in DnaA box 1, and four base pair deletions in: the intergenic XmaIII site, DnaA box 2, DnaA box 3 and DnaA box 4. Relative copy numbers were calculated from total DNA preprared from strains in early exponential phase (e) (OD_{600} = 0.1) and late stationary phase (s) (OD_{600} = 7.0) as the ratio of plasmid signal to chromosome signal.


Relative Copy Number
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pBR5e
pBR5s
wt oriCe
wt oriCs
box1e
box1s
XmIIIe
XmIIIs
box2e
box2s
box3e
box3s
box4e
box4s
Rm5004 strains

CHAPTER 4. THE SINORHIZOBIUM MELILOTI REPLICATION INITIATOR DnaA

Purification of the *S. meliloti* DnaA protein

To initiate a biochemical investigation of the *S. meliloti* chromosomal origin we sought to purify the protein responsible for the initiation of chromosome replication in many bacteria (Skarstad and Boye, 1994). Initially DnaA was overexpressed as a translational fusion to six histidine residues (pBADHisA) but the overexpressed protein was predominantly represented in the insoluble fraction of the *E. coli* cell lysate. DnaA was then overexpressed as a translational fusion to the *Schistosoma japonicum* glutathione S-transferase (pGEX5X-1). This overexpressed fusion protein was soluble however for unknown reasons it would not efficiently bind to the glutathione agarose resin. DnaA was subsequently overexpressed with an N-terminal translational fusion to a thrombin recognition sequence, six histidine residues and the *E. coli* NusA protein. (pET431a). NusA is predicted to be the most soluble *E. coli* protein and is advantageous in solubilizing

many insoluble target proteins not only because of its solubility but also because of its non toxic high expression levels (Davis *et al.*, 1999).

Optimal conditions for fusion protein expression in BL21 STAR was determined to be growth of the culture (J1027) at 37°C to an OD600 = 0.6 before the addition of the lactose analogue IPTG to a final concentration of 0.3 mM. The culture was then grown for an additional 2.5 hours at a reduced temperature of 30°C. A cell lystae was prepared with lysozyme treatment and sonication. The fusion was then purified via nickel affinity chromatography. The imidazole was removed from the eluted sample with dialysis and the NusAHis$_6$ tag was liberated from DnaA with a thrombin protease digestion. Purified DnaA has an additional 18 N-terminal amino acids after the cleavage with thrombin. The NusAHis$_6$ tag was then purified away from DnaA by nickel affinity chromatography. Figure 16 shows a typical purification of *S. meliloti* DnaA.

DnaA binds to putative DnaA boxes in the *S. meliloti oriC*

Mutaions made in the putative DnaA binding sites in the *hemE - Y02793* intergenic region abolish replication of

plasmid borne *oriC*. Purified DnaA was used in an electrophoretic mobility shift assay to test the ability of DnaA to bind to the *oriC* locus.

The six DNA sequences that were used in DnaA binding experiments included: a 190 base pair probe that contained all three DnaA boxes in the *hemE - Y02793* intergenic region (DnaA boxes 2, 3, and 4), a 178 base pair probe with the same sequence as the 190 base pair probe with four base pair deletions in all 3 DnaA boxes, a 117 base pair probe containing DnaA box 5 downstream of *hemE*, a 197 base pair fragment from the *dnaA* promoter that contains a predicted DnaA box that overlaps the translational start site of the *dnaA* gene, a 233 base pair *repA2* promoter probe and a 125 base pair *repA1* promoter probe containing 2 unusual DnaA boxes. Complexes were resolved on a 4% polyacrylamide gel and were only detected when the gel was run at 144 V/cm for 10 minutes once the binding reactions had been added to the wells and then reduced to 9 V/cm for an additional 2.5 hours.

Two complexes were resolved with the 190 base pair probe (Figure 17) however in some cases using this probe resulted in the formation of 3 complexes which is consistent with the number of bands that should result

based on the predicted number of binding sites in the probe. The 178 base pair probe was created with three rounds of site-directed mutagensis on pTH838 to generate a template that could be used for PCR amplification of the probe. This was a very useful target DNA in the electrophoretic mobility shift assay because with comparison to the complexes formed with the 190 base pair probe it can be deduced that that the mutations that prevent replication from the cloned *oriC* also prevent wildtype interaction of the DnaA protein with the *hemE* - *Y02793* intergenic region. One faint band was detected with the 178 base pair probe in the presence of 300 ng and 500 ng of protein suggesting that DnaA is still able to interact with this target DNA. A faint band is observed only in lanes with protein added to the 117 base pair probe containing DnaA box 5. No complexes appeared to be formed with the 197 base pair *dnaA* promoter or the 125 base pair *repA1* promoter. A complex was formed on the 233 base pair *repA2* promoter. Band intensities for all shift bands that appear in Figure 17 are provided in Table 9. Band intensities increase with the increasing amounts of protein added to the binding reaction.

A mutation in the DnaA box upstream of the *repA2* gene moderately reduces *repA2* transcription

The results of the electrophoretic mobility shift assay revealed that DnaA was interacting with sequence upstream of the *repA2* gene. We decided further investigate this binding site because of its location at 158 base pairs upstream of the translational start site of *repA2*. We had previously detected promoter activity within 100 base pairs of the translational start site of the *repA2* gene and therefore hypothesized that DnaA may be acting a transcriptional regulator of the *repA2B2C2* operon. We cloned 518 base pairs upstream of the *repA2* gene into the transcriptional *gfp* reporter plasmid pOT1. Site-directed mutatgenesis was then done on this plasmid (pTH1465) to create a four base pair deletion in the DnaA binding site. DnaA box 2 (in *oriC*) and the binding site upstream of *repA2* have the same sequence (TCATCCACA) and the same four base pairs have been removed in both mutant binding sites.
The reporter plasmids were then transferred into *A. tumefaciens* At123 via conjugation because this region exerts strong incompatibility against the pSymA megaplasmid and thus promoter strength had to be assayed in a surrogate strain. The amount of Green Fluorescent Protein was

quantified with a Tecan Safire Fluorimeter/Spectrophotometer and specific activity was calculated by dividing fluorescent values by the optical density at 600 nm of the tested culture. These values are reported graphically in Figure 18. The four base pair deletion in the DnaA box 158 base pairs upstream of the translation start site of *repA2* mildly reduced the transcriptional activity of the *repA2* gene.

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

We speculated that the low copy number (less than one) of minichromosomes replicating from *oriC* may be due to a limited amount of DnaA in the *S. meliloti* cell and thus became interested in the effect of overexpressing DnaA in *S. meliloti*. The *dnaA* gene including 20 upstream base pairs (containing a predicted ribosome binding site) was PCR amplified from genomic DNA using a sense primer that had three engineered stop codons in all three reading frames to prevent a translational fusion to the LacZα peptide. The PCR product was directionally cloned into pBBR1MCS5 thus *dnaA* gene expression is under the transcriptional control of the *E. coli lac* promoter.

Colonies harbouring plasmids that containing the *dnaA* insert were selected for in a conventional blue white screen on LB Gm Xgal plates. A single white colony containing the cloned *dnaA* gene in pBBR1MCS5 (pTH1091) was streak purified on a LB Gm Xgal plates and the strain maintained a white colour and uniform colony size throughout the purification. A frozen permanent was made (J1037) and when this frozen permanent was struck out onto LB Gm Xgal plates both blue and white colonies with streaky growth was observed. Suspecting contamination the pTH1091 plasmid was retransformed into *E. coli* DH5α and plated onto LB Gm Xgal and all transformant colonies were white with uniform size, a single colony was streak purified maintaining the white colony colour and a new frozen permanent was prepared. Yet again when the frozen permanent was struck onto LB Gm Xgal plates both blue and white colonies with streaky growth was observed. We decided to look at these transformant colonies under the microscope to see if overexpression of the *S. meliloti* DnaA protein had any morphological consequences in *E. coli*. Light microscopy of crystal violet stained *E. coli* cells revealed that expression of *S. meliloti* DnaA fom P*lac* caused many of the *E. coli* cells in the culture to grow as

long filaments. The overexpressing *E. coli* strains were further examined with the Environmental Scanning Electron Microscope (Figure 19). This filamentous phenotype was observed from all colony types that resulted from streaking out the frozen permanent.

The pTH1091 overexpressing plasmid was transferred into Rm1021 and Rm5004 with a transconjugation frequency into both strains of 10^{-4} transconjugants per recipient cell. The empty pBBR1MCS5 plasmid transferred into both strains at a much greater frequency of 10^{-1} transconjugants per recipient cell. When the mating spots were serial diluted and plated onto selective media pTH1091 transconjugant colony formation took approximately six days as opposed to four days for the pBBR1MCS5 transconjugants. The Rm1021 (pTH1091) and Rm5004 (pTH1091) transconjugants were of two types, most of the colonies were small however some of the transconjugant colonies were much larger and appeared to develop normally in four days. Cells in both types of colonies were examined with the light microscope. Cells from the large colonies were the same size and shape as wildtype cells. Cells from the small colonies were of a much different nature and the vast majority of the cells in the colony appeared to be growing as complex filaments much

larger than wildtype cells. Several small colonies were used to inoculate LBmc Sm Gm and the culture was grown for 6 hours and then prepared for examination with the Enivronmental Scanning Electron Microscope. If overnight cultures were grown from a small pTH1091 transconjugant all cells appeared indistinguishable from wildtype cells presumably do to the strong pressure to inactivate the DnaA overexpression. This strong pressure to inactivate either the P*lac* promoter or the *dnaA* gene itself is likely the reason for the presence of the large pTH1091 transconjugant colonies. The block in cell division is very striking as a result of DnaA overexpression, with many of the cells up to 15 µm in length as oppose to the wildtype cells of 1 µm. The *S. meliloti* filaments are complex because in most cases they have many branches and swollen areas flanked by regions that appear to be partially sepatated. Many of the cells that are overexpressing DnaA appear to look very similar to the Y shaped differentiated bacteroids that are found inside plant cells (Figure 19).

Figure 16. 10% SDS PAGE showing the purification of *S. melioti* DnaA (57 kDa) from an overexpressing *E. coli* cell lysate (J1027). DnaA was overexpressed as a fusion protein to *E. coli* NusA with a His_6 tag (pET431a). Lanes: 1, induced J1027 cell lysate; 2, an elution from a Ni column after purification; 3, thrombin digested fusion protein liberating the $NusAHis_6$ tag. DnaA was subsequently purified away from the $NusAHis_6$ tag via nickel affinity chromatography. Lanes 4, is purified DnaA and lanes 6 - 8 are 20 mM, 40 mM and 250 mM imidazole washes respectivley showing the removed $NusAHis_6$ tag.

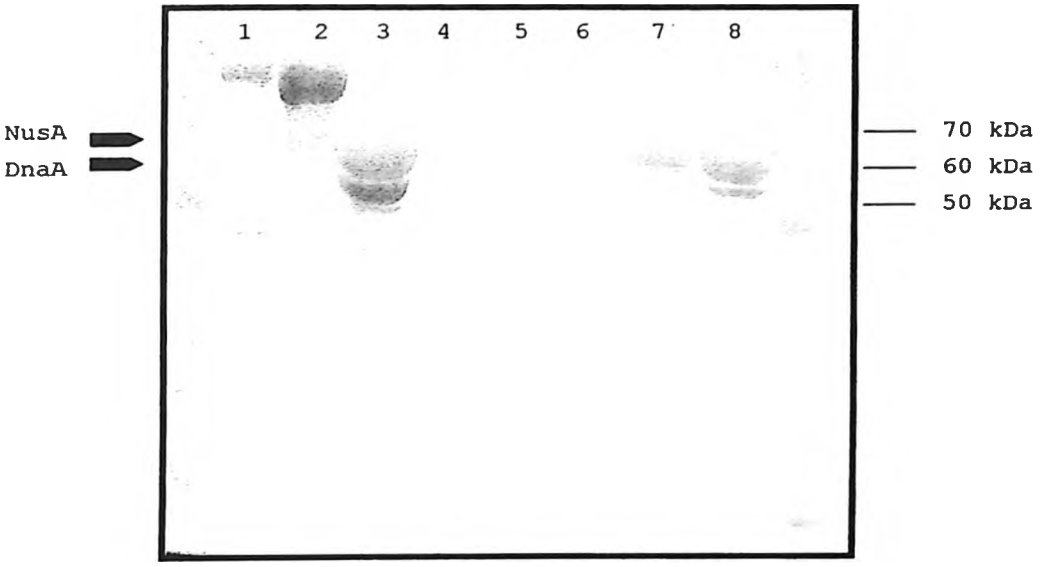

1
2
3
4
5
6
7
8
NusA
DnaA
70 kDa
60 kDa
50 kDa

Figure 17. Electrophoretic Mobility Shift Assay with target DNA containing predicted DnaA boxes and purified *S. meilioti* 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 ng, 300 ng and 500 ng) and the DNA fragments were also loaded without protein added. The size and description of the DNA target loaded is indicated above the lanes. The first lane on the left for each DNA target is DNA without protein added and the next three lanes are the DNA sample incubated with the amount of purified DnaA shown below the lanes.

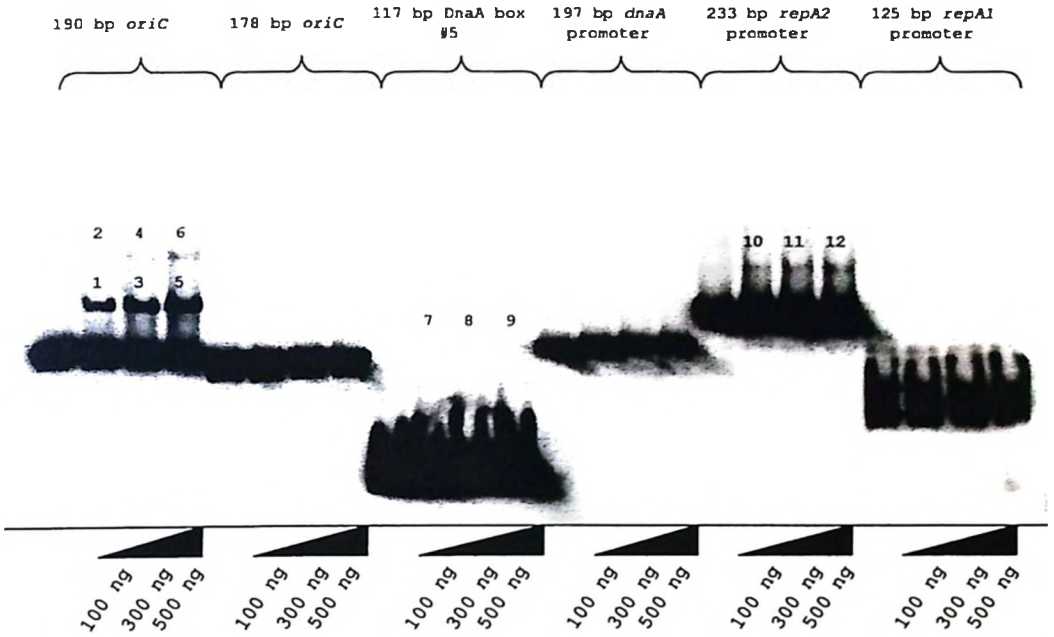

190 bp oriC
178 bp oriC
117 bp DnaA box #5
197 bp dnaA promoter
233 bp repA2 promoter
125 bp repA1 promoter
1
2
3
4
5
6
7
8
9
10
11
12
100 ng
300 ng
500 ng

Table 9. Integrated intensities of all protein complexs in Figure 17. The shifted band number appears above the band in Figure 17. The values were calculated from the same number of pixels for each band using the ImageQuant 5.2 software.

Shifted complex	Intensity
1	9.5×10^5
2	6.9×10^4
3	2.0×10^6
4	2.1×10^5
5	2.6×10^6
6	3.6×10^5
7	9.2×10^4
8	1.0×10^5
9	3.5×10^5
10	6.4×10^5
11	7.1×10^5
12	7.6×10^5

Figure 18. Wildtype and mutant *repA2* promoter activities. A 518 base pair sequence upstream of the *repA2* gene was cloned into the transcriptional reporter plasmid pOT1 (pTH1465). Four base pairs from a DnaA binding site 158 base pairs upstream of the *repA2* translational start site were deleted with site-directed mutagenesis on pTH1465. All plasmids that were sequenced after the mutagenesis contained the desired mutation however only one contained only this mutation upstream of *repA2* (pTH1527) and all others (pTH1524, pTH1525, pTH1526, pTH1528 and pTH1529) contained the four base pair deletion but mutations elsewhere in the 518 base pair insert. The cloned wildtype *repA2* promoter and all mutant constructs were transferred into At123 and the amount of Green Flourescent Protein was assayed and is expressed in specific activity (fluorescence / $absorbance_{600nm}$).

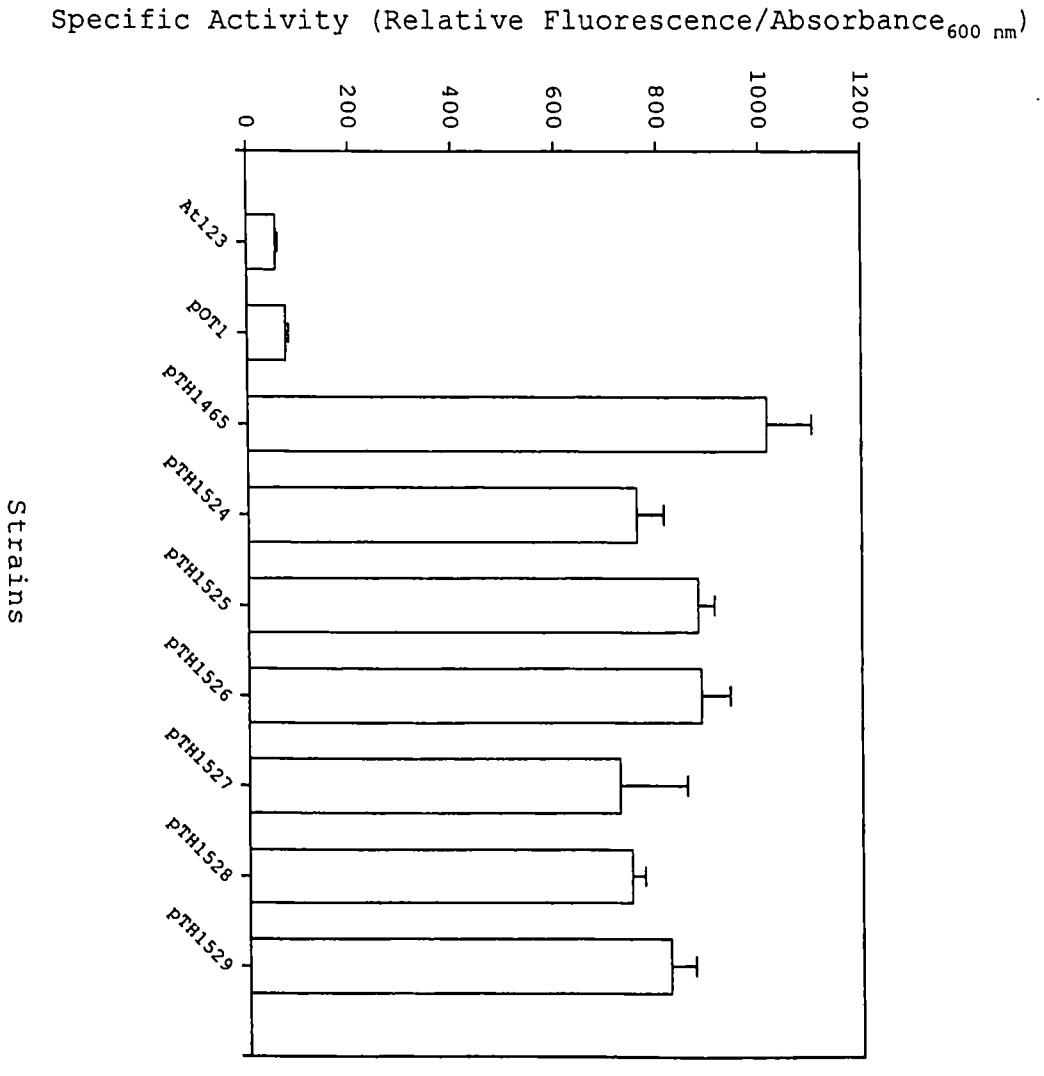
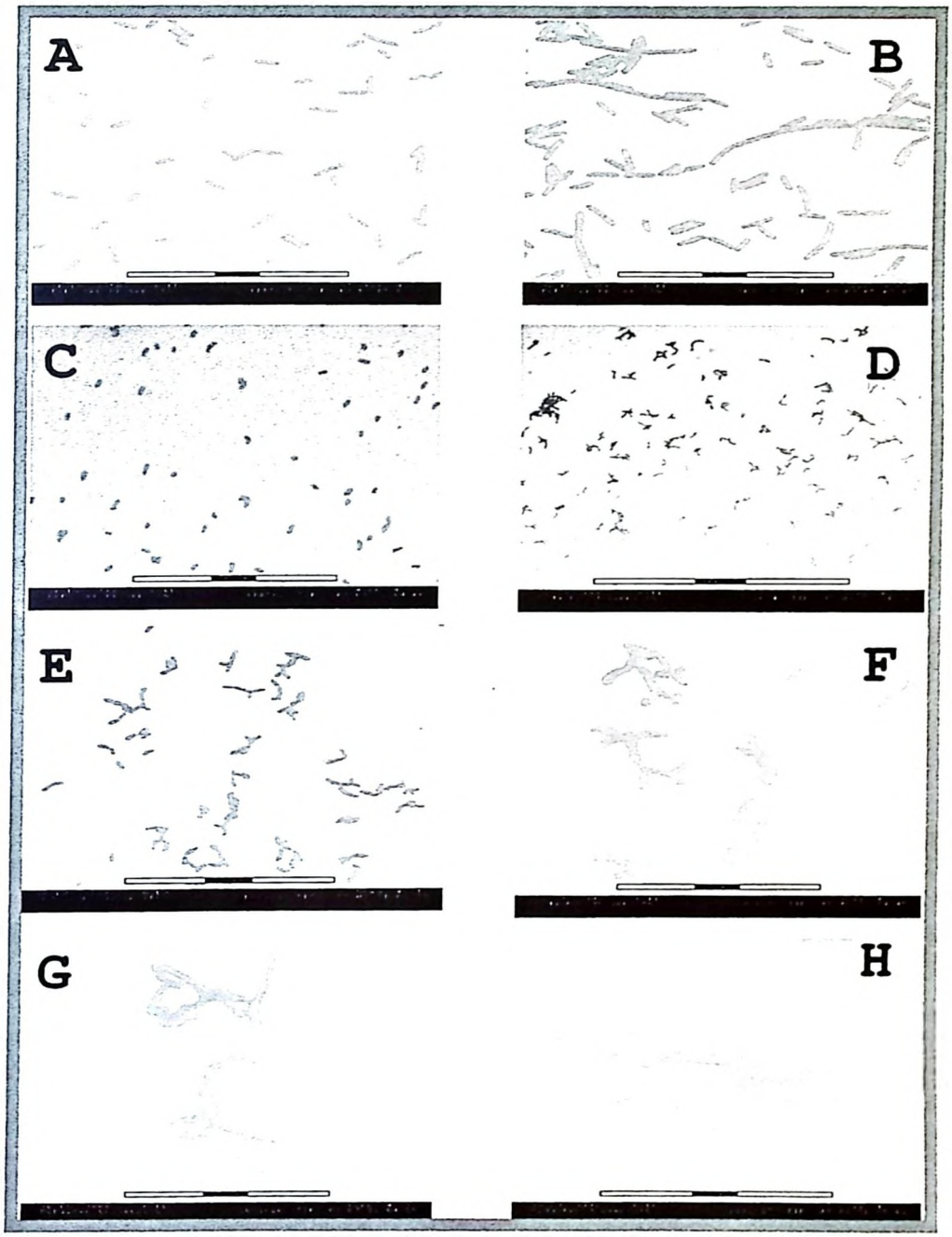

Specific Activity (Relative Fluorescence/Absorbance600 nm)
0
200
400
600
800
1000
1200
Atc123
pOT1
pTH1465
pTH1524
pTH1525
pTH1526
pTH1527
pTH1528
pTH1529
Strains

Figure 19. Environmental Scanning Electron Micrographs of *E. coli and S. meliloti* cells overexpressing *S. meliloti* DnaA from the *E. coli lac* promoter (P*lac*) in plasmid pBBR1MCS5. Panel A (1950x) shows *E. coli* DH5α harbouring pBBR1MCS5 and panel B (1950x) shows *E. coli* DH5α expressing DnaA from P*lac*. Panel C (1500x) shows wildtype Rm1021 harbouring the pBBR1MCS5 plasmid and panels: D, 550x; E, 1500x; F, 3000x; G, 3000x; and H, 5000x are wildtype Rm1021 cells expressing DnaA from the pTH1091 overexpression plasmid.


A
B
C
D
E
F
G
H

CHAPTER 5. DISCUSSION

This thesis reports the cloning and characterization of the *S. meliloti* chromosomal origin of replication (*oriC*). The identification of this chromosomal origin of replication is the first to be localized in a multipartite bacterial genome. The position of the origin on the chromosome provides insight into chromosomal gene dosage, genes closely linked to the origin will have an increased gene dosage for parts of the cell cycle. This work has expanded our knowledge on alphaproteo-bacterial origins as this is the second chromosome origin to be characterized from this class of bacteria. A better understanding of how the genetic material of these organisms is replicated may ultimately become useful in providing treatments for both plant and animal diseases.

The ColE1 suicide plasmid pUCP30T is able to autonomously replicate in an *S. meliloti recA*::Tn5 strain (Rm5004) when the 3 kb region spanning the *hemE* and *Y02793* genes is cloned into the plasmid. This recombinant plasmid (pTH838) is efficiently mobilized out of Rm5004 into an *E.*

coli host whereas a donor strain in which pUCP30T is integrated into the chromosome shows no mobilization. The ability of pTH838 to be maintained as a plasmid and not as a cointegrant (as shown with a southern blot) strongly suggests that pTH838 carries a sequence sufficient for autonomous replication. This ARS is encoded on the *S. meliloti* chromosome and thus it is very likely that this sequence cloned in pTH838 represents the chromosomal origin of replication.

The *oriC* is the third autonomously replicating sequence to be localized in the *S. meliloti* genome. Margolin and Long (1993) isolated the first autonomously replicating sequence from *S. meliloti* and it was located on the pSymB megaplasmid. A library was made of *EcoRI* or *HindIII*-cleaved total genomic DNA cloned into a derivative of pBR322, pZC9. The library was transferred into an *S. meliloti* recombination-deficient *recA* strain. Autonomously replicating plasmid DNA from the transconjugants was mated back into *E. coli* and plasmids were analyzed by restriction. Plasmid DNA was also prepared from the transconjugants (undetectable on EtBr stained agarose gels) and used to transform *E. coli*. The smallest fragment capable of supporting autonomous replication was 0.8 kb and

contained three putative DnaA boxes. A southern blot using the minimal 0.8 kb ARS to probe transconjugant total DNA revealed similar intensities of the autonomously replicating plasmid and pSymB signals suggesting that mini-pSymB was under the same copy number control as the resident megaplasmid. The ARS was transductionally mapped to approximately 12 kb from the Ω5007 insertion. This was an unexpected finding considering that this region can be deleted from the megaplasmid (Charles and Finan, 1991). This ARS could only replicate in *A. tumefaciens* strains that harboured the pSymB megaplasmid suggesting a trans acting factor is required for the autonomous replication ability of this sequence (Margolin and Long, 1993). To extend this observation we have only ever detected *S. meliloti* transconjugants when this sequence (cloned onto a suicide plasmid) is transferred into strains that contain this homologous sequence on pSymB. We have never detected transconjugants in *S. meliloti* strains in which the region coding for this apparent ARS has been deleted (MacLellan, 2004) suggesting that a trans acting factor encoded outside the 0.8 kb sequence is required for replication or that a homologous sequence is required for a recombination event. The strategy employed by Margolin and Long (1993) to detect

origins from the *S. meliloti* genome should have detected a 3.5 kb EcoRI ARS representing the chromosome origin. However, it is likely that because of the six days required for small transconjugant colonies to form when *oriC* plasmids are transferred into *S. meliloti*, the chromosome origin was missed in this screen.

The second autonomously replicating sequence reported for *S. meliloti* was also located on the pSymB megaplasmid. A 60 kb segment of the megaplasmid was rescued as a plasmid in *E. coli* using oriT-directed recombination (Chain *et al.*, 2000). The *repABC* genes were present within this 60 kb segment of pSymB. RepA and RepB are likely involved in plasmid partitioning and RepC is presumed to play a role in the replication initiation reaction at the pSymB origin of replication. This 60 kb was further subcloned revealing that only *repC* and part of *repB* is required for autonomous replication of the suicide plasmid pUCP30T in *A. tumefaciens*.

The identification of the ARS reported in this work is the first of its type in *S. meliloti* as it is encoded on the chromosome. To test the sequence limits required for the autonomous replication ability of the sequence from the *S. meliloti* chromosome we used the formation of *S. meliloti*

Rm5004 transconjugant colonies upon transfer of various recombinant *oriC* plasmids. These experiments suggest that the size of *oriC* is much larger than the other well characterized minimal alphaproteo-bacterial origin from *C. crescentus* of 437 bp (Marczynski, Lentine, and Shapiro, 1995). The minimal size of the *S. meliloti* oriC is 1802 base pairs (Figure 3). This size may be significantly reduced if the *hemE* open reading frame is not required but a deletion analysis has not been done. The 5′ sequence limits of *oriC* have been mapped to within 19 base pairs downstream of *hemE* and encoded within the 19 base pairs is a perfect match to the DnaA box (TGATCCACA) found in the *C. crescentus* chromosome origin. Four other DnaA boxes that match the expanded *E. coli* DnaA binding consensus sequence (Schaefer and Messer, 1991) are present in the 3 kb PCR product cloned in pTH838. Three of these binding sites fall into the 477 base pair *hemE* - *Y02793* intergenic region. Site-directed mutagenesis creating either three or four base pair deletions in the five predicted binding sites indicated that all DnaA boxes located in the intergenic region are required for Rm5004 transconjugant colony formation.

The mutations in the DnaA boxes in the *hemE - Y02793* intergenic region as well as a four base pair deletion in an XmaIII recognition site were tested to determine if they altered the *hemE* and *Y02793* promoter activities. Initially the β-glucuronidase assay on Rm5004 strains containing recombinant pFUS1 reporter plasmids with wildtype and mutant intergenic sequences cloned in both orientations revealed that all mutations in the intergenic region appeared to alter *hemE* transcription with little effect on the *Y02793* promoter. We were unsure what effect cloning the intergenic sequences into pFUS1 would have on the copy number or the reporter and hence the gene dosage of *gusA*. Therefore GusA activity was corrected by dividing the specific activity (Miller units) by the relative copy number of the reporter plasmid (determined by quantification of band intensities in a Southern blot). The corrected units revealed that all mutations in the intergenic region reduced transcription of *hemE*. It is unclear if the variability in copy number of the recombinant pFUS1 plasmids was due to the cloned DNA or if pFUS1 is naturally maintained in *S. meliloti* cell in variable numbers. Recall that all of the mutations in the *hemE - Y02793* intergenic region in the context of the 3 kb

oriC sequence abolish transconjugant colony formation when these mutant plasmids are transferred into Rm5004 (Figure 4) and thus it is likely that appropriate levels of *hemE* transcription are required for replication of the *S. meliloti* chromosome.

In *C. crescentus* an origin-internal *hemE* promoter is important for the regulation of DNA replication and all mutations that reduce *hemE* transcription from either the weak or the strong promoter also impair replication suggesting that transcription of *hemE* is required for replication. The strong *hemE* promoter (originating inside *Cori*) has been implicated as a developmentally regulated promoter because there is preferential transcription from this promoter at the stalked cell pole (cell undergoing DNA replication) and when transcription from this strong promoter is impaired such that it is equally expressed at both cell poles, the control of replication is lost in progeny cells (Marczynski, Lentine, and Shapiro, 1995).

A possible reason why the mutations that prevent DnaA binding in the *S. meliloti oriC* also reduce transcription of *hemE* is the requirement for DnaA to melt the adjacent A/T rich region of DNA in turn promoting the assembly of transcriptional macninery upstream of the *hemE* gene. It

would be interesting to determine if multiple *hemE* promoters exist as they do in *C. crescentus*. Perhaps a transcript from one of these promoters may be acting as an RNA primer for replication from *oriC* as is speculated to be the case on the *C. crescentus* chromosome.

Transconjugant colony formation upon transfer of recombinant *oriC* plasmids into Rm5004 always resulted in both small and large colonies with both types taking longer to form on selective plates (6 days) than *S. meliloti* pUCP30T cointegrante recipients. Both small and large transconjugant colonies were observed when either small or large colonies were streak purified on LB Sm Gm plates. Total DNA was prepared from purified small colonies and large colonies. DNA was restricted and probed in a Southern blot. The intensity of the bands corresponding to the pTH838 plasmid and chromosomal signal were used to calculate a relative copy number of the autonomously replicating minichromosome (Figure 9). The result of this experiment suggests small and large colony size on selective media correlats with the copy number of the *oriC* plasmid encoding gentamicin resistance. The average relative copy number of the pTH838 plasmid in the three small and the three large transconjugants is 0.053 and

0.135 copies per chromosome respectively. These low copy number values were corroborated when transconjugant colonies were grown under non-selective conditions and then screened for retention of the gentamicin resistance encoded by the pTH838 plasmid molecule (Table 8). The very low copy number of pTH838 in both colony types together with the rapid loss of these plasmids shows that the pTH838 minichromosome is not stably maintained in *S. meliloti*. The reason for slow colony formation of a strain harbouring a plasmid encoding gentamicin resistance with a copy number of less than one is likely because after cell division events many daughter cells are produced in the cell population that do not contain a pTH838 molecule however still have the enzyme required for gentamicin resistance in the cytoplasm. Subsequent cell divisions of plasmid-less cells would ultimately diffuse out the gentamycin acetyl transferase from the cytoplasm resulting in gentamicin sensitivity. This mechanism would account for the delayed Rm5004 (pTH838) transconjugant colony formation.

Altered colony morphology has been reported for C. crescentus cells harbouring minichromosomes. Mutations were made in *C. crescentus Cori* plasmids (cannot replicate without the functional chromosome origin) and then the

plasmids were introduced into *C. crescentus*. Plasmids were scored into three classes: Rep+, Rep +/- and Rep-. Rep+ plasmids were indistinguishable from unmutated plasmids because they had: a high transformation efficiency, large colony morphology, and efficient *Cori* plasmid extraction from liquid culture. Rep+/- plasmids had similar transformation efficiencies, colonies were very tiny and plasmid yields were low from liquid culture (undetectable with EtBr staining). Another difference between Rep+ and Rep+/- *Cori* plasmids is that Rep+ plasmid can be maintained indefinitely by subculturing whereas Rep+/- plasmids quickly lost their autonomous replication potential and integrated into the chromosome. Rep- plasmids gave transformation efficiencies equal to background. Thus it appears that mutations that impaired replication in the Rep+/- plasmids do so in a way that leads to a reduced copy number and therefore tiny colonies (Marczynski, Lentine, and Shapiro, 1995) which parallels our observation of Rm5004 (pTH838) transconjugant colony formation.

It is clear that many of the requirements for minichromosome replication are not necessary for replication of the origin located on the chromosome. Models to explain replication initiation of the chromosome

created by studying plasmids dependent on *oriC* for replication may in fact be laboratory aritifacts. Examples of such artifacts are the requirements for the DNA bending protiens HU and IHF for plasmid replication which are not required for chromosome replication. It has been speculated that the reason for this is that plasmids are less capable of adopting the same DNA topology as the chromosome such as extensive negative supercoiling, which is required for efficient replication initiation on the chromosome (Asai *et al.*, 1998). The *S. meliloti* minichromosome studied in this work is obviously not behaving the same as the chromosomal copy of the origin because of the low number of minichromosomes relative to chromosomes in the Rm5004 (pTH838) transconjugants. Perhaps the pTH838 minichromosomes are not able to efficiently initiate replication because of plasmid constraints on DNA topology.

The *E. coli oriC* is the best studied chromosome origin however the high copy number of minichromsomes of ~ 38 per cell is unusual. The *pseudomonad* chromosome origins were the second class of chromsome origins to be characterized after the enteric species. Copy numbers of *pseudomonas* minichromosomes have been reported as low as 0.7 copies per

chromosome with a loss frequency of 10 -20% per generation on non-selective media (Yee and Smith, 1990).

It has been noted that the ColE1 origin exerts a strong interferring effect on *M. tuberculosis oriC* minichromosomes. The copy number of *M. tuberculosis* minichromosomes was determined to be ~ 17 copies per chromosome in a similar way that the copy number was calculated for *S. meliloti* minichromosomes in this study. However, if the ColE1 *ori* sequence was included on the *M. tuberculosis* minichromosome the copy number was reduced to 0.6 - 1.0 per chromosome suggesting that the ColE1 origin exerts a strong effect on minichromosome stability (Qin, Madiraju, and Rajagopalan, 1999). The mechanism of this interference in unknown but we do not rule out the possibility that the ColE1 origin in pUCP30T may be the cause of the instability of the pTH838 minichromosome.

We speculated that the low copy number may also be due to an incompatibility phenomenon associated with titration of host replication factors such as DnaA. The cloned chromosome *oriC* region does not exert strong incompatibility against the *S. meliloti* genome. Cloning of this region into the broad host range plasmid pBBR1MCS5 did not reduce the copy number as in the case with *B. subitlis*

in which cloning the *oriC* onto a high copy number plasmid significantly reduced the copy number (Seiki, Ogasawara, and Yoshikawa, 1981).

The replication initiator DnaA was used in an electrophoretic mobility shift assay with DNA targets from: the chromosome origin, the pSymB *repA1* promoter, the pSymA *repA2* promoter and the *dnaA* promoter. Two or three complexes of DnaA bound to a 190 base pair *oriC* probe that contains all three predicted DnaA boxes in the *hemE* - *Y02793* intergenic region were resolved. Resolution of two versus three complexes in this experiment was likely due to the amount of protein added to the binding reaction. The specificity of DnaA binding was investigated by testing a 178 base pair target with the same sequence as the 190 base pair probe but contained four base pair deletions in the three DnaA boxes. This target DNA did not efficiently form complexes with the DnaA protein and is consistent with the prediction that the mutations lay in DnaA binding sites. Binding of DnaA to the *S. meliloti oriC* is the first experimental evidence that the replication initiator is interacting with an alpha proteobacterial chromosome origin. It is noteworthy that no complexes were detected with the *dnaA* promoter fragment containing the sequence

(TCATCCGCA) matching the expanded consensus sequence overlapping the ATG translational start site for the *dnaA* gene. The dnaA gene is autoregulated in *E. coli* (Atlung, Clausen, and Hansen, 1985;Braun, O'Day, and Wright, 1985) and *B. subilis* (Ogura *et al.*, 2001) by binding to the *dnaA* promoter region but apparently does not autoregulate its expression in *Pseudomonas putida* (Ingmer and Atlung, 1992).

Interestingly, a complex was detected with the *repA2* promoter. This promoter carries the sequence TCATCCACA, which matches the sequences of a DnaA box in the chromosome origin. The location of this sequence at 158 base pairs upstream of the translational start site of the *repA2* gene is consistent with the distance from the translational start site that DnaA boxes can be located in genes influence by DnaA in *E. coli* (Messer and Weigel, 1997). The relevance of the DnaA binding site in modulating transcription of the *repA2* gene was tested using the Green Fluorescent Protein as a transcriptional reporter. A four base pair deletion in the DnaA box only modestly reduced the activity of the *repA2* promoter (Figure 18). This slight difference may be significant because it is well known that the levels of replication proteins must be

tightly regulated to ensure the proper timing of replication initiation.

A transcriptional termination assay revealed that the *E. coli* DnaA protein binds with different affinities to different DnaA box sequences and this was used to expand the DnaA box consensus sequence (Schaefer and Messer, 1991). Sequences adjacent to DnaA boxes have the potential to increase the affinity of DnaA for the binding sites by 50 times (Schaper and Messer, 1995). However, the precise sequence of the binding site is of primary importance and potentially the topology of this sequence (Schaper and Messer, 1995). It has been shown that the affinity of DnaA for a fragment of the *S. lividans dnaA* promoter region containing two DnaA boxes is ten times higher than its affinity for a single DnaA box suggesting cooperative binding (Majka *et al.*, 1999;Majka, Zakrzewska-Czerwinska, and Messer, 2001). The kinetics of the interaction of *S. meliloti* DnaA with the DnaA boxes in *oriC* and the *repA2* promoter needs more analysis because it is not known how cooperative binding, the sequence context, and DNA topology play a role in the DNA protein interaction. The interaction of DnaA with the *repA2* promoter is much weaker than with *oriC* however this is not uncommon. An example of

such an interaction is DnaA binding to the *mioC* promoter (upstream of the *E. coli oriC*) which has been classified as a weak interaction (Kd = 51 +/- 38 nM) and if the native 6 nucleotides are replaced on each side of the DnaA box the interaction becomes non-specific (Schaper and Messer, 1995).

One of the most interesting results of this work is the ultrastructural transformation resulting from the overexpression of the *S. meliloti* DnaA protein in both *E. coli* and *S. meliloti*. The overexpression of DnaA in *E. coli* stimulates the initiation reaction at *oriC* (Atlung, Lobner-Olesen, and Hansen, 1987;Skarstad *et al.*, 1989) and results in a loss of the correct timing of replication initiation in the cell cycle (Pierucci, Rickert, and Helmstetter, 1989). DnaA overexpression in *E. coli* has been shown to lead to filamentation and increased cell death when overexpressed in a *recA* mutant (Grigorian *et al.*, 2003). Filamentation of *Mycobacterium smegmatis* cells has also been reported in DnaA overexpressing strains (Greendyke *et al.*, 2002). The complex cell division phenotype of *S. meliloti* resulting from DnaA overexpression is consistent with the phenotype observed from: overexpression of an essential DNA methyltransferase CcrM

(Wright, Stephens, and Shapiro, 1997), overexpression o FtsZ (*S. meliloti* encodes two copies of *ftsZ* (Margolin an Long, 1994)), treatment with DNA-damaging agents (Latch an Margolin, 1997) and lack of MinE expression (unpublishe data). It appears that a block in cell division in *S. meliloti* results in a very complex change in cell morphology. The phenotype resulting from overexpression of DnaA in *S. meliloti* is consistent with an intimate connection between DNA replication and cell division.

S. meliloti offers itself as an interesting organism in which to study DNA replication not only because of the genome architecture but because many of the replication genes identified in *E. coli* have yet to be discovered in *S. meliloti*. Genes encoding DNA polymerase II (*polB*), several DNA polymerase III accessory subunits (θ, δ, ψ, and χ), and proteins such as DnaT, DnaC, PriB and PriC are missing in *S. meliloti* (Capela *et al.*, 2001). *S. meliloti* does not posses a SeqA homologue which in *E. coli* sequesters hemimethylated *oriC* DNA to the membrane to prevent initiation of replication. However, methylation of DNA appears to be important in controlling DNA replication initiation in *S. meliloti*. The CcrM methyltransferase (highly conserved in the alpha subdivision of

proteobacteria) is essential in *S. meliloti* and wher overexpressed cells contain up to three genome equivalents of DNA (Wright, Stephens, and Shapiro, 1997). It is likely that CtrA may be acting as a negative regulator of replication initiation in *S. meliloti* as opposed to a protein such as SeqA. Recently, the GcrA global regulator in *C. crescentus* was identified and was found to regulate the expression directly or indirectly of 125 genes including *ctrA* and *dnaA*. GcrA activates the *ctrA* P1 promoter and the levels of DnaA increased after depletion of GcrA suggesting that it is negatively regulating *dnaA* expression. This discovery implicates GcrA and CtrA as the central genetic circuit that spatially and temporally controls the regulators of DNA replication and cellular differention in *C. crescentus* (Holtzendorff *et al.*, 2004). Interestingly, *S. meliloti* encoded a gene (*Y02139*) that is located on the chromosome downstream of *phoB* on the opposite strand and has 48% amino acid identity to *C. crescentus* GrcA.

One of the first steps in characterizing a replicon is to identify the replication origin. This work has provided the experimental evidence to support to location of the *S. meliloti* chromosome origin, the first chromosome origin

localized in the Rhizobiaceae. Experimental evidence has localized another alpha proteobacterial chromosome origin from *C. crescentus* and this facilitated the identification of the *S. meliloti* chromosome origin. Other groups also used the location of the *C. crescentus* origin to localize the chromosome origins from *Rikettsia prowazekii* (Brassinga *et al.*, 2002) and *Brucella abortus* (Bellefontaine *et al.*, 2002) but have only provided information regarding the conservation of CtrA binding upstream of the *hemE* gene and have never reported the identification of an autonomously replicating sequence. The detection of an autonomously replicating sequence from the *S. meliloti* chromosome is the second autonomously replicating sequence detected from an alpha proteobacterial chromosome and confirms that origins from this group of bacteria may represent a distinct class of bacterial origins. We now have the potential to study how *S. meliloti* controls replication initiation reactions of multiple origins.

APPENDIX 1.

Purification of *S. meliloti* CtrA and the Identification of putative CtrA binding sites

CtrA is considered a key molecular switch in controlling both replication initiation at the chromosome origin and cell division in *C. crescentus*. Recently, it has been shown that CtrA binding sites are conserved in the *hemE* promoters of *B. abortus* (Bellefontaine et al., 2002) and *R. prowazekii* (Brassinga *et al.*, 2002), the putative chromosome origins of these alpha-proteobacteria. Therefore, it is likely that CtrA is involved in *S. meliloti* chromosome replication.

The role of CtrA in replication initiation of the *S. meliloti oriC* was not investigated in this work however *S. meliloti* CtrA was purified using the same strategy as the purification of DnaA. CtrA could only be liberated from the NusAHis$_6$ tag if *ctrA* was cloned downstream of the thrombin recognition site (pTH1178), when *ctrA* was cloned into the SmaI site of pET431a (pTH1174) to maintain the amino acid sequence of the thrombin recognition sequence

the fusion protein could not be digested with thrombin. Using an expanded CtrA box consensus TTN_9TT instead of the proposed *S. meliloti* CtrA box consensus $TAAN_7TTAAC$ (Barnett *et al.*, 2001) which was determined by the protection of the *S. meliloti ctrA* promoter by *C. crescentus* CtrA reveals five putative binding sites in the *hemE - Y02793* intergenic region and two binding sites downstream of the *repC* gene on pSymB. The binding sites downstream of *repC* are in a non-protein coding sequence essential for replication. CtrA binding sites are often coincident with promoters and thus the presence of these binding sites downstream of *repC* may reflect the presence of promoters driving the expression of antisense RNA molecules involved in replication initiation of pSymB. The binding sites may also be functioning to control the timing of replication initiation of the pSymB megaplasmid because replication does not occur when CtrA is bound to the replication origin in *C. crescentus*. CtrA binding sites were not detected in the pSymA replicator region. The function of CtrA in replication initiation of the two essential *S. meliloti* replicons remains unknown.

APPENDIX 2.

Contributions to this Thesis

Andrea Sartor as an undergraduate student helped in the contruction of some of the plasmids used to map the sequence limits of the *oriC*. She also made the site-directed mutations in the pTH1465 plasmid (constructed by Shawn MacLellan) and preformed the Green Flourescent Protein Assays.

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