Chapter 1

General Introduction

Large Natural Virulence Plasmids of *Enterobacteriaceae*

CONTENTS

1. INTRODUCTION

Plasmids are extrachromosomal elements of DNA. They can be found in all three domains of the living world, in *Archaea*, *Bacteria* and *Eukarya*. Plasmids may constitute a substantial amount of the total genetic content of an organism, representing more than 25% of the genetic material of the cell in some members of *Archaea* (39, 80, 100).

The majority of gram-negative and gram-positive bacteria harbour plasmids. These extrachromosomal elements encode a remarkable array of phenotypic traits of medical, agricultural, environmental and commercial importance (37). Encoded traits include resistances to heavy metals, supplementary metabolic pathways and pathways for degradation of xenobiotics, as well as virulence factors and resistances to antibiotics (46). Plasmids can have the machinery to transfer themselves and other parts of bacterial genome horizontally and thus facilitate the introduction of genes into different species, genera, or sometimes even families (21). Similarly, plasmids can incorporate and deliver genes by recombination or transposition and by this means increase the genetic exchange in- and between bacterial populations (80).

Besides their clinical, biotechnological and environmental relevance, plasmids are famous for their contribution to the advance of basic science. Plasmid research has been at the basis of milestone discoveries, such as the discovery of antisense RNA and the replication and segregation of chromosomes (80). Last but not least, plasmids are important elements in cloning strategies. They are easily isolated, dissected, reassembled and introduced into various hosts and therefore they are used as vectors in gene technology (99).

2. COMMON CHARACTERISTICS OF PLASMIDS

Plasmids are known to be highly diverse; they differ in their size, copy number and genetic make up. Most plasmids consist of covalently closed double-stranded circular DNA molecules, while some plasmids occur as linear double-stranded DNA molecules (46). The linear plasmids have been discovered in *Streptomyces* (35, 49, 60) and *Borrelia* species (10), as well as in yeasts and filamentous fungi, where there are located mostly in mitochondria (25, 57). Two types of linear plasmids exist; the so-called hairpin plasmids with covalently closed ends and those with proteins bound to their 5' termini (55).

The size of plasmids can vary from approximately 300 bp to over 2400 kb (46). Small plasmids are usually present in the host cell in many copies (up to 100), while large plasmids are present in one to two copies per cell (6, 96). Based on the overall genetic content, two types of plasmids are distinguished. One type, designated as non-conjugative or non-transmissible, has genes for the initiation and regulation of its replication but does not possess a functional set of genes that are required for conjugal transfer. The second type of plasmid is the conjugative or self-transmissible that carries, apart from genes needed for autonomous replication, also genes that are involved in conjugation (37).

2.1. Replication

Regardless of plasmid size, the basic replicon of a plasmid generally consist of a contiguous set of information that includes a definable origin, where DNA replication initiates (*ori*), and one or more adjoining controlling elements. All this information is often contained within a segment that is 3 kb or less in size. The *ori*, harboured within several-hundred bp, contains recognition sites for plasmid

and host proteins involved in replication initiation. Most plasmids have a single origin of replication, however replicons with two or even three *ori* can be also found. In the majority of replicons a structural gene, often designated as *rep*, is present that encodes a plasmid-specific protein required for the initiation of replication (37). Larger plasmids often contain more than one replication region (15).

Most of the linear plasmids replicate using a mechanism involving a protein bound to the 5'-end of each strand and that acts in the priming of DNA synthesis (6). Linear plasmids with hairpins at their ends replicate via concatemeric intermediates (80).

For circular plasmids three general replication mechanisms are known: theta type, strand displacement and rolling circle (80). DNA replication through the theta mechanism begins with the melting of the parental strands at the *ori* with help of the Rep protein, a specific plasmid-encoded initiator protein, and DnaA proteins. Then, a primer RNA is synthesised by either RNA polymerase or a primase, and DNA synthesis is initiated by covalent extension of the primer RNA (51). The main replicative helicase of the cell catalyses the further unwinding of the strands. The DNA synthesis of both strands is coupled and occurs continuously on one strand (leading strand) and discontinuously on the other strand (lagging strand). DNA polymerase III is required for elongation of plasmid DNA replication. In addition, DNA polymerase I can participate in the early synthesis of the leading strand. The termination of the synthesis occurs at particular sequences, the *ter* sequences, which are the binding sites of the proteins that promote termination of plasmid replication. In the case that catenates occur, topoisomerases are resolving them. Additional features found in many origins of theta-replicating plasmids are an adjacent AT-rich region, one or more *dnaA* boxes, directly repeated sequences (termed iterons), Dam methylation sequences, and binding sites for factors like IHF (integration host factor) or FIS (factor for inversion stimulation). Replication by the theta-type mechanism is widespread among plasmids from gram-negative bacteria, but it can be also found in plasmids of gram-positive bacteria. The DNA synthesis can start from one or from several origins, and replication can be either uni- or bidirectional (80).

The strand displacement mechanism of replication was mostly studied on the promiscuous plasmids of the IncQ family, whose prototype is RSF1010. Members of this family require three plasmid-encoded proteins for initiation of DNA replication; RepA, RepB and RepC. The replication is bidirectional, starting at *ori*. In these plasmids, the origin of replication includes iterons, a GC-rich stretch and a AT-rich segment, and two palindromic sequences, *ssiA* and *ssiB* (80). Iterons are the RepC-binding sites (32) and the *ssiA* and *ssiB* sequences are specially recognised by RepB, the primase (43). Replication starts, when *ssiA* and *ssiB* are exposed as single-stranded regions. The melting of the DNA strand is dependent on RepC and RepA, a DNA helicase, and is facilitated by the AT-rich region. Priming of DNA synthesis is catalysed by RepB and synthesis of each one of the strands occurs continuously and results in the displacement of the complementary strand. Replication of the displaced strand is initiated at the exposed *ssi*. Due to activities of the three plasmid replication proteins (RepA, RepB and RepC), the replication is independent of host-encoded factors like RNA polymerase, DnaA, DnaB, what may account for the broad-host range character of plasmid harbouring this kind of replicons (80).

Replication by the rolling-circle mechanism is unidirectional and since the synthesis of the leading and lagging strand is uncoupled, it is asymmetric. One of the most relevant features of this type of replication is that the newly synthesised leading plus strand remains covalently bound to the parental plus strand (80). Replication is initiated by the plasmid-encoded Rep protein, which introduces a site-specific nick on the plus strand, at a region termed double-stranded origin (*dso*). The Rep protein becomes covalently attached to the 5' phosphate at the nick site. The 3'-OH end of the nick is used as a primer for leading-strand synthesis. For the DNA synthesis host proteins, DNA polymerase III, single-stranded DNA-binding proteins and a helicase, are needed (48). The end products of leading-

strand replication are a double-stranded DNA molecule constituted by the parental minus strand and the newly synthesised plus strand, and a single-stranded DNA intermediate, which corresponds to the parental plus strand. Finally, the parental plus strand is converted into double-stranded DNA by host proteins initiating the synthesis at the single-stranded origin (*sso*). The *ori* of plasmids with a rolling-circle mechanism harbours the *rep* gene, the *dso* region, which consists of two loci: the *bind* for binding of Rep protein and the *nic*, where the Rep protein introduces the initial nick, and one or two *sso* regions. Replication by rolling-circle is widespread among multicopy plasmids of a size that is usually smaller than 10 kb (80).

2.2. Copy number control mechanisms

Each plasmid has a characteristic copy number within the host. The copy number may vary in different hosts, but within a given host and under fixed growth conditions, any particular plasmid has a characteristic copy number (80). To define and maintain this copy number, plasmids use negative regulatory circuits (65) consisting of plasmid-encoded control elements that regulate the initiation of the replication step (80).

There are three general types of plasmid copy number control systems, depending on the type of negative control element that is used (81). The first is found in plasmids that contain a series of direct repeats, iterons, located within the *ori*. To these direct repeats the plasmid-specific replication initiation protein Rep binds and thereby, on one hand, the initiation of replication is enabled, but on the other hand, also controlled (37). As iterons function by binding Rep proteins, it was initially thought that iterons titrate Rep proteins and thereby make them limiting for replication (91). However increasing Rep beyond the physiological concentration was of little consequence to the copy number in several plasmids (19, 28, 63, 91) and therefore handcuffing was proposed as a new, more reasonable, model for negative control (12).

The second type of control is based on a small, diffusible RNA molecule, named countertranscribed RNA (ctRNA). This ctRNA acts as an antisense transcript that hybridises to a complementary region of an essential RNA (81) and thus negatively regulates a key step in the replication of the plasmid (37). Control systems using ctRNAs (93) are widespread among plasmids that replicate by different mechanisms, but share a similar genetic structure in the control region, namely the presence of two oppositely oriented promoters that direct the synthesis of an RNA essential for replication and of the inhibitor ctRNA, respectively. An important feature of this kind of control systems is that the rate of synthesis of the inhibitor ctRNA is much higher than that of the essential RNA. In addition, the ctRNAs are synthesised from a constitutive promoter and have a short half-life, so that their intracellular concentration stays nearly proportional to the copy number. Differences between various replicons regulated by ctRNAs are found in the step of inhibition. Some plasmids inhibit the maturation of the primer essential for replication, others inhibit the synthesis of the essential *rep* mRNA or the *rep* translation process (81).

The third type of copy number control is based on a combined action of a ctRNA and a protein, often designated Cop (81). Within this last group, there are two categories. In one of them, the ctRNA plays the main regulatory role, whereas the protein has been proposed as only an auxiliary element. In the second category, both elements, but acting on different targets, could correct the fluctuation in the copy number (79).

2.3. Stable maintenance mechanisms

Plasmids ensure their hereditary stability either by maintaining a high copy number and/or by specific genetic mechanisms including multimer resolution, equipartitioning, and post-segregational killing.

The occurrence of plasmid multimers is a frequent event that is most likely often caused by defects in the termination of replication and homologous recombination of monomers. Formation of multimers can have serious consequences, particularly in the case of a low-copy-number plasmid. Therefore a number of different plasmids possess a multimer resolution system (*mrs*) that is site specific and will resolve dimers and multimers. In general the *mrs* consists of a recombinase that acts on a specific site and resolves the multimers (37). Some plasmids encode their own recombinase, but others use the chromosomally encoded Xer proteins. Plasmids carrying transposons with a functional resolvase can utilise the transposon resolvase for this role (86).

A second important feature is the adequate distribution of plasmid copies to daughter cells. For this purpose, plasmids may carry partitioning loci that are involved in the active distribution of the plasmid copies (38, 62). Partitioning loci (*par*) consist of three essential components: two genes encoding *trans*-acting proteins and a *cis*-acting centromere-like site to which the partitioning proteins bind (59) and that results in the formation of a nucleoprotein complex (31). Both *par* genes are located in an operon. The upstream *par* gene encodes an ATPase that is essential to the DNA segregation process, whereas the downstream gene encodes a protein that binds to the centromere-like region. The ATPase functions as an adaptor between a host-encoded component and the partition complex and thereby tethers plasmids to specific subcellular sites (31).

The post-segregational killing or plasmid addiction systems result in death of plasmid-free segregants (30, 44). A number of different plasmid-encoded addiction systems have evolved. They are divided into two types, depending on whether the toxin responsible for the killing of the plasmid-free segregants is excreted and acts extracellularly, or whether the toxin functions intracellularly on the cell that has lost the plasmid. Examples of the first type include the plasmid-encoded secreted bactericidal polypeptides bacteriocins and microcins. The bacteria that produce these polypeptides are resistant against their action, while the bacteria in the population that have lost the plasmid are killed (37). The killing is achieved after adsorbtion of the active polypeptide to specific receptors located on the external surface of sensitive bacteria through the formation of channels in the cytoplasmic membrane, degradation of cellular DNA and/or inhibition of protein synthesis (64). Examples of the second type of plasmid addiction include stabilisation systems with involvement of a plasmid-encoded toxin and an antitoxin (antidote). The bactericidal activity of some of these systems is based on the different stability of the toxin and the antitoxin protein with the antitoxin being far less stable. When bacteria loose the plasmid no new antitoxin is produced and the bacteria are killed. The killing of other systems is based on the lack of the plasmid-encoded antisense RNA, which prevents the translation of the toxin protein mRNA (37).

It has been also proposed that plasmid-encoded restriction-modification systems may exert post-segregational killing in addition to their role in defending the cell against the incoming foreign DNA. Under this scenario, the descendants, that have lost the plasmid, will contain fewer and fewer molecules of the modification enzyme, while the restriction enzyme will persist in the cell. Eventually, the capacity of the modification enzyme to modify the many sites needed to protect the newly replicated chromosomes from the restriction enzyme will become inadequate and the chromosomal DNA will be cleaved at the unmodified sites, thus the cell will be killed (50).

Of the different mechanisms that control plasmid stability, equipartitioning is generally used by low- or intermediate-copy-number plasmids. High-copy-number plasmids seem to rely primarily on

random partitioning. Multimer resolution systems are necessary if multimer formation occurs with significant frequency. Postsegregational killing appears to be primarily used by low- and intermediate-copy-number plasmids as a fail-safe mechanism (37).

2.4. Incompatibility

Plasmids are grouped on the basis of their inability to co-exist with other plasmids, that is to say, by their incompatibility (Inc) group. Two plasmids are said to belong to the same incompatibility group if the stability of one is diminished by the presence of the other. The basis of incompatibility is widely assumed to be competition for limited resources required for plasmid maintenance (99). It is clear, that sharing of any function required for the regulation of plasmid replication by two plasmids is likely to result in incompatibility (37). Several different incompatibility elements can often be isolated from a single plasmid, each involved in a different aspect of plasmid maintenance. Well established elements of incompatibility are the diffusible ctRNAs, which can act not only on the plasmid encoding it, but also on a co-resident plasmid with the same type of replicon (83). Well known elements of incompatibility are also the iterons (89). In general, the dominant incompatibility element, the one that determines the Inc group to which the plasmid is assigned, controls the copy number (99).

3. PLASMID TRANSFER

Plasmids seem to have expanded their habitat by developing machineries that enable their spread to different hosts. An important mechanism in this regard is conjugation, which involves DNA transfer following the establishment of direct contact between a donor and a recipient cell (11, 36, 74). Commonly, genes encoding conjugative-transfer functions are located on plasmids, termed conjugative or self-transmissible plasmids (21).

The initial step in bacterial conjugation involves the formation of physical contact between the donor and recipient cells. This contact is established through thin, tube-like extracellular filaments, the conjugative pili, protruding from the donor cell. The tip of the pilus binds to a receptor on the recipient cell. A depolymerisation step is thought to pull donor and recipient cell together, thus allowing the cell envelopes to engage in intimate contact – a mating pair or a mating aggregate is formed (47). This contact is stabilised in a manner that renders the aggregate more resistant to shear forces (1, 2, 54). To establish the actual DNA transfer, the conjugative plasmid DNA in the donor cell is relaxed at the origin of transfer (*oriT*) by proteins belonging to the DNA transfer and relaxation system, and channelled into the periplasm through the lumen of a hexameric protein. Mating pair formation (Mpf) proteins, which span the cell envelope are required to transfer the single-stranded plasmid DNA into the recipient cell (47). Electrophysiological studies have shown that the presence of Mpf proteins enhances the permeability of the host cell envelope (16). In both cells, the donor and recipient cell, recircularisation of plasmid DNA occurs and the DNA synthesis of the complementary strand is performed by the host enzymes (21). The end result of conjugation are two cells harbouring the conjugative plasmid and being capable of plasmid transfer (23).

The F plasmid was the first plasmid discovered to be able to conjugate and it is considered the paradigm for plasmid-specified transfer systems (23). In plasmid F, the transfer (*tra*) region that encodes the conjugation machinery is 33.5 kb in size and consists of approximately 40 genes that are needed for regulation of transfer, for synthesis and assembly of the pili, for the cutting and transfer of the DNA, for the stabilisation of the mating pair aggregate and for the surface exclusion. The whole *tra*

region has several promoters. The main promoter is the P_{traYZ}, from which the transcription of a polycistronic mRNA encoding the products needed for conjugal transfer is initiated. The regulation of conjugation is exerted by a complex network involving host and plasmid encoded factors. The main positive regulator of the P_{traYZ} promoter, and thus of conjugal transfer, is the plasmid encoded TraJ. For maximal P_{traYZ} activity, the chromosomal transcription activator SfrA (ArcA) and also the plasmid encoded TraY are required (23, 29). TraJ itself is regulated by a process termed fertility inhibition that is accomplished by the combined actions of two *tra* gene products, FinP and FinO. FinP is a plasmid-specific antisense RNA molecule that is complementary to a part of the 5' untranslated region of *traJ* mRNA. The binding of FinP to *traJ* mRNA's complementary sequence prevents translation of *traJ* mRNA and leads to repression of plasmid transfer. The product of *finO* gene is a protein that has no direct influence on *traJ* expression but promotes the duplex formation between *finP* gene RNA and *traJ* mRNA. FinO establishes this by blocking FinP antisense RNA decay i.e. by increasing the effective concentration of FinP RNA (23). At this time, no positive regulators of *traJ* promoter have been identified. Factors that have been suggested to influence F plasmid conjugation include temperature, growth phase, intracellular levels of cAMP, CpxA, SfrB (21), and IHF (24).

In addition to self-transfer, the transfer systems of conjugative plasmids often facilitate the transfer of other DNA sequences that are present in the donor cell. One mechanisms of transfer involves the independent transfer of non-conjugative, mobilisable plasmids that are co-resident in the donor cell. These plasmids usually harbour an *oriT* and several adjacent genes that code for proteins needed for nicking at *oriT*, strand separation and mobilisation of DNA (56). Besides via plasmid mobilisation, DNA sequences can also be transferred after integration of sequences from the bacterial chromosome, transposons, and/or foreign plasmids into the conjugative plasmid (21). Since conjugative processes enable bacteria to transfer different DNA not only between members of their own kingdom, but also to fungi, plants and even mammalian cells (95), it can be stated that bacterial conjugation efficiently mediates horizontal gene transfer in a highly promiscuous manner (47). Therefore conjugation is a phenomenon of fundamental evolutionary, ecological and, since it is a means of transfer and spread of antibiotic resistances and virulence determinants, also of medical importance (21).

4. PLASMID EVOLUTION

The variety of natural plasmids that are present in different hosts suggests that they behave as an entity that aims to propagate and conquer the world. Study of plasmid evolution reveals a number of successful strategies that are consistent with this scenario. The main strategy seems to be to sequester and incorporate new valuable genetic information.

It can be hypothesised that each plasmid starts small, as a replicon, and later acquires more genetic information. It may acquire properties that make the replicon more efficient such as an increased efficiency of replication, more sophisticated control circuits that regulate copy number, and mechanisms that link plasmid replication to cell growth. Furthermore, functions that promote stable inheritance and propagation may become incorporated into the plasmid. A further increase in the ability to survive and multiply may be achieved by acquiring the ability to spread between bacteria and for this purpose the mobilisation systems and mechanisms that enables conjugative transfer may have evolved (86). Since a plasmid continued existence depends on the survival of its host, in a next step in their evolution plasmids may sequester and incorporate additional genetic information that may be of great advantage to the host cell, for instance, by acquiring genes that promote bacterial survival in an existing environment or in a new niche. Traits that have been found associated with plasmids include resistances against antibiotics, heavy metals, lysing enzymes, UV, phages, or bacteriocins, but

also factors that alter energy metabolism, virulence or pathogenicity (e.g. toxins, transport systems, colonisation factors, serum resistance, capsule and iron transport systems), and properties that are relevant for symbiosis, host specificity and nodulation (46).

A good example of successful evolution are the ColV plasmids, which carry several of the above mentioned properties including several replication regions, copy number control mechanisms, colicin V, plasmid transfer-related functions, the aerobactin uptake system, increased serum survival, resistance to phagocytosis, change in motility, hydrophobicity, and intestinal epithelial cell adherence (94).

5. LARGE NATURAL VIRULENCE PLASMIDS OF *ENTEROBACTERIACEAE*

One of the most evolved class of plasmids seems to be the large natural plasmids that can be found in bacteria belonging to the family of *Enterobacteriaceae*. This subgroup of facultatively anaerobic gram-negative rods consists of 32 genera (*Arsenophonus*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Photorhabdus*, *Pragia*, *Proteus*, *Providencia*, *Rahnella*, *Salmonella*, *Serratia*, *Shigella*, *Tatumella*, *Trabulsiella*, *Xenorhabdus*, *Yersinia* and *Yokenella*) (20, 40). These bacteria are distributed world-wide and are found in soil, water, fruits, vegetables as well as in a wide variety of living creatures ranging from flowering plants and insects to humans (40). *Enterobacteriaceae* have also been associated with more or less serious infections such as abscesses, pneumonia, meningitis, septicaemia, urinary tract and intestinal infections. The *Enterobacteriaceae* are thought to account for nearly 50% of septicaemia cases, more than 70% of urinary tract infections and a significant percentage of intestinal infections, and represent over 50% of the clinically significant bacteria in clinical microbiology laboratories (20). Medically most important genera are *Shigella*, *Salmonella*, *Yersinia*, *Escherichia*, *Klebsiella*, *Proteus*, *Enterobacter*, and *Serratia*.

In recent years, it has become evident that the pathogenicity associated with *Enterobacteriaceae* is often encoded by both chromosomal and extrachromosomal elements including large natural plasmids (18). The involvement of these plasmids in bacterial virulence as well as antibiotic resistance has renewed the interest in their properties and evolution. At this time the nucleotide sequence of a series of large natural plasmids of *Enterobacteriaceae* have been completely determined, including of plasmid F, pO157, pB171 and R721 of *Escherichia coli* (*E. coli*), ColIb-P9, R100, pWR501 and pCP301 of *Shigella*, R64, R27 and pSLT of *Salmonella*, R751 of *Enterobacter aerogenes*, and pYVe227, pYVe8081, pCD1 and pMT1 of *Yersinia. Citrobacter*, *Klebsiella*, *Edwardsiella*, and *Providencia* also may carry large plasmids, but their complete sequences are not yet available. The general characteristics of several typical, clinically important large natural plasmids of *Enterobacteriaceae* are summarised in Table 1. In this Table also two plasmids are presented that are not directly connected with disease, but that have been leading study objects for biology of plasmids: the narrow-host range F plasmid, and the promiscuous R751 plasmid. To illustrate importance of plasmids for virulence, the characteristics of several of individual large natural plasmids are discussed below (see also Table 1).

Plasmid pO157 is found in most enterohemorrhagic *Escherichia coli* O157:H7 strains (9). Virulence factors that are encoded on this plasmid are EHEC-haemolysin (69, 70, 97, 98), O157:H7 toxin (9) and *katP,* which encodes a catalase-peroxidase that protects bacterium against oxidative stress (8). pO157 has also a gene *espP*, coding for an extracellular serine protease. Whether this protease plays a role in pathogenicity is still unknown (8). The *etp* loci found on pO157 are coding for Type II secretion pathway related proteins (71). Although the operon *etp* appears to contain all the necessary genes

Table 1: Important plasmids of *Enterobacteriaceae*

Plasmid[a]	Size (bp)	Replication regions[b]	Stable maintenance systems
F (AP001918) *E. coli*	99,159	RepFIA (f) RepFIB (f) RepFIC	*flm*, *srn*, and *ccd* post-segregational killing systems; *sop* partitioning system; *psi* inhibition of SOS system
pO157 (AF074613) *E. coli*	92,077	RepFIIA (f) RepFIB (f) RepFIA	*flm*, and *ccd* post-segregational killing systems; *kfr*, and *klc* stable maintenance systems; *sop* partitioning system; *psi* inhibition of SOS system
pB171 (AB024946) *E. coli*	68,817	RepFIIA (f) RepFIB (f)	*ccd* post-segregational killing system; *stb*, and *vag* stable maintenance systems
pWR501 (AF348706) *S. flexneri*	221,851	RepFIIA (f)	*ccd* post-segregational killing system; *par* partitioning system; *mvp* maintenance system; *psi* inhibition of SOS system
R27 (AF250878) *S. typhi*	180,461	RepHI1A (f) RepHI1B (f) RepFIA	*stm* post-segregational killing system; *par* partitioning system; *stb* stable maintenance system
R751 (U67194) broad host range	53,425	TrfA (f)	*inc*, and *kor* partitioning systems; *kle*, *klc*, and *kfr* stable maintenance systems
pYVe8081 (AF336309) *Y. enterocolitica*	67,720	IncL/M similar replication region (f)	*sop*, and *spy* partitioning systems

[a] For each plasmid the Genbank Accession number (in brackets) and its host are given .
[b] The functional replication region is marked with (f).
[c] Conjugal transfer regions of pO157 and pWR501 are incomplete. The conserved (partial) genes that are present are indicated.

Transposable elements	Conjugal transfer region[c]	Virulence factors	Other properties	References
Tn*1000* IS*2*, IS*3*	*tra*	none	*pif* for inhibition of T7 development	21, 22, 23, 33, 41, 42, 52, 58, 67
Tn*801* IS*3*, IS*21*, IS*91*, IS*600*, IS*629*, IS*911*	*traI*, *traX*, *finO*	Ap^r (Tn*801*); EHEC-haemolysin; O157:H7 toxin; *katP* catalase-peroxidase	*espP* extracellular serine protease; *etp* type II secretion pathway	8, 9, 34, 67, 69, 70, 71, 73, 98
IS*1*, IS*3*, IS*10*, IS*21*, IS*30*, IS*91*, IS*100*, IS*258*, IS*629*, IS*630*, IS*679*, IS*911*, IS*1491*	none	*bfp* pili	*imp* UV protection; glutamate racemase amino acid antiporter	78, 88
IS*1*, IS*2*, IS*3*, IS*4*, IS*10*, IS*21*, IS*91*, IS*100*, IS*150*, IS*600*, IS*629*, IS*630*, IS911, IS*1294*, IS*1328*, IS*1353*, IS*Sfl1*, IS*Sfl2*, IS*Sfl3*, IS*Sfl4*	*traD, traI*, *traX*, *finO*	*ipa-mxi-spa* pathogenicity island needed for invasion; *shet2* toxin; *mkaD* intracellular growth determinant	*rfuB* UDP-sugar hydrolase for O-antigen biosynthesis	68, 92
Tn*10* IS*1*, IS*2*, IS*30*	*tra1, tra2*	Tc^r (Tn*10*)		26, 27, 61, 72
Tn*4321*	*tra, trb*	polyketide antibiotic resistance (Tn*4321*)		9, 45, 75, 76, 87
Tn*1*-like IS*1541C*, IS*Yen1*, IS*Yen1*-like, IS*4*-like, IS*4F*-like, IS*1327*-like, IS*1328*-like, IS*1353*-like, IS*1400*, IS*1400*-like, IS*1477a*-like, IS*5377*-like, IS*pG5*-like	none	*yop* outer proteins; type III secretion system; V antigen	low-calcium response	3, 17, 77

needed for exoprotein secretion, its ability to secrete proteins has not yet been demonstrated and the correlation with disease is also unclear (9).

The plasmid pB171 is an EAF (EPEC adherence factor) plasmid, found in the enteropathogenic *Escherichia coli* (EPEC) strain B171. Epidemiological studies of *Escherichia coli*-associated diarrhea in children have shown that the localised adherence phenotype is correlated with EPEC strains that harbour large plasmids. This family of related plasmids has been denoted EAF plasmids (4). Plasmid pB171 harbours a locus *bfp* that encodes the bundle-forming pili that are produced within adherent microcolonies of EPEC (78). Plasmid pB171 also carries a homologues of the *impB* gene present on the virulence plasmids of *Salmonella* and *Shigella*. In these pathogens, the *impCAB* operon is involved in UV protection and mutation. Whether the single gene *impB* found on pB171 has a similar function is unknown. Another locus that was identified on pB171 showed to be homologous with a family of amino acid antiporter protein genes of *E. coli*, *Shigella flexneri* and *Lactococcus lactis*. These antiporter proteins are necessary for glutamate-dependent acid resistance. pB171 also carries a gene that appears to belong to the glutamate racemase family of genes. The glutamate racemases are participating in the biosynthesis of D-glutamate, an essential component of the bacterial peptideglycan (88).

The plasmid pWR501 had been derived, by insertion of selectable marker Tn*501*, from the native virulence plasmid pWR100. This plasmid has been found in the *S. flexneri* wild type serotype 5a strain M90T (92). *Shigella* spp. cause bacillary dysentery in humans by invading and replicating in epithelial cells of the colon (20). The entire set of genes critical for invasion of epithelial cells is contained on a large 220 kb plasmid, termed the virulence plasmid or the invasion plasmid. Such a plasmid is present in all pathogenic strains (90) and the pWR100 is such virulence plasmid. pWR501 contains the typical pathogenicity island, encompassing the *ipa-mxi-spa* loci needed for invasion of *Shigella* into epithelial cells. Other virulence genes are distributed throughout the plasmid and include five alleles *ipaH* gene, and one allele each of *virG*, *virA*, *icsP*, *virF*, *virK*, *msbB*, *sepA*, *ipgH*, *shet2*, *phoN-Sf*, *trcA*, and an apyrase gene. Other proteins with significant sequence similarity to known virulence-associated proteins are a protein similar to toxin ShET2, a *Shigella flexneri* bacterial factor leading to release of proinflammatory cytokines and osmotic leak of the mucosal epithelium, a protein similar to the *Salmonella* serovar Typhimurium intracellular growth and virulence determinant MkaD, a protein similar to the *E. coli* lipopolysaccharide biosynthesis-related protein RfuB, and a protein similar to a UDP-sugar hydrolase (92). Experimental data have shown that pWR501 has only remnants of *tra* region and thus is not capable of self-transfer by conjugation. However, it can be conjugated in the presence of conjugative plasmids (68). Another interesting feature of this plasmid is, that it is the first described plasmid with high proportion of IS elements (92).

Plasmid R27, the prototype IncHI1 plasmid, was discovered in *Salmonella typhi* (*S. typhi*). *S. typhi* is the causative agent of typhoid fever. Multiple antibiotic-resistant *S. typhi* has contributed significantly to the persistence of typhoid fever. The plasmid-encoded multiple drug resistance is always encoded by plasmids of the incompatibility group H (IncH) (72). R27 is a self-transmissible plasmid, which is capable of transfer between members of *Enterobacteriaceae* and several other gram-negative organisms. IncH1 plasmids are characterised by an unusual thermosensitive mode of transfer, with optimal transfer between 22 and 28 °C and no transfer at 37 °C (53). Two separate and distinct regions of R27 are responsible for conjugative transfer, Tra1 and Tra2 (84). Tra1 region contains genes required for DNA translocation across the membrane during conjugation as well as initial replication events after the plasmid has entered the recipient cell. Tra2 region contains genes for pilus production and also for mating pair formation (66, 85). No genes directly related to pathogenesis were identified on R27 (72).

Plasmid R751 (45), a self-transmissible promiscuous plasmid, is the best studied IncPβ plasmid (87). R751 harbours a cryptic transposon Tn*4321* (76). This is a composite transposon with IS,

IS*4321L* and IS*4321R*, at its ends. In-between the two IS elements, a protein is encoded. The possible role of this protein could be in inactivation of a polyketide antibiotic and thus conferring resistance (87). For conjugative transfer two regions are needed, *tra* and *trb* operon. The *tra* operon codes for a primase, a DNA-relaxase, *oriT* recognising and binding proteins and genes, whose products are needed for mating pair formation, relaxosome stabilisation and DNA transfer. The *trb* operon codes for genes, whose products are needed for regulation, for mating pair formation, for entry exclusion and for pili formation (U67194). A unique feature of the IncP plasmids is the co-ordinated regulation of replication and transfer functions, exerted by the *kor* genes (87).

Another well studied plasmid is pYVe8081 (77) that was found in a *Yersinia enterocolitica* serotype 0:8, which is associated with more invasive disease (7, 13). Essential virulence proteins encoded by genes that are carried on pYVe8081 include the Yops (*Yersinia* outer proteins), the type III secretion system, the V antigen. The virulence plasmid encodes also the low-calcium response (82), which refers to a complex response to in vitro growth conditions of a temperature of 37 °C and extracellular calcium concentration less than 2.5 mM Ca^{2+} (14).

Overall, the presence of a multitude of virulence genes, but also the variable presence of genes with related function on different large natural plasmids, suggests that these plasmids are highly dynamic and of great medical relevance.

6. AIM AND SCOPE OF THIS THESIS

Current knowledge on natural plasmids indicates that they are important elements in the development of a variety of bacterial infections and that they play an important role in the dissemination of virulence determinants between bacterial populations. The composition of the various plasmids suggests that plasmid evolution involves the continuous reshuffling of genetic information between plasmids and chromosomal elements (5) resulting in the generation of novel, perhaps better host-adapted plasmids.

Considering the importance of large natural plasmids, we focussed our studies for this thesis on a recently identified large natural conjugative *E. coli* plasmid, pRK100, which was isolated from an *E. coli* strain causing a urinary tract infection (UTI) in humans (101). The primary objectives of the work described in this thesis were:

1. Characterisation of the genetic organisation of pRK100 and construction of a pRK100 plasmid map based on the obtained genetic information.
2. Identification of the origin(s) of replication and evolutionary origin of pRK100.
3. Identification of molecular mechanisms that drive and regulate the activity of *tra* region in pRK100, and, hence, control the conjugal transfer machinery.

The results of the experiments performed to characterise the self-transmissibility and general genetic make-up of the plasmid are presented in Chapter 2. In Chapter 3, the origin and relatedness of plasmid pRK100 with other large plasmids and the mosaicism of pRK100 are described. In the studies on the genetic regulation of the conjugal transfer, we focussed on the identification of positive regulators of TraJ, a principal regulator of the conjugation event. In Chapters 4 and 5, for the first time, evidence is provided that in large natural plasmids the activity of the *traJ* promoter is regulated by the DNA binding protein CRP and further fine-tuned by the global regulators H-NS and Lrp. In the General discussion (Chapter 6), the major findings of this work are taken together and discussed.

7. REFERENCES

1. **Achtman, M.,** 1975. Mating aggregates in *Escherichia coli* conjugation. *J Bacteriol* **123**:505–515.
2. **Achtman, M., G. Morelli, and S. Schwuchow.** 1978. Cell-cell interactions in conjugating *Escherichia coli*: role of F pili and fate of mating aggregates. *J Bacteriol* **135**:1053–1061.
3. **Athanasopoulos, V., J. Praszkier, and A. J. Pittard.** 1995. The replication of an IncL/M plasmid is subject to antisense control. *J Bacteriol* **177**:4730–4741.
4. **Baldini, M. M., J. B. Kaper, M. M. Levine, D. C. Candy, and H. W. Moon.** 1983. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. *J Pediatr Gastroenterol Nutr* **2**:534–538.
5. **Boyd, E. F., C. W. Hill, S. M. Rich, and D. L. Hartl.** 1996. Mosaic structure of plasmids from natural populations of *Escherichia coli*. *Genetics* **143**:1091–1100.
6. **Brock, T. D., M. T. Madigan, J. M. Martinko, and J. Parker.** 1994. Biology of microorganisms. 7th edition. Prentice-Hall International, Inc., London.
7. **Brubaker, R. R.** 1991. Factors promoting acute and chronic diseases caused by yersiniae. *Clin Microbiol Rev* **4**:309–324.
8. **Brunder, W., H. Schmidt, and H. Karch.** 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology* **142**:3305–3315.
9. **Burland, V., Y. Shao, N. T. Perna, G. Plunkett, H. J. Sofia, and F. R. Blattner.** 1998. The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Res* **26**: 4196–4204.
10. **Casjens, S., M. Delange, H. L. Ley 3rd, P. Rosa, and W. M. Huang.** 1995. Linear chromosomes of Lyme disease agent spirochetes: genetic diversity and conservation of gene order. *J Bacteriol* **177**:2769–2780.
11. **Cavalli, L. L., E. Lederberg, and J. M. Lederberg.** 1953. An effective factor controlling sex compatibility in *Bacterium coli*. *J Gen Microbiol* **8**:89–103.
12. **Chattoraj, D. K.** 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. *Mol Microbiol* **37**:467–476.
13. **Cornelis, G., Y. Laroche, G. Balligand, M. P. Sory, and G. Wauters.** 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. *Rev Infect Dis* **9**:64–87.
14. **Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M.-P. Sory, and I. Stainier.** 1998. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev* **62**:1315–1352.
15. **Couturier, M., F. Bex, P. L. Bergquist, and W. K. Maas.** 1988. Identification and classification of bacterial plasmids. *Microbiol Rev* **52**:375–395.
16. **Daugelavicius, R., J. K. Bamford, A. M. Grahn, E. Lanka, and D. H. Bamford.** 1997. The IncP plasmid-encoded cell envelope-associated DNA transfer complex increases cell permeability. *J Bacteriol* **179**: 5195–5202.
17. **Davey, R. B., P. I. Bird, S. M. Nikoletti, J. Praszkier, and J. Pittard.** 1984. The use of mini-Gal plasmids for rapid incompatibility grouping of conjugative R plasmids. *Plasmid* **11**:234–242.
18. **Dorman, C. J**. 1994. Genetics of bacterial virulence. Blackwell Scientific Publications, Oxford.
19. **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.
20. **Farmer III, J. J.** 1999. *Enterobacteriaceae:* Introduction and identification, p. 442–458. *In* P. R. Murray, *et al.* (editors), Manual of Clinical Microbiology, 7th edition. ASM Press, Washington, D. C.
21. **Firth, N., K. Ippen-Ihler, and R. A. Skurray.** 1996. Structure and function of the F factor and mechanism of conjugation, p. 2377–2401. *In* F. C. Neidhardt, *et al.* (editors), *Escherichia coli* and *Salmonella*, Cellular and molecular biology, 2nd edition. ASM Press, Washington, D. C.
22. **Frame, R., and J. O. Bishop.** 1971. The number of sex-factors per chromosome in *Escherichia coli*. *Biochem J* **121**:93–103.
23. **Frost, L., K. Ippen-Ihler, and R. A. Skurray.** 1994. Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiol Rev* **58**:162–210.
24. **Frost, L. S., and J. Manchak.** 1998. F- phenocopies: characterization of expression of the F transfer region in stationary phase. *Microbiology* **144**:2579–2587.

25. **Fukuhara, H.** 1995. Linear DNA plasmids of yeasts. *FEMS Microbiol Lett* **131**:1–9.
26. **Gabant, P., P. Newnham, D. Taylor, and M. Couturier.** 1993. Isolation and location on the R27 map of two replicons and an incompatibility determinant specific for IncHI1 plasmids. *J Bacteriol* **175**:7697–7701.
27. **Gabant, P., A. O. Chahdi, and M. Couturier.** 1994. Nucleotide sequence and replication characteristics of RepHI1B: a replicon specific to the IncHI1 plasmids. *Plasmid* **31**:111–120.
28. **Garcia de Viedma, D., R. Giraldo, G. Rivas, E. Fernandez-Tresguerres, and R. Diaz-Orejas.** 1996. A leucine zipper motif determines different functions in a DNA replication protein. *EMBO J* **15**:925–934.
29. **Gaudin, H. M., and P. M. Silverman.** 1993. Contributions of promoter context and structure to regulated expression of the F plasmid *traY* promoter in *Escherichia coli* K-12. *Mol Microbiol* **8**:335–342.
30. **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.
31. **Gerdes, K., J. Møller-Jensen, and R. B. Jensen.** 2000. Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol* **37**:455–466.
32. **Gruss, A., and S. D. Ehrlich.** 1988. Insertion of foreign DNA into plasmids from gram-positive bacteria induces formation of high-molecular-weight plasmid multimers. *J Bacteriol* **170**:1183–1190.
33. **Guyer, M. S.** 1978. The γδ sequence of F is an insertion sequence. *J Mol Biol* **126**:347–365.
34. **Hales, B. A., C. A. Hart, R. M. Batt, and J. R. Saunders.** 1992. The large plasmids found in enterohemorrhagic and enteropathogenic *Escherichia coli* constitute a related series of transfer-defective Inc F-IIA replicons. *Plasmid* **28**:183–193.
35. **Hayakawa, T., T. Tanaka, K. Sakaguchi, N. Otake, and H. Yonehara.** 1979. A linear plasmid-like DNA in *Streptomyces* sp. producing lankacidin group antibiotics. *J Gen Appl Microbiol* **25**:255–260.
36. **Hayes, W.** 1953. Observations on a transmissible agent determining sexual differentiation in *Bacterium coli*. *J Gen Microbiol* **8**:72–88.
37. **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, *et al.* (editors), *Escherichia coli* and *Salmonella*, Cellular and molecular biology, 2nd edition. ASM Press, Washington, D. C.
38. **Hiraga, S.** 1992. Chromosome and plasmid partition in *Escherichia coli*. *Annu Rev Biochem* **61**:283–306.
39. **Holmes, M. L., F. Pfeifer, and M. L. Dyall-Smith.** 1995. Analysis of the halobacterial plasmid pHK2 minimal replicon. *Gene* **153**:117–121.
40. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergery's manual of determinative bacteriology, 9th edition. Williams & Wilkins, Baltimore.
41. **Hu, S., E. Otsubo, N. Davidson, and H. Saedler.** 1975. Electron microscope heteroduplex studies of sequence relations among bacterial plasmids: identification and mapping of the insertion sequences IS*1* and IS*2* in F and R plasmids. *J Bacteriol* **122**:764–775.
42. **Hu, S., K. Ptashne, S. N. Cohen, and N. Davidson.** 1975. αβ sequence of F is IS*31*. *J Bacteriol* **123**: 687–692.
43. **Ingmer, H., and S. N. Cohen.** 1993. The pSC101 par locus alters protein-DNA interactions in vivo at the plasmid replication origin. *J Bacteriol* **175**:6046–6048.
44. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. *Mol Microbiol* **17**:205–210.
45. **Jobanputra, R. S., and N. Datta.** 1974. Trimethoprim R factors in enterobacteria from clinical specimens. *J Med Microbiol* **7**:169–177.
46. **Kado, C. I.** 1998. Origin and evolution of plasmids. *Antoine van Leeuwenhoek* **73**:117–126.
47. **Kalkum, M., R. Eisenbrandt, R. Lurz, and E. Lanka.** 2002. Tying rings for sex. *Trends Microbiol* **10**: 382–387.
48. **Khan, S. A.** 2000. Plasmid rolling-circle replication: recent developments. *Mol Microbiol* **37**:477–484.
49. **Kinashi, H., M. Shimaji, and A. Sakai.** 1987. Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. *Nature* **328**:454–456.
50. **Kobayashi, I.** 1998. Selfishness and death: raison d'être of restriction, recombination and mitochondria. *Trends Genet* **14**:368–374.
51. **Kornberg, A., and T. Baker.** 1992. DNA replication, 2nd edition. Freeman, New York.

52. **Lane, H. E.** 1981. Replication and incompatibility of F and plasmids in the IncFI Group. *Plasmid* **5**: 100–126.
53. **Maher, D., and D. E. Taylor.** 1993. Host range and transfer efficiency of incompatibility group HI plasmids. *Can J Microbiol* **39**:581–587.
54. **Manning, P. A., G. Morelli, and M. Achtman.** 1981. TraG protein of the F sex factor of *Escherichia coli* K-12 and its role in conjugation. *Proc Natl Acad Sci USA* **78**:7487–7491.
55. **Meinhardt, F., R. Schaffrath, and M. Larsen.** 1997. Microbial linear plasmids. *Appl Microbiol Biotechnol* **47**:329–336.
56. **Meyer, R.** 2000. Identification of the *mob* genes of plasmid pSC101 and characterization of a hybrid pSC101-R1162 system for conjugal mobilization. *J Bacteriol* **182**:4875–4881.
57. **Miyashita, S., H. Hirochika, J. E. Ikeda, and T. Hashiba.** 1990. Linear plasmid DNAs of the plant pathogenic fungus *Rhizoctonia solani* with unique terminal structures. *Mol Gen Genet* **220**:165–171.
58. **Molineux, I. J.** 1991. Host-parasite interactions: recent developments in the genetics of abortive phage infections. *New Biol* **3**:230–236.
59. **Møller-Jensen, J., R. B. Jensen, and K. Gerdes.** 2000. Plasmid and chromosomal segregation in prokaryotes. *Trends Microbiol* **8**:313–320.
60. **Netolitzky, D. J., X. Wu, S. E. Jensen, and K. L. Roy.** 1995. Giant linear plasmids of beta-lactam antibiotic producing *Streptomyces*. *FEMS Microbiol Lett* **131**:27–34.
61. **Newnham, P. J., and D. E. Taylor.** 1994. Molecular analysis of RepHI1A, a minimal replicon of the IncHI1 plasmid R27. *Mol Microbiol* **11**:757–768.
62. **Nordstrom, K., and S. J. Austin.** 1989. Mechanisms that contribute to the stable segregation of plasmids. *Annu Rev Genet* **23**:37–69.
63. **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.
64. **Pugsley, A. P.** 1984. The ins and outs of colicins. Part II. Lethal action, immunity and ecological implications. *Microbiol Sci* **1**:203–205.
65. **Rasooly, A., and R. P. Novick.** 1993. Replication-specific inactivation of the pT181 plasmid initiator protein. *Science* **262**:1048–1050.
66. **Rooker, M. M., C. Sherburne, T. D. Lawley, and D. E. Taylor.** 1999. Characterization of the Tra2 region of the IncHI1 plasmid R27. *Plasmid* **41**:226–239.
67. **Saadi, S., W. K. Maas, and P. L. Bergquist.** 1984. RepFIC, a basic replicon of IncFI plasmids that has homology with a basic replicon of IncFII plasmids. *Plasmid* **12**:61–64.
68. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect Immun* **35**:852–860.
69. **Schmidt, H., L. Beutin, and H. Karch.** 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect Immun* **63**:1055–1061.
70. **Schmidt, H., C. Kernbach, and H. Karch.** 1996. Analysis of the EHEC *hly* operon and its location in the physical map of the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology* **142**: 907–914.
71. **Schmidt, H., B. Henkel, and H. Karch.** 1997. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic *Escherichia coli* O157 strains. *FEMS Microbiol Lett* **148**:265–272.
72. **Sherburne, C. K., T. D. Lawley, M. W. Gilmour, F. R. Blattner, V. Burland, E. Grotbeck, D. J. Rose, and D. E. Taylor.** 2000. The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res* **28**:2177–2186.
73. **Silva, R. M., S. Saadi, and W. K. Maas.** 1988. A basic replicon of virulence-associated plasmids of *Shigella* spp. and enteroinvasive *Escherichia coli* is homologous with a basic replicon in plasmids of IncF groups. *Infect Immun* **56**:836–842.
74. **Singleton, P., and D. Sainsbury.** 1999. Dictionary of microbiology and molecular biology, 2nd edition. John Wiley & Sons, Chichester, USA.
75. **Smith, C. A., and C. M. Thomas.** 1985. Comparison of the nucleotide sequences of the vegetative replication origins of broad host range IncP plasmids R751 and RK2 reveals conserved features of probable functional importance. *Nucleic Acids Res* **13**:557–572.

76. **Smith, C. A., M. Pinkney, D. G. Guiney, and C. M. Thomas.** 1993. The ancestral IncP replication system consisted of contiguous *oriV* and *trfA* segments as deduced from a comparison of the nucleotide sequences of diverse IncP plasmids. *J Gen Microbiol* **139:**1761–1766.
77. **Snellings, N. J., M. Popek, and L. E. Lindler.** 2001. Complete DNA sequence of *Yersinia enterocolitica* serotype 0:8 Low-calcium-response plasmid reveals a new virulence plasmid-associated replicon. *Infect Immun* **69**:4627–4638.
78. **Sohel, I, J. L. Puente, S. W. Ramer, D. Bieber, C. Y. Wu, and G. K. Schoolnik.** 1996. Enteropathogenic *Escherichia coli*: identification of a gene cluster coding for bundle-forming pilus morphogenesis. *J Bacteriol* **178**:2613–2628.
79. **Solar, G. del, 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 RNA II. *Mol Microbiol* **18**:913–924.
80. **Solar, G. del, R. Giraldo, M. J. Ruiz-Echevarria, M. Espinosa, and R. Diaz-Orejas.** 1998. Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**:434–464.
81. **Solar, G. del, and M. Espinosa.** 2000. Plasmid copy number control: an ever-growing story. *Mol Microbiol* **37**:492–500.
82. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca2+ in the *Yersinia* low-Ca2+ response. *Mol Microbiol* **8**:1005–1010.
83. **Tamm, J., and B. Polisky.** 1983. Structural analysis of RNA molecules involved in plasmid copy number control. *Nucleic Acids Res* **11**:6381–6397.
84. **Taylor, D. E., E. C. Brose, S. Kwan, and W. Yan.** 1985. Mapping of transfer regions within incompatibility group HI plasmid R27. *J Bacteriol* **162**:1221–1226.
85. **Taylor, D. E., P. J. Newnham, C. Sherburne, T. D. Lawley, and M. M. Rooker.** 1999. Sequencing and characterization of *Salmonella typhi* plasmid R27 (incompatibility group HI1) *trhC*, a transfer gene encoding a potential nucleoside triphosphate-binding domain. *Plasmid* **41**:207–218.
86. **Thomas, C. M.** 2000. Paradigms of plasmid organization. *Mol Microbiol* **37**:485–491.
87. **Thorsted, P. B., D. P. Macartney, P. Akhtar, A. S. Haines, N. Ali, P. Davidson, T. Stafford, M. J. Pocklington, W. Pansegrau, B. M. Wilkins, E. Lanka, and C. M. Thomas.** 1998. Complete sequence of the IncPβ plasmid R751: implications for evolution and organisation of the IncP backbone. *J Mol Biol* **282**:969–990.
88. **Tobe, T., T. Hayashi, C.-G. Han, G. K. Schoolnik, E. Ohtsubo, and C. Sasakawa.** 1999. Complete DNA sequence and structural analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid. *Infect Immun* **67**:5455–5462.
89. **Tolun, A., and D. R. Helinski.** 1981. Direct repeats of the F plasmid *incC* region express F incompatibility. *Cell* **24**:687–694.
90. **Tran van Nhieu, G., and P. J. Sansonetti.** 1999. Mechanism of *Shigella* entry into epithelial cells. *Curr Opin Microbiol* **2**:51–55.
91. **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.
92. **Venkatesan, M. M., M. B. Goldberg, D. J. Rose, E. J. Grotbeck, V. Burland, and F. R. Blattner.** 2001. Complete DNA sequence and analysis of the large virulence plasmid of *Shigella flexneri*. *Infect Immun* **69**: 3271–3285.
93. **Wagner, E. G. H., and S. Brantl.** 1998. Kissing and RNA stability in antisense control of plasmid replication. *Trends Biochem Sci* **23**:451–454.
94. **Waters, V. L., and J. H. Crosa.** 1991. Colicin V virulence plasmids. *Microbiol Rev* **55**:437–450.
95. **Waters. V. L.** 2001. Conjugation between bacterial and mammalian cells. *Nat Genet* **29**:375–376.
96. **Watson, J. D., N. H. Hopkins, J. W. Roberts, J. Argetsinger Steitz, and A. M. Weiner.** 1988. Molecular biology of the gene, 4th edition. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.
97. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. *Mol Microbiol* 5:521–528.
98. **Welch, R. A., C. Forestier, A. Lobo, S. Pellett, W. Thomas Jr., and G. Rowe.** 1992. The synthesis and function of the *Escherichia coli* hemolysin and related RTX exotoxins. *FEMS Microbiol Immun* **5**:29–36.

99. **Yarmolinsky, M. B.** 2000. A pot-pourri of plasmid paradoxes: effects of a second copy. *Mol Microbiol* **38**: 1–7.
100. **Zillig, W., H. P. Arnold, I. Holz, D. Prangishvili, A. Schweier, K. Stedman, Q. She, H. Phan, R. Garrett, and J. K. Kristjansson.** 1998. Genetic elements in the extremely thermophilic archaeon *Sulfolobus*. *Extremophiles* **2**:131–140.
101. **Žgur-Bertok, D., E. Modrič, and M. Grabnar.** 1990. Aerobactin uptake system, ColV production, and drug resistance encoded by a plasmid from an urinary tract infection *Escherichia coli* strain of human origin. *Can J Microbiol* **36**:297–299.