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Replication and maintenance of plasmids in Bacillus subtilis

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Replication and maintenance of plasmids in Bacillus subtilis

Wilfried Meijer

Replication and maintenance of plasmids in *Bacillus subtilis*

To the members of the graduation committee
Ter attentie van de leden van de promotiecommissie

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Prof. dr. L. Dijkhuizen
Prof. dr. S.D. Ehrlich

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RIJKSUNIVERSITEIT GRONINGEN

Replication and maintenance of plasmids in Bacillus subtilis

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenshappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus Dr. F. van der Woude
in het openbaar te verdedigen op
vrijdag 13 oktober 1995
des namiddags te 1.15 uur precies

door

Wilhelmus Johannes Jozef Meijer

geboren op 19 mei 1966
te Raalte

Promotor : Prof. Dr. G. Venema

Co-promotor: Dr. S. Bron

Aan Pa en Ma

Cover: Identification of replication intermediates of a plasmid replicating via either the theta mechanism (top) or the rolling circle mechanism (bottom) .

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Chapter I

Introduction

1: General Introduction

The genus Bacillus incorporates many species of Gram-positive, rod-shaped, aerobic, endospore-forming bacteria. Most species normally inhabit the soil or decaying plant material. Several features make bacilli important organisms for scientific and applied purposes. Several bacilli secrete large amounts of industrially important enzymes into the medium (e.g. α-amylases and proteases) and they can grow on simple and cheap media. Bacillus thuringiensis species produce, intracellularly, valuable insecticidal endotoxins. In addition, the lack of endotoxin production by most Bacillus species is a considerable advantage in the industrial use of these bacteria.

The paradigm for research on bacilli is Bacillus subtilis which, next to the Gram-negative bacterium Escherichia coli, is the best-characterized prokaryotic organism on the genetic, biochemical and physiological level. An extensive overview of the current knowledge of bacilli has recently been published (Sonenshein et al., 1993). An additional advantage of bacilli is that apart from the pathogens B.anthracis and B.cereus (active on certain mammals, including humans), and the insect pathogens B.larvae, B.popilliae, B.sphaericus and B.thuringiensis, most other Bacillus species are regarded as non-pathogenic. In fact, some species (e.g. B.subtilis var. natto) are used in the fermentation of steamed soy-beans and the product, natto, is a regular food component, especially in South-East Asia. The importance of B.subtilis is also illustrated by the fact that several laboratories in Europe and Japan work together in a project aimed at the determination of the DNA sequence of the complete chromosome of this bacterium ($4.2*10^6$ bp) which will be completed within the next years.

The availability of several transfer systems for the introduction of homologous and heterologous DNA into B.subtilis that may result in the permanent change of the cell's genotype offers the possibility to (i), adapt the bacterium for specific industrial purposes; and (ii), study fundamental questions by altering specific genetic loci or introducing native or heterologous DNA. The DNA transfer regimes used for B.subtilis include: transformation via natural competence, protoplast transformation, transduction and electroporation.

Important tools for recombinant DNA technology in bacteria are gene cloning and expression vectors. Cloning vectors are frequently based on autonomously replicating units, called plasmids, which can be present in a cell in addition to the chromosome. Plasmids and their derived cloning vectors play an eminent role in almost all fields of molecular genetics. A historical overview of the importance of plasmids in molecular genetics was given by Cohen (1993), one of the pioneers in the field of genetic engineering. The highly transformable B.subtilis strain 168, which has become the standard strain for most studies, does not harbour endogenous plasmids, nor do most other isolates of B.subtilis. The endogenous plasmids identified in B.subtilis, mostly in industrial strains, do not confer easily selectable phenotypes on their hosts. This is the reason why most cloning vectors used today in this bacterium are based on plasmids which were originally isolated from other Gram-positive bacteria, like staphylococci and streptococci (Dubnau, 1983; Novick, 1989; Gruss and Ehrlich, 1989; Jannière et al., 1993). Several of these plasmids replicate autonomously, and express their antibiotic resistance genes in B.subtilis (Ehrlich, 1977; Lacks et al., 1986; Novick, 1989; Bron, 1990). Although

successful cloning has been reported, it is now clear that these plasmids are not optimal for B.subtilis A frequently observed problem is a high level of plasmid instability (Dubnau, 1983; Bron and Luxen, 1985; Ehrlich et al., 1986; Bron et al., 1991b; Meijer et al., 1995a; Meijer et al., 1995b). Two types of plasmid instability can be distinguished: structural and segregational instability. Structural instability refers to DNA rearrangements that result in structural alterations of the plasmid, mostly deletions. Segregational instability refers to the loss of the entire plasmid population from the cell under non-selective growth conditions. The mechanisms underlying these instabilities are only partly understood.

The major aim of the studies described in this thesis is to increase the understanding of the factors causing plasmid instability, in particular, those involved in plasmid maintenance. In addition, the knowledge obtained has been used used for the construction of improved cloning vectors for B.subtilis Since segregational plasmid stability is closely associated with plasmid replication, a considerable part of this thesis deals with studies on plasmid replication in B.subtilis In the following sections the current knowledge of the two major known plasmid replication mechanisms, and the parameters involved in plasmid maintenance will be described, followed by an outline of this thesis.

2: Plasmid replication

2.A: Two mechanisms of plasmid replication

Plasmids have been isolated from a wide range of prokaryotic organisms and the analysis of their mode of replication led to the identification of two mechanisms of replication. One group of plasmids replicates via the so-called rolling-circle mechanism (RCM) and the other via the theta mechanism (TM). Whereas RCM is used by most small plasmids (< ~ 12 kb), all known larger plasmids replicate by the TM. Remarkably, many RCM plasmids have been identified in Gram-positive bacteria but, so far, only a limited number of RCM plasmids has been isolated from Gram-negative bacteria (Kleanthous et al., 1991; Yasukawa et al., 1991; see also Table 1). RCM plasmids have been studied extensively (for reviews see Gruss and Ehrlich, 1989; Novick, 1989; Bron, 1990; Novick, 1991; Koonin and Ilyina, 1993). Although theta-replicating plasmids were initially mainly identified in Gram-negative hosts, such plasmids have now also been described for Gram-positive bacteria. With the exception of the pAMß1-family of Gram-positive theta plasmids (Brantl et al., 1990; Brantl and Behnke, 1992; Bruand et al., 1993; Jannière et al., 1993; Le Chatelier et al., 1993; Ceglowski et al., 1993a; Ceglowski et al., 1993b; Rojo and Alonso, 1994; Le Chatelier et al., 1994; Brantl et al., 1994; Brantl, 1994), most studies on theta plasmids have been performed with plasmids from Gram-negative bacteria (Kornberg and Baker, 1992; Scott, 1984; Kües and Stahl, 1989; Marians, 1992). A description of the main replication features of RCM and TM plasmids is given in the following section.

Table 1. Rolling-circle plasmids and their original host

Replicon	size (kb)	Original host	Reference
pT181	4.4	Staphylococcus aureus	Khan and Novick, 1983
pC221	4.6	Staphylococcus aureus	Novick, 1989
pC223	4.6	Staphylococcus aureus	Novick, 1989
pS194	4.4	Staphylococcus aureus	Novick, 1989
pUB112	4.1	Staphylococcus aureus	Novick, 1989
pCW7	4.2	Staphylococcus aureus	Novick, 1989
pHD2	2.1	Bacillus thuringiensis	McDowell and Mann, 1991
pC194	2.9	Staphylococcus aureus	Horinuchi and Weisblum, 1982b
pUB110	4.5	Staphylococcus aureus	Gruss and Ehrlich, 1989
pOX6	3.2	Staphylococcus aureus	Novick, 1989
pTA1015	5.8	Bacillus subtilis	This thesis, chapter VII
pTA1020	6.8	Bacillus subtilis	Uozumi et al., 1980
pTA1030	7.3	Bacillus subtilis	Uozumi et al., 1980
pTA1040	7.8	Bacillus subtilis	This thesis, chapter VII
pTA1050	8.2	Bacillus subtilis	Uozumi et al., 1980
pTA1060	8.7	Bacillus subtilis	This thesis, chapter VII
pBAA1	6.8	Bacillus subtilis	Devine et al., 1989
pFTB14	8.2	Bacillus amyloliquefaciens	Murai et al., 1987
pBC16	4.6	Bacillus cereus	Gruss et al., 1987
pBC1	1.6	Bacillus coagulans	de Rossi et al., 1992
pCB101	6.0	Clostridium butyricum	unpublished
pLP1	2.1	Lactobacillus plantaricum	Bouia et al., 1989
pIJ101	8.8	Streptomyces lividans	Kendell and Cohen, 1988
pSN22	11.0	Streptomyces nigrifaciens	Kataoka et al., 1994a
pC30iL	2.1	Lactobacillus plantarum	Skaugen, 1989
pTD1	2.6	Treponema denticola	MacDougall et al., 1992
pKYM	2.1	Shigella sonnei	Yasukawa et al., 1991
pLAB1000	3.3	Lactobacillus hilgardii	Josson et al., 1990
pWGB32	2.4	Staphylococcus aureus	Grinius et al., 1992

pVA380-1	4.2	Streptococcus ferus	LeBlanc et al., 1992
pRF1	4.2	Plectonema	Perkins and Barnum, 1992
p8014-2	1.9	Lactobacillus plantarum	Leer et al., 1992
p353-2	2.4	Lactobacillus pentosus	Leer et al., 1992
pTB913	4.5	Thermophilic Bacillus	van der Lelie et al., 1989
pSK89	2.4	Staphylococcus aureus	Littlejohn et al., 1991
pST1	2.1	Streptococcus thermophilus	Janzen et al., 1992
pMA1	-	Microcystis aeruginosa	unpublished
pNostoc	-	Nostoc sp.	unpublished
pE194	3.7	Staphylococcus aureus	Horinuchi and Weisblum, 1982a
pNE131	2.4	Staphylococcus epidermis	Lampson and Parisi, 1986
pE3692	2.4	Staphylococcus aureus	Wieckiewicz and Wojcik, 1989
pMV158	5.5	Streptococcus agalactiae	Lacks et al., 1986
pWV01	2.2	Lactococcus lactis subsp. cremoris	Leenhouts et al., 1991
pSH71	2.2	Lactococcus lactis	de Vos, 1987
pFX2	2.2	Lactococcus lactis	Xu et al., 1989
pLB4	3.5	Lactobacillus plantarum	Bates and Gilbert, 1989
pA1	2.8	Lactobacillus plantarum	Vujcic and Topisirovic, 1993
pKMK1	1.9	Mycoplasma mycoides	King and Dybvig, 1992
pHPK255	1.5	Heliobacter pylori	Kleanthous et al., 1991
pSN2	1.3	Staphylococcus aureus	Novick, 1989
pE12	2.2	Staphylococcus aureus	Novick, 1989
pE5	2.1	Staphylococcus aureus	Projan et al., 1987
pT48	2.1	Staphylococcus aureus	Novick, 1989
pTCS1	1.3	Staphylococcus aureus	Novick, 1989
pNE131	2.1	Staphylococcus epidermis	Gruss and Ehrlich, 1989
pIM13	2.1	Bacillus subtilis	Projan et al., 1987
pTX14-3	7.5	Bacillus thuringiensis	Madsen et al., 1993

The plasmids shown in bold are representative plasmids of the various groups

2.B: Rolling-circle replication

2.B.I Structure of small Gram-positive plasmids

A characteristic feature of RCM replication is the generation of single-stranded (ss) DNA intermediates (Te Riele et al., 1986a; Te Riele et al., 1986b). Typically, RCM plasmids are similar in their structural and functional organization (Projan and Novick, 1988; Gruss and Ehrlich, 1989; Bron, 1990). A prototype of a small RCM plasmid is presented in Fig. 1. All RCM plasmids contain several interchangeable modules, which frequently show considerable homology at the DNA and/or deduced protein level. The relative order of the modules varies among the different plasmids. Apart from a few exceptions, all genes in these plasmids are transcribed in the same direction, which coincides with the direction of replication (leading strand synthesis; see below). An essential module comprizes a gene, denoted rep, which encodes the Rep protein, and its cognate double-strand origin (DSO). Another module contains a non-transcribed region characterized by strong dyad symmetry. This region forms the single-strand origin (SSO, formerly denoted as minus origin) and functions as the major initiation site for lagging-strand synthesis. These two modules, involved in replication, are present on all RCM plasmids. In addition, many RCM plasmids contain a module comprizing a gene, denoted mob or pre, which is involved in conjugative mobilization or site-specific plasmid recombination, respectively. A fourth module, comprizing one or more antibiotic resistance markers, is present on many of the S.aureus and Streptococcus sp. plasmids (in cryptic plasmids this module is absent). Besides these common modules, plasmid-specific unique modules can be present. Several Bacillus plasmids contain such modules, which are likely to be involved in the adaptation of the host to specific growth conditions (see chapters VI and VII of this thesis). Unique sequences present on the relatively large Streptomyces plasmids pIJ101 and pSN22 are mainly involved in plasmid transfer and pock formation (Kataoka et al., 1994b).

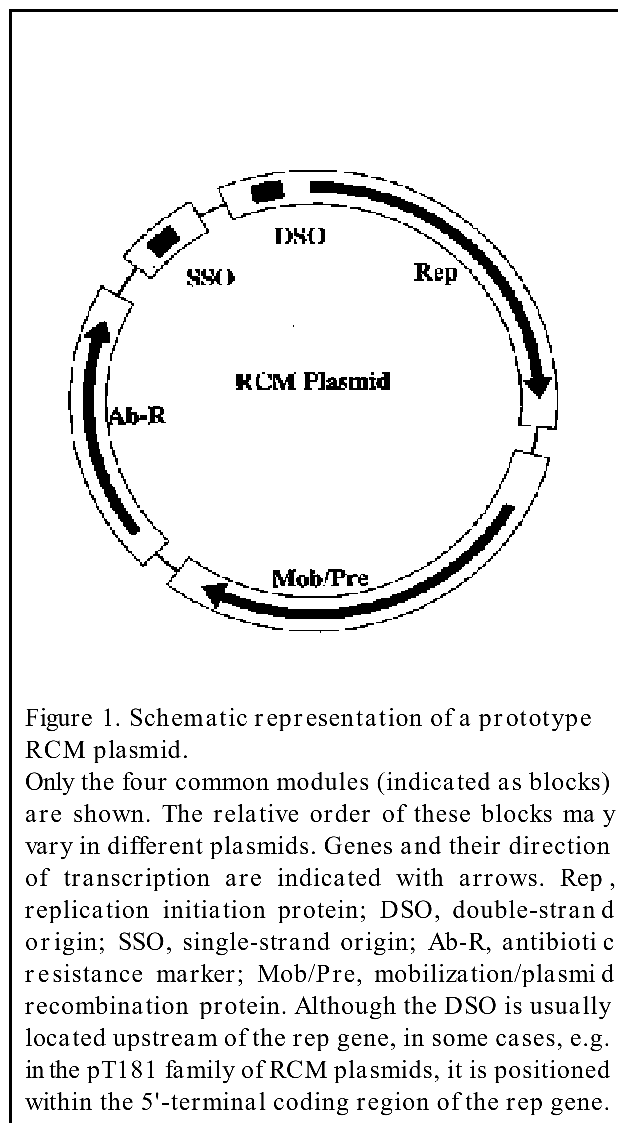

Figure 1. Schematic representation of a prototype RCM plasmid.
Only the four common modules (indicated as blocks) are shown. The relative order of these blocks may vary in different plasmids. Genes and their direction of transcription are indicated with arrows. Rep, replication initiation protein; DSO, double-strand origin; SSO, single-strand origin; Ab-R, antibiotic resistance marker; Mob/Pre, mobilization/plasmid recombination protein. Although the DSO is usually located upstream of the rep gene, in some cases, e.g. in the pT181 family of RCM plasmids, it is positioned within the 5'-terminal coding region of the rep gene.

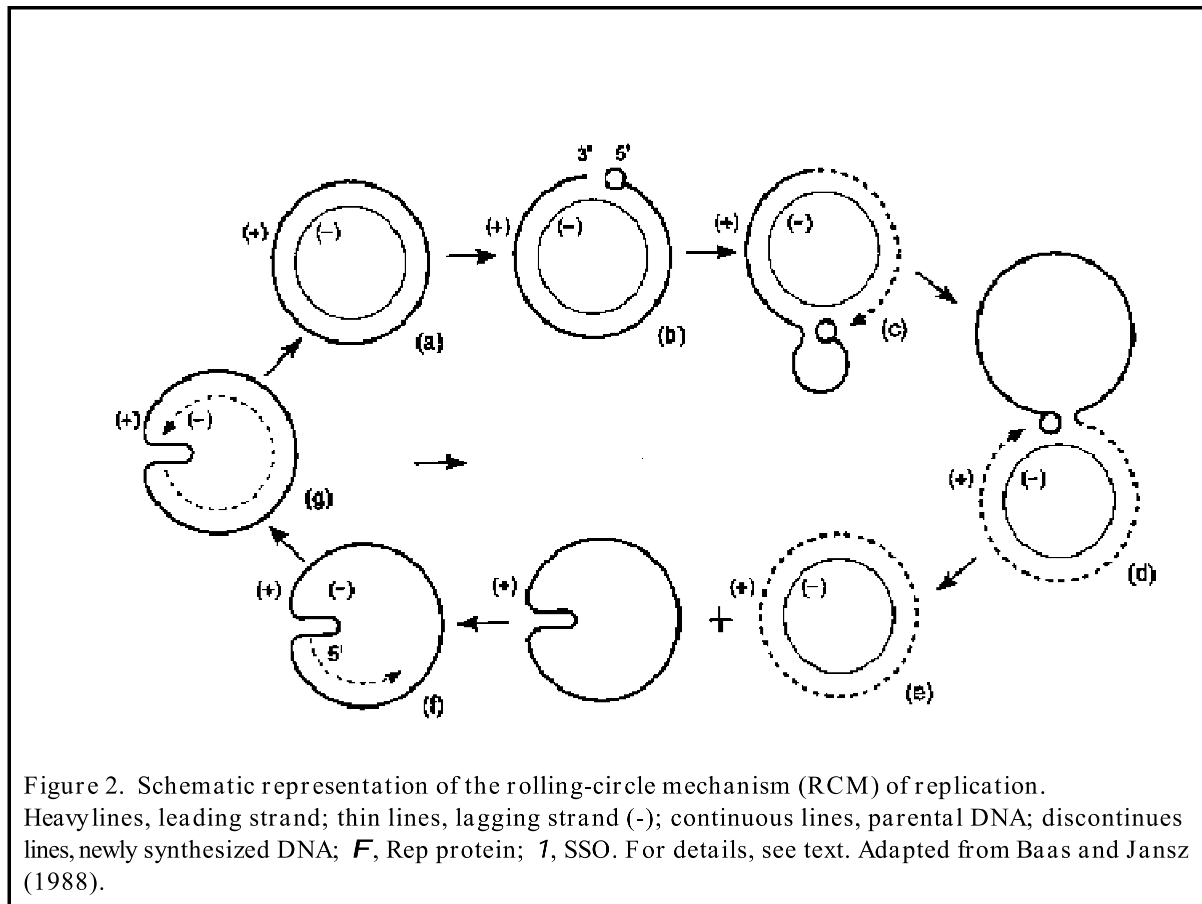

Figure 2. Schematic representation of the rolling-circle mechanism (RCM) of replication. Heavy lines, leading strand; thin lines, lagging strand (-); continuous lines, parental DNA; discontinues lines, newly synthesized DNA; *F*, Rep protein; *1*, SSO. For details, see text. Adapted from Baas and Jansz (1988).

2.B.II Rolling-circle mechanism of replication

RCM replication is one of the basic mechanisms by which circular replicons propagate themselves. Besides the vast number of Gram-positive RCM plasmids (Table 1), such plasmids have recently also been detected in Gram-negative bacteria (Yasukawa et al., 1991; Kleanthous et al., 1991); the spirochaete, Treponema denticola (MacDougall et al., 1992); and the cyanobacteria Nostoc (Waltson et al.), Microcystis aeruginosa (Ochiai et al.) and Plectonema (Perkins and Barnum, 1992). The RCM of replication was initially described for small isometric and filamentous ssDNA bacteriophages, such as ΦX174 and M13 (Baas and Jansz, 1988), and temperate dsDNA bacteriophages of the P2 family [reviewed by (Bertani and Six, 1988)]. Recently, evidence has been reported that also the plant gemini-viruses use this type of replication (Stenger et al., 1991; Saunders et al., 1991). Whereas the main principles of RCM are uniform, differences exist, however, between different genomes. The various steps in rolling-circle replication of Gram-positive plasmids will be described in connection with the model shown in Fig. 2. Details of differences between RCM plasmids and ssDNA phages and the effects of impaired replication on plasmid maintenance will be described thereafter.

Rolling-circle replication is initiated by the binding of the plasmid-encoded replication protein (Rep) to the plasmid and subsequent introduction of a site- and strand-specific nick (Fig. 2b). The region comprizing the binding- and nick-site is called the double-strand origin (DSO). At least with some, and probably all RCM plasmids, Rep remains covalently linked to

the 5'-end of the nicked strand via a phospho-tyrosine linkage (Thomas et al., 1988; Gros et al., 1989). A new leading strand is synthesized through extension of the free 3'OH-end at the nick-site, which results in the displacement of the original leading strand. Since the Rep protein travels along the template, this results in the formation of a looped rolling-circle (Fig. 2c). As the replication intermediates show up as typically σ-shaped molecules in the electron microscope, this replication mechanism has also been called σ-replication. Displacement of the original leading strand continues until the DSO is reached (Fig. 2d). Next, the Rep protein cleaves the leading strand at this site once more and the displaced parental strand is concomitantly ligated, yielding a single-stranded circle and a duplex plasmid molecule (Fig. 2e). Thus, Rep mediates both the initiation and the termination of leading strand synthesis during RCM replication. One round of replication is completed by the conversion of the ssDNA replication intermediate into a duplex plasmid molecule (Fig. 2f,g). Hence, leading and lagging strand synthesis are separated in time and this replication mechanism has, therefore, also been referred to as asymmetric replication. Initiation of lagging strand synthesis normally takes place at a specific plasmid region which is characterized by a high potential to form secondary structures (represented by ∩ in Fig. 2). This lagging strand initiation signal, denoted single-strand origin (SSO), is recognized by host factors that mediate the synthesis of an RNA primer from which strand elongation can occur.

2.B.IIa Rep proteins

In all well-explored systems, the Rep proteins interact with their cognate DSOs at which they have DNA binding, nicking/closing and topoisomerase-like activities (Koepsel et al., 1985; de la Campa et al., 1990). Based on sequence similarities of DSOs and deduced amino acid sequences of Rep proteins, the currently known RCM plasmids can be divided into five groups (Table 1). The five groups are represented by the prototype plasmids pT181, pC194, pE194, pSN2 and pTX14-3. Using the criteria used for plasmid classification, the ssDNA phage ΦX174 would belong to the group of pC194 plasmids (Jannière et al., 1993). Despite the homologies between the Rep proteins of RCM plasmids and ΦX174, important differences exist in the way they control the copy number of their cognate replicons. Within the life cycle of phages, like ΦX174, many replicas of the phage genome have to be made before the host cell lyses. However, with plasmids, which exist in harmony with their host for numerous generations, a tight regulation of the plasmid copy numbers is required. Since Rep is the sole enzyme required for replication initiation, it is also a major factor in determining the plasmid copy number. Accordingly, the expression of the Rep proteins is strictly regulated (Gruss and Ehrlich, 1989; Novick, 1989; del Solar and Espinosa, 1992). Regulation can take place at several levels. For the well-studied plasmid pT181 it is known that expression of its Rep protein, RepC, is primarily regulated via an antisense-mediated control mechanism (Kumar and Novick, 1985). The antisense (countertranscript) RNA (ctRNA), causes premature termination (attenuation) of RepC mRNA synthesis (Novick et al., 1989). Similar control mechanisms have been proposed for pC194 and pUB110, both members of the pC194 group of plasmids (Alonso and Tailor, 1987; Maciag et al., 1988). Expression of the Rep proteins of

members of the pE194 group of plasmids, such as pE194 and pMV158, is regulated at the level of both transcription and translation (del Solar and Espinosa, 1992; Kwak and Weisblum, 1994). With these plasmids, two genes, one encoding a small repressor protein and the other the Rep protein, are cotranscribed from the same promoter. The repressor regulates transcription of the operon through the binding to operator sequences that overlap with the promoter. Regulation of translation of the Rep protein occurs through the binding of a small ctRNA to regions of the Rep mRNA which include the ribosomal binding site. The regulation of Rep synthesis of members from the other two groups of RCM plasmids, pSN2 and pTX14-3, has not yet been studied.

With all RCM plasmids, a strict regulation of the expression of the Rep protein would be useless if Rep would not be inactivated after the completion of one round of replication. An obvious stage in the replication cycle for this inactivation to occur would be the release of Rep from the DNA during termination of leading strand synthesis. Studies, mainly performed in the laboratory of R. Novick, have indeed shown that Rep proteins of different members of the pT181 group of plasmids are inactivated following (or during) replication (Rasooly and Novick, 1993; Rasooly et al., 1994a). These authors showed that initiation of replication by RepC of pT181 is started by a homodimer of the Rep protein. However, after termination, a heterodimer RepC/RepC* is released which is inactive as a topoisomerase and can not initiate new rounds of replication. The RepC* subunit is modified through the covalent attachment of a single-stranded oligodeoxynucleotide whose sequence corresponds to the 10-12 nucleotides which are located immediately 3' to the nick site in the origin (Rasooly et al., 1994b). A similar mechanism seems to be used by pUB110 except that RepU molecules may consist of protein monomers (Müller et al., 1995).

As mentioned above, for phages like ΦX174 it is important to make many replicas before the host cell lyses. This is achieved by recycling of the replication protein. In fact, after termination, the replication protein CisA of ΦX174 can reinitiate a next round of replication without being released from the DNA. This is achieved through the presence of two neighbouring tyrosines in the active site which are alternately linked to the 5'-end of the cleaved DNA strand (van Mansfeld et al., 1986). In contrast, Rep proteins of RCM plasmids contain only one tyrosine in the active site (Gros et al., 1994). Activities of the Rep proteins of filamentous phages, which also use the RCM of replication, differ even more from those of RCM plasmids in that their initiator proteins do not form covalent bonds with the DNA. Instead, these proteins weakly bind to the DNA strand opposite to the nicked one and do not travel along the template. In these cases replication does not proceed via so-called looped rolling circles but via rolling circles with loose tails (Geider et al., 1984).

2.B.IIb Lagging strand initiation signals

Lagging strand initiation sites of RCM plasmids, called SSOs, are characterized by a high level of dyad symmetry. Based on sequence homologies most SSOs can be divided into three groups: palA (Gruss et al., 1987; del Solar et al., 1987; this thesis, chapters II and III), palU (Boe et al., 1989; van der Lelie et al., 1989; this thesis, chapters II and III), and palT (Bron et al., 1987; Chang et al., 1987; Devine et al., 1989; this thesis, chapter V).

SSOs of the palA-type form a heterogeneous group with only limited sequence similarity (Gruss et al., 1987). The palU-type SSOs, however, show a high level of sequence similarity (van der Lelie et al., 1989); and the palT-type SSOs exist of two subfamilies, palT1 and palT2, which

are 77% identical (this thesis, chapter V). Recently, SSOs have been described that cannot be classified in one of the above-mentioned groups. For instance, Leer et al. (1992) described a novel type of SSO present on the small Lactobacillus plasmid p8014-2; Madsen et al. (1993) have identified an SSO showing similarity to the palT type SSO on the Bacillus thuringiensis plasmid pTX14-3; and Seegers et al. (1995) have analyzed another novel SSO, designated palW, from the lactococcal plasmid pWVO1. A characteristic feature of all SSOs is that their activity is orientation-dependent. SSOs are usually only functional in their original host or closely related bacteria (Gruss et al., 1987; del Solar et al., 1987; Gruss and Ehrlich, 1989; this thesis, chapter III). In this respect, palU is an exception: activity of this SSO has been shown in S.aureus (Boe et al., 1989), B.subtilis (Bron et al., 1988a; Viret and Alonso, 1988; Boe et al., 1989; van der Lelie et al., 1989; this thesis, chapter III), L.lactis (this thesis, chapter II) and Streptococcus pneumoniae (Kramer et al., 1995). ssDNA conversion of most RCM plasmids is inhibited by rifampicin, an RNA polymerase-specific inhibitor (Boe et al., 1989; Birch and Khan, 1992; Seery and Devine, 1993; Dempsey et al., 1995). This indicates that conversion of the ssDNA intermediates requires the host-encoded RNA polymerase (RNAP). Most likely, the RNA polymerase synthesizes an RNA primer on the SSO which is then used for the initiation of lagging strand synthesis. Such an RNAP-dependent mechanism has been demonstrated for pT181, pE194, pSN2 and pUB110 by in vitro replication studies (Birch and Khan, 1992; Dempsey et al., 1995). However, SSO-activity of pTX14-3 in B.subtilis (Boe et al., 1991), and palW of pWV01 in L.lactis (Leenhouts et al., 1991; Seegers et al., 1995), is not, or only partly inhibited by rifampicin, indicating that ssDNA conversion in these cases can occur via another, RNAP-independent, route.

For the ssDNA phages, three different modes of initiation of lagging strand synthesis have been described, which differ in the requirements for host functions (Baas and Jansz, 1988). One of these modes, used by filamentous phages like M13, also requires the host-encoded RNAP. However, G4-type phages require the host primase, and for ssDNA conversion of ss ΦX174 DNA the formation of a primosome complex at the primosome assembly site is required. At present, no conclusive data are available for the possible involvement of the host primase or the primosome complex in the ssDNA conversion initiated by the SSO of pTX14-3 or pWV01. However, the observed sequence homology between the ΦX174 primosome assembly site and palW of pWV01 may suggest that the host primosome complex is involved in ssDNA conversion in L.lactis (Leenhouts et al., 1991).

Recently, the palA SSO of pMV158, although not active as an ssDNA conversion signal in B.subtilis, has been described to be involved in plasmid maintenance in this bacterium (del Solar et al., 1993; this thesis, chapter III). The presumed mechanism underlying the positive effects of palA on plasmid stability in B.subtilis is described in section 3.A; "Maintenance of rolling-circle plasmids".

2.B.IIc Alternative mechanisms of lagging strand synthesis

In the absence of functional SSOs, ssDNA replication intermediates are usually easily detected (Gruss et al., 1987; del Solar et al., 1987; this thesis, chapters II, III, IV and V). Despite the lack of a functional ssDNA conversion signal RCM plasmids are still able to replicate, indicating that alternative mechanisms of ssDNA conversion must exist (Gruss et al., 1987; del Solar et al., 1987; Viret and Alonso, 1988; this thesis, chapters II, III and IV). Viret and Alonso (1988) analyzed replication and ssDNA accumulation of plasmid pUB110 and its derivative pBT32, which lacks the palU SSO, in an isogenic set of temperature-sensitive replication-defective B.subtilis strains. These authors observed that, in contrast to intact pUB110, plasmid pBT32 was unable to transform the B.subtilis strains dnaD23 and dnaF33. In addition, it was observed that pBT32, but not pUB110, accumulated higher amounts of ssDNA at the non-permissive compared to the permissive temperature in the B.subtilis dnaB19 and dnaB37 strains. From these results it was concluded that dnaD, dnaF and dnaB are involved in an alternative, SSO-independent, pathway for the conversion of pUB110 ssDNA. The DNA sequences of the dnaB gene (Hoshino et al., 1987) and the dnaD (Bruand et al., 1995b) genes are known. Although these genes show no homology with known sequences in the databanks, Bruand et al. (1995b) suggested that their products are components of the B.subtilis primosome complex. The role of dnaB, dnaD and dnaF in the SSO-independent conversion of pUB110 ssDNA has not yet been studied in detail. The observation that replication of a derivative of the endogenous B.subtilis plasmid pBS2 lacking its SSO also requires the dnaB gene (Forough et al., 1987), indicates that the dnaB gene product may be generally involved in alternative ssDNA conversion.

The involvement of another host-encoded enzyme, RecA (a key enzyme involved in DNA repair and homologous recombination), in the conversion of ssDNA molecules of pMV158 derivatives lacking an SSO, is described in this thesis, chapter IV. In addition to RecA, a plasmid region encoding an RNA transcript, RNAI, which is complementary to part of the ssDNA replication intermediate, was shown to be also involved in the alternative pathway for lagging strand synthesis. A model for this alternative ssDNA conversion mechanism is presented in chapter IV. In short, it is proposed that plasmid-specified RNA molecules which are complementary to ss plasmid replication intermediates anneal to the ssDNA molecules in a RecA-mediated process. Subsequently, the hybridized RNA molecules function as initiation signals for ssDNA conversion analogous to the situation in the SSO-dependent pathway. The proposed mechanism may also explain results obtained by Viret and Alonso (1988) who reported that pUB110 derivatives lacking a functional SSO accumulate high amounts of ssDNA in RecA-deficient B.subtilis strains. This may imply that this alternative ssDNA conversion pathway is not limited to pMV158 derivatives and may be a general ssDNA conversion mechanism in B.subtilis for RCM plasmids lacking a functional SSO.

2.B.IId Host-range of RCM plasmids

Many RCM plasmids are able to replicate in several different Gram-positive bacteria. Since the main prerequisites for their establishment in a certain host are the synthesis of functional Rep protein and the conversion of at least a fraction of the ssDNA replication intermediates, impairment of either of these processes will limit the plasmids host range. Most Gram-positive RCM plasmids cannot be established in the Gram-negative bacterium

E.coli. The following observations indicate that inefficient ssDNA conversion rather than lack of expression of the Rep protein is the major factor preventing establishment in E.coli. For instance, the S.aureus plasmid pC194 can, by itself, not be established in E.coli. However, Goze and Ehrlich (1980) showed that a hybrid between pC194 and pBR322, the latter containing an efficient lagging strand initiation site for E. coli, is able to replicate in this bacterium under conditions in whch pBR322 replication is prevented. Further, although the B.thuringiensis plasmid pTX14-3 cannot be established in E.coli, its Rep protein is expressed in this bacterium from its own promoter (Andrup et al., 1994). Finally, the Rep protein of the RCM plasmid pKYM, isolated from the Gram-negative bacterium Shigella sonnei, shows a strong homology to Rep proteins of Gram-positive plasmids (Yasukawa et al., 1991). However, instead of an SSO which is characteristic for Gram-positive plasmids, pKYM contains a specific Gram-negative lagging strand initiation signal showing 74% identity with the ssDNA conversion signals of the filamentous phages fd, f1 and M13 (Kodaira et al., 1995).

A limited number of Gram-positive plasmids, e.g. pMV158/pLS1 (Lacks et al., 1986) and pWV01 (Kok et al., 1984), belonging to the pE194 group of RCM plasmids, are exceptional in that they are able to replicate also in E.coli. Apparently, in these cases Rep is functionally expressed and ssDNA molecules are converted to double-stranded plasmid molecules. A possible explanation for the wide host range of these plasmids is that their SSOs are functional in E.coli. Both pMV158 and pWV01 contain a palA-type SSO for which homology with ssDNA conversion signals of E.coli phages has been reported (del Solar et al., 1987; Leenhouts et al., 1991). It can, therefore, not be excluded that these SSOs are functional in E.coli. However, rather large amounts of pMV158 ssDNA accumulate in E.coli even when its palA SSO is present (del Solar et al., 1987), which argues against the idea that palA is functional in E.coli. Unlike most other RCM plasmids, pMV158 contains a second SSO which is of the palU type (van der Lelie et al., 1989; this thesis, chapters II and III). Although it is unknown whether this SSO is functional in E.coli, pMV158 and pWV01 derivatives lacking their SSOs are still able to replicate in E.coli suggesting that ssDNA conversion occurs via an alternative pathway. In this context, it may be relevant to note that pMV158 is unable to replicate in RecA-deficient E.coli strains (Lacks et al., 1986), and that pWV01 can only be introduced in RecA-deficient E.coli strains with extremely low efficiencies. Analyses of the rare transformants revealed a decrease in double-stranded plasmid copy numbers and a drastic increase in ssDNA accumulation (de Vos and Simons, 1994). This strongly suggests that RecA is involved in the host range of these plasmids, most likely via a RecA-mediated ssDNA conversion pathway similar to that described above for ssDNA conversion of pMV158 derivatives lacking their SSOs in B.subtilis (this thesis, chapter IV).

Another feature contributing to the rather wide host range of RCM plasmids is the presence of a mob/pre gene on many of these plasmids. The plasmid-location of these genes may also explain the large number of related plasmids isolated from many different Gram-positive bacteria and other organisms. Mob genes encode a protein (Mob) which is involved in the conjugative transfer of these plasmids, and pre genes encode a protein (Pre; plasmid recombination enzyme) involved in cointegrate formation between two RCM plasmids at specific sites, called recombination sites A (RS_A) and B (RS_B) (Novick et al., 1984; Gennaro et al., 1987). Whereas through conjugative mobilization many RCM plasmids can be horizontally

transferred to related strains and even to different species (Koehler and Thorne, 1987), the site-specific recombination activity results in cointegrates with other co-resident RCM plasmids. Subsequent resolution of the composite plasmid at other sites will result in recombinant progeny. This may be a general mechanism which can account for the presence of common modules on different plasmids (Gruss and Ehrlich, 1989; Novick, 1989). The protein sequences of Mob/Pre proteins specified by different RCM plasmids share extensive homology (Gruss and Ehrlich, 1989; Novick, 1989; this thesis, chapter VII). In addition, sequences homologous to the RS_A sites, which are present upstream of the pre genes (Novick et al., 1984; Gennaro et al., 1987), are also present upstream of the mob genes of the plasmids pBC16 (Selinger et al., 1990), pUB110 (van der Lelie et al., 1989), pTB913 (van der Lelie et al., 1989), pLAB1000 (Josson et al., 1990), pMV158 (van der Lelie et al., 1990), pGI2 (Mahillon and Seurinck, 1988), pTA1015 (this thesis, chapter VII), and pTA1040 (this thesis, chapter VII). Oskam et al.(1991) have shown that mutations in the RS_A site of pUB110 decrease the mobilization frequencies of these plasmids, and Selinger et al. (1990) provided evidence that the RS_A sites of pBC16 and pUB110 function as the origin of transfer. Although the protein sequences of Mob/Pre proteins are homologous and both types of enzymes act on highly homologous target sequences, the Pre proteins do not seem to be capable of mobilizing plasmids (Koehler and Thorne, 1987; Selinger et al., 1990).

2.C: Theta replication

Two major features distinguish theta-replicating plasmids from those that use the RCM of replication. First, theta plasmids do not generate ss DNA replication intermediates and second, all plasmids studied so far that are larger than about 12 kb appear to replicate via the TM. This, however, does not mean that all small plasmids use the RCM of replication: the 3.8 kb lactococcal plasmid pWV02 uses the TM (Kiewiet et al., 1993a). The size of TM plasmids can vary from as small as 4 kb to over 300 kb. This type of replication derives its name from the Θ-shape of the replication intermediates, as identified by electron-microscopy. The TM of replication is also used by prokaryotic and eukaryotic chromosomes and several phages and eukaryotic viruses. Here, we will focus on the description of theta replication as used by plasmids. A general and more extensive overview of theta replication by different replicons has been published by Kornberg and Baker (1992). The enzymology of prokaryotic DNA replication has been reviewed by Baker and Wickner (1992), and initiation of chromosome replication in B.subtilisby Yoshikawa and Wake (1993).

Studies on plasmid replication, in particular plasmids from Gram-negative bacteria, have, apart from intrinsic scientific interest, offered attractive alternatives for the analysis of chromosomal replication. Advantages are that plasmids are dispensable for cell survival, are relatively small, and are usually present in multiple copies per cell. For reviews we refer to Scott (1984), Kües and Stahl (1989), Marians (1992) and Kornberg and Baker (1992). Compared to the Gram-negative theta plasmids, studies on theta-replicating plasmids from Gram-positive bacteria have a relative short history. An overview on the replication mechanism of pAMβ1 and

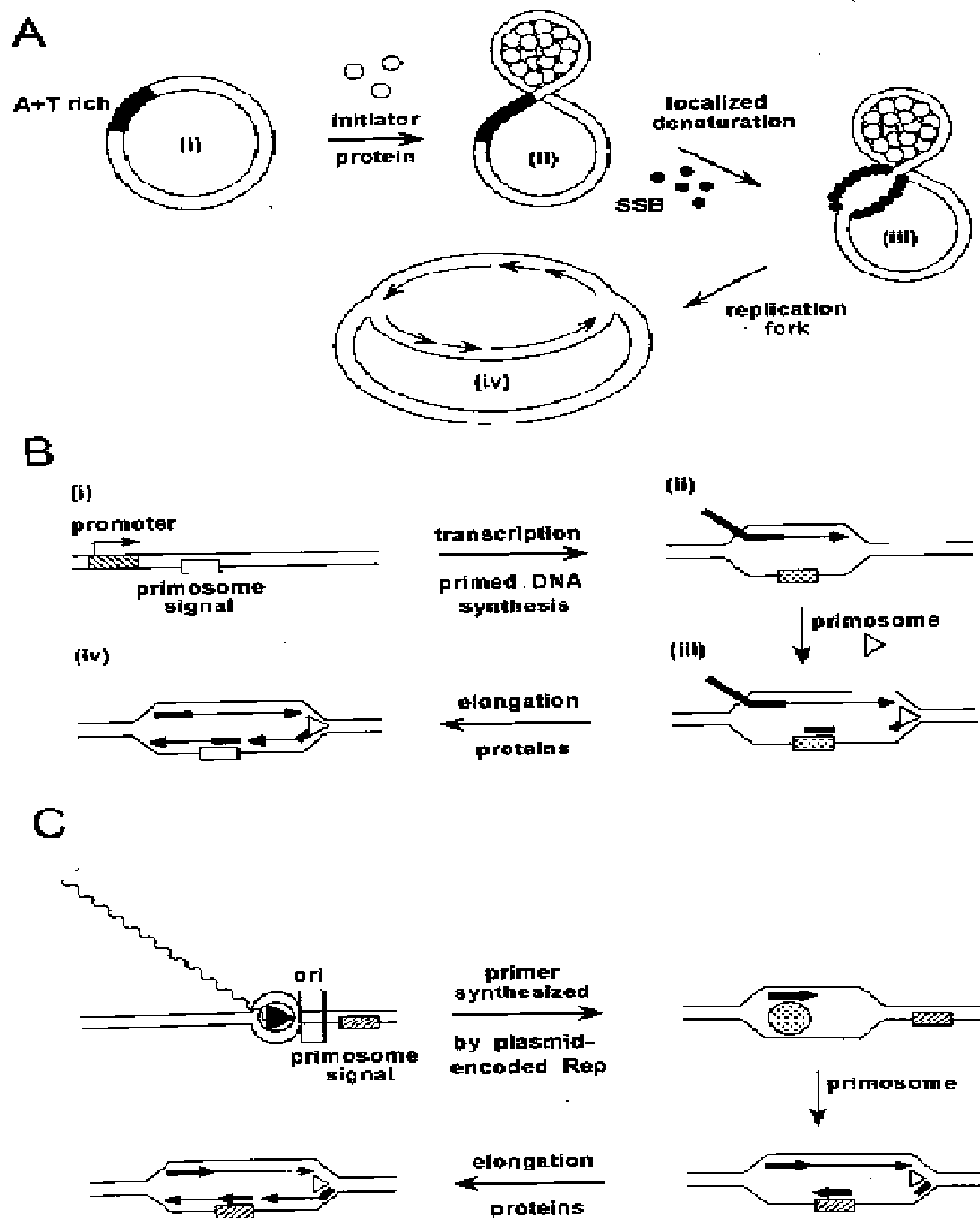

Figure 3. Schematic representation of the three known mechanisms of initiation of theta replication.
(A), (B) and (C) represent the mechanisms corresponding to class A, B and C type plasmids as described in the text. Continuous and discontinuous arrows represent newly synthesized leading and lagging strands, respectively. (A) and (B) are adapted from Marians (1992).

related plasmids, which are currently the most thoroughly studied Gram-positive theta plasmids, was published by Jannière et al. (1993). The isolation and characterization of a theta replicon (pLS20) from B.subtilisis described in this thesis, chapters VIII and IX. To our knowledge, this is the first detailed report on a theta plasmid from B. subtilis

2.C.I Different mechanisms for the initiation of theta replication

All theta-replicating plasmids initiate DNA replication at specific sequences, called origins. The origin is the site where the duplex DNA is opened, allowing the subsequent formation of replication fork(s). Although theta-replicating plasmids generally contain a single origin, exceptions are known. For instance, R6K contains three origins all located within a 4 kb region of the plasmid (Crosa, 1980), and the F plasmid contains two origins located close to each other (Kornberg and Baker, 1992). So far, three different scenarios for the initiation of theta replication, i.e. opening of the origin region, have been studied in detail. These are schematically shown in Fig. 3.

The first scenario (Fig. 3A) involves the association of a sequence-specific DNA-binding protein to a series of direct repeats representing its cognate binding site to form a nucleoprotein complex at the origin. This protein, encoded by the plasmid, is called replication initiator (Rep) protein. Next, the bound Rep protein mediates the opening of an A+T-rich region directly adjacent to the nucleoprotein complex. The protein(s) required for replication can assemble on the single-stranded DNA region, which are probably coated with single-strand binding protein (SSB). The region containing the direct repeats recognized by Rep, the A+T-rich stretch, and one or two DnaA boxes which are also present in this region (see below), is called oriA. Examples of plasmids that use the oriA-mediated replication are the Gram-negative plasmids pSC101 (Cohen et al., 1973; Cohen and Chang, 1977; Manen and Caro, 1991; Manen et al., 1994), R1 (Bernarder et al., 1992), R6K (Miron et al., 1994), and F (Kawasaki et al., 1992). Preliminary results suggest that also the large family of related lactococcal plasmids (Seegers et al., 1994), exemplified by pWV02 (Kiewiet et al., 1993a), use this mechanism of replication initiation. Plasmids falling is this group will be denoted as class A plasmids.

In the second scenario, which is used by the E.coli colicin-producing plasmid ColE1 (Kingsbury and Helinski, 1970), the initial denaturation step at the origin occurs by a strand displacement reaction (Fig. 3B) caused by the synthesis of an RNA transcript. After the initial RNA transcript has been processed by the host-encoded RNase H protein, the RNA molecule is used as a primer for leading strand synthesis, which is initially carried out by DNA polymerase I (PolI) and later by the DNA polymerase III holoenzyme (Itoh and Tomizawa, 1978). PolI is an essential enzyme in the initiation of this process and plasmids using this mode of replication are therefore characterized by their inability to be established in polI mutant strains. During the initial elongation of the leading strand by PolI, creating a D-loop structure of increasing size, a specific DNA sequence at the original lagging strand becomes single-stranded. This site, called primosome assembly site (pas), is the functional equivalent of the SSOs of RCM plasmids. Once single-stranded, it initiates lagging strand synthesis after being loaded by the primosome complex. Plasmid ColE1 and its derivative pBR322 use this replication initiation mechanism and they constitute the class B of theta plasmids.

Class C of theta plasmids contains a group of at least 17 related, colicin-

Table 2. Classification of theta plasmids based on replication initiation

Class		Represented by	Rep	oriA-like structure	PolI-dependent
Gram-neg and Gram-pos	A	R1, pSC101, F, RK2, P1, R6K, pWV02	+	+	-
Gram-neg	B	ColE1	-	-	+
	C	ColE2, ColE3	+	-	+
Gram-pos	D	pAMß1, pIP501, pSM19035	+	-	+
	E	pLS20	-	+	-

Gram-pos: Gram-positive bacteria; Gram-neg: Gram-negative bacteria; Rep: Replication initiation protein;
PolI: host-encoded DNA polymerase I

producing plasmids, collectively called ColE2-type plasmids (Hiraga et al., 1994). The well-studied ColE2-P9 and ColE3-CA38 plasmids encode a Rep protein essential for replication (Horii and Itoh, 1988; Itoh and Horii, 1989; Kido et al., 1991) and these plasmids require the host-encoded PolI (Kingsbury and Helinski, 1970; Tacon and Sherratt, 1976). Recently, important aspects of this replication mechanism have been elucidated: the Rep proteins bind to their cognate origin which is located directly downstream of the *rep* gene (Kido et al., 1991) and synthesize a unique primer RNA (ppApGpA) which is used for the initiation of leading strand synthesis by PolI (Takechi et al., 1995; Takechi and Itoh, 1995). This type of replication is schematically shown in Fig. 3C.

Based on factors specified by the plasmid or the host, three major differences can be distinguished between the three replication-initiation mechanisms discussed above (summarized in Table 2). These are: (i) the requirement for a plasmid-encoded Rep protein (class A and C); (ii) the presence of an *ori*A structure (class A); and (iii) the dependance on the host-encoded PolI (class B and C). These differences in requirements for initiation of replication can be used for the classification of other theta-replicating plasmids. Based on these criteria two other groups of plasmids, denoted class D and E can be distinguished (Table 2).

The pAMß1 family of plasmids constitutes the class D plasmids (Bruand et al., 1993). In addition to pAMß1 (Swinfield et al., 1990), the related plasmids pIP501 (Brantl et al., 1990) and pSM19035 (Ceglowski et al., 1993a), all isolated from Gram-positive hosts, belong to this class. Although their structural organization resembles that of the class A replicons (pAMß1 encodes a Rep protein and

contains an oriA-like structure), these plasmids constitute a separate class. The oriA-like structure is dispensable for replication, and pAMß1 requires PolI for the initiation of replication (Bruand et al., 1993). The exact position of the origin has been determined; it is located just downstream of the rep gene (Bruand et al., 1991). A model for the mechanism of initiation of replication of pAMß1 was presented by Bruand (1993). In pAMß1, a new type of lagging strand initiation signal has also been identified which is located ~ 150 bp downstream from its origin of replication (Bruand et al., 1995a). The structural organization of pAMß1-type plasmids resembles that of the ColE2-type plasmids. Since the exact role of the essential Rep protein of the pAMß1-type plasmids in replication initiation is not yet known, the possibility that these plasmids use the same mechanism as the ColE2 plasmids can not be excluded at present.

In chapter VIII of this thesis we describe that the replicon of the cryptic B.subtilis plasmid pLS20 cannot be classified into one of the four classes mentioned. The origin-containing region of pLS20 does not encode a Rep protein and replication does not require PolI. Based on these criteria, pLS20 has been placed in a novel, fifth, class of theta plasmids.

2.C.Ia Involvement of DnaA in theta replication

Replication of class A plasmids is initiated by the binding of the plasmid-encoded Rep protein to its cognate binding sites. A similar initiation mechanism is used by prokaryotic chromosomes. In these cases, the protein involved in nucleoprotein complex formation is the host-encoded DnaA which binds to a specific nonamer sequence, 5'-TTATCCACA-3', called the DnaA-box. DnaA protein was reported to be essential for the replication of the mini-plasmids of P1 and F (both belonging to class A). With these plasmids, DnaA is not involved in the unwinding of the DNA strands but probably in guiding the replicative helicase (DnaB) to the denatured A+T rich region (Marians, 1992). DnaA is also involved in the replication of plasmid pSC101 (class A) by an unknown mechanism (Kornberg and Baker, 1992). Also the origin region of plasmid ColE1 (class B) contains a DnaA-box near the pas site. Seufert and Messer (1987) have shown that binding of the DnaA protein to the DnaA-box can substitute for the function of the pas site. Moreover, DnaA-boxes have been identified in the origin regions of the Gram-positive plasmids of the pAMß1 family (class D) (Bruand et al., 1993), the pWV02 family (class A) (Kiewiet et al., 1993a), and pLS20 (class E) (this thesis, chapter VIII). Although DnaA-boxes are dispensable for pAMß1 replication (Bruand et al., 1993), it can not be excluded that they are involved in replication of pWV02 and/or pLS20. Thus, in addition to the essential role of the DnaA protein in chromosome replication, this protein seems to be essential for replication initiation of at least some theta plasmids.

2.C.Ib Establishment of a moving replication fork

Once formed, a replication fork has four basic components: the DNA polymerase required for nascent strand synthesis, a DNA helicase required for unwinding the parental duplex DNA, a primase required to initiate Okazaki fragment synthesis, and the SSB protein to coat exposed single-stranded template DNA (Marians, 1992). A fundamental asymmetry exists concerning the enzymatic requirements for DNA synthesis at the fork because of the antiparallel nature of the template strands and the fact that DNA polymerases synthesize DNA in the 5'→3' direction only. As a consequence, only the leading strand can be synthesized in a

continuous fashion. For this process to occur, the leading strand DNA polymerase, if processive enough, needs to be introduced to the replication fork only once. The lagging strand, however, can only be synthesized discontinuously in small fragments of about 2 kb in length (Okazaki fragments). Thus, a replication fork appears to require two distinct DNA polymerases, one highly processive and one only moderately processive. DNA polymerases cannot initiate DNA synthesis de novo. Other enzymes, primases, synthesize short RNA primers, which are then utilized by the lagging strand polymerase for Okazaki fragment synthesis. To ensure that the primase has ready access to the template when and where it is needed, its association with the replication fork is mediated by the DNA helicase acting on the lagging strand template to unwind the parental duplex in the 5'→3' direction. This mobile complex of helicase and primase has been termed the primosome. As a consequence, in contrast to RCM replication, leading and lagging strand synthesis are coupled in theta replication.

2.C.Ic Direction of replication and termination of replication

Once initiated, replication can proceed either unidirectionally or bidirectionally. In bidirectional replication the two replication forks move away from the origin in opposite directions (Fig. 3A). Consequently, the moving forks will, under normal physiological conditions, fuse more or less diametrically opposite of the origin. In unidirectional replication only one replication fork is present that moves away from the origin. After one round of replication, this fork will meet the initially created fork at the origin region. Whereas replication of bacterial chromosomes is bidirectional (Masters and Broda, 1971; Harford, 1975), most theta plasmids replicate unidirectionally. Exceptions do exist, however. An example is the F-plasmid which contains two closely located origins, oriV and oriS. One of these, oriS, controls unidirectional synthesis and the other, oriV, bidirectional replication (Kornberg and Baker, 1992). Another exception is plasmid R6K which is discussed below. In most cases the mechanisms determining the direction of replication are not known. However, with R6K, R1, R100 and presumably pLS20, replication is probably forced to proceed unidirectionally due to the arrest of the moving replication fork in one of the two directions. The sites responsible for arresting a moving replication fork are called terminator sites. Besides their presence on at least some plasmids, terminator sites are also present on the chromosomes of E.coli (Hill et al., 1988; Hidaka et al., 1988; Hidaka et al., 1992) and B.subtilis (Carrigan et al., 1987). Although the DNA sequences of the terminator sites of E.coli and B.subtilis are different, both consist of specific DNA sequences of 20 to 30 bp which are only functional when they bind a specific host-encoded DNA-binding protein (Hill, 1992). The chromosomally-located genes from E.coli (Hill et al., 1989) and B.subtilis (Carrigan et al., 1987) encoding these DNA-binding proteins have been cloned and sequenced. The respective gene from E.coli has been designated tus (terminus utilization substance), and that from B.subtilis rtp (replication terminator protein). Although the primary structure of the corresponding proteins and their cognate DNA binding sequences show little similarity (Bussiere et al., 1995), both nucleoprotein complexes are able to arrest an entering fork in a polar fashion. Fork arrest may occur because the nucleoprotein complexes form a unidirectional "clamp" on the DNA that prevents other proteins from passing the terminator site. In addition to the clamp-like activity of the termination proteins, it has also been reported that both bacterial DNA-terminator protein

complexes described here act as specific polar contrahelicases (Khatri et al., 1989; Lee et al., 1989; Kaul et al., 1994; Sahoo et al., 1995). For more detailed reviews on replication termination processes we refer to Hill (1992), Yoshikawa and Wake (1993) and Baker (1995).

Interestingly, the sequences of the plasmid-located replication terminator sites on the Gram-negative plasmids R6K (Bastia et al., 1981), R100 (Hill, 1992), R1 (Hill, 1992), and P307 (Saadi et al., 1987), are highly homologous to sequences of the E.colichromosomal terminators. Moreover, the sequence of the replication terminator of the B.subtilis plasmid pLS20 is homologous to that of the B.subtilis chromosomal terminators (this thesis, chapter IX). The pLS20 terminator differs from all others in the sense that it has bidirectional activity: it can arrest a replication fork entering from either direction (this thesis, chapter IX). The terminators on R100, R1 and pLS20 are located in close proximity to the origin of replication and are likely to force unidirectional replication. The R6K terminus, however, is located asymmetrically with respect to the origin. In this case, unidirectional replication proceeds from the origin to the terminus where the fork is arrested. A second replication fork is then initiated from the same origin that proceeds in the opposite direction and meets the arrested fork at the terminus region (Lovett et al., 1975).

The position of the B.subtilis and E.coli replication terminators on their chromosome is shown in Fig. 4. For E.coli six different terminator sites (TerA-TerF) have been identified which are asymmetrically distributed in a region comprizing approximately 25% of the chromosome. These sites are located more or less diametrically opposite of the origin of replication (Hill, 1992). These terminators, which have polar activity, are positioned in such a way that they funtion as a replication fork trap, assuring that the approaching replication forks meet within a restricted region of the chromosome. A similar situation occurs in B.subtilis Also in this bacterium the terminus region, which is located approximately opposite of oriC, contains six polar terminators (TerI-TerVI) which are arranged in a similar way as those in E.coli (Franks et al., 1995). Another feature shared by both organisms is that one of the inner terminators (TerI [formerly denoted IRI] in B.subtilis and TerB in E.coli) is localized in close proximity to the promoter of the gene encoding the cognate terminator protein (RTP in B.subtilis, Tus in E.coli). As a consequence, expression of these genes are negatively autoregulated by binding of the terminator protein (Ahn et al., 1993; Roecklein and Kuempel, 1992). In the chromosome of B.subtilisspecific loci have been identified at considerable distances (approximately 200 kb) from both sides of the origin which arrest replication fork movement only during stringent response conditions (Henckes et al., 1989; Levine et al., 1991). Under relaxed conditions the arrested forks are released and replication is continued (Levine et al., 1995). Since the arrest of replication fork movement depends on RTP, it was assumed that these sites are terminator sites (Levine et al., 1995). Probably, the DNA sequences of these terminators are less conserved than the highly homologous sequences of TerI through TerVI. This assumption is based on the observation that, whereas TerII through TerVI are detected in Southern hybridizations performed under stringent conditions with TerI as a probe, the oriC flanking terminators are not (Franks et al., 1995).

Although the chromosomal replication terminators of E.coli and B.subtilis, as well as those on plasmids, are dispensable for replication (Iismaa and Wake, 1987; Henson and Kuempel, 1985; Hill, 1992; this thesis, chapter VIII), it seems clear that replication termination processes are involved in regulation of DNA replication. Hiasa and Marians (1994) suggested that replication termination functions to prevent over-replication of DNA. Using in vitro assays these authors showed that in the absence of a functional Ter-Tus complex multimeric DNA forms were generated. Evidence was presented that the generation of multimeric DNA forms was inhibited by preventing the DnaB helicase to continue to unwind DNA after one round of replication of the duplex DNA consisting of an original template strand and a nascent strand. Possibly, preventing over-replication is also a function of the plasmid-located terminators since deletion of plasmid Ter sites has been associated with the accumulation of plasmid multimers (Baker, 1995). Experimental support for this idea is not yet available, however. A possible second function of terminators can be derived from the observation that RecA-dependent homologous recombination occurs 10-100 times more frequently in the terminus region than in other regions of the chromosome (Louarn et al., 1994; Horiuchi et al., 1994). Ter sites may constitute efficient loading sites for the homologous recombination machinery (Horiuchi et al., 1994). The recent elucidation of the crystal structure of RTP from B.subtilis(Bussiere et al., 1995) and the identification of a distinct terminator with bidirectional activity (this thesis, chapter IX) will undoubtedly contribute to the ongoing research on the function of replication terminators.

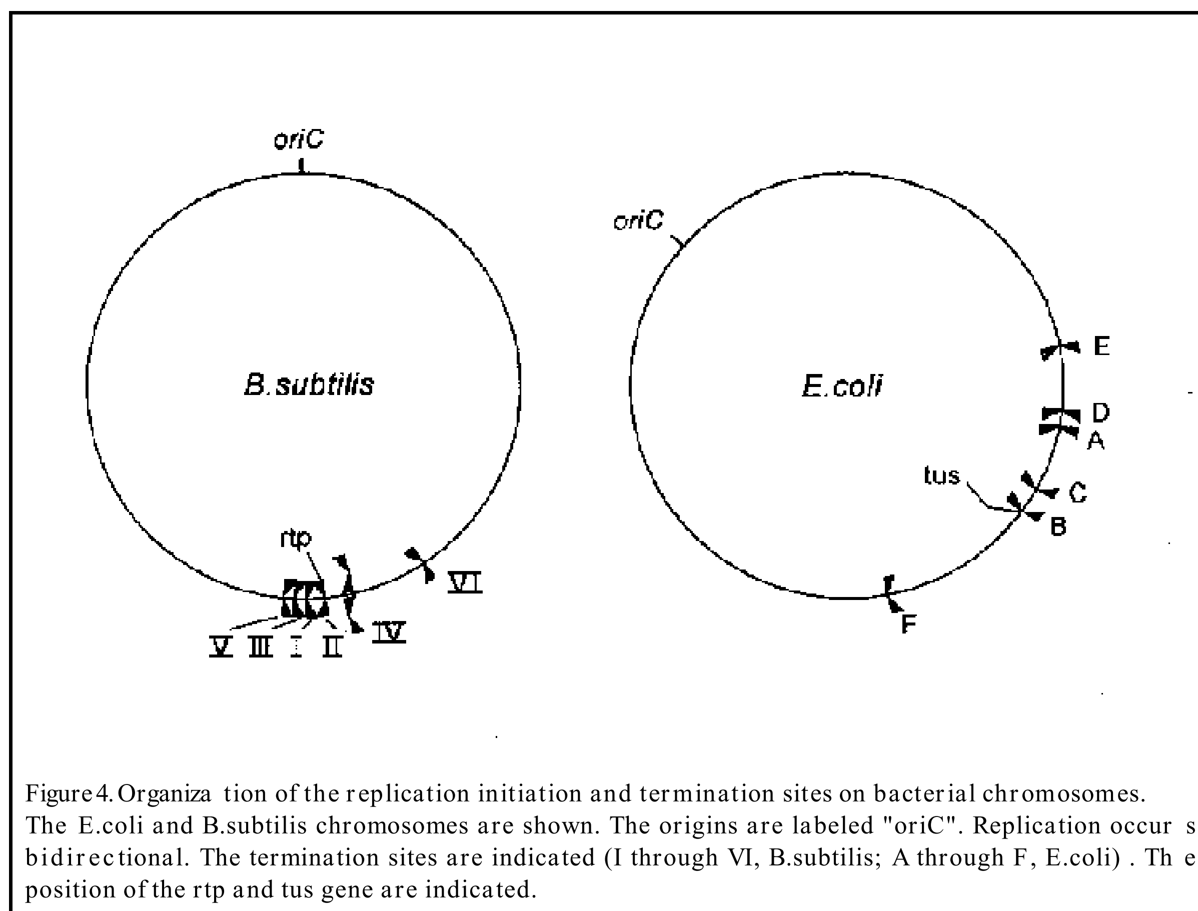

Figure 4. Organization of the replication initiation and termination sites on bacterial chromosomes. The E.coli and B.subtilis chromosomes are shown. The origins are labeled "oriC". Replication occurs bidirectional. The termination sites are indicated (I through VI, B.subtilis; A through F, E.coli) . The position of the rtp and tus gene are indicated.

2.C.Id Host-range of theta replicons

Large differences exist in the host-ranges of different theta plasmids. Unlike certain RCM plasmids, none of the known theta plasmids seems to be able to replicate in both Gram-positive and Gram-negative bacteria. Plasmids belonging to class A can have either a broad host-range (e.g. RK2, RSF1010), or a narrow host-range (e.g. pSC101, pWV02). ColE1 and the ColE2 group of plasmids, constituting classes B and C, have a narrow host-range. The Gram-positive pAMß1 family of plasmids has a wide host-range. So far, the host-range of pLS20 has not been determined.

Plasmid replication depends on host enzymes and plasmid-located cis and trans determinants. Effective interactions between host and plasmid determinants are key elements in determining the host-range of a plasmid. These factors are at present only partly understood. For an overview of known factors involved in host-ranges of Gram-negative plasmids we refer to Kües and Stahl (1989).

3:Plasmid instability

Plasmid instability is a phenomenon frequently observed with recombinant DNA in Gram-positive hosts and poses problems of both fundamental and applied nature. Two types of plasmid instability can be distinguished. The first is structural instability and the second segregational instability.

Structural instability refers to DNA rearrangements that result in structural alterations of the plasmid. Frequently, these alterations consist of DNA deletions. DNA rearrangements can occur via several error-prone mechanisms, such as slipped-mispairing and breakage-reunion, and the insertion or excision of DNA fragments via transposons and insertion elements. Slipped-mispairing, also referred to as copy-choice replication errors, results from fork slippage between short direct repeats (DR), causing the deletion of the region between the repeats and one of the repeats (Ehrlich et al., 1986; Peeters et al., 1988; Bron et al., 1991a). Deletions between non-repeated sequences are usually considered to result from breakage-reunion errors (Ehrlich, 1989; Ehrlich et al., 1993). Structural rearrangements can also be induced by pauses during replication (Bierne and Michel, 1994), or by transcription of two divergently oriented genes (C. Cordes, personal communication).

Segregational instability refers to the loss of the entire plasmid population from the cell. In B.subtilis this can occur both during vegetative growth or during sporulation. In the first case, none of the plasmids is segregated into one of the daughter cells after division, and in the second case none of the plasmids is received by the spore. As described below, separate mechanisms exist that ensure high segregational stability during vegetative growth and sporulation.

Experiments described in this thesis

are focused on segregational stability during vegetative growth. At least two factors are important for high segregational plasmid stability in bacterial cells. The first is faithful plasmid replication. On the average, the plasmid population should be doubled at least once per cell cycle. The second is the accurate partitioning of plasmids so that each daughter cell receives at least one plasmid copy during cell division. Under a given set of conditions plasmids are maintained at more or less constant numbers per cell. Some plasmids are maintained at high copy numbers, others at only a few copies per cell. If their copy number is high enough to ensure stable inheritance via random distribution during cell division, high stability mainly requires faithful replication. Low-copy-number plasmids require the presence of additional mechanisms to ensure their faithful distribution to the daughter cells.

Segregational stability is measured by analyzing plasmid maintenance under non-selective growth conditions. Although plasmid maintenance can also be affected by parameters other than plasmid segregation during cell division (see below), this assay is adequate for studying segregational plasmid stability. Wherever plasmid stability, or maintenance, is mentioned in the remainder of this chapter, this refers to plasmid maintenance during vegetative growth, unless mentioned otherwise.

3.A Maintenance of rolling-circle plasmids

Several plasmids that use the RCM of replication have been fully sequenced. So far, no specific genes have been identified ensuring that each daughter cell receives at least one plasmid copy during cell division. Therefore, these plasmids seem to be partioned randomly. An important prerequisite for stable maintenance of these plasmids is, therefore, their faithful replication. As described in section II.B, faithful RCM replication requires a functional Rep protein and a cognate DSO, necessary for replication initiation, and a functional SSO, necessary for efficient ssDNA conversion. The absence of a functional SSO impairs faithful RCM replication which can be revealed as the accumulation of of ssDNA replication intermediates. Inefficient ssDNA conversion indeed causes plasmid instability (Gruss et al., 1987; del Solar et al., 1987; Chang et al., 1987; Viret and Alonso, 1988; Devine et al., 1989; Boe et al., 1989; Bron et al., 1991b; this thesis, chapters II and III, IV and V). In some cases, in particular with certain plasmids in staphylococci and streptoccoci, the absence of a functional SSO results in a decrease of plasmid copy numbers, which may explain the reduced plasmid maintenance (Gruss et al., 1987; del Solar et al., 1987). However, especially in B.subtilis, no clear differences in plasmid copy numbers were observed with several plasmids containing or lacking a functional SSO (Viret and Alonso, 1988; Boe et al., 1989; del Solar et al., 1993; this thesis, chapter III). In addition, with the plasmids studied, the level of accumulation of ssDNA was relatively low and the plasmids were maintained rather stably in B. subtilis. Apparently, in these cases ssDNA conversion still occurred rather efficiently. This implies that ssDNA conversion may occur via an alternative, SSO-independent, route. The observation that pUB110 and pMV158 derivatives lacking a functional SSO accumulate far higher amounts of ssDNA in B.subtilis RecA-deficient compared to RecA-proficient strains (Viret and Alonso, 1988; this thesis, chapters III and IV) suggests that RecA is involved in an alternative pathway for ssDNA conversion. A possible RecA-stimulated pathway of ssDNA conversion was analyzed in detail for pMV158 derivatives lacking a functional SSO (this chapter, section II.B.2c and

chapter IV). As described above (section II.B.2d), the proposed RecA-dependent ssDNA conversion pathway may also be involved in the host-range of certain plasmids. In chapter III of this thesis we describe that, compared to the situation in a B.subtilis RecA-proficient strain, in B.subtilis RecA-deficient strains pMV158 derivatives lacking a functional SSO accumulate higher amounts of ssDNA and are maintained far more unstably. This suggests that a direct correlation exists between ssDNA accumulation and plasmid maintenance. Although the accumulation of large amounts of ssDNA indeed seems to be correlated with high levels of plasmid instability, such a correlation between the amount of ssDNA and plasmid maintenance was not always observed, in particular when only moderate or low amounts of ssDNA are present (del Solar et al., 1993; this thesis, chapters II and III). Apparently, also parameters other than ssDNA accumulation affect plasmid maintenance. Several of these parameters will be discussed in the following paragraph.

The palA-type SSO of pMV158 is not, or only poorly, functional as an ssDNA conversion signal in B.subtilis (del Solar et al., 1987; del Solar et al., 1993; Kramer et al., 1995; this thesis chapter III). However, its presence has a positive effect on plasmid maintenance of pMV158 derivatives lacking a functional SSO (del Solar et al., 1993; this thesis chapter III). Del Solar et al. (1993) have identified stretches of DNA within the palA sequence that show homology with the par region of the E.coli theta plasmid pSC101. The latter region is involved in the stable inheritance of this plasmid (Meacock and Cohen, 1980; Manen and Caro, 1991; Ingmer and Cohen, 1993). The par locus is a binding site for the host-specified DNA gyrase (Wahle and Kornberg, 1988), an enzyme that can alter the superhelical conformation of DNA molecules. For pSC101 it was shown that the level of DNA supercoiling has a major effect on plasmid maintenance. Deletion of the par region from pSC101 resulted in a decrease of overall superhelical density of this plasmid and increased plasmid loss rates (Miller et al., 1990). Conceivably, the palA-type SSO of pMV158 likewise contains a gyrase-binding site, which could explain its positive effect on plasmid maintenance. Beaucage et al. (1991) have shown that alterations in superhelicity caused by promoter activities also affect the maintenance of pSC101. In this respect, it may be relevant to note that the S.aureus RCM plasmids pT181 and pE194 have a lower superhelical density in B.subtilis than in S.aureus (Novick et al., 1986). Both plasmids are maintained unstably only in B.subtilis. Although both plasmids accumulate high amounts of ssDNA in B.subtilis, which may cause plasmid instability, (part of) the instability may also be a consequence of altered superhelicity.

Other parameters that affect plasmid maintenance are deleterious products encoded by the plasmid which reduce the growth rate of the host. Typically, in these cases the kinetics of plasmid loss show a biphasic pattern. Whereas the initial rates of plasmid loss are moderate, the plasmid-containing cells are rapidly outgrown by plasmid-free cells once the latter are present in the culture (Boe et al., 1987; this thesis, chapter III). Several processes may underlie the production of deleterious substances. One of these is the production of linear, tandemly-repeated head-to-tail plasmid molecules. These plasmid species, which migrate in agarose gels at positions of high molecular weight, have been designated as HMW DNA (high-molecular-weight DNA). HMW DNA is considered for be toxic to B.subtilis cells (Leonhardt and Alonso, 1991). The generation of this type of plasmid molecules is stimulated by the insertion of, in particular, heterologous DNA fragments into RCM plasmids (Gruss and Ehrlich, 1988). Bron and Luxen (1985) and Bron et al. (1988b) reported that the

size of the inserts in pUB110 derivatives is inversely correlated with plasmid maintenance. The plasmids used in these studies indeed accumulated HMW DNA (Gruss and Ehrlich, 1988). Also in L.lactis, a correlation between insert size and plasmid instability was observed with RCM plasmids, and this effect was attributed to the generation of HMW DNA (Kiewiet et al., 1993b). However, the production of HMW DNA does not always result in plasmid instability. Gruss and Ehrlich (1988) reported that although the introduction of certain foreign DNA fragments into pUB110, other than those used by Bron and coworkers, resulted in the generation of HMW DNA in B.subtilis, these plasmids were maintained stably. A major difference between the plasmids used by Bron and coworkers (1988b) and those used by Gruss and Ehrlich (1988) is that only in the latter studies the pUB110 derivatives still contained their palU-type SSO. Also, Viret and Alonso (1987) have reported that HMW DNA is generated when foreign DNA is inserted into RCM plasmids. These authors have shown that, in some cases, HMW DNA accumulation was correlated with plasmid instability. As in the studies performed by Bron et al. (1988b), HMW DNA accumulation and plasmid instability was observed with plasmids lacking a functional SSO. In B.subtilis, it therefore seems that the accumulation of HMW DNA results in plasmid instability only when RCM plasmids lack a functional SSO. It should be noted that the accumulation of HMW DNA can also affect plasmid copy numbers (Bron and Luxen, 1985; Bron et al., 1988b; Kiewiet et al., 1993b) which (partly) may account for the observed instability.

It cannot be excluded that (part of) the instability described in this section is caused by the expression of deleterious gene products synthesized from insert-located genes. That this idea is realistic, is illustrated by results obtained by Leonhardt (1990). The stability of a pUB110 derivative, from which a toxic product was synthesized, was compared with a nearly identical plasmid that differed by only one basepair, which resulted in a lower plasmid copy number. The high-copy number variant was maintained less stably than the low-copy number plasmid. This was attributed to the higher production of toxic products by the high-copy number variant causing a growth disadvantage to these cells. These studies show that the maintenance of randomly distributed plasmids is not in all cases improved if the plasmid copy number is increased. Additional examples of a lack of correlation between plasmid copy number and plasmid maintenance have been reported (del Solar et al., 1993; this thesis, chapter III). In the work of the latter authors, the maintenance of RCM plasmids lacking a functional SSO could not be improved by increasing the plasmid copy numbers to values which, assuming random partitioning, should guarantee full stability. Two possible explanations can account for these observations (this thesis chapter III). First, the high-copy number plasmids, which lack a functional SSO, will accumulate higher amounts of ssDNA which may be detrimental to the cell (Bron et al., 1991b). Second, subpopulations of cells may be present within the culture which have a higher than average probability to become plasmid-free. The latter explanation has gained experimental support from studies in E.coli (Tolker-Nielsen and Boe, 1994). The generation of such subpopulations may be caused by clustering of plasmids in the cell or by a high variation in plasmid copy numbers between individual cells.

Other factors that can affect the stability of RCM plasmids are host mutations, especially those involved in recombination processes. An example is the effect of RecA on the maintenance of plasmids lacking a functional SSO (this thesis, chapter IV). Alonso et al. (1987)

described that pC194 derivatives, in addition to being unstable in recA mutants, are also unstably maintained in B.subtilis addAB and recF mutants [the B.subtilis addAB genes are the functional equivalents of the E.coli recBCD genes (Kooistra et al., 1993)]. Interestingly, mutations in addAB or recF seem to result in increased amounts of HMW DNA, which has been shown to interfere with plasmid maintenance (Viret and Alonso, 1987). Thus, the effects of mutations in certain genes involved in recombination may be explained by effects on the generation of HMW DNA. In addition to effects on plasmid maintenance, it has also been reported that deletion of the addAB genes greatly enhances structural instability (Meima et al., 1995). It is conceivable that high levels of structural instability can reduce plasmid maintenance through the deletion of essential replication functions. In fact, Leonhardt (1990) has described that certain structurally unstable pUB110 derivatives are also segregationally unstable.

Tanaka and Sueoka (1983), have described that specific regions of plasmid pUB110 bind, at least in vitro, to B.subtilis membrane fractions. Four such membrane binding areas, designated binding area (BA) 1 through 4, were detected. Two of these are located within the rep gene. Interestingly, BA3 encompasses the palU-type SSO. The fourth binding area, BA4, is located within the mob gene required for conjugative mobilization (Selinger et al., 1990). Conceivably, plasmid partitioning might occur via membrane binding, but this hypothesis has not been explored in detail. Bron et al. (1988b) have reported that pUB110 derivatives containing large inserts are less stably maintained when these plasmids lack the BA4 region. Moreover, Seyler et al. (1993) reported that deletion of the BA4 region results in the generation of plasmid-free spores. Together, these results could indicate that specific plasmid regions may affect plasmid stability through an interaction with the membrane.

In summary, so far, no plasmid-located genes other than rep have been identified on RCM plasmids which encode functions that assure stable plasmid maintenance. Many of the parameters studied which affect the maintenance of these plasmids are related, directly or indirectly, to plasmid replication. Another main conclusion is that large inserts have a strong negative effect on plasmid maintenance of RCM plasmids (Bron et al., 1988a; Gruss and Ehrlich, 1988; Bron et al., 1991b; Kiewiet et al., 1993b). Apparently, faithful RCM replication becomes problematic when the plasmids are larger than approximately 12 kb. This may also be the reason why all RCM plasmids described so far do not exceed the 12 kb size limit. As described in the following section, the cloning of large DNA fragments should, therefore, preferably be carried out in theta-replicating plasmids.

3.B Maintenance of theta plasmids

Obviously, also proper inheritance of theta-replicating plasmids requires faithful replication. Generally, natural theta plasmids with very low copy numbers are maintained in an extremely stable way. Loss rates as low as 10^{-7} per cell division have been observed (Nordström and Aagaard-Hansen, 1984). In contrast to the intact plasmids, deletion derivatives containing solely the information required for autonomous replication are frequently unstably maintained, indicating that the parental plasmid contains specific stability functions ensuring stable inheritance. Regions that stabilize theta plasmids have been found in several systems and it is clear that enhanced plasmid maintenance result from different mechanisms. In addition to regions that specify typical partition functions, that is functions that guarantee the active distribution of plasmid molecules to daughter cells upon cell division,

functions have been identified on plasmids that mediate the resolution of plasmid oligomers, or the killing of plasmid-free cells. A particular plasmid can specify different functions affecting maintenance. Thus the high levels of maintenance of naturally occurring plasmids frequently appear to be due to the concerted action of multiple systems. Most information concerning such stability functions is available from Gram-negative plasmids. Based on their mode of action, stability functions can be divided into two classes: (i), elements that improve random distribution; (ii), elements that ensure a better than random distribution. For recent reviews we refer to Nordström and Austin (1989); Williams and Thomas (1992); and Hiraga (1992).

(I) Elements that improve random distribution

Plasmid multimerization results in a reduction of the number of segregating units. Therefore, maintenance of multicopy plasmids that are segregated randomly will be optimal when all plasmid molecules are in the monomeric form. The process by which multimeric plasmid forms are converted into monomers is called resolution. Resolution functions have been identified on multi-copy and low-copy plasmids. Since the result of one round of theta replication is a dimer which cannot be partitioned to both daughter cells, it has been speculated that resolution functions may be present on all natural theta plasmids (Nordström and Austin, 1989). In the examples studied so far, resolution occurs through efficient site-specific recombination. Two different classes of site-specific recombination processes can be distinguished: (i), the recombination site and the recombinase-encoding gene are both plasmid-located; and (ii), the recombination site is plasmid-located but the recombinase is host-encoded. Examples of the first class have been found on the Gram-negative plasmids F and P1, both present in the cell at very low copy numbers. The F plasmid contains the D gene which encodes the recombinase that acts on the rsfF site (O'Connor and Malamy, 1984; Lane et al., 1986); P1 contains the lox-cre system, where Cre is the recombinase and loxP the site at which it acts (Austin et al., 1981). Also the site-specific resolvase systems present on the Gram-positive plasmids pAMß1, pIP501, pIP404 and pSM19035 belong to this class (Swinfield et al., 1991; Pujol et al., 1994; Garnier et al., 1987; Ceglowski et al., 1993a; Ceglowski et al., 1993b; Rojo and Alonso, 1994).

An example of the second class is the cer-xer system of the Gram-negative multi-copy plasmid ColE1. In this case, the plasmid-located recombination site, cer, is recognized by the host-encoded XerCD recombinases (Summers and Sherratt, 1984; Blakely and Sherratt, 1994). Since all plasmids from the ColE2 group of plasmids contain a sequence highly homologous to the ColE1 cer site, it is likely that these plasmids use a similar resolution mechanism (Hiraga et al., 1994).

One might reason that high-copy number plasmids would not require efficient resolution systems because random segregation would assure full stability in these cases. If distributed randomly during cell division, the probability (P_0) that plasmid-free daughter cells are formed can be estimated from the equation $P_0=2^{(1-n)}$ in which n represents the plasmid copy number at the time of cell division. Accordingly, the probability that a cell containing 30 plasmid monomers would generate a plasmid-free daughter is expected to be $1.x10^{-9}$. However, many reports indicate that high-copy number plasmids are far more unstable than predicted. Recently, Summers et al. (1993) have shown that low levels of plasmid dimers may be responsible for the observed

high instability. These authors argued, that, as a consequence of plasmid origins being chosen at random for replication, the probability that a dimer will replicate is twice that of a monomer. Consequently, the proportion of dimers within a cell will increase rapidly, resulting in subpopulations of cells containing dimers only. These have an increased probability of generating plasmid-free offspring. Summers et al. (1993) have designated this concept "the dimer catastrophe hypothesis". This concept does explain why efficient resolution systems are important, also for high-copy number plasmids.

Another stability determinant that belongs to this class is the cis-acting par locus of plasmid pSC101. Originally, it was assumed that this locus constituted part of an active partitioning system (Meacock and Cohen, 1980). However, the 374 bp par locus does not encode a protein, and it is now known that plasmid derivatives lacking the par locus have a decreased overall superhelical density compared to intact pSC101 (Miller et al., 1990). It was shown that the par locus is a binding site for the host-encoded DNA gyrase (Wahle and Kornberg, 1988), and Miller et al. (1990) have suggested that altered levels of supercoiling would underlie the par-mediated effects on the stability of pSC101. Support for this hypothesis is the observation that the stability of derivatives lacking the par locus can be increased by increasing the negative superhelicity through the introduction of certain active promoters (Beaucage et al., 1991). Recently, Conley and Cohen (1995) described that mutations in the 5'-half of the pSC101 repA gene can also stabilize plasmid derivatives lacking par. The authors suggested that the repA mutations compensate for the par deletion by enabling the formation of the origin/RepA nucleoprotein complex under suboptimal conditions. This complex would have a role in partitioning different from its known role in DNA replication.

(II) Elements that ensure a better than random distribution

Elements that actively enhance stable plasmid maintenance have been identified and studied in detail with several low-copy-number E.coli plasmids. Based on their mode of action, these can be divided in two groups. The first involves the so-called "killer systems", which act by killing plasmid-free segregants, and the second group involves the so-called "true partition systems", which act by ensuring that each daughter cell receives at least one plasmid copy during cell division.

Killer systems have been identified on several plasmids. The R1 plasmid contains the hok-sok system, in which two RNAs are involved. One of these, hok, is a stable mRNA which encodes a toxic protein, Hok. The other, sok, is an unstable antisense RNA which prevents translation of the hok mRNA (Gerdes and Molin, 1986; Gerdes et al., 1986). The following mechanism has been proposed for the hok-sok killing system. Daughter cells, including plasmid-free descendants, will inherit hok/sok RNA molecules from the mother cells. The sok RNA molecules decay rapidly and, since in plasmid-free daughter cells no sok RNA will be synthesized de novo, this will lead to derepression of hok mRNA translation and the production of the lethal Hok protein in plasmid-free descendants (Gerdes et al., 1990a). The regulation of this ingenious system has been studied in detail (Thisted and Gerdes, 1992; Thisted et al., 1994a; Thisted et al., 1994b). A homologous system on the F plasmid has been termed flm (Loh et al., 1988), or stm (Golub and Panzer, 1988), and several other homologous systems are known (reviewed by Gerdes et al. (1990b). In addition to the flm or stm locus, the F plasmid contains the ccd system which, likewise, is a killer system for plasmid-free

cells. In this case, the toxic product and its unstable antagonizing effector are both proteins (Hiraga et al., 1986; Tam and Kline, 1989; Bernard and Couturier, 1991). Systems homologous to ccd have been identified on R100/NR1 [denoted the pem system (Tsuchimoto et al., 1988; Tsuchimoto and Ohtsubo, 1989)], and on R1 [the kis/kid system (Bravo et al., 1987)]. As a consequence of the killing of plasmid-free segregants the growth rates of the host population are reduced (Jaffé et al., 1985).

True partition systems ensure that each daughter cell receives at least on plasmid copy during cell division. Like "killer systems", "true partition systems" have been identified on several plasmids. The systems present on the F plasmid, [the sop system (Austin and Abeles, 1983a; Biek and Shi, 1994)], and on the P1 prophage plasmid, [the par system; (Austin and Abeles, 1983b; Abeles et al., 1985; Davis et al., 1990; Hayes et al., 1994; Hayes and Austin, 1994; Davey and Funnell, 1994)] have been studied most extensively. True partition systems have also been identified on P7, RK2, R1, NR1/R100, the *Agrobacterium* plasmids pTAR, pTiB6S3, pRiA4b, the *Chlamydia trachomatis* plasmid pCHL1, and the *Shigella* virulence plasmid pSS120 (Williams and Thomas, 1992; Hiraga, 1992). Plasmids sharing the same active partition system will interfere with each other's ability to be partitioned correctly, and thus show incompatibility towards each other (Austin and Nordström, 1990). In general, these systems consist of two *trans*-acting polypeptides and a cis-acting centromere-like site, all of which are essential for partition. The *trans*-acting proteins assemble to a nucleoprotein complex at the cis-site. Miller and Kline (1979) described that F plasmid DNA could be recovered from a folded bacterial chromosome fraction. Thus partition might occur via attachment to the chromosomes. Ezaki et al. (1991) tested this hypothesis by using a mukB null-mutant host strain harbouring mini-F plasmids containing the sop system. The mukB mutant used was defective in chromosome partitioning and frequently generated anucleate cells. Analysis showed, however, that the mini-F plasmids segregated faithfully in nucleate as well as anucleate cells. This demonstrates that active partitioning does not require the host chromosome. Despite extensive biochemical and functional analyses, the precise mechanism by which true partition systems act remains unclear.

Evidence that large low-copy number plasmids from Gram-positive bacteria have similar active partitioning systems is limited. The streptococcal plasmid pSM19035 seems to have a partition-like system (Ceglowski et al., 1993a; Ceglowski et al., 1993b). Recently, Weaver and Tritle (1994) identified a stability determinant on the 60 kb *Enterococcus faecalis* plasmid pAD1. This determinant was delineated to a region of no more than 720 bp which was unlikely to encode proteins. The high stability was correlated with the synthesis of two small RNA molecules and the authors suggested that this locus encodes an active partition system which functions by a unique, so far unknown, mechanism. Finally, Lereclus and Arantes (1992) have described a stability-promoting gene, *spbA*, present on the *B.thuringiensis* theta plasmid pTH1030. In addition to the stabilizing effect of *spbA* on the maintenance of pTH1030 during vegetative growth, this locus is particularly effective during sporulation. The *spbA* gene encodes a small protein of 15 kDa. No homology was observed between the *spbA* gene product and other known proteins, and the mechanism by which the *spbA* gene operates is unclear.

4: Bacillus plasmids

Several plasmids have been isolated from B.subtilis, mainly from industrial strains. Since most cloning vectors used for B.subtilis are based on RCM plasmids from other Gram-positive bacteria, like staphylococci and streptococci, the endogenous B.subtilis plasmids have attained relatively little attention. Only during the last few years has it become clear that some of the stability problems observed with RCM vectors based on non-native plasmids are caused by non-optimal plasmid-host interactions. Support for the supposition that endogenous plasmids of B.subtilis are superior to non-native plasmids with respect to structural stability has been obtained by Haima et al. (1990). These authors showed that large heterologous DNA fragments (up to at least 29 kb) could be cloned in the B.subtilis-derived plasmid pHP10, and that the recombinant plasmids were structurally stably maintained in this bacterium. This E.coli/B.subtilis shuttle plasmid is based on the replication functions of the cryptic B.subtilis RCM plasmid pTA1060 and the E.coli pUC origin. An improved version of this plasmid, pHB201, contains, besides the palT SSO of pTA1060, also the lacZα-complementation system. The latter system enables the blue-white screening assay on X-Gal-containing agar plates in lacZΔM15 B.subtilis background. It was also shown that recombinant plasmids based on the replication functions of pTA1060 are maintained far better as compared to S.aureus plasmids, even when the former lack their native SSO (our own unpublished results).

As mentioned above, several plasmids have been identified in industrial B.subtilis strains. Based on their size and restriction profiles, Uozumi et al. (1980)

Table 3. Classification of cryptic RCM plasmids from B.subtilis

family	size[a]	plasmids with identical sizes and restriction profiles
pTA1015	5.8	pTA1015, pTA1010 (=pLS15), pTA1011 (=pLS17), pTA1012, pTA1013 (=pLS19), pTA1014, pTA1016, pTA1017 (=pLS24), pTA1018 (=pLS26), pTA1019, pUH1 through pUH8
pTA1020	6.6	pTA1020, pTA1021 (=pLS28), pTA1022 (pLS30), pTA1023, pBAA1
pTA1060	8.7	pTA1060 (=pLS11), pTA1061 (=pLS12)
pTA1040	7.7	pTA1040 (=pLS13)
pTA1030	7.2	pTA1030, pTA1031
pTA1050	8.2	pTA1050 (=pLS14)
pFTB14	8.2	pFTB14

a: plasmid size in kb. pTA-series according to Uozumi et al. (1980); pLS-series according to Tanaka and Koshikawa (1977), and Tanaka et al. (1977); pUH1 series according to Hara et al. (1983); pFTB14 according to Murai et al. (1987)

could classify the small B.subtilis plasmids, known at that time, into six groups of which pTA1015, pTA1020, pTA1030, pTA1040, pTA1050 and pTA1060 are representatives. Later, Murai et al. (1987) extended this classification by a seventh group, represented by pFTB14 isolated from Bacillus amyloliquefaciens Other cryptic Bacillus plasmids have been identified by different research groups (Lovett and Bramucci, 1975; Tanaka and Koshikawa, 1977; Tanaka et al., 1977; Le Hégarat and Anagnostopoulos, 1977; Bernhard et al., 1978; Hara et al., 1983; Hara et al., 1986; Hara et al., 1993). Based on their size and restriction pattern most of these plasmids can also be classified in one of these seven described groups. The various classified plasmids are listed in Table 3. Most likely, all plasmids within a group are identical or at least highly homologous to each other (this thesis, chapters V and VII). Some of these plasmids have been studied in more detail by the cloning and sequencing of the primary replication functions, i.e. the rep gene and the DSO. Examples of these are pBAA1 (Devine et al., 1989), pUH1 (Hara et al., 1991), pLS11 (Hara et al., 1992b), pBS2 (Darabi et al., 1989) and pFTB14 (Murai et al., 1987). The primary replication functions of all of these plasmids are highly related and these plasmids belong to the pC194 family of RCM plasmids (Table 1; this thesis, chapter VII)). In addition, the SSOs of some of these plasmids have been analyzed: pTA1060 (Bron et al., 1987), pLS11 (Chang et al., 1987), and pBAA1 (Seery and Devine, 1993). A comparative study of SSOs from representative plasmids of each of the six classes defined by Uozumi et al. (1980) is described in this thesis (chapter V). The identification of a rep gene, a putative DSO and an SSO, together with the observed production of ssDNA replication intermediates, indicates that the plasmids concerned use the RCM of replication.

Compared to most of the RCM plasmids from S.aureus, the B.subtilis RCM plasmids are somewhat larger (Table 1) which suggests that, in addition to the typical RCM modules, additional genes may be present on the latter. So far, knowledge of the presumed additional genes was limited. Since most of these plasmids were isolated from industrial strains, it is conceivable that the additional genes encode industrially important traits. In fact, industrially-important traits can be encoded by plasmids as exemplified in lactic acid bacteria that are used in dairy industry (McKay, 1983). To obtain support for the possible presence of industrially important traits on endogenous B.subtilis plasmids we determined the complete nucleotide sequence of three such plasmids, pTA1015, pTA1040 and pTA1060. Additional motivations for these analyses were that elements, involved in the observed stable maintenance of pTA1060 derivatives, might be identified in these studies. Moreover, the information obtained would contribute to the understanding of cryptic B.subtilis plasmids in general. The outcome of these studies are presented in chapter VII and will only be briefly summarized here. All three pTA-plasmids contain two highly homologous genes. One, the rep gene, encodes the typical RCM replication initiator protein. The other gene encodes a putative-DNA binding protein of which the function is unknown. In addition, both pTA1015 and pTA1040 contain a module consisting of two genes, one encoding a putative exported protein with unknown function; and the other encoding a functional type I signal peptidase (for detailed analyses, see chapter VI). Moreover, pTA1040 and pTA1060 share a homologous, but non-identical, transcription unit which shares homology with the B.subtilis chromosomally-encoded rap (= gsi) genes A and B. The latter genes encode aspartate phosphatases involved in the control of post-exponential developmental processes in B.subtilis

(Perego et al., 1994). In particular, these gene products postpone the onset of sporulation (Mueller et al., 1992; Mueller and Sonenshein, 1992; Perego et al., 1994). The plasmid-located homologues are also likely to delay the onset of sporulation of host cells. Especially the plasmid encoded sip and rap genes are of potential industrial importance.

One further conclusion is that pTA1015 is identical to pUH1, a plasmid of which the complete sequence was determined previously (Hara et al., 1992c). These authors concluded that pUH1 contains a gene, gtp, encoding the γ-glutamyltranspeptidase (γ-GTP) which is involved in "natto" production (Hara et al., 1992a). "Natto" is a traditional fermented food in Japan and other South-East Asian countries, and is produced by growing B.subtilis (natto) on steamed soybeans. The gtp gene present on pUH1 was claimed to be responsible for γ-polyglutamate production which is a major constituent of "natto" (Hara et al., 1992a). However, whereas the pUH1-located γ-gtp gene shares no homology with a cloned γ-gtp gene from the chromosome of B.subtilis strain SJ138 (Ryoichi et al., 1991), this plasmid region has a high level of homology with mob genes identified on several other RCM plasmids. In fact, we have shown that the corresponding region of pTA1015 encodes a functional Mob protein by which the plasmid can be conjugatively mobilized. Therefore, it is unlikely that the corresponding region on pUH1 encodes a γ-GTP (this thesis, chapter VII). A highly homologous mob gene is also present on pTA1060, but not on pTA1040.

As mentioned before, large inserts can be cloned more efficiently in theta plasmids, compared to most RCM plasmids. In addition, the recombination frequencies between short direct repeats are much lower in theta plasmids (Jannière et al., 1990; Bron et al., 1991b). These superior characteristics of theta plasmids have led to an increased interest in Gram-positive theta plasmids during recent years. So far, knowledge about native Bacillus theta plasmids was very limited. In several reports the presence of large (ranging from 15 to approximately 160 kb) plasmids in different Bacillus species has been reported (Le Hégarat and Anagnostopoulos, 1977; Tanaka and Koshikawa, 1977; Rostas et al., 1980; von Tersch and Carlton, 1983; Battisti et al., 1985; Baum and Gilbert, 1991; Baum and Gonzalez, 1992). As judged from their size, these large plasmids are expected to use the TM of replication. However, preliminary characterizations of the replication region have only been carried out with a few of these plasmids, several of which were isolated from B.thuringiensis (Baum et al., 1990; Baum and Gilbert, 1991; Arantes and Lereclus, 1991; Lereclus and Arantes, 1992), and one (pTB19) which was originally isolated from Bacillus stearothermophilus (Imanaka et al., 1986). The structural organization of the replication regions of the 43, 44 and 60 mDa plasmids from B.thuringiensis (Baum and Gilbert, 1991) and the B.stearothermophilus plasmid pTB19 (Imanaka et al., 1986), seems to be similar to that of the well-studied pAMß1 family of plasmids. Contrary to pAMß1 and the other Bacillus plasmids mentioned above, the replication region of the B.thuringiensis plasmid pTH1030 does not contain a typical rep gene (Lereclus and Arantes, 1992).

To the best of our knowledge, replication origins from theta-replicating B.subtilis plasmids were not studied so far. We reasoned that, as with RCM plasmids, endogenous theta-replicating plasmids from B.subtilis would be optimally adapted to this host, and would, therefore, show superior stability properties compared to non-native theta plasmids. This consideration, together with the intrinsic interest in the replication mechanism of endogenous B.subtilis theta plasmids, prompted us to clone and analyze the

replication region of the B.subtilis (natto) plasmid pLS20. We cloned and analyzed a 3.1 kb region containing the replication functions of this plasmid. The results of these studies are presented in chapters VIII and IX. In short, the replication region was delineated to 1.1 kb, which appeared to lack a typical rep gene. Moreover, the pLS20 minireplicon shows no homology with any other known replicon. The essential region for replication contains several inverted repeat structures. Replication does not require the host-encoded PolI or RecA proteins. Based on these characteristics, the pLS20 replicon cannot be classified into one of the four families of theta-replicating plasmids defined by Bruand et al. (1993). Moreover, a replication terminator was identified just outside of the replicaton region of pLS20. In vivo and in vitro characterization of this terminator, terLS20, is described in chapter IX. Two features of this terminator are novel: (i), it is the first replication terminator identified on a Gram-positive plasmid and (ii), it is the first identified terminator that has bidirectional activity.

Outline of this thesis

The major topic of the work described in this thesis is the analysis of host- and plasmid-associated functions involved in plasmid replication and plasmid maintenance in B. subtilis In addition, in this work the identification and characterization of plasmid-located genes, which do not seem to be involved in plasmid replication or maintenance, is described. Most of the studies have been carried out with native plasmids in B.subtilis Both rolling-circle-type and theta-type plasmids were used.

In chapters II and III , the ssDNA conversion activity of the pMV158-located palA- and palU-type SSOs in L.lactis and B.subtilis is described, in relation to the effects of ssDNA accumulation on plasmid maintenance in these bacteria. In chapter III, also drastic effects of the host-encoded RecA enzyme on ssDNA accumulation and plasmid maintenance of pMV158 derivatives lacking a functional SSO are described. Apparently, RecA stimulates the initiation of lagging strand synthesis of pMV158 derivatives lacking a functional SSO. In chapter IV, the analysis of the mechanism underlying the RecA-mediated effects is presented. It was shown that RecA is involved in an alternative, SSO-independent, ssDNA conversion pathway.

In chapter V, the cloning, sequencing and characterization of SSOs present on a set of representative B.subtilis RCM plasmids is reported. For these studies, a special-purpose vector, pWM100, was constructed which is based on the replication functions of the broad host-range plasmid pMV158.

In chapter VI the identification and characterization of a new structural module present on the cryptic B.subtilis RCM plasmids pTA1015 and pTA1040 is described. This module comprizes two genes, one encoding a putative export

protein and the other encoding a functional type I signal peptidase.

The sequence determination of the complete genomes of three cryptic B.subtilis RCM plasmids, pTA1015 (5.8 kb), pTA1040 (7.7 kb) and pTA1060 (8.7 kb), and the analyses of several of the identified genes, are described in chapter VII.

The last two experimental chapters deal with the analysis of the B.subtilistheta plasmid pLS20. In chapter VIII the cloning, sequencing and analysis of the replication region of pLS20 is decribed. Chapter IX reports the in vivoand in vitro characterization of the bifunctional replication terminator, TerLS20, which is located on pLS20 just outside the region required for replication.

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Figure legends

Fig. 1. Schematic representation of a prototype RCM plasmid. Only the four common modules (indicated as blocks) are shown. The relative order of these blocks may vary in different plasmids. Genes and their direction of transcription are indicated with arrows. Rep, replication initiation protein; DSO, double-strand origin; SSO, single-strand origin; Ab-R, antibiotic resistance marker; Mob/Pre, mobilization/plasmid recombination protein. Although the DSO is usually located upstream of the rep gene, in some cases, e.g. in the pT181 family of RCM plasmids, it is positioned within the 5'-terminal coding region of the rep gene.

Fig. 2. Schematic representation of the rolling-circle mechanism (RCM) of replication. Heavy lines, leading strand; thin lines, lagging strand (-); continuous lines, parental DNA; discontinues lines, newly synthesized DNA; ○, Rep protein; ∩, SSO. For details, see text. Adapted from Baas and Jansz (1988).

Fig. 3. Schematic representation of the three known mechanisms of initiation of theta replication. (A), (B) and (C) represent the mechanisms corresponding to class A, B and C type plasmids as described in the text. Continuous and discontinuous arrows represent newly synthesized leading and lagging strands, respectively. (A) and (B) are adapted from Marians (1992).

Fig. 4. Organization of the replication initiation and termination sites on bacterial chromosomes and plasmids. The E.coli and B.subtilis chromosomes and the Gram-negative plasmids R6K, R1 and R100 and the Gram-positive plasmid pLS20 are shown. The origins are labeled "ori", and the direction of replication is indicated with arrows. The termination sites are labeled Ter. The hatched T-like symbols denote the polarity of the site; replication forks meeting the flat side (the top of T) are arrested. The termination sites are not to scale. The figure is partly adapted from Baker (1995).

Table 1. Rolling-circle plasmids and their original host

Replicon	size (kb)	Original host	Reference
pT181	4.4	Staphylococcus aureus	(Khan and Novick, 1983)
pC221	4.6	Staphylococcus aureus	(Novick, 1989)
pC223	4.6	Staphylococcus aureus	(Novick, 1989)
pS194	4.4	Staphylococcus aureus	(Novick, 1989)
pUB112	4.1	Staphylococcus aureus	(Novick, 1989)
pCW7	4.2	Staphylococcus aureus	(Novick, 1989)
pHD2	2.1	Bacillus thuringiensis	(McDowell and Mann, 1991)
pC194	2.9	Staphylococcus aureus	(Horinuchi and Weisblum, 1982b)
pUB110	4.5	Staphylococcus aureus	(Gruss and Ehrlich, 1989)
pOX6	3.2	Staphylococcus aureus	(Novick, 1989)
pTA1015	5.8	Bacillus subtilis	This thesis, chapter VII
pTA1020	6.8	Bacillus subtilis	(Uozumi et al., 1980)
pTA1030	7.3	Bacillus subtilis	(Uozumi et al., 1980)
pTA1040	7.8	Bacillus subtilis	This thesis, chapter VII
pTA1050	8.2	Bacillus subtilis	(Uozumi et al., 1980)
pTA1060	8.7	Bacillus subtilis	This thesis, chapter VII
pBAA1	6.8	Bacillus subtilis	(Devine et al., 1989)
pFTB14	8.2	Bacillus amyloliquefaciens	(Murai et al., 1987)
pBC16	4.6	Bacillus cereus	(Gruss et al., 1987)
pBC1	1.6	Bacillus coagulans	(de Rossi et al., 1992)
pCB101	6.0	Clostridium butyricum	unpublished
pLP1	2.1	Lactobacillus plantaricum	(Bouia et al., 1989)
pIJ101	8.8	Streptomyces lividans	(Kendell and Cohen, 1988)
pSN22	11.0	Streptomyces nigrifaciens	(Kataoka et al., 1994a)
pJV1	??	Streptomyces phaeochromogenes	(Servin-Gonzalez, 1993)
pC30iL	2.1	Lactobacillus plantarum	(Skaugen, 1989)
pTD1	2.6	Treponema denticola	(MacDougall et al., 1992)
pKYM	2.1	Shigella sonnei	(Yasukawa et al., 1991)
pLAB1000	3.3	Lactobacillus hilgardii	(Josson et al., 1990)

pWGB32	2.4	Staphylococcus aureus	(Grinius et al., 1992)
pVA380-1	4.2	Streptococcus ferus	(LeBlanc et al., 1992)
pRF1	4.2	Plectonema	(Perkins and Barnum, 1992)
p8014-2	1.9	Lactobacillus plantarum	(Leer et al., 1992)
p353-2	2.4	Lactobacillus pentosus	(Leer et al., 1992)
pTB913	4.5	Thermophilic Bacillus	(van der Lelie et al., 1989)
pRBH1	?	Bacillus subtilis	(Muller et al., 1986)
pSK89	?	Staphylococcus aureus	(Littlejohn et al., 1991)
pSBBM3XM	?	Staphylococcus xylosus	(Hansson et al., 1992)
pSB2424	?	Streptomyces cyanogenus	(Bolotin et al., 1985)
pST1	?	Streptococcus thermophilus	(Janzen et al., 1992)
pMA1	?	Microcystis aeruginosa	unpublished
pNostoc	?	Nostoc sp.	unpublished
pE194	3.7	Staphylococcus aureus	(Horinuchi and Weisblum, 1982a)
pNE131	2.4	Staphylococcus epidermis	(Lampson and Parisi, 1986)
pE3692	2.4	Staphylococcus aureus	(Wieckiewicz and Wojcik, 1989)
pMV158	5.5	Streptococcus agalactiae	(Lacks et al., 1986)
pWV01	2.2	Lactococcus lactis subsp. cremoris	(Leenhouts et al., 1991)
pSH71	2.2	Lactococcus lactis	(de Vos, 1987)
pFX2	2.2	Lactococcus lactis	(Xu et al., 1989)
pLB4	3.5	Lactobacillus plantarum	(Bates and Gilbert, 1989)
pA1	2.8	Lactobacillus plantarum	(Vujcic and Topisirovic, 1993)
pADB201	1.7	Mycoplasma mycoides	(Gruss and Ehrlich, 1989)
pKMK1	1.9	Mycoplasma mycoides	(King and Dybvig, 1992)
pHPK255	1.5	Heliobacter pylori	(Kleanthous et al., 1991)
pSN2	1.3	Staphylococcus aureus	(Novick, 1989)
pE12	2.2	Staphylococcus aureus	(Novick, 1989)
pE5	2.1	Staphylococcus aureus	(Projan et al., 1987)
pT48	2.1	Staphylococcus aureus	(Novick, 1989)
pTCS1	1.3	Staphylococcus aureus	(Novick, 1989)
pNE131	2.1	Staphylococcus epidermis	(Gruss and Ehrlich, 1989)
pIM13	2.1	Bacillus subtilis	(Projan et al., 1987)
pTX14-3	7.5	Bacillus thuringiensis	(Madsen et al., 1993)

The plasmids shown in bold are representative plasmids of the various groups