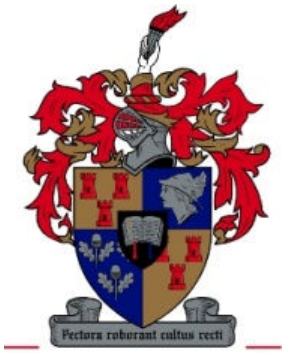
CHARACTERISATION OF PLASMID p31T1 ISOLATED FROM *AEROMONAS*



UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

By

Inge Laubscher

Thesis presented in partial fulfillment of the requirements for the Degree of Master of Science in Microbiology at the University of Stellenbosch

Supervisor: Prof DE Rawlings

Co-supervisor: Dr SM Deane

December 2013

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: 7 January 2013

ABSTRACT

Plasmids are an integral part of the horizontal gene pool and, therefore, are the main vectors for the spread of antibiotic and heavy metal resistance genes in the environment. Functional and taxonomic characterization of novel plasmids is, therefore, central to our general understanding of plasmid biology and their contribution to microbial evolution. Two 14-kb mobilizable plasmids, p31T1 and p36T2, conferring resistance to tetracycline were isolated from the opportunistic fish pathogens *Aeromonas sobria* and *Aeromonas hydrophila* and were found to have indistinguishable restriction fragment length polymorphism (RFLP) patterns (Marx, MSc Thesis). DNA sequence analysis of the two isogenic plasmids (only p36T2 was sequenced) revealed the presence of 18 putative open reading frames (ORFs), of which the *tetAR* tetracycline resistance genes, associated with a truncated Tn*1721,* were the only ORFs with significant similarity to known sequences within the NCBI database. Putative functions were assigned to 10 of the ORFs based on their distant homology with proteins of known function. Six of the 18 ORFs, spanning 5.7-kb, were found to comprise the minimal region required for replication (minimal replicon) by means of deletion analysis using derivatives of p31T1. Of the six ORFs, ORF2 and ORF4 were found to be essential for plasmid replication. Inactivation of ORF3 resulted in an increase of plasmid copy number (PCN) from ~3 to ~7 plasmids per chromosome and a decrease in plasmid stability from ~80 % to 16 % over approximately 127 generations (7 days). Furthermore, by means of β-galactosidase promoter fusion assays it was shown that ORF3 autoregulated its own promoter. These results, therefore, suggested that although ORF3 was not essential for replication, it may be involved in plasmid copy number regulation and control. Host range analysis indicated that p31T1 was able to replicate in two other members of the γ-proteobacteria group (*Escherichia coli* and *Pseudomonas putida*) but was unable to do so in an α-proteobacterium strain, thus suggesting a limited host range. Furthermore, p31T1 was mobilized only at low frequencies (5.4 x 10^{-5} transconjugants per donor) by an IncP-1 conjugative system though it is possible that the mobilization system of these plasmids is adapted to

function optimally with alternate conjugative systems. Given the unique PCN, stability, host range and mobilization characteristics determined for p31T1 and that no other plasmid replication and mobilization systems with significant sequence similarity to these plasmids have yet been identified, it is likely that these two plasmids are the first representative members of a new family of plasmids found within aquaculture-associated *Aeromonas* species and which are involved in the spread of tetracycline resistance.

OPSOMMING

Plasmiede vorm ‘n integrale deel van die horisontale geen poel en vorm daarom die hoof vektore vir die verspreiding van antibiotika- en swaarmetaal-weerstandbiedende gene in die omgewing. Funksionele en taksonomiese karakterisering van nuwe plasmiede is belangrik in die begrip van plasmied biologie en hul bydrae tot mikrobiese evolusie. Twee 14-kb mobiliseerbare plasmiedes, p31T1 en p36T2, met tetrasiklien weerstandigheid was vanaf die opportunistiese vis patogene *Aeromonas sobria* en *Aeromonas hydrophila* geïsoleer en het identiese restriksie fragment lengte polimorfisme (RFLP) patrone. DNA volgorde analise van die twee isogeniese plasmiede (slegs die volgorde van p36T2 was bepaal) het die teenwoordigheid van 18 moontlike oop leesrame (OLR) getoon. Die *tetAR* tetrasiklien weerstandbiedende gene, wat met ‘n verkorte Tn*1721* transposon geassosieerd is, was die enigste OLR wat beduidende volgorde ooreenkoms met bekende volgordes binne die NCBI databasis getoon het. Moontlike funksies was toegeken aan 10 van die OLRe en was gebasseer op vêrlangse homologie met proteïene met bekende funksies. Ses van die 18 OLRe strek oor ‘n 5.7-kb minimale replikon fragment wat benodig word vir replisering en is deur middel van delesie analises van p31T1 derivate gevind. Van hierdie ses OLRe, word OLR2 en OLR4 benodig vir plasmied replisering. Inaktivering van OLR3 het ‘n toename in plasmied kopiegetal (PKG) vanaf ~3 tot ~7 plasmiede per kromosoom en ‘n afname in stabiliteit vanaf ~80% tot 16% oor 127 generasies (7 dae) tot gevolg gehad. Verder kon daar deur middel van β-galaktosidase fusie analises getoon word dat OLR3 sy eie promotor outoreguleer. Hierdie resultate stel dus voor dat alhoewel OLR3 nie benodig was vir replikasie nie, mag dit dalk by plasmied kopiegetal regulering en beheer betrokke wees. Bakteriële gasheer analises het getoon dat p31T1 in 2 addisionele lede van die γ-proteobakterieë groep (*Escherichia coli* en *Pseudomonas putida*) kon repliseer, maar nie in ‘n α-proteobacterium nie. Verder kon p31T1 teen ‘n lae frekwensie (5.4×10^5) gemobiliseer word deur ‘n IncP-1 konjugasie sisteem, maar dit mag wees dat die mobilisering eerder optimaal kan plaasvind met ‘n alternatiewe konjugasie sisteem. Na aanleiding van die unieke PKG, stabiliteit, gasheer en mobilisering eienskappe wat

vir p31T1 bepaal is en die feit dat geen ander replisering en mobilisering sisteme met noemenswaardige volgorde homologie tot hierdie plasmiede gevind kon word nie, blyk dit dat hierdie van die eerste lede van ‘n nuwe familie van plasmiede binne die akwakultuur-geassosieerde *Aeromonas* spesies is, wat betrokke is by die verspreiding van tetrasiklien weerstandbiedendheid.

ACKNOWLEDGEMENTS

I would firstly like to show appreciation to my Mother and Father for their love and support through my entire tertiary career and for lending me the opportunity to study at a University institution.

My friends have played a vital role in my MSc career and have always been there to listen, understand, support and lighten up the challenging times.

Furthermore, I need to thank my supervisor Prof Rawlings, for his willingness to help and his open door policy. He has given me much support and tought me to persist and never give up. Dr Shelly Deane, my co-supervisor, has helped me to grow as a scientist and I thank her for her confidence in me.

Special mentioning is given to Dr Wesley Loftie-Eaton. He has always believed in me and brought the best out of me as a scientist. I thank him for his motivation and support and the much provided help he has lended me throughout my post-graduate career.

Finally, I thank the NRF and Prof Rawlings for funding, which is highly appreciated.

TABLE OF CONTENTS

Chapter 1

Literature Review

1. INTRODUCTION

1.1 An Introduction to Plasmid Classification

It was in 1952 that Lederberg assigned the term plasmid to all "extrachromosomal hereditary determinants". F-plasmids were the first discovered and were soon followed by the discovery of Col (colicinogenic) and R (resistance) plasmids. The first classification scheme was related to the fi^+ (fertility inhibition) and fi^- phenotypes exhibited by some R plasmids which either have the ability to inhibit transfer of F plasmids or not. These terms were later changed to F-like and I-like plasmids, respectively, when it was found that there is a correlation between the fi type and the type of sex pili. With the discovery of non-transferable plasmids in the 1960s this classification scheme could no longer be used and was subsequently abandoned. A different classification scheme was required which led to the development of incompatibility grouping in the early 1970's by Datta and Hedges (1972). Incompatibility classification is based on the fact that two plasmids with closely related determinants responsible for their stable maintenance cannot co-exist within the same cell. Such plasmids are said to be incompatible and thus fall into the same incompatibility group (Inc). Incompatibility testing is performed by introducing a plasmid into a host with a resident plasmid. The plasmids need to have different antibiotic selectable markers as antibiotic selection is maintained for the entering plasmid while the presence of the resident plasmid is monitored concurrently. If the resident plasmid is eliminated the two plasmids are said to be incompatible. The problems with incompatibility testing are the availability of suitable selectable markers, cell surface exclusion properties where some hosts will inhibit the entry of a new plasmid, the presence of more than one replicon on a plasmid and the possibility of genetic changes that could cause an altered incompatibility state. Furthermore, for incompatibility between two plasmids to be tested, they need to replicate in the same host. Since many plasmids are either narrow host-range or have a limited host range it may not be possible to test their incompatibility. This led to the development of replicon typing by Couturier *et al*. (1988) who made a bank of Rep probes derived from 19 basic replicons (Inc groups), which carry at least

one incompatibility determinant and range between 304 and 2250 bp. These probes contained sequences made up of copy number control elements or partition sequences and were used for plasmid specific DNA hybridizations. Some complications with this method have to be taken into consideration. For example, variation between two replicons does not occur over the entire length of the replicon and therefore probe design needs to be precise so as to differentiate between the different replicons. Also cross-hybridization could be seen with different Inc groups that had a common replication control mechanism and which were confirmed to be related by sequence comparisons. Replicon typing should, therefore, be used to assign plasmids to the larger group and not to specific Inc groups. PCR-based replicon typing (PBRT) is more widely used (Gotz *et al*., 1996) and more recently a Degenerate primer MOB typing system was developed by designing a set of primers for classifying plasmids based on their relaxase protein phylogenies (Alvarado *et al*., 2012). This method, however, applies only to transmissible plasmids which were previously placed into 6 MOB families based on relaxase homology (Garcillán-Barcia *et al*., 2011). With the ease of sequencing, DNA sequence homology analysis is more common in the identification of new plasmids.

1.2 Plasmid Host Range

Plasmids can further be classified based on their host range as either narrow host-range (NHR) or broad host-range (BHR). Host range refers to all the bacteria a plasmid can replicate in and is dependent on different factors. BHR plasmids like RK2 and RSF1010 can be transferred to a wide range of Gram-negative bacteria by means of conjugation, transformation or transduction. RSF1010 has also been shown to replicate in Gram-positive bacteria (Gormley and Davies, 1991). NHR plasmids such as ColEI, pBR322, pET and pUC only replicate in *Escherichia coli* or other closely related bacteria.

Plasmids have developed several strategies in achieving broad host-range. These include independence from host replication factors, genetic adaptability of the initiator

proteins and the origins, initiator protein interactions and host communication, and the presence of more than one replicon on the same plasmid. Since plasmids RK2 and RSF1010 are the best studied examples of the BHR phenomenon these strategies will be discussed with referral to these two plasmids primarily.

Independence from host replication factors. RSF1010 is an excellent example of host independence. It has been shown to replicate in at least 31 different Gram-negative species (Frey and Bagdasarian., 1989). The plasmid encodes a RepC (initiator), RepB (primase) and RepA (helicase) making it independent of host DnaA, DnaB, DnaC and DnaG. Furthermore RSF1010 replicates by means of a unique single strand displacement mode of replication which adds to its broad host-range (Scherzinger *et al*., 1991).

Adaptability of the origins of replication and sequence organization. Origin structure and topology plays an important role in establishment of a plasmid. In RK2 the integrity of five iterons is essential for establishment in *E. coli*, *Pseudomonas putida* and *Pseudomonas aeruginosa*. Sequences located upstream of these iterons, however, are required only in *E. coli* and *P. putida*. The presence of all four DnaA boxes is needed for replication in *E. coli*, *P. putida* and *Azotobacter vinelandii,* but not for *P. aeruginosa* (Doran *et al*., 1999). Two proximal DnaA boxes are required for replication in *E. coli* and *P. putida* while only the fourth box is required in *A. vinelandii*. The sequence integrity of the fourth box is vital for replication in all three organisms (Doran *et al*., 1999). The positioning of the DnaA boxes relative to the rest of the minimal replicon is important for stable replication and insertions are not tolerated (Doran *et al*., 1998). Similarly, sequences outside the minimal replicon of RSF1010 are dispensable for replication in *E. coli* but not for *P. putida*. The single strand intitiation (*ssi*) sequences of RSF1010 used for replication in *E. coli* and *P. aeruginosa* differ. In *P. aeruginosa ssiB* is required whilst in *E. coli* both *ssiA* and *ssiB* are required. These sites can be substituted by other priming sites such as a DnaA box for example. Sequence organization in plasmids thus appears to play a role in host range determination (Meyer *et al*., 1982).

Initiator protein interactions and host communication. A plasmid will not be established in a new host if there is no adequate interaction between plasmid and host-specific factors. For example, in RK2 it is important that the replication initiator (*rep*) gene is expressed in the new host. Mutations within *rep* can lead to changes in a plasmid's host range since this can possibly improve the Rep-host factor interactions. An example is mutations that occurred in the C-terminal end of the TrfA replication initiator protein of RK2, which brought about a shift in this plasmid's host range (Cereghino and Helinski, 1993; del Solar *et al*., 1996; del Solar *et al*., 1998). RK2 has two forms of the replication initiator TrfA referred to as TrfA33 and TrfA44 for the short and long versions of the protein, respectively. The short TrfA allows for replication in *Pseudomonas putida* and *E. coli* (Durland and Helinski, 1987; Shingler and Thomas, 1989), whereas the long protein is required for replication in *Pseudomonas aeruginosa* (Fang and Helinski, 1991). This emphasizes the importance of efficient interaction with host replication factors for plasmid establishment.

2. Plasmid Replication and Control

2.1 Replication Mechanisms

Two replication types exist for circular bacterial plasmids. These include the theta mode of replication, which is generally known to be more prevalent in Gram-negative bacteria, and rolling circle replication (RCR) often found in Gram-positive bacteria (del Solar *et al*., 1998). A variation in the theta mode of replication, namely single strand displacement replication, is often seen as a third type of replication which is generally associated with the broad host IncQ plasmid family. Although linear plasmids have been identified and investigated (Hinnebusch and Tilly, 1993), the focus will be aimed at the replication of circular bacterial plasmids for the purpose of this study.

2.1.1 Theta Replication

Although extensively studied in Gram-negative bacteria, this mode of replication is also described for plasmids belonging to Gram-positive bacteria, such as *Enterococcus* (pAMβ1) (Bruand *et al*., 1991), *Lactococcus* (pWVO2) (Kiewiet *et al*., 1993) and *Bacillus subtilis* (pLS20) (Meijer *et al*., 1995b). The name of this replication type is derived from the observation that these plasmids form θ shaped structures during replication when visualised by electron microscopy (del Solar *et al*., 1998). Plasmids belonging to this group are usually >12kb in size and do not produce single strand intermediates. Replication occurs unidirectionally or bidirectionally (Helinski *et al*., 1996). Although variations exist among different plasmids belonging to this group, the general outline for replication remains the same. This involves melting of parental strands at the origin of replication, primer RNA (pRNA) synthesis and extension of pRNA for initiation of DNA synthesis.

Origin of Replication. The origin of replication is a *cis*-acting region where replication is initiated by melting of the DNA strands to allow complementary strand synthesis. In general this region contains specific sequences to which the initiator protein (Rep) can bind. Also many origins often contain additional AT-rich regions, which allows for strand opening, and *dnaA* boxes, required for host DnaA initiator binding (Bramhill and Kornberg, 1988, Kornberg and Baker, 1992). Furthermore, Dam methylation sequences, such as those found in the *oriC* of *E. coli* chromosome are also found in some plasmids such as P1 (Brendler *et al*., 1991a, Brendler *et al*., 1991b). These sequences, however, have no role in replication but rather function in post replication (Abeles *et al.,* 1993). Furthermore, binding sites for host encoded proteins such as integration host factor (IHF) and factor for inversion stimulation (FIS), may exist and play a role in DNA bending and promote protein interactions (Rep/host, host/host, Rep-Rep) (Krüger *et al*., 2004). In broad terms, origins of replication can be classified into

those containing iterons and iteron-independent origins and they can be quite different in their organizational structures as depicted in figure 1.1.

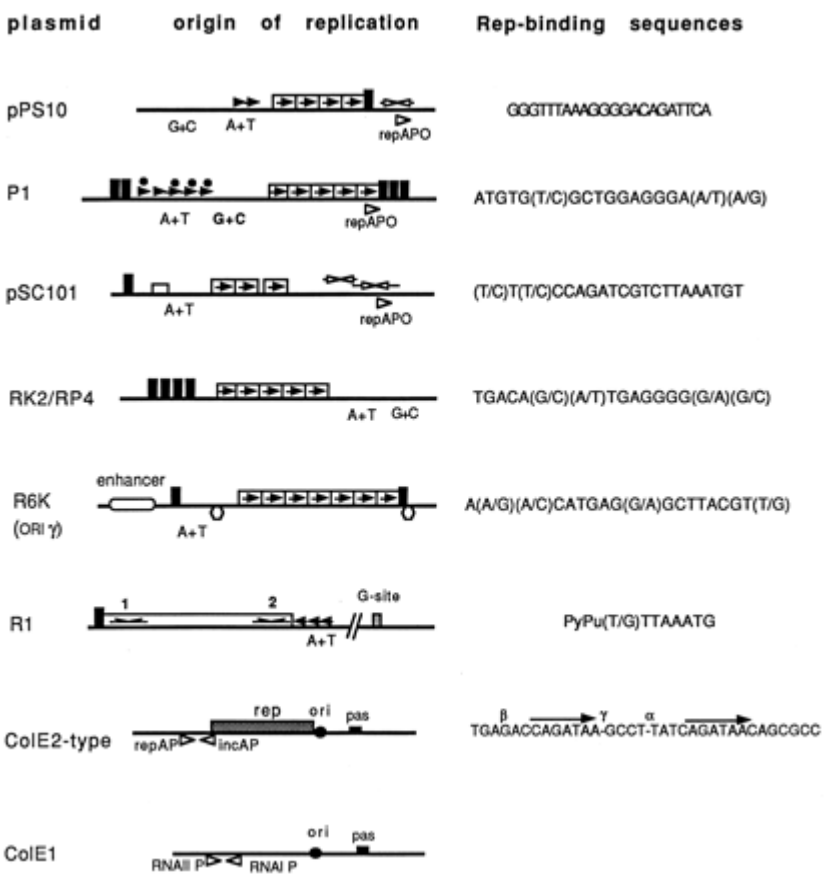


FIG. 1.1. A comparison of the origins of replication of different theta-replicating plasmids from Gram-negative bacteria. Boxed arrows (iterons); open arrows above the map (inverted repeats with partial homology to iterons); solid arrow heads (repeats in AT-rich regions); open arrow heads below the maps (promoters); open rectangles (IHF binding sites); solid rectangles (*dnaA* boxes); hexagons (FIS binding sites); solid circles (*dam* methylation sites); *pas* (primase assembly sites). Arrows 1 and 2 in R1 indicate imperfect palindromes. From del Solar *et al*. (1998).

Iterons are a series of direct repeats of 17-22bp adjacent or separated by intervening sequences, usually arranged in tandem at 11-bp intervals. The double stranded helix has a major and minor groove. The major groove is on the outside and accessible to

DNA binding proteins. The minor groove is buried deeper inside the helix and therefore not accessible. It takes ~11-bp to complete one full turn, and by having the iterons spaced 11 nucleotides or multiples of 11-bp apart these binding sites are always spaced in the major groove. The reason for this arrangement with respect to the helix is to allow Rep proteins to bind to these repeats on the same side of the helix so that they become aligned. Iteron sequences are usually highly conserved but variable sequences also occur. For different plasmids, dissimilar iterons in a specific origin seem to have a consensus motif, 5'-TGAGnG-3' (McEachern *et al*., 1985; Miao *et al*., 1995). A second functional sequence area, but which varies in nucleotide sequence between iteron families, occur one helix turn apart from the first conserved area and is important for Rep recognition (Chattoraj and Schneider, 1997). A third sequence area is a spacer region in the minor groove which faces Rep and is variable among plasmids of the same family and different replicons (Chattoraj and Schneider, 1997). Iterons are essential for initiation of replication and play a role in plasmid copy number control. They have been identified in a number of replicons such as P1 (Abeles *et al*., 1995), F (Murotsu *et al*., 1981; Tolun and Helinski, 1981), pSC101 (Churchward *et al*., 1983), R6K (Germino and Bastia, 1983a; Germino and Bastia, 1983b; Stalker *et al*., 1979; Stalker *et al*., 1982), Rts1(Kamio and Terawaki, 1983), RK2/RP4 (Papp *et al*., 1993; Stalker *et al*., 1981) pSa (Tait *et al*., 1983) and pPS10 (Fernández-Tresguerres *et al*., 1995; Giraldo *et al*., 1992; Nieto *et al*., 1992). Origins contain several iterons, although not all are necessarily required for a given origin. Plasmid R6K contains 7 iterons and removal of one has no effect on replication, however replication efficiency is reduced with the removal of two and completely halted after the removal of three iterons (Kolter and Helinski, 1978). Some plasmids like P1, F, RK2, R6K and Rts1 carry iterons outside the origin of replication (*oriV*). These are known as auxiliary iterons and assist in regulation of replication.

In some origins, such as for R1, ColE1 and pLS20 iterons are absent. These plasmids also require a Rep protein (plasmid-encoded initiator) and DNA polymerase I (synthesis of RNA primer), to initiate plasmid replication. Unlike iteron-containing plasmids, however, initiation of replication is under the control of antisense RNA elements (discussed later).

Rep proteins. Rep initiator proteins are similar to the DnaA proteins involved in bacterial chromosome replication in that they recognize specific sequences at the origin of replication and bind to them to form a nucleoprotein complex (Rep-DNA, Rep-Rep, Rep-host proteins) (Bramhill and Kornberg, 1988). Rep proteins are generally known to act *in trans* but in some systems they act *in cis* with respect to the origin (Helinski *et al*., 1996). Although involved in replication initiation they serve an additional role by regulating their own synthesis and, therefore, the frequency of initiation. Rep proteins are known to exist in a monomer-dimer equilibrium, particularly in iteron-containing plasmids. Monomers actively bind iterons for initiation of replication while dimers limit Rep availability for replication. Dimerization and dissociation of Rep proteins involve conformational changes promoted by chaperones. Chaperone involvement was demonstrated for the RepA of plasmid P1 (Wickner *et al*., 1991a; Wickner *et al*., 1991b) and RepE of the F plasmid (Ezaki *et al*., 1989; Kawasaki *et al*., 1990). Different interfaces of the Rep protein are responsible for monomer-dimerization and interaction with the DNA. A model for conformational change and how monomers or dimer interfaces interact has been developed for the RepE initiator of F plasmid and is illustrated in figure 1.2 (Chattoraj, 2000). The leucine-zipper (LZ) motif is involved in protein-protein interactions and, therefore, dimerization, whereas the α-helix-turn-α-helix (HTH) domain mediates DNA binding (del Solar *et. al*. 1998).

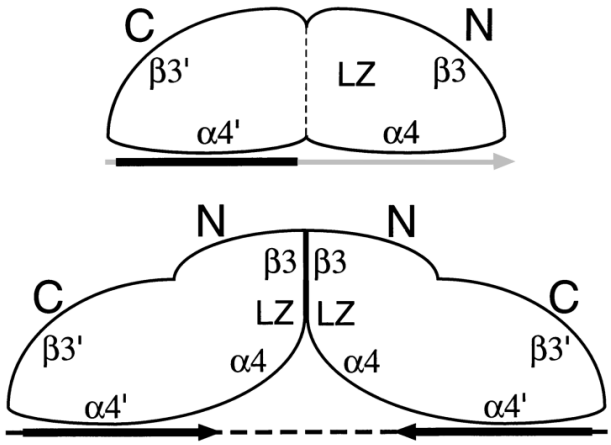


FIG. 1.2. Rep monomer and dimer binding of F plasmid. The top figure represents a monomer bound to an iteron. The black bar on the iteron represents the invert repeat (IR) region (bottom figure). The α4' and α4 helices contact two consecutive major grooves on the same face of an iteron. The bottom figure shows the RepE dimer bound to the *repE* operator invert repeats (IR). The α4' helix contacts the IR sequences and the α4 helices are not in contact with the DNA. From Chattoraj (2000).

Several host encoded factors are known to interact with initiator proteins. For example host-encoded DnaJ is required for the initiation of replication of plasmid P1 (Wickner, 1990). To interact with the DnaA box present in the *oriV* during initiation of replication of plasmid R1 the host DnaA must associate with the initiator RepA (Masai and Arai, 1987). As another example interaction of host-encoded DnaA, DnaB and DnaG with π protein of R6K is required for π-mediated initiation of replication (Ratnakar *et al*., 1996). For host-encoded chaperones such as DnaK, DnaJ and GrpE it has been shown that their interaction with the Rep proteins play a role in the conversion of dimers to monomers, by inducing conformational changes in initiator dimers (Wickner *et al*., 1992).

Rep proteins autorepress their own synthesis by binding to regions in the *rep* promoter area in addition to sequences in the *oriV*. One form of the protein can be involved in both autoregulation and initiation of replication or alternatively different forms such as monomers and dimers can play separate roles (Chattoraj, 2000).

Initiation of replication and elongation. Depending on the requirement of plasmid-encoded initiator, host initiator elements or both, replication initiation of theta replicating plasmids can be classified into five different classes (Bruand *et al*., 1993; Meijer *et al*., 1995b):

Class A theta replication is dependent on the plasmid-encoded initiator protein Rep for initiation as well as the host initiator. The Rep and host-encoded DnaA (promotes DNA unwinding) proteins bind to the iterons, thus leading to the formation of a nucleoprotein complex. Strand opening of the adjacent A+T rich region occurs and the replication proteins (DNA Polymerase III holoenzyme, DnaB helicase and primase) assemble to form a replication fork (del Solar *et al*., 1998). DNA Pol I (DNA Polymerase I) is not required for Class A theta replication. Examples include pSC101 (Hasunuma and

Sekiguchi, 1977; Vocke and Bastia, 1983; Stenzel *et al*., 1991), R1 (Diaz *et al*., 1981; Kollek *et al*., 1978; Masai *et al*., 1983; Uhlin and Nordström, 1978; Bernander *et al*., 1992), P1 (Abeles *et al*., 1984; Hansen and Yarmolinsky, 1986; Wickner and Chattoraj, 1987; Wickner *et al*., 1991a), R6K (Filutowicz *et al*., 1994; Kelley and Bastia, 1992) and F (Kawasaki *et al*., 1992). The characteristic structure of the origin of replication of these plasmids, designated *oriA*, resembles that of the chromosomal *oriC* and includes direct repeats, an AT-rich region and DnaA boxes (Bramhill and Kornberg, 1988).

Class B theta replication is initiated independenly of a plasmid encoded initiator protein and lacks *oriA*. ColEI is the best characterised plasmid in this group. Its replication is host dependent and it is, therefore, characterised as a narrow host-range plasmid (del Solar *et al*., 1998). Instead of a plasmid-encoded replication initiator the initiation step in ColEI replication requires host-encoded RNA polymerase (RNAP) which synthesizes an RNA transcript. This transcript is subsequently processed by RNaseH to form an RNA primer. The RNaseH catalytic activity leads to the formation of a free 3'OH end for leading strand synthesis by DNA Pol I until the primosome assembly site (*pas*) is exposed on the lagging strand. The primosome (helicase and primase) is assembled and the DNA is replicated by discontinuous complimentary strand synthesis. DNA Pol I is replaced with DNA Polymerase III (DNA Pol III) holoenzyme since the latter is more processive than the first. DNA Pol III synthesizes DNA at 1000 nucleotides per second (Kelman and O'Donnell, 1995), compared to DNA Pol I which synthesizes at 20 nucleotides per second.

Class C theta replication requires a plasmid-encoded initiator together with DNA Pol I to bind the origin of replication on the plasmid and synthesize a RNA primer (primase activity) for leading strand synthesis (Takechi *et al*., 1995). Plasmids ColE2 and ColE3 belong to this group and they do not carry an *oriA*-like structure (Kingsbury and Helinski, 1970; Tacon and Sheratt, 1976; Yasueda *et al*., 1989; Itoh and Horii, 1989).

Class D theta replication is similar to Class C. This mechanism requires Rep and DNA Pol I, however, although an *oriA*-like structure is present it is not required for replication (Bruand *et al*., 1993). An example of a plasmid that utilizes this *oriA*-independent mechanism is pAMβ1 (isolated from Gram-positive bacteria). Although its mechanism of

replication bears similarity to that of Class C plasmids, it replication system bears no homology to that of ColE2 plasmids and pAMβ1 was, therefore, classified into this new class.

Class E initiation resembles a novel mechanism which is independent of both plasmid encoded-initiator and DNA Pol I. This type of replication is observed in plasmid pLS20 which was isolated from the Gram-positive *Bacillus subtilis* (Meijer *et al*., 1995b).

2.1.2 Strand displacement replication

Strand displacement replication is a form of theta replication unique to the IncQ family of plasmids and is often seen as a third replication group aside from theta- and rolling circle replication. This plasmid family, with RSF1010 as the prototype plasmid, has a broad host-range. What makes this type of replication distinct from the theta mode is the requirement of three plasmid-encoded proteins for initiation of replication. Furthermore, replication proceeds continuously in both directions from single strand initiation sites (*ssi*) located in opposite strands (Sakai and Komano, 1996; Scherzinger *et al*., 1991).

Origin of replication (*oriV*). The *oriV* of RSF1010 contains 3 identical 20-bp repeats, a GC- and AT-rich sequence stretch and two palindromic sequences, *ssiA* and *ssiB,* situated on opposing strands. The iterons are bound by the initiator to induce strand opening (Haring and Scherzinger, 1989) while the invert repeats (IR) comprising the *ssi* sites can form hairpin loops which are essential for primer formation.

Rep proteins. As previously mentioned, three Rep proteins are involved in single strand displacement replication. The initiator, RepC, exists as a dimer and interacts with the iterons and possibly the RepA helicase. RepA is a hexamer with ATPase and helicase activities, respectively. The RepB primase exist as two forms, RepB and RepB', which are transcribed from two alternative in-frame start codons. The larger primase form is a MobA-RepB fusion. MobA is a relaxase and this fusion protein is required for conjugative mobilisation (Geibel *et al*., 2009). The smaller primase form is required for replication, although it can be substituted by the larger version (Scherzinger

et al., 1991). Although IncQ plasmids require host-encoded DNA Pol III holoenzyme and gyrase, other host-encoded proteins such as DnaA, DnaB, DnaC and DnaG are dispensable due to the presence of a plasmid-encoded helicase (RepA) and primase (RepB), (Frey and Bagdasarian, 1989; Haring and Scherzinger, 1989; Scherzinger *et al*., 1984).

Replication initiation. Initiation of replication is induced by RepC (figure 1.3. A) binding to the iterons in the *oriV* (Scherzinger *et al*., 1991). This leads to bending of the DNA and subsequent strand opening at the adjacent AT-rich region and recruitment of the RepA helicase (figure 1.3. B). The RepA catalyzes strand separation/melting in the 5'-3' direction. Once the *ssi* sites are exposed as single strands a hairpin/stemloop structure is exposed which is required for RepB primase assembly and primer synthesis (figure 1.3. C), (Miao *et al*., 1993). DNA Pol III holoenzyme is finally recruited and the RepA helicase facilitates continuous replication in the 5' to 3' direction while the parental strand is displaced as a D-loop (figure 1.3. D). The end products are single strand displaced circles and double strand supercoiled circles. The displaced single strand circle can contain either of the *ssiA* or *ssiB* sequences, which is then used to synthesize the complementary strand and form a double stranded circle.

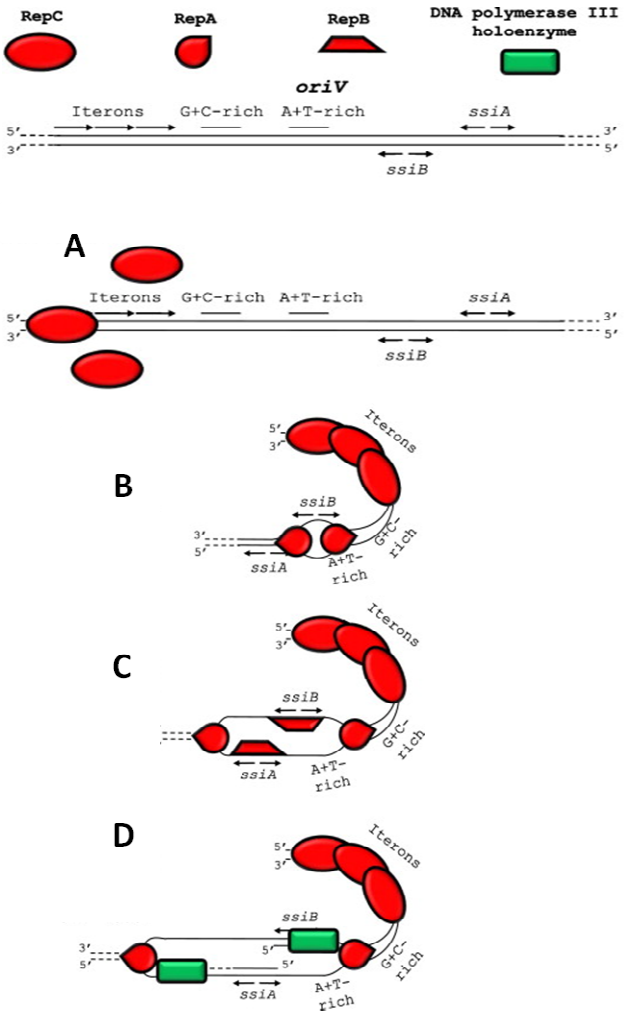


FIG. 1.3. Single strand displacement replication as seen in RSF1010. See text for details of the mechanism. Adapted from Loftie-Eaton and Rawlings (2012).

2.1.3 Rolling Circle Replication

Rolling circle replication (RCR) was originally observed in the ssDNA bacteriophages of *E. coli* (Baas, 1985; Baas *et al*., 1988; Eisenberg *et al*., 1979; Reinberg *et al*., 1983; Sims *et al*., 1979; Zinder and Horiuchi, 1985). Early studies on the *Staphylococcus aureus* plasmids pT181 and pC194 led to the discovery of rolling circle replication approximately 27 years ago (Koepsel 1985A; Koepsel 1985b; te Riele 1986a; te Riele 1986b). Plasmid replication via this mode is generally associated with Gram-positive

bacteria, however, it has also been observed in Gram-negative bacteria (e.g. pKYM) (Yasukawa *et al*., 1991), Archaea (eg pGT5) (Marsin and Forterre, 1999), animal paroviruses (Berns, 1990; Cossons *et al*., 1996) and mitochondrial DNA in plants (Backert *et al*., 1997). Rolling circle plasmids are small, usually <10-kb in size due to the possible limitations posed by efficiency of rolling circle (RC) mode of replication (Khan, 2004) and structural instabilities inherent in large RC molecules (Helinski *et al*., 1996). Not all small plasmids utilize a RC mode of replication. The small Gram-positive plasmids pRJF1 and pWV02 use a theta mode of replication (Hefford *et al*., 1993; Kiewiet *et al*., 1993). In RCR plasmids replication occurs in both a unidirectional and asymmetric manner, meaning that leading- and lagging-strand synthesis are uncoupled (del Solar *et al*., 1993b; Espinosa *et al*., 1995; Gruss and Ehrlich, 1989; Khan, 1996; Khan, 1997; Novick, 1989).

Based on homology in their initiator proteins and double stranded origin (*dso*) sequences, rolling circle replication (RCR) plasmids can be grouped into at least seven major families, namely, pT181, pC194/pUB110, pE194/pLS1, pSN2, pGA1, pG13 and pTX14-3 (Khan, 2004). Since more than 200 RCR plasmids have been identified the need for grouping of these plasmids into various families is apparent (Khan, 2005).

Origins of replication. RCR plasmids contain two types of origins, namely a double strand origin (*dso*) for leading strand replication and a single strand origin (*sso*) for lagging strand replication.

The *dso* region consists of a nick site (*nic*) at which a single DNA strand is cleaved as well as a plasmid-specific Rep protein binding site (*bind*). The *nic* site sequence is highly conserved within the plasmid families while the binding sequence is less well-conserved. The *nic* sites are known to form a secondary structure in the pT181 and pMV158 families and it is thought that this feature allows for the efficient recruitment of the Rep initiator to allow nicking at the *nic* site (Gros *et al*., 1987, Moscoso *et al*., 1995a). The *nic* site within the *dso*'s of pT181 plasmid family is located adjacent to the binding region, which in turn carries an invert repeat region (figure 1.4). In contrast, the *nic* site of pMV158-like plasmids is separated from the Rep binding site by a spacer

region of 14 to 95-bp and the Rep binding site consists of three iterons as opposed to an inverted repeat (Moscoso *et al*., 1995a; Moscoso *et al*., 1995b).

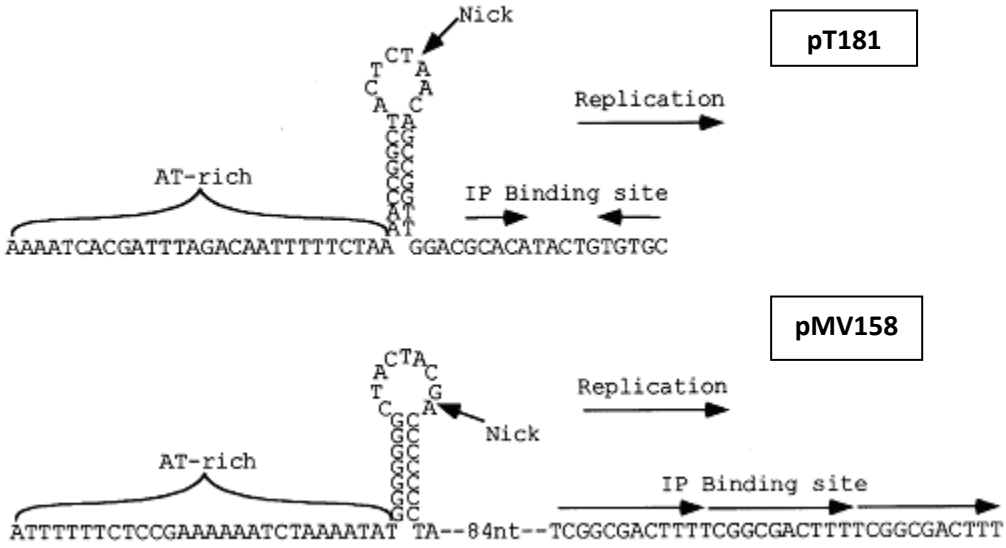


FIG. 1.4. The pT181 and pM158 *dso* structure organization. IP (initiator protein). From Helinski *et al*. (1996).

The *sso* regions are activated only at the commencement of lagging strand synthesis. For this reason lagging strand synthesis will not commence before leading strand synthesis is completed and rolling-circle replication can, therefore, be regarded as assymetrical. The *sso* regions are not conserved between families with imperfect palindromic regions thereby forming imperfect secondary structures. (Gruss and Ehrlick, 1989; del Solar *et al*., 1998). Different *sso*'s have been classified into *ssoA*, *ssoT*, *ssoU* and *ssoW* groups based on structural and sequence similarities (Andrup *et al*., 1994, Boe *et al*., 1989, del Solar *et al*., 1993a; Kramer *et al*., 1995, Madsen *et al*., 1993; Meijer *et al*., 1995a; Seegers *et al*., 1995; Zaman *et al*., 1993). The *ssoA* and *ssoW* type origins function only in their native hosts whereas *ssoU* and *ssoT* are broad host-range in nature. Most *sso*s are dependent on RNA polymerase for primer synthesis, but *ssoW*

for example, can allow primer synthesis, independent of RNA polymerase, to some extent (Khan, 1997). Some plasmids such as pMV158 and pUB110 carry both *ssoA* and *ssoU* type origins. *ssoA* allows for efficient replication in its native host, whereas *ssoU* makes replication possible in additional hosts. To avoid complication in such multiple origin plasmids one *sso* might be functionally dominant over the other (Khan, 2000).

Rep proteins. In addition to DNA binding activity, the Rep initiator proteins of RCR plasmids also have a DNA strand transferase activity which can cleave and ligate DNA similar to type I topoisomerase (del Solar *et al*., 1998). A nucleophilic attack on the *nic* site by Rep generates a 3'-OH end for the initiation of replication by serving as a primer for leading strand synthesis from the *dso* (Khan, 1997). The mechanism by which these activities proceed, however, seems to be different between Rep initiators. The RepC of pT181 exists as a dimer and is involved in both initiation and termination of replication. A Tyr residue is responsible for nicking of the 5'-ApT-3' sequence at the *nic* site of the *dso* after which the Rep protein remains covalently attached to the 5'-end of the DNA (phosphotyrosine bond) (Thomas *et al*., 1990). The RepB of plasmid pMV158, on the other hand, exists as a hexamer and also causes cleaving at the *nic* site, but it does not remain covalently bound to the DNA (Moscoso *et al*., 1995a). In yet another example the Rep initiator of plasmid pC194 acts as a monomer and contains a Tyr and Glu residue which both contain nicking activity, however, the first is involved in replication initiation and attachment of the RepA, whereas the last is responsible for cleavage during termination and release of the initiator (Noirot-Gros *et al*., 1994). After its release, the Rep is inactivated through formation of heterodimers (Rasooly and Novick, 1993; Rasooly *et al*., 1994a; Rasooly *et al*., 1994b). Each Rep protein is only utilized once for replication and the heterodimer differs from the homodimer in that it contains a modified protein in which the active Tyr is absent (Rasooly and Rasooly, 1996).

Replication Overview. Replication initiates with the Rep protein binding to the plasmid *bind* region (figure 1.5). This generates a cruciform protrusion and introduces a nick at the *nic* site of the *dso* on the parental (+) strand by means of a nucleophilic attack on the phosphodiester bond (del Solar *et al*., 1998). This exposes a 3'-OH group that is used as a primer for leading strand synthesis. The host-encoded helicase enters the

complex and promotes opening of the DNA strands whereafter DNA Pol III initiates synthesis of the leading strand. During elongation the + strand is displaced as a single strand and coated with host-encoded single strand binding (SSB) proteins. Leading strand synthesis continues until the repaired *dso* is reached. Replication is terminated when the replication machinery reaches the *dso* and specific interactions at the origin displaces the + strand as a single strand entirely. The remaining single stranded parental DNA, coated by host SSB proteins, is then converted to dsDNA formed upon initiation of lagging strand synthesis at the single stranded origin site(s) (*sso*), and is mediated by host-encoded RNAP, with DNA Pol I and DNA Pol III involved in further synthesis.

.

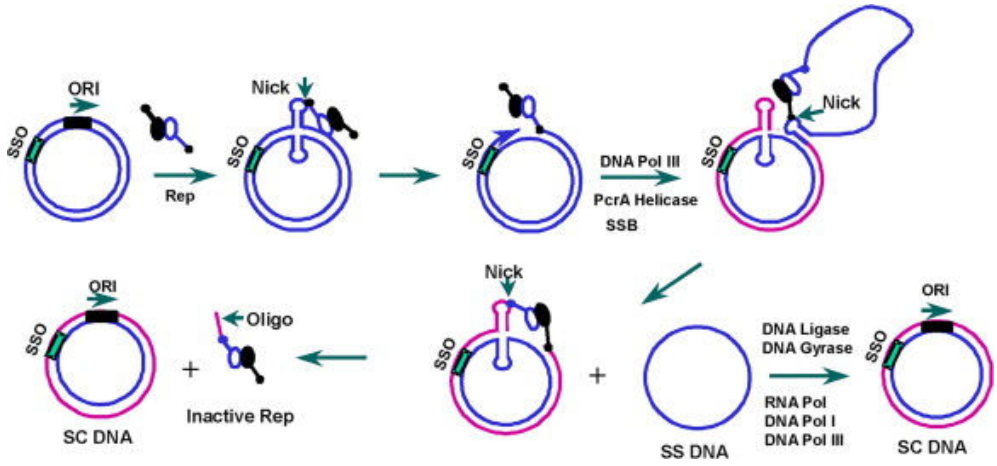


FIG. 1.5. Model of rolling circle replication based on studies from pT181. From Khan (2005).

Plasmid pT181 as an example. Plasmid pT181 is the best studied plasmid within the RCR plasmid groups and will, therefore, be used as an example to illustrate the rolling circle mode of replication.

Initiation of leading strand synthesis starts with binding of the RepC homodimer to its cognate *bind* site (Rasooly and Novick, 1993; Rasooly *et al*., 1994a; Wang *et al*., 1993). An enhancer molecule, namely *cmp,* increases the binding efficiency of RepC (Gennaro and Novick., 1986; Herniquez *et al*., 1993; Zhang *et al*., 1997). The binding of RepC to

the IRIII invert repeat causes bending of the DNA and a conformational change in the RepC homodimer which in turn brings the *nic* site closer to the active Tyr of the RepC protein (Koepsel and Khan, 1986). This brings about DNA melting facilitated by the invert repeats within the AT-rich region (IRI). Formation of a cruciform structure at IR II enables the nucleophilic attack by RepC on the *nic* site. Subsequently an initiation complex is formed with a free 3'-OH end and recruitment of the host DNA Pol III and helicase. (Helinski *et al*., 1996; Chang *et al*., 2002; Ruiz-Maso *et al*., 2006; O'Donnell, 2006; Iordanescu and Basheer, 1991; Iordanescu, 1993).

Leading strand synthesis generates a displaced parental strand which is subsequently converted to double stranded DNA by means of lagging strand synthesis. Initiation of lagging strand synthesis occurs at the *ssoA* site in a manner that is independent of plasmid-encoded functions. A highly conserved recombination site, RS_B, is the RNAP binding site for primer RNA synthesis (Kramer et al., 1997; Kramer et al., 1998). A further central conserved sequence (5'-TAGCG(T/A)-3') referred to as the CS-6 site, acts as a transcriptional terminator for RNA primer synthesis (Kramer *et al*., 1997; del Solar *et al*., 1998). Dna Pol I and later DNA Pol III are involved in further synthesis of the duplex strand.

Termination of Rolling Circle Replication. An additional round of (nascent) leading strand synthesis is initiated before the *nic* site in the IRII hairpin is reconstituted in the *dso* of the newly replicated, displaced single strand (figure. 1.6) (del Solar *et al*., 1998). The RepC monomer (A) which is not involved in initiation of replication cleaves the DNA at the *dso* and remains covalently bound to the 5'-end of the newly synthesized DNA strand (figure 1.6.c). This reaction leads to the release of the 3'-OH end of the parental strand. The released 3'-OH-end causes a nucleophilic attack on the tyrosyl-phosphodiester bond between the 5'-end of the parental strand and the RepC monomer (B) that was involved in initiation. This causes the release of the single strand parental intermediate DNA molecule which is subsequently converted to its dsDNA form as explained previously (figure 1.6.g). The leading strand is extended further thereby creating a new *nic* site at which the RepC subunit (B) (which was involved in initiation) can generate another nucleophilic attack (figure 1.6.d). A free 3'-OH end is generated

on the newly synthesized nascent leading strand and it attacks the tyrosyl-phosphodiester bond of the RepC subunit (which is not involved in initiation) that is covalently bound to the 5'-end of the nascent strand (figure 1.6.e). A newly synthesized double-stranded DNA molecule is released together with an inactive RepC* heterodimer (figure 1.6.f). The attachment of an oligonucleotide to the RepC monomer that was involved in initiation causes it to be unavailable for reinitiation of replication (Rasooly and Novick, 1993; del Solar *et al*., 1998).

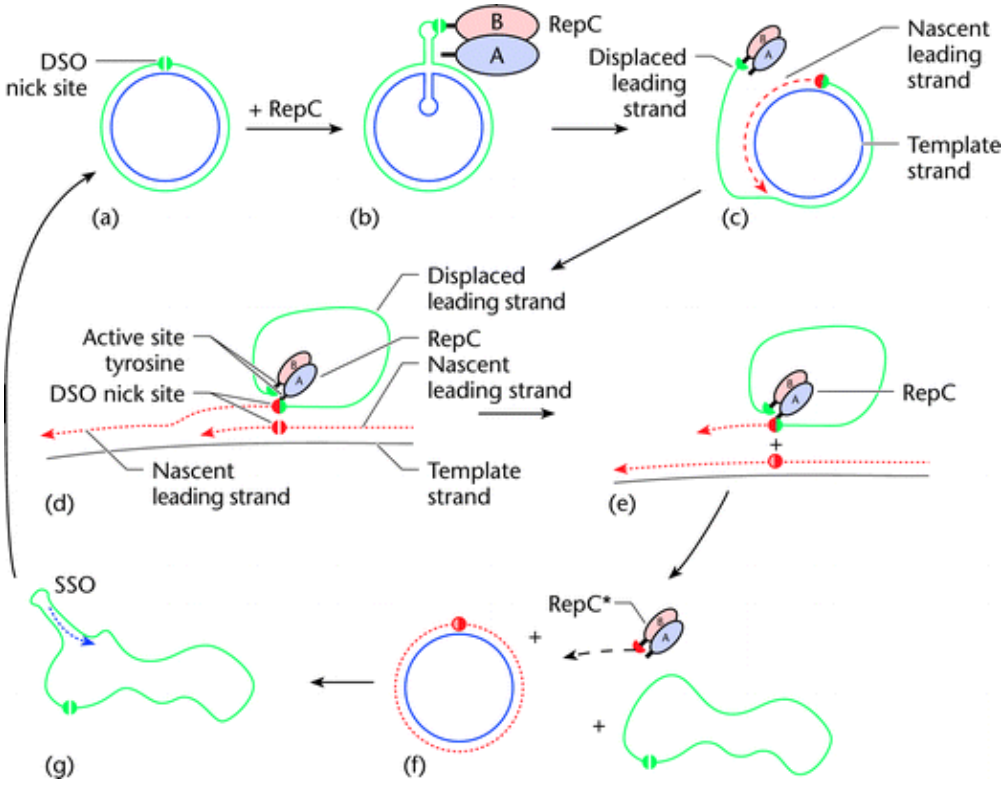


FIG. 1.6. Putative mechanism of rolling circle replication termination. See text for details. From Novick (2002).

2.2 Regulation of replication/Copy number control

Plasmid copy number can vary between different bacteria. It is crucial, however, that a steady state copy number be maintained to avoid plasmid loss or runaway replication

which can subsequently lead to a decrease in host fitness. Different systems such as autoregulation of initiator protein, direct control by means of active monomer/inactive dimer equilibrium, iteron control and antisense RNA control are employed to maintain a plasmid's steady state copy number and any fluctuations thereof (figure 1.7).

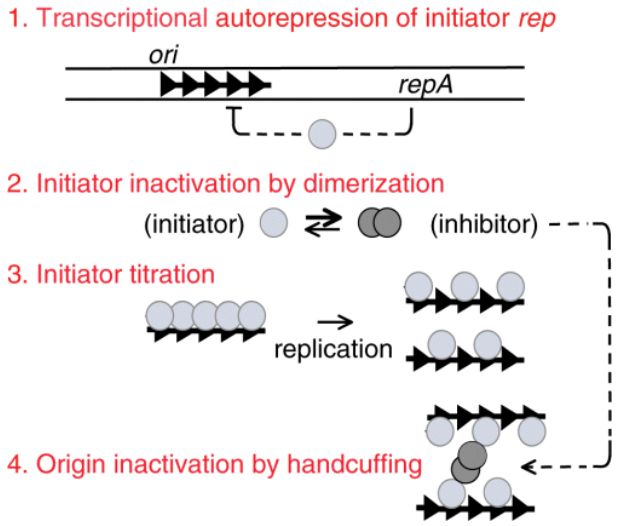


FIG. 1.7. Different mechanisms of replication initiation control of iteron-containing plasmids. 1) Replication initiator monomers in P1 for example serve a dual role by initiating and autorepressing its promoter. 2) Initiator proteins exist in monomer and dimer form, but dimers bind iterons weakly, and they can bind to an invert repeat which is partially homologous to iterons, therefore they can repress promoters in some plasmids. Dimers are believed to serve as inhibitors by taking part in handcuffing. 3) The initiator protein can be titrated by daughter iterons or iteron arrays outside the origin leading to the iterons not being saturated for replication initiation, thereby preventing initiation. 4) Origin pairing by monomer bound iterons and dimer bridge formation (handcuffing) further inhibits replication initiation. From Paulsson and Chattoraj (2006).

2.2.1 Autoregulation and direct control of Rep availability

The Rep initiator can act as an autorepressor by binding to its own operator region. The Rep protein exists in either a monomer or dimer form. Monomers bind to iterons in the origin and initiate replication, but the role of dimers is not well known. Dimers are inactive in binding iterons, but they do bind the operators of *rep* genes of some plasmids

e.g. F plasmid and pSC101 (Manen *et al*., 1991; Ishiai *et al*., 1994; Urh *et al*., 1998). In these plasmids the dimer binds to invert repeats (half iterons) which are partially homologous to the iterons, thereby autoregulating Rep expression (Germino and Bastia, 1983b; Vocke and Bastia, 1983). Experiments using prophage P1 suggested that autoregulation of *rep* expression by dimerized Rep proteins alone is insufficient for copy number control (Sozhamannan and Chattoraj, 1993). In this plasmid the origin iterons overlap with the Rep operator and the binding of monomers to the origin leads to initiation and repression. Rep dimers are, however, still formed and require chaperones for iteron binding. In this case dimer formation possibly plays a direct role in limiting the Rep availability and disrupts initiation. Aside from the role of Rep dimers in autoregulation of its own transcription, dimers are also involved in inhibition of replication as has been demonstrated for RK2/RP4 (Toukdarian and Helinski, 1998) and R6K (Krüger *et al*., 2004)

2.2.2 Iteron control

Iterons function as negative regulators of replication as unsaturated binding of initiators at the iterons results in inhibition of replication. This topic has however been under much discussion with regards to the mechanism by which replication is negatively regulated or inhibited. Two models have been proposed for the inhibitory regulation of iteron-containing plasmids.

In a model known as the "**titration model**", iterons titrate initiators, thereby making them rate limiting for replication (Uga *et al*., 1999). An increase in iteron concentration *in cis* or *in trans* caused a decrease in copy number (Helinski *et al*, 1996). Since iterons sequestered the Rep proteins it was thought that the amount of replication events was a direct consequence of Rep concentration and *rep* expression is constitutive. In light of this, the model could not explain why, in the case of RK2/RP4, a 200-fold increase in TrfA initiator concentration only caused a 30% copy number increase (Durland and Helinski, 1990). Nor was a significant effect on copy number of R6K observed when the π protein concentration was decreased two-fold (Filutowicz and Rakowski, 1998). A similar observation, where an excess RepA did not affect copy number significantly, was made for pPS10 and P1 (Pal and Chattoraj, 1988; García de Viedma, 1996).

Therefore, as the effect of titration could not be overcome by derepression, an autoregulatory mechanism together with titration would be dispensable for strict copy number control.

In light of this an alternative "**Handcuffing model**" was proposed. In this model two arrays of iterons are bridged together either on the same plasmid ("looping") or on different plasmids ("handcuffing") (Gasset-Rosa *et al*., 2008) and has been demonstrated for plasmid P1 (Pal and Chattoraj., 1988) and R6K (McEachern *et al*., 1989). Handcuffing has a negative effect on replication due to the steric hindrance it exhibits on origins by inhibiting origin melting and thereby replication. Based on the role of dimers vs monomers, three alternative models have been proposed for origin pairing or handcuffing. The first is the dimer bridge model described for R6K (Urh *et al*., 1998; Kunnimalaiyaan *et al*., 2005). In this model the one winged helix domain of one of the monomers, namely WH2, binds to the DNA and the other domain, WH1, remains free (figure 1.8). This WH1 subunit can then bind to a WH2-WH1 complex bound to DNA on an iteron array of another plasmid. A second monomer-monomer interaction model was described for mini-F of *E. coli* where a direct interaction between monomers, bound to the *ori*2 iterons (initiation of replication) and *inc*C iterons (regulation of replication), leads to origin pairing (Uga *et al*., 1999). In the third model two monomer bound iteron arrays are bridged by dimers forming a tetramer bridge as was demonstrated for P1 (Das and Chattoraj, 2004), TrfA of RK2 (Toukdarian and Helinski, 1998) and RepE of F (Zzaman and Bastia, 2005).

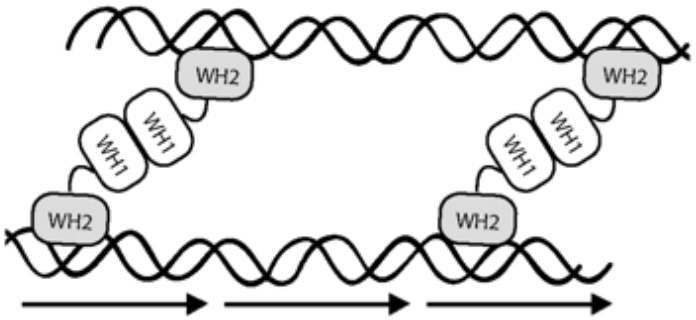


FIG. 1.8. Dimer bridge model for R6K replication inactivation by bridging two iterons (Kunnimalaiyaan *et al*., 2005).

2.2.3 Antisense RNA control

Control by antisense RNA operates by negative feedback wherein a constitutively counter-transcribed and unstable RNA inhibitor molecule binds to the target *rep* mRNA, thereby controlling the Rep availability and limits plasmid copy number (Brantl, 2004). The concentration of antisense RNA is dependent on plasmid copy number. A higher copy number leads to increased levels of antisense RNA, causing inhibition of the function of an initiator protein (at mRNA level) or a RNA primer. The RNA, therefore, detects and regulates copy number by inhibition. Antisense RNAs can act alone or in concert with a protein. The protein can have an auxiliary role (e.g. R1 and ColE1) or have a function in control together with the antisense RNA (e.g. pMV158) (Brantl, 2004). Whether by individual antisense RNAs, or in conjunction with a repressor protein, inhibition can be accomplished on different levels such as i) transcriptional attenuation, ii) translation inhibition and iii) inhibition of primer processing/formation (del Solar and Espinosa., 2000).

i) Transcriptional Attenuation (pT181 and *inc*18) – antisense RNA

Regulation by transcriptional attenuation has been identified exclusively in plasmids replicating in Gram-positive bacteria such as the pT181 and the Inc18 plasmid families (Novick *et al*., 1989; Brantl *et al*., 1993). Two potential stem loop structures can form in the Rep mRNA. Directly upstream of the ribosomal binding site (RBS) of the *rep* transcript are two invert repeats which can form a rho-independent terminator (looping between repeats a and b) to induce premature termination of transcription (figure 1.9). Repeats A and B are situated further upstream from a and b. Repeats A and a, on the other hand, have the ability to pair up in the absence of antisense RNA. This results in the folding of an alternative secondary structure which inhibits the formation of a terminator and replication can occur. Binding of an antisense RNA to the mRNA region overlapping the A and B repeats prevents secondary structure formation in this area, leaving repeats a and b to form a rho-independent terminator.

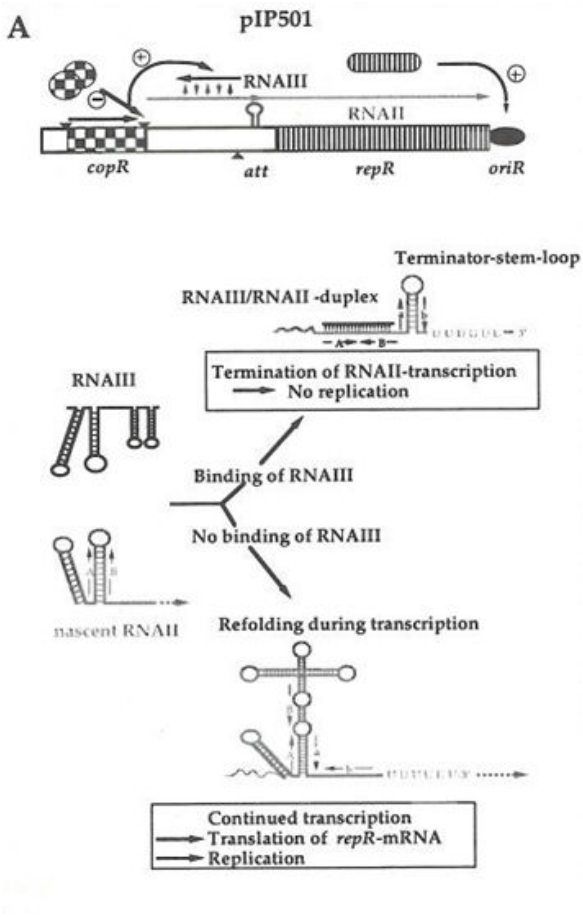


FIG. 1.9. Transcriptional attenuation in pIP501. CopR acts as a transcriptional repressor and regulates *repR* transcription for expression of *repR* initiator. The antisense RNA III is synthesized and interacts with the leader region of *repR* mRNA causing transcriptional attenuation at the attenuator (*att*) site, therefore no *repR* expression. From Brantl (2004).

ii) Translation Inhibition of

a. leader peptide (R1) – antisense RNA + auxiliary proteins

This type of regulation is similar to the mechanism employed by ColE1 type plasmids. It also uses a antisense RNA (CopA) and an auxiliary protein (CopB) to regulate its expression, however regulation occurs at the translational level where the antisense RNA inhibits Rep translation (del Solar and Espinosa., 2000). RepA can be transcribed from two promoters, P1 and P2 (figure 1.10). Expression of *repA* is translationally coupled to the leader peptide Tap (Blomberg *et al*., 1992) and forms a *repA*-mRNA leader template which is synthesized from the P2 promoter. CopA, the main regulator,

blocks translation of this template by binding to the leader mRNA region and thereby indirectly blocks *repA* translation (Malmgren *et al*., 1997). CopB is an auxiliary protein that is co-transcribed with *tap* and *repA* from the P1 promoter and is a transcriptional repressor of promoter P2. Initially expression of *repA* is initiated from the P2 promoter until the plasmid copy number reaches its steady state and then CopB represses this promoter and the *repA* gene is transcribed from promoter P1. At steady state CopA is adequate for regulation.

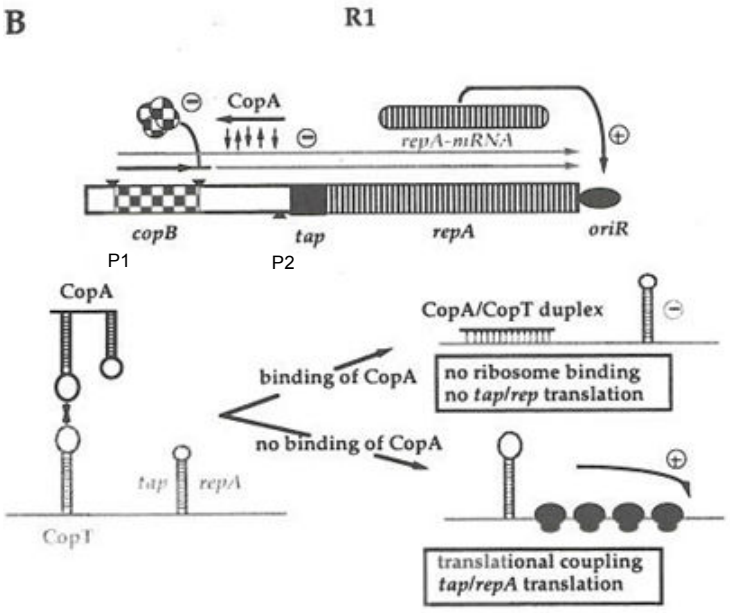


FIG. 1.10. Plasmid copy number control in R1. CopA antisense RNA blocks translation of the *tap* leader peptide and that of *repA*. CopB acts as an auxiliary protein and represses promoter of P2 (*repA* promoter), required for initial expression of *repA.* Ribosome structures are symbolized as black caps. From Brantl (2004).

b. formation of pseudoknot (ColIb-P9) – antisense RNA

The best characterized example of this type of replication control is for plasmid ColIb-P9 (Brantl, 2004). An antisense RNA acts as the only regulator and inhibits the formation of a pseudoknot which is needed for the translation of the Rep initiator. A leader peptide, RepY, needs to be translated to allow RepZ synthesis. Two stem-loop structures are found within the *repZ* mRNA (figure 1.11) (Asano and Mizobuchi, 1998). Structure III is

situated in the middle of the *repY* leader peptide and structure I is located upstream of the ribosome binding site (RBS) of *repY*. The RBS of *repZ* and a sequence complementary to a region in the loop of structure I are masked within structure III. Termination of *repY* translation unfolds structure III and structure I can pair with its complementary sequence by means of pseudoknot formation (Asano *et al*., 1991; Wilson *et al*., 1993). The ribosomes can bind to the exposed RBS of *repZ* and translation is initiated. Inhibition of this translation is mediated by the binding of antisense RNA (Inc RNA or RNA I) to its complementary region in structure I. The consequence is the inhibition of pseudoknot formation and *repY* translation is blocked directly and *repZ* is, therefore, also repressed.

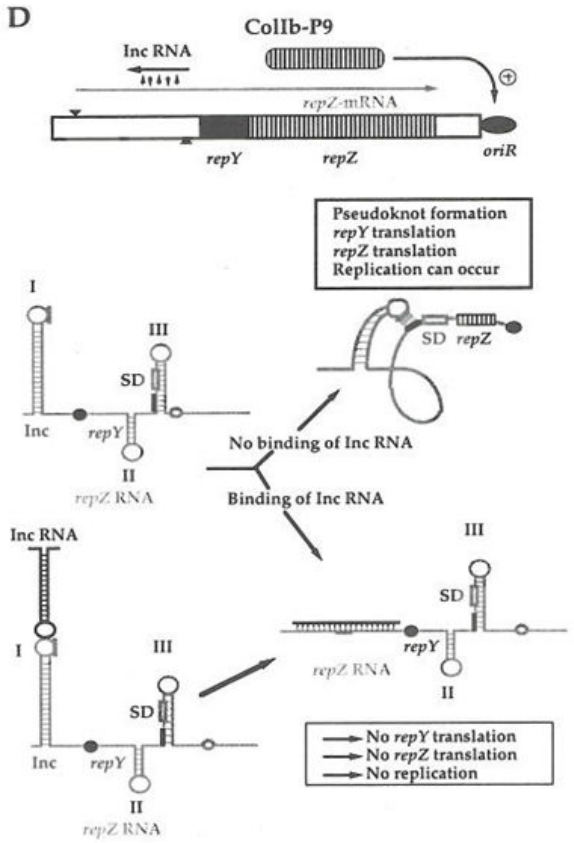


FIG. 1.11. Depiction of ColIb-P9 replication inhibition and activation by pseudoknot formation. Pseudoknot formation leads to translation from *repY* leader peptide. This causes unfolding of the structure which masks the SD of *repZ* and the *repZ* replication initiator is translated. Interaction with IncRNA hinders pseudoknot formation and thus inhibits translation of leader *repY* and *repZ*. Closed circle (*repY* start codon); Open circle (*repY* stop codon). From Brantl, 2004.

c. Rep synthesis (pMV158 antisense RNA + protein) + (ColE2 antisense RNA)

The synthesis of the pMV158 RepB initiator is dually regulated by an antisense RNA (RNA II) and a transcriptional repressor (CopG), (figure. 1.12) (del Solar *et al*., 1995). RNA II pairs with the RBS of *repB* mRNA, thereby directly inhibiting translation (Brantl, 2004). CopG controls replication by acting as a transcriptional repressor and binds to its own promoter. Since CopG and *repB* are co-transcribed from the same promoter, repression of CopG also represses RepB synthesis.

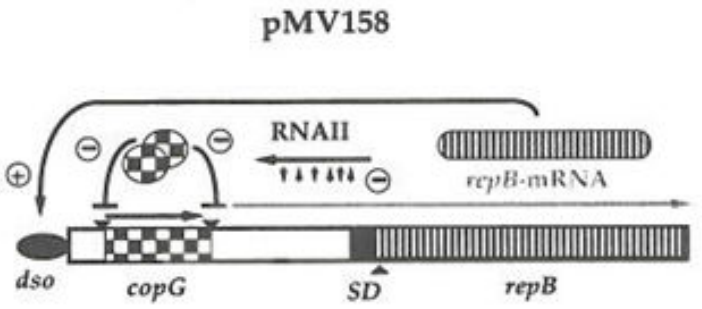


FIG. 1.12. pMV158 replication control. The antisense RNA II is complementary to the *repB* RBS (or Shine Dalgarno, SD), thereby inhibiting ribosome binding. CopG represses its own promoter and the *repB* promoter. From Brantl, 2004.

The Rep protein of ColE2 is a primase and is the only plasmid-encoded factor needed for initiation of replication (Takechi *et al*., 1995). Expression of *rep* is controlled by RNAI antisense RNA which is complementary to and can pair with the 5' region of *rep*-mRNA to inhibit translation.

iii) Inhibition of primer formation (ColE1) – antisense RNA + auxiliary proteins

ColE1 is an example of a plasmid that utilizes this type of regulation. As previously described, leading strand synthesis of this plasmid is initiated at the origin by RNAP. It synthesizes a preprimer RNA (RNAII) and a DNA-RNA complex is subsequently formed (figure 1.13). RNase H cleaves the duplex and a free 3'-OH end is generated which serves as an initiation point for DNA polymerase I to continue replication (del Solar and

Espinosa., 2000). A D-loop is formed during elongation by DNA Pol I and exposes a *pas* (primosome assembly site) sequence from which lagging strand synthesis is initiated (Brantl, 2004). Regulation of the ColE1 replication is mediated by an antisense RNA (RNAI) which is complementary to the primer region and constitutively transcribed (Lacatena and Cesareni, 1981; Tomizawa and Itoh, 1981). A weak interaction is formed between RNAI and RNAII ("kissing complex"). Later an RNA duplex which prevents refolding of RNAII into its active secondary structure, therefore no DNA-RNA hybrid can be formed and no maturation of primer. The Rom protein, sometimes referred to as Rop, acts as an auxiliary protein by improving the stable complex formation between RNAI and RNAII. Rom does not seem to be required for control but deletions of this protein showed an increase in copy number for slow growing cells (Atlung *et al*., 1999). Three roles have been proposed for Rom. The Rom concentration is proportional to copy number, therefore Rom presence would allow for an improved response in copy number fluctuations. Secondly, if there is a high RNAI concentration then Rom would make the chance of replication to occur close to zero. This is because in the absence of Rom the duplex formation between RNAI and RNAIII cannot take place sufficiently for full inhibition to occur. Thirdly, Rom could simply act as a backup in the case where copy number is reduced severely.

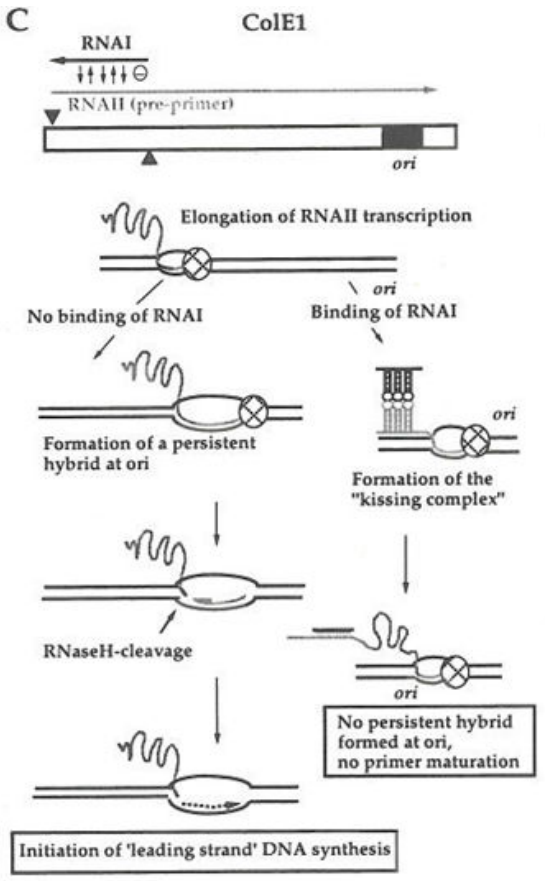


FIG. 1.13. Copy number control in ColE1. Preprimer RNAII synthesis is essential for replication. No interaction with RNAI allows stable hybrid formation with template DNA and primer maturation which leads to replication activation. In the presence of RNAI an RNAI-RNAII interaction inhibits formation of a DNA-RNA hybrid and no primer maturation or replication. From Brantl (2004).

2.2.4. Novel type of replication control

A novel plasmid replication regulation type was described by Burian *et al*., (1999) that does not involve iterons or antisense RNA and only involves the replication initiator protein in initiation and repression of replication of a small cryptic plasmid, pKL1, of *E. coli*. The plasmid encodes a *cop* region which carries the *rep* promoter and two Rep binding sites, namely BD1 and BD2. Rep monomers/dimers bind the BD1 site, whereas Rep oligomers bind BD2 preferentially. Binding of Rep to both sites initiate replication. This results in an increase in plasmid copy number as well as Rep concentration. At

high concentrations oligomerization of Rep is favoured, resulting in displacement of the monomer/dimers from the BD2 site and thus autorepression of *rep*.

3. Plasmid Maintenance Mechanisms

Plasmids have a region referred to as the minimal replicon and is defined as the minimal plasmid segment that can support normal replication and maintain its copy number. This region plays an important role in regulating copy number fluctuations (Ebersbach and Gerdes, 2005). In addition to replication control mechanisms some plasmids might employ additional elements or strategies to ensure stable maintenance. Such strategies may include site-specific recombination (multimer resolution), addiction systems and or active partitioning (Funnell and Slavcev, 2004). Most if not all plasmids utilize a site-specific recombination mechanism, since plasmid dimer formation is inevitable. High copy number plasmids in general rely on random distribution to daughter cells. Low copy number plasmids, however, employ active maintenance mechanisms to ensure the stable inheritance of these plasmids.

3.1 Helper elements:

3.1.1 Multimer Resolution Systems/Site-specific recombination

High copy number plasmids in general rely on random segregation for equal distribution of plasmids between daughter cells. Plasmid multimers or oligomers are, however, formed during replication. Plasmid multimers would eventually lead to a decrease in the number of plasmid monomers available for segregation and subsequent plasmid loss. In addition it is also known that plasmid dimers are selected 2-fold more frequently than its monomer counterpart for replication and thus accumulate. This “dimer catastrophe” is generally resolved by site-specific recombination systems (Summers *et al*., 1993). Site-specific recombination systems encode a site-specific recombinase (resolvase) and a *cis*-acting *res* site. Recombination between two *res* sites utilizing a site-specific recombination mechanism resolves the dimer into two monomers. Such a site-specific

recombination system can be entirely encoded on the plasmid or the plasmid may only contain a *res* site and utilize the host resolvase. The recombinases can be of the serine- or tyrosine-recombinase type, for example the ParA/*res* of RK2/RP4 and the Cre/loxP of P1, respectively. Many plasmids carry transposons such as Tn*3* which encode site-specific recombination systems and it is thought that these transposon-encoded systems may also be involved in plasmid monomerization (Grindley, 2002).

3.2 Active Systems:

3.2.1 Partitioning

Plasmid partitioning is an active system which ensures that both daughter cells acquire at least one plasmid copy during cell division. It relies on the function of a plasmid encoded partition (*par*) locus (Ebersbach and Gerdes, 2005). In general, the *par* locus encodes two *trans*-acting proteins and a *cis*-acting centromere-like site. The two Par proteins are firstly DNA-binding proteins which recognise and specifically bind repeats in the centromere-like site and secondly NTPases, which provides the energy for the attachment and movement of plasmids to specific locations within the host (Funnell and Slavcev, 2004). The centromere-like site serves as a recognition site for the assembly of the partitioning complex. The genetic organisation of most *par* loci can, however, differ as depicted in figure 1.14.

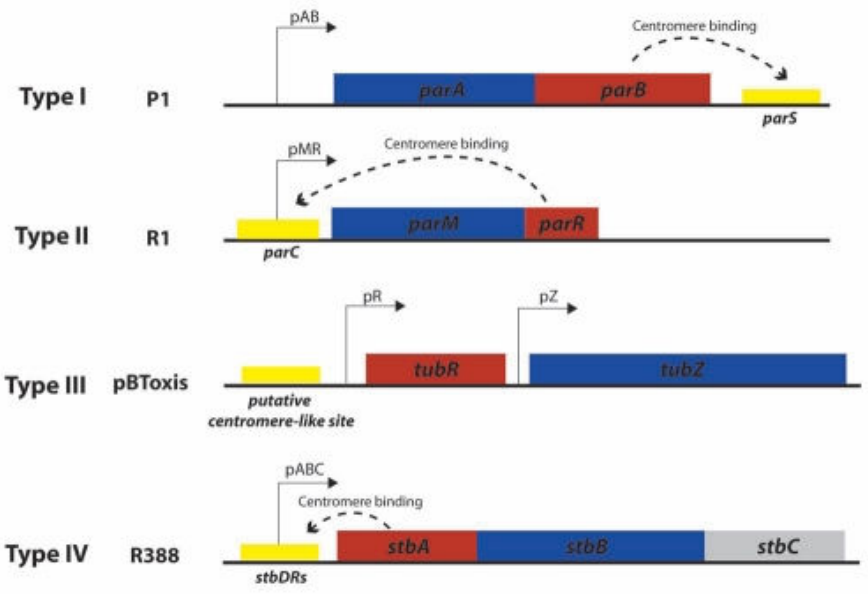


FIG. 1.14. Genetic organization of the four main types of partition segregation systems, representing the prototype plasmid in each group. Motor proteins (blue), DNA-binding proteins (red), *cis*-acting sites (yellow). From Guynet and de la Cruz (2011).

Partitioning systems are divided into four types, based on the type of NTPases they encode (Guynet and de la Cruz, 2011). Type I is characterized by Walker-type partition NTPases, whereas the NTPases of Type II and III are actin- and tubulin-like, respectively (Salje *et al*., 2010). Type I partitioning loci can be further subdivided based on the size and sequence of the binding proteins and NTPases (Gerdes *et al*., 2010). These include types 1a and 1b, both of which are plasmid encoded, and a third class which is chromosomally encoded (Salje, 2010). A more recently discovered Type III system was found to be encoded on the *Bacillus* plasmid pBtoxis (Larsen *et al*., 2007). Two other novel segregation systems, encoded on plasmids pSK1 (Simpson *et al*., 2003) and R388 (Guynet *et al*., 2011) have also been described and were classified as Type IV par systems. These two systems are different compared to the three “classic” types of partition systems since they do not encode the classical *par* system configuration and seem to require only a single protein for their segregational ability. The mechanism by which pSK1 elicits segregation is unknown and will not be discussed

in further detail. A “Pilot-fish” mechanism was, however, proposed for plasmid R388 and will be discussed below.

3.2.1.1 Type I partitioning systems: Plasmids P1 and F

The prototype plasmids for this subdivision of partitioning are plasmids P1 and F from *E. coli* and were the first partitioning systems to be identified almost 30 years ago (Ogura and Hiraga 1983 and Austin and Abeles 1983). These plasmids have, therefore, been studied extensively and are the most prevalent of all partitioning types.

The *par* loci of P1 and F are referred to as Par and Sop (stabilization of plasmid), respectively. The P1 *par* locus encodes a ParA (ATPase), ParB (DNA binding protein) and *parS* (centromere-like site). The ParA, ParB and *parS* counterparts of plasmid F are referred to as SopA, SopB and *sopC,* respectively.

Centromere-like sites and CBPs (centromere binding proteins). The *parS* site of P1 has a BoxA and BoxB motif that is recognised by ParB. An interaction site for host-encoded IHF is present and when bound by IHF a bend in *parS* is induced which increases the affinity of ParB for *parS* (Ebersbach and Gerdes, 2005). IHF is, however, not essential for partitioning and although there are four A-box motifs, only the invert repeat motifs adjacent to the IHF site are required for *par* functioning.

The *sopC* site of F contains 12 direct repeats each of which contain an invert repeat sequence and is recognized by SopB. Only one direct repeat is, however, required for partitioning (Ebersbach and Gerdes, 2005). SopB has a coating function and forms a wrapped nucleoprotein complex with *sopC*. This coating or wrapping function is necessary to stimulate polymerisation of SopA ATPase and drives plasmid segregation (Schumacher, 2012). Growing evidence suggests that plasmids are grouped together during the cell cycle, and this is mediated by ParA/SopA which pair up plasmids that are coupled by ParB/SopB (Funnell and Slavcev, 2004) at the centromeres. It has been proposed that P1 plasmid segregation is enhanced by pairing sister plasmids in close proximity which are then recognized by the partition complex and moved apart (Sengupta *et al*., 2010).

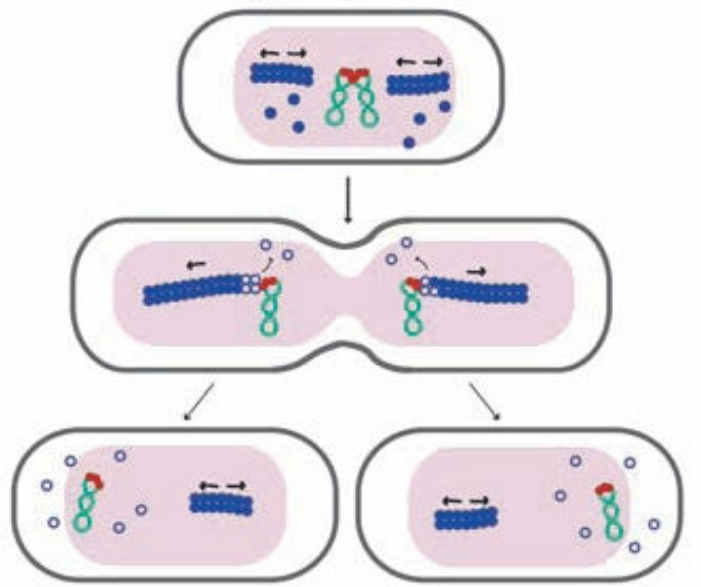
NTPases. ParA NTPases have been shown to move dynamically over the nucleoid (Hirano *et al*., 1998; Quisel *et al*., 1999; Marston and Errington, 1999; Ebersbach and Gerdes, 2001; Lim *et al*., 2005; Hatano *et al*., 2007; Pratto *et al*., 2008; Castaing *et al*., 2008) and thus play an important role in plasmid segregation by providing the motive force required to pull plasmids apart (Ebersbach and Gerdes, 2005). The ParB/*parS* partition complex is essential for the recruitment of ParA ATPase. ATP binding and subsequent hydrolysis by ParA supplies the energy for segregation to occur.

In daughter cells plasmids P1 and F localize themselves at midcell position (Gordon *et al*., 1997; Niki and Hiraga, 1997). After replication they relocate to quarter cell positions. This ensures that, in the case of more than one plasmid, there will be at least one plasmid copy in each half of the cell prior to cell division. Different models have been proposed for the mechanism by which Type I plasmids segregate. Among these are a "Pulling" mechanism (Ringgaard *et al*., 2009) and more recently a diffusion ratchet model has been proposed for plasmid P1 (Vecchiarelli *et al*., 2010).

"Pulling" mechanism of segregation. A ParA pulling mechanism for segregation was proposed by Ringgaard and colleagues (2009) for Type I *par* systems. They described this mechanism based on studies on pB171. As previously mentioned, the binding of ATP to ParA is important for the formation of ParA filaments. ParA, in the presence of ATP, binds to nucleoid DNA as dimers and ParA filaments are formed by polymerisation (figure 1.15). This filament continues to grow or polymerise until it reaches a plasmid *parC* site bound by ParB. The interaction between ParB and ParA-ATP stimulates the ATPase activity of ParA and subsequent ATP hydrolysis to ADP leads to depolymerisation (retraction). The ParA-ADP complex is released from the DNA (nucleoid) and the adjacent free ParA-ATP filament is now accessible for interaction with the ParB/*parC* partition complex. During depolymerisation the plasmid can either remain attached to the ParA filament or disconnect itself. The growing ParA filament is associated with the nucleoid. ATP hydrolysis (depolymerisation) causes ParA filaments to change into their ADP form which detaches from the nucleoid and a ParA-free segment is subsequently formed on the nucleoid. During the hydrolysis the plasmid is pulled to one end of the cell by an action which resembles that of a shift from one ParA-

ATP dimer to the next ParA-ATP dimer. A new polymerisation event can occur from the generated ParA-free segment on the nucleoid by rejuvenating ParA-ADP to ParA-ATP and then progress towards the other end and contact a different plasmid.

A



B

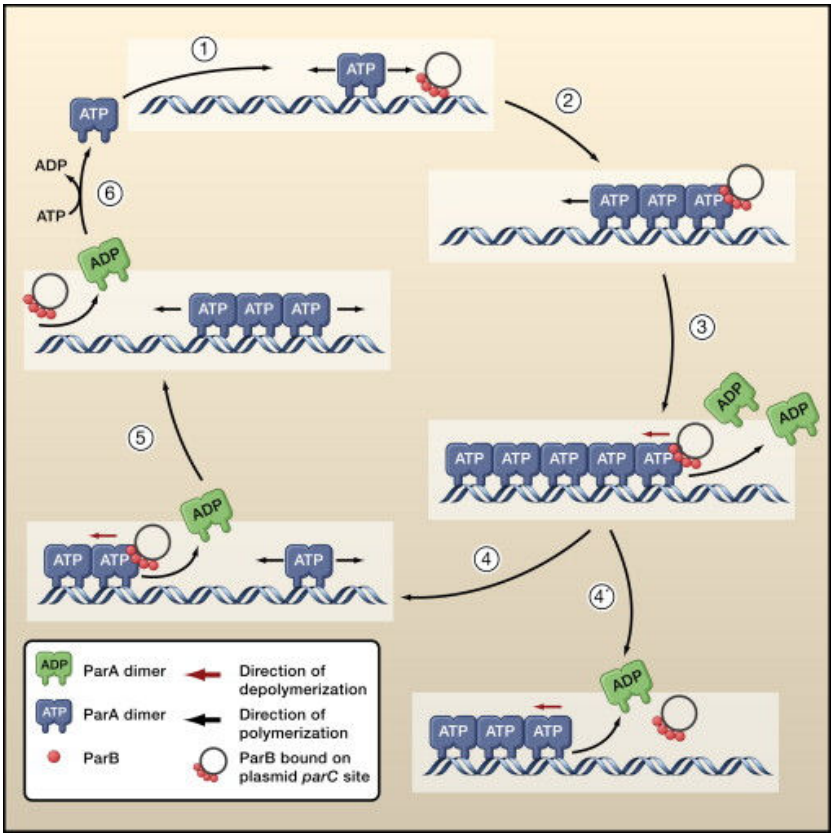


FIG. 1.15. A. Pulling mechanism of plasmid partitioning depicted as it occurs in the bacterial cell. See text for details. NTP-bound ParA motor proteins (blue circles); adapter proteins ParB bound to centromere-like site (red circles); NDP form of ParA motor protein (open blue circles) From Guynet and de la Cruz (2011). B. Molecular model of the ParA pulling mechanism depicted as it occurs on the nucleoid level. 1) Binding of ParA-ATP to nucleoid and bidirectional filament polymerization; 2) filament contacts ParB-parC complex on plasmid; 3) ParB stimulates ATPase activity of ParA-ATP which is converted to ParA-ADP and released from DNA. A new ParA-ATP end is available for interaction with the partition complex. The depolymerisation event has one of two outcomes: 4') the plasmid can be dropped off or 4) remain attached to the end of the depolymerizing filament; 5) If the plasmid is released new ParA-ATP units can assemble on the nucleoid zone free of ParA-ATP subunits and the released plasmid can interact with a new filament; 6) ParA-ADP is converted to ParA-ATP and another cycle can occur. From Guynet and de la Cruz (2011); Gerdes *et al*. (2010).

Diffusion-ratchet mechanism. The Diffusion-ratchet model was described by Vecchiarelli *et al*. (2010) for plasmid P1 in contrast to the pulling mechanism described for pB171. In this model the ParB-*parS* partition complex has a higher affinity for active ParA-ATP molecules. ATP hydrolysis occurs once ParB associates with nucleoid bound ParA-ATP. ATP hydrolysis results in dissociation of ParA from the nucleoid and inactive ParA-ADP is released to diffuse throughout the cell. The ParA molecule becomes activated again by exchanging its ADP for ATP and undergoes a conformational change that subsequently enables it to bind the nucleoid again at a random position. The decrease in the local concentration of nucleoid-bound ParA stimulates the ParB-*parS* to glide to the areas with higher nucleoid bound ParA concentration. The plasmid movement is thus generated by the gradient of available nucleoid-bound ParA. Once ParB-*parS* reaches the ends of the nucleoid it changes its direction of movement and move back to the other end in a similar gradient dependent manner. Figure 1.16 illustrates the diffusion-ratchet mechanism.

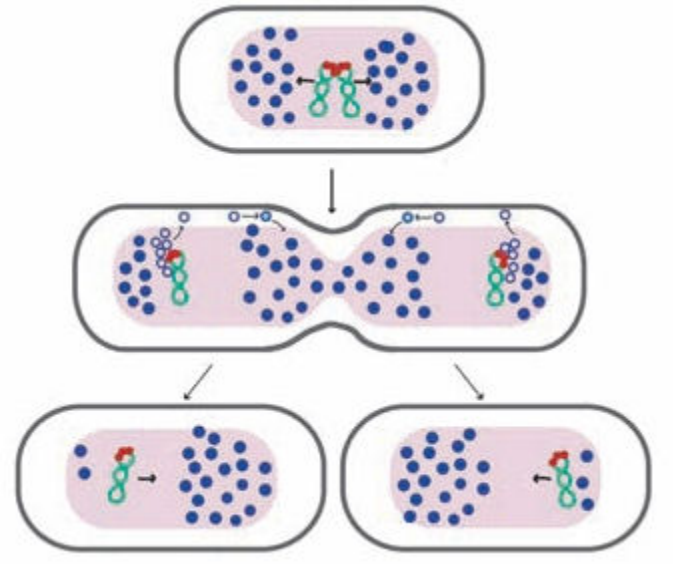


FIG. 1.16. Diffusion-ratchet mechanism of plasmid segregation. See text for details. ParB adapter bound to centromere-like site (red circles); ParA-ATP active motor protein (dark blue circles); ParA-ADP motor protein (open blue circles); ParA-ATP inactive motor protein (light blue circles). From Guynet and de la Cruz (2011).

3.2.1.2 Type II partition: Plasmid R1

It has been more than 25 years since the *parMRC* locus of plasmid R1 was discovered (Gerdes *et al*., 1985). This locus encodes a ParM (motor protein), ParR (binding protein) and the *cis*-acting *parC* site. To date various *parMRC* loci have been identified on plasmids from Gram-negative and Gram-positive bacteria, but not for bacterial chromosomes (Ebersbach and Gerdes, 2001; Becker *et al*., 2006; Schumacher, 2007).

Centromere site (*par*C) and CBP (ParR). Both ParR and *parC* are required for ParM filament formation, since overproduction of ParM alone was not sufficient (Gerdes *et al*., 2010). The centromere site, *parC*, consists of 10 repeat sequences, all of which are required for partitioning. Binding of ParR to *parC* results in plasmid pairing (Jensen and Gerdes, 1997; Jensen *et al*., 1998; Weitao *et al*., 2000). The promoter region of *parMRC* is located within *parC* and, therefore, binding of ParR to *parC* also autoregulates transcription of the operon.

NTPase (ParM). ParM forms actin-like filaments that segregate plasmids in a mitotic like fashion (Møller-Jensen *et al*., 2002; Møller-Jensen *et al*., 2003). ParM filament formation requires ParR/*parC* binding and polymerisation at the end of the filament is dependent on ATP hydrolysis. Bidirectional polymerisation of the ParM drives paired or clustered plasmids to opposite cell poles (Garner *et al*., 2004; Popp *et al*., 2007). Although polymerisation occurs bidirectionally the disassembly step or depolymerisation occurs unidirectionally and is reminiscent of the dynamic instability of these growing and shrinking microtubules. Both elongation and shortening of ParM polymers requires ATP binding and hydrolysis (Møller-Jensen *et al*., 2002; Garner *et al*., 2004). ParM is dynamically unstable. ParM-ATP is needed for polymerisation to occur at the filament ends. Therefore, to prevent depolymerisation the filament ends are capped with ParM-ATP. Over time the ParM-ATP subunits are converted to ParM-ADP due to ATP hydrolysis. If ATP hydrolysis reaches the cap, no more ParM-ATP monomers are added and thus results in depolymerisation to occur from the end. This dynamic unstable property of ParM filaments means that filaments stabilized at one end can search for other plasmids with a ParR/*parC* complex while stabilisation at both ends allow for the active segregation of paired plasmids to opposite cell poles via polymerisation.

“Pushing” mechanism of segregation. Plasmid segregation by the ParMRC system works by a molecular mechanism of search and capture as shown in figure 1.17 (Salje *et al*., 2010). ParM filaments grow by means of insertional polymerisation in search of ParR-*parC* partition complexes in the presence of ATP. This leads to one of two outcomes. Growing filaments that reach a partition complex are capped by it and continues to polymerize in a bipolar fashion (pushing mechanism). Alternatively if a partition complex is not found ParM filaments undergoes catastrophic disassembly due to ATP hydrolysis and continues the search for a ParR-*parC* partition complexes. Once a growing filament capped by the ParR-*parC* complex reaches the poles of the cell, depolymerisation or catastrophic disassembly also occurs.

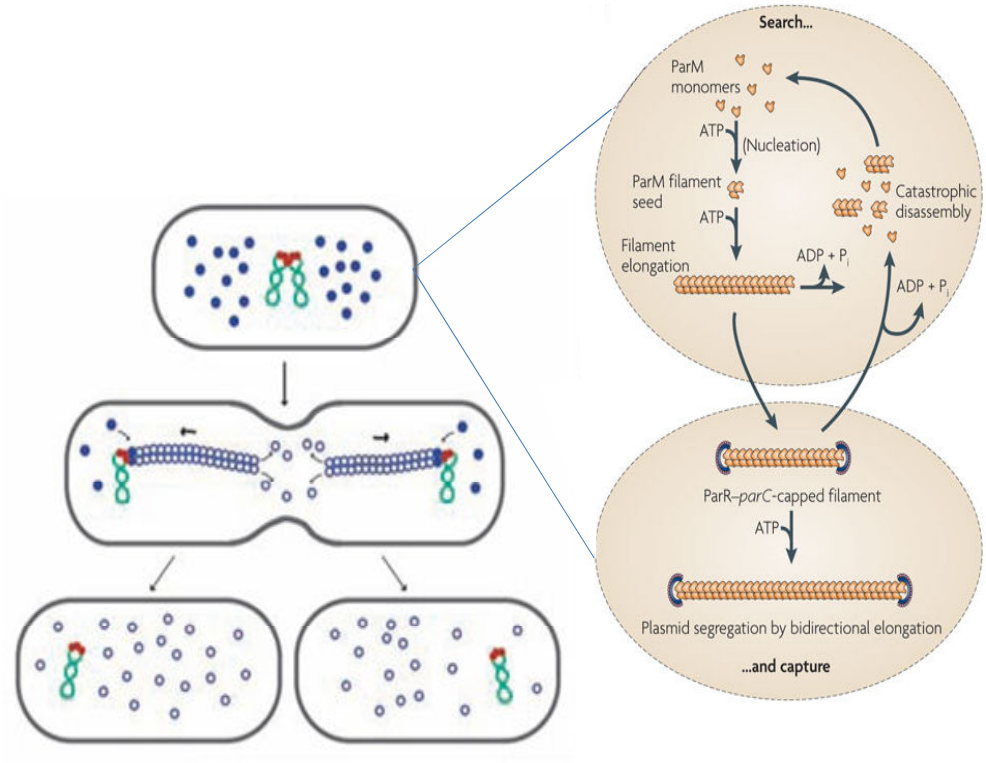


FIG. 1.17. Pushing mechanism of plasmid segregation. ParR proteins (red circles) bind to *parC* centromere-like site of newly replicated plasmid molecules. This serves as an initiation point for ParM filamentation with the insertion of ParM-ATP motor proteins (blue circles) on the filament ends, thereby pushing plasmids apart. The conversion of ParM-ATP to ParM-ADP (open blue circles) destabilizes the filaments and another ParM-ATP molecule can be inserted on the end. Adapted from Salje *et al*. (2010) and Guynet and de la Cruz (2011).

3.2.1.3 Type III partition: Plasmid pBtoxis

Plasmid pBtoxis encodes a partitioning locus consisting of two proteins, TubZ and TubR and a *cis*-acting site. TubZ is a GTPase and a deep branching member of the tubulin/FtsZ superfamily of GTPases. TubR is a DNA-binding protein which binds to the *cis*-acting site of four iterons (Tang *et al*., 2007). These iterons are also associated with replication.

"Treadmilling" mechanism. The mechanism of Type III segregation has been described as "treadmilling" (Larsen *et al*., 2007), "tramming" (Schumacher, 2012) or "pulling" (Guynet and de la Cruz, 2011) in nature.

Firstly, TubR binds to the *cis*-acting site forming the TubR-pBtoxis complex while the C-terminal tail of TubZ polymer binds to this TubR-pBtoxis complex (figure 1.18). GTP hydrolysis of the TubZ polymer allows for elongation at one end and retraction at the other (dynamic filaments), thereby causing the treadmilling-like movement and subsequent translocation of the TubR-pBtoxis complex to the poles of the cell. It is thought that bending of the TubZ filaments upon reaching the cell pole causes detachment of TubR-pBtoxis.

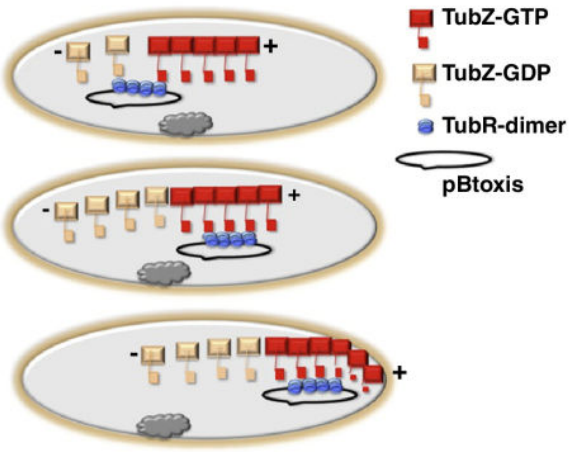


FIG. 1.18. Tramming or treadmilling mechanism of pBtoxis plasmid segregation. From Schumacher (2012).

3.2.1.4 Type IV partition: R388

It was recently demonstrated that plasmid R388 utilizes a novel system for segregation. A single plasmid-encoded StbA protein binds to a *cis*-acting *stbDR* site. Since StbA is not an NTPase it is assumed that R388 uses either a host encoded motor protein or segregates independently of a motor protein. Guynet *et al*. (2011) showed that StbA is the only protein in the *stbABC* gene cluster required for segregation of R388 in *E. coli*.

Though the mechanism by which segregation is brought about is unknown, it is thought to act in a “pilot-fish” manner. StbA binds to the *stbDRs* and this complex has been proposed to pair up with the host nucleoid (figure 1.19). The complex acts as a “pilot-fish” and the plasmid benefits from the chromosome segregation by being passively segregated in concert with the host DNA.

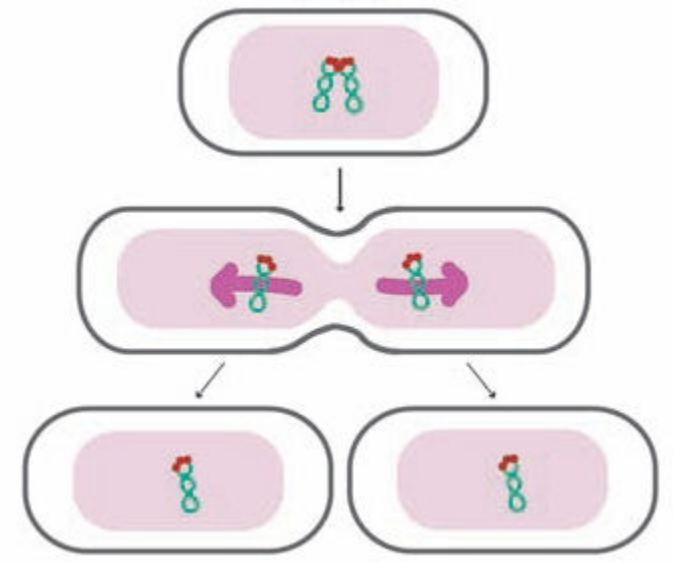


FIG. 1.19. ‘Pilot-fish’ mechanism of R388 plasmid segregation. See text for details. Adaptor protein bound to centromere-like site (red circles); direction of host chromosome segregation (pink arrows). From Guynet and de la Cruz (2011).

3.2.2 Post-segregational killing/Plasmid addiction systems

Partitioning functions, random distribution (replication) and multimer resolution systems do not guarantee that all daughter cells will inherit at least one copy of the plasmid (figure 1.20). Even in the presence of these systems plasmid-free cells are often formed. Such plasmid-free segregants may outcompete the plasmid-containing cells under non-selective conditions. Thus, to address this problem a post-segregational killing system is employed to inhibit the propagation of plasmid-free daughter cells. Many terms have been adopted in describing the post-segregational killing mechanism.

Toxin-antitoxin (TA), killer system, killing-antikilling, poison-antidote, plasmid addiction system and programmed cell death are all used interchangeably (Zielenkiewicz and Ceglowski, 2001).

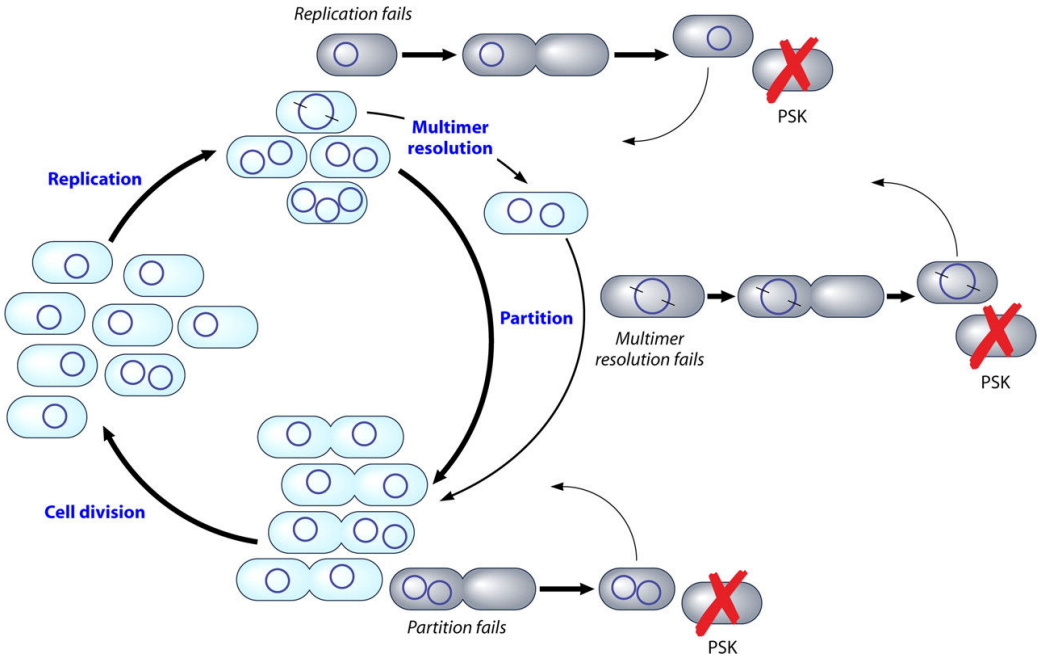


FIG. 1.20. An overview of the combined functioning of plasmid stability systems. Blue cells represent low copy number containing cells. Replication increases plasmid copy number in each cell to ensure that the daughter cells will at least inherit one plasmid copy. Multimer resolution systems resolve plasmid dimers into monomers. Partition systems actively segregate plasmids to daughter cells after replication. The grey cells represent the cases in which the replication, multimer resolution and partition systems have failed and if present the post-segregational killing system is triggered. From Sengupta and Austin (2011).

Plasmid addiction modules have been identified in and described for various plasmids and they employ similar strategies to combat propagation of plasmid free cells. The system typically consists of two components, a stable toxin (protein) and an unstable antitoxin which can be either a protein or an antisense RNA (Jensen and Gerdes, 1995). Toxin-antitoxin systems have been catagorized into three groups (figure 1.21), namely Proteic Plasmid Addiction Systems (PPAS), in which both toxin and antitoxin are proteins, Antisense-RNA-regulated addiction systems, where the mRNA antitoxin interacts with the mRNA of the protein toxin and the more recently discovered addiction

system in which the RNA antitoxin directly interacts with the toxin protein (Yamaguchi and Inouye, 2011).

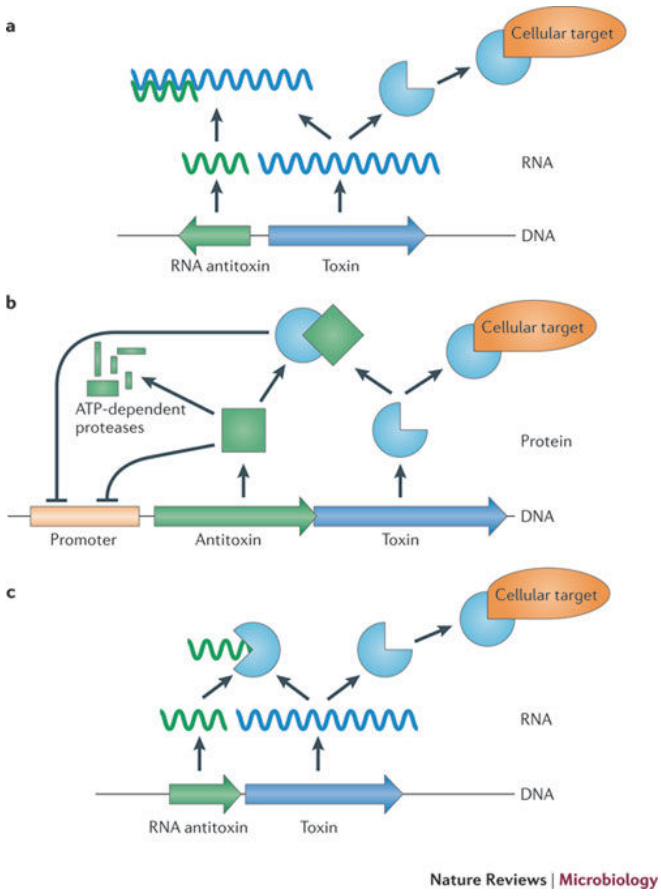


FIG. 1.21. Models for regulation of different types of TA systems. a) Type I TA regulation. The RNA antitoxin binds to the toxin mRNA thereby inhibiting translation of the toxin mRNA; b) Type II TA regulation. Antitoxin proteins bind to toxin protein to neutralize their toxic effect. The toxin-antitoxin complex or antitoxin alone (weak) can autoregulate the TA system. Antitoxin proteins can be cleaved by ATP-dependent proteases under stress conditions and the toxin is released to attack the cell; c) Type III TA regulation. The RNA antitoxin interacts directly with protein toxin and inhibits the toxic effect of the protein. From Yamaguchi and Inouye (2011).

3.2.2.1 Type I – Antisense-RNA-regulated Addiction System

The toxin gene expression is regulated by an antisense RNA molecule which is also encoded on the same locus and therefore can be seen as the antitoxin. If the plasmid is lost, the plasmid-born gene is also lost and the antisense RNA is degraded by RNase, thus leaving the long-lived toxin and growth is inhibited/the cell is killed.

The *hok/sok* locus of plasmid R1 has been extensively studied and described by Gerdes *et al*. (1997). The locus consists of three genes, the *hok* (host killing) toxin, *mok* (modulation of killing) regulator of translation and *sok* (suppression of killing) antitoxin (Zielenkiewicz and Ceglowski, 2001). Sok is an antisense RNA complementary to the leader region of *hok* mRNA comprising the *mok* gene (figure 1.22). *Sok* mRNA is very unstable compared to *hok* mRNA. This ensures rapid depletion of *sok* mRNA in plasmid free cells and the excess remaining Hok toxin brings about host killing. The *hok* mRNA is present in two forms in a plasmid-carrying cell, namely a stable full length mRNA in an inert conformation and a shorter active version. The primary full length version is unavailable for translation. This is due to the presence of a fold-back-inhibition (*fbi*) element that leads to pairing of the 3' end with the 5' end and blocks translation and antisense RNA binding (Thisted *et al*., 1994). Processing of this full length mRNA into its mature form renders the *hok* mRNA available for antisense RNA binding and translation. In plasmid-containing cells, *sok* mRNA binds to the leader region of mature *hok* mRNA which also includes the *mok* gene which is translationally coupled to *hok*. The RNA hybrids are cleaved by RNaseIII, thus indirectly inhibiting *hok* mRNA translation. Excess Hok toxin causes plasmid-free cells to die, due to the decreased membrane potential, respiration arrest and small molecule efflux (Gerdes *et al*., 1986).

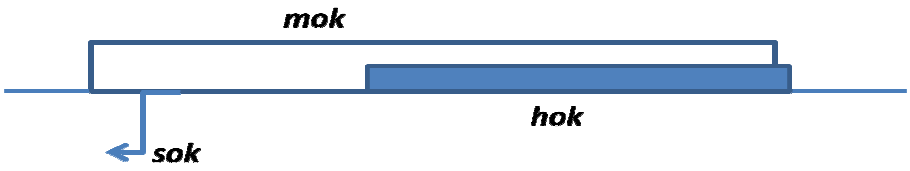


FIG. 1.22. *hok*/*sok* locus of plasmid R1. From Gerdes *et al*., 1990.

3.2.2.2 Type II – Proteic Plasmid Addiction Systems

PPAS require direct interaction between a proteic toxin and antitoxin to neutralize the effect of the toxic protein in plasmid containing cells (Gerdes *et al*., 2005). The antitoxin has a much shorter half-life compared to the toxin. When the plasmid-encoded genes of these proteins are no longer present due to plasmid loss the antitoxin is rapidly degraded through proteolytic action, thus leaving the longer-lived toxin to kill/inhibit cell growth. PPAS have a similar genetic layout compared to other TA systems in that the toxin and antitoxin is arranged in an operon, with antitoxin transcription preceding that of the toxin. The exception to this layout is the TA system of plasmid pRts1 in which the toxin precedes the antitoxin. Nine families of toxins, encoded on either plasmids or chromosomes have been identified based on sequence homology (Pandey and Gerdes, 2005). For the purpose of this review, only one of the best characterised plasmid encoded toxin-antitoxin systems will be summarized.

***ccd* locus of plasmid F.** One of the best characterised PPAS is the *ccd* locus of the F plasmid. F plasmid maintains a copy number of 1-2 copies per chromosomes and employs multiple maintenance mechanisms such as site-specific resolution, an active partition system and three addiction modules (Nordström and Austin, 1989). Two of the TA systems, namely the *flm* (F leading maintenance) and *srnB* (stable RNA degradation) loci are regulated by antisense RNAs (Golub and Panzer 1988, Ohnishi *et al*., 1977 and Nielsen *et al*., 1991). The third *ccd* locus (control of cell death), however, is an interactive protein system (Van Melderen *et al*., 1994). The *ccdA* locus consists of the *ccdA/letA/H* and *ccdB/letB/G* genes encoding the antitoxin and toxin, respectively, as well as a *resD* gene encoding a resolvase (Bex *et al*., 1983; Miki *et al*., 1984; Lane *et al*., 1986). CcdA couples with CcdB to neutralize the toxic effects of CcdB. The *ccd* operon is autoregulated by the CcdA:CcdB complex to ensure that it maintains adequate levels of the inactive poison for killing of plasmid free segregants. CcdA has an increased half life when bound to CcdB, but as it decays the CcdA-CcdB complex will also break up and is unable to repress the operon. Autorepression is, therefore, relieved and more CcdA and CcdB is produced to replenish the intracellular pool. The CcdA and CcdB protein levels are not replenished in plasmid-free segregants and as a

result the toxic activity of CcdB activated. This leads to CcdB binding the GyrA subunit of DNA gyrase, thereby causing decreased supercoiling and induction of the SOS response which in turn leads to filament formation. Cell killing is brought about by complex formation between the CcdB bound to inactive gyrase and DNA, thereby, blocking replication forks (Bernard and Couturier, 1992; Bernard *et al*., 1993; Maki *et al*., 1992; Maki *et al*., 1996).

3.2.2.3 Type III – Direct Protein-RNA interaction systems

Type III toxin-antitoxin systems rely on a direct protein-RNA interaction in which the protein is a toxin and a RNA molecule acts as an antitoxin. The *toxIN* locus of plasmid pECA1039 of the Gram-negative phytopathogen *Pectobacterium atrosepticum* (previously *Erwinia carotovora* subspecies *atroseptica*) was the first such system to be described (Fineran *et al*., 2009; Blower *et al*., 2012). It also encodes a phage abortive infection system (Abi). The protein ToxN toxin is inactivated *in vivo* by the ToxI RNA. The exact mechanism by which the cell inhibition effect is achieved is not clear as yet. However, ToxN functions through a bacteriostatic mechanism and it was further determined that the inhibitory effect of ToxN requires the formation of a trimeric complex with ToxI where three ToxI monomers bind to three ToxN monomers. ToxI is a non-coding pseudoknot of 36 nucleotides. Type III TA systems can be identified by the occurrence of repetitive sequences upstream of the toxin gene interspersed with a transcriptional terminator.

4. Plasmid incompatibility

Incompatibility is the phenomenon where two plasmids which are closely related cannot be stably maintained in the same cell. The outcome is that one of the two plasmids are lost from the cell (Scaife and Gross, 1962). The incompatibility phenotype occurs because one of the plasmids fails to replicate when it is in the presence of another related plasmid. Two types of plasmid incompatibility are described by Novick (1987). Symmetric incompatibility is where either of the plasmids are lost at equivalent probability while in vectorial incompatibility one plasmid is more likely to be lost

compared to the other. Incompatibility can be a result of the inability of either the replication system or partition system to distinguish between the plasmids. During replication the control elements which negatively regulate the replication frequency of one plasmid continues to function even in the presence of a second plasmid to such an extent that plasmid replication is not often enough for stable maintenance of either plasmid. Partition instability, on the other hand, refers to the mutual/common instability of two plasmids with the same partitioning complex (Ogura and Hiraga, 1983; Kusukawa *et al*., 1987) and is a vectorial incompatibility type. A mixed pairing model (Funnell, 2005) for partition instability suggests that plasmids will pair as mixed or relative pairs and that they can be segregated in such a way that the progeny will disinherit one of the plasmid types. Ebersbach *et al*., (2005) proposed a model in which they suggest that random-positioning over the length of the cell rather than random pairing results in incompatibility. The more partition-competent plasmid will thus occupy the mid-cell position more frequently and is more likely to be inherited compared to the incompatible, more polar plasmid. A plasmid with a larger partition complex (larger centromere) is more likely to be positioned at the cell center while plasmids with smaller centromeres are more likely to be lost.

5. PLASMID MOBILISATION

Plasmid conjugation or mobilization plays an important role in horizontal gene transfer. It allows for plasmid spread to new hosts and allows them to penetrate new environments and adapt to newly encountered environments. A conjugation event can ensure and increase persistence of a bacterial cell within a population by invading hosts that have lost the plasmid (Lawley *et al*., 2004). Such conjugative systems consist of three components: the relaxosome, transferosome and the coupling protein (Lawley *et al*., 2004). The relaxosome consists of a protein complex, encoded by the Dtr (DNA transfer and replication) component which is involved in the processing of the DNA at the origin of transfer (*oriT*) and the transferosome is a Type IV secretion system (T4SS) encoded by the Mpf (mating-pair formation) component which is responsible for pilus assembly. These two systems are coupled by the coupling protein.

Transferable plasmids can be grouped as either conjugative (self-transmissible) or mobilizable. Self-transmissible plasmids encode the *oriT*, Dtr and Mpf elements required for self-transfer. Conjugative plasmids assist in the transfer of mobilizable plasmids, which lack the Mpf component. Self-transmissible plasmids are large (~40-500-kb) since they encode all the transfer components (~20-35-kb) required for their own transfer. In contrast, mobilizable plasmids tend to be smaller (<15-kb) since they encode only the *oriT* and Dtr component.

5.1 Relaxosome/ Dtr component

The relaxosome is a complex of proteins which together with the relaxase protein binds at the *cis*-acting *oriT* and forms part of the Dtr component. The proteins in the complex, in addition to the relaxase, likely serve as helpers for relaxase binding to the *oriT* or strand separation at the *oriT* (e.g. helicase). The proteins can also interact with the coupling protein, which is responsible for distributing a signal to the relaxase to stimulate origin nicking.

***oriT*.** The *oriT* region can be 38 – 500-bp in size and contain direct- and invert-repeats for binding of proteins which alter the DNA structure (TraK/IHF) to bring the *nic* site in contact with the relaxase and transport proteins (Lawley *et al*., 2004). The relaxase make a single stranded nick at this site to initiate transfer. The two ends are religated again after transfer. An inverted repeat located 5' to the *nic* site is required for termination of transfer (Gao *et al*., 1994).

Relaxase. Six classes or groups of relaxases or MOB families have been characterized, namely MOB(F), MOB(H), MOB(Q), MOB(C), MOB(P) and MOB(V) (Garcillán-Barcia *et al*., 2011). The relaxase DNA endonuclease catalyzes a single stranded nick at the *nic* site in the *oriT* to initiate the transfer process and is also responsible for sealing the nick after transfer. This process is similar to the rolling circle replication mechanism in which the Rep protein performs an almost similar function. After nicking of the *oriT* the relaxase remains covalently attached to the 5'-end of the single stranded DNA upon which a helicase enters to separate the strands. The relaxase is transferred together with the single stranded DNA into the recipient cell via the pilus and the linear DNA is

recircularized at the *nic* site. The plasmid exists as a single stranded circle in the recipient cell until complementary strand synthesis is initiated.

Primase. Although not part of the relaxosome, a plasmid encoded DNA primase often comprises part of the Dtr component of broad host-range plasmids. The nick at the *oriT* serves as a primer to complement the non-transferred strand in a manner similar to rolling circle replication (see section 2.1.3) and, therefore, a plasmid-encoded primase is not required in the donor cell during transfer. The plasmid-encoded primase is, however, transferred to and required in the recipient cell for replication of the single stranded DNA independent of the host primase. This independence from host-encoded functions contributes to the promiscuous nature and broad host-range of some plasmids. Primases of F, RP4 and I1 conjugation systems have been shown to be transported into a recipient cell (Rees and Wilkins., 1990, Wilkins and Thomas, 2000).

RP4 relaxosome formation. The Dtr region of RP4 is encoded by the Tra1 region. Three genes are required for relaxosome formation, these encode TraJ, TraI and TraH (+ TraK chaperone) as shown in figure 1.23. TraJ binds to a palindromic sequence upstream of the *nic* site in the *oriT*. This is followed by binding of the TraI relaxase which cleaves the *oriT* at the *nic* site and remains covalently attached to the 5' end of the single strand DNA. TraI, together with the covalently attached plasmid DNA binds to the TraG coupling protein. TraH is thought to stabilize the relaxosome by binding to TraI-TraJ-DNA complex. Binding of TraK at a position downstream to the *nic* site influences superhelicity at the *oriT* and positions the relaxosome so that an increased DNA amount can be captured in the nicked state.

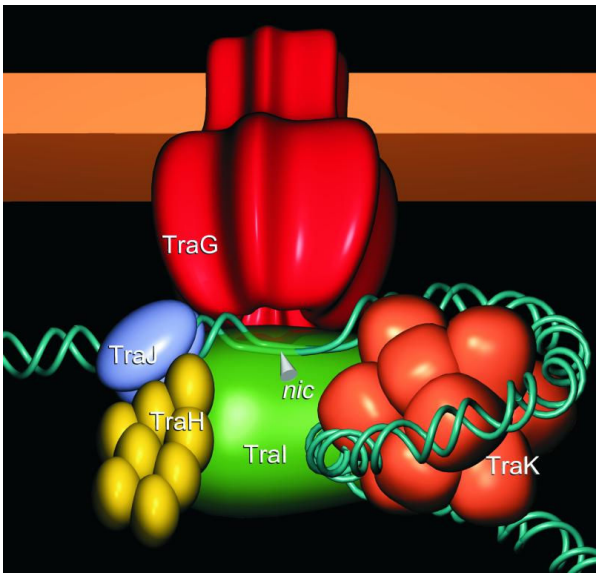


FIG. 1.23. Proposed structure and model for the RP4 relaxosome. See text for details. Schröder *et al*. (2002).

5.2 Transferosome/ Mpf system

The mating pair formation system mediates the contact between donor and recipient cells. Although the well-studied F plasmid is frequently used as the model for a self-transmissible plasmid, RP4 will be used in this description. The RP4 Tra2 region encodes all the components required for mating pair formation (Lessl *et al*., 1992) and consists of 16 ORFs (*trb*A – *trb*P).

Pilus. The conjugative pilus is randomly distributed on the cell surface as a thin filament and occurs at low numbers in general (Frost, 2009). The pilus diameter ranges between 6 – 11 nm, F-like (IncF, H, T, J) and P-like (IncP, N, W, I) pili are distinguished from each other with F-pili being long and flexible and the P-like pili short and rigid. The RP4 TrbC pilin subunit, encoded by *trb*C in the Tra2 region, is a 15 kDa prepilin polypeptide. It is processed three times to form a mature 7.5 kDa circular product with covalently linked N- and C-terminals. The linked terminals are cleaved by the actions of LepI (cleavage) and TraF, encoded in the Tra1 region, (removal of 4 amino acids at C-

terminus and pilin cyclization). TrbD-L, but not, TrbK are also required for assembly of the pilus.

Pore. A conjugation pore extends between the cytoplasmic membranes of the donor and recipient cells and is crucial for plasmid transfer to recipient cells. The structure is inserted into the outer membrane of a recipient cell and extends into the inner membrane so as to ensure that DNA is delivered to the cytoplasm. A competent recipient cell is therefore required and it has been shown, for example, that the presence of certain proteins in the recipient cell increases mobilization of RSF1010 by the Ti plasmid (Bohne *et al*., 1998).

Mating pair formation. The TrbB protein of RP4 is associated with the inner membrane as well as a soluble ATPase, (figure 1.24) (Krause *et al*., 2000a; Krause *et al*., 2000b). Binding of NTPs to TrbB stabilizes its conformation upon which it either acts as a chaperone for unfolded Mpf components or facilitates transfer of the nucleoprotein complex. TrbE is a NTPase associated with the inner membrane and is involved in the transport or positioning of other Mpf components and energizes the DNA transfer process.

TrbH is an outer membrane lipoprotein which helps with anchoring of the transmembrane complex in the outer membrane (Grahn *et al*., 2000; Harris *et al*., 2001). TrbK is a small inner membrane lipoprotein involved in entry exclusion. TrbN is a transglycosylase involved in lysis of the peptidoglycan cell wall so that other components of the Mpf can stretch across the cell membrane. It could also facilitate the passage of the nucleoprotein complex through this layer.

Coupling proteins. An element found common to all conjugative systems is the presence of a coupling protein and facilitates transfer across Type IV secretion systems (T4SS) (Lawley *et al*., 2004). These proteins are thought to use the energy from NTP hydrolysis to couple the relaxosome with the transferosome and pump the DNA, which is covalently attached to a relaxase, through the T4SS (Gomis-Ruth *et al*., 2002; Lanka and Wilkins, 1995).

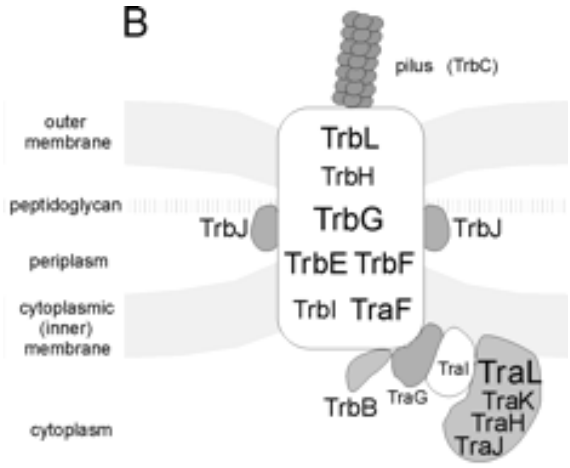


FIG. 1.24. The Mpf complex assembly of RP4 and the relaxosome coupled to the Mpf complex by TraG coupling protein. See text for details. From Grahn *et al*. (2000).

5.3 General mechanism of plasmid transfer with focus on RP4

The general mechanism of plasmid DNA transfer to a recipient cell is depicted in figure 1.25. The first step involves the formation of the relaxosome. The relaxosome is formed by the TraI relaxase which forms a complex with TraJ and the complex is stabilized by TraH (Frost, 2009). During the next (step 2, figure 1.25) the relaxase cleaves the *oriT* at the *nic* site and remains bound to the 5'-end. TraK causes bending of DNA at the *oriT* and forms a nucleosome-like structure for initiation of conjugative DNA replication (step 3, figure 1.25). No IHF is needed. The cleaved DNA is distributed to the TraG coupling protein by a TrbB ATPase located at the inner membrane (step 4, figure 1.25). DNA transfer occurs in a 5' to 3' direction and terminates after one round of transfer (step 5, figure 1.25). The invert repeat adjacent to the *nic* site of the *oriT* is required for termination during a religation reaction by the relaxase. Replication of the complementary DNA strands is mediated by DNA Pol III and is discontinuous in the recipient and in the donor cell it occurs either continuous from a free 3'-end in the donor or from an RNA primer. It has been shown that a primase protein (TraC encoded by Tra1), is transferred simultaneously with the DNA during transfer. This protein probably initiates DNA synthesis or replication in the recipient by forming a primer for replication initiation.

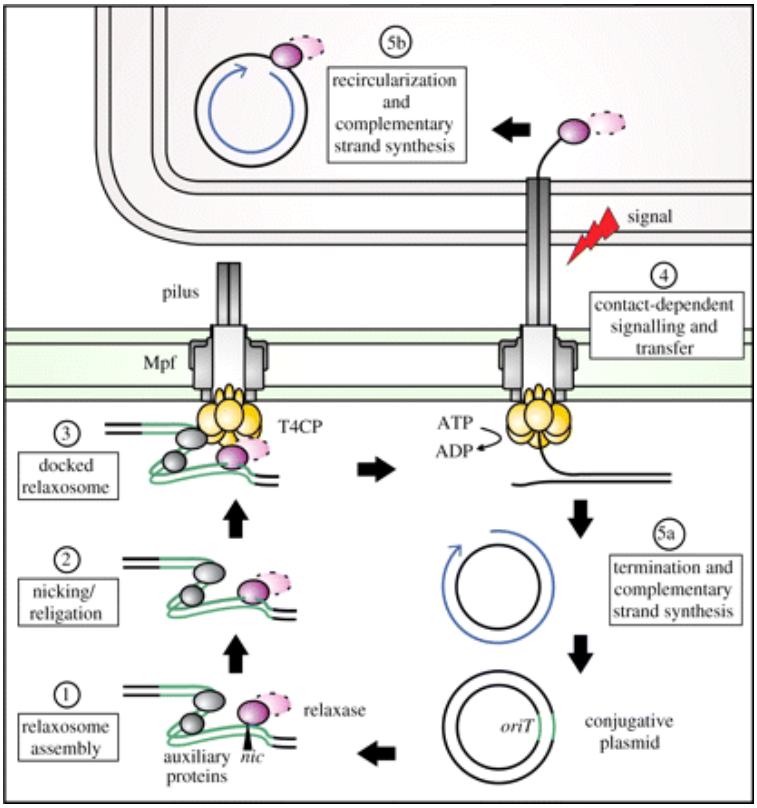


FIG. 1.25. General mechanism of plasmid transfer. T4CP (yellow); secretion substrate and DNA nicking relaxase (pink); some relaxases are fused to a helicase/primase to facilitate conjugation (dotted oval). See text for details. From Zechner *et al*. (2012).

5.4 Type IV Secretion Systems

Type IV Secretion Systems (T4SS) are transport systems of proteins and nucleic acids such as in conjugation and toxin secretion (e.g. Pertussis toxin). Different classification schemes are used based on their function, the conjugative plasmid incompatibility group they represent and an alternative classification scheme (see below). T4SS have been subdivided into four classes (Lawley *et al*., 2004; Juhas *et al*., 2007). These include the P-family, F-familiy, I-family and GI-family, where the first three are representative of the respective incompatibility groups of the conjugative plasmids they represent and the fourth group is the GI-family associated with genomic-islands. In the alternative classification scheme the original F- and P-families are grouped as Type IVA and the I-family belongs to the Type IVB system (Christie *et al*., 2005; Juhas *et al*., 2007). The GI-

family is classified as Type IVC in this alternative scheme (Zhang *et al*., 2012). Based on function these systems can be grouped into three, namely conjugation-, effector protein translocation- and contact independent-secretion systems (Zechner *et al*., 2012).

The VirB-D4 type system of *Agrobacterium tumefaciens* is the prototype of Type IVA T4SS (F-family and P-family). The T-DNA transfer system of *A. tumefaciens*, together with the plasmid transfer systems of F, RP4 and R388 are well-studied examples and all are grouped as Type IVA T4SS. Plasmids encoding conjugative systems belonging to this group of T4SS have homologues to most of the components of the VirB-D4 system as shown in Table 1.1 and figure 1.26.

TABLE 1.1. Protein homologies between conjugative T4SS

Function	**VirB/D4**	**IncPα (RP4)**	**IncF**	**IncW (R388)**
Coupling protein	VirD4	TraG	TraD	TrwB
Relaxase	VirD2	TraI	TraI	TrwC
Lysozyme/SLT	VirB1	TrbN	P19[A]/ORF169	TrwN[B]
Pilin	VirB2	TrbC	TraA	TrwL[B]
Pore, pilus assembly	VirB3	TrbD	TraL	TrwM[B]
Pilus assembly, ATPase	VirB4	TrbE	TraC	TrwK
Pore, pilus assembly	VirB5	TrbF[A]	TraE	TrwJ
Pore, pilus assembly, Mps	VirB6	TrbL	TraG	TrwI
Pore, lipoprotein	VirB7	TrbH	TraV	TrwH
Pore	VirB8	TrbF		TrwG
Pore (secretin)	VirB9	TrbG	TraK	TrwF
Pore (TonB-like)	VirB10	TrbI	TraB	TrwE
Secretion, Transport ATPase	VirB11	TrbB		TrwD
Pilin cyclase		TraF		
Acetylase		TrbP	TraX	

Lawley *et al*. (2004); A. From Frost (2009); B. From Schröder and Lanka (2005).

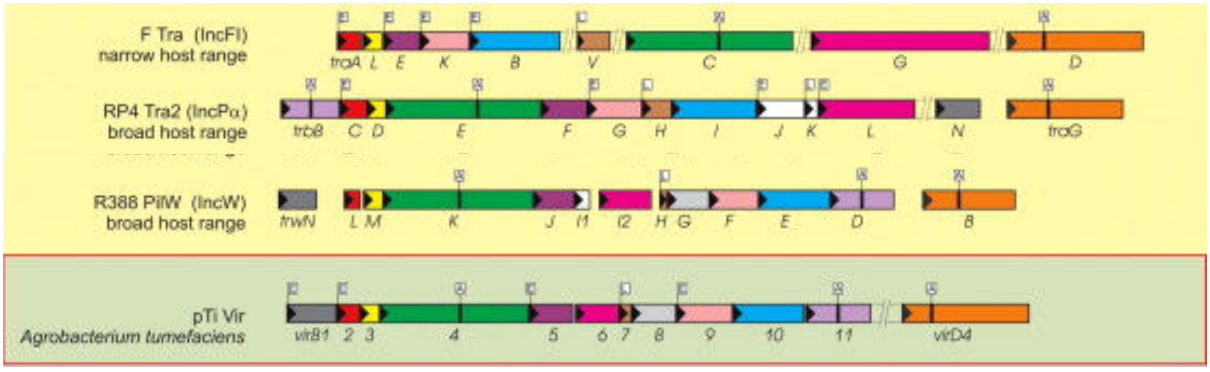


FIG. 1.26. Protein homologies between the T4SS of F, RP4, R388 and pTi. Homologous genes are shown in identical colours. Schröder and Lanka 2005

6. AIMS OF THIS STUDY

The 14-kb mobilizable plasmids p31T1 and p36T2 are related plasmids since they were shown to have identical restriction profiles (Marx, MSc thesis). They were isolated from *Aeromonas sobria* and *Aeromonas hydrophila*, respectively, and reported to carry erythromycin, nalidixic acid and tetracycline resistance. A tetracycline resistance transposon Tn*1721* was identified by means of Southern hybridization. Furthermore they are able to autonomously replicate in an *Escherichia coli polA*$^-$ mutant strain suggesting the ability to replicate in the absence of DNA Polymerase I. Southern hybridizations with the *repB* gene of plasmid RSF1010 as a probe and *repC* gene of pRAS3.1 were used to classify these plasmids as possible IncQ-like. Positive hybridization signals were obtained for the *repC* probe but none for *repB*. To further investigate the possibility of IncQ-related plasmids, probes for the three-*mob* system (*mobABC*) of RSF1010 (IncQ) and five-*mob* system (*mobABCDE*) of pTC-F14 (IncQ) was designed but no positive hybridization signals were obtained. The transfer frequency of p31T1 and p36T2 was low and measured to 2.21 x 10^{-5} and 3.59 x 10^{-2} transconjugants per donor respectively, obtained over a 16 h mating period, which was much lower than for pRAS3.1. These results suggest a novel mobilisation system. Nothing is known further regarding the replication system or maintenance ability of these plasmids.

The current study therefore focussed on plasmid p31T1 which was further investigated with regard to:

1) the possible assignment of putative functions to open reading frames (ORFs) based on sequence analysis,
2) the determination of putative functions of replication associated ORFs and their regulation by means of biological analysis
3) copy number
4) the stable maintenance of p31T1
5) the possible identification of an origin of transfer (*oriT*) required for mobilization.

Chapter 2

General Features and Biology of p31T1

Chapter 2: General Features and Biology of p31T1

2.1 INTRODUCTION

Plasmids can be studied from a fundamental and applied point of view. The plasmid biologist would focus on replication, maintenance and mobilization systems and contribute to the overall in depth understanding of the functioning of such systems whereas a clinical and environmental biologist would be most likely interested in the accessory components and how they function in the environment, medical and industrial settings. Plasmids are categorized based on the sequence similarity, genetic organization and phenotypic properties of their replicon (Fernandéz Lopez, 2006) and can be further characterized based on several properties such as their size, modes of replication and transmission, host ranges and accessory genes.

Plasmids are widely spread among *Aeromonas* species and one investigation showed the presence of plasmids to be present within 11% and 40% of environmental *A. hydrophila* and *Aeromonas veronii* biovar *sobria* isolates, respectively (Brown *et al*., 1997). The characterization of plasmids from *Aeromonas* species in aquaculture systems is important both from a fundamental and applied view. The fish from these aquacultures are aimed for human consumption and the spread of plasmids encoding resistance to antibiotics could have serious implications in potential infectious disease outbreaks. It would, therefore, be beneficial to understand the molecular biology of such plasmids. This would include the mechanism of replication, stability and mode of spread. Several studies have focused on the prevalence of plasmids within *Aeromonas* species but few studies have examined the plasmids in more depth with regard to replication and mobilization (Casas *et al*., 2005; L'Abée-Lund and Sørum, 2002; Rhodes *et al*., 2000; Rhodes *et al*., 2004; Sørum *et al*., 2003, Boyd *et al*., 2003; Loftie-Eaton and Rawlings, 2009; Loftie-Eaton and Rawlings, 2010).

Two tetracycline resistant *Aeromonas* strains, namely *Aeromonas sobria* and *Aeromonas hydrophila* strains, which are known opportunistic fish pathogens (Thune *et al*., 1993; Austin and Adams, 1996), were isolated from Tilapia fish in South Africa. The

tetracycline resistance phenotype was found to be associated with two novel plasmids, p31T1 and p36T2, from *A. sobria* and *A. hydrophila,* respectively (Marx, MSc thesis). These plasmids were found to contain a Tn*1721* associated *tetA* gene. Tetracycline resistance genes are often found in *Aeromonas* species for example Rhodes *et al*. (2000) identified a *tetA* gene in 58.8% of plasmids found in *Aeromonas* species.

Plasmids p31T1 and p36T2 are similar in that both are 14-kb in size and have identical restriction profiles. Furthermore they were shown to be mobilizable and before this study nothing was known regarding their replication abilities. The aim of the work reported in this chapter was therefore to characterize p31T1 based on sequence analysis as well as host range, copy number, stability and mobilization.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial strains, media and growth conditions. Bacterial strains used are described in the table in Addendum C. *Agrobacterium tumefaciens* and *Pseudomonas putida* were grown in LB media at 30°C supplemented with antibiotics as required in the following concentrations, tetracycline (30 µg/ml) and kanamycin (50 µg/ml). *E. coli* was grown in LB media at 37°C and antibiotics added in the following concentrations tetracycline (10 µg/ml), kanamycin (50 µg/ml), ampicillin (100 µg/ml), chloramphenicol (20 µg/ml).

2.2.2. DNA techniques, sequencing and analysis. Plasmid miniprep extractions were performed using standard methods (Sambrook *et al*., 1989; Ausubel *et al*., 1993) Plasmids were isolated using the alkaline lysis method, Pure Yield™ Plasmid Miniprep System (Promega) or Nucleobond® AX (Macherey-Nagel) kit. Sequencing was performed using an ABI PRISM^{TM} 377 automated DNA sequencer according to the dideoxy chain termination method (Sanger and Coulson, 1975). The p31T1 sequence was analysed using DNAMAN (Lynnon Biosoft), BLAST (http://www.ncbi.nlm.nih.gov), Glimmer3 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi), VectorNTI (Invitrogen) and DNADynamo (BlueTractorSoftware Ltd.).

2.2.3. PCR amplification. Amplification was carried out in an Eppendorf Mastercycler® personal PCR cycler and cycle parameters were kept standard at 95°C 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s and extension at 72°C. The final two steps were carried out at 72°C for 5 min and a hold step at 22°C. The variable annealing temperatures and extension times were dependent on the primers used and the amplicon size and were adapted accordingly. Primer sets used are described in the table in Addendum D.

2.2.4. *Agrobacterium tumefaciens* electroporation. Electrocompetent cells and electroporation procedures were prepared according to the method of Weigel *et al*. (2006).

2.2.5. *Pseudomonas putida* electroporation. Electroporation of *P. putida* was carried out according to the method described by Iwasaki *et al*. (1994).

2.2.6. Absolute copy number determination using Real-Time qPCR. Whole genome extractions of cells containing the respective test plasmid constructs were performed using the QIAmp DNA Mini Kit (QIAGEN). These *E. coli* cultures were grown overnight at 37°C and the following morning diluted 1:100 into fresh LB broth and grown at 37°C until the OD_{600} reached ~0.8. DNA extractions were performed using the QIAmp DNA Mini Kit in which the DNA of 2 ml of culture was purified. The total DNA was eluted by running 30 µl of the elution buffer twice over the column so as to achieve a final volume of 60 µl.

Realtime qPCR was performed using a similar procedure to that of Lee *et al*. (2006). The p31T1 plasmid and chromosomal D-1-deoxyxylulose 5-phosphate synthase gene (*dxs*) were amplified using the Minrep31T1 and *dxs* primer sets, respectively. A StepOne Plus cycler system (Applied Biosystems) and Kapa Universal FAST SYBR Green *Taq* (Kapa) was used. Reaction conditions and cycle parameters were as follows, an initial hold stage at 95°C for 20s followed by a 40 cycle cycling stage of 95°C at 3 s, and 54°C at 30 s. Following amplification a melt curve stage at an initial 95°C for 15 s was followed by increments of 0.3°C with a hold step of 60 s at each step from 60°C to 95°C and maintained at 95°C for 15 s.

Plasmid copy number was calculated as the number of plasmids per chromosome as described by Lee *et al*. (2006). In order to obtain the copy number of p31T1, it was required to generate standard curves from which the concentration of plasmid or chromosome DNA could be extrapolated. Plasmid (p31T1) and chromosome specific templates were required. For the chromosomal template the *E. coli dxs* gene was amplified using the *dxs* primer set and cloned into pGem®-T Easy (Promega) to generate pGemdxs. Plasmids were extracted by harvesting 2 ml of an overnight culture at 37°C with further preparation using the Wizard prep or Pure Yield Miniprep kits (Promega). The final elution volumes were 100 µl. Serial dilutions were prepared of the plasmid preparations using 0.8 ng as a starting concentration and diluting 10x up to 10^{-5}. From each dilution 5 µl were added into the SYBR Green reaction mix. The amount of molecules added was determined by the equation used by Lee *et al*. (2006) which was derived from Whelan *et al*. (2003):

$$DNA\ (copy) = \frac{6.02x10^{23}\ (copy/mol)\,x\ DNA\ amount\ (g)}{DNA\ length\ \ (dp)\,x\ 660\ (g/mol/dp)}$$

The PCR efficiency was calculated using the equation of Rasmussen (2001):

$$E = 10^{-1/slope} - 1$$

2.2.7. Stability Assays. Single *E. coli* DH5α colonies containing the p31T1 plasmid were inoculated into 5 ml LB broth with antibiotic selection and grown overnight at 30°C. The next day and following every 24 h for an additional 6 d, ~1000 cells were transferred into fresh LB broth without antibiotic selection. Serial dilutions of the respective cultures were made in PBS and spread onto LB agar with and without antibiotic selection. These dilutions were also used initially to determine the volume of

cells needed to be transferred into fresh media. Plate counts were recorded the following day after which 50 colonies from the non-selective plates were replicaplated onto the selective and non-selective media respectively. The percentage plasmid retention was recorded for every ~20 generations. The number of generations per day was calculated using the following formula:

$$Number\ of\ generations\ per\ day = \left(\frac{log10(CFUf) - Log10(CFUi)}{0.301}\right)$$

$CFUi = CFU/ml\ at\ beginning\ of\ 24h\ growth\ cycle$

$CFUf = CFU/ml\ at\ end\ of\ 24h\ growth\ cycle$

2.2.8. Mating assays. *E. coli* strains S17.1 and either ACSH50I^{q} or DH5α served as donor and recipient strains respectively. *E. coli* S17.1 donor transformed with the respective plasmids and recipient strains were inoculated into 5 ml LB broth with appropriate antibiotic selection and incubated overnight at 37°C. Untransformed *E. coli* S17.1 was also inoculated, since this strain together with the recipient strains served as negative controls in the experiment. Cells (2 ml) were harvested the next morning using a benchtop centrifuge at 8000 rpm for 2 min and were then washed three times in 1x PBS and finally resuspended in 1 ml PBS. The resuspended cells were diluted 1:9 and the absorbance was measured at OD_{600}. The absorbance values were standardized to 1 from the original suspension. 100 µl of a 1:10 donor-to-recipient mixture was spotted onto a 0.2 µm mating filter (Supor®-200, Pall Corporation) which was placed onto a LA plate. Donors and recipients were allowed to mate for 16 h at 37°C after which the filters were removed and placed into 10 ml PBS and vortexed to suspend the cells. From the suspension, 8 ml of cells were collected by centrifugation and resuspended in 1 ml PBS. Serial dilutions were plated onto donor- and recipient-selective LA plates, incubated overnight at 37°C and the amount of transconjugants per donor was calculated. Minipreps of transconjugants and restriction enzyme digestion with *Kpn*I confirmed the presence of p31T1.

2.3. RESULTS

2.3.1. Sequence analysis of p31T1. Sequence analysis of p31T1 (Fig. 2.1) using a combination of Glimmer 3, DNAMAN (Lynnon Biosoft) and Vector NTI (Invitrogen), revealed 18 putative open reading frames (ORFs). The full sequence with annotation is reported in Addendum A. Putative functions were assigned based on BLAST (http://www.ncbi.nlm.nih.gov) analysis for all ORFs except four. The functions and descriptions of the putative ORFs of p31T1 are shown in the table in Addendum B. A short discussion of some of the important ORFs will follow in this section.

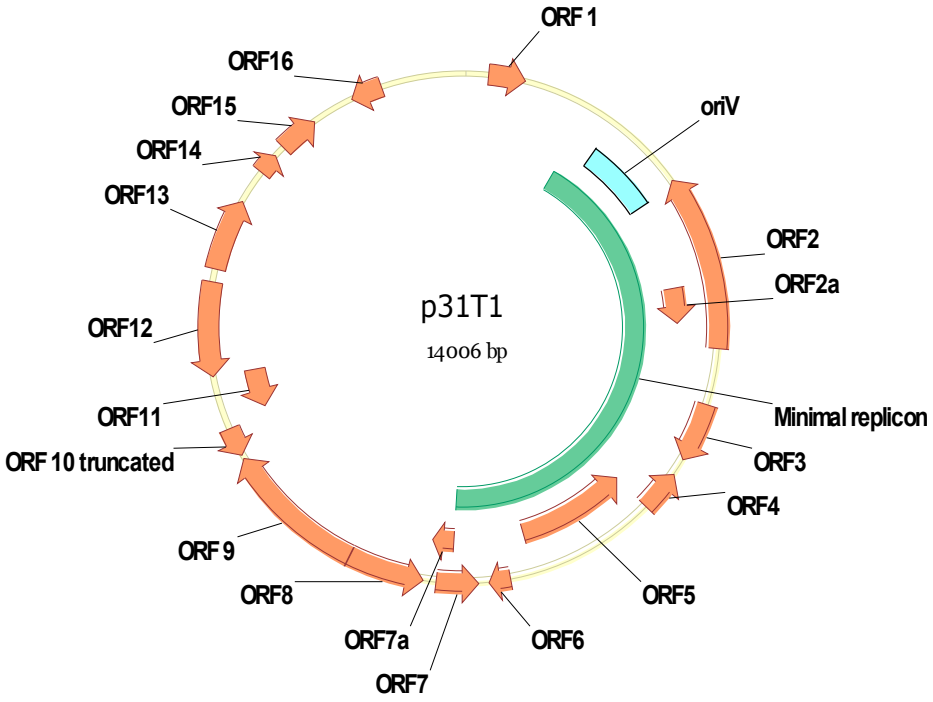


FIG. 2.1. Plasmid map of p31T1 depicting the 17 putative ORFs. The minimal replicon region is indicated in green and the origin of replication in light blue.

ORF2 is a putative primase (38% identity over 89% protein coverage) overlapped by a smaller ORF2a which is transcribed in the opposite direction. This smaller ORF2a contains a putative mobilization region spanning 59 amino acids (aa) of the 123 aa ORF. ORF3 shows homology to the *copG* family of regulators (34% identity over 92%

protein coverage) which act as repressors and control plasmid copy number. They contain a ribbon-helix-helix (RHH) domain involved in dimerization and DNA-binding.

ORF5 is a putative *traC*-like primase (46% identity over 98% protein coverage), but also has similarity to an AAA+ ATPase (44% identity over 98% protein coverage), thus suggesting a possible role in mobilization. Three other ORFs have also been identified with a possible role in mobilization, namely ORF2a, ORF6 and ORF16. As previously mentioned ORF2a contains a putative MobA/MobL domain (30% identity over 82% protein coverage), ORF6 is a putative relaxase/Mob protein (35% identity over 65% protein coverage) and ORF16 gave a strong hit to MobC (78% identity over 98% protein coverage). A relaxase and an *oriT* is an absolute requirement for mobilization, whereas MobC acts only as a helper element to improve mobilization. It is known that AAA+ ATPases play a role in initiation of replication in bacteria (Duderstadt and Berger, 2008). The bacterial DnaA replication initiator, for example, has a domain III region which comprises an AAA+ module. Whether ORF5 is involved in initiation of replication or mobilization remains to be determined.

Positive hybridization signals with *tetA* and Tn*1721* probes were obtained for both p31T1 and p36T2 (Marx, MSc thesis). The plasmid sequence later confirmed the presence of the antibiotic resistance genes *tetR* and *tetA* and was similar to that of pRAS3 (L'Abée-Lund and Sørum, 2002). The transposon element on p31T1 was further confirmed by the identification of a TnpR-like resolvase (ORF13) with 66% identity over 94% protein coverage belonging to the serine-recombinase superfamily which is similar to Tn*3*. The resolvase is likely required to catalyze site-specific recombination during transposition. Furthermore a site-specific recombinase (ORF7) was also identified (100% identity) along with an N-terminal truncated *pecM*-like gene (ORF10). PecM (*pecM* gene product) is known to be associated with some transposons belonging to the Tn*1712* family (Pasquali *et al*., 2005). Tn*3* and Tn*1721* belong to the Tn*3* family of transposons (Sherratt, 1989). It was shown that Tn*3* encodes a site-specific recombination system independent of *tnpA* but requiring *tnpR*, further substantiating the results (Kostriken *et al*., 1981). Transpositioning of Tn*3* and of members of the Tn*3* family generate target duplications of 5-bp and contain 38-bp inverted repeats flanking

the transposon, however no such repeats could be identified on p31T1. The alignment of the 2500-bp segment of p31T1 with regions associated with transposon Tn*1721* and the tetracycline resistance genes are depicted in figure 2.2. The *tetA* and *tetR* gene of p31T1 shows 99% sequence identity to those of Tn*1721* (bottom figure 2.2). Alignment of the 2500-bp p31T1 sequence with sequences of *Aeromonas allosaccharophila* and *E. coli* O104:H4 showed the presence of an N-terminal truncated *pecM*-like gene and an N-terminal truncated *tnpA* gene (top and middle figures 2.2).

FIG.2.2. Nucleotide BLAST results of the whole p31T1 sequence aligned to the database. Top figure represents the alignment with *Aeromonas allosaccharophila* and the representative ORFS which align with the p31T1 sequence (grey bar) spanning positions 7253 – 9755 of p31T1. This segment includes the truncated *pecM*, *tetA*, *tetR* and truncated *tnpA* genes. Middle figure: *E. coli* O104:H4 strain and the ORFs which align with a segment of the p31T1 sequence (grey bar). The 2500 bp aligned segment of p31T1 extends from positions 7253 to 9755 on p31T1. This segment has 99% identity to a truncated transporter permease protein (O3K_00375), tetracycline efflux protein (O3K_00380), tetracycline repressor protein (O3K_00385) and truncated transposase (O3K_390). Bottom figure shows the alignment of the 2500 bp p31T1 segment (grey bar) with Tn*1721*, corresponding to the *tetA* and *tetR* gene regions of Tn*1721*.

2.3.2. The host range of p31T1 is not limited to *Aeromonas*. Plasmid p31T1 was isolated from *Aeromonas sobria* and it has also been shown to propagate in *E. coli* (Marx Thesis). To further investigate the host range of p31T1 it was transformed into *A. tumefaciens* (α-proteobacteria) and *P. putida* (γ-proteobacteria) by means of electroporation. The broad host-range IncQ plasmid RSF1010K was used as a positive control in these experiments since it was previously shown to replicate in these organisms (Bohne *et al*., 1998; Nagahari and Sakaguchi, 1978). RSF1010K was successfully transferred into *A. tumefaciens*, however, no transformants were observed on selective plates for p31T1. In contrast, p31T1 could be transferred into *P. putida* and its ability to exist as an extrachromosomal unit was confirmed by plasmid extraction.

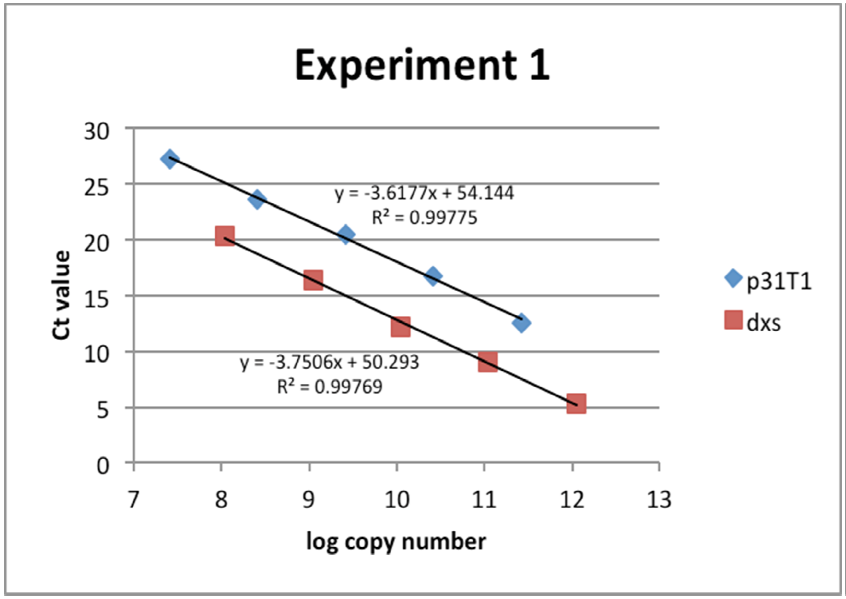
2.3.3. Copy number of p31T1. The copy number of p31T1 was determined by Real-Time qPCR in separate experiments to be ~3 plasmids per chromosome. This was done by estimating the number of molecules (x-value) of plasmids and chromosomes within total DNA preparation from a standard curve using the determined Ct values (y-value) (fig. 2.3). The standard curves consisted of ten-fold serial dilutions starting at 2.6 x 10^{14} and 1.1 x 10^{15} molecules per reaction for p31T1 and pGemdxs, respectively. The amplification efficiencies and number of plasmid and chromosome molecules per reaction were calculated as described by Lee *et al*. (2006) and are summarized in the table 2.1.

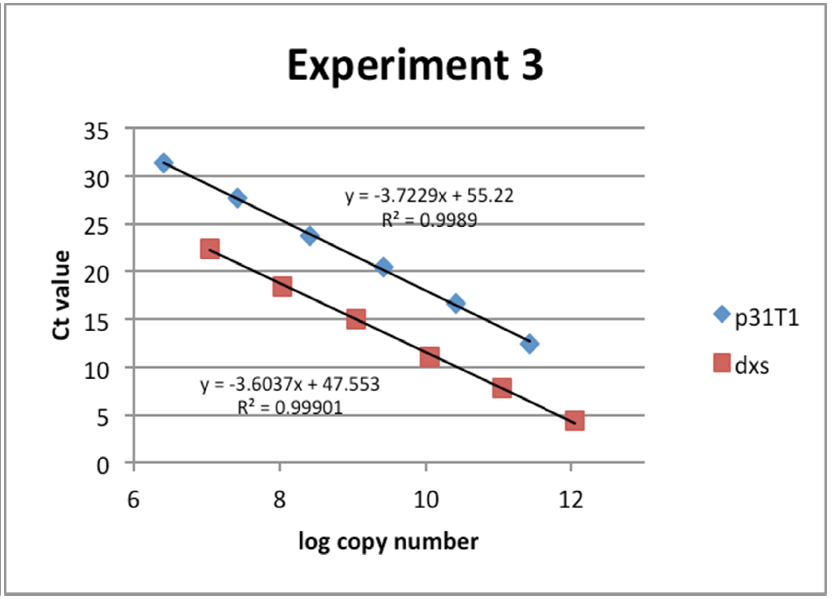
TABLE 2.1. Absolute quantification results as determined by real-time qPCR

	Efficiency		Ct value		Copies		Plasmids/ Chromosome
	p31T1	dxs	p31T1	dxs	p31T1	dxs	
Experiment 1	82%	85%	20.71 ± 0.26	17.35 ± 0.18	$1.76 \times 10^{9} \pm 3.01 \times 10^{8}$	$6.07 \times 10^{8} \pm 6.49 \times 10^{7}$	2.90
Experiment 2	89%	85%	22.81 ± 0.35	19.46 ± 0.28	$2.04 \times 10^{9} \pm 4.22 \times 10^{8}$	$6.52 \times 10^{8} \pm 1.16 \times 10^{8}$	3.12
Experiment 3	86%	89%	20.71 ± 0.21	15.86 ± 0.19	$1.86 \times 10^{9} \pm 2.47 \times 10^{8}$	$6.16 \times 10^{8} \pm 7.55 \times 10^{7}$	3.27

Amplification efficiencies should ideally be between 90-100% (-3.6 > slope > -3.1). Although the efficiencies in these experiments were slightly less than 90%, the amplification still produced a linear standard curve (with R^2 values shown on the graphs in figure 2.3) High R^2 values gave confidence in the fit of the data. A higher efficiency

would likely not have had a marked influence on copy number as slight variations in efficiency would still result in a copy number calculation of ~3 plasmids per chromosome.





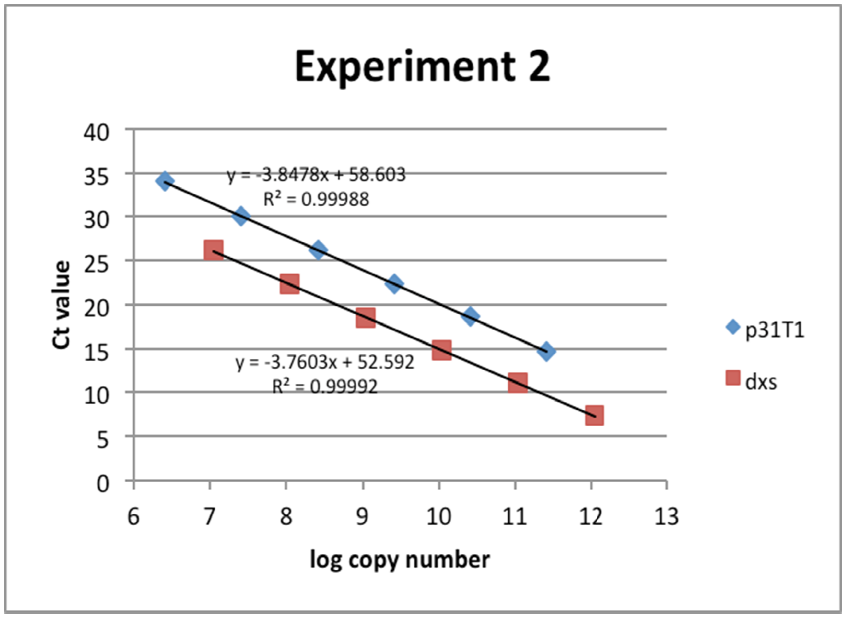


FIG.2.3. Standard curves for three separate experiments in plasmid copy number determination by absolute quantification. In experiment 1 five standard dilution points were used, but in experiment 2 and 3 an extra dilution point was included as this had an increased efficiency impact as seen in Table 2.1.

2.3.4. p31T1 segregational stability. After 127 generations plasmid p31T1 was maintained at 81% plasmid retention as depicted in the graph in figure 2.4.

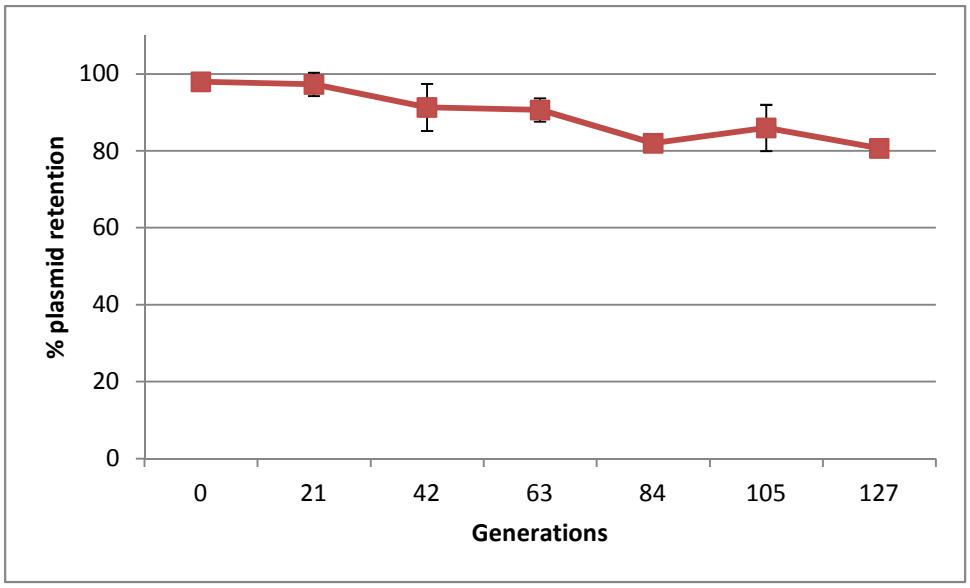


FIG. 2.4. Plasmid stability of p31T1 over a 7 day period representing 127 generations. Three samples were tested in triplicate with a confidence interval of 95% for standard deviation determinations.

2.3.5. p31T1 is mobilized by an IncP-1 conjugative system. Plasmid p31T1 was found to be mobilized by the RP4 conjugative system which has been integrated onto the chromosome of a streptomycin resistant *E. coli* S17.1 donor strain. Chloramphenicol resistant *E. coli* ACSH50I^{q} and nalidixic acid resistant DH5α strains were used as recipients and transconjugants were selected on agar plates containing either chloramphenicol or nalidixic acid (strain-selective) in combination with tetracycline (plasmid-selective). When *E. coli* DH5α was used as recipient no transconjugants were obtained, however, p31T1 was transferred at a frequency of 5.383 (± 2.28) x 10^{-5} transconjugants per donor when *E. coli* ACSH50I^{q} was the recipient. The presence of p31T1 in these transconjugants was confirmed by plasmid extraction and restriction profiling (figure 2.5).

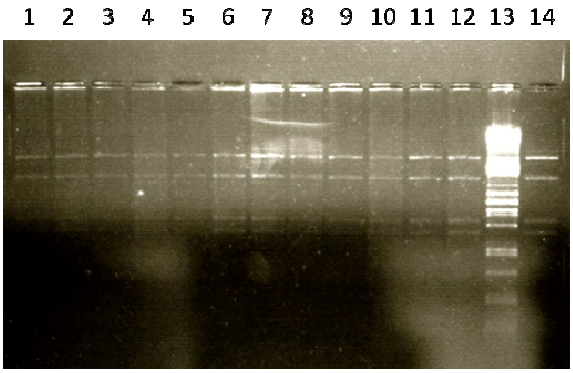


FIG. 2.5. Plasmid extractions of p31T1 from transconjugants. Three matings were performed of which 4 colonies were picked and plasmid DNA extracted. Plasmid preparations were evaluated by restriction profiling using *Kpn*I (lanes 1-12). In addition p31T1 DNA was digested with *Kpn*I to serve as a control (lane 14). Lambda DNA digested with *Pst*I served as a marker (lane 13).

2.4. DISCUSSION

From the 18 putative identified ORFs, only 13 could be assigned putative functions based on BLAST analysis. p31T1 is a medium-sized low copy number plasmid although a higher copy number would have been expected as being more typical of a plasmid of this size. Maintenance at such a low copy number requires tight regulation both at copy number and segregational level. The presence of a putative resolvase and site-specific recombinase is suggestive of a multimer resolution system which could play an important role in maintenance. No other stability system with clear similarity to previously reported systems could be identified on p31T1 based on sequence analysis. ORF3 however aligned with a hypothetical protein with a RHH region of the *copG* family of protein repressors. CopG is the prototype for the family of Cop repressors. It acts as a transcriptional repressor and mutations outside the C-terminal domain has negative influence on the global structure and leads to decreased half-life of the altered CopG protein (Acebo *et al*., 1998).

Transposition events require the catalytic activities encoded by transposase and resolvase genes and occur either by a copy-and-paste mechanism (replicative) or a cut-and-paste mechanism (non-replicative). The presence of a *tetAR* tetracycline

resistance operon, a truncated *pecM*-like gene and truncated *tnpA*, which are associated with the 11.1-kb transposon Tn*1721* suggests that these genes were acquired during a transposon event. ORF7 which partly overlaps with the truncated *tnpA* gene (ORF7a) is a putative site-specific recombinase (100% identity covering 21% of a 126 amino acid protein). Site-specific recombinases fall into one of two categories, either serine- or tyrosine-recombinases (Grindley *et al*., 2006). They differ based on the specific amino acid they use to employ a nucleophilic attack on the specific target DNA site. The *tetA* and *tetR* genes encode for a membrane associated efflux protein and repressor protein which regulates the action of TetA respectively. PecM is known to regulate the synthesis of virulence factor in *Erwinia chrysanthemi* (Pasquali *et al*., 2005) and is truncated at the N-terminal in p31T1, therefore it may play no functional role. Tn*1721* is a member of the Tn*3* transposon family which follows replicative transpositioning (Sherratt, 1989). The transposase catalyses recombination between a donor plasmid carrying the transposon and a target plasmid by forming a donor-target plasmid cointegrate. The cointegrate structure is resolved by the action of the resolvase protein. A putative *tnpR* resolvase gene (ORF13) could be identified and belongs to the serine-recombinase family. This gene however is located distantly from the Tn*1721*-associated genes (1424-bp downstream of the truncated *pecM* gene). The N-terminal domain of such enzymes carry a catalytic domain and a small HTH binding domain is present further downstream. Transposon Tn*1721* is known to be flanked by 38-bp inverted repeats. Upon transpositioning into a target site, the transposon becomes flanked by 5-bp direct repeat duplications of the target site. No such inverted repeats or direct repeats could be identified in p31T1. Furthermore, in this case the transposase is truncated by 8 amino acids at the N-terminal and it is thus unclear how such a transposition event could have taken place in p31T1.

Four putative ORFs were identified to be possibly involved in mobilization. They are not located in close proximity to one another and the identification of a possible mobilization region based on sequence analysis was therefore difficult and inconclusive. ORF2a gave a hit to a putative plasmid mobilization protein with 30% identity over 82% protein coverage. From the the total 123 amino acids for this protein, a MobA/MobL region was identified covering only 59 amino acids. MobA is from the *E. coli* RSF1010 plasmid and

MobL from the *Acidithiobacillus ferrooxidans* plasmid pTF1 and are relaxases with site-specific DNA strand transferase activity that cleave at *oriT* sites (Scherzinger *et al*., 1993; Drolet and Lau, 1992; Zatyka and Thomas, 1998). A smaller ORF6 (72 amino acids), showed a 35% identity over 65% protein coverage, to a putative relaxase/mobilization protein. The true roles for ORF2a and ORF6 are questionable, since ORF6 is very small and both ORFs generate very low BLAST scores. A more reliable BLAST alignment was obtained for ORF16, which aligned to putative MobC proteins (78% over 98% protein coverage). It is interesting to note that Marx (MSc thesis), did not obtain any positive hybridization signals for a three MobABC or five MobABCDE system from RSF1010 and pTC-F14, respectively. However sequence analysis in the present study show a strong positive hit for a putative MobC protein. The MobC helps MobA with strand separation at the *oriT* and thus improves the nicking activity of MobA. In the absence of MobC, plasmid R1162 (similar to RSF1010) is mobilized at a much lower frequency (Brasch and Meyer, 1986), indicating that MobC acts as a helper element. Another element although not essential to mobilization is the occurrence of a transfer-associated primase. The IncI and IncP groups have been shown to transfer primases between *E. coli* strains and these are referred to as TraC proteins (Miele *et al*., 1991). Plasmid RP4 (IncP-1) has two forms of *traC* from different start sites (Lanka *et al*., 1984), a 116-kDa and 81-kDa counterpart. The C-terminal domain is likely to encode for the primase domain. ORF5 gave a BLAST hit to a hypothetical protein from a plasmid isolated from *Klebsiella pneumoniae* (46% over 98% protein coverage) which contains a TOPRIM region (topoisomerase primase domain) which are characteristic of DnaG and *traC*-like primases. Its role as a putative primase is not exclusive since sequence similarity towards an AAA ATPase, which could be involved in replication initiation, was also found (44% identity over 98% protein coverage). A putative primase from *Citrobacter* aligned with 38% identity over 89% coverage of the ORF2 protein.

The low mating frequency of p31T1 could be explained by the RP4 conjugative system not being the optimal system for effective mating of this plasmid. A high mating frequency would be required to eliminate plasmid loss at a population level, but this plasmid still seems able to maintain the ability to establish within a population if required

in spite of its low mating frequency. The mobilization potential in conjunction with a stability system ensures that this plasmid be maintained on an intercellular and intracellular level respectively. Active stability systems and copy number control on a genetic or cellular level is more important than maintenance of a plasmid through transfer since the absence of such systems would lead to the absolute loss of a plasmid which in turn would then not even allow for transfer to progress. Since p31T1 was shown to have a low copy number and can be maintained over several generations in the absence of selection it is evident that this plasmid is capable of maintaining on cellular and population level. It furthermore suggests the presence of an active stability system since random segregation would not be sufficient for such a low copy number plasmid and this is investigated in chapter 3. A putative *oriT* site could not be identified through sequence analysis and an attempt to screen for an *oriT* bank was also unsuccessful.

p31T1 is able to establish itself within a bacterial population since we showed that it was able to transfer by means of conjugation. It needs to encode for its own maintenance systems in order to establish and persist in other bacterial strains especially at such a low copy number and to ensure intracellular stability. Host range experiments showed that p31T1 could indeed replicate in *E. coli* and *P. putida* aside from its original host *Aeromonas*. Based on this limited sample, it seems capable of replication in the γ-proteobacteria but not in the single α-proteobacterium tested.

BLAST analysis of ORFs encoded on p31T1 did not reveal any characteristic ORF homology to proteins involved in replication apart from a putative RepA-like protein (ORF1) and a CopG-like ORF3. The gene encoding the RepA-like protein however is located outside the identified minimal replicon (see chapter 3). It seems, therefore, that plasmid p31T1 has a novel replication control circuitry and as replication proteins are usually well conserved this finding could indicate a new replication system, which is further explored in chapter 3.

Chapter 3

Characterisation and Analysis of the p31T1 Replication Region

Chapter 3: Characterisation and analysis of the p31T1 replication region

3.1. INTRODUCTION

An essential part of plasmid persistence is its ability to replicate and be stably maintained in one or more hosts. Stringent control of these functions is crucial to ensure that a plasmid is not lost. A basic or minimal replicon is identified as the smallest part of a plasmid able to undergo autonomous replication and be stably maintained. This minimal replicon typically carries three distinct traits, namely an *oriV*, a replication initiator protein and functions to regulate plasmid copy number (del Solar *et al*., 1998).

Minimal replicons are usually constructed by cloning partial DNA fragments of the plasmid into a vector, its function is tested by transforming it into a host within which the vector replicon is unable to replicate. Replication of the construct is, therefore, dependent upon the function of the minimal replicon derived from the plasmid. The minimal replicon is subsequently sequenced and analyzed for sequence homology.

Each plasmid has a characteristic copy number, though it can differ between different hosts. It is important that a plasmid maintain its copy number so as to prevent loss due to runaway replication or failed stability systems. Although high copy number plasmids usually rely on random distribution for stable inheritance during vegetative growth of the host, lower copy number plasmids require stability systems to ensure stable segregation to daughter cells. Plasmid carriage generally imparts a metabolic burden upon its host, therefore, in order to persist under non-selective conditions a plasmid must control its copy number so as to not render the host uncompetitive (Watve *et al*., 2010).

Regulation of plasmid copy number occurs on different levels. Two general mechanisms for replication control can be distinguished, namely regulation by antisense RNA or control by iterons (see Chapter 1 section 2.2). It can, therefore, be assumed that the initiation of replication of a non-iteron containing plasmid is under control of either an antisense RNA dependent- (see Chapter 1 section 2.2.3) or a novel mechanism.

Copy number regulation is often studied by means of real time PCR. Relative quantification compares the quantity of a target gene relative to a reference gene (Pfaffl, 2004). Thus the gene expression of a mutant plasmid, for example, can be compared to its wild type counterpart. With the StepOnePlus system (Applied biosystems) relative quantification is carried out using Relative Standard Curve quantification or Comparative C_T ($\Delta\Delta C_T$ method), (Pfaffl *et al*., 2002). For the Relative Standard Curve method, standard curves are used to interpolate target quantity in both the sample and reference sample and the unknown sample target quantity is then compared to the reference target quantity. This method is best suited for PCR assays with suboptimal PCR efficiencies and where the PCR efficiencies of the target and endogenous control do not need to be similar. The comparative Ct method does not make use of a standard curve. Instead amplification of the target and endogenous control in both the unknown and reference sample is measured and the data is normalized using the endogenous control. The normalized target amount for each sample is thus compared to the normalized target amount in the reference sample and the results are given as a fold-change. This method is best suited for high-throughput relative quantification experiments where many genes and many samples are involved. The drawback to this method is that the PCR efficiencies of the target and endogenous control need to be equal and low PCR efficiencies may produce inaccurate results. The endogenous control is used to account for variability between samples and to normalize the data. Such controls are usually housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase (Huggett *et al*., 2005), but can be any gene which is constitutively expressed at similar levels during treated and untreated conditions.

Promoter regulation is an important consideration in the understanding of plasmid copy number control, and can be studied indirectly using reporter genes fused to the promoter of interest. Prokaryotic promoters have a characteristic structure. They contain core promoter elements with two consensus sequences, namely TATAAT for the -10 box and TTGACA for the -35 box which is located 10 and 35 basepairs upstream of the transcription start site, respectively (Burgess and Anthony, 2001). These sequences are however highly variable between promoters and are specific for recognition by the *E. coli* σ^{70} transcription factor. Promoter studies can give important clues and suggestions

on how and when a promoter is regulated and whether the promoter is autoregulated or under global control.

From the results obtained in chapter 2 it was evident that p31T1 is a low copy number plasmid able to transfer by means of mobilization in *E. coli* and capable of replication in *E. coli* and *P. putida*. Such a low copy number plasmid cannot rely on simple copy number regulation systems or random distribution for its maintenance and distribution into daughter cells since it will most certainly be lost in such a case. With this in mind further investigation into plasmid copy number and stability was employed as described in this chapter.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Addendum C. *E. coli* DH5α and EC100D *pir*$^{+}$ strains were grown in LB-media at 37°C. Appropriate antibiotic selection was added as required at the following concentrations, kanamycin (50 µg/ml), tetracycline (10 µg/ml), chloramphenicol (20 µg/ml) and/or ampicillin (100 µg/ml)

3.2.2. DNA manipulations, sequencing and general techniques. Plasmid DNA preparations, cloning, restriction enzyme digestions and gel electrophoresis were performed according to the standard methods described by Sambrook *et al*. (1989) and Ausubel *et al*. (1993). DNA was purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) and general DNA clean-up was done using the DNA Clean & Concentrator™ - 5 Kit (Zymo Research Corp.). Plasmid DNA was isolated using either the alkaline lysis method (Engebrecht *et al*., 2001), Pure Yield™ Plasmid Miniprep System (Promega) or Nucleobond® AX (Macherey-Nagel) kit. Sequencing was performed using an ABI PRISM^{TM} 377 automated DNA sequencer according to the dideoxy chain termination method.

3.2.3. PCR. All PCRs were performed as described in chapter 2 section 2.2.3., unless otherwise stated.

3.2.4. Mutants of minimal replicon ORFs. The R6K-minrep31T1 construct (Addendum C) was manipulated to generate mutants of ORFs 2, 3, 4 and 5, and were designated R6K-Minrep::ORF2, R6K-Minrep::ORF3, R6K-Minrep::ORF4 and R6K-Minrep::ORF5, respectively. These constructs were obtained by restriction enzyme digestion of R6K-Minrep31T1 with *Sal*I (R6K-Minrep::ORF2), *Spe*I (R6K-Minrep::ORF3), *Xcm*I (R6K-Minrep::ORF4) and *Xma*I (R6K-Minrep::ORF5), (Fermentas and Roche), removing the resulting nucleotide overhangs using T4 DNA Polymerase (Roche) to create frame shift mutations and religating the linear DNA with T4 DNA ligase (Roche) (see figure 3.2. for restriction map). The resulting constructs were transformed into an *E. coli* EC100D strain (supports the replication of the R6K replicon) and verified with restriction analysis or sequencing after purification using the Pure Yield Plasmid Miniprep Kit (Promega).

3.2.5. Cloning of ORF2 behind the pBAD28 promoter. PCR of ORF2 from p31T1 using Roche High Fidelity *Taq* Polymerase, was performed under the following thermal conditions, 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 100 s, and a final extension step at 72°C for 2 min and a hold step at 4°C. The PCR product was run on a 0.8% agarose gel, the 1.6 kb fragment excised and purified using the QIAquick Gel Extraction Kit (Qiagen) and subsequently cloned into pGem®-TEasy cloning vector to generate pGem-T Easy-ORF2. The 1-kb *Sac*I-*Xba*I fragment was subcloned from pGem-T Easy-ORF2 into pBAD28 (Addendum C) to generate pBAD28-ORF2. Sequencing confirmed that no PCR errors were present and *E. coli* DH5α cells containing pBAD28-ORF2 was made competent. The R6K-Minrep::ORF2 was put *in trans* of pBAD28-ORF2 and tested for replication in *E. coli* DH5α.

3.2.6. Cloning ORF3 behind pBAD28 promoter. The *orf3* gene was amplified from R6K-Minrep31T1 using the pBAD28-ORF3 primer set and Faststart High Fidelity *Taq* Polymerase (Roche), ligated into pGem®-T Easy vector and transformed into *E. coli* DH5α. The sequence of *orf3* was confirmed as free from PCR errors by sequencing with pUC/M13 primers. The 500-bp ORF3 fragment was cloned from pGem-T Easy-ORF3 into the *Sac*I-*Xba*I sites of pBAD28. pBAD28-ORF3 construct encodes chloramphenicol- and ampicillin resistance genes. As pBAD28-ORF3 was required to be *in trans* of other constructs that also encode an ampicillin resistance gene the *bla* gene on pBAD28-

ORF3 was inactivated by blunting and religating the DNA after restriction digestion using *Apa*LI. This created a frameshift mutation in the *bla* gene and rendered host cells sensitive to ampicillin.

3.2.7. Cloning of ORF4 behind the pBAD28 promoter. A 922-bp *Sma*I-*Spe*I fragment spanning ORF4 was ligated to a 5.8-kb *Sma*I-*Xba*I fragment of pBAD28 and generated pBAD28-ORF4.

3.2.8. Mapping minimal *oriV*. Initial identification and subcloning of the *oriV* into pUCBM21 and EZ-Tn5™ was done by Vos (Honours Thesis). This pUCBM21-*oriV* construct (figure 3.5) was used to further subclone three smaller segments of the *oriV* in an attempt to identify the minimum region required for *oriV* function. The 360-bp and 1000-bp *Pvu*II fragments of the pUCBM21-*oriV* construct was ligated to the *Sma*I site of dephosphorylated pBluescript SK+ to yield constructs SKoriV360 and SKoriV1000. A 750-bp *Taq*I-*Taq*I fragment from pUCBM21-*oriV* was ligated to the *Cla*I site of pBluescript SK+ and the construct was named SKoriV750. The 360-bp *oriV* fragment from SKoriV360 was transferred into EZ-Tn5™ (Epicentre) using a *Sal*I–*Xba*I cloning strategy to give R6KoriV360. A similar cloning strategy was used to transfer the 750-bp *oriV* fragment from SKoriV750 into R6K to generate R6KoriV750 while a *Bam*HI-*Apa*I *oriV* fragment from SKoriV1000 was transferred into the *Bam*HI-*Pst*I sites of R6K to generate R6KoriV1000.

3.2.9. Relative plasmid copy number determination using Real-time qPCR. The plasmid copy numbers of R6K-Minrep31T1 and R6K-Minrep::ORF3 was determined relative to p31T1 using the relative standard curve method which is similar to the standard curve method described in chapter 2. Three samples of each total DNA preparation were tested in triplicate.

3.2.10. Relative plasmid copy number determination using agarose gel densitometry. *E. coli* DH5α was transformed with p31T1, R6K-Minrep31T1, R6K-MinrepORF3, pRAS3.1.74 and pUC19, respectively. The plasmid-containing cultures were inoculated into LB-broth containing the appropriate antibiotics and grown overnight at 37°C. The saturated cultures were diluted 1:100 into fresh LB-broth the following

morning and grown at 37°C until the OD_{600} reached ~0.8. Cells (6 ml) were harvested by centrifugation and resuspended in 6 ml phosphate buffer saline (PBS). The OD_{600} of all the cultures except the pUC19-containing culture were standardized by dilution with PBS until the values were nearly identical. The cultures were mixed with 0.5 ml pUC19-containing culture and plasmid DNA was extracted using the Pure Yield Miniprep kit (Promega). The plasmid DNA was digested with suitable restriction endonucleases to linearize the pUC19 control and generate at least one band for each plasmid in question that would be similar in size for all the samples. Plasmid p31T1 was digested with *Eco*RI and *Nhe*I, R6K-Minrep31T1 and R6K-Minrep::ORF3 with *Sal*I and pRAS3.1.74 with *Bam*HI and *Hin*dIII. Samples were run on 0.8% agarose gel in TBE buffer.

3.2.11. Construction of promoter-*lacZ* fusion constructs. Putative promoter regions within the replicon region of plasmid p31T1 were amplified by means of PCR. The primer pairs used were ORF2fus, ORF3fus (short), ORF3fus (long), ORF4fus and ORF5fus (Addendum D) and are specific to putative promoter regions of the respective ORFs as denoted in the primer names. The PCR products were cloned into pGem®-T Easy (Promega) and sequenced using pGem®-T Easy-specific pUC/M13 primers to exclude any possible PCR errors. The promoter-reporter (*lacZ*) fusion was made by an in-frame ligation of the putative promoter regions from pGem®-T Easy into the *Bam*HI-*Eco*RI sites of pMC1403. The fusions were confirmed to be in-frame by sequencing using the LacZPri sequencing primer. The promoter fusion constructs were transformed into *E. coli* CSH50I^{q} and the relevant plasmids were provided *in trans* for purposes of β-galactosidase assays.

3.2.12. β-galactosidase assays. β-galactosidase activity was measured using an adaptation of the method of Miller (1972). Cultures containing the appropriate plasmids were grown overnight at 37°C with antibiotic selection. Overnight cultures were diluted 1:100 into fresh pre-warmed LB-broth containing 0.2% L-arabinose and the relevant antibiotics and grown for 3 h at 37°C. After 3 h, 200 µl of each culture was transferred in triplicate into a 96-well Microplate (Greiner Bio-one, USA) and the OD_{600} was recorded using a Biorad Microplate reader. Duplicates of all the cultures were diluted in a 1:1 ratio into Z-buffer to a final volume of 1 ml except for the ORF3lacZ (short) fusion construct

and the cultures which had the p31T1, R6K-Minrep31T1 and R6K-Minrep::ORF3 *in trans* of the ORF3lacZ (short) fusion construct. These cultures were diluted in a 1:9 culture to Z-buffer ratio. After the dilutions were prepared the cultures were vortexed for 10 s in the presence of 1% v/v toluene which was subsequently allowed to evaporate at 37°C for approximately 30 min. The dilutions were then transferred into a 24-well Flat Bottom Plate (Costar, Corning Incorporated, USA) and calibrated at 28°C. The assays were initiated by addition of 250 µl of a 4 mg/ml stock concentration of *o*-nitrophenyl-β-D-galactoside (ONPG) and the time was recorded sequentially. The reaction was stopped by addition of 500 µl 1M Na_2CO_3 when a yellow straw-like colour could be observed and the reaction time noted. After the assay was stopped, 200 µl of each reaction was transferred in triplicate into a 96-well Microplate (Greiner Bio-one, USA) and the optical density was recorded at 420 nm and 550 nm in a Biorad Microplate Reader. The β-galactosidase activity was expressed as Miller Units according to the equation by Miller (1972):

$$Miller\ Units = 1000\ x\ \frac{OD_{420} - 1.75\ x\ OD_{550}}{t\ x\ v\ x\ OD_{600}}$$

t = time in minutes

v = volume of culture used in assay (ml)

3.2.13. Stability Assays. Performed as described in chapter 2 section 2.2.7.

3.2.14. Cloning ORF3 into pOU82. The ORF3 was first cloned from R6K-Minrep31T1 into pBluescript SK+ to form an intermediate construct before cloning it into pOU82. To do this R6K-Minrep31T1 was digested with *Dra*I and *Nae*I in the presence of pBR322 and the 1190-bp ORF3 fragment was cloned into the *Sma*I site of pBluescript SK+ to yield SKORF3. The presence of an active *Nae*I site on pBR322 allows for improved digestion of the *Nae*I site on R6K-Minrep31T1 (http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/site_preferences.asp) as at least two

copies of its recognition sequence are required (http://www.fermentas.com/en/products/all/fastdigest-restriction-enzymes/fd152-naei?print). The 1.2-kb *Bam*HI-*Eco*RI fragment from SKORF3 was subsequently cloned into the *Bam*HI-*Eco*RI sites of pOU82 (figure 3.1) to yield pOU82-ORF3.

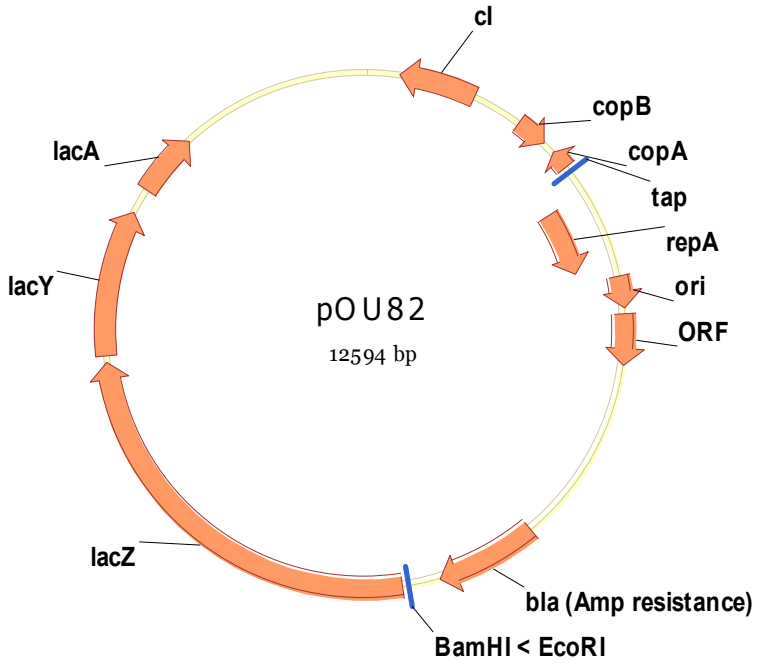


FIG.3.1. Plasmid vector map of the unstable test vector pOU82.

3.2.15. pOU82 Stability Assays. An adaptation of the stability assay protocol of Cooper and Heinemann (2000), utilizing the unstable pOU82-test vector was performed. Newly transformed *E. coli* DH5α colonies containing the test plasmids were inoculated into 5 ml LB-broth with antibiotic selection and incubated overnight at 30°C. The following day ~1000 cells were transferred into fresh LB-broth and every 24 h thereafter for 4 d without antibiotic selection. Serial dilutions were prepared in PBS and spread onto LB-agar containing 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and grown at 37°C. Colony counts were recorded and the percentage of plasmid-containing cells were determined by comparing the amount of plasmid containing (blue) and plasmid-free (white) colonies each day.

3.3. RESULTS

3.3.1. The minimal replicon resides on a 5.7-kb fragment of p31T1. The minimal replicon of p31T1 was identified previously by Vos (Honours Thesis). A partial *Sau3*A-bank ranging in insert size from 4.5-kb to 6-kb of plasmid p31T1 was constructed previously by Vos (Honours Thesis). This bank was cloned into the pEcoR252 suicide vector and transformed into *E. coli* GW125α. Four clones were selected and the inserts were sequenced and subcloned into EZ-Tn5™ with *E. coli* EC100D *pir*+ as a host. These R6K constructs were tested for replication in *E. coli* DH5α. The correct construct was identified and further deletion cloning was performed to identify the minimal region required for replication. It was determined to span a 5.7-kb region of p31T1 as depicted in the plasmid map of p31T1 (Chapter 2, figure 2.1) This region includes ORFs 2, 3, 4, 5, 6 and 2a as well as the *oriV* region and the construct referred to as R6K-Minrep31T1 (figure 3.2) for further studies.

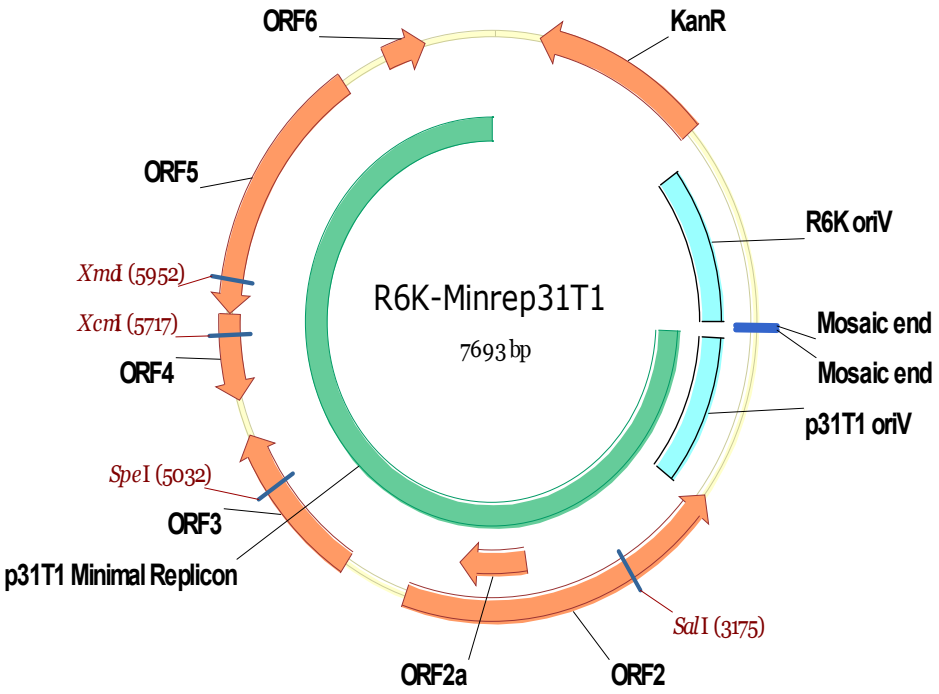


FIG.3.2. Plasmid map of the minimal replicon of p31T1 (green segment) ligated to EZ::Tn5 containing a kanamycin resistance marker. The R6K *oriγ* and the p31T1 origin of replication are depicted in light blue. KanR (kanamycin resistance marker).

3.3.2. ORFs 2 and 4 are required for autonomous replication in *E. coli* DH5α. The mutants were confirmed by restriction analysis as shown in figure 3.3. The ORF4 mutant was further confirmed by sequencing analysis.

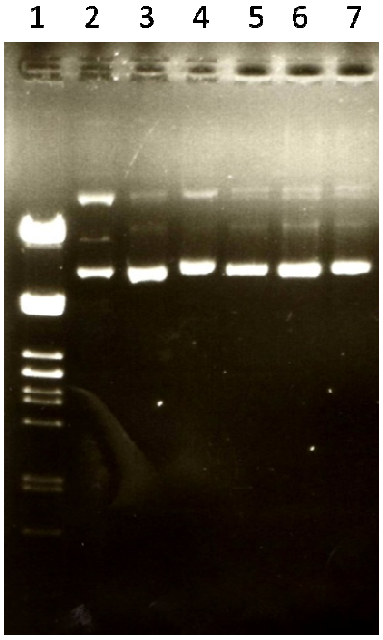


FIG.3.3. Restriction analysis of the R6KMinrep::ORF2, R6K-Minrep::ORF3 and R6K-Minrep::ORF5 constructs. Lane 1, Lambda *Pst*I marker; Lane 2, R6K-Minrep::ORF2 (*Sal*I digest); Lane 3, R6K-Minrep::ORF3 (*Spe*I digest); Lane 4, R6K-Minrep::ORF5 (*Xma*I digest); Lane 5, R6K-Minrep::ORF2 (uncut); Lane 6, R6K-Minrep::ORF3 (uncut); Lane 7, R6K-Minrep::ORF5 (uncut).

All mutants had the ability to replicate in *E. coli* EC100D since autonomous replication was from the vector EZ-Tn5™ R6K γ *ori* replicon. This replicon can function only in *E. coli* EC100D *pir*$^{+}$, and, therefore, is a suitable system for screening the replication phenotypes of the mutant versions of the R6K-Minrep31T1 plasmid in a host other than EC100D *pir*$^{+}$. The lack of viable colonies after transforming R6K-Minrep::ORF2 and R6K-Minrep::ORF4 into *E. coli* DH5α indicated that ORFs 2 and 4 were essential for replication of the p31T1 minimal replicon. ORF5 is located upstream of ORF4 and when interrupted, replication was also abolished. However, when a functional ORF4 (pBAD28-ORF4) was provided *in trans* replication was restored. This suggests that ORF5 is not essential for replication and confirms the requirement of ORF4.

Alternatively ORF5 mutant could possibly still function since it is truncated by only 140-bp at the C-terminal end of the 1149-bp ORF5 gene. Construct R6K-Minrep::ORF3 carrying the ORF3 mutant was the only construct with an interrupted ORF which could still replicate in *E. coli* DH5α. The mutation within ORF3 resulted in a truncated ORF3 towards the C-terminal end, as shown in figure 3.4 the putative ribbon-helix-helix (RHH) domain is retained (underlined). This shortened protein might therefore still function to some extent. Knockouts of ORFs 2a and 6 were not made. These two ORFs were only later identified as putative ORFs using the Glimmer 3 software in combination with BLAST analysis. Since they are also very small (123 aa and 72 aa, respectively) a true role for them is highly speculative.

```
1          ATGCAGCGCAAAAGCCCGACATTCAGCCTACGTCTACCCGCCGACCTGCTCGAGCAGACG
1           M  Q  R  K  S  P  T  F  S  L  R  L  P  A  D  L  L  E  Q  T

61         AACGAGCTGGCCGAGAAAACGAACCGTACCCGCACCGACGTGATTACCGACGCACTCCGT
21          N  E  L  A  E  K  T  N  R  T  R  T  D  V  I  T  D  A  L  R

121        GCATACCTTGGCATACCAGAGCCGCAGGGGGAGAGCGGCAACCGCCTCGACCTGATGGTG
41          A  Y  L  G  I  P  E  P  Q  G  E  S  G  N  R  L  D  L  M  V

181        GAGCTGCTGCAGGACATCTCGAATACGCTGAAACACAATGTGCTACAGAAGCCAACAGGA
61          E  L  L  Q  D  I  S  N  T  L  K  H  N  V  L  Q  K  P  T  G

241        CGCGCTACAAGGCCACGGGCTGAAAAGCTGATAACTCATCTGAAACACAAAACGCAGCAC
81          R  A  T  R  P  R  A  E  K  L  I  T  H  L  K  H  K  T  Q  H

301        AGCGCCCCGCTGGCGCTGAATTTTCGGGGCATTACGACGAGGCCGAGGTGATGGCCACCA
101         S  A  P  L  A  L  N  F  R  G  I  T  T  R  P  R  *  W  P  P

361        TACGGCGCATGAGGGAGGAGCAGCGCGATCGGGGATTCCGCTACGACAACAAAGCTATCG
121         Y  G  A  *  G  R  S  S  A  I  G  D  S  A  T  T  T  K  L  S

421        CCCAGGCGCTGAACGAGGCAGGGCTCCTGCAGTCGAACGGTCGCCTATGGAACAACGACC
141         P  R  R  *  T  R  Q  G  S  C  S  R  T  V  A  Y  G  T  T  T

481        GCATCAACACGGTGATCACCCGCCGGATGCCTGACCTGAAGTAA
161         A  S  T  R  *  S  P  A  G  C  L  T  *  S
```

FIG.3.4. ORF3mutant sequence displaying the interrupted protein of ORF3 (short) with newly generated stop codons (*) and therefore the truncated version at the TGA stop (*). The putative RHH domain remains intact (underlined).

3.3.3. Minimal *oriV*. Vos (Honours Thesis) previously identified a 1.19-kb fragment of p31T1 which contains the *oriV* by means of sequence analysis and subcloning (figure 3.5). In this study further subcloning and complementation assays allowed for the identification of a 750-bp fragment spanning the *oriV* region and sequence analysis allowed the identification of inverted- and direct-repeats and an AT-rich region which are characteristic of an *oriV* (figure 3.6), (del Solar *et al*., 1998). The R6KoriV750 construct was able to replicate in *E. coli* DH5α when p31T1 was provided *in trans*, but could not replicate independently (control). Both the R6KoriV360 and R6KoriV1000 constructs were, however, unable to replicate when p31T1 was provided *in trans*. This suggests that the 360- and 1000-bp *Pvu*II fragments, which partially overlap with the 750-bp *Taq*I-*Taq*I *oriV* region, do not carry the *oriV* region or only parts thereof.

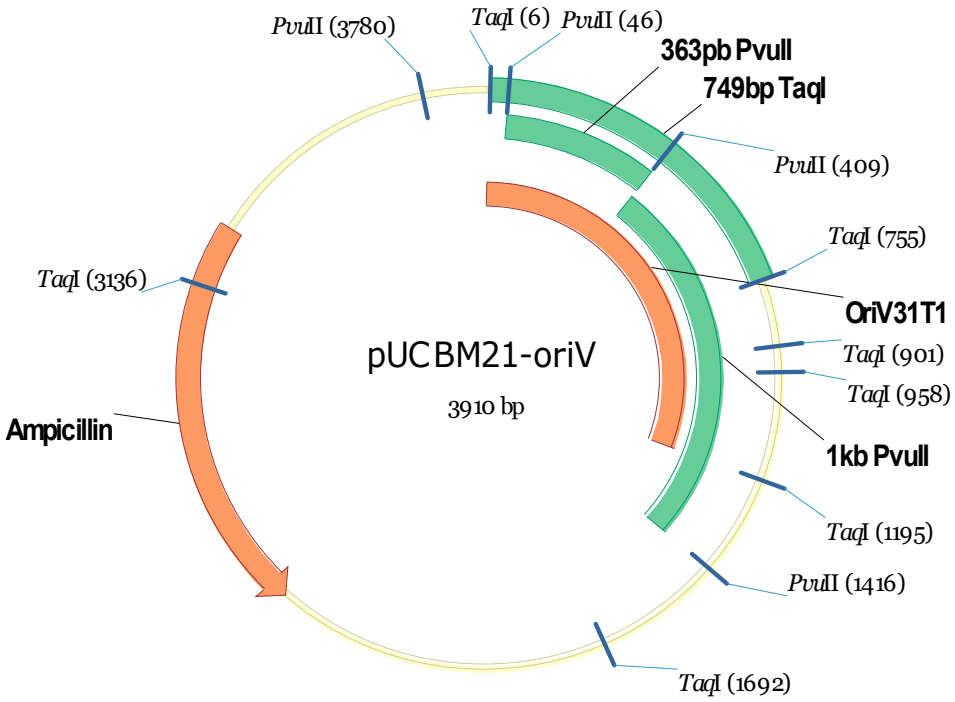


FIG.3.5. The *oriV* region of p31T1 cloned into pUCBM21 as done by Vos (Honours Thesis). Green segments indicate the different fragments which were subcloned into EZ-Tn5™ and screened for replication when p31T1 was provided *in trans*. The 363-bp *Pvu*II, 1-kb *Pvu*II and 749-bp *Taq*I fragments were subcloned first into pBluescript SK+ and then into EZ-Tn5™ to generate R6KoriV360, R6KoriV1000 and R6KoriV750 respectively.

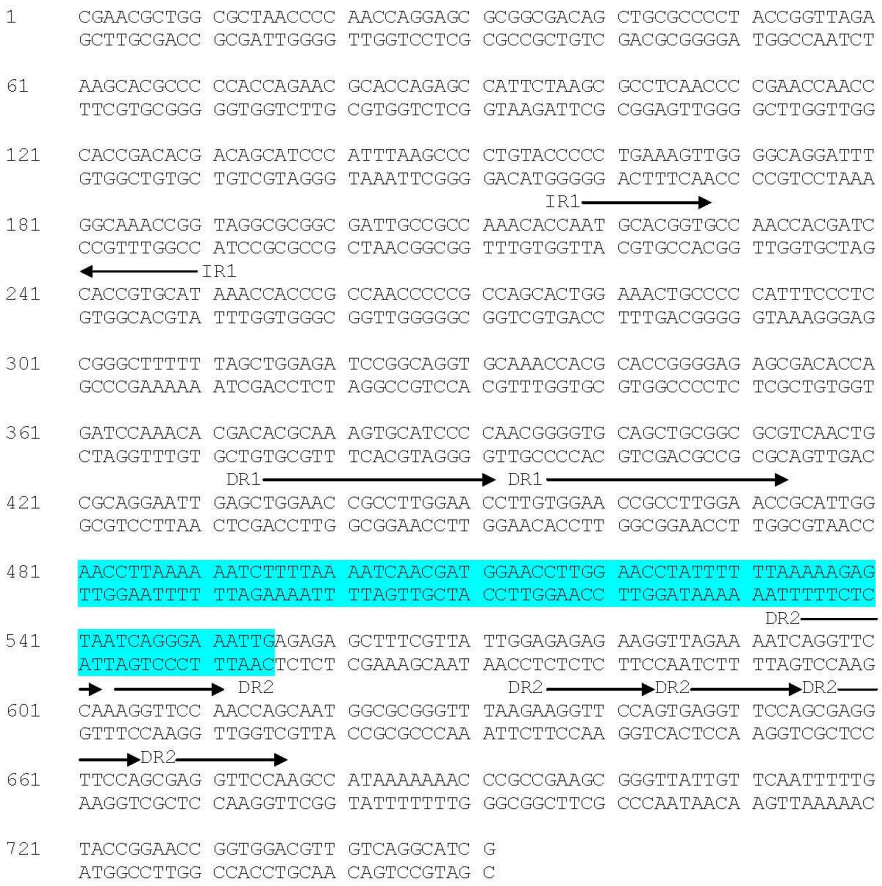


FIG.3.6. Sequence annotation of the 750-bp *oriV* region of p31T1. The blue shaded region represents an AT-rich region. IR (inverted repeats); DR (direct repeats)

3.3.4. R6K-Minrep::ORF3 has an increased plasmid copy number relative to the wild type p31T1 plasmid. Two individual Real-Time PCR experiments utilizing different analysis software packages (ABI StepOnePlus software, Applied Biosystems and Rest 2009 software, Qiagen) were used for analysis of two quantitative real-time PCR experiments for relative plasmid copy number determination. ABI software gave a calculated 2- to 3.2-fold increase in copy number for R6K-Minrep::ORF3 (figure 3.7). A 1.5- to 1.75-fold increase was observed for R6K-Minrep31T1. The copy number of R6K-Minrep::ORF3 was significantly ($p<0.05$) upregulated by a mean factor of 2- to 2.5-fold compared to the wild type p31T1 plasmid when analysed with REST software (figure 3.8). The 1.3- to 1.6-fold difference observed between the wild type p31T1 and minimal

replicon counterparts were not significant ($p>0.05$). The REST software is preferred since it takes statistical accuracy of the data into account, and therefore the discrimination between significant and non-significant data can be taken into account.

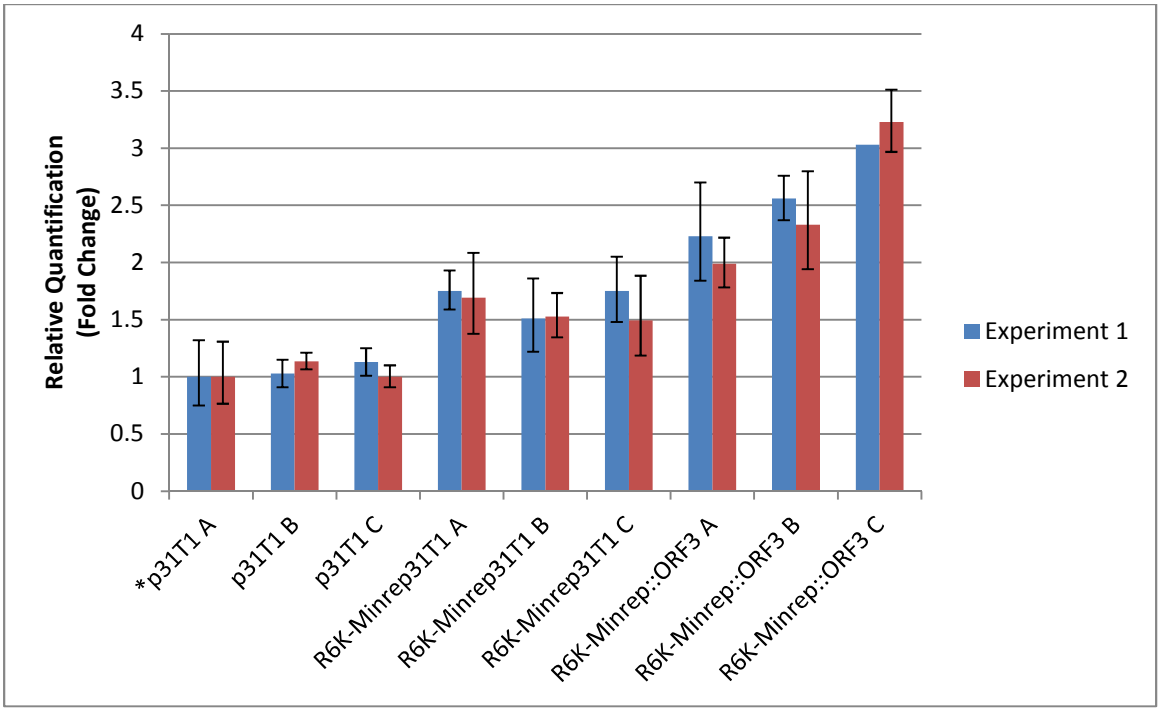


FIG.3.7. Relative quantification results as obtained with StepOnePlus (Applied Biosystems) software. The R6K-Minrep31T1 and R6K-Minrep::ORF3 constructs were tested relative to the p31T1 wild type. Three samples A, B, and C for each construct was tested in triplicate for each experiment (9 data points per experiment).The sample *p31T1 A served as reference sample.The average values R6K-Minrep31T1 and R6K-Minrep::ORF3 relative to p31T1 was obtained with both experimental data combined and were 1.54 ± 0.12 and 2.44 ± 0.48, respectively. Three samples were tested in triplicate for each experiment.

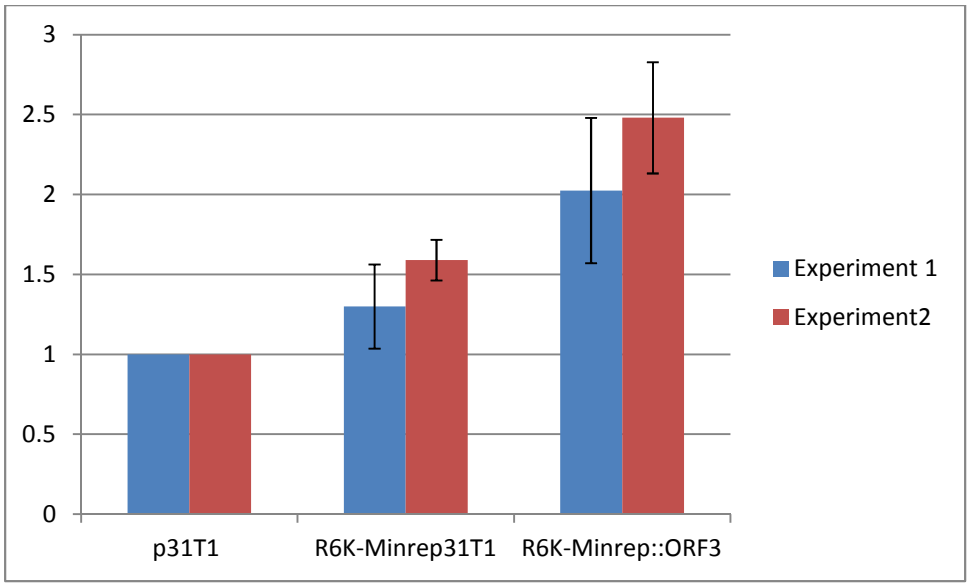


FIG.3.8. Relative quantification results for two separate experiments as analyzed with REST2009 software (Qiagen). The R6K-Minrep31T1 and R6K-Minrep::ORF3 constructs were tested relative to p31T1. An average 1.45±0.2 fold and 2.25±0.3 fold increase was observed for R6K-Minrep31T1 and R6K-Minrep::ORF3 respectively.

A gel densitometry method was used as a second approach to verify the Real-Time PCR results (figure 3.9). Plasmid p31T1 and its mutants were extracted from approximately equal amounts of cells. Equal volumes of *E. coli* DH5α (pUC19) were added to each sample to serve as an endogenous control for plasmid extraction efficiency. The plasmid DNA was treated with restriction enzymes to obtain DNA fragments of approximately equal size and from which relative abundances could be extrapolated. The band volumes were normalized based upon the linearized pUC19 DNA using GelQuant.NET (BiochemLabSolutions) and calculated as shown in Table 3.1. From this it was estimated that R6K-Minrep::ORF3 was approximately 2.5-fold more abundant than p31T1, thus suggesting a 2.5-fold higher copy number and was comparable to the relative quantification results obtained with Real-Time PCR. A derivative of the IncQ-2 plasmid pRAS3.1 with a copy number of 15 copies per chromosome (Loftie-Eaton and Rawlings, 2010) had a 5.9 fold increase relative to p31T1. It can therefore be estimated that the copy number of p31T1 is roughly 2.5

plasmids per chromosome. This corresponds to the absolute quantitative results obtained for real-time PCR (~3 plasmids per chromosome) in chapter 2.

TABLE.3.1 Gel densitometry band volume calculations				
	p31T1	**R6K-Minrep31T1**	**R6K-Minrep::ORF3**	**pRAS3.1.74**
Corresponding pUC19 gel band volume	2210	2121	3136	2697
Band volume	3994	5212	14295	28856
Correction factor	1.42	1.48	1	1.16
Corrected Volume	5671	7714	14295	33473
Fold difference	1	1.36	2.52	5.90

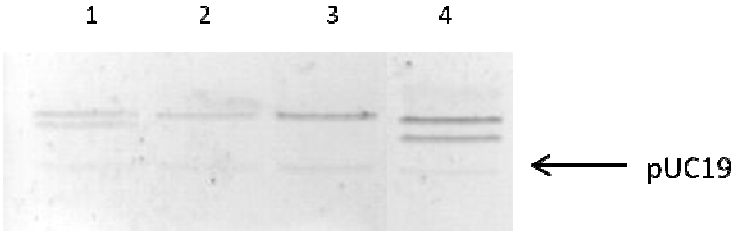


FIG.3.9. Relative quantification results as obtained with agarose gel densitometry analysis. Lane 1. p31T1; Lane 2 R6K-Minrep31T1; Lane 3 R6K-Minrep::ORF3. Lane 4. pRAS3.1.74. The feint lower band in both figures represent pUC19. The band sizes for the p31T1 constructs and the pRAS3.1.74 plasmid are the same and are therefore comparable.

3.3.5. ORF3 is a repressor of its own promoter. Putative promoter regions of ORFs 2, 3 (long), 3 (short), 4, and 5, namely ORF2fus, ORF3fus (long), ORF3fus (short), ORF4fus and ORF5fus were PCR-cloned in front of the *lacZ* ORF of pMC1403 to yield constructs ORF2lacZ, ORF3lacZ (long), ORF3lacZ (short), ORF4lacZ and ORF5lacZ. The regions that were PCR amplified and subcloned are depicted in figure 3.10.

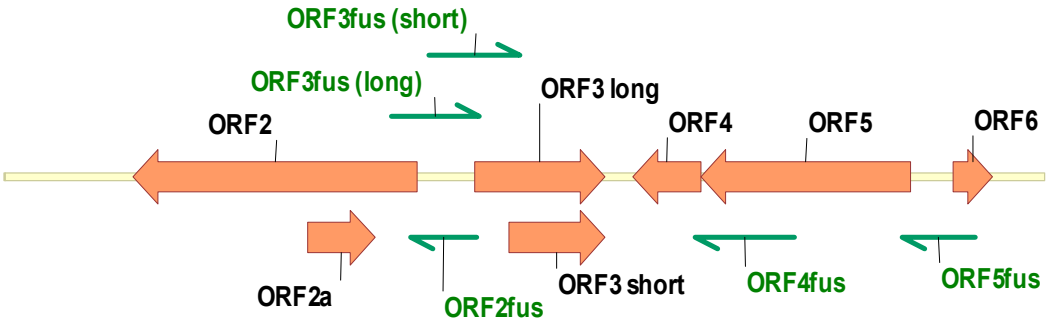


FIG. 3.10. Putative promoter segments (green) from the minimal replicon ORFs (orange) which were subcloned into pMC1403 for β-galactosidase assays.

The putative promoters were then tested for their ability to express the *lacZ* gene by means of β–galactosidase assays (figure 3.11). The pMC1403 vector gave β-galactosidase activity of 16 Miller Units. No significant β-galactosidase activity was observed when provided with the putative promoters from ORF4 (18 Miller Units) and ORF5 (32 Miller Units). The activities of the putative promoters of ORF2lacZ and ORF3lacZ (long) were determined to be 18 and 14 Miller Units respectively. Therefore the activity of the putative ORF2, ORF3(long), ORF4 and ORF5 promoters were considered to be negligible. The placement of ORF4lacZ and ORF5lacZ *in trans* of p31T1 gave Miller Units of 15.98 and 24.84 respectively. Similarly the *in trans* placement of R6K-Minrep31T1 with these two promoter fusions gave Miller values of 17 and 23 respectively. The placement of p31T1 and R6K-Minrep31T1 *in trans* of ORF2lacZ and ORF3lacZ (long) did not deliver any viable cells, however, when transformed individually viable colonies could be observed. Therefore ORF2lacZ and ORF3lacZ (long) could not be tested with the *in trans* placement of p31T1 or R6K-Minrep31T1.

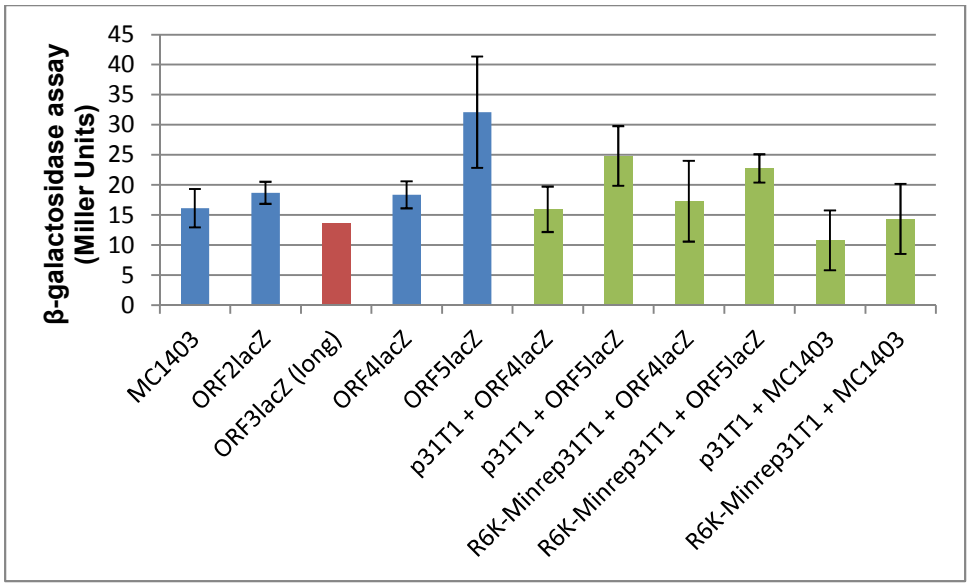


FIG.3.11. β-galactosidase assay results for ORF2lacZ, ORF4lacZ and ORF5lacZ. Results are also shown for p31T1 and R6K-Minrep31T1 placed *in trans* of ORF4lacZ and ORF5lacZ. ORF2lacZ could not be tested *in trans* of p31T1 or R6K-Minrep31T1 and was therefore excluded. MC1403 was included as a control and did not contain any cloned promoter fragment.Blue bars (3 data points), Green bars (2 data points) and Red bar (1 data point, therefore no Standard Deviation was determined).

Careful inspection of the ORF3 DNA sequence led to the finding of an alternative ATG start for ORF3, located 188 nucleotides downstream of the GTG start of ORF3 (long), (see figure 3.10). This newly identified putative promoter region contains an AGGAG sequence 6-bp upstream of the ATG start codon which resembles that of the AGGAGG consensus sequence of the *E. coli* ribosomal binding site (RBS). ORF3lacZ (short) was constructed by cloning this newly identified promoter region, ORF3fus (short), (see figure 3.10) in front of the *lacZ* gene of pMC1403. The ORF3lacZ (short) promoter-*lacZ* fusion construct yielded β–galactosidase activity of ~1298 Miller units. When p31T1 was provided *in trans* of ORF3lacZ (short) activity was reduced to 447 Miller Units implying a decreased promoter activity. The β–galactosidase activity of ORF3lacZ (short) was slightly less repressed (555 Miller Units) when R6K-Minrep31T1 was provided *in trans*.

To test whether the ORF3 protein was responsible for repression of the ORF3 (short) promoter, the R6K-Minrep::ORF3 construct was provided *in trans* to the ORF3lacZ

(short) fusion construct. The activity of the promoter in the presence of the mutant was slightly higher (694 Miller Units) compared to when p31T1 plasmid was provided *in trans* of ORF3lacZ (short), (figure 3.12) and showed a similar level of activity to when R6K-Minrep31T1 was provided *in trans* of ORF3lacZ (short). It was next investigated whether an excess supply of ORF3 protein had an effect on ORF3lacZ (short) promoter activity. For this reason ORF3 was cloned behind the P_{ara} L-arabinose inducible promoter on the pBAD28 expression vector. Overexpression of ORF3 led to complete repression of promoter activity (figure 3.12).

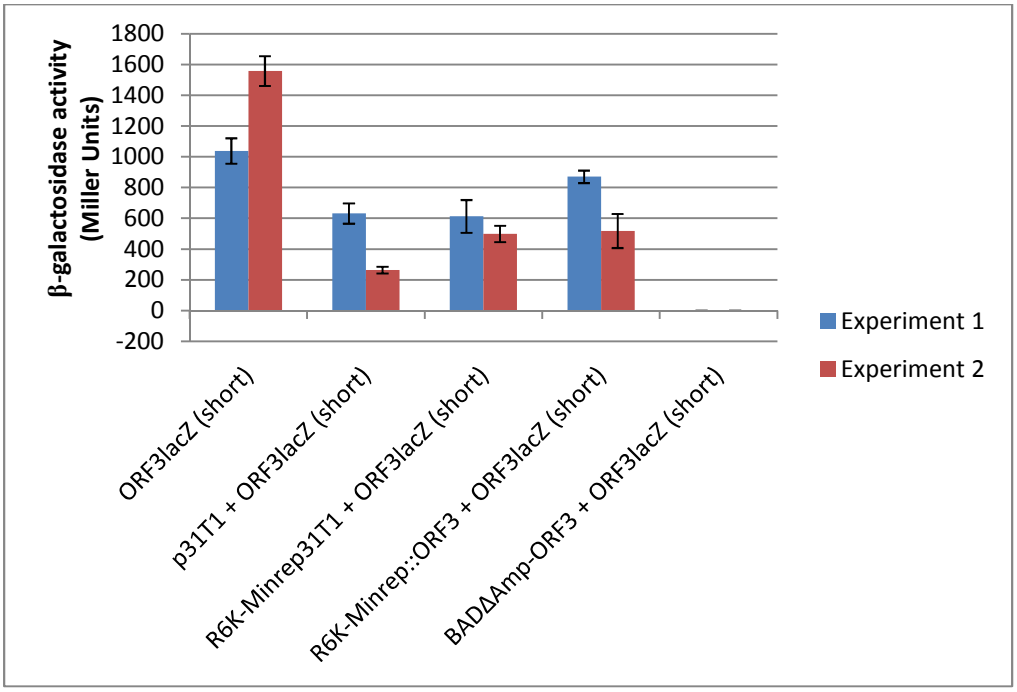


FIG.3.12. β-galactosidase assay results of two different experiments, depicting the single ORF3fusion (short) plasmid and when the representative p31T1 plasmid and its counterparts were placed *in trans*.

3.3.6. ORF3 is a stability determinant. The stability of wild type p31T1 relative to its minimal replicon counterpart (R6K-Minrep31T1) and ORF3 mutant (R6K-Minrep::ORF3) was determined by scoring the ratio of plasmid-containing to plasmid-free colonies over 7 days (~127 generations) after serial batch cultures in the absence of antibiotics (figure 3.13). The wild type plasmid p31T1 was retained in 80.67% ± 1.15 of the cells after

~127 generations while R6K-Minrep31T1 was only slightly less stable at 74% ± 5.66. ORF3 could be inactivated without complete loss of replication and, therefore, was further tested for its possible contribution to plasmid stability. As shown in figure 3.13. inactivation of ORF3 led to a significant decrease in plasmid stability such that the ORF3 mutant was retained in only 16% ± 8.7 of the cells after ~127 generations. This indicated that ORF3 is required for stable plasmid maintenance.

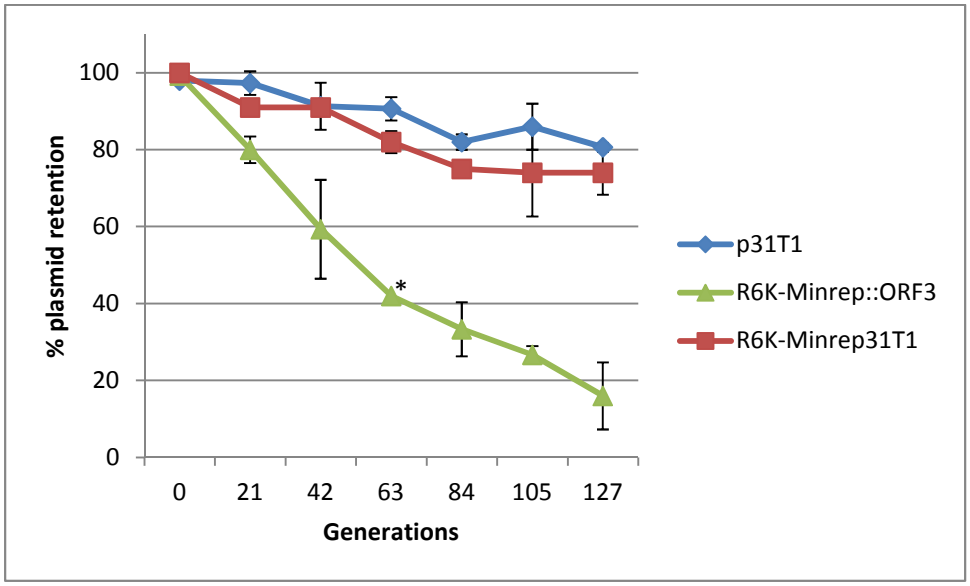


FIG.3.13. Stability assay of plasmids p31T1 (blue), R6K-Minrep31T1 (red) and R6K-Minrep::ORF3 (green). The mutant shows a significant plasmid loss frequency relative to the wild type p31T1 and the minimal replicon version of p31T1. Plasmid stability was measured in triplicate.

*No standard deviation due to the availability of only one data point as a result of skewed plate counts.

In order to confirm the involvement of ORF3 in plasmid stability, a 1191-bp fragment containing the 525-bp ORF3 (short) gene was cloned onto the unstable pOU82 test vector. Plasmid pOU82 encodes a β-galactosidase enzyme (LacZ) and, therefore, plasmid-containing and plasmid-free cells could be distinguished from each other as blue and white colonies, respectively, when plated onto X-gal. When ORF3 was cloned into pOU82 it was lost at a more gradual rate, with 60% ± 7.5 of the population retaining

the plasmid after ~79 generations compared to complete loss of the pOU82 control plasmid (figure 3.14).

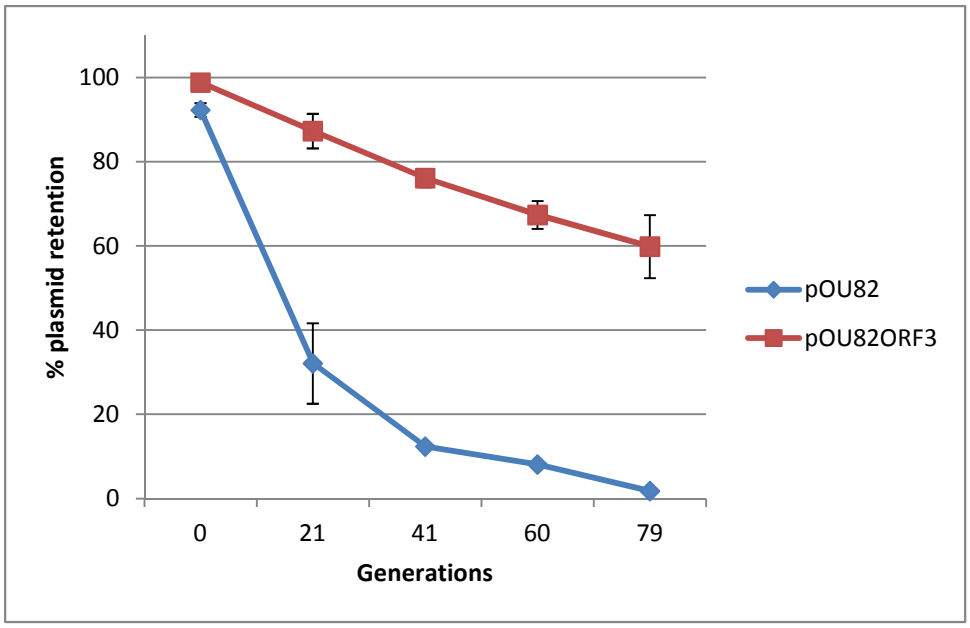


FIG.3.14. pOU82 stability assays. ORF3 improves pOU82 stability in the absence of antibiotic selection. Plasmid stability was performed in triplicate.

3.4. DISCUSSION

Plasmid p31T1 contains 18 putative ORFs of which 6 were found to be associated with a minimal replicon spanning 5.7-kb of the plasmid. An *oriV* region could also be identified adjacent to ORF2. The minimal replicon ORFs 2, 3, 4, and 5 were investigated and although ORF2a and 6 also form part of this area, no experimental studies were performed on them since they were not initially identified as possible ORFs when Glimmer version 2 was used compared with the more recently released Glimmer version 3. ORFs 2 and 4 are absolutely essential for replication based on mutational analysis. The stop codon of ORF5 overlaps with the putative GTG start of ORF4 and therefore they seem to be translationally coupled. Furthermore, inactivation of ORF5 lead to a complete inhibition of replication and was restored when ORF4, (pBAD28-

ORF4), was provided *in trans*, suggesting that ORF5 is not essential for replication. pBAD28-ORF4 could complement the ORF5 knockout *in trans*, even when uninduced with L-arabinose, suggesting that only small amounts are required to function *in trans*. Similarly a knockout of ORF2 lead to inactivation of replication.This knockout mutant could be trans-complemented by an intact ORF2 cloned behind the P_{ara} L-arabinose inducible promoter of pBAD28 to generate pBAD28-ORF2. However, the pBAD28-ORF2 construct required induction with L-arabinose and therefore higher expression levels were needed to complement the ORF2 knockout *in trans*. Both ORF2 and ORF4 are essential for replication and function *in trans* and it is likely that one of these ORFs might function as a replication initiator. The ORF3 mutant was the only ORF which could independently function to support autonomous replication of p31T1. The emphasis and further investigation was placed on ORF3 and its purpose in replication.

By determining the plasmid copy number of the WT p31T1 plasmid and derivatives in which ORFs have been inactivated, as well as from promoter studies using a *lacZ* reporter gene it was evident that ORF3 plays an important regulatory role in both plasmid copy number regulation and stability. It has been shown using the RepX/*orfX* in various *Lactococcus lactis* plasmids as a model system that a single ORF can have a dual role in both plasmid copy number regulation and stability (Frére *et al*., 1993; Gravesen *et al*., 1995; Hayes *et al*., 1991). The exact role of *orfX* remains undetermined, however it was shown that in some cases it was dispensable for replication (Gravesen *et al*., 1995). Plasmid copy number assays showed a 2- to 2.5-fold increase in plasmid copy number for the R6K-Minrep::ORF3 construct containing the mutated ORF3 gene. The differences in plasmid copy number observed between the wild type p31T1 and R6K-Minrep31T1 minimal replicon counterpart was not significant ($p>0.05$), suggesting that the higher copy number that was observed when ORF3 was interrupted was not as a result of other essential ORFs being missing.

Stability assays showed that there was a slight difference in stability between p31T1 and R6K-Minrep31T1 (6%). This could imply that there might be additional stability determinants outside the minimal replicon or the difference could be as a result of different selections used (tetracycline vs kanamycin). R6K-Minrep::ORF3 also showed

an increased instability phenotype. Furthermore cloning the 1191-bp fragment containing the intact ORF3 region into pOU82 significantly increased the stability of this unstable vector from 1.8% to 60% after ~79 generations and thus seems to be an active stability determinant. This ORF3 region which was cloned into pOU82 is depicted in figure 3.15 and could resemble a possible operon arrangement consisting of ORF3 leader and ORF3 (long). The ORF3 leader peptide occurs in-frame with ORF3 (long). ORF3 has two possible translational start sites, however an active promoter region could only be shown for ORF3 (short), therefore the the longer protein version ORF3 (long) may not exist, since it does not show any characteristic RBS and β-galactosidase assays could not be performed for this longer ORF due to the inability of obtaining transformants when the ORF3lacZ (long) construct was placed *in trans* of either p31T1 or R6K-Minrep31T1. A functional role for the ORF3 leader protein (54 amino acids) is also speculative since no function could be assigned based on BLAST analysis and no characteristic RBS could be identified. This operon configuration for ORF3 and the ORF3 leader proteins could suggest a possible partitioning function or toxin-antitoxin system, since these systems consist of at least two proteins in an operon configuration. The coding of a toxin-antitoxin system by this region is unlikely. If ORF3 were to be a toxin or antitoxin, then deletion of ORF3 would lead to the accumulation of plasmid free cells (toxin deletion) or have a deleterious effect on cell survival (antitoxin deletion). ORF3 is a putative DNA-binding protein since it carries a ribbon-helix-helix domain and could therefore resemble a partitioning protein which would bind to a *cis*-acting site or partitioning site, however no such site could be identified on p31T1. If the plasmid does rely on active partitioning for its stable maintenance, R6K-Minrep::ORF3 construct would have had to switch from active partitioning to being reliant upon random distribution which could explain why the plasmid would become unstable even though there is an increase in the plasmid copy number. The increase in copy number (runaway replication) can also be explained if the copy number regulation and active partitioning systems are somehow interconnected. Whether ORF3 functions independently or in an operon configuration remains to be determined and the true role for the elements in this putative operon are not well understood.

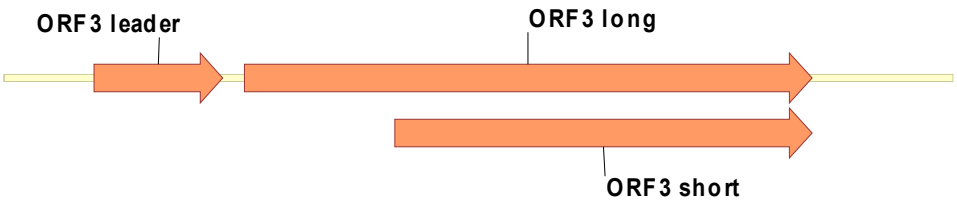


FIG.3.15. The region spanning the ORF3 gene region of p31T1 that was subcloned into pOU82. ORF3 leader and ORF3 (long) are putative and their possible true role in stability needs confirmation.

ORF3 is a repressor of its own promoter, thereby autoregulated, as was evident when the ORF3 promoter was down-regulated during β–galactosidase assays in the presence of ORF3. Furthermore, inactivation of ORF3 resulted in an increase in plasmid copy number and this too is indicative of negative feedback regulation. We know that the ORF3 promoter is active (ORF3lacZ shows strong β-galactosidase activity ~1298 Miller Units). When p31T1 or R6K-Minrep31T1 was provided *in trans* there was a decreased expression in β-galactosidase activity or promoter repression but not entirely. This could indicate that the ORF3 promoter is not completely shut down at physiological concentrations and a low level of ORF3 promoter expression is required to fulfil its maintenance function. An inactivation of ORF3 (R6K-Minrep::ORF3) caused a derepression of ORF3 promoter but not to completely unrepressed levels. The mutated ORF3 might therefore still preserve some of its DNA binding activity since it was only truncated and retains a putative RHH domain, which has been shown with other RHH containing proteins to be involved in dimerization and DNA-binding (Hernández-Arriaga *et al*., 2009). Alternatively another protein in conjunction with ORF3 could be required for ORF3 promoter repression. A complete repression of the ORF3 promoter was brought about when pBAD28ΔAmp-ORF3 was placed *in trans* of ORF3lacZ. This total shut-off only on overexpression of ORF3 protein supports the view that physiological concentrations are not sufficient for complete repression of the ORF3 promoter. Transformation of *E. coli* containing p31T1 or R6K-Minrep31T1 resident with either ORF2lacZ or ORF3lacZ (long) *in trans* was not possible due to some undefined incompatibility or toxicity and therefore β-galactosidase assays could not be performed

with ORF2lacZ or ORF3lacZ (long). This observation suggested that the overlapping promoter regions of ORF2 and ORF3 (long) contain an area which titrate important proteins either plasmid or chromosome encoded.

The results of the work reported in this chapter indicated that ORF3 is a key determinant both in regulation of plasmid copy number and stability. Whether ORF3 acts as a partitioning locus, multimeric resolution system or plasmid addiction-like system remains to be determined. The role of ORF2 and ORF4 remain unknown, but their importance in replication is evident. Future research is required to answer questions such as whether the regulation of plasmid copy number is dependent on antisense RNAs; which protein, ORF2 or ORF4, serves as the initiator of replication and whether there are any other plasmid stability determinants located outside of the minimal replicon that could improve stability?

Chapter 4

General Discussion and Conclusion

Chapter 4: General Discussion and Conclusion

Plasmids are selfish genetic elements which in general are a burden to the host, however under certain circumstances they can render the host which harbours them competitive in order to survive in harsh environmental conditions for example. Accessory determinants such as antibiotic resistance and heavy metal resistance are often encoded on plasmids and are spread by mobile genetic elements such as transposons. This is an important function of plasmids. It is beneficial to a plasmid to be mobilizable since gene delivery and spread through interspecies transfer by conjugation, transformation and transduction can occur more efficiently (Bruun *et al*., 2003). The spread of resistance plasmids between the clinical and environmental setting has been shown for *Aeromonas* (Rhodes *et al*., 2000). Plasmids are diverse with respect to their size, copy number and phenotype (Osborn *et al*., 2000) and it is important that we characterize them since they carry determinants of clinical, environmental and industrial importance. By studying plasmids from a molecular perspective we obtain a better understanding of how these entities operate, persist and adapt within certain environmental and clinical settings.

The main aims of this study were to characterize p31T1 based on sequence analysis and further investigate the replicon ORFs and their regulation. General plasmid characteristics such as copy number, stability and mobilization were also explored.

Plasmid p31T1 is a 14-kb mobilizable plasmid harbouring tetracycline resistance that was isolated from an *Aeromonas sobria* strain (Marx, MSc Thesis). It has a similar restriction profile to another plasmid, p36T2, which was isolated from *Aeromonas hydrophila*. Since the two plasmids were isolated from two different strains of *Aeromonas* that was found within the same aquaculture environment the plasmids were likely transferred between these two strains by means of conjugation. The replication of p31T1 in *E. coli* and *Pseudomonas putida* aside from its original *Aeromonas* host was demonstrated in this study and as all of these bacteria are γ–proteobacteria it may be that the plasmid is capable of replication more widely within this group. However, the

inability to replicate in the α-proteobacterium, *Agrobacterium*, suggests that p31T1 is not a true broad host-range plasmid. It is likely that p31T1 has the ability to persist within a mixed bacterial population and therefore maintain itself through intra- and interspecies-transfer. p31T1 was captured by means of a standard alkaline lysis protocol by Marx (MSc Thesis) and it was also shown in this study to be mobilizable between different *E.coli* strains, although at a very low frequency (5.38 (± 2.28) x 10^{-5} transconjugants per donor) by an IncP-1 conjugative system. Different conjugative systems were previously shown to transfer mobilizable plasmids at different frequencies/efficiencies. Van Zyl (MSc Thesis) showed that plasmid pTC-F14 was more sufficiently transferred by *E. coli* S17.1 with the RP4 (IncPα) plasmid integrated onto the chromosome at a frequency of 2.8 x 10^{-3} transconjugants per donor, compared to plasmid R751 (IncPβ) which gave a 100 fold lower frequency at 1.2 x 10^{-5}. Plasmid R388 (IncW) did not give any detectable result. In this study matings with R773 (IncF) and R46 (IncN) conjugative plasmids with p31T1Cm (tetracycline resistance genes of p31T1 disrupted with chloramphenicol resistant marker) *in trans* were tested for mobilization using *E. coli* EC100D Rif as a donor and *E. coli* DH5α as a recipient. No transfer could be observed for p31T1 or either of the conjugative plasmids. Whether a conjugative system other than IncP-1 would be more suitable for p31T1 transfer remains to be determined. Mapping of a putative *oriT* region for p31T1 was also unsuccessful. An attempt was made to screen a partial *Sau*3A bank of p31T1 in a non-mobilizable pUC19 vector. The transformation of pUC19 into *E. coli* S17.1 donor and mobilization of the bank *in trans* of p31T1 into *E. coli* ACSH50I^{q} as a recipient, appeared to give possible transconjugants on recipient selective plates. However, analysis of six putative transconjugants did not give a common p31T1 insert sequence. How pUC19 derivatives were transferred is unclear.

p31T1 has possibly acquired its tetracycline resistance by means of transposition which was most likely promoted by a conjugation event. Transposons have the ability to jump between plasmids or between plasmids and chromosomes by either a replicative (copy-and-paste) (Weinert *et al*., 1984) or non-replicative (cut-and-paste) mechanism (Berg *et al*., 1984). The minimum requirement for movement of a transposon is a self-encoded transposase enzyme which recognizes specific terminal repeats required for excision/insertion. Transposition events into a plasmid or chromosome can have either

a beneficial or deleterious consequence, depending on where the transposon is inserted and what accessory DNA is carried by the transposon. The *tetA* and *tetR* genes on p31T1 are identical to those carried on Tn*1721* (100% DNA sequence identity). Furthermore, a truncated transposase lies adjacent to *tetR* while an N-terminal truncated *pecM* ORF, followed by two ORFs and a *tnpR* resolvase lies adjacent to the *tetA*. No 38-bp inverted repeats or any 5-bp direct repeats characteristic of Tn*1721* nor any other putative repeats possibly associated with a transposon could be identified in the flanking regions of the transposase and resolvase genes (Schöffl *et al*., 1981). The acquired transposon is, therefore, most likely inactive.

It was determined that p31T1 was maintained at a low copy number of ~3 plasmids per chromosome in *E. coli*, but nevertheless seemed to be stable in the absence of selection. This implies that an active stability system has to be present on p31T1 since low copy number plasmids cannot rely on random distribution for stable inheritance. Such a plasmid would be outcompeted in an environmental setting without selective pressure by plasmid-free segregants. In contrast a low copy number plasmid imposes a lower metabolic burden on the host. Low copy number is, therefore, optimal for long term survival of a plasmid. A copy-up mutant plasmid would have a selective intercellular advantage over a short term period as it would out replicate the low copy number variant (Watve *et al*., 2010). This raises the phenomena of intra- and inter-host selection (Paulsson, 2002). With intra-host selection over-replicating plasmids will more likely be selected for replication, however, cells containing such plasmids would impose an increased metabolic burden and most likely be out competed by cells which harbour low copy number plasmids or no plasmids at all. The latter phenomenon is referred to as inter-host selection. The importance of active stability systems for intracellular maintenance of low copy number plasmids is, therefore, emphasized.

The p31T1 minimal replicon spans 5.7-kb of the plasmid (36%) and represents 6 of the 18 putatively identified ORFs. The minimal replicon therefore has the ability to carry a minimum of 8.3-kb of additional DNA. All the replication regulatory elements needs to be harbored on this segment and it seems that a stability system is also located within this region since stability assays between p31T1 and the R6K-Minrep31T1 minimal

replicon construct did not give a large variation in stability (6%). One putative ORF residing within the minimal replicon (ORF3) was shown to play a role in plasmid maintenance and plasmid copy number control. Inactivation of ORF3 resulted in an ~2.35 fold increase in copy number and a decrease in plasmid stability from ~80% to ~16% plasmid retention over 127 generations (7 days). Maintaining a low copy number is a mechanism of survival for p31T1 and ORF3 ensures that plasmid copy number does not deviate from steady state concentration. The question remains why an inactivated ORF3 would have a decreased stability yet the copy number is increased? A possible explanation for this is that in the absence of a stability system, the plasmids are not properly distributed within the cytoplasm in a manner that favours distribution into daughter cells but may be clumped together in one location. Thus the number of units to be partitioned is reduced.

Given its size (14-kb) plasmid p31T1 was expected to have a higher copy number than the experimentally determined ~3 plasmids per chromosome. Large plasmids are usually maintained at very low copy numbers (example, F-plasmid, 1-2 copies per chromosome) so as to not be a metabolic burden to the host while small plasmids (<10-kb) are often maintained at much higher copy numbers (>20 copies) (Nordström and Austin, 1989; Providenti *et al*., 2006). A 14-kb IncQ-like, broad host-range plasmid isolated from *Acidithiobacillus caldus*, pTC-F14, was shown to have a moderate copy number of 12-16 plasmids per chromosome in both *E. coli* and *A. caldus* (Gardner *et al*., 2001). Two other 11.8-kb plasmids isolated from *Aeromonas salmonicida*, namely pRAS3.1 and pRAS3.2 had an even higher copy number of 45 ± 13 and 30 ± 5 plasmids per chromosome respectively (Loftie-Eaton and Rawlings, 2009). Plasmid p31T1 would presumably have a very stringent regulatory system for replication and its control and to lower the burden it imposes on the host. Higher copy number plasmids can to some extent rely on random distribution for their stable maintenance, but p31T1 cannot follow such a route. Also high copy number plasmids require increased amounts of regulator protein in order to down-regulate replication.

The strong activity of the ORF3 promoter was shown to be controlled by negative autoregulation mediated by ORF3. Negative autoregulation mechanisms cause a

reduction in the steady state levels of the gene product and limits fluctuations between cells (Semsey *et al*., 2009). This has a stabilizing effect within regulatory networks and allows for a faster response time (Rosenfeld *et al*., 2002). Disruption of a gene which autoregulates itself results in increased promoter activity due to its inability to repress its own promoter. Sequence analysis indicated that ORF3 contains a RHH-like domain common to the CopG family of transcriptional regulators and, therefore, it is likely that ORF3 functions as a transcriptional regulator. The activities of the putative promoters of ORF2 and ORF3 (long) were very low and could not be assayed for β-galactosidase activity when p31T1 and R6K-Minrep31T1 were placed *in trans* since no viable cells were obtained. The reason for the inability to co-transform the plasmids is uncertain, but it is possible that titration of important proteins by the high copy number of the promoter region occurred when placed *in trans*. If, for example, ORF2 was a putative replication initiator that also binds its own promoter, this could explain initiator titration by the promoter region which would subsequently affect replication of p31T1 negatively.

Analysis using RibEx web based software (Abreu-Goodger and Merino, 2005) and ARNold (http://rna.igmors.u-psud.fr/toolbox /arnold/index.php), allowed the identification of putative secondary structures and/or riboswitches possibly involved in regulation of certain ORFs within the minimal replicon of p31T1. These secondary structures are illustrated in figure 4.1. The likelihood of occurrence of these structures are reflected in their respective ΔG values, chances of structures C, D, E and F formation are much higher than for the A and B structures. This can also be justified when observing the structure layout of the putative hairpin formations.

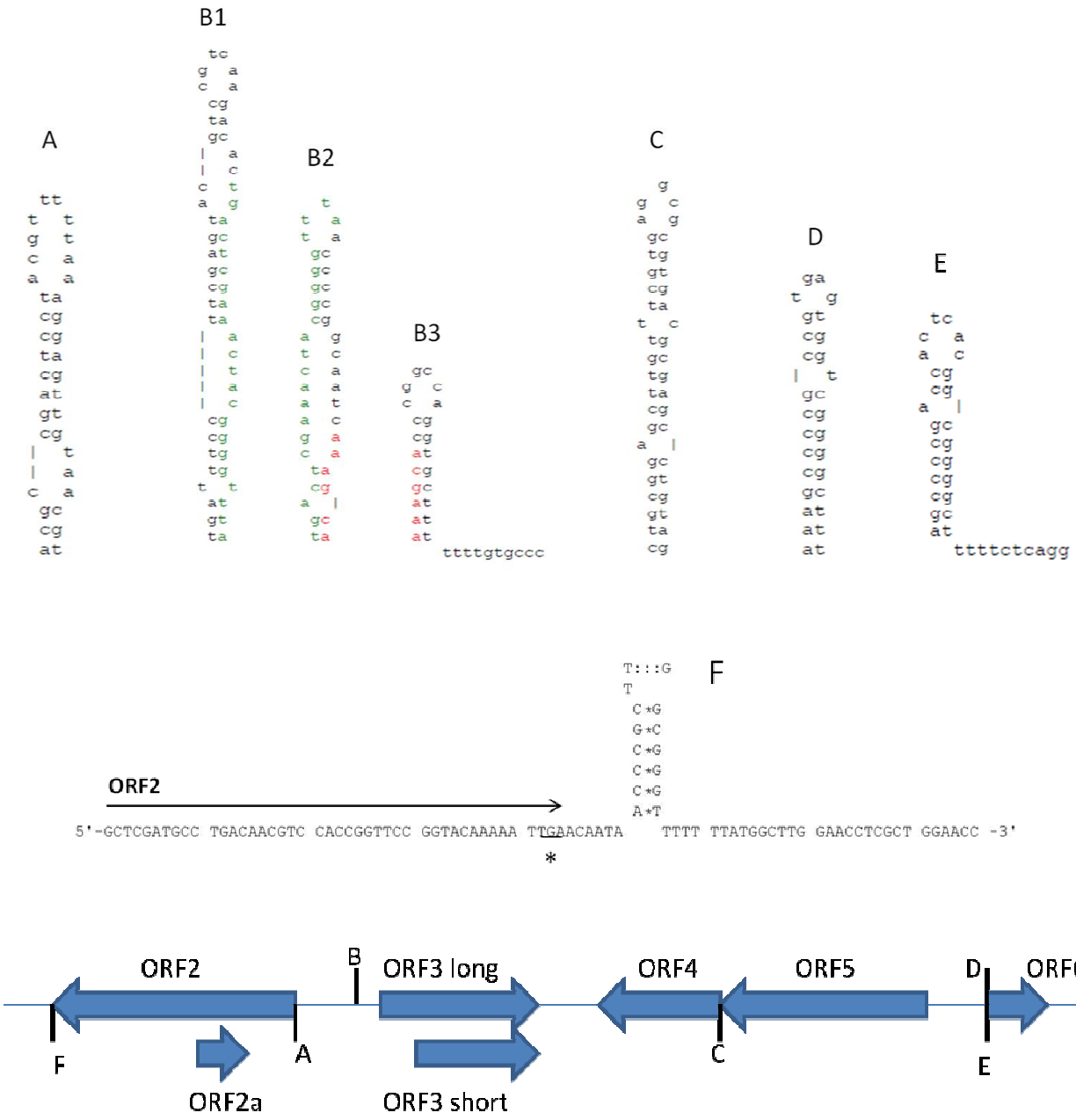


FIG.4.1. Putative secondary structures (top) with a possible role in replication and/or transcription regulation and a physical map (below) to illustrate their location. A. Stem-loop preceding ORF2 (ΔG = -10.2 kcal/mol); B1. Anti-antiterminator stem-loop (ΔG = -10.9 kcal/mol); B2. Antiterminator stem-loop (ΔG = -9.29 kcal/mol); B3. Terminator stem-loop (ΔG = -10.0 kcal/mol); C. Stem-loop preceding ORF4 (ΔG = -17.2 kcal/mol); D. Secondary structure preceding ORF6 (ΔG = -18.2 kcal/mol); E. Stem-loop structure (ΔG = -16.3 kcal/mol). F. Transcriptional attenuator as identified by ARNold (ΔG = -12.7 kcal/mol). Green and red base-pairs represent the corresponding regions of similarity.

Transcriptional attenuation can be rho-independent (intrinsic) or rho-dependent (Ciampi, 2006). Rho-independent termination involves the formation of a GC-rich hairpin structure followed by a set of 6-8 U's. Rho-dependent termination in contrast requires the rho factor for efficient termination of transcription and is found at the start, end or within the coding sequence of genes (Ciampi 2006). Both mechanisms cause the generation of shorter transcripts. Rho-independent terminators have been identified to occur intergenic and intragenic (Ramamoorthy *et al*., 2005). Structure B is representative of a putative riboswitch element. It was emphasized in chapter 3 section 3.4 that ORF3 could be located in an operon configuration with an ORF3 leader peptide. The leader peptide is perfectly in-frame with ORF3 (long) and therefore an intergenic riboswitch regulation mechanism is not unlikely for this "operon". This type of regulation could be similar to that described for plasmid pT181 or pIP501 (chapter 1, section 2.2.3.) The likelihood of structures C and D forming is very high based on the structure formation and ΔG values. The D hairpin perfectly overshadows the putative RBS of ORF6 and could act as an antisense RNA. Although ORF4 does not have a strong putative RBS sequence the presence of a hairpin structure preceding this ORF4 could hint to a translational coupling between ORF4 and ORF5, and is likely to form (high ΔG). Structure E, is a putative rho-independent transcriptional terminator, and is found in the same vicinity as structure D but on opposite strands. The ORF2 gene region was analyzed with ARNold software and the end of the gene showed strong formation of a putative rho-independent terminator (ΔG= -12.7) and the string of 6 T's (U's in the case of mRNA) is apparent.

ORF2 could be regulated by an antisense RNA that may pair with the RBS of ORF2 RNA thereby directly inhibiting translation (similar to ColE2). A transcriptional repressor, similar to CopG in pMV158, could also bind ORF2 promoter and cause repression and act as an accessory protein (possibly the ORF3 protein in the case of p31T1?). CopG belongs to the RHH class of DNA-binding proteins (Hernández-Arriaga *et al*., 2009). ORF3 protein had clear similarity to a RHH domain of the CopG family of transcriptional repressors (spanning 35 amino acids of the total 176 amino acid protein with 34% identity).

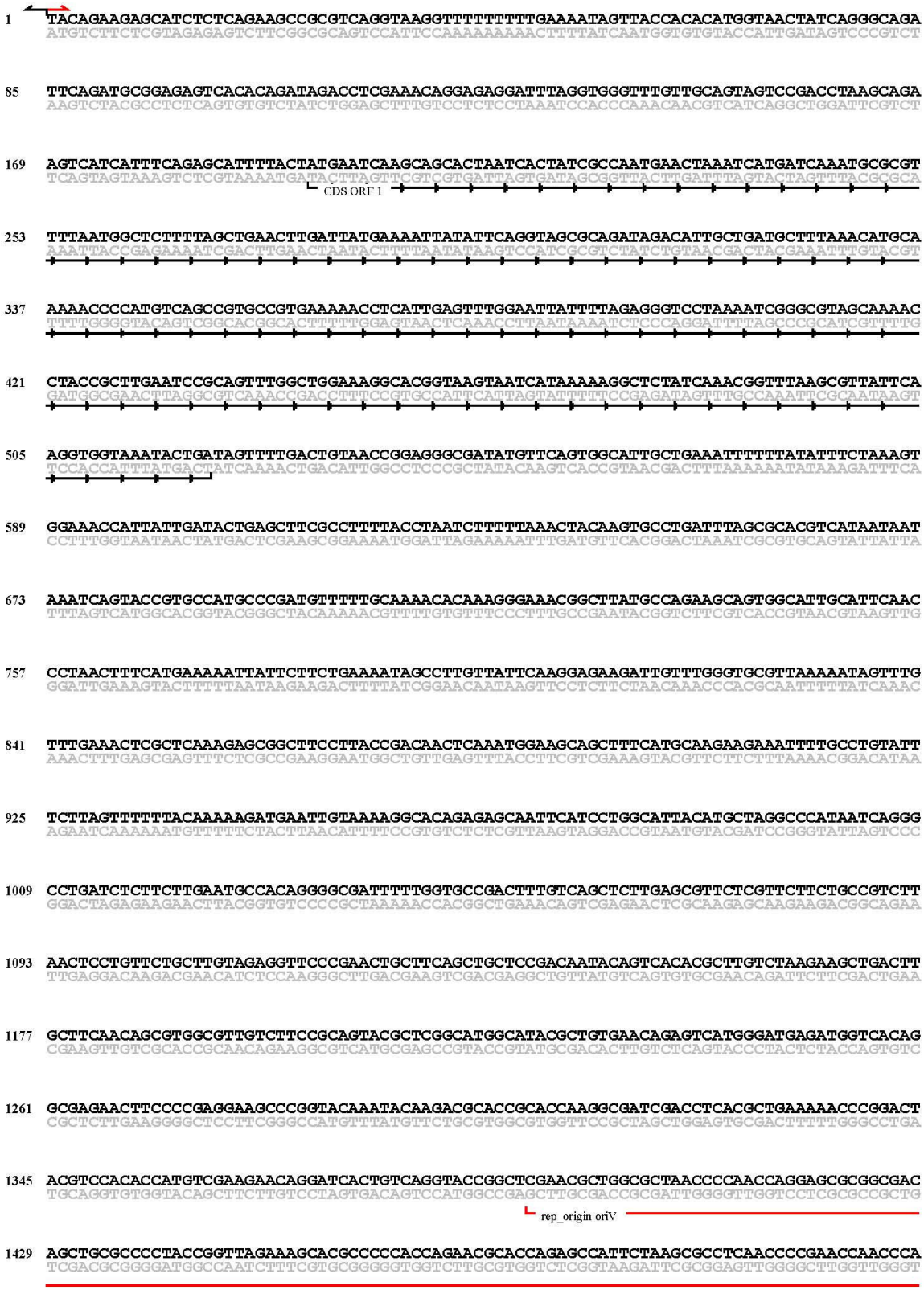
Further studies on p31T1 are required to answer some key questions. For example:

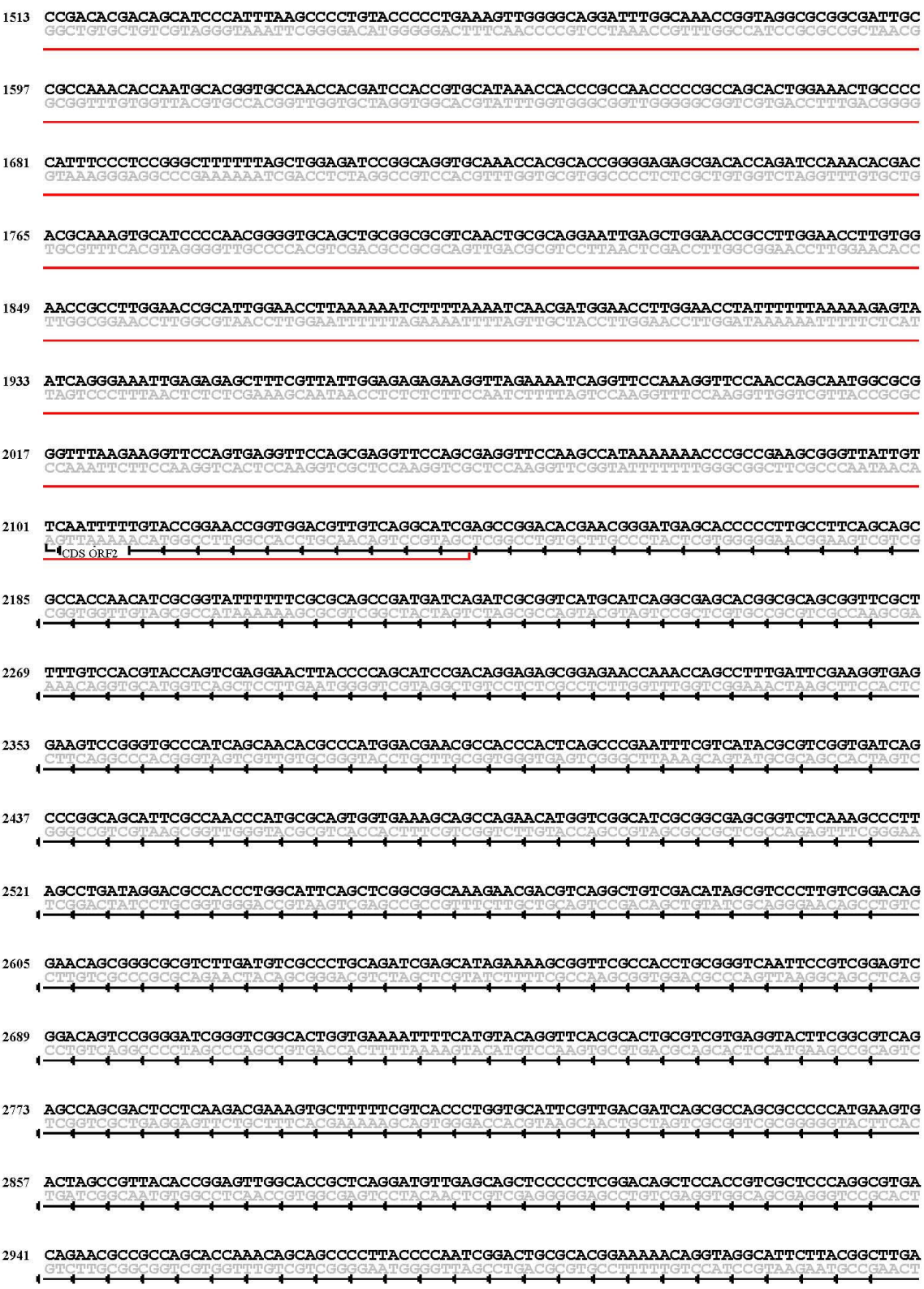
1) whether regulation of antisense RNA is involved
2) if additional stability determinants other than ORF3 exist
3) how does the main mechanism for plasmid copy number control operate and the interplay between proteins and DNA involved in this regulation
4) which protein serves as the replication initiator
5) is ORF3 regulated or does it function in a possible operon configuration
6) whether ORF2 or ORF5 is a primase

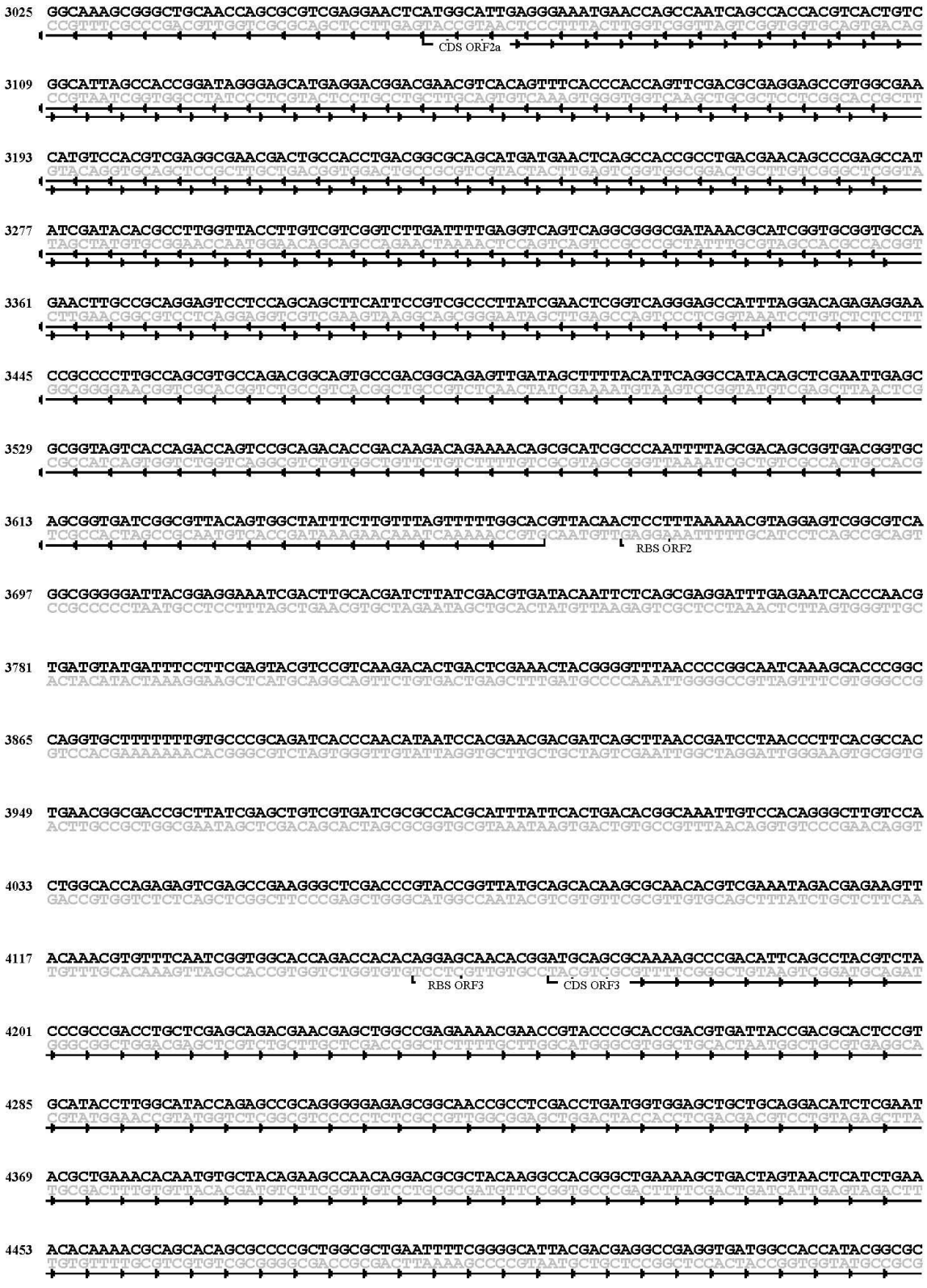
Plasmids are sequenced regularly but it is not often that they are studied more in depth using molecular biology approaches to elucidate the plasmid characteristics and functions. The biological findings of this study would help other researchers who discover relatives of p31T1 to better characterize their plasmids. Plasmid p31T1 proved to be a very interesting plasmid with little identity to previously identified plasmids. This makes it an ideal candidate for further studies which could extend our knowledge of plasmid biology diversity. The replicon and mobilization systems of p31T1 seem unique and possibly need to be placed in a new family. The misuse of tetracyclines in aquaculture practices for the control of furunculosis within fish has led to the emergence of plasmid encoded tetracycline resistance within these pathogens (Smith *et al*., 1994; Adams *et al*., 1998; Sandaa and Enger, 1994; Sandaa and Enger, 1996). Plasmid p31T1 is an example of such a plasmid. This is problematic and poses a threat for the use of tetracyclines in these practices. In fact, tetracycline resistance within *Aeromonas salmonicida* increased from 4% to more than 50% from ~1980 to ~1990 in Scottish fish farms (Aoki *et al*., 1983; Richards *et al*., 1992). Although p31T1 was first shown to be resistant to erythromycin and nalidixic acid in addition to tetracycline, no such genes could be identified, however nalidixic acid resistance could also be associated with mutations (Crumplin, 1987). The molecular characterization of such plasmids like p31T1 which contribute to the *Aeromonas* virulence, and how such a plasmid persists in the presence and absence of antibiotic selection could help facilitate better aquaculture farming practices and lead to the development of alternative treatment solutions.

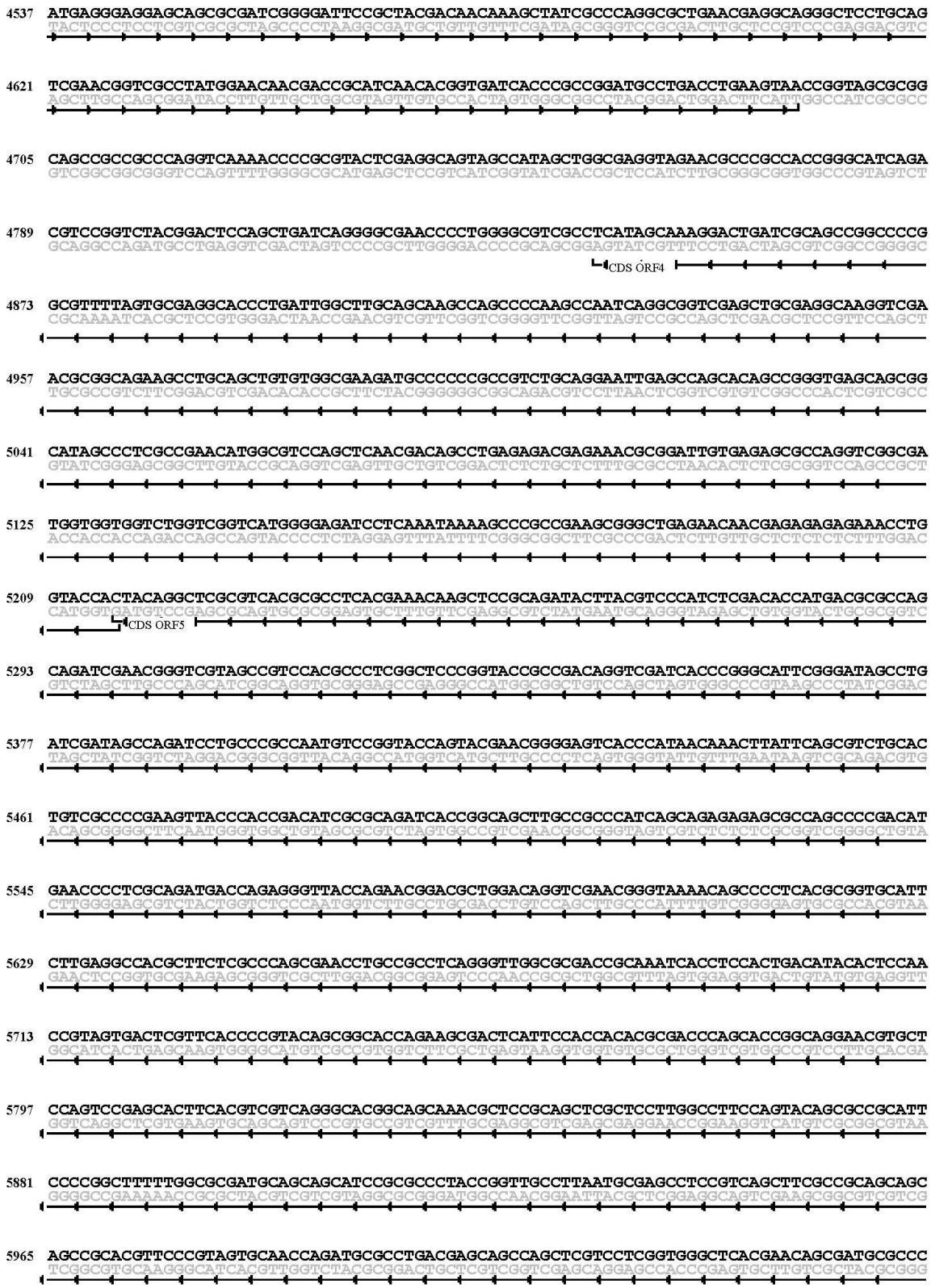
Addendum A

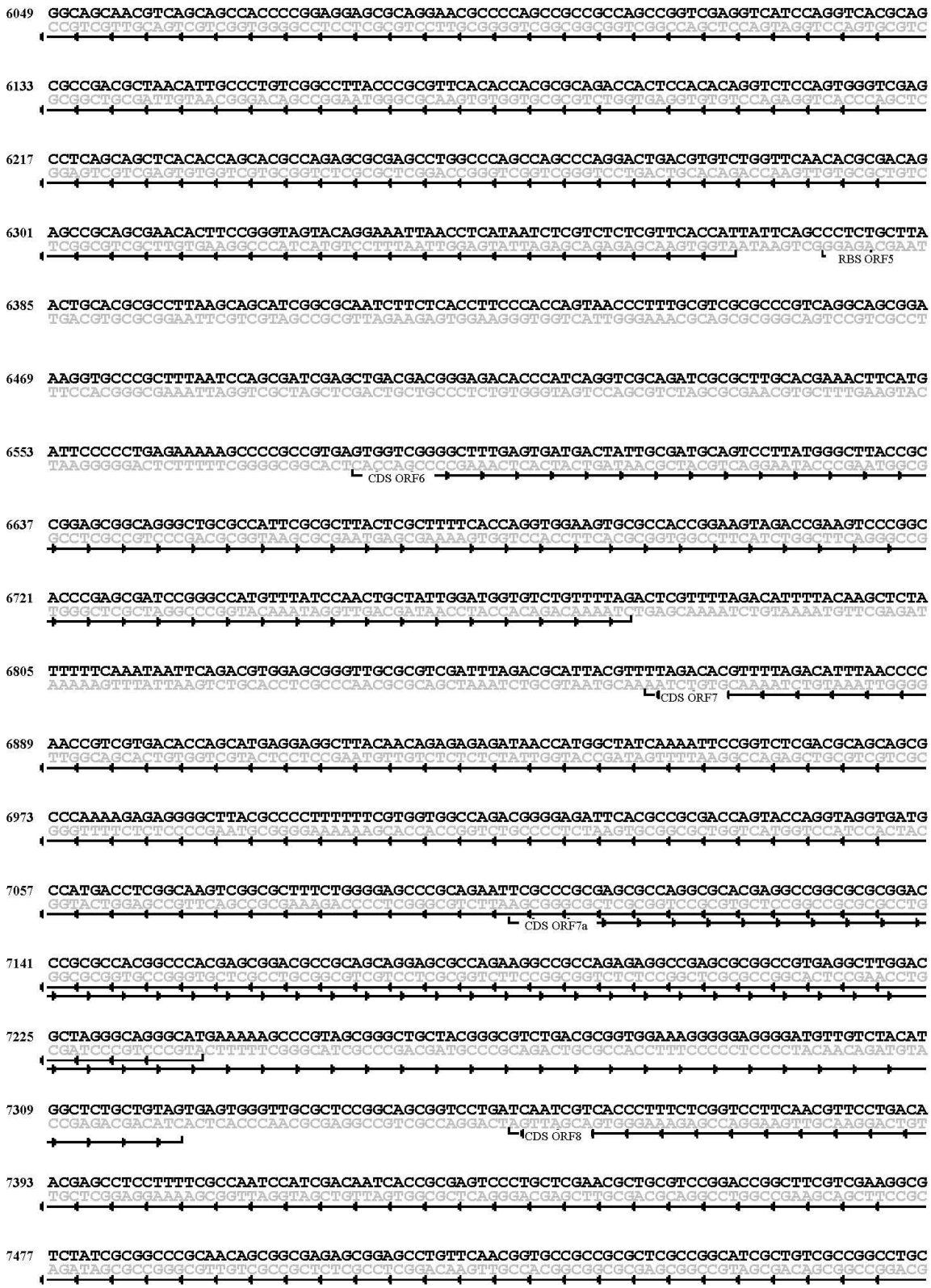
p31T1 Sequence Annotation

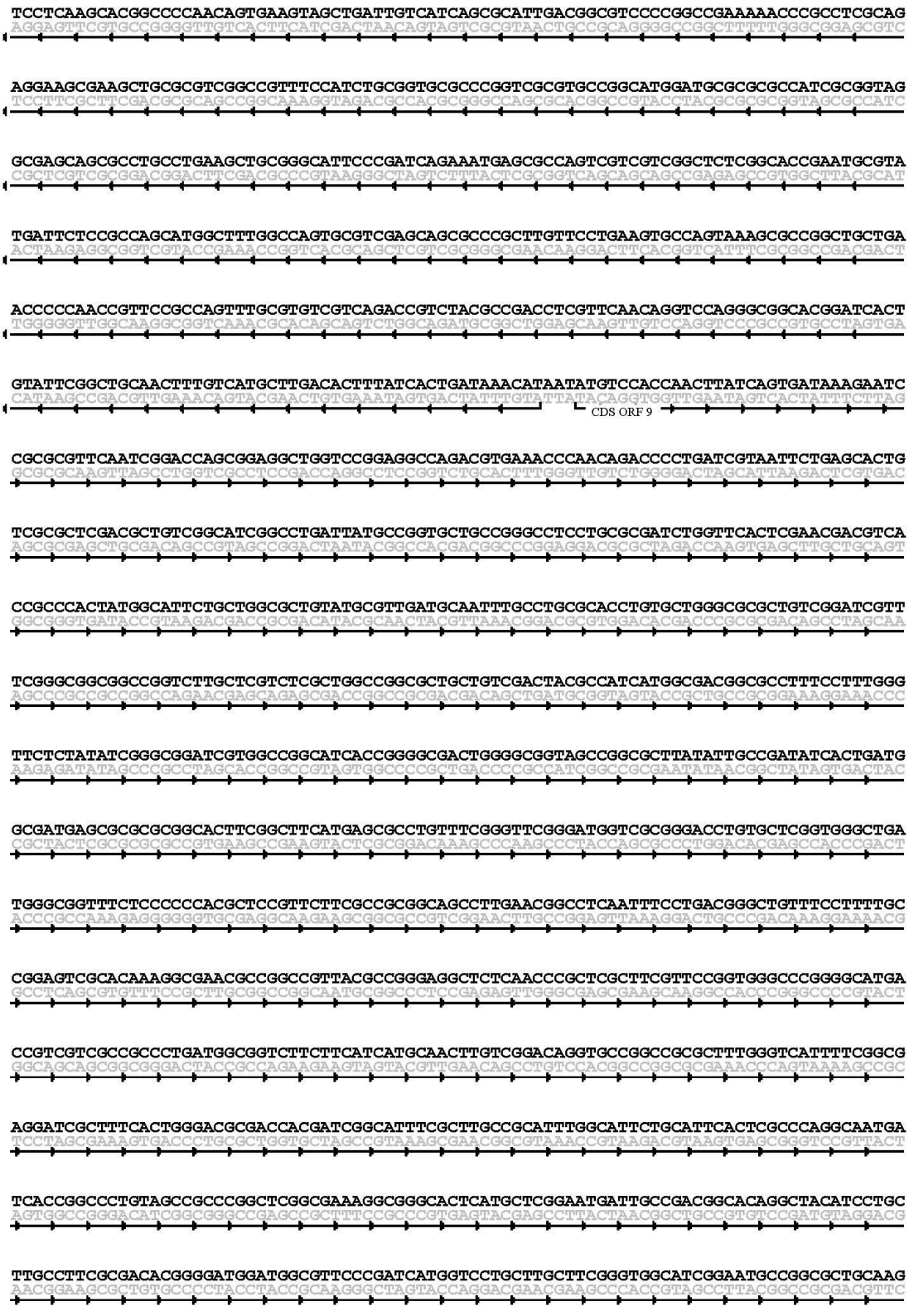


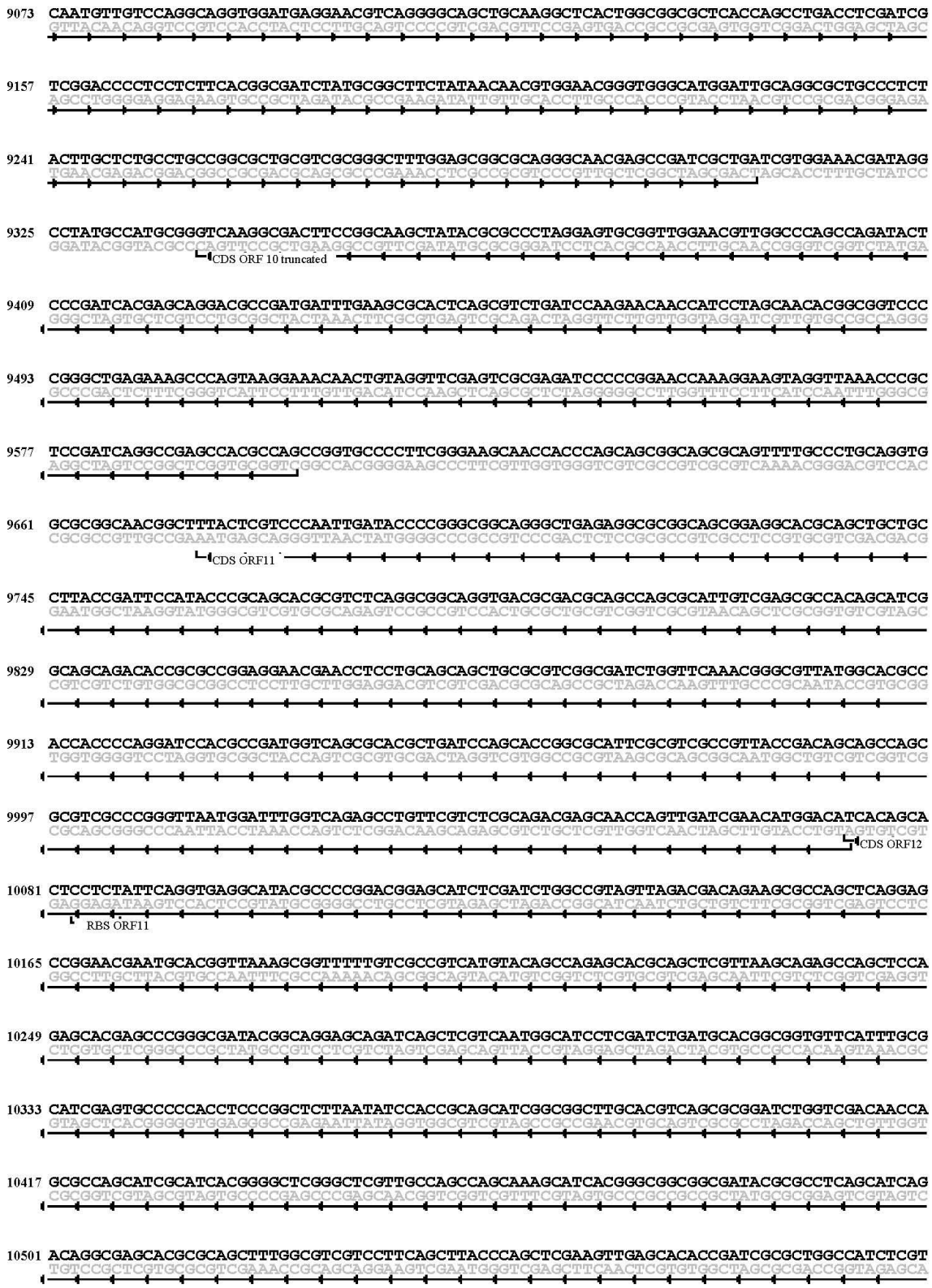


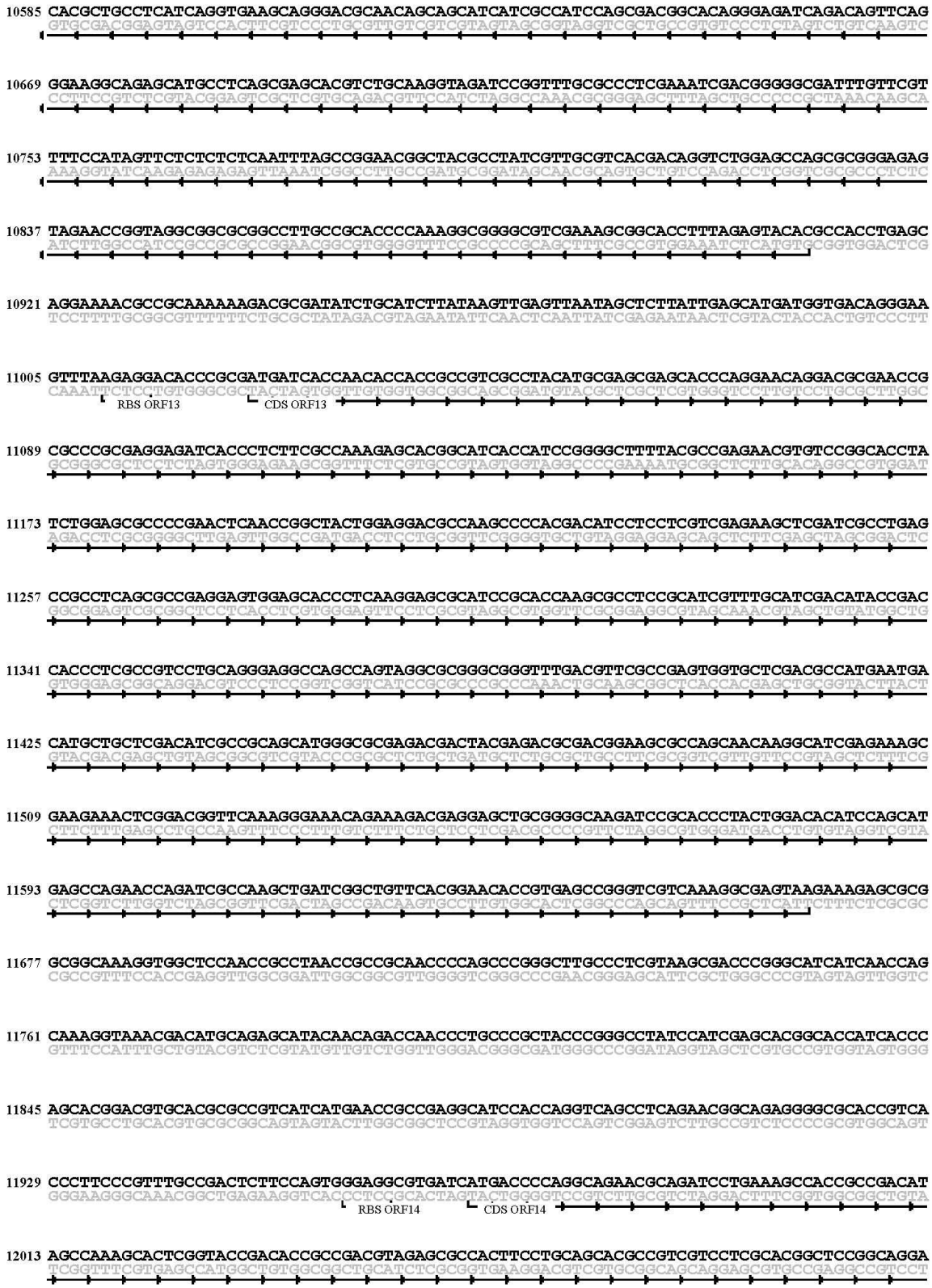


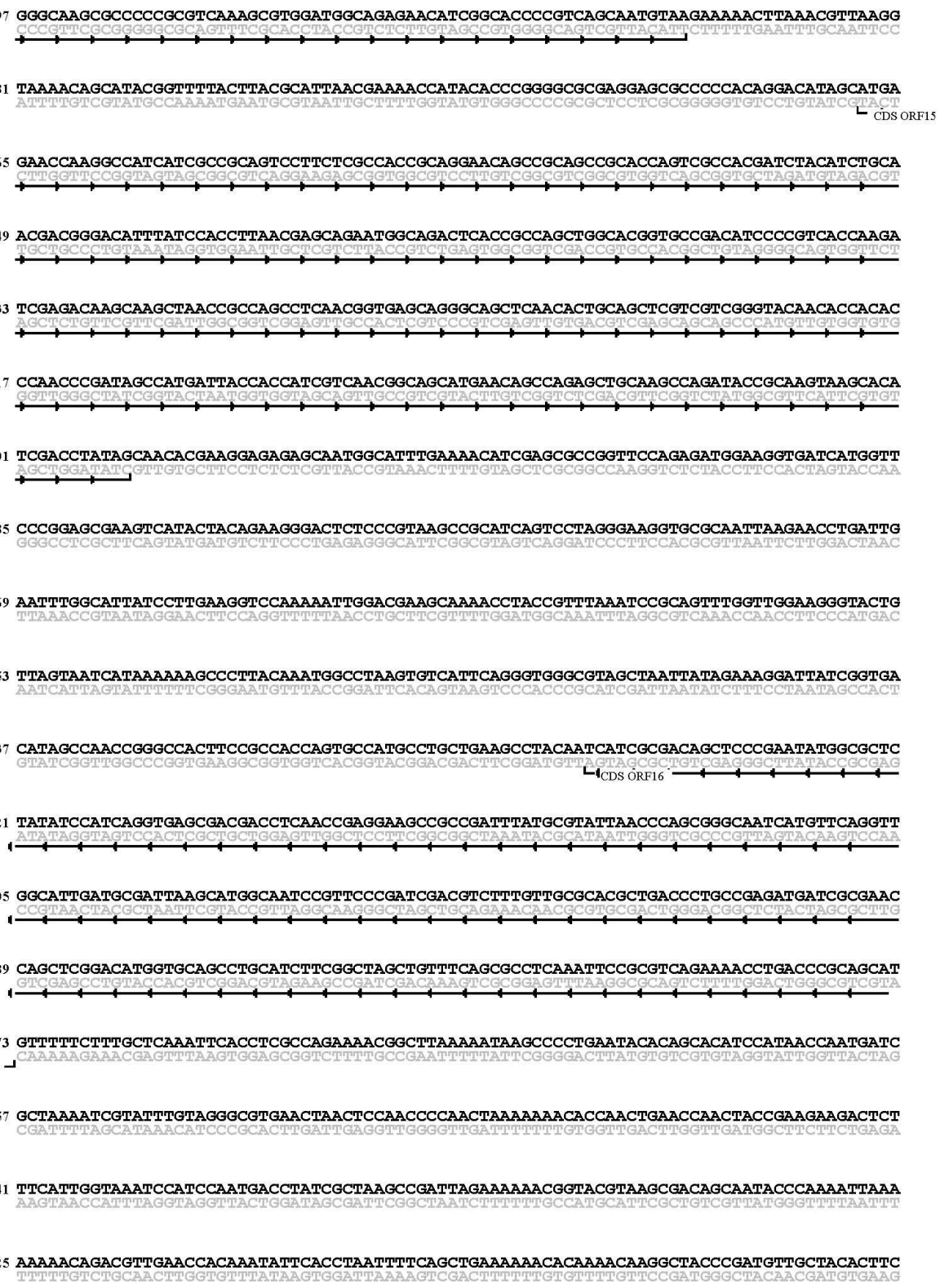












13609 GCTACGCTCAATGCTGCACATCGGGCCTTGCAATGGGCGACTTCGCCTCCCCTTGACCCCTCCCTTGCAGCAGTCGCTTTGATC
CGATGCGAGTTACGACGTGTAGCCCGGAACGTTACCCGCTGAAGCGGAGGGGAACTGGGGAGGGAACGTCGTCAGCGAAACTAG

13693 CTTCGCCCAGGGCAACGGCGTCTGTCAGGTGGATGGCCTCACTGCGTGACACCACCCACCCCTGACAAGCGTGTTTTGTCCAGA
GAAGCGGGTCCCGTTGCCGCAGACAGTCCACCTACCGGAGTGACGCACTGTGGTGGGTGGGGACTGTTCGCACAAAACAGGTCT

13777 GAGTCGTCAAGGGTGAAATACACTCGCTACGCTCGCCCTTGACGAAATCAGGCAATACCAGCGCCTTTTGAATGAGCAGGTACC
CTCAGCAGTTCCCACTTTATGTGAGCGATGCGAGCGGGAACTGCTTTAGTCCGTTATGGTCGCGGAAAACTTACTCGTCCATGG

13861 GCTCGCCGCAGTTCCGGCTGAGGCGTACCGCAGCGCAGCGAGGAACGAACAGCCGGAACGAGGAAGCGGAATATCATCTGAACG
CGAGCGGCGTCAAGGCCGACTCCGCATGGCGTCGCGTCGCTCCTTGCTTGTCGGCCTTGCTCCTTCGCCTTATAGTAGACTTGC

13945 GGCATAGAAATGCGTTCGCTCCGTGTGACATATACTGTAATCAGCAACGTGCAGTAACAAGA
CCGTATCTTTACGCAAGCGAGGCACACTGTATATGACATTAGTCGTTGCACGTCATTGTTCT

Addendum B

p31T1 Sequence Analysis Table

ORF position (bp)	Size	Putative RBS	Proposed function/related protein	Superfamily/domain hits and region of protein	% Identity (part of protein	Protein coverage	BLAST e-value	NCBI accession nr
ORF1 **194-520**	109aa 327bp	Poor	RepA *Salmonella enterica* subsp *enterica* serovar Montevideo	HTH (pfam13463) 40-78aa winged helix DNA binding domain	99/107 93%	1-107 aa 99%	$7x10^{-67}$	ZP12162451.1
ORF2 **3660-2101**	520aa 1560bp GTG start	AGGAG	putative primase *Citrobacter* sp. A1		179/471 38%	51-514aa 89%	$4x10^{-92}$	ZP10409347.1
ORF2a **3061-3432**	123aa 369bp	Poor	putative plasmid mobilization protein – MobA/MobL region (31-90aa of ORF2a) *Xanthomonas fuscans* subsp *aurantifoli*		32/105 30%	14-115aa 82%	1.6	ZP06703579.1
ORF3 **4165-4692**	176aa 528bp	AGGAG	hypothetical protein – region RHH protein copG family *Citrobacter* sp. A1	RHH (pfam01402) 10-45aa copG family; protein repressor; homodimeric RHH; helix-turn-helix involved in dimerization	57/168 34%	2-163aa 92%	$3x10^{-10}$	ZP10409348.1
ORF4 **5215-4841**	125aa 375bp GTG start	Poor	1) hypothetical protein *Yersinia enterocolitica* 2) hypothetical protein *Citrobacter* sp. A1 3) hypothetical protein *Klebsiella pneumoniae*		36/82 44% 35/75 47% 34/75 45%	24-102aa 63% 24-96aa 58% 24-96aa 58%	$7x10^{-10}$ $2x10^{-09}$ $2x10^{-09}$	YP002643129.1 ZP10409335.1 YP002286974.1

ORF5 **6366-5215**	384aa 1152bp	AGAGG	1)hypothetical protein – TOPRIM region; Topoisomerase primase domain; DnaG type primases; *traC*-like *Klebsiella pneumonia* 2) hypothetical protein *Yersinia enterocolitica* 3)AAA ATPase *Citrobacter* sp. A1		177/389 46% 170/388 44% 170/390 44%	2-380aa 98% 2-379aa 98% 2-379aa 98%	$2x10^{-94}$ $1x10^{-88}$ $1x10^{-86}$	YP002286973.1 YP002643130.1 ZP10409336.1
ORF6 **6598-6816**	72aa 216bp GTG start	poor	putative relaxase/mobilization protein *Serratia symbiotica* str *Tucson*		19/55 35%	10-56aa 65%	4.3	ZP08039630.1
ORF7 **7239-6862**	126aa 378bp	Poor	putative site-specific recombinase *Escherichia coli*		27/27 100%	1-27aa 21%	$2x10^{-08}$	YP002527533.1
ORF7a **7253-7472**	221-bp truncated at N-terminal by 22-bp	Poor	1)transposase *Escherichia coli* O104:H4 2) *tnpA* delta 5; putative relaxase *Aeromonas allosaccharophila*		221/221 100% 221/221 100%	1-221 bp 100% 1-221 bp 100%	$3x10^{-108}$ $3x10^{-108}$	CP003289.1 HM453327.1
ORF8 **8030-7353**	226aa 678bp	Poor	TetR *Escherichia coli*		99/100 99%	1-226aa 100%	$9x10^{-116}$	YP025723.1
ORF9 **8034-9308**	425aa 1275bp	Poor	TetA *Escherichia coli*		100/100 100%	1-425aa 100%	0.0	ABF71536.1
ORF10 **9600-9339**	86aa truncated at N-terminal by 207aa	Poor	PecM-like protein *Klebsiella pneumoniae*	EamA like transporter family (pfam00892) 1-68aa	86/86 100%	1-86aa 100%	$4x10^{-50}$	BAM29024.1

ORF11 **10073-9675**	133aa 399bp	AGGAG	low temperature protein A *Citrobacter* sp. A1		56/123 46%	8-130aa 93%	$4x10^{-13}$	ZP10409340.1
ORF12 **10909-10073**	279aa 837bp GTG start	Poor	1) Emm-like cell surface protein CspZ.2 – region Spc24 subunit of Ndc80 (Spc24 involved in *S. cerevisiae* chromosome segregation) *Streptococcus equi* subsp *zooepidemicus* 2) hypothetical protein – region chromosome segregation *Leishmania mexicana*		33/122 27% 23/60 38%	92-209aa 42% 87-143aa 20%	0.65 3.3	YP002124236.1 XP003872792.1
ORF13 **11024-11665**	214aa 642bp	AGAGG	Resolvase/TnpR-like protein 1) hypothetical protein *Yersinia enterocolitica* 3) TnpR *Salmonella enterica* subsp. *enterica* 4) Transposon Tn3 resolvase *Escherichia coli*	1) Ser Recombinase superfamily (cd03767) 10-158aa partitioning resolvase subfamily; similar to resolvase found in RP4 par region; similar to Tn3; catalyze site-specific recombination 2) HTH (pfam13936) 170-210aa found in transferases likely involved in DNA binding	 134/202 66% 105/203 52% 105/205 51%	 10-211aa 94% 9-211aa 95% 9-213aa 96%	 $6x10^{-88}$ $3x10^{-59}$ $9x10^{-59}$	 YP002643121.1 ZP02669871.1 YP006953666.1
ORF14 **11969-12160**	64aa 192bp	GGGAG	no significant BLAST hits high Glimmer score (6.50)		N/A	N/A	N/A	
ORF15 **12261-12611**	117aa 351bp	Poor	no significant BLAST hits high Glimmer score (2.43)		N/A	N/A	N/A	
ORF16 **13272-12991**	94aa 282bp	Poor	MobC protein *Rahnella* sp. WMR104	MobC (pfam05713) $3.06x10^{-04}$ 51-93aa	72/92 78%	1-92 98%	$3x10^{-44}$	YP006960812.1

Addendum C

Strains and Plasmids used in this study

Bacterial strains and plasmids used in this study

Strains	***Description***	***Reference/source***
Agrobacterium tumefaciens	Prototrophic	Rawlings culture collection
Escherichia coli ACSH50I^{q}	*rspL* Δ(*lac-pro*) (F' *traD36 proAB lacI*^{q} ΔM15) Cm^{R}	Smith and Rawlings, 1998
E. coli CSH50I^{q}	*rspL* Δ(*lac-pro*) (F' *traD36 proAB lacI*^{q} ΔM15)	Smith and Rawlings, 1998
E. coli DH5α	φ80*dlacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U196*	Promega Corp
E. coli EC100D *pir*$^{+}$	*F*$^{-}$ *mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (Str*^{R}*) nupG pir*$^{+}$*(DHFR)*	Epicentre® Biotechnologies
E. coli S17.1	*recA pro hsdR* (RP4-2 Tc::Mu Km::Tn7)	Simon *et al*., 1983
Pseudomonas putida	Prototrophic	Rawlings culture collection

Plasmids	***Description***	***Reference/source***
EZ-Tn5™	Km^{R}, R6K γ-*ori*	Epicentre® Biotechnologies
ORF2lacZ	In-frame cloning of ORF2 promoter from pGemORF2fus into the *Bam*HI-*Eco*RI sites of pMC1403	This study
ORF3lacZ (short)	In-frame cloning of ORF3 (short) promoter from pGemORF3fus(short) into *Bam*HI-*Eco*RI sites of pMC1403	This study
ORF3lacZ (long)	In-frame cloning of ORF3 (long) promoter from pGemORF3fus(long) into *Bam*HI-*Eco*RI sites of pMC1403	This study
ORF4lacZ	In-frame cloning of ORF4 promoter from pGemORF4fus into *Bam*HI-*Eco*RI sites of pMC1403	This study
ORF5lacZ	In-frame cloning of ORF4 promoter from pGemORF5fus into *Bam*HI-*Eco*RI sites of pMC1403	This study

p31T1	14-kb natural plasmid isolated from *Aeromonas sobria*	Marx (MSc thesis)
p36T2	14-kb natural plasmid isolated from *Aeromonas hydrophila*	Marx (MSc thesis)
pBAD28	Ap^R, Cm^R, arabinose-inducible expression vector, pACYC184 replicon	Guzman *et al*., 1995
pBAD28-ORF2	1-kb *Sac*I-*Xba*I fragment subcloned from pGem-T Easy-ORF2 into pBAD28	This study
pBAD28-ORF3	500bp *Sac*I-*Xba*I fragment subcloned from pGem-T Easy-ORF3 into pBAD28	This study
pBAD28-ORF4	922-bp *Sma*I-*Spe*I fragment from R6K-Minrep31T1 cloned into pBAD28	This study
pBAD28ΔAmp-ORF3	pBAD28-ORF3 digested with *Apa*LI, blunted and religated	This study
pBluescript KS (±)	Ap^R, *LacZ'*, ColE1 replicon, vector	Stratagene
pBluescript SK (±)	Ap^R, *LacZ'*, ColE1 replicon, vector	Stratagene
pBR322	Ap^R, Tc^R, ColE1 replicon, cloning vector	Bolivar *et al*., 1977
pGem®-T	Ap^R, T-tailed PCR product cloning vector	Promega Corp
pGem®-T Easy	Ap^R, T-tailed PCR product cloning vector	Promega Corp
pGem-T Easy-ORF2	1.6-kb ORF2 PCR product from p31T1 cloned into pGem®-T Easy	This study
pGem-T Easy-ORF3	500-bp *Sac*I-*Xba*I fragment from R6K-Minrep31T1 cloned into pGem®-T Easy	This study
pGemORF2fus	ORF2 promoter region from p31T1 PCR-cloned into pGem®-T Easy	This study
pGemORF3fus(short)	ORF3 (short) promoter region from p31T1 PCR-cloned into pGem®-T Easy	This study
pGemORF3fus(long)	ORF3 (long) promoter region from p31T1 PCR-cloned into pGem®-T Easy	This study
pGemORF4fus	ORF4 promoter region from p31T1 PCR-cloned into pGem®-T Easy	This study
pGemORF5fus	ORF5 promoter region from p31T1 PCR-cloned into pGem®-T Easy	This study
pGemdxs	290-bp chromosomal *dxs* gene product PCR cloned into pGem®-T Easy	This study
pMC1403	Ap^R, promoterless *lacZYA* operon, ColE1 replicon	Casadaban *et al*., 1983

pOU82	Ap^R, *LacZYA*, R1 replicon	Gerdes *et al*., 1985
pOU82-ORF3	1.2-kb *Bam*HI-*Eco*RI fragment from SKORF3 cloned into pOU82	This study
pRAS3.1.74	Tc^r; pRAS3.1.75 derivative with four 6-bp repeats from pRAS3.2 by exchanging the 2.9-kb *Hind*III-*Pvu*I region	Loftie-Eaton and Rawlings, 2010
pUC19	Ap^R, *LacZ'*, ColE1 replicon, cloning vector	Yanisch-Perron *et al*., 1985
pUCBM21	Ap^R, *LacZ'*, ColE1 replicon, cloning vector	Roche Molecular Biochemicals
pUCBM21-*oriV*	1.2-kb *oriV* fragment of p31T1 cloned into pUCBM21	Vos, Hons thesis
R6K-Minrep31T1	5.7kb minimal replicon fragment of p31T1 cloned into EZ-Tn5	This study
R6K-Minrep::ORF2	R6K-Minrep31T1 digested with *Sal*I, blunted and religated	This study
R6K-Minrep::ORF3	R6K-Minrep31T1 digested with *Spe*I, blunted and religated	This study
R6K-Minrep::ORF4	R6K-Minrep31T1 digested with *Xcm*I, blunted and religated	This study
R6K-Minrep::ORF5	R6K-Minrep31T1 digested with *Xma*I, blunted and religated	This study
R6KoriV360	360bp *Sal*I-*Xba*I fragment cloned from SKoriV360 into EZ-Tn5	This study
R6KoriV750	360bp *Sal*I-*Xba*I fragment cloned from SKoriV750 into EZ-Tn5	This study
R6KoriV1000	1kb *Sal*I-*Xba*I fragment cloned from SKoriV1000 into EZ-Tn5	This study
RSF1010K	Km^R, 1-1704bp of RSF1010 replaced by Tn903	G. Ziegelin
SKORF3	1.19kb *Dra*I-*Nae*I fragment of R6K-Minrep31T1 cloned into pBluescript SK+	This study
SKoriV360	360bp *Pvu*II fragment cloned from pUCBM21-*oriV* into pBluescript SK+	This study
SKoriV750	750bp *Taq*I fragment cloned from pUCBM21-*oriV* into pBluescript SK+	This study
SKoriV1000	1kb *Pvu*II fragment cloned from pUCBM21-*oriV* into pBluescript SK+	This study

Addendum D

Primers used in this study

Primers used in this study		
Primer	***Primer sequence***	***Source***
dxs fwd	TGCTGGTGATTCTCAACGA	This study
dxs rev	TCGCGCATGTTCTTTAGC	This study
LACZPRI	CGCCAGCTGGCGAAAGGGGG	A. Smith
ORF2fus fwd	GAATTCGCGCGATCACGACAG	This study
ORF2fus rev	GGATCCGGCGTTACAGTGGC	This study
ORF3fus(short) fwd	GGAATTCGCACGATCTTATCG	This study
ORF3fus(short) rev	AGGATCCGTCTGCTCGAGC	This study
ORF3fus(long) fwd	GAATTCTCGAATTGAGCGCGG	This study
ORF3fus(long) rev	GGATCCAGTGAATAAATGCGTGG	This study
ORF4fus fwd	GAATTCAACGAGTCACTACG	This study
ORF4fus rev	GGATCCGAGAACAACGAG	This study
ORF5fus fwd	GAATTCGGGACTTCGGTCTAC	This study
ORF5fus rev	GGATCCGGGTAGTACAGGA	This study
pUC/M13 fwd	GTTTTCCCAGTCACGAC	Promega
pUC/M13 rev	CAGGAAACAGCTATGAC	Promega
Minrep31T1 fwd	CAGGTAGGCATTCTTACGGC	This study
Minrep31T1 rev	CAAGGCGTGTATCGATATGG	This study
pBAD28ORF2 fwd	TAGAGCTCGCCGACTCCTACG	This study
pBAD28ORF2 rev	TATCTAGAAGGTTCCAGTGAG	This study
pBAD28ORF3 fwd	GAGCTCAGGAGCAACACG	This study
pBAD28ORF3 rev	TCTAGAGGTTACTTCAGGTC	This study

Literature Cited

LITERATURE CITED

Abeles, A. L., K. M. Snyder, and D. K. Chattoraj. 1984. P1 plasmid replication: replicon structure. J.Mol.Biol. **173**:307-324.

Abeles, A. L., T. Brendler, and S. J. Austin. 1993. Evidence of two levels of control of P1 *oriR* and host *oriC* relication origins by DNA adenine methylation. J.Bacteriol. **175**:7801-7807.

Abeles, A. L., L. D. Reaves, B. Youngre-Grimes, and S. J. Austin. 1995. Control of P1 plasmid replication by iterons. Mol.Microbiol. **18**:903-912.

Abreu-Goodger, C. and E. Merino. 2005. RibEx: a web server for locating riboswitches and other conserved bacterial regulatory elements. Nucl.Acids Res. **33** :W690-W692 doi:10.1093/nar/gki445.

Acebo, P., M. Garcia de Lacoba, G. Rivas, J. M. Andreu, M. Espinosa, and G. del Solar. 1998. Structural features of the plasmid pMV158-encoded transcriptional repressor CopG, a protein sharing similarities with both Helix-Turn-Helix and β-Sheet DNA binding proteins. Proteins: Structure, Function and Genetics **32**:248-261.

Adams, C. A., B. Austin, P. G. Meaden, and D. McIntosh. 1998. Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. Appl.Environ.Microbiol. **64**:4194-4201.

Alvarado, A., M. P. Garcillán-Barcia, and F. de la Cruz. 2012. A Degenerate Primer MOB Typing (DPMT) Method to Classify Gamma-Proteobacterial Plasmids in Clinical and Environmental Settings. PLos ONE **7**:e40438.

Andrup, L., J. Damgaard, K. Wasserman, L. Boe, F. G. Madsen, and F. G. Hansen. 1994. Complete nucleotide sequence of the *Bacillus thuringiensis* subsp. *israelensis* plasmid pTX14-3 and its correlation with biological properties. Plasmid **31**:72-88.

Aoki, T., T. Kitao, N. Iemura, Y. Mitoma, and T. Nomura. 1983. The susceptibility of *Aeromonas salmonicida* strains isolated in cultured and wild salmonids to various chemotherapeutants. Bull.Jpn.Soc.Sci.Fish. **49**:17-22.

Asano, K., H. Moriwaki, and K. Mizobuchi. 1991. Positive and negative regulations of plasmid ColIb-P9 *repZ* gene expression at the translational level. J.Biol.Chem. **266**:3774-3781.

Asano, K. and K. Mizobuchi. 1998. An RNA pseudoknot as the molecular switch for translation of the *repZ* gene encoding the replication initiator of Inclα plasmid ColIb-P9. J.Biol.Chem. **273**:11815-11825.

Atlung, T., B. B. Christensen, and F. G. Hansen. 1999. Role of the Rom protein in copy number control of plasmid pBR322 at different growth rates in *Escherichia coli* K-12. Plasmid **41**:110-119.

Austin, B. and C. Adams. 1996. Fish Pathogens, p. 197-243. *In* B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (eds.), The genus *Aeromonas*. John Wiley & Sons, Chichester.

Austin, S. and A. Abeles. 1983. Partition of unit-copy miniplasmids to daughter cells. I. P1 and F miniplasmids contain discrete, interchangeable sequences sufficient to promote equipartition. J.Mol.Biol. **169**:353-372.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1993. *Current protocols in molecular biology*. Wiley Interscience, New York.

Baas, P. D. 1985. DNA replication of single-stranded *Escherichia coli* DNA phages. Biochim.Biophys.Acta **825**:111-139.

Baas, P. D. and H. S. Jansz. 1988. Single-stranded DNA phage origins. Curr.Top.Microbiol.Immunol. **136**:31-70.

Backert, S., K. MeiBner, and T. Borner. 1997. Unique features of the mitochondrial rollling circle-plasmid mp1 from the higher plant *Chenopodium album*. Nucl.Acids Res. **25**:582-589.

Becker, E., N. C. Herrera, F. Q. Gunderson, A. I. Derman, A. L. Dance, J. Sims, R. A. Larsen, and J. Pogliano. 2006. DNA segregation by the bacterial actin AlfA during *Bacillus subtilis* growth and development. EMBO J. **25**:5919-5931.

Berg, D. E., J. Lodge, C. Sasakawa, D. K. Nag, S. H. Phadnis, K. Weston-Hafer, and G. F. Carle. 1984. Transposon Tn5: specific sequence recognition and conservative transposition . Cold Spring Harb.Symp.Quant.Biol. **49**:215-226.

Bernander, R., M. Krabbe, and K. Nordström. 1992. Mapping of the *in vivo* start site for leading strand DNA synthesis in plasmid R1. EMBO J. **11**:4481-4487.

Bernard, P. and M. Couturier. 1992. Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. J.Mol.Biol. **226**:735-745.

Bernard, P., K. E. Kezdy, L. Van Melderen, J. Steyaert, L. Wyns, M. L. Pato, P. N. Higgins, and M. Couturier. 1993. The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. J.Mol.Biol. **234**:534-541.

Berns, K. I. 1990. Parovirus replication. Microbiol.Rev. **54**:316-329.

Bex, F., H. Karoui, L. Rokeach, P. Dreze, L. Garcia, and M. Couturier. 1983. Mini-F encoded proteins: identification of a new 10.5 kilodalton species. EMBO J. **2**:1853-1861.

Blomberg, P., K. Nordström, and E. G. Wagner. 1992. Replication control of plasmid R1: RepA synthesis is regulated by CopA RNA through inhibition of leader peptide translation. EMBO J **11**:2675-2683.

Blower, T. R., F. L. Short, F. Rao, K. Mizuguchi, X. Y. Pei, P. C. Fineran, B. F. Luisi, and G. P. C. Salmond. 2012. Identification and classification of bacterial Type III toxin–antitoxin systems encoded in chromosomal and plasmid genomes. Nucl. Acids Res. **40**:6158-6173.

Boe, L., M. F. Gros, H. te Riele, S. D. Ehrlich, and A. Gruss. 1989. Replication origins of single-stranded-DNA plasmid pUB110. J.Bacteriol. **171**:3366-3372.

Bohne, J., A. Yim, and A. N. Binns. 1998. The Ti plasmid increases the efficiency of *Agrobacterium tumefaciens* as a recipient in *virB*-mediated conjugal transfer of an IncQ plasmid. Proc.Natl.Acad.Sci.USA **95**:7057-7062.

Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicle. II. A multipurpose cloning system. Gene **2**:95-113.

Boyd, J., J. Williams, B. Curtis, C. Kozera, R. Singh, and M. Reith. 2003. Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. Plasmid **50**:131-144.

Bramhill, D. and A. Kornberg. 1988. A model for initiation at origins of DNA replication. Cell **54**:915-918.

Brantl, S., E. Birch-Hirschfeld, and D. Behnke. 1993. RepR protein expression on plasmid pIP501 is controlled by an antisense RNA-mediated transcription attenuation mechanism. J.Bacteriol. **175**:4052-4061.

Brantl, S. 2004. Plasmid Replication Control by Antisense RNAs, p. 47-62. *In* B. E. Funnell and G. J. Phillips (eds.), Plasmid Biology. ASM Press, Washington, DC.

Brasch, M. A. and R. J. Meyer. 1986. Genetic organization of plasmid R1162 DNA involved in conjugative mobilization. J.Bacteriol. **167**:703-710.

Brendler, T., A. Abeles, and S. J. Austin. 1991. Critical sequences in the core of the P1 plasmid replication origin. J.Bacteriol. **173**:3935-3942.

Brendler, T. G., A. L. Abeles, L. D. Reaves, and S. J. Austin. 1991. Unique sequence requirements for the P1 plasmid replication origin. Res.Microbiol. **142**:209-216.

Brown, R. L., K. Sanderson, and S. M. Kirov. 1997. Plasmids and *Aeromonas* virulence. FEMS Immunol. Med. Microbiol. **17**:217-223.

Bruand, C., S. D. Ehrlich, and L. Jannière. 1991. Unidirectional theta replication of the structurally stable *Enterococcus faecalis* plasmid pAMβ1. EMBO J. **10**:2171-2177.

Bruand, C., E. Le Chatelier, S. D. Ehrlich, and L. Jannière. 1993. A fourth class of theta-replicating plasmids: the AMβ1 family from Gram-positive bacteria. Proc.Natl.Acad.Sci.USA **90**:11668-11672.

Bruun, M. S., A. S. Schmidt, I. Dalsgaard, and J. L. Larsen. 2003. Conjugal transfer of large plasmids conferring oxytetracycline (OTC) resistance: Transfer between environmental Aeromonads, fish-pathogenic bacteria and *Escherichia coli*. J.Aquat.Anim.Health **15**:69-79.

Burgess, R. R. and L. Anthony. 2001. How sigma docks to RNA polymerase and what sigma does. Curr.Opin.Microbiol. **4**:126-131.

Burian, J., S. Stuchlik, and W. W. Kay. 1999. Replication control of a small cryptic plasmid of *Escherichia coli*. J.Mol.Biol. **294**:49-65.

Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou. 1983. β–galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100**:293-308.

Casas, C., E. C. Anderson, K. K. Ojo, I. Keith, D. Whelan, D. Rainnie, and M. C. Roberts. 2005. Characterization of pRAS1-like plasmids from atypical North American psychrophilic *Aeromonas salmonicida*. FEMS Microbiol.Lett. **242**:59-63.

Castaing, J. P., J. Y. Bouet, and D. Lane. 2008. F plasmid partition depends on interaction of SopA with non-specific DNA. Mol.Microbiol. **70**:1000-1011.

Cereghino, J. L. and D. R. Helinski. 1993. Essentiality of the three carboxyl-terminal amino acids of the plasmid RK2 replication initiation protein TrfA for DNA binding and replication activity in gram-negative bacteria. J.Biol.Chem. **268**:24926-24932.

Chang, T. L., A. Naqvi, S. P. Anand, M. G. Kramer, R. Munshi, and S. A. Khan. 2002. Biochemical characterization of the *Staphylococcus aureus* PcrA helicase and its role in plasmid rolling-circle replication. J.Biol.Chem. **277**:45880-45886.

Chattoraj, D. K. and T. D. Schneider. 1997. Replication control of plasmid P1 and its host chromosome: the common ground. Prog.Nucl.Acid Res.Mol.Biol. **57**:145-186.

Chattoraj, D. K. 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol.Microbiol. **37** :467-476.

Christie, P. J., K. Atmakuri, V. Krishnamoorthy, S. Jakubowski, and E. Cascales. 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu.Rev.Microbiol. **59**:451-485.

Churchward, G., P. Linder, and L. Caro. 1983. The nucleotide sequence of replication and maintenance functions encoded by plasmid pSC101. Nucl.Acids Res. **11**:5645-5659.

Ciampi, M. S. 2006. Rho-dependent terminators and transcription termination. Microbiol. **152**:2515-2528.

Cooper, T. F. and J. A. Heinemann. 2000. Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. Proc.Natl.Acad.Sci.USA **97**:12643-12648.

Cossons, N., M. Zannis-Hadjopoulos, P. Tam, C. R. Astell, and E. A. Faust. 1996. The effect of regulatory sequence elements upon the initiation of DNA replication of the minute virus of mice. Virology **224**:320-325.

Couturier, M., F. Bex, P. L. Bergquist, and W. K. Maas. 1988. Identification and Classification of Bacterial Plasmids. Microbiol.Rev. **52**:375-395.

Das, N. and D. K. Chattoraj. 2004. Origin pairing ('handcuffing') and unpairing in the control of P1 plasmid replication. Mol.Microbiol. **54**:836-849.

Datta, N. and R. W. Hedges. 1972. R factors identified in Paris, some conferring gentamycin resistance, constitute a new compatibility group. Ann.Inst.Pasteur (Paris) **123**:849-852.

del Solar, G., M. G. Kramer, S. Ballester, and M. Espinosa. 1993a. Replication of the promiscuous plasmid pLS1: a region encompassing the minus origin of replication is associated with stable plasmid inheritance. Mol.Gen.Genet. **241**:97-105.

del Solar, G., M. Moscoso, and M. Espinosa. 1993b. Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls. Mol.Microbiol. **8**:789-796.

del Solar, G., P. Acebo, and M. Espinosa. 1995. Replication control of plasmid pLS1: efficient regulation of plasmid copy number is exerted by the combined action of two plasmid components, CopG and RNAII. Mol.Microbiol. **18**:913-924.

del Solar, G., J. C. Alonso, M. Espinosa, and R. Diaz-Orejas. 1996. Broad-host-range plasmid replication: an open question. Mol.Microbiol. **21**:661-666.

del Solar, G., R. Giraldo, M. J. Ruiz-Echevarría, M. Espinosa, and R. Díaz-Orejas. 1998. Replication and control of circular bacterial plasmids. Microbiol.Mol.Biol.Rev. **62**:434-464.

del Solar, G. and M. Espinosa. 2000. Plasmid copy number control: an ever-growing story. Mol.Microbiol. **37**:492-500.

Díaz, R., K. Nordström, and W. L. Staudenbauer. 1981. Plasmid R1 DNA replication dependent on protein synthesis in cell-free extracts of *E. coli*. Nature **289**:326-328.

Doran, K. S., I. Konieczny, and D. R. Helinski. 1998. Replication origin of the broad host range plasmid RK2: positioning of various motifs is critical for initiation of replication. J.Biol.Chem. **273**:8447-8453.

Doran, K. S., D. R. Helinski, and I. Konieczny. 1999. Host-dependent requirement for specific DnaA boxes for plasmid RK2 replication. Mol.Microbiol. **33**:490-498.

Drolet, M. and P. C. Lau. 1992. Mobilization protein-DNA binding and divergent transcription at the transfer origin of the *Thiobacillus ferrooxidans* pTF1 plasmid. Mol.Microbiol. **6**:1061-1071.

Durland, R. H. and D. R. Helinski. 1987. The sequence encoding the 43-kilodalton *trfA* protein is required for efficient replication or maintenance of minimal RK2 replicons in *Pseudomonas aeruginosa*. Plasmid **18**:164-169.

Durland, R. H. and D. R. Helinski. 1990. Replication of the broad-host-range plasmid RK2: direct measurement of intracellular concentrations of the essential TrfA replication proteins and their effect on plasmid copy number. J.Bacteriol. **172**:3849-3858.

Ebersbach, G. and K. Gerdes. 2001. The double *par* locus of virulence factor pB171: DNA segregation is correlated with oscillation of ParA. Proc.Natl.Acad.Sci.USA **98**:15078-15083.

Ebersbach, G., D. J. Sherratt, and K. Gerdes. 2005. Partition-associated incompatibility caused by random assortment of pure plasmid clusters. Mol.Microbiol. **56**:1430-1440.

Ebersbach, G. and K. Gerdes. 2005. Plasmid segregation mechanisms. Annu.Rev.Genet. **39**:453-479.

Eisenberg, S., J. F. Scott, and A. Kornberg. 1979. Enzymatic replication of ϕX174 duplex circles: continuous synthesis. Cold Spring Harb.Symp.Quant.Biol. **43**:295-302.

Engebrecht, J., R. Brent, and M. A. Kaderbhai. 2001. Minipreps of Plasmid DNA. *Current Protocols in Molecular Biology* **Unit 1.6.**.

Espinosa, M., G. del Solar, F. Rojo, and J. C. Alonso. 1995. Plasmid rolling circle replication and its control. FEMS Microbiol.Lett. **130**:111-120.

Ezaki, B., T. Ogura, H. Mori, H. Niki, and S. Hiraga. 1989. Involvement of DnaK protein in mini-F plasmid replication: temperature-sensitive *seg* mutations are located in the *dnaK* gene. Mol.Gen.Genet. **218**:183-189.

Fang, F. C. and D. R. Helinski. 1991. Broad-host-range properties of plasmid RK2: importance of overlapping genes encoding the plasmid replication initiation protein TrfA. J.Bacteriol. **173**:5861-5868.

Fernández-López, R., M. P. Garcillán-Barcia, C. Revilla, M. Lázaro, L. Vielva, and F. de la Cruz. 2006. Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. FEMS Microbiol.Rev. **30**:942-966.

Fernández-Tresguerres, M. E., M. Martín, D. García de Viedma, R. Giraldo, and R. Díaz-Orejas. 1995. Host growth temperature and a conservative amino acid substitution in the replication protein of pPS10 influence plasmid host range. J.Bacteriol. **177**:4377-4384.

Filutowicz, M., S. Dellis, I. Levchenko, M. Urh, F. Wu, and D. York. 1994. Regulation of replication in an iteron-containing DNA molecule. Prog.Nucl.Acid Res.Mol.Biol. **48**:239-273.

Filutowicz, M. and S. A. Rakowski. 1998. Regulatory implications of protein assemblies at the γ origin of plasmid R6K - a review. Gene **223**:195-204.

Fineran, P. C., T. R. Blower, I. J. Foulds, D. P. Humphreys, K. S. Lilley, and G. P. C. Salmond. 2009. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. Proc.Natl.Acad.Sci.USA **106**:894-899.

Frere, J., M. Novel, and G. Novel. 1993. Molecular analysis of the *Lactococcus lactis* subspecies *lactis* CNRZ270 bidirectional theta replicating lactose plasmid pUCL22. Mol.Microbiol. **10**:1113-1124.

Frey, J. and M. Bagdasarian. 1989. The molecular biology of IncQ plasmids, p. 79-93. *In* C. M. Thomas (ed.), Promiscuous Plasmids of Gram Negative Bacteria. Academic Press, Ltd, London, United Kingdom.

Frost, L. S. 2009. Conjugation, Bacterial, p. 294-308. *In* M. Schaechter (ed.), Desk encyclopedia of microbiology. Academic Press, San Diego, CA, USA.

Funnell, B. E. and R. A. Slavcev. 2004. Partition Systems of Bacterial Plasmids, p. 81-103. *In* B. E. Funnell and G. J. Phillips (eds.), Plasmid Biology. ASM Press, Washington, DC.

Funnell, B. E. 2005. Partition-mediated plasmid pairing. Plasmid **53**:119-125.

Galkin, V. E., A. Orlova, C. Rivera, R. D. Mullins, and E. H. Egelman. 2009. Structural polymorphism of the ParM filament and dynamic instability. Structure **17**:1253-1264.

Gao, Q., Y. Luo, and R. C. Deonier. 1994. Initiation and termination of DNA transfer at F plasmid *oriT*. Mol.Microbiol. **11**:449-458.

Garcillán-Barcia, M. P., A. Alvarado, and F. de la Cruz. 2011. Identification of bacterial plasmids based on mobility and plasmid population biology. FEMS Microbiol.Rev. **35**:936-956.

García de Viedma, D., R. Giraldo, G. Rivas, E. Fernández-Tresguerres, and R. Díaz-Orejas. 1996. A leucine zipper motif determines different functions in a DNA replication protein. EMBO J. **15**:925-934.

Gardner, M. N., S. M. Deane, and D. E. Rawlings. 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J.Bacteriol. **183**:3303-3309.

Garner, E. C., C. S. Campbell, and R. D. Mullins. 2004. Dynamic instability in a DNA-segregating prokaryotic actin homolog. Science **306**:1021-1025.

Gasset-Rosa, F., T. Díaz-López, R. Lurz, A. Prieto, M. E. Fernández-Tresguerres, and R. Giraldo. 2008. Negative regulation of pPS10 plasmid replication: origin pairing by zipping-up DNA-bound RepA monomers. Mol.Microbiol. **68**:560-572.

Geibel, S., S. Banchenko, M. Engel, E. Lanka, and W. Saenger. 2009. Structure and function of primase RepB' encoded by broad-host-range plasmid RSF1010 that replicates exclusively in leading-strand mode. Proc.Natl.Acad.Sci.USA **106**:7810-7815.

Gennaro, M. and R. P. Novick. 1986. cmp, a cis-acting plasmid locus that increases interaction between replication origin and initiator protein. J.Bacteriol. **168**:160-166.

Gerdes, K., J. E. Larsen, and S. Molin. 1985. Stable inheritance of plasmid R1 requires two different loci. J.Bacteriol. **161**:292-298.

Gerdes, K., F. W. Bech, S. T. Jørgensen, A. Lobner-Oleson, P. B. Rasmussen, T. Atlung, L. Boe, O. Karlström, S. Molin, and K. von Meyenburg. 1986. Mechanism of post-segregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli relB* operon. EMBO J. **5**:2023-2029.

Gerdes, K., L. K. Poulson, T. Thisted, A. K. Nielsen, J. Martinussen, and P. H. Andreasen. 1990. The *hok* killer gene family in gram-negative bacteria. New Biol. **2**:946-956.

Gerdes, K., A. P. Gultyaev, T. Franch, K. Pedersen, and N. D. Mikkelsen. 1997. Antisense RNA-regulated programmed cell death. Annu.Rev.Genet. **31**:1-31.

Gerdes, K., S. K. Christensen, and A. Løbner-Oleson. 2005. Prokaryotic toxin-antitoxin stress response loci. Nat.Rev.Microbiol. **3**:371-382.

Gerdes, K., M. Howard, and F. Szardenings. 2010. Pushing and Pulling in Prokaryotic DNA Segregation. Cell **141**:927-942.

Germino, J. and D. Bastia. 1983a. The replication initiator protein of plasmid R6K tagged with beta-galactoside shows sequence-specific DNA-binding. Cell **32**:131-140.

Germino, J. and D. Bastia. 1983b. Interaction of the plasmid R6K-encoded replication initiator protein with its binding sites on DNA. Cell **34**:125-134.

Giraldo, R., M. Martín, M. E. Fernández-Tresguerres, C. Nieto, and R. Díaz. 1992. Mutations within the minimal replicon of plasmid pPS10 increase its host range, p. 225-237. *In* P. Hughes, E. Fanning, and M. Kohiyama (eds.), DNA replication: the regulatory mechanisms. Springer-Verlag KG, Berlin, Germany.

Golub, E. I. and H. A. Panzer. 1988. The F factor of *Escherichia coli* carries a locus of stable plasmid inheritance *stm*, similar to the *parB* locus of plasmid R1. Mol.Gen.Genet. **214**:353-357.

Gomis-Ruth, F. X., F. de la Cruz, and M. Coll. 2002. Structure and role of coupling proteins in conjugal DNA transfer. Res.Microbiol. **153**:199-204.

Gordon, G. S., D. Sitnikov, C. D. Webb, A. Teleman, A. Straight, R. Losick, A. W. Murray, and A. Wright. 1997. Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. Cell **90**:1113-1121.

Gormley, E. P. and J. Davies. 1991. Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. J.Bacteriol. **173**:6705-6708.

Gotz, A., R. Pukall, E. Smit, E. Tietze, R. Prager, H. Tschäpe, J. D. van Elsas, and K. Small. 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Appl.Environ.Microbiol. **62**:2621-2628.

Grahn, A. M., J. Haase, D. H. Bamford, and E. Lanka. 2000. Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner membrane and outer membranes of donor cells: implications for related macromolecule transport systems. J.Bacteriol. **182**:1564-1574.

Gravesen, A., J. Josephsen, A. von Wright, and F. K. Vogensen. 1995. Characterization of the replicon from the lactococcal theta-replicating plasmid pJW563. Plasmid **34**:105-118.

Grindley, N. D. F. 2002. The movement of Tn*3*-like elements: transposition and cointegrate resolution, p. 272-302. *In* N. L. Craig, R. Craigie, M. Gellert, and A. M. Lambowitz (eds.), Mobile DNA II. ASM Press, Washington, DC.

Grindley, N. D. F., K. L. Whiteson, and P. A. Rice. 2006. Mechanisms of site-specific recombination. Annu.Rev.Biochem. **75**:567-605.

Gros, M. F., H. te Riele, and S. D. Ehrlich. 1987. Rolling circle replication of single-stranded DNA plasmid pC194. EMBO J. **6**:3863-3869.

Gruss, A. and S. D. Ehrlich. 1989. The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. Microbiol.Rev. **53**:231-241.

Guynet, C. and F. de la Cruz. 2011. Plasmid segregation without partition. Mob.Genet.Elements **1**:236-241.

Guynet, C., A. Cuevas, G. Moncalián, and F. de la Cruz. 2011. The *stb* operon balances the requirements for vegetative stability and conjugative transfer of plasmid R388. PLos Genet. **7:e1002073**:1-12.

Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J.Bacteriol. **177**:4121-4130.

Hansen, E. B. and M. B. Yarmolinsky. 1986. Host participation in plasmid maintenance: dependence upon *dnaA* of replicons derived from P1 and F. Proc.Natl.Acad.Sci.USA **83**:4423-4427.

Haring, V. and E. Scherzinger. 1989. Replication proteins of the IncQ plasmid RSF1010, p. 95-124. *In* C. M. Thomas (ed.), Promiscuous plasmids of Gram-negative bacteria. Academic Press, Ltd, London, United Kingdom.

Harris, R. L., V. Hombs, and P. M. Silverman. 2001. Evidence that F-plasmid proteins TraV, TraK and TraB assemble into an envelope-spanning structure in *Escherichia coli*. Mol.Microbiol. **42**:757-766.

Hasunuma, K. and M. Sekiguchi. 1977. Replication of plasmid pSC101 in *Escherichia coli* K12: requirement for *dnaA* function. Mol.Gen.Genet. **154**:225-230.

Hatano, T., Y. Yamaichi, and H. Niki. 2007. Oscillating focus of SopA associated with filamentous structure guides partitioning of F plasmid. Mol.Microbiol. **64**:1198-1213.

Hayes, F., P. Vos, G. F. Fitzgerald, W. M. de Vos, and C. Daly. 1991. Molecular organization of the minimal replicon of novel, narrow-host-range, lactococcal plasmid pCI305. Plasmid **25**:16-26.

Hefford, M. A., R. M. Teather, and R. J. Forster. 1993. The complete nucleotide sequence of a small cryptic plasmid from a rumen bacterium of the genus *Butyrivibrio*. Plasmid **29**:63-69.

Helinski, D. R., A. E. Toukdarian, and R. P. Novick. 1996. Replication control and other stable maintenance mechanisms of plasmids, p. 2295-2324. *In* F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, e. C. C. Lin, K. B. Low, and B. Magasanik (eds.), *Escherichia coli and Salmonella*. ASM Press, Washington, DC.

Hernández-Arriaga, A. M., T. S. Rubio-Lepe, M. Espinosa, and G. del Solar. 2009. Repressor CopG prevents access of RNA polymerase to promoter and actively dissociates open complexes. Nucl.Acids Res. **37**:4799-4811.

Herniquez, V., V. Milisavljevic, J. D. Khan, and M. L. Gennaro. 1993. Sequence and structure of *cmp*, the replication enhancer of the *Staphylococcus aureus* plasmid pT181. Gene **134**:93-98.

Hinnebusch, J. and K. Tilly. 1993. Linear plasmids and chromosomes in bacteria. Mol.Microbiol. **10**:917-922.

Hirano, M., H. Mori, T. Onogi, M. Yamazoe, H. Niki, T. Ogura, and S. Hiraga. 1998. Autoregulation of the partition genes of the mini-F plasmid and the intracellular localization of their products in *Escherichia coli*. Mol.Gen.Genet. **257**:392-403.

Huggett, J., K. Dheda, S. Bustin, and A. Zumla. 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. **6**:279-284.

Iordanescu, S. and R. Basheer. 1991. The *Staphylococcus aureus* mutation *pcrA3* leads to the accumulation of pT181 replication initiator complexes. J.Mol.Biol. **221**:1183-1189.

Iordanescu, S. 1993. Plasmid pT181-linked suppressors of the *Staphylococcus aureus pcrA3* chromosomal mutation. J.Bacteriol. **175**:3916-3917.

Ishiai, M., C. Wada, Y. Kawasaki, and T. Yura. 1994. Replication initiator protein RepE of mini-F plasmid: functional differentiation between monomers (initiator) and dimers (autogenous repressor). Proc.Natl.Acad.Sci.USA **91**:3839-3843.

Itoh, T. and T. Horii. 1989. Replication of ColE2 and ColE3 plasmids: *in vitro* replication dependent on plasmid-coded proteins. Mol.Gen.Genet. **219**:249-255.

Iwasaki, K., H. Uchiyama, O. Yagi, T. Kurabayashi, K. Ishizuka, and Y. Takamura. 1994. Transformation of *Pseudomonas putida* by electroporation. Biosci.Biotechnol.Biochem. **58**:851-854.

Jensen, R. B. and K. Gerdes. 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol.Microbiol. **17**:205-210.

Jensen, R. B. and K. Gerdes. 1997. Partitioning of plasmid R1. The ParM protein exhibits ATPase activity and interacts with the centromere-like ParR-*par*C complex. J.Mol.Biol. **269**:505-513.

Jensen, R. B., R. Lurz, and K. Gerdes. 1998. Mechanism of DNA segregation in prokaryotes: replicon pairing by *parC* of plasmid R1. Proc.Natl.Acad.Sci.USA **95**:8550-8555.

Juhas, M., D. W. Crook, I. D. Dimopoulou, G. Lunter, R. M. Harding, D. J. Ferguson, and D. W. Hood. 2007. Novel type IV secretion system involved in propagation of genomic islands. J.Bacteriol. **189**:761-771.

Kamio, Y. and Y. Terawaki. 1983. Nucleotide sequence of an incompatibility region of mini-Rts1 that contains five direct repeats. J.Bacteriol. **155**:1185-1191.

Kawasaki, Y., C. Wada, and T. Yura. 1990. Roles of *Escherichia coli* heat shock proteins DnaK, DnaJ and GrpE in mini-F plasmid replication. Mol.Gen.Genet. **220**:277-282.

Kawasaki, Y., C. Wada, and T. Yura. 1992. Binding of RepE initiator protein to mini-F DNA origin (*ori2*). Enhancing effects of *repE* mutations and DnaJ heat shock protein. J.Biol.Chem. **267**:11520-11524.

Kelley, W. L. and D. Bastia. 1992. Activation *in vivo* of the minimal replication origin beta of plasmid R6K requires a small target sequence essential for DNA looping. New Biol. **4**:569-580.

Kelman, Z. and M. O'Donnell. 1995. DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. Annu.Rev.Biochem. **64**:171-200.

Khan, S. 2004. Rolling-Circle Replication, p. 63-78. *In* B. E. Funnell and G. J. Phillips (eds.), Plasmid Biology. ASM Press, Washington, DC.

Khan, S. A. 1996. Mechanism of replication and copy number control of plasmids in Gram-positive bacteria. Genet.Eng.(N.Y.) **18**:183-201.

Khan, S. A. 1997. Rolling-circle replication of bacterial plasmids. Microbiol.Mol.Biol.Rev. **61**:442-455.

Khan, S. A. 2000. Plasmid rolling-circle replication: recent developments. Mol.Microbiol. **37**:477-484.

Khan, S. A. 2005. Plasmid rolling-circle replication: highlights of two decades of research. Plasmid **53**:126-136.

Kiewiet, R., S. Bron, K. de Jonge, G. Venema, and J. F. M. L. Seegers. 1993. Theta replication of the lactococcal plasmid pWVO2. Mol.Microbiol. **10**:319-327.

Kingsbury, D. T. and D. R. Helinski. 1970. DNA polymerase as a requirement for the maintenance of the bacterial plasmid colicinogenic factor E1. Biochem.Biophys.Res.Commun. **41**:1538-1544.

Koepsel, R. and S. Khan. 1986. Static and initiator protein-enhanced bending of DNA at a replication origin. Science **233**:1316-1318.

Koepsel, R. R., R. W. Murray, W. D. Rosenblum, and S. A. Khan. 1985a. Purification of pT181-encoded *repC* protein required for the initiation of plasmid replication. J.Biol.Chem. **260**:8571-8577.

Koepsel, R. R., R. W. Murray, W. D. Rosenblum, and S. A. Khan. 1985b. The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase-like activities. Proc.Natl.Acad.Sci.USA **82**:6845-6849.

Kollek, R., W. Oertel, and W. Goebel. 1978. Isolation and characterization of the minimal fragment required for autonomous replication ("basic replicon") of a copy mutant (pKN102) of the antibiotic resistance factor R1. Mol.Gen.Genet. **162**:51-57.

Kolter, R. and D. R. Helinski. 1978. The activity of the replication terminus of plasmid R6K in hybrid replicons in *Escherichia coli* . J.Mol.Biol. **124**:425-441.

Kornberg, A. and T. Baker. 1992. DNA replication. W. H. Freeman & Co., New York, N.Y.

Kostriken, R., C. Morita, and F. Heffron. 1981. Transposon Tn*3* encodes a site-specific recombination system: Identification of essential sequences, genes, and actual site of recombination. Proc.Natl.Acad.Sci.USA **78**:4041-4045.

Kramer, M. G., G. del Solar, and M. Espinosa. 1995. Lagging-strand origins of the promiscuous plasmid pMV158: physical and functional characterization. Microbiology **141**:655-662.

Kramer, M. G., S. A. Khan, and M. Espinosa. 1997. Plasmid rolling circle replication: identification of the RNA polymerase-directed primer RNA and requirement for DNA polymerase I for lagging strand synthesis. EMBO J. **16**:5784-5795.

Kramer, M. G., S. A. Khan, and M. Espinosa. 1998. Lagging-strand replication from the *ssoA* origin of plasmid pMV158 in *Streptococcus pneumoniae*: *in vivo* and *in vitro* influences of mutations in two conserved *ssoA* regions. J.Bacteriol. **180**:83-89.

Krause, S., M. Bárcena, W. Pansegrau, R. Lurz, J. M. Carazo, and E. Lanka. 2000a. Sequence-related protein export NTP-ases encoded by the conjugative transfer region of RP4 and by the *cag* pathogenicity island of *Helicobacter pylori* share similar hexameric ring structures. Proc.Natl.Acad.Sci.USA **97**:3067-3072.

Krause, S., W. Pansegrau, R. Lurz, F. de la Cruz, and E. Lanka. 2000b. Enzymology of type IV macromolecule secretion systems: the conjugative transfer regions of plasmids RP4 and R388 and the *cag* pathogenicity island of *Helicobacter pylori* encode structurally and functionally related nucleoside triphosphate hydrolases. J.Bacteriol. **182**:2761-2770.

Krüger, R., S. A. Rakowski, and M. Filutowicz. 2004. Participating Elements in the Replication of Iteron-Containing Plasmids, p. 25-45. *In* B. E. Funnell and G. J. Phillips (eds.), Plasmid Biology. ASM Press, Washington, DC.

Kunnimalaiyaan, S., R. B. Inman, S. A. Rakowski, and M. Filutowicz. 2005. Role of π dimers in coupling ('handcuffing) of plasmid R6K's γ *ori* iterons. J.Bacteriol. **187**:3779-3785.

Kusukawa, N., H. Mori, A. Kondo, and S. Hiraga. 1987. Partitioning of the F plasmid: overproduction of an essential protein for partition inhibits plasmid maintenance. Mol.Gen.Genet. **208**:365-372.

L'Abée-Lund, T. M. and H. Sørum. 2002. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid **47**:172-181.

Lacatena, R. M. and G. Cesareni. 1981. Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication. Nature **294**:623-626.

Lane, D., R. de Feyter, M. Kennedy, S. H. Phua, and D. Semon. 1986. D protein of miniF plasmid acts as a repressor of transcription and as a site-specific resolvase. Nucl.Acids Res. **14**:9713-9728.

Lanka, E., R. Lurz, M. Kröger, and J. P. Fürste. 1984. Plasmid RP4 encodes two forms of a DNA primase. Mol.Gen.Genet. **194**:65-72.

Lanka, E. and B. M. Wilkins. 1995. DNA processing reactions in bacterial conjugation. Annu.Rev.Biochem. **64**:141-169.

Larsen, R. A., C. Cusumano, A. Fujioka, G. Lim-Fong, P. Patterson, and J. Pogliano. 2007. Treadmilling of a prokaryotic tubulin-like protein, TubZ, required for plasmid stability in *Bacillus thuringiensis*. Genes Dev. **21**:1340-1352.

Lawley, T., B. M. Wilkins, and L. S. Frost. 2004. Bacterial Conjugation in Gram-Negative Bacteria, p. 203-226. *In* B. E. Funnell and G. J. Phillips (eds.), Plasmid Biology. ASM Press, Washington, D.C.

Lederberg, J. 1952. Cell genetics and hereditary symbiosis. Physiol.Rev. **32**:403-430.

Lee, C., J. Kim, S. G. Shin, and S. Hwang. 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J.Biotech. **123**:273-280.

Lessl, M., D. Balzer, R. Lurz, V. L. Waters, D. G. Guiney, and E. Lanka. 1992. Dissection of IncP conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region *in trans*. J.Bacteriol. **174**:2493-2500.

Lim, G. E., A. I. Derman, and J. Pogliano. 2005. Bacterial DNA segregation by dynamic SopA polymers. Proc.Natl.Acad.Sci.USA **102**:17658-17663.

Loftie-Eaton, W. and D. E. Rawlings. 2009. Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. J.Bacteriol. **191**:6436-6446.

Loftie-Eaton, W. and D. E. Rawlings. 2010. Evolutionary competitiveness of two natural variants of the IncQ-Like plasmids, pRAS3.1 and pRAS3.2. J.Bacteriol. **192**:6182-6190.

Loftie-Eaton, W. and D. E. Rawlings. 2012. Diversity, biology and evolution of IncQ-family plasmids. Plasmid **67**:15-34.

Madsen, S. M., L. Andrup, and L. Boe. 1993. Fine mapping and DNA sequence of replication functions of *Bacillus thuringiensis* plasmid pTX14-3. Plasmid **30**:119-130.

Maki, S., S. Takiguchi, T. Miki, and T. Horiuchi. 1992. Modulation of DNA supercoiling activity of *Escherichia coli* DNA gyrase by F plasmid proteins. Antagonistic actions of LetA (CcdA) and LetD (CcdB) proteins. J.Biol.Chem. **267**:12244-12251.

Maki, S., S. Takiguchi, T. Horiuchi, K. Sekimizu, and T. Miki. 1996. Partner switching mechanisms in inactivation and rejuvenation of *Escherichia coli* DNA gyrase by F plasmid proteins LetD (CcdB) and LetA (CcdA). J.Mol.Biol. **256**:473-482.

Malmgren, C., E. G. H. Wagner, C. Ehresmann, B. Ehresmann, and P. Romby. 1997. Antisense RNA control of plasmid R1 replication. The dominant product of the antisense RNA-mRNA binding is not a full RNA duplex. J.Biol.Chem. **272**:12508-12512.

Manen, D. and L. Caro. 1991. The replication of plasmid pSC101. Mol.Microbiol. **5**:233-237.

Marsin, S. and P. Forterre. 1999. The active site of the rolling circle replication protein Rep75 is involved in site-specific nuclease, ligase and nucleotidyl transferase activities. Mol.Microbiol. **33**:537-545.

Marston, A. L. and J. Errington. 1999. Dynamic movement of the ParA-like Soj protein of *B. subtilis* and its dual role in nucleoid organization and developmental regulation. Mol.Cell **4**:673-682.

Marx, I. 2007. Characterization of the plasmids isolated from *Aeromonas* spp. obtained from South African aquaculture systems. Thesis (MSc) University of Stellenbosch.

Masai, H., Y. Kaziro, and K. Arai. 1983. Definition of *oriR*, the minimum DNA segment essential for initiation of R1 plasmid replication in vitro. Proc.Natl.Acad.Sci.USA **80**:6814-6818.

Masai, M. and K. Arai. 1987. RepA and DnaA proteins are required for initiation of R1 plasmid replication *in vitro* and interact with the *oriR* sequence. Proc.Natl.Acad.Sci.USA **84**:4781-4785.

McEachern, M. J., M. Filutowicz, and D. R. Helinski. 1985. Mutations in direct repeat sequences and in a conserved sequence adjacent to the repeats result in a defective replication origin in plasmid R6K. Proc.Natl.Acad.Sci.USA **82**:1480-1484.

McEachern, M. J., M. A. Bott, P. A. Tooker, and D. R. Helinski. 1989. Negative control of plasmid R6K replication: possible role of intermolecular coupling of replication origins. Proc.Natl.Acad.Sci.USA **86**:7942-7946.

Meijer, W. J. J., G. Venema, and S. Bron. 1995a. Characterization of single strand origins of cryptic rolling-circle plasmids from *Bacillus subtilis*. Nucl. Acids Res. **23**:612-619.

Meijer, W. J. J., A. J. de Boer, S. van Tongeren, G. Venema, and S. Bron. 1995b. Characterization of the replication region of the *Bacillus subtilis* plasmid pLS20: a novel type of replicon. Nucl.Acids Res. **23**:3214-3223.

Meyer, R., R. Laux, G. Boch, M. Hinds, R. Bayly, and J. A. Shapiro. 1982. Broad-host-range IncP-4 plasmid R1162: effects of deletions and insertions on plasmid maintenance and host range. J.Bacteriol. **152**:140-150.

Miao, D. M., Y. Honda, K. Tanaka, A. Higashi, T. Nakamura, Y. Taguchi, H. Sakai, T. Komano, and M. Bagdasarian. 1993. A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad host range plasmid RSF1010. Nucl.Acids Res. **21**:4900-4903.

Miao, D. M., H. Sakai, S. Okamoto, K. Tanaka, M. Okuda, Y. Honda, T. Komano, and M. Bagdasarian. 1995. The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. Nucl.Acids Res. **23**:3295-3300.

Miele, L., B. Strack, V. Kruft, and E. Lanka. 1991. Gene organization and nucleotide sequence of the primase region of IncP plasmids RP4 and R751. DNA Seq. **2**:145-162.

Miki, T., Z. T. Chang, and T. Horiuchi. 1984. Control of cell division by sex factor F in *Escherichia coli*. II. Identification of genes for inhibitor protein and trigger protein on the 42.84-43.6 F segment. J.Mol.Biol. **174**:627-646.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Moscoso, M., G. del Solar, and M. Espinosa. 1995a. Specific nicking-closing activity of the initiator of replication protein RepB of plasmid pMV158 on supercoiled or single-stranded DNA. J.Biol.Chem. **270**:3772-3779.

Moscoso, M., G. del Solar, and M. Espinosa. 1995b. *In vitro* recognition of the replication origin of pLS1 and of plasmids of the pLS1 family by the RepB initiator protein. J.Bacteriol. **177**:7041-7049.

Møller-Jensen, J., R. B. Jensen, J. Lowe, and K. Gerdes. 2002. Prokaryotic DNA segregation by an actin-like filament. EMBO J. **21**:3119-3127.

Møller-Jensen, J., J. Borch, M. Dam, R. B. Jensen, P. Roepstorff, and K. Gerdes. 2003. Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. Mol.Cell **12**:1477-1487.

Murotsu, T., K. Matsubara, H. Sugisaki, and M. Takanami. 1981. Nine unique repeating sequences in a region essential for replication and incompatibility of the mini-F plasmid. Gene **15**:257-271.

Nagahari, K. and K. Sakaguchi. 1978. RSF1010 plasmid as a potentially useful vector in *Pseudomonas* species. J.Bacteriol. **133**:1527-1529.

Nielsen, A. K., P. Thorsted, T. Thisted, E. G. H. Wagner, and K. Gerdes. 1991. The rifampicin-inducible genes *srnB* from F and *pnd* from R483 are regulated by antisense RNAs and mediate plasmid maintenance by killing of plasmid-free segregants. Mol.Microbiol. **5**:1961-1973.

Nieto, C., R. Giraldo, E. Fernández-Tresguerres, and R. Díaz. 1992. Genetic and functional analysis of the basic replicon of pPS10, a plasmid specific for *Pseudomonas* isolated from *Pseudomonas syringae patovar savastanoi*. J.Mol.Biol. **223**:415-426.

Niki, H. and S. Hiraga. 1997. Subcellular distribution of actively partitioning F plasmid during the cell division cycle in *E. coli*. Cell **90**:951-957.

Noirot-Gros, M. F., V. Bidnenko, and S. D. Ehrlich. 1994. Active site of the replication protein of the rolling circle plasmid pC194. EMBO J. **13**:4412-4420.

Nordström, K. and S. J. Austin. 1989. Mechanisms that contribute to the stable segregation of plasmids. Annu.Rev.Genet. **23**:37-69.

Novick, R. 2002. Plasmids. eLS.. Chichester, John Wiley & Sons Ltd. http://www.els.net[doi:10.1038/npg.els.0001490]

Novick, R. P. 1987. Plasmid incompatibility. Microbiol.Rev. **51**:381-395.

Novick, R. P. 1989. Staphylococcal plasmids and their replication. Annu.Rev.Microbiol. **43**:537-565.

O'Donnell, M. 2006. Replisome architecture and dynamics in *Escherichia coli*. J.Biol.Chem. **281**:10653-10656.

Ogura, T. and S. Hiraga. 1983. Partition mechanism of F plasmid: two plasmid gene-encoded products and a *cis*-acting region are involved in partition. Cell **32**:351-360.

Ohnishi, Y., H. Iguma, T. Ono, H. Nagaishi, and A. J. Clark. 1977. Genetic mapping of the F plasmid gene that promotes degradation of stable ribonucleic acid in *Escherichia coli*. J.Bacteriol. **132**:784-789.

Osborn, M., S. Bron, N. Firth, S. Holsappel, A. Huddleston, R. Kiewiet, W. Meijer, J. Seegers, R. Skurray, P. Terpstra, C. M. Thomas, P. Thorsted, E. Tietze, and S. L. Turner. 2000. The evolution of bacterial plasmids, p. 301-347. *In* C. M. Thomas (ed.), The horizontal gene pool: Bacterial plasmids and gene spread. Harwood Academic Publishers, Amsterdam.

Pal, S. K. and D. K. Chattoraj. 1988. P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites. J.Bacteriol. **170**:3554-3560.

Pandey, D. P. and K. Gerdes. 2005. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucl.Acids Res. **33**:966-976.

Papp, P. P., D. K. Chattoraj, and T. D. Schneider. 1993. Information analysis of sequences that bind the replication initiator RepA. J.Mol.Biol. **233**:219-230.

Pasquali, F., C. Kehrenberg, C. Manfreda, and S. Schwarz . 2005. Physical linkage of Tn*3* and part of Tn*1721* in a tetracycline and ampicillin resistance plasmid from *Salmonella Typhimurium*. J.Antimicrob.Chemother. **55**:562-565.

Paulsson, J. 2002. Multilevel selection on plasmid replication. Genetics **161**:1373-1384.

Paulsson, J. and D. K. Chattoraj. 2006. Origin inactivation in bacterial DNA replication control. Mol.Microbiol. **61**:9-15.

Pfaffl, M. W., G. W. Horgan, and L. Dempfle. 2002. Relative expression software tool (REST(C)) for the group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl.Acids Res. **30**:e36.

Pfaffl, M. W. 2004. Quantification strategies in real-time PCR, p. 87-112. *In* S. A. Bustin (ed.), A-Z of quantitative PCR. International University Line (IUL), La Jolla, CA, USA.

Popp, D., A. Yamamoto, M. Iwasa, A. Narita, K. Maeda, and Y. Maeda. 2007. Concerning the dynamic instability of actin homolog ParM. Biochem.Biophys.Res.Commun. **353**:109-114.

Pratto, F., A. Cicek, W. A. Weihofen, R. Lurz, W. Saenger, and J. C. Alonso. 2008. *Streptococcus pyogenes* pSM19035 requires dynamic assembly of ATP-bound ParA and ParB on *parS* DNA during plasmid segregation. Nucl.Acids Res. **36**:3676-3689.

Providenti, M. A., J. M. O'Brien, R. J. Ewing, E. S. Paterson, and M. L. Smith. 2006. The copy-number of plasmids and other genetic elements can be determined by SYBR-Green-based quantitative real-time PCR. J.Microbiol.Methods **65**:476-487.

Quisel, J. D., D. C. Lin, and A. D. Grossman. 1999. Control of development by altered localization of a transcription factor in *B. subtilis*. Mol.Cell **4**:665-672.

Ramamoorthy, R., N. A. McClain, A. Gautam, and D. Scholl-Meeker. 2005. Expression of the *bmpB* gene of *Borrelia burgdorferi* is modulated by two distinct transcription termination events. J.Bacteriol. **187**:2592-2600.

Rasmussen, R. 2001. Quantification on the LightCycler, p. 21-34. *In* S. Meuer, C. Wittwer, and K. Nakagawara (eds.), Rapid Cycle Real-time PCR, Methods and Applications. Springer-Verlag, Berlin, Germany.

Rasooly, A. and R. P. Novick. 1993. Replication-specific inactivation of the pT181 plasmid initiator protein. Science **262**:1048-1050.

Rasooly, A., P. Wang, and R. P. Novick. 1994a. Replication-specific conversion of the *Staphylococcus aureus* pT181 initiator protein from an active homodimer to an inactive heterodimer. EMBO J. **13**:5245-5251.

Rasooly, A., S. J. Projan, and R. P. Novick. 1994b. Plasmids of the pT181 family show replication-specific initiator protein modification. J.Bacteriol. **176**:2450-2453.

Rasooly, A. and R. S. Rasooly. 1996. Modification of the plasmid initiator protein RepC active site during replication. FEMS Microbiol.Lett. **145**:245-253.

Ratnakar, P. V., B. K. Mohanty, M. Lobert, and D. Bastia . 1996. The replication initiator protein Pi of plasmid R6K specifically interacts with the host-encoded helicase DnaB. Proc.Natl.Acad.Sci.USA **93**:5522-5526.

Rees, C. E. and B. M. Wilkins. 1990. Protein transfer into the recipient cell during bacterial conjugation: studies with F and RP4. Mol.Microbiol. **4**:1199-1205.

Reinberg, D., S. L. Zipursky, P. Weisbeek, D. Brown, and J. Hurwitz. 1983. Studies on the ϕX174 gene A protein-mediated termination of leading strand DNA synthesis. J.Biol.Chem. **258**:529-537.

Rhodes, G., G. Huys, J. Swings, P. McGann, M. Hiney, P. Smith, and R. W. Pickup. 2000. Distribution of oxytetracycline resistance plasmid between Aeromonads in hospital and aquaculture environments: Implication of Tn*1721* in dissemination of the tetracycline resistance determinant Tet A. Appl.Environ.Microbiol. **66**:3883-3890.

Rhodes, G., J. Parkhill, C. Bird, K. Ambrose, M. C. Jones, G. Huys, J. Swings, and R. W. Pickup. 2004. Complete nucleotide sequence of the conjugative tetracycline resistance plasmid pFBAOT6, a member of the group of IncU plasmids with global ubiquity. Appl.Environ.Microbiol. **70**:7497-7510.

Richards, R. H., V. Inglis, G. N. Frerichs, and S. D. Miller. 1992. Variation in antibiotic resistance patterns of *Aeromonas salmonicida* isolated from Atlantic salmon *Salmo salar* L. in Scotland, p. 276-284. *In* C. Michel and D. Alderman (eds.), Chemotherapy in aquaculture: from theory to reality. Paris, France: Office International des Epizooties.

Ringgaard, S., J. van Zon, M. Howard, and K. Gerdes. 2009. Movement and equipositioning of plasmids by ParA filament disassembly. Proc.Natl.Acad.Sci.USA **106**:19369-19374.

Rosenfeld, N., M. B. Elowitz, and U. Alon. 2002. Negative autoregulation speeds the response times of transcription networks. J.Mol.Biol. **323**:785-793.

Ruiz-Masó, J. A., S. P. Anand, M. Espinosa, S. A. Khan, and G. del Solar. 2006. Genetic and biochemical characterization of the *Streptococcus pneumoniae* PcrA helicase and its role in plasmid rolling circle replication. J.Bacteriol. **188**:7416-7425.

Sakai, H. and T. Komano. 1996. DNA replication of the IncQ broad-host-range plasmids in gram-negative bacteria. Biosci.Biotechnol.Biochem. **60**:377-382.

Salje, J. 2010. Plasmid segregation: how to survive as an extra piece of DNA. Crit.Rev.Biochem.Mol.Biol. **45**:296-317.

Salje, J., P. Gayathri, and J. Lõwe. 2010. The ParMRC system: molecular mechanisms of plasmid segregation by actin-like filaments. Nat.Rev.Microbiol. **8**:683-692.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandaa, R. and Ø. Enger. 1994. Transfer in marine sediments of the naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. Appl.Environ.Microbiol. **60**:4234-4238.

Sandaa, R. and Ø. Enger. 1996. High frequency transfer of a broad host range plasmid present in an atypical strain of the fish pathogen *Aeromonas salmonicida*. Dis.Aquat.Org. **24**:71-75.

Sanger, F. and A. R. Coulson. 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J.Mol.Biol. **94**:441-448.

Scaife, J. and J. D. Gross. 1962. Inhibition of multiplication of an Flac factor in Hfr cells of *Escherichia coli* K-12. Biochem.Biophys.Res.Commun. **7**:403-407.

Scherzinger, E., M. M. Bagdasarian, P. Scholz, R. Lurz, B. Rückert, and M. Bagdasarian. 1984. Replication of the broad host-range plasmid RSF1010: requirement for three plasmid-encoded proteins. Proc.Natl.Acad.Sci.USA **81**:654-658.

Scherzinger, E., V. Haring, R. Lurz, and S. Otto. 1991. Plasmid RSF1010 DNA replication *in vitro* promoted by purified RSF1010 RepA, RepB and RepC proteins. Nucl.Acids Res. **19**:1203-1211.

Scherzinger, E., V. Kruft, and S. Otto. 1993. Purification of the large mobilization protein of plasmid RSF1010 and characterization of its site-specific DNA-cleaving/DNA-joining activity. Eur.J.Biochem. **217**:929-938.

Schöffl, F., W. Arnold, A. Pühler, J. Altenbuchner, and R. Schmitt. 1981. The tetracycline resistance transposons Tn*1721* and Tn*1771* have three 38-base-pair repeats and generate five-base-pair direct repeats. Mol.Gen.Genet. **181**:87-94.

Schröder, G., S. Krause, E. L. Zechner, B. Traxler, H. Yeo, R. Lurz, G. Waksman, and E. Lanka. 2002. TraG-like proteins of DNA transfer systems and of the *Helicobacter pylori* type IV secretion system: inner membrane gate for exported substrates? J.Bacteriol. **184**:2767-2779.

Schröder, G. and E. Lanka. 2005. The mating pair formation system of conjugative plasmids - A versatile secretion machinery for transfer of proteins and DNA. Plasmid **54**:1-25.

Schumacher, M. A. 2007. Structural biology of plasmid segregation proteins. Curr.Opin.Struct.Biol. **17**:103-109.

Schumacher, M. A. 2012. Bacterial plasmid partition machinery: a minimalist approach to survival. Curr.Opin.Struct.Biol. **22**:72-79.

Seegers, J. F. M. L., W. J. J. Meijer, G. Venema, S. Bron, A. C. Zhao, and S. A. Khan. 1995. Structural and functional analysis of the single-strand origin of replication from the lactococcal plasmid pWVO1. Mol.Gen.Genet. **249**:43-50.

Semsey, S., S. Krishna, J. Erdõssy, P. Horváth, L. Orosz, K. Sneppen, and S. Adhya. 2009. Dominant negative autoregulation limits steady-state repression levels in gene networks. J.Bacteriol. **191**:4487-4491.

Sengupta, M., H. J. Nielsen, B. Youngren, and S. Austin. 2010. P1 plasmid segregation: accurate redistribution by dynamic plasmid pairing and separation. J.Bacteriol. **192**:1175-1183.

Sengupta, M. and S. Austin. 2011. Prevalence and significance of plasmid maintenance functions in the virulence plasmids of pathogenic bacteria. Infect.Immun. **79**:2502-2509.

Sherratt, D. 1989. Tn*3* and related transposable elements: site-specific recombination and transposition, p. 163-184. *In* D. E. Berg and M. M. Howe (eds.), Mobile DNA. ASM Press, Washington, DC, USA.

Shingler, V. and C. M. Thomas. 1989. Analysis of nonpolar insertion mutations in the *trfA* gene of IncP plasmid RK2 which affect its broad-host-range property. Biochim.Biophys.Acta **1007**:301-308.

Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram negative bacteria. Nat.Biotech. **1**:784-791.

Simpson, A. E., R. A. Skurray, and N. Firth. 2003. A single gene on the Staphylococcal multiresistance plasmid pSK1 encodes a novel partitioning system. J.Bacteriol. **185**:2143-2152.

Sims, J., S. Koths, and D. Dressler. 1979. Single-stranded phage replication: positive- and negative-strand DNA synthesis. Cold Spring Harb.Symp.Quant.Biol. **43**:349-365.

Smith, A. S. and D. E. Rawlings. 1998. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the lon protease. J.Bacteriol. **180**:5458-5462.

Smith, P., M. Hiney, and O. Samuelson. 1994. Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning. Annu.Rev.Fish Dis. **4**:273-313.

Sozhamannan, S. and D. K. Chattoraj. 1993. Heat shock proteins DnaJ, DnaK, and GrpE stimulate P1 plasmid replication by promoting initiator binding to the origin. J.Bacteriol. **175**:3546-3555.

Sørum, H., T. M. L'Abée-Lund, A. Solberg, and A. Wold. 2003. Integron-containing IncU R plasmids pRAS1 and pAR-32 from the fish pathogen *Aeromonas salmonicida*. Antimicrob. Agents Chemother. **47**:1285-1290.

Stalker, D. M., R. Kolter, and D. R. Helinski. 1979. Nucleotide sequence of the region of an origin of the antibiotic resistance plasmid R6K. Proc.Natl.Acad.Sci.USA **76**:1150-1154.

Stalker, D. M., C. M. Thomas, and D. R. Helinski. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol.Gen.Genet. **181**:8-12.

Stalker, D. M., R. Kolter, and D. R. Helinski. 1982. Plasmid R6K DNA replication. I. Complete nucleotide sequence of an autonomously replicating segment. J.Mol.Biol. **161**:33-43.

Stenzel, T. T., T. MacAllister, and D. Bastia. 1991. Cooperativity at a distance promoted by the combined action of two replication initiator proteins and a DNA bending protein at the replication origin of pSC101. Genes Dev. **5**:1453-1463.

Summers, D. K., C. W. H. Beton, and H. L. Withers. 1993. Multicopy plasmid instability: the dimer catastrophe hypothesis. Mol.Microbiol. **8**:1031-1038.

Tacon, W. and D. J. Sheratt. 1976. ColE plasmid replication in DNA polymerase I-deficient strains of *Escherichia coli*. Mol.Gen.Genet. **147**:331-335.

Tait, R. C., C. I. Kado, and R. L. Rodriquez. 1983. A comparison of the origin of replication of pSa with R6K. Mol.Gen.Genet. **192**:32-38.

Takechi, S., H. Matsui, and T. Itoh. 1995. Primer RNA synthesis by plasmid-specified Rep protein for initiation of ColE2 DNA replication. EMBO J. **14**:5141-5147.

Tang, M., D. K. Bideshi, H. Park, and B. A. Federici. 2007. Iteron-binding ORF157 and FtsZ-like ORF156 proteins encoded by pBtoxis play a role in its replication in *Bacillus thuringiensis* subsp. *israelensis*. J.Bacteriol. **189**:8053-8058.

te Riele, H., B. Michel, and S. D. Ehrlich. 1986a. Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. Proc.Natl.Acad.Sci. USA **83**:2541-2545.

te Riele, H., B. Michel, and S. D. Ehrlich. 1986b. Are single-stranded circles intermediate in plasmid DNA replication? EMBO J. **5**:631-637.

Thisted, T., N. S. Sørensen, E. G. Wagner, and K. Gerdes. 1994. Mechanism of post-segregational killing: Sok antisense RNA interacts with Hok mRNA via its 5'-end single-stranded leader and competes with the 3'-end of Hok mRNA for binding to the *mok* translational initiation region. EMBO J. **13**:1960-1968.

Thomas, C. D., D. F. Balson, and W. V. Shaw. 1990. *In vitro* studies of the initiation of staphylococcal plasmid replication. J.Biol.Chem. **265**:5519-5530.

Thune, R. L., L. A. Stanley, and R. K. Cooper. 1993. Pathogenesis of gram-negative bacterial infections in warmwater fish. Annu.Rev.Fish Diseases **3**:37-68.

Tolun, A. and D. R. Helinski. 1981. Direct repeats of the F plasmid *incC* region express F incompatibility. Cell **24**:687-694.

Tomizawa, J., T. Itoh, G. Selzer, and T. Som. 1981. Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. Proc.Natl.Acad.Sci.USA **78**:1421-1425.

Toukdarian, A. E. and D. R. Helinski. 1998. TrfA dimers play a role in copy-number control of RK2 replication. Gene **223**:205-211.

Uga, H., F. Matsunaga, and C. Wada. 1999. Regulation of DNA replication by iterons: an interaction between the *ori2* and *incC* regions mediated by RepE-bound iterons inhibits DNA replication of mini-F plasmid in *Escherichia coli*. EMBO J. **18**:3856-3867.

Uhlin, B. E. and K. Nordström. 1978. A runaway-replication mutant of plasmid R1drd-19: temperature-dependent loss of copy number control. Mol.Gen.Genet. **165**:167-179.

Urh, M., J. Wu, K. Wu, K. Forest, R. B. Inman, and M. Filutowicz. 1998. Assemblies of replication initiator protein on symmetric and asymmetric DNA sequences depend on multiple protein oligomerization surfaces. J.Mol.Biol. **283**:619-631.

Van Melderen, L., P. Bernard, and M. Couturier. 1994. Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. Mol.Microbiol. **11**:1151-1157.

Van Zyl, L.J. 2003 Analysis of the mobilization region of the broad host-range IncQ-like plasmid, pTC-F14, and its ability to interact with a related plasmid, pTF-FC2. Thesis (MSc). University of Stellenbosch.

Vecchiarelli, A. G., Y. W. Han, X. Tan, M. Mizuuchi, R. Ghirlando, C. Biertümpfel, B. E. Funnell, and K. Mizuuchi. 2010. ATP control of dynamic ParA-DNA interactions: a key role for the nucleoid in plasmid partition. Mol.Microbiol. **78**:78-91.

Vocke, C. and D. Bastia. 1983. DNA-protein interaction at the origin of DNA replication of the plasmid pSC101. Cell **35**:495-502.

Wang, P. Z., S. Projan, V. Herniquez, and R. Novick. 1993. Origin recognition specificity in pT181 plasmids is determined by a functionally asymmetric palindromic DNA element. EMBO J. **12**:45-52.

Watve, M. M., N. Dahanukar, and M. G. Watve. 2010. Sociobiological control of plasmid copy number in bacteria. PLos ONE e9329 **5**.

Weigel, D. and J. Glazebrook. 2006. Transformation of *Agrobacterium* using electroporation. Cold Spring Harb.Protoc.doi:10.1101/pdb.prot4665 .

Weinert, T. A., K. M. Derbyshire, F. M. Hugson, and N. D. F. Grindley. 1984. Replicative and conservative transpositional recombination of insertion sequences. Cold Spring Harb.Symp.Quant.Biol. **49**:251-260.

Weitao, T., S. Dasgupta, and K. Nordstrom. 2000. Plasmid R1 is present as clusters in the cells of *Escherichia coli*. Plasmid **43**:200-204.

Whelan, J. A., N. B. Russel, and M. A. Whelan. 2003. A method for the absolute quantification of cDNA using real time PCR. J.Immunol.Meth. **278**:261-269.

Wickner, S., J. Hoskins, and K. McKenney. 1991a. Monomerization of RepA dimers by heat shock proteins activates binding to DNA replication origin. Proc.Natl.Acad.Sci.USA **88**:7903-7907.

Wickner, S., J. Hoskins, and K. McKenney. 1991b. Function of DnaJ and DnaK as chaperones in origin-specific DNA binding by RepA. Nature **350**:165-167.

Wickner, S., D. Skowyra, J. Hoskins, and K. McKenney. 1992. DnaJ, DnaK, and GrpE heat shock proteins are required in *ori*P1 DNA replication solely at the RepA monomerization step. Proc.Natl.Acad.Sci.USA **89**:10345-10349.

Wickner, S. H. and D. Chattoraj. 1987. Replication of mini-P1 plasmid DNA *in vitro* requires two initiation proteins, encoded by the *repA* gene of phage P1 and the *dnaA* gene of *Escherichia coli*. Proc.Natl.Acad.Sci.USA **84**:3668-3672.

Wickner, S. H. 1990. Three *Escherichia coli* heat shock proteins are required for P1 plasmid DNA replication: formation of an active complex between *E. coli* DnaJ protein and the P1 initiator protein. Proc.Natl.Acad.Sci.USA **87**:2690-2694.

Wilkins, B. M. and A. T. Thomas. 2000. DNA-independent transport of plasmid primase protein between bacteria by the I1 conjugation system. Mol.Microbiol. **38**:650-657.

Wilson, I. W., J. Praszkier, and A. J. Pittard. 1993. Mutations affecting pseudoknot control of the replication of B group plasmids. J.Bacteriol. **175**:6476-6483.

Yamaguchi, Y. and M. Inouye. 2011. Regulation of growth and death in *Escherichia coli* by toxin-antitoxin systems. Nat.Rev.Microbiol. **9**:779-790.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103-119.

Yasueda, H., T. Horii, and T. Itoh. 1989. Structural and functional organization of ColE2 and ColE3 replicons. Mol.Gen.Genet. **215**:209-216.

Yasukawa, H., T. Hase, A. Sakai, and Y. Masamune. 1991. Rolling-circle replication of the plasmid pKYM isolated from a gram-negative bacterium. Proc.Natl.Acad.Sci. USA **88**:10282-10286.

Zaman, S., L. Radnedge, H. Richards, and J. M. Ward. 1993. Analysis of the site for second-strand initiation during replication of the *Streptomyces* plasmid pIJ101. J.Gen.Microbiol. **139**:669-676.

Zatyka, M. and C. M. Thomas. 1998. Control of genes for conjugative transfer of plasmids and other mobile elements. FEMS Microbiol.Rev. **21**:291-319.

Zechner, E. L., S. Lang, and J. F. Schildbach. 2012. Assembly and mechanisms of bacterial type IV secretion machines. Philos.Trans.R.Soc.Lond.B.Biol.Sci. **367**:1073-1087.

Zhang, Q., S. Soares de Oliveira, R. Colangeli, and M. L. Gennaro. 1997. Binding of a novel host factor to the pT181 plasmid replication enhancer. J.Bacteriol. **179**:684-688.

Zhang, W., C. Rong, C. Chen, and G. F. Gao. 2012. Type-IVC secretion system: A novel subclass of Type IV Secretion System (T4SS) common existing in Gram-Positive genus *Streptococcus*. PLos ONE **7**:e46390.

Zielenkiewicz, U. and P. Ceglowski. 2001. Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems. Acta Biochim.Pol. **48**:1003-1023.

Zinder, N. D. and K. Horiuchi. 1985. Multiregulatory element of filamentous bacteriophages. Microbiol.Rev. **49**:101-106.

Zzaman, S. and D. Bastia. 2005. Oligomeric initiator protein-mediated DNA looping negatively regulates plasmid replication *in vitro* by preventing origin melting. Mol.Cell **20**:833-843.