Mosaic structure and regulation of conjugal transfer of the *Escherichia coli* plasmid pRK100

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Mosaic structure and regulation of conjugal transfer of the *Escherichia coli* plasmid pRK100

Mozaiekstructuur en regulatie van de overdracht door conjugatie van het *Escherichia coli* plasmide pRK100
(met een samenvatting in het Nederlands)

Mozaičnost in uravnavanje konjugacijskega prenosa plazmida pRK100 bakterije *Escherichia coli*
(s povzetkom v slovenščini)

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"Like many bacteria before me I found that,
once picked up, plasmids were hard to put down."
David Summers

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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.

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Chapter 2

Escherichia coli ColV Plasmid pRK100: Genetic Organisation, Stability and Conjugal Transfer

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ABSTRACT

Uropathogenic *Escherichia coli* strains express chromosomal and plasmid-encoded virulence-associated factors such as specific adhesins, toxins and iron-uptake systems. A ColV plasmid (pRK100) of a uropathogenic strain and its host KS533 were studied. The host strain encodes the K1 capsule, and P and S fimbriae, but neither haemolysin nor the cytotoxic necrotising factor CNF1, indicating that this strain does not harbour a larger pathogenicity island. A restriction map of pRK100 was constructed on the basis of hybridisation experiments and nucleotide sequencing. pRK100 harbours ColV, the conserved replication region RepFIB, the aerobactin-uptake system, a RepFIC replicon and additionally ColIa as well as transposon Tn*5431*. The location of the RepFIC replicon was similar to that in plasmid F. ColV plasmids and F thus share a region spanning more than half the length of plasmid F. Even though their replication and transfer regions are homologous, ColV plasmids are found only in *E. coli* strains. Among the four other species tested, conjugal transfer of pRK100 was demonstrated, with low frequency, only to *Klebsiella pneumoniae*, suggesting that a natural barrier effectively bars transfer. *In vitro* stability of the plasmid with integration into the chromosome to ensure maintenance in the presence of an incompatible plasmid was demonstrated.

Keywords: ColV plasmid, plasmid pRK100, stability, conjugal transfer

1. INTRODUCTION

Pathogenic strains of *Escherichia coli* can cause intestinal and extraintestinal infections as well as new-born meningitis (22). They encode various virulence determinants - adhesins, toxins, capsules, invasins and other virulence factors - on the chromosome, on plasmids, or on the genomes of bacteriophages. Virulence genes can be organised into large chromosomal blocks termed pathogenicity islands (4).

Plasmids are self-replicating and are normally not essential for bacterial growth; however, they often encode antibiotic resistances, colicins and various virulence determinants. Conjugative plasmids, in particular, facilitate the exchange and spread of resistances to antibiotics and chemicals, virulence factors and metabolic properties. The ColV plasmids form a heterogeneous group of large plasmids belonging to the IncFI incompatibility group and encoding the production of colicin V (30). ColV plasmids are harboured primarily by virulent enteric bacteria and encode several virulence-associated properties. Colicin V is a small molecule, but is not SOS inducible and is therefore actually a microcin (7). Recent studies in a chicken embryo model system have shown that an avirulent wild type avian *E. coli* strain transformed with the cloned colicin V genes became virulent, demonstrating that colicin V has a direct role in virulence enhancement (32). Most colicin V plasmids also encode the aerobactin-uptake system and increased serum survival as well as resistance to phagocytosis. They could serve as models for the study of the evolution and molecular biology of other virulence plasmids.

ColV plasmids can harbour more than one replicon. The main replicon is homologous to the RepFIB of plasmid F. This replication region, along with the aerobactin-uptake system, is highly conserved. Some ColV plasmids also carry a replicon homologous to RepFIA of plasmid F and it has been reported that pColV-K30, the prototypic ColV plasmid, also carries an incomplete RepFIC (30) even though its location has not been determined.

Colicin V production was first described more than 70 years ago (14), indicating that ColV plasmids were present in natural *E. coli* populations well before the widespread use of antibiotics. Use of antibiotics has probably selected for carriage of additional virulence and resistance determinants on ColV plasmids.

pRK100 is an approximately 145 kb conjugative plasmid, which was discovered in a uropathogenic *E. coli* strain (34). The plasmid encodes colicins V and Ia, the aerobactin-uptake system and the 16.1 kb transposon Tn*5431* with ampicillin and tetracycline resistance determinants (35, 36). The present work was carried out to study pRK100, to prepare its map, and to gain insight into its evolution, conjugational transfer and stability.

2. MATERIAL AND METHODS

Bacterial strains, plasmids and media

The *E. coli* strains and plasmids used in this study are listed in Table 1. KS533 is a uropathogenic strain, serotype rough:K1:H7 (determined by I. Ørskov), isolated at the Institute of Microbiology, Medical Faculty of Ljubljana. It harbours a large (145 kb) conjugative plasmid, pRK100 (34). *E. coli* HB101 was used as the recipient strain for the conjugative transfer of plasmids pRK100 and RSF2001, a kanamycin-resistant derivative of plasmid F. *E. coli* DH5*a* was used as recipient for recombinant plasmids.

Plasmid pColVK-30, whose map has already been published (29), was used for comparison of probe binding in the hybridisation experiments for IS*1*, RepFIA, RepFIB, RepFIC, the aerobactin-up-

take system and the *tra* region. Plasmids of the Couturier bank of rep probes were used for replicon typing.

The *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* strains used as recipients in conjugation experiments are clinical isolates. The *Salmonella typhimurium* recipient was LT2.

***Table 1. E. coli* strains and plasmids**

Strain or plasmid	Relevant properties	Source or reference*
Strains		
KS533	Rough:K1:H7 harbouring pRK100	34
HB101	*hsdR hsdM recA13 supE44 leuB6 lacZ proA2*	D. Ehrlich[1]
DH5α	*thi-1 recA hsdR17 lac*	A. Francky[2]
C600	*thi-1 thr-1 leuB6 lacY1* Sm^R	B. Bachmann[3]
AB1133	Sensitive to all colicins	B. Bachmann
KH1038	Sensitive only to ColV	K. G. Hardy[4]
KH1044	Resistant to ColV	K. G. Hardy
Plasmids		
pRK100	ColV plasmid with Tn*5431*	34
pColV-K30	ColV	17
RSF2001	F plasmid with Kn^R	16
pUC19	Ap^R	33
pAlter	Tc^R	Promega
pTC72#24	IS*1*	M. Chandler[5]
pABN1	Aerobactin-uptake system	11
pHK11	Colicin V	13
pED100	*tra* operon	31
pPKL4	Fimbriae type I	23
pRHU845	*pap*/*prs*	23
pANN801-13	*sfa*/*foc*	23
pUBK2404	RepFIB	10
pUBL2440	RepFIC	10
pUBL2422	Rep9	10
pFDH1	pAlter with the 20 kb *Hin*dIII fragment of pRK100	This study
pFDH2	pAlter with the 20 and 12 kb *Hin*dIII fragments of pRK100	This study
pFDS1	pAlter with the 17.5 kb *Sal*I fragment of pRK100	This study
pUX1	pUC19 with 0.9 kb *Eco*RI–*Hin*dIII of pFDH1	This study
pUV55	pUC19 with the 5 kb *Eco*RI fragment of pFDH2	This study
pUX5	pUC19 with the 3.6 kb *Eco*RI fragment of pFDH1	This study
pUX600	pUC19 with 0.6 kb *Pst*I fragment of pUX5	This study

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[2]National Institute of Chemistry, Ljubljana, Slovenia
[3]*E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, CT, USA
[4]Biogen SA, Geneva, Switzerland
[5]Centre de Recherche de Biochimie et Génétique Cellulaire du CNRS, Toulouse, France

Strains were grown in Luria-Bertani (LB) medium. When necessary, LB was supplemented with: ampicillin (Ap, 100 μg/ml); tetracycline (Tc, 10 μg/ml); streptomycin (Sm, 100 μg/ml); chloramphenicol (Cm, 10 μg/ml); kanamycin (Kn, 30 μg/ml). For selection of *S. typhimurium*, *K. pneumoniae*, *Ent. cloacae*, *P. aeruginosa* transconjugants, Simmons citrate media (8) supplemented with the appropriate antibiotics was used.

General DNA manipulation techniques

Prior to DNA manipulation, plasmids pRK100 and pColVK-30 were transferred to *E. coli* HB101. Isolation of plasmid and chromosomal DNA, large-scale isolation of CsCl-purified pRK100, and ligation and transformation experiments were performed as described by Sambrook, *et al.* (27). To visualise shorter restriction fragments pRK100 DNA was concentrated by ultrafiltration through a Centricon concentrator (Amicon) prior to restriction enzyme cleavage. Plasmids pRK100 and pColVK-30 were cleaved with *Eco*RI, *Hin*dIII, *Sal*I, *Xho*I and by double cleavages. Plasmids carrying cloned inserts of pRK100 were additionally cleaved with *Acc*I, *Bam*HI, *Cla*I, *Dra*I, *Pst*I and *Pvu*II. Restriction endonuclease digestions were carried out according to the instructions of the supplier. DNA fragments were purified from agarose gels using the Geneclean II system (Bio101).

Agarose gel electrophoresis and restriction fragment size determination

Restriction enzyme digests of pRK100 were separated by constant field (CFGE) and pulsed-field (PFGE) gel electrophoresis. For separation and size determination of restriction fragments ranging from 0.2 to 7 kb, CFGE with 0.5–2% submarine agarose gels was used. Larger fragments were separated by PFGE (Pharmacia Biotech, Gene Navigator System).

DNA hybridisation for pRK100-encoded determinants

DNA labelling and hybridisation experiments were carried out using the DIG DNA labelling and detection kit (Boehringer). Hybridisation experiments were performed to detect the aerobactin-uptake system, ColV, the *tra* operon, replicons – first to determine, which replication regions were present and subsequently their position (RepFIB, RepFIC, Rep9) – IS*1*sequences and chromosomal P, type I and S-fimbriae. The following labelled probes were used. For the aerobactin-uptake system the 2.7 kb *Sal*I–*Bam*HI fragment of pABN1 (11) with genes *iucA* and *iucB* was used, and for ColV the 0.5 kb *Pvu*II–*Bgl*II fragment of pHK11 with *cvaC* and the overlapping *cvi* genes (13). All *Eco*RI restriction fragments of pRK100 were labelled and used to probe the Couturier bank of rep plasmids (10): for RepFIB the 1.2 kb *Pst*I fragment of pUBL2404; for RepFIC the 0.9 kb *Eco*RI–*Hin*dIII fragment of pUBL2440; for Rep9 the 0.5 kb *Pst*I fragment of pUBL2422 (10). For the *tra* operon, *Eco*RI fragments of pED100 and the 8.3 kb *Eco*RI restriction fragment f6 of pED100 (31) were used, and for detection of IS*1* the 2.9 kb *Pvu*II fragment of pTC72#24 (kindly provided by M. Chandler).

The 0.9 kb *Eco*RI–*Hin*dIII fragment of pFDH1 and the three longest (27, 20 and 17 kb) *Eco*RI fragments of pRK100 were also individually labelled and used to probe *Eco*RI, *Hin*dIII, *Sal*I, *Xho*I and double digestions of pRK100.

Stringent conditions were used for all hybridisation experiments described in this report.

Colicin production

ColV and ColIa production was determined using the overlay method (25) with indicator strains of the Pugsley collection of colicinogenic strains and with strains KH1038 (sensitive only to ColV), KH1044 (resistant to ColV) and AB1133 (sensitive to all colicins).

Resistance to the bactericidal action of serum

E. coli C600 and C600 harbouring pRK100, as well as *E. coli* KS533 with and without pRK100, were tested for serum survival or resistance. Serum survival was tested in the presence of 1%, 2% and 3% human serum (19).

Plasmid stability and curing

Plasmid stability was studied by inoculation of the strain into LB medium without antibiotics and incubating at 37 °C with shaking. The next day the cell suspension was diluted into fresh LB medium and again incubated at 37 °C. After 21 passages the cell suspension was diluted and plated. Colonies were then transferred to grids and tested for antibiotic resistances and colicin production.

Plasmid stability was further tested by introducing pED100, a conjugative derivative of plasmid F also of the IncF1 incompatibility group, into KS533.

Plasmid curing was also performed with acridine orange and SDS treatment (15).

Conjugational transfer experiments

For conjugational transfer of plasmids the donor and recipient strains were overstreaked on an LB plate and incubated overnight at 37 °C. Following the mating period, a portion of the mating mixture was removed from the growth surface with a sterile rod and was streaked on a selection plate. For liquid mating, overnight cultures of donor and recipient strains were diluted 50-fold in LB liquid medium. The donor strain was incubated at 37 °C for 3 h without shaking and the recipient strain for 2 h with shaking. A mating mixture consisting of 0.5 ml of the donor and 4.5 ml of the recipient cultures was prepared and incubated for 20 h at 37 °C. Transconjugants were selected on Simmons citrate agar plates, supplemented with Tc and Ap when testing for transfer of pRK100 and with Kn when testing for transfer of plasmid RSF2001. *E. coli* transconjugants were selected on LB media supplemented with Sm, Ap and Tc for transfer of pRK100 or Sm and Kn for transfer of RSF2001. Negative controls were prepared by plating donor and recipient strains separately on selective media. Transconjugants were screened for plasmid DNA.

Production of type I fimbriae, P fimbriae, S fimbriae, CNF1 and haemolysin

Cleaved KS533 chromosomal DNA was probed for type I fimbriae with the labelled 6 kb *Pst*I fragment of pPKL4, for *pap/prs* with the 4 kb *Hin*dIII–*Eco*RI fragment of pRHU845 and for *sfa*/*foc* with the 6 kb *Cla*I–*Eco*RV fragment of pANN801-13. PCR was carried out with primers specific for the cytotoxic necrotising factor CNF1: CNF1 primer 1 (CTGACTTGCCGTGGTTTAGTCGG) and CNF1 primer 2 (TACACTATTGACATGCTGCCCGGA). PCR was carried out in the following steps: heating at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1.5 min, extension at 72 °C for 2 min, and the final extension for 5 min at 72 °C.

Haemolysin production was tested by plating onto LB plates containing 2% washed sheep erythrocytes.

DNA sequencing

Single- and double-stranded sequencing of cloned pRK100 restriction fragments was performed using the Sequenase version 2.0 Sequencing Kit (USB) and the Silver sequencing kit (Promega). Primers were the commercially available M13/pUC forward and reverse sequencing primers.

3. RESULTS

Location of Tn*5431* and flanking regions

Previously, we determined that pRK100 carries transposon Tn*5431*, encoding resistances to Tc and Ap (35). To determine the position of Tn*5431* on pRK100, three clones carrying the antibiotic resistances of Tn*5431*, pFDH1, pFDH2 and pFDS1, were isolated (Fig. 1). pFDH1 harbours the 20 kb *Hin*dIII fragment, pFDH2 two consecutive *Hin*dIII fragments, 20 kb and 12 kb long (isolated by cloning partially *Hin*dIII-digested pRK100 DNA), while pFDS1 carries the 17.5 kb *Sal*I fragment. Using restriction mapping, hybridisation experiments, and testing with indicator strains for colicin activity it was determined that pFDH1 and pFDS1 harbour an incomplete Tn*5431* and that each plasmid carries one flanking region. pFDH1 carries ColIa, pFDS1 carries ColV, and pFDH2 carries the entire Tn*5431*, ColV and ColIa.

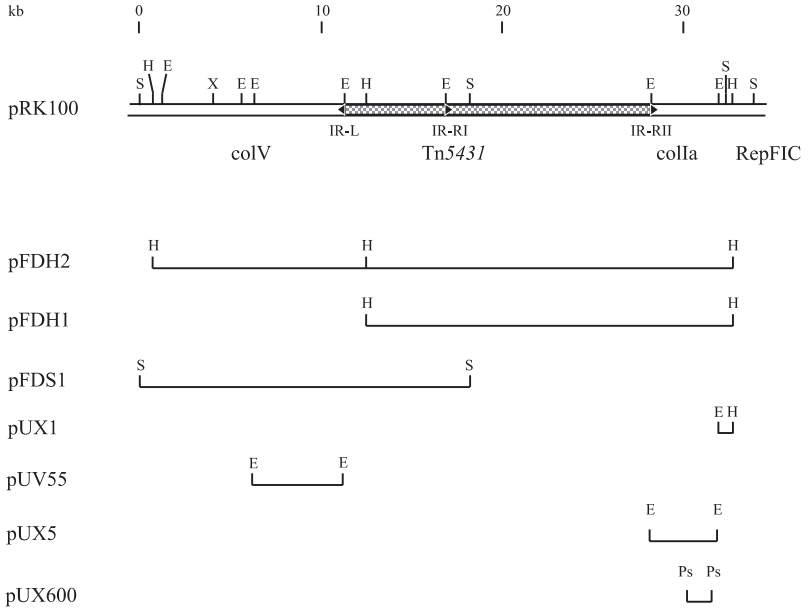

Fig. 1. **Schematic representation of the pRK100 region encoding ColV, Tn*5431*, ColIa, RepFIC and the restriction enzyme maps of clones harbouring restriction fragments of this region**
Restriction sites: E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; S, *Sal*I; X, *Xho*I. *Pst*I restriction sites are shown only for pUX600. The hatched area denotes Tn*5431*, with the arrowheads designating inverted repeats (IR).

To confirm the hybridisation and colicin typing results, plasmids pUV55 and pUX5, with 5 kb and 3.6 kb *Eco*RI fragments of pFDH2, respectively were prepared (Figs 1 and 2). Subsequently, the 0.6 kb *Pst*I restriction fragment of pUX5 was subcloned to generate pUX600. Single-stranded sequencing of the pUV55 insert with the reverse sequencing primer and of the pUX5 insert with the forward sequencing primer showed that Tn*5431* was inserted into ColIa at nucleotide position 3424 (determined by alignment with the sequence deposited under EMBL accession number M13819) of the conserved ORF2, which is not involved in ColIa production. Upstream of the IR-L of Tn*5431* are sequences from nucleotide positions 3425 to 3727 of the 3727 bp deposited sequence. Downstream of the Tn*5431* IR-RII are the colicin Ia immunity and structural genes. Single-stranded sequencing

of the 0.6 kb *Pst*I insert of pUX600 with the forward and reverse sequencing primers demonstrated conservation of sequences of the ColIa structural gene, while the ORFI sequences are not conserved. Alignment of the nucleotide sequence obtained by single-stranded sequencing of pUV55 DNA with the forward primer and the ColV sequence deposited under EMBL accession number X57525 showed conservation of nucleotide sequences of the colicin V *cvaC* structural gene and the *cvi* immunity gene. Nucleotide sequencing also showed characteristic 5 bp repeats of target DNA at both Tn*5431* ends, confirming that transposition had occurred.

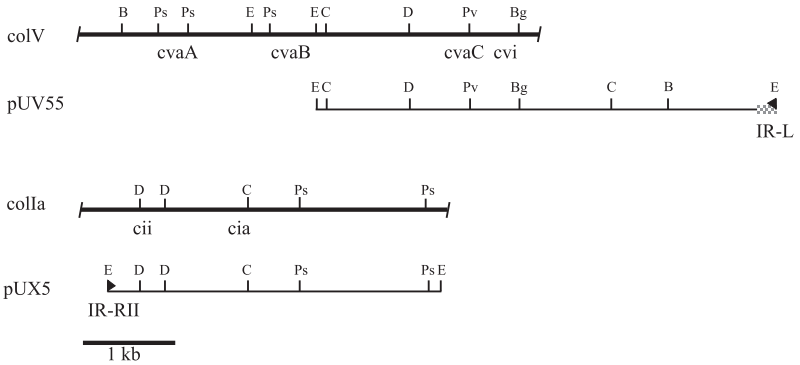

Fig. 2. **Comparison of ColV and ColIa restriction maps**
Top: comparison of the restriction map of ColV, prepared on the basis of sequences deposited under accession numbers X57525 and X57524 and denoted with a heavy line, with the pUV55 restriction map. The shaded line at the right-hand end of the pUV55 map denotes the conserved sequences in the direct vicinity of the ColIa immunity gene from nucleotide positions 3425–3727. The arrowhead denotes the left inverted repeat (IR-L) of Tn*5431*. Bottom: comparison of the restriction map of ColIa, prepared on the basis of the deposited sequence and denoted with a heavy line, with the restriction map of pUX5. The arrowhead denotes the right inverted repeat (IR-RII) of Tn*5431*. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; Pv, *Pvu*II.

Altogether these results demonstrated that Tn*5431* had transposed into a region encoding ColIa and ColV. Tn*5431* is flanked on one side by ColV and on the other by ColIa.

Replication regions, aerobactin system and IS*1*

Waters & Crosa (28) reported conservation of the replication region RepFIB among all ColV plasmids investigated and, further, that most encode the aerobactin iron-uptake system with an upstream and downstream insertion sequence IS*1*. To detect and map these nucleotide sequences we performed hybridisations of specific labelled probes with restriction enzyme digests of pRK100 and, for comparison, with fragments of the prototypic plasmid pColV-K30.

Comparison of the hybridisation signals demonstrated conservation in pRK100 of the replicon RepFIB, a basic replicon found in large plasmids of the IncFI group (12), the aerobactin-uptake system and the two IS*1* sequences (Figs 3, 4 and 5, respectively). Some but not all ColV plasmids also have the RepFIA homologous replicon downstream of the aerobactin-uptake system. Since large plasmids are known to carry more than one replicon, first all *Eco*RI fragments of pRK100 were labelled and used to probe the Couturier bank of rep plasmids. The probe did not hybridise with the 917 bp *Eco*RI fragment of plasmid pUBL2154 harbouring RepFIA, demonstrating that pRK100 does not have this replicon. On the other hand the probe hybridised with fragments corresponding to RepFIC

(the 967 bp *Eco*RI–*Hin*dIII fragment of pUBL2440), Rep9 (the 539 bp *Pst*I fragment of pULB2422), RepFIIA *copA* (the 543 bp *Pst*I fragment of pUBL2401) and RepFIIA *copB* (the 540 bp *Pst*I fragment of pULB2402). All four are known to cross-hybridise. To determine their positions, the labelled Rep-FIC and Rep9 fragments were then used to probe restriction enzyme digests of pRK100. Both probes hybridised to the same two *Eco*RI, *Sal*I and *Hin*dIII fragments, indicating that a replicon(s) of this family is present at two positions of pRK100 (Fig. 6).

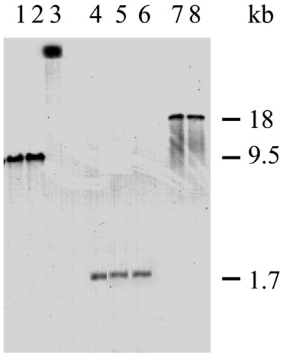

Fig. 3. **Southern blot hybridisation, with the Rep-FIB-specific probe, of pRK100**
pRK100 digested with: lane 1, *Eco*RI; lane 2, *Xho*I/*Eco*RI; lane 3, *Xho*I; lane 4, *Hin*dIII; lane 5, *Xho*I/*Hin*dIII; lane 6, *Eco*RI/*Hin*dIII; lane 7, *Sal*I; lane 8, *Xho*I/*Sal*I.

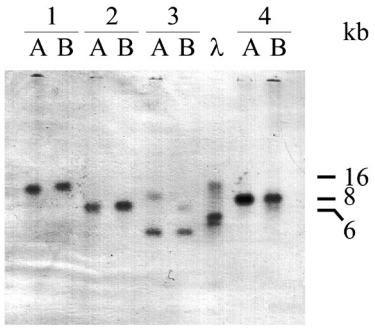

Fig. 4. **Southern blot hybridisation, with the probe specific for the aerobactin-uptake system, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1, *Hin*dIII; 2, *Eco*RI/*Hin*dIII; 3, *Bam*HI/*Hin*dIII; 4, *Eco*RI. Lane λ, labelled marker λ DNA digested with *Eco*RI/*Hin*dIII.

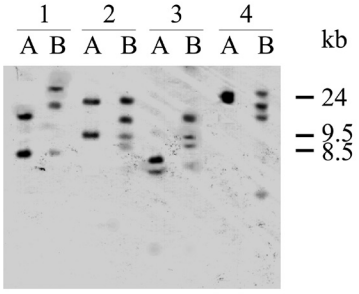

Fig. 5. **Southern blot hybridisation, with the IS*1*-specific probe, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1 *Hin*dIII; 2, *Eco*RI; 3, *Eco*RI/*Hin*dIII; 4, *Sal*I.

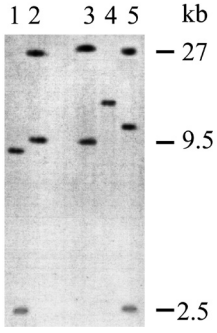

Fig. 6. **Southern blot hybridisation, with the Rep-FIC-specific probe, of pRK100**
pRK100 digested with: lane 1, *Eco*RI/*Sal*I; lane 2, *Eco*RI/*Hin*dIII; lane 3, *Eco*RI; lane 4, *Hin*dIII; lane 5, *Sal*I.

tra region and construction of the pRK100 map

The pRK100 *tra* region was determined by hybridisation of the labelled pED100 *Eco*RI fragments harbouring the *tra* operon of plasmid F and the labelled 8.3 kb *Eco*RI fragment f6, to restriction fragments of pRK100 and pColV-K30 (Figs 7 and 8, respectively).

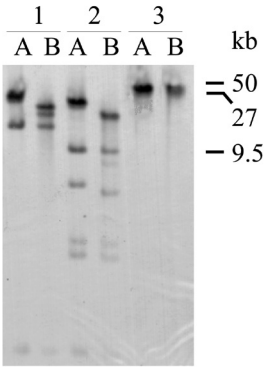

Fig. 7. **Southern blot hybridisation, with *Eco*RI fragments of the F factor *tra* operon, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1, *Eco*RI; 2, *Eco*RI/*Sal*I; 3, *Hin*dIII.

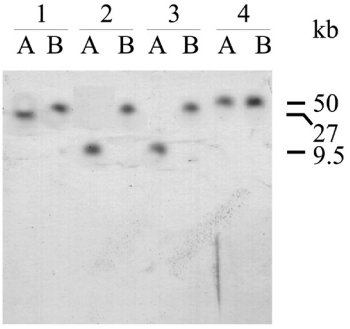

Fig. 8. **Southern blot hybridisation, with fragment f6 of the F factor *tra* operon specific probe, of pRK100 and pColV-K30**
pRK100 (lanes A) and pColV-K30 (lanes B) digested with: 1, *Sal*I; 2, *Eco*RI/HindIII; 3, *Eco*RI; 4, *Hin*dIII.

Comparison of the hybridisation results for the *tra* region with the results obtained with the labelled 0.9 kb *Eco*RI–*Hin*dIII fragment of pFDH1 enabled us to deduce the position of the *tra* operon with regard to ColIa. Further comparison of the above results with the RepFIC and Rep9 hybridisation patterns enabled us to deduce that a RepFIC replicon is downstream of ColIa. This was confirmed by single-stranded nucleotide sequencing with the forward sequencing primer of the pUX1 insert. Subsequently, by analysing the hybridisation results obtained with probes specific for the aerobactin system, IS*1* sequences, RepFIC and with the labelled 0.9 kb *Eco*RI–*Hin*dIII pFDH fragment, we were able to deduce the position of the *tra* operon on pRK100.

*Xho*I cleaves pRK100 at only two sites, resulting in two fragments, of 26 kb and 120 kb. One site was mapped in the ColV region, while the other was deduced from the fact that neither Tn*5431* nor pFDH1 has a *Xho*I site and from fragment patterns of double restrictions.

On the basis of the above-mentioned results together with the fragment patterns of double restrictions, hybridisation experiments using the three longest individually labelled *Eco*RI fragments (27, 20 and 17 kb), and comparison of probe binding to pColV-K30 we were able to complete the restriction map (Fig. 9).

Increased serum survival conferred by pRK100

ColV plasmids have been shown to enhance serum resistance. TraT, the surface exclusion protein of the plasmid transfer system, and the *iss* (increased serum survival) locus, which is linked to the colicin V genes, have been implicated (3). Serum survival was tested for the original clinical strain KS533, strain C600 and C600 harbouring pRK100 in the presence of 1%, 2% and 3% human serum.

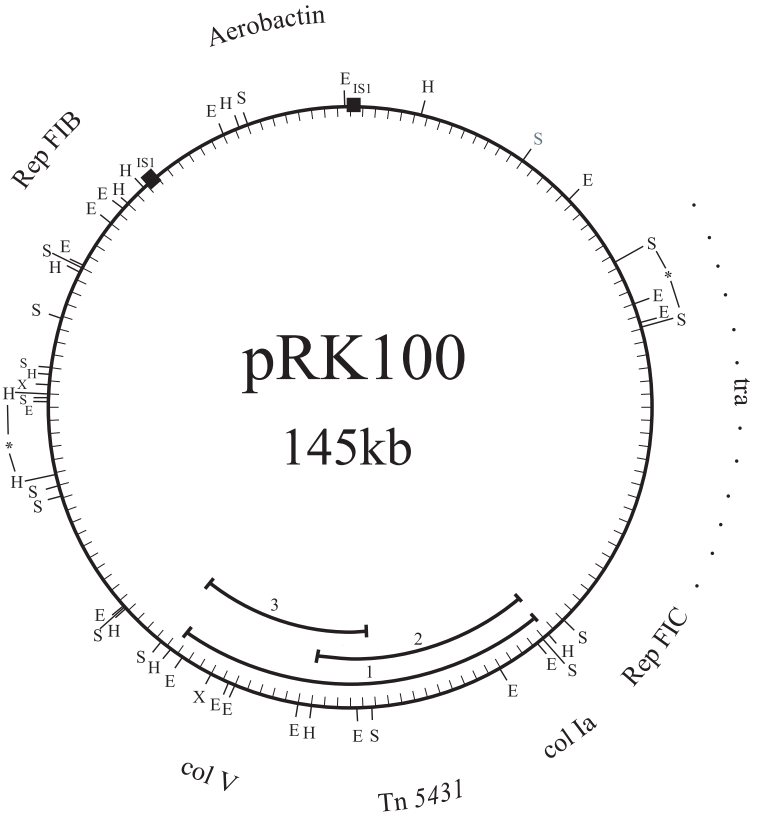

Fig. 9. **Map of pRK100**
On the inside border of the map are the boundaries of clones: 1, pFDH2; 2, pFDH1; 3, pFDHS1. The black boxes denote the two IS*1* insertion sequences. The asterisks in the bracketed area denote a region containing several small *Hin*dIII and *Sal*I fragments.

Table 2. **Survival in human serum of strains with and without pRK100**
Percentage survival is expressed relative to that of bacteria incubated in buffer without serum. The experiment was repeated three times and the results presented are mean values.

Strain	Percentage survival in 1%, 2% and 3% serum:		
	1%	2%	3%
C600	0.4	0.03	0
C600 (pRK100)	1.97	0.13	0
KS533	800	789	679
KS533 (pRK100)	802	800	683

The results (Table 2) show increased survival of C600 harbouring pRK100 in the presence of 1% and 2% serum (five- and fourfold, respectively) while both strains were killed in the presence of 3% serum. In contrast, both the clinical strain KS533 with plasmid pRK100 and strain KS533 without the plasmid exhibited growth at all serum concentrations tested. The K1 capsule thus offers much greater protection than any plasmid-encoded determinant.

Stability and conjugal transfer to other Gram-negative bacteria

Following 21 passages of pRK100 without antibiotic selection 1200 colonies were transferred to grids (LB plates) and tested for antibiotic resistances and colicinogenicity. All colonies retained the characteristics of the initial strain. Stability of pRK100 was also studied by introduction of an incompatible plasmid, pED100, a Cm^R derivative of F. A total of 152 colonies expressing the characteristics of both plasmids, resistances to Ap, Tc, Cm and colicinogenicity, were subsequently further subcultured by replica plating first onto an LB plate and then to an LB plate supplemented with Cm. This procedure was repeated seven times, during which some colonies exhibited gradual loss of the Cm^R character. None of the Cm^R colonies exhibited loss of pRK100. Gel electrophoresis of the isolated plasmid DNA of strains, which stably maintained colicinogenicity and antibiotic resistances of both plasmids (Ap, Tc, Cm), showed the presence of both plasmids or only F plasmid DNA, indicating integration of pRK100 into the chromosome. Only culturing strain KS533 in the presence of SDS yielded two colonies cured of plasmid pRK100.

To determine whether pRK100 can be transferred and maintained in other Gram-negative bacteria, conjugal transfer was attempted to *S. typhimurium*, *K. pneumoniae*, *P. aeruginosa* and *Ent. cloacae*. With plate mating only a small number of *K. pneumoniae* transconjugants harbouring pRK100 were isolated. Liquid matings were also carried out and the transfer frequencies of pRK100 and RSF2001 were compared (Table 3). pRK100 and RSF2001 were both transferred at low frequency to *K. pneumoniae*. On the other hand while the transfer frequency of RSF2001 to *S. typhimurium* was comparable with that to *E. coli*, transfer of pRK100 to *S. typhimurium* was never detected. Transfer of RSF2001 to *Ent. cloacae* was detected only with plate mating.

Table 3. **Plasmid transfer frequency to different species**

Conjugal transfer frequencies are expressed as the proportion of transconjugants to recipients (means of three independent experiments). –, No transconjugants were isolated; ±, a small number of transconjugants were isolated only with plate mating; NT, not tested.

Plasmid	Transfer frequency to				
	E. coli	*S. typhimurium*	*K. pneumoniae*	*Ent. cloacae*	*P. aeruginosa*
pRK100	2.4×10^{-2}	–	1.2×10^{-6}	–	–
RSF2001*	1.1×10^{-2}	3.7×10^{-2}	3.7×10^{-7}	±	NT

* RSF2001 is plasmid F with Kn^R

Chromosomal virulence-associated genes of strain KS533

Hybridisation experiments demonstrated the presence of nucleotide sequences specific for type I, P and S fimbriae. PCR was carried out with primers specific for CNF1 but amplification of the corresponding fragment was not detected. KS533 does not produce haemolysin as determined by growth on blood agar plates.

4. DISCUSSION

Using hybridisation and nucleotide sequencing a map of plasmid pRK100 and its characteristics was constructed. Transposon Tn*5431* has transposed into the direct vicinity of the ColIa immunity and structural genes. Prior to transposition the ColV and ColIa genes were thus linked. The ColV operon has so far been determined only on plasmids of the IncF1 incompatibility group, which share replicon RepFIB, colicin V genes and – in most of the plasmids examined – the aerobactin-uptake

system. On the other hand, examination of ColIa-harbouring plasmids of the *E. coli* Reference Collection (ECOR) showed that ColIa is present on large plasmids with little homology. It was therefore suggested that the ColIa operon or some larger fragment has been transferred between distinct plasmid lineages (26). On pRK100 the conserved ColIa sequences are on one side separated from ColV by approximately 2.6 kb and on the other side, upstream of the conserved RepFIC sequences, by approximately 1.5 kb. Together with the ColIa sequences this is roughly the size of the fragment homologous to different ColIa-carrying plasmids (26). Sequences involved in ColIa transfer could be present in the vicinity of the ColIa and ColV determinants of pRK100.

Using specific probes we demonstrated that pRK100 harbours RepFIB and RepFIC. It is not yet known whether pRK100 carries a complete and functional RepFIC replicon. However, when pRK100 was challenged with an incompatible plasmid, integration of pRK100 into the chromosome or the coexistence of both plasmids was observed, indicating that pRK100 has two functional replication regions. Further, on the basis of binding of probes specific for RepFIC and the *tra* region we demonstrated that in pRK100 the *tra* region is, as in plasmid F, linked to the RepFIC replication region. Other ColV plasmids, including pColV-K30, also harbour RepFIC sequences; however, their locations have not been determined (2).

Colicinogenicity and conjugal transfer contribute to plasmid stability in bacterial populations. Cells, which have lost the plasmid are no longer immune and can be killed by plasmid-harbouring cells. Further, cells, which have lost the plasmid can act as recipients in conjugal transfer. However, our experiments demonstrated pronounced *in vitro* stability of pRK100. Colonies cured of the plasmid were isolated only by SDS treatment. Further, the spontaneous loss of pRK100 upon sub-culturing and storage was never observed. Introduction of an incompatible plasmid could result in integration of pRK100 into the chromosome, probably by recombination between chromosomal and plasmid insertion sequences. Integration of plasmids is known to reduce the expression of plasmid-encoded factors due to differential supercoiling of the integrated plasmid DNA (24). It might also be viewed as a means by which selfish DNA avoids elimination in conditions detrimental to maintenance. The influence of environmental signals in the regulation of integration will be investigated. On plasmids such as pRK100, the maintenance of genes is stable while having, with regard to chromosomal genes, the additional advantage of being able to disseminate through a population by conjugation.

As well as plasmid-encoded determinants, strain KS533 also has chromosomal virulence determinants important for eliciting extraintestinal infections. The K1 capsule is poorly immunogenic and is responsible for immuno-tolerance by the host. Type P fimbriae mediate the initial binding of uropathogenic *E. coli* to its host receptor.

Fifteen per cent of the ECOR strains possess F-related plasmids (5). The *Salmonella* Reference Collection A (SARA) also has approximately the same percentage of F-like plasmids and it has been inferred that F plasmid transfer is an important mechanism of interspecies recombination (6). *Salmonella* IncF1 R plasmids are known to carry the IS*1*-bound aerobactin-uptake system (9) and some *Salmonella* isolates harbour colicin plasmids (1). Besides, some *E. coli* virulence factors might have originated in some other species (20). On the other hand, ColV plasmids have been determined only in *E. coli* even though the replication and transfer regions of plasmid F and ColV plasmids are homologous and exhibit a high degree of sequence conservation (12). It could be that for some reason DNA restriction and modification is more efficient in reducing or eliminating recombinants carrying ColV plasmid than F plasmid recombinants. In our plasmid-transfer experiments only *K. pneumoniae* transconjugants harbouring pRK100 were isolated. By comparing the host range of plasmids RSF2001 and pRK100 we see that the latter cannot be transferred to *S. typhimurium* or *Ent. cloacae* even though both plasmids are transferred to another *E. coli* strain with approximately the same frequencies. The absence of the RepFIA replication region in pRK100 could also be responsible for the plasmid's

limited host range when compared with plasmid F. Plasmid host range is important particularly for transferable plasmids. Conjugal transfer of plasmid-encoded virulence properties to another strain or species could provide quantum leaps toward virulence. The role of RepFIA in plasmid host range will be investigated. As environmental stimuli regulate gene expression and gene transfer (18), experiments will be carried out to determine the *in vivo* stability and transfer of pRK100.

The maps of plasmids pRK100, F and two other known ColV plasmids – pColV-K30 and pColV-B188 – are presented in Fig. 10.

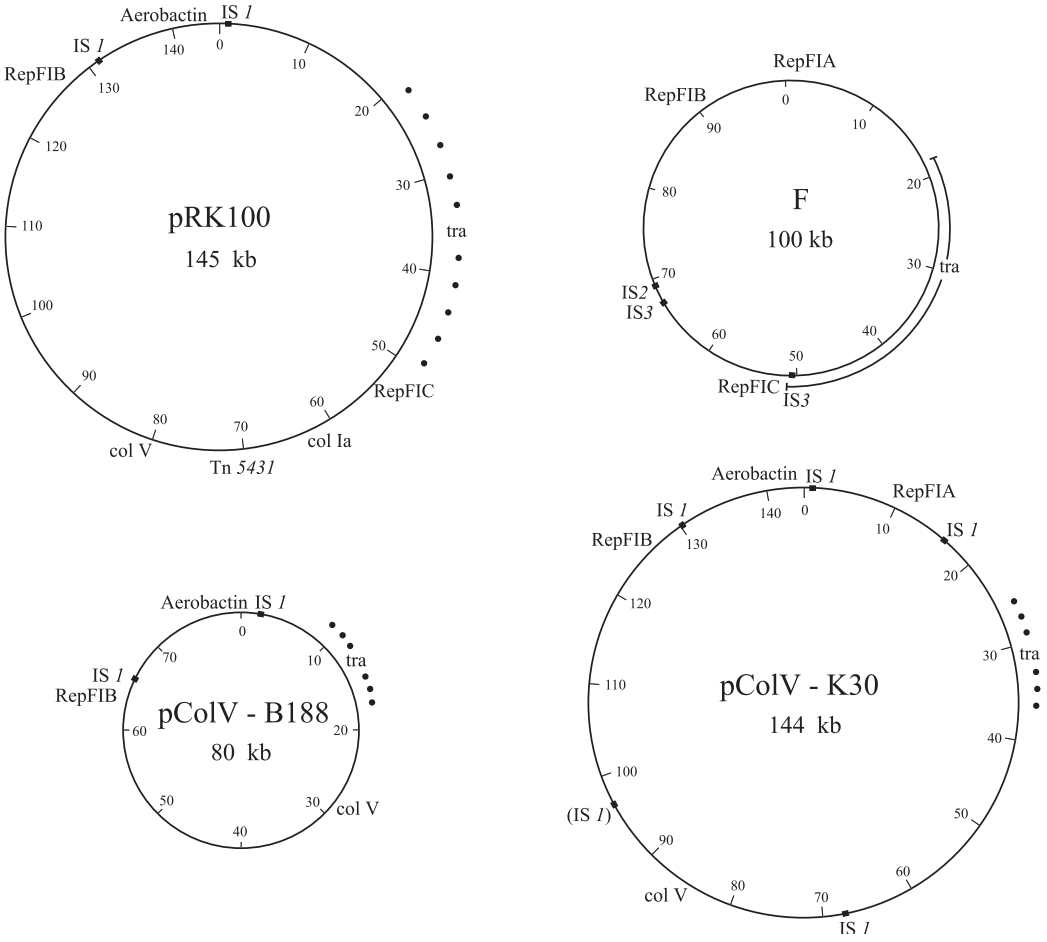

Fig. 10. **Comparison of the map of pRK100 with those of plasmid F (adapted from Neville, *et al.* (21)) and the ColV plasmids pColV-K30 and pColV-B188 (29)**
The location of insertion sequences (IS) is shown by black boxes. The IS*1* copy, which is believed to be partial, is labelled in parentheses. Dotted lines indicate that the location of the *tra* region is approximate, within the limits of available restriction enzyme sites. Numbers within the maps indicate kilobase co-ordinates.

The *tra* regions of the ColV plasmids are approximate, but with regard to the well-studied *tra* region of plasmid F should span about 30 kb. The aerobactin iron-uptake system, which is encoded by most of the ColV plasmids, is found on plasmids and chromosomes among *E. coli*, *Shigella* and

Salmonella species. The ubiquity of the system can be explained by IS*1*-mediated genetic mobility via recombination or probably less frequently by transposition (30). By comparing the maps of the ColV plasmids and plasmid F it can be inferred that acquisition of IS*1* sequences could have been important in the evolution of ColV plasmids. It can be envisaged that the aerobactin system integrated via IS*1*-mediated recombination into the region between the RepFIB and RepFIA replicons of an ancestral F-like plasmid. Recombination between two copies of IS*1* could result in deletion of the RepFIA replication region.

It is evident that pRK100 evolved in several steps. First a segment encoding the ColIa operon was transferred from one plasmid to a ColV plasmid, then transposon Tn*5431* transposed into sequences in the direct vicinity of the ColIa operon. By comparing pRK100 with plasmids F and pColV-K30 (Fig. 10) we see that deletion of the RepFIA replication region could have occurred by IS*1*-mediated recombination. However, other rearrangements must have taken place, as sequences homologous to the RepFIC probe were also detected in the region between RepFIB and the ColV genes. A pronounced clustering of virulence-related properties is also evident in pRK100 when compared with other ColV plasmids. An insertion hotspot could be present in the ColIa region and/or there could be a constraint on insertions in the region between the RepFIB and colicin V genes. The presence of transposable antibiotic resistances on a ColV plasmid is a reflection of the widespread use of antibiotics. The aerobactin iron-uptake system, the *tra* region, the ColV and ColIa genes, and the transposable antibiotic resistance genes together with the replication regions could be considered a stable transferable pathogenicity island.

Plasmid pRK100 and the constructed map will also enable further studies of sequences involved in transfer of the ColIa genes, the role of certain replicons in plasmid host range and gene clustering on large plasmids encoding virulence properties.

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Chapter 3

Mosaicism of the Large Natural *Escherichia coli* Plasmid pRK100

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ABSTRACT

In search for the evolutionary origin of the conjugative F-like plasmid pRK100, we determined the plasmid's functional replication region(s) and performed targeted genetic analysis of the plasmid. Construction of minireplicons via ligation of Tn*1725* with plasmid fragments and targeted cloning of putative replication regions, followed by sequence analysis indicated two functional replication regions, a plasmid F-like RepFIB and a plasmid R1-like RepFIIA replication region. Partial nucleotide sequencing of regions of the plasmid revealed genes that encode a putative enterochelin iron uptake system previously associated with an *Escherichia coli* pathogenicity island, PAI III_{536}, and the pColV-like aerobactin genes. In addition, a homologue of the plasmid R100-like *rmoA* gene was found that exhibits strong similarity to the Hha/YmoA class of modulators of gene expression. PCR and hybridisation experiments further demonstrated that pRK100 harbours IS*2* and IS*3* insertion sequences that may have facilitated in the acquisition of elements from other DNA molecules. These data together with the previous identification of a F-like *tra* region and a pColIa-like colicin Ia, indicate that pRK100 has a highly mosaic structure with elements derived from many different known large natural plasmids, and also from chromosome.

Key words: replication region, RepFIB, RepFIIA, F-like plasmid, ColV-like plasmid, construction of minireplicons, transposable element, iron uptake system

1. INTRODUCTION

Plasmids are self-replicating extrachromosomal DNA elements that may embody 1% to more than 10% of the genome of a given bacterial strain. To propagate and maintain themselves, plasmids have developed sophisticated replication functions that are encoded by distinct gene replication regions. These regions typically contain the origin of replication (generically termed *ori*), a gene encoding the protein involved in the initiation of replication, and genes whose products are required for the control of replication (18). To further ensure their stability in a bacterial population plasmids also encode systems responsible for resolution of plasmid multimers, equipartitioning at cell division, and post-segregational killing of plasmid free cells that arise in a population.

Conjugative plasmids can transfer themselves to other bacteria via the plasmid-encoded conjugation machinery. In addition they can facilitate the transfer of non-mobilisable genetic elements. Conjugative plasmids have been demonstrated to be important gene transfer vehicles in the spread of resistances to antibiotics and chemicals as well as of metabolic properties and virulence genes. The growing evidence from genome sequence analysis that microbial evolution has to an important degree been shaped by horizontal gene transfer has further renewed the interest in plasmid conjugation and the properties of large natural conjugative plasmids.

Genetic and functional analyses of the various kinds of large natural conjugative plasmids that have been isolated suggest that they are generally larger than 40 kb in size and may have evolved from the integration into small plasmids of distinct chromosomal or plasmid elements and/or fusion of plasmids. Major classes of large natural plasmids are the IncFI and IncFII plasmids. This strict classification however, may turn out to be rather artificial as members of the plasmid families appear to have exchanged genetic information.

In order to learn more about the dynamic nature and the evolution of large natural conjugative plasmids, we investigated the plasmid pRK100. pRK100 is a 145 kb F-like conjugative plasmid that was discovered in a uropathogenic *Escherichia coli* strain. The plasmid harbours the transposon Tn*5431* that carries genes that confer resistance to ampicillin and tetracycline, and genes that encode the colicins V and Ia and an aerobactin iron uptake system (1), suggesting that it has acquired genetic elements from different origins. In the present study, we further investigated the mosaicism of pRK100 by determining the active origin(s) of replication, and targeted hybridisation and sequencing based on typical characteristics of other large natural plasmids.

2. MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study and their characteristics are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium with aeration at 37 °C. Ampicillin (Ap, 100 μg/ml), tetracycline (Tc, 10 μg/ml), kanamycin (Km, 30 μg/ml), chloramphenicol (Cm, 50 μg/ml), and nalidixic acid (Nal, 25 μg/ml) were added to the growth media, when appropriate.

Table 1. **Bacterial strains and plasmids**

Strain or DNA	Relevant features	Reference or source*
Strains		
KS533	Rough:K1:H7 harbouring pRK100	23
HB101	*hsdR hsdM recA13 supE44 leuB6 lacZ proA2*	D. Ehrlich[1]
CL225	HB101 harbouring pRK100	D. Žgur-Bertok
DH5α	Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17 deoR*	BRL Life
	thi-1 supE44 gyrA96 relA1	Technologies
RU4404	MM294::Tn*1725* Cm^r *thi endA hsdR*	21
RU4405	MM294::Tn*1731* Tc^r *thi endA hsdR*	21
PC1994	*thyA argA bio pheA endA polA*	NCCB
Plasmids		
pMW2	plasmid with kanamycin cassette; Ap^r, Kn^r	M. Wösten[2]
pBluescript SK–	*E. coli* vector plasmid; Ap^r	Stratagene
pGEM-T Easy	T-vector for cloning of PCR products; Ap^r	Promega
pRK100	colV colIa natural plasmid; Ap^r, Tc^r	1
pS2	pBluescript SK- with the 1731 bp *Sal*I fragment of pRK100	This study
pS3	pBluescript SK- with the 2156 bp *Sal*I fragment of pRK100	This study
pS10	pBluescript SK- with the 2062 bp *Sal*I fragment of pRK100	This study
pSA11	pGEM-T Easy with the 1204 bp PCR product of pS3 (Sal11-A/Sal11-B)	This study
pSA14	pGEM-T Easy with the 839 bp PCR product of pS10 (Sal14-A/Sal14-B)	This study

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General DNA manipulation techniques

Plasmid DNA isolation, ligation, transformation and stringent hybridisation experiments were performed using standard methods (17). Restriction endonuclease digestions were carried out as specified by the manufacturer (Promega, Madison, WI). DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). DNA sequencing was performed using the ABI dye terminator cycle sequencing kit in an ABI PRISM™ 310 Genetic Analyzer automated sequencer. Sequences were analysed using Lasergene software (DNAstar, Inc.). The primers used for sequencing are listed in Table 2.

PCR amplification

The polymerase chain reactions (PCR) were performed in a 50 µl PCR reaction mixture with 20 pmol of forward and reverse primer (Table 2), 5 µl of purified template DNA, 0.2 mM of dNTP mixture (Pharmacia), 0.625 U Taq DNA polymerase (Promega) and 1× PCR buffer (Promega). The PCR conditions are given in Table 2. Each PCR started with a prolonged denaturation step (4 min, 94 °C), and ended with a prolonged extension step (10 min, 72 °C) after the last cycle. The pGEM-T Easy system (Promega) was used for cloning of the PCR products.

Table 2. **Oligonucleotide primers and PCR conditions**

Oligonucleotide primer*	Nucleotide sequence	PCR conditions
for RepFIIA PCR:		
FinO-1	5'-CGGATAAAGGCAGAACTTCAGGC-3'	(94 °C-1:00, 58 °C-0:30,
Sal14-B	5'-GAAAGGCGGCACTCTGTTGT-3'	72 °C-4:00) 30×
Sal11-A	5'-CCGGAAAGTTATATGACAGT-3'	(94 °C-1:00, 55 °C-0:30,
Sal14-B	5'-GAAAGGCGGCACTCTGTTGT-3	72 °C-3:00) 35×
for RepFIIA sequencing:		
Sal11-B	5'-GCTACGCCACAAAGTAAAGT-3'	
Sal14-A	5'-CCCTGAAGTGACCTCCTCTG-3'	
565-PS3	5'-CTCAATGAGGAAGTCACTGC-3'	
568-PS10	5'-AAACCTATTCACTGCCTGTC-3'	
for RepFIB PCR:		
RepA-f[1]	5'-GGAATTCTCGCTGCAAACCTTGTCACT-3'	(94 °C-0:30, 63 °C-0:30,
RepA-r[1]	5'-GGAATTCGGAGATCCTGCGTACACTGCCT-3'	72 °C-1:30) 30×
RepFIB-5	5'-GCGGACAATCCAAATGGTGA-3'	(94 °C-1:00, 55 °C-0:30,
RepFIB-6	5'-ATATGCTGTTCGCCACCCTC-3'	72 °C-3:00) 30×
for RepFIIA sequencing:		
FIB2-1	5'-CAATAACAACACCGTACAACC-3'	
FIB2-2	5'-ACAGTTTATGTTCAGCGGGAT-3'	
RepFIB-3	5'-TATCAGTAACATGCCACAGC-3'	
RepFIB-4	5'-TCCCGTAACCTGATGCTGAG-3'	
for RepFIC PCR:		
RepC-1	5'-TTCATCTAGTTTGGCGACGAGG-3'	(94 °C-1:00, 63 °C-1:00,
RepC-2	5'-GCTGTCTTTCGGGCTGATTTCT-3'	72 °C-2:00) 30×
for RepZ PCR:		
RepZ-1	5'-CCATATAACGCAGTACACTGGA-3'	(94 °C-1:00, 58 °C-0:30,
RepC-2	5'-GCTGTCTTTCGGGCTGATTTCG-3'	72 °C-1:30) 30×
for RepFIA PCR:		
RepFIA-f[2]	5'-CTCACTGAGGCGGCATATAGTC-3'	(94 °C-0:30, 63 °C-0:30,
RepFIA-r[2]	5'-ATGGAAGTGATATCGCGGAAGG-3'	72 °C-1:00) 30×
for aerobactin PCR:		
aer1	5'-TACCGGATTGTCATATGCAGACCGT-3'	(94 °C-0:30, 62 °C-0:30,
aer2	5'-AATATCTTCCTCCAGTCCGGAGAAG-3'	72 °C-0:50) 30×
for *traM* and *finP* PCR:		
FinP-1	5'-TATTGAGAAGCGTCGACAGG-3'	(94 °C-1:00, 55 °C-1:00,
FinP-2	5'-TGACGAACATGAGCAGCATC-3'	72 °C-1:00) 30×
for *traJ* PCR:		
PtraJ-1	5'-CGGGATCC-TCCAAAAAATGATGATGAAT-3'	(94 °C-1:00, 55 °C-0:30,
TraYN-2	5'-GCAGAACGTGTACCAAATCTT-3'	72 °C-1:00) 30×
for *traY* PCR:		
TraY-f[1]	5'-GGAATTCAAGATTTGGTACACGTTCTGC-3'	(94 °C-1:00, 63 °C-1:00,
TraY-r[1]	5'-GGAATTCCTTCCTCTTTATCTGCCTCCC-3'	72 °C-2:00) 30×
for *traD* PCR:		
TraD-f[1]	5'-GGAATTCCAGATTGCGTCCATGCGTATCC-3'	(94 °C-1:00, 63 °C-1:00,
TraD-r[1]	5'-GGAATTCATCACCACACATATCACCGCGC-3'	72 °C-1:00) 30×

Oligonucleotide primer*	Nucleotide sequence	PCR conditions
for *traI* PCR:		
TraI-1	5'-ACAGCGAATATACGTGACGG-3'	(94 °C-0:30, 57 °C-0:30,
FinO-4	5'-CGTGGTGACATTGATGATGG-3'	72 °C-3:00) 30×
for *finO* PCR:		
FinO-f[1]	5'-GGAATTCGAAGCGACCGGTACTGACACTG-3'	(94 °C-1:00, 63 °C-1:00,
FinO-r[1]	5'-GGAATTCGCCTGAAGTTCTGCCTTTATCCG-3	72 °C-2:00) 30×
for *ccdB* PCR:		
CcdB-1	5'-GAGAGCCGTTATCGTCTGTT-3'	(94 °C-1:00, 55 °C-1:00,
CcdB-2	5'-CTGAGATCAGCCACTTCTTC-3'	72 °C-1:00) 30×
for IS*2* PCR:		
IS2-1	5'-ACTTAACCCATTACAAGCCCGC-3'	(94 °C-0:30, 61 °C-0:30,
IS2-2	5'-AACCTGCTGTACCGCCATCGAA-3'	72 °C-1:00) 30×
for IS*3* PCR:		
IS3-1	5'-AGTTCAGCATCAAAGCAATGTG-3'	(94 °C-0:30, 59 °C-0:30,
IS3-2	5'-GCAGGCATTATCGTAGCAGCAA-3'	72 °C-1:00) 30×

*[1] Nucleotide sequence is based on Boyd, *et al.* (4)

[2] Nucleotide sequence is based on Mulec, *et al.* (12)

Construction of minireplicons

To construct minireplicons using transposon Tn*1725* (21), pRK100 (Ap^r, Tc^r) was first conjugally transferred to strain RU4404 that carries Tn*1725* (Cm^r) on the chromosome (21). Transconjugants were selected on LB plates with Cm, Ap, and Tc and incubated at 30 °C for several days to allow transposition of Tn*1725* into pRK100. pRK100 plasmids with inserted transposon (pRK100::Tn*1725*) were selected by conjugal transfer to DH5α (Nal^r) on LB plates containing Cm, Ap, Tc, and Nal. Several pRK100::Tn*1725* plasmids from independent experiments were isolated, partially digested with *Sal*I, self-ligated with T4-ligase (Gibco), and introduced into DH5α by electrotransformation. Transformants carrying minireplicons were selected on LB plates containing Cm.

Construction of minireplicons using PCR products of the putative replication regions was carried out by either cloning the PCR product into pGEM-T Easy, or by joining the DNA polymerase I polished PCR product with the *Sma*I fragment of pMW2 that harbours the kanamycin resistance gene. Transformants carrying minireplicons were selected on LB plates containing Km.

The obtained minireplicons were analysed by restriction enzyme digestion analysis, PCR and sequencing, as described above.

3. RESULTS

Identification of a functional RepFIB region

To determine the functional replication region(s) of pRK100, we initially utilised the randomly inserting transposon Tn*1725* (Cm^r) (21). For this purpose, pRK100 was transferred into the Tn*1725* carrying *E. coli* strain RU4404. After induction of the transposition event by growth at 30°C, pRK100 was re-isolated from strain RU4404, and partially digested. Self-ligation of the obtained plasmid fragments and re-introduction of the ligated plasmid fragments into DH5α, yielded several Cm resistant transformants. Plasmid analysis indicated that these transformants carried Tn*1725* (Cm^r) containing minireplicons with sizes between 19.3 and 24 kb.

Assuming that pRK100 contains a replication region that resembles one of the identified *rep* regions in other large natural plasmids, the pRK100 derived functional replication region of the minireplicons was investigated by PCR using RepFIIA, RepFIA, RepFIB, RepFIC, and RepZ specific primers (Table 2). Partial nucleotide sequencing of the obtained PCR products indicated that all of the minireplicons, which were obtained in independent experiments, harboured a RepFIB-like replication region.

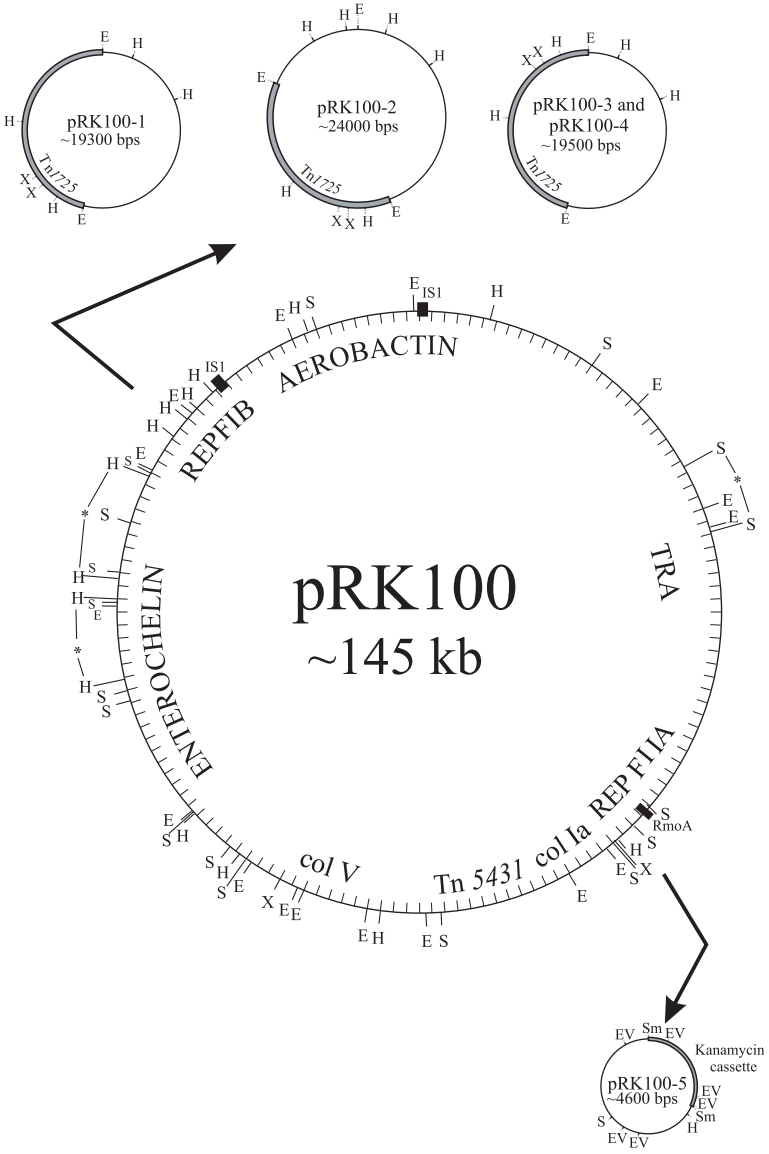

Fig. 1. **Map of pRK100 and the constructed minireplicons**
The large map depicts the known coding regions of pRK100 and the position of the newly identified replication regions. The smaller maps represent the RepFIIA (pRK100-5) and RepFIB (pRK100-1, pRK100-2, pRK100-3, pRK100-4) minireplicons. The restriction sites for *Eco*RI (E), *Eco*RV (EV), *Sal*I (S), *Hin*dIII (H), *Sma*I (Sm) and *Xho*I (X) are indicated.

Further nucleotide sequencing of the pRK100-1 RepFIB replicon using primer walking showed that RepFIB of pRK100 was highly similar (96%) to RepFIB replication regions of the plasmids F, pColV-K30, pO157, R124 and other related plasmids (data not shown). Restriction analysis of the obtained RepFIB minireplicons located the RepFIB region in proximity of the aerobactin encoding region on the pRK100 plasmid map (Fig. 1).

Identification of a functional RepFIIA region

Previous hybridisation experiments suggested that pRK100 may carry a RepFIC replication region downstream of the transfer *tra* region (1). In order to further investigate the nature and functionality of this region, which was not obtained as a minireplicon with Tn*1725* approach, we PCR amplified the region with the primers FinO-1 and Sal14-B that encompass the downstream *finO* gene of the *tra* operon and the downstream ColIa region of plasmid pRK100, respectively (Fig. 1). This yielded a 4.5 kb PCR product, which was sequenced using primer walking. Sequence analysis using the BLAST algorithm (www.ncbi.nlm.nih.gov.) revealed that the obtained sequence was 95% similar to the RepFIIA replication region of plasmid R1 of *Escherichia coli* and 94% similar to that of the virulence plasmid pWR100 of *Shigella flexneri*.

To assess the possible functionality of the newly identified RepFIIA region of the pRK100, we amplified the region with the primers Sal11-A and Sal14-B (Table 2) and attempted to clone the amplified fragment into pGEM-T Easy. Introduction of these constructs into either a *polA*+ or *polA*– *E. coli* background yielded no transformants. As an alternative approach, the RepFIIA PCR product was ligated to the kanamycin resistance cassette derived from the plasmid pMW2. Electrotransformation of these constructs into *E. coli* DH5α yielded several Km resistant transformants. Restriction enzyme digestion analysis, PCR and nucleotide sequencing confirmed the successful construction of the RepFIIA minireplicon (Fig. 1). Together, these results strongly suggest that pRK100 has two intact replicons, RepFIB and RepFIIA.

Heterogeneous origin of pRK100

The presence of two intact replication regions, RepFIB and RepFIIA, with high similarity to those present on the large natural plasmids F, pColV-K30, pO157 and R124, and pWR100 and R1, respectively, and the mapping of RepFIB and RepFIIA to different parts of pRK100 (Fig. 1), suggested that pRK100 may constitute a plasmid chimera composed of elements from different plasmids. To obtain further evidence for the mosaic nature and origin of pRK100, we tested the plasmid for the presence of several other large natural plasmid specific regions, and sequenced several thus far unidentified DNA regions.

Since large F-like plasmids frequently harbour multiple insertion sequences that may facilitate in the acquisition of elements from other plasmids, PCR reactions with primers specific for either IS*2* or IS*3* were performed. PCR products were obtained in both reactions indicating that at least one copy of each insertion sequence is carried by pRK100. PCR amplification and hybridisation experiments aimed at the detection of *ccdB*, which encodes the CcdB protein of the post-segregational CcdAB killing system in F plasmid (9), yielded no positive results (data not shown), indicating that pRK100 has not acquired this killing system.

To gain more information about the nature and origin of the region upstream of RepFIB, we cloned and partially sequenced a 1731 bp *Sal*I restriction fragment (pS2). Hybridisation experiments mapped this fragment to the correct region (data not shown). Sequence analysis indicated that the fragment carried two adjacent open reading frames that were 95% and 98% similar at the amino acid level to the *iroD* and *iroC* genes, respectively, of the uropathogenic *E. coli* strain 536 pathogenicity island PAI III_{536} (Genbank accession number X16664). The *iroD* gene encodes a putative ferric ente-

rochelin esterase, while the *iroC* gene product is an ABC transport protein. These data suggest that this part of pRK100 may be of chromosomal origin.

Nucleotide sequence analysis of a 2156 bp *Sal*I restriction fragment (pS3) that mapped by hybridisation to a region downstream of the *tra* region (Fig. 1) revealed an open reading frame that was 95% identical to the RmoA protein encoded on plasmid R100. In R100, this protein is a modulator of R100 conjugal transfer (15). The protein sequence of R100 RmoA is highly homologous to chromosomally encoded protein Hha (14) that downregulates the expression of the virulence factor haemolysin in *E. coli* (13), and to YmoA that modulates the expression of Yop proteins and the YadA adhesin in *Yersinia enterocolitica* (5). Both Hha and YmoA belong to a protein family that downregulates gene expression in enterobacteria (11).

Sequence analysis of the PCR product obtained with primers aer1 and aer2 demonstrated the presence on pRK100 of genes encoding an aerobactin iron uptake system consistent with earlier reported DNA hybridisation data (1). BLAST analysis of the obtained aerobactin showed that the region was 100% identical to the sequence found on *E. coli* pColV-K30, and 97% identical to sequences found on the *Shigella flexneri* SHI-2 pathogenicity island and the *Shigella boydii* 0-1392 aerobactin island SHI-3.

Mosaic nature of pRK100

The newly identified traits of pRK100 described above, together with the previous identification of a F-like *tra* region and a pColIa-like colicin Ia (1), indicate that pRK100 has a highly mosaic structure with elements derived from the chromosome and other known large natural plasmids. In Figure 2, the mosaic nature of pRK100 as well as that of other large natural plasmids is illustrated by a systematic comparison of the identified pRK100 elements with sequences present in other large natural plasmids.

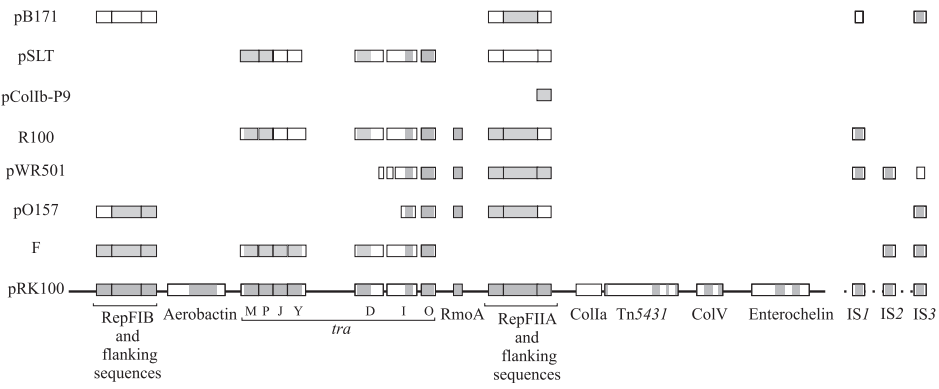

Fig. 2. **Mosaicism of pRK100**

The obtained pRK100 sequences with the assigned functions were analysed using the BLAST algorithm for their similarity with other plasmids. In the Figure, the regions of pRK100 that are present in one or more of the other plasmids are marked as boxes (note that the differences in box sizes are only approximate). The shaded parts of the boxes are very similar (>95%) to the obtained pRK100 nucleotide sequence. The nonshaded parts represent sequences that were not compared due to the fact that the corresponding pRK100 regions were not sequenced. The smaller size of distinct boxes in some of the other plasmids denotes that part of the region is missing. Only the presence of IS sequences is denoted. Note that only large natural plasmids whose complete nucleotide sequences are available in Genbank are represented.

4. DISCUSSION

Conjugative plasmids are increasingly recognised as important vehicles of horizontal gene transfer and knowledge of their evolution and mechanism of action is important in understanding the spread of traits of medical and ecological significance. The data obtained in the present study indicate the large natural conjugative plasmid pRK100 as a plasmid mosaic that is composed of a diverse set of plasmid and chromosomal genetic elements. The most prominent evidence of the highly mosaic plasmid structure of pRK100 was the demonstration of the presence of two intact replication regions, RepFIB and RepFIIA. The RepFIB replication region was found to be most similar (96%) to the RepFIB replication region of plasmid F and other related plasmids. In these plasmids, this region typically contains a single initiator gene *repA* that is flanked by a series of repeat elements. The *repA* promoter is located within the upstream repeat elements and the expression of the gene is autoregulated by the binding of RepA to the repeats, which presumably prevents RNA polymerase access to the promoter (19).

The sequence of the RepFIIA replication region of pRK100 was most similar (95%) to the RepFIIA of plasmid R1. IncFII replicons (originally designated RepFIIA) typically consist of *repA2* encoding a repressor, the *copA* gene that encodes an antisense RNA molecule, a *repA1* gene whose protein initiates plasmid replication by binding to the downstream *ori*, the *tapA* gene encoding a short leader peptide, and a *repA4* region. In this system, the RepA2 repressor is assumed to regulate transcription of *repA1* mRNA, while the antisense RNA CopA, which is complementary to the leader region of *repA1* mRNA (CopT), regulates translation. When CopA binds to CopT, *tapA,* which is necessary for RepA1 synthesis, is not expressed (3). The *repA4* appears to be important for the stability of plasmid maintenance (10).

The presence of two replication regions with distinctive mechanisms of action within one large natural plasmid is not unique and has previously been reported for several plasmids, such as pO157 and pB171 (2). The successful construction of minireplicons carrying either RepFIB or RepFIIA indicates that both replication regions of pRK100 are functional in *E. coli*. The identification of the replication regions required different experimental strategies. The RepFIB minireplicon was isolated by religation of plasmid pRK100 digestion fragments carrying Tn*1725*. The fact that we were not able to isolate RepFIIA minireplicons using this approach may be due to the apparent preference of Tn*3*-like transposons (such as Tn*1725*) (8) for the RepFIIA replicon (16). The alternative approach of ligating the PCR generated RepFIIA fragment with DNA carrying a kanamycin resistance gene circumvented this problem and resulted in the formation of functional RepFIIA minireplicons. The functionality of the identified replication regions in the setting of pRK100 remains to be defined.

Previous DNA hybridisation experiments suggested that pRK100 may harbour a RepFIC replication region belonging to the IncFII extended family of replicons (1). The inability to obtain a PCR product with primers specific for RepFIC indicated that the RepFIC probe may have cross-hybridised with another replication region. Indeed it is known that the used pULB2440 derived RepFIC probe is not highly specific and thus may hybridise with different replication regions including Rep9, RepFIIA, RepI1, RepB/O and RepK (6). The mapping of RepFIIA to the same position in pRK100, as previously suggested for RepFIC, further supports the idea that pRK100 carries RepFIIA and not RepFIC.

The presence of genes that exhibit a strong similarity with sequences present on of a wide variety of other plasmids further underlined the mosaic nature of pRK100 and suggests that during its evolution pRK100 apparently sequestered genetic information from many different sources. Typical examples of the mosaicism of pRK100 are the *tra* region that enables conjugal transfer and the RepFIB replication region that appear to be acquired from a F-like plasmid, the replication region RepFIIA that is probably derived from a R1-like plasmid, the aerobactin uptake system and the colicin V deter-

minants likely originating from a pColV-like plasmid, the *Shigella flexneri* SHI-2 pathogenicity island and/or the *Shigella boydii* 0-1392 aerobactin island SHI-3, and the colicin Ia encoding region derived from a ColIa harbouring plasmid into which transposon Tn*5431* had inserted (24). Particularly noteworthy is the newly identified putative enterochelin-based iron uptake system on pRK100 that is most similar (98%) to sequences on the pathogenicity island PAI III_{536} of the uropathogenic *E. coli* strain 536. This PAI also harbours the *cvaB* gene required for colicin V export (7). The two convergent operons required for colicin V synthesis, export and immunity are encoded by ColV plasmids (22) as well as by pRK100.

An additional noteworthy finding was that partial nucleotide sequencing revealed that pRK100 carries a gene that is very similar to the *rmoA* gene on plasmid R100 (Fig. 2). This in conjunction with the finding that *rmoA* is situated between the promoter distal region of the *tra* operon and the RepFIIA replication region in both pRK100 and R100 suggests that these plasmid regions are related. RmoA seems to be involved in modulation of plasmid transfer in response to some environmental factors (15) and comparative sequence analysis has shown the same gene organisation in a number of other characterised F-like plasmids. The DNA sequences of the corresponding region in plasmid R1 remains to be defined.

The backbone of pRK100 has previously been suggested to be similar to that of plasmid F (1). Our data suggest that this may be only partially true as we found no evidence for the presence on pRK100 of the CcdAB post-segregational killing system of plasmid F. It can be imagined that in pRK100 the colicinogenicity and conjugal transfer are the important factors in the maintaining plasmid stability among the population. Under this scenario, bacteria that have lost the plasmid are no longer immune and can be killed by plasmid-harbouring cells producing the colicin, while bacteria that have lost the plasmid can serve as recipients in conjugal transfer. An additional factor that may contribute to persistence of the plasmid is the presence of Tn*5431*. The resolvase of the transposon may function as a multimer resolution system and thus prevent dimerisation of plasmids and unequal plasmid distribution to daughter cells (20).

The mechanism(s) that contributed to the mosaicism of pRK100, are unknown. However, hybridisation and PCR experiments showed that pRK100 harbours several insertion sequences including IS*1* (1) and, as demonstrated in this study, IS*2* and IS*3* elements that can mediate recombination and rearrangement events. Some of the genes, such as the enterochelin uptake system, appear to originate from a chromosomal pathogenicity island. Pathogenicity islands (PAI) range in size from approximately 20-120 kb and have been proposed to be horizontally transferred via temperate bacteriophages. Extensive analysis of the PAIs of uropathogenic *E. coli* strains indicate that they contain mosaic sequences (7). The discovery of plasmid encoded enterochelin and aerobactin may imply that conjugative plasmids could be involved in the flux of chromosomal PAI sequences and, possibly, their horizontal transfer. To this end, the highly mosaic plasmid structure of pRK100 may be considered as an important player in the development of genome plasticity by acting as an intermediate in the transfer of a variety of virulence, antibiotic resistance, or metabolic traits between populations.

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Chapter 4

The Cyclic AMP-CRP Complex Regulates the Activity of the *traJ* Promoter of the *Escherichia coli* Conjugative Plasmid pRK100

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In press

ABSTRACT

The TraJ protein is a central activator of F-like plasmid conjugal transfer. In search for regulators of *traJ* expression, we studied the possible regulatory role of the cAMP-CRP complex in *traJ* transcription using a *traJ-lacZ* reporter system. Comparison of the enzyme activities in the wild type *E. coli* strain MC4100 with that in a *cya* and *crp* mutant indicated that disruption of the formation of the cAMP-CRP complex negatively influenced the activity of the *traJ* promoter of the F-like plasmid pRK100. The defect in the *cya* mutant was partially restored by the addition of exogenous cAMP. Competitive RT-PCR using RNA isolated from the wild type and mutant strains showed that the cAMP-CRP complex influenced the level of TraJ transcript. Electrophoretic mobility shift assays with purified CRP demonstrated direct binding of CRP to the *traJ* promoter region. DNaseI footprint experiments mapped the CRP binding site around position –67.5 upstream of the assumed *traJ* promoter. Targeted mutagenesis of the *traJ* promoter region confirmed the location of the CRP binding site. Consistent with the demonstrated regulation of TraJ by the cAMP-CRP complex, mutants with defects in *cya* or *crp* exhibited reduced conjugal transfer from pRK100.

Key words: gene regulation, *traJ*, cAMP, CRP, conjugal transfer, F-like plasmid

1. INTRODUCTION

Conjugation leads to the transfer of genetic material from one bacterium to another and is directed by conjugative plasmids. One family of conjugative plasmids are the F-like plasmids present in *Escherichia coli* and related species. F-like plasmids carry an ~ 33 kb long transfer (*tra*) region that harbours approximately 40 genes responsible for conjugal transfer (Fig. 1A). The expression of the *tra* genes is tightly regulated by both plasmid and chromosomally encoded proteins (8), although subtle differences exist among the various F-like plasmids (5, 25). The main plasmid-encoded positive regulator of conjugation is the 27 kD protein TraJ, which is required for the initiation of high levels of transcription from the major *tra* promoter, P_{traY}. Full activation of P_{traY} of plasmids F and R1 also requires the chromosomally encoded ArcA protein, which is part of the ArcA/B two component system that responds to oxygen (30). In plasmid F and R100, the TraY protein further stimulates its own promoter and this autoactivation enhances the synthesis of proteins that form the scaffolding of the conjugation machinery. TraY also induces DNA bending and stimulates nicking at the origin of transfer in co-operation with IHF (22). This leads to the expression of the gene *traM*, which is essential for DNA transfer of F and R1. Eventually, TraM autorepression limits the activity of the *tra* operon. In R1 and R100, but not in F, *traJ* expression has been shown to be linked to *traM* (5, 25).

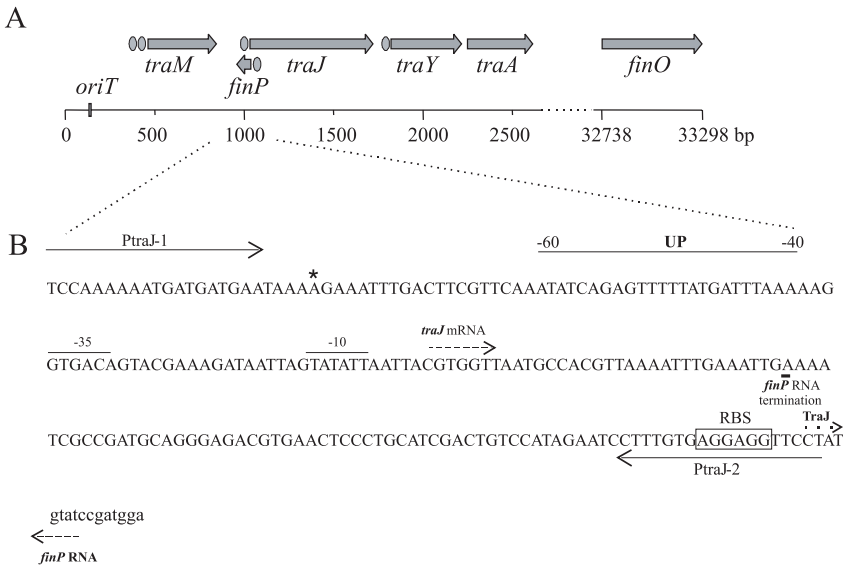

Fig. 1. **Schematic representation of the F-like plasmid transfer region**
(A) F-plasmid *tra* operon. Schematic presentation of the first 2700 bp and the last 600 bp of the 33.3 kb-sized *tra* operon (11). The origin of transfer (*oriT*), the *traM*, *traJ*, *traY*, *traA* and *finO* genes and the *finP* antisense RNA are indicated. Promoters are indicated as filled circles.
(B) Genetic organisation of the *traJ* promoter region of the F-like plasmid pRK100. The –35 and –10 promoter regions, the positions of transcription initiation (*traJ* mRNA and *finP* RNA) and termination (*finP* RNA termination), the ribosomal binding site (RBS) as well as the initiation of the TraJ translation (TraJ) are indicated. The upstream element (UP) from –40 till –60 is also depicted. Binding sites of the oligonucleotide primers PtraJ-1 and PtraJ-2 are marked with solid arrows. The indicated sequence of the pRK100 plasmid differs at one base (indicated by an asterisk) from the published sequence of the same region of the F plasmid (Genbank number U01159).

Even though TraJ is a central positive regulator of the transfer region, the knowledge of the mechanisms that regulate the expression of the *traJ* gene itself, is limited. TraJ expression is regulated at the translational level through the fertility inhibition FinOP system (7, 10). Fertility inhibition is imposed by the action of two *tra* gene products: FinP, the antisense RNA molecule complementary to the 5' untranslated region of *traJ* mRNA, and FinO, which increases the concentration of FinP and thereby promotes the formation of the *traJ*-FinP duplex. This *traJ*-FinP duplex is degraded by RNase III, thereby decreasing the amount of the *traJ* mRNA, and hence TraJ protein (15). The combined actions of FinO and FinP repress F transfer by 100 – 1000-fold while FinP, by itself, represses F transfer by only 6-fold (18). Further, it has been demonstrated for plasmid F that in *cpx* mutant strains the TraJ protein level is reduced (29).

Considering the importance of TraJ in bacterial conjugation promoted by F and other F-like plasmids, we focussed our work on the discovery of mechanisms of transcriptional regulation of *traJ* expression. In these studies, we used pRK100, a ~145-kb natural conjugative F-like plasmid isolated from a uropathogenic *Escherichia coli* strain as a model system. This plasmid has been partially characterised (1) and its *tra* region has been partially sequenced. At the nucleotide level, the sequenced *tra* genes (*traM, finP*, *traJ*, *traY*, *traD*, *finO*) were most similar to genes of the plasmid F (4). The regulation of the pRK100 *traJ* promoter was studied using a reporter system consisting of a transcriptional fusion of the *traJ* promoter and the *lacZ* gene. The expression of this construct was studied in the absence of the FinOP fertility inhibition system to facilitate the identification of factors acting at the level of *traJ* transcription initiation. Our data indicate that the expression of the *traJ* gene varies with the growth cycle and that the cyclic AMP (cAMP)-CRP complex is a positive regulator of *traJ* transcription. The regulatory role of this complex was supported by results from gel retardation assays, DNaseI footprinting experiments, targeted mutagenesis of the CRP binding site, and mating assays.

2. MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium with aeration at 37 °C, unless stated otherwise. When appropriate, bacteria were grown in minimal medium M63 (24) supplemented with 0.2% glucose, 0.1% casamino acids and 1 μg/ml thiamine. Conditioned medium was prepared by growing strain MC4100 in LB medium (12 h, 37 °C with aeration), removal of the bacteria by centrifugation, and filter sterilisation of the medium. Conditioned medium was used within 24 h. Starvation for glucose, casamino acids or phosphate was achieved by adding only 1/5 of the usual concentration of these compounds to M63 medium. When appropriate, cAMP (10 mM) was added to LB. Ampicillin (100 μg/ml) was added to the growth media, as needed.

General DNA manipulation techniques

Plasmid DNA isolation, ligation, and transformation experiments were performed using standard methods (27). Restriction endonuclease digestions were carried out as specified by the manufacturer (Promega, Boehringer). DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen). DNA sequencing was performed using a dye rhodamine terminator cycling reaction and an ABI PRISM™ 310 Genetic Analyser.

Table 1. **Bacterial strains and plasmids**

Strains	**Genotype**		**Source or reference***
MC4100	*ara*D139 Δ(*argF-lac*)U169 *rps*L150 *rel*A1 *flb*B5301 *pts*F25 *deo*C1		3
RH74	MC4100 Δ*cya851 ilv::*Tn*10*		20
SBS688	MC4100 Δ*crp*39		13
DH5α	*thi*-1 *hsdR*17 *gyrA*96 *recA*1 *endA*1 *glnV*44 *relA*1 Φ80d*lacZ*ΔM15 *phoA*8		NCCB
Plasmids	**Properties / Vector**	**Insert**	**Source or reference**
pCB267	*lacZ* promoter probe vector		28
pTJ1	pCB267	210 bp *traJ* promoter	This paper
pGEM-T Easy	T-vector for cloning of PCR products		Promega
pUC19	Multi-purpose cloning vector		33
pINP2	pGEM-T Easy	158 bp Δ*lacZ*	This paper
pYZCRP	CRP encoding		R. H. Ebright[1]

*[1] Department of Chemistry and Waksman Institute, Rutgers University, New Jersey, USA

Construction of the *traJ-lacZ* fusions

A 210 bp fragment of pRK100 containing 208 nucleotides upstream of the *traJ* translation initiation site and the first two nucleotides of the *traJ* start codon ATG was amplified by PCR using primers PtraJ-1 (5'-CGGGATCC-TCCAAAAAATGATGATGAAT-3') and PtraJ-2 (5'-GCTCTAGA-ATAG-GAACCTCCTCACAAAG-3') (Fig. 1B). PtraJ-1 and PtraJ-2 contained *Bam*HI and *Xba*I restriction sites to facilitate cloning into the plasmid pCB267 (28), generating pTJ1. To generate mutations in the CRP binding site, primers with defined nucleotide substitutions were used in the PCR reactions. For mutagenesis of the left part of the CRP binding site (TTTGA → GTCGA) the primer PtraJ-1-ML (5'-CGGGATCC-TCCAAAAAATGATGATGAATAAAAGAAA**GTCGA**CTTCGTTCAAATATCA-GAG-3') was used in combination with PtraJ-2, while for mutagenesis of the right part of the CRP binding site (TCAAA → ATCGA) the primer PtraJ-1-MR (5'-CGGGATCC-TCCAAAAAATGATGA-TGAATAAAAGAAATTTGACTTCGT**ATCGA**TATCAGAG-3') was used in combination with PtraJ-2. The nucleotides that were altered are given in bold. To enable cloning into pCB267 both primers, PtraJ-1-ML and PtraJ-1-MR contained a *Bam*HI restriction site. DNA sequencing confirmed that the correct fragments had been cloned.

β-Galactosidase assays

For measurements of β-galactosidase activity, relevant strains were grown (37 °C) overnight in LB medium, diluted (1/500) into fresh LB or M63 medium, re-grown to an OD_{600} of 1, and again diluted (1/500) into fresh LB medium, M63 or conditioned medium. Samples were periodically removed and assayed for β-galactosidase activity. β-Galactosidase assays were performed essentially as described (21) with bacteria treated with sodium dodecyl sulfate (SDS)-chloroform and washed with Z buffer and *o*-Nitrophenyl-β-D-galacto-pyranoside (ONPG) as a substrate. Enzyme activity was defined in Miller units (MU) (21).

Generation of Δ*lacZ* RNA for use as RT-PCR competitive template

A 215 bp fragment of the *lacZ* gene was generated by PCR using plasmid pTJ1 as a template and the primers lacZ-1 (5'- ACGATGCGCCCATCTACACC-3') and lacZ-2 (5'- ACGACTGTCCT-GGCCGTAAC-3'). The generated DNA fragment was digested with *Mse*I and the restriction fragments were ligated with T4-ligase (Amersham Pharmacia Biotech). PCR amplification of the ligation

mixture with the primers lacZ-1 and lacZ-2 yielded several products, including the desired 158 bp Δ*lacZ* product. This fragment was isolated from a 4% NuSieve 3:1 agarose gel (BMA, BioWhittaker Molecular Applications) and cloned into pGEM-T Easy vector (Promega), generating the plasmid pINP2. Sequence analysis confirmed that, apart from the deletion, no other base changes had been introduced. pINP2 was cut with the restriction endonuclease *Pst*I and used in the Riboprobe In Vitro Transcription System (Promega) as the template for the Δ*lacZ* RNA. The amount of *in vitro* Δ*lacZ* RNA transcript was determined spectrophotometerically.

RNA isolation

RNA was isolated from log-phase grown bacteria passaged twice as indicated above, and then grown in LB medium for 2 hours. For each sample, RNA from the same amount of bacteria was purified with RNAzol™B (Campro Scientific). After isopropanol precipitation, RNA was dissolved in 40 µl of H_2O, and stored at −70 °C. Prior to analysis of the *traJ-lacZ* mRNA in the competitive RT-PCR (see below), samples were checked for DNA contamination by PCR using oligonucleotide primers lacZ-1 and lacZ-2. If DNA contamination was detected, samples were treated with RQ1 RNase-Free DNase (Promega) until no contamination of DNA was detected.

Competitive RT-PCR

In the competitive RT-PCR (12), 1 µl of DNA-free RNA sample of the relevant strain and 1 µl of competitive template (either 50, 5 or 0.5 pg of Δ*lacZ* RNA) were used together with the lacZ-1 and lacZ-2 oligonucleotide primers in the Access RT-PCR System (Promega). The RT-PCR involved 45 min of reverse transcription at 48 °C, 2 min of AMV reverse transcriptase inactivation and RNA/cDNA/oligonucleotide primer denaturation at 94 °C, and 40 cycles of denaturation (30 s at 94 °C), annealing (30 s at 58 °C) and extension (30 s at 72 °C), followed by one final extension step (10 min at 72 °C). The RT-PCR products were separated on 2% agarose gels. The competitive RT-PCR was performed twice on two different samples and gave similar results in all tests. The absence of DNA was verified by running the competitive RT-PCR with RNA as template but with the addition of water instead of reverse transcriptase.

EMSA

The electrophoretic mobility shift assay (EMSA) was performed as described (2). The 226 bp PCR product, *ptraJ,* obtained with oligonucleotide primers PtraJ-1 and PtraJ-2 (see above), was labelled with [α-^{32}P] at the *Bam*HI site using the Klenow enzyme (USB). CRP binding reactions were performed essentially as described (31). In brief, 20 ng of labelled DNA fragment were mixed with variable amounts of purified CRP (generously provided by G. S. Lloyd and S. Busby) in the presence of cAMP (18 mM) in a final volume of 20 µl, and incubated for 30 minutes at 37 °C. The 214 bp *Bam*HI–*Pvu*II fragment of pUC19 carrying the *lacZ* promoter was used as a positive control. After the binding reaction, 1 µl of loading buffer (0.1% brom-phenol blue and 50% glycerol in water) was added and samples were loaded onto a 5% PAGE gel and run at a constant voltage (240 V). The gels were transferred to Whatman 3MM paper, dried, and autoradiographed.

Mobility shift assays using agarose gels were performed as described (16) except that the reaction volume was 20 µl and incubations were performed for 15 min at 37 °C. The 100 bp DNA ladder (MBI Fermentas) was used as competitor DNA.

DNase I footprinting

DNaseI footprint experiments were essentially performed as described (32). For these experiments, the 226 bp *PtraJ* fragment was amplified using the non-labelled oligonucleotide primer PtraJ-1

and the T4 polynucleotide kinase (Gibco BRL) [γ-^{32}P]ATP end-labelled primer PtraJ-2. From the labelled PCR product, 3 µl were used in a 20 µl binding reaction with variable amounts of purified CRP protein as described for the EMSA. After incubation, 0.06 U of DNaseI (Amersham Pharmacia) was added and the mixture was incubated for 3 min at room temperature. The reaction was stopped by the addition of 1 µl of 60 mM EDTA followed by 10 min of incubation at 65 °C. DNA fragments were purified with QIAquick Nucleotide Removal Kit (Qiagen) and eluted in 30 µl of H_2O. Six µl of the final sample were analysed by denaturing polyacrylamide 6% gel (National Diagnostics) electrophoresis run at a constant power 60 W with a DNA sequence ladder in parallel. The DNA sequence ladder was generated with the appropriate primer using the Sequenase Version 2.0 DNA Sequencing Kit (USB).

Mating assay

Conjugation experiments were essentially carried out as described (9) except that the strains were grown in LB and overnight cultures of donor and recipient strains were diluted 100-fold and incubated for 2 hours with aeration at 37 °C, and that the mating mixture consisted of 0.05 ml of the donor and 0.45 ml of the recipient culture and 0.5 ml of fresh LB. When appropriate the mating mixture was supplemented with cAMP (10 mM). Mating was performed for 2 hours at 37 °C. Transconjugants were selected on LB media supplemented with the appropriate antibiotics. Conjugal transfer frequencies were expressed as the portion of transconjugants to recipient or donor cells.

3. RESULTS

Glucose starvation enhances *traJ* expression in a CRP dependent fashion

Measurement of β-galactosidase activity in *E. coli* strain MC4100 carrying the *traJ-lacZ* transcriptional fusion at various stages of growth in LB medium, demonstrated a gradual increase in enzyme activity with the duration of growth (Fig. 2A). To determine whether the increase in activity was caused by a depletion of nutrients, we assayed β-galactosidase activity after transfer of log-phase grown bacteria to conditioned LB medium. In this medium, in which the *E. coli* were unable to grow (Fig. 2A), a much stronger increase in enzyme activity was found, which was already apparent at 1 h of incubation and which reached its peak at 4 h of incubation (Fig. 2A).

The factors in the medium responsible for the induction of β-galactosidase activity were sought by monitoring bacterial enzyme activities during growth in M63 minimal medium with variable amounts of glucose, casamino acids or phosphate. These experiments indicated that *traJ-lacZ* transcription varied with the availability of glucose in the medium (Fig. 2B), while starvation for casamino acids or phosphate had no effect (data not shown).

Since a well established bacterial response to the level of glucose is via the cAMP-CRP complex, we measured *traJ-lacZ* expression levels in a *cya* and *crp* mutant background. As illustrated in Fig. 2C, β-galactosidase activity in both mutants was considerably lower than in the parent strain. Complementation experiments demonstrated a strong increase in β-galactosidase activity in the *cya* mutant when grown in the presence of 10 mM cAMP (Fig. 2D), while no significant effects were observed for the parental strain (data not shown). Complementation of the *crp* mutant via introduction of a plasmid encoding CRP (pYZCRP) was not successful as it led to a loss of the pTJ1 plasmid from the cell. Overall, the results support the notion that the cAMP-CRP complex is required for the induction of *traJ* expression during conditions of glucose starvation.

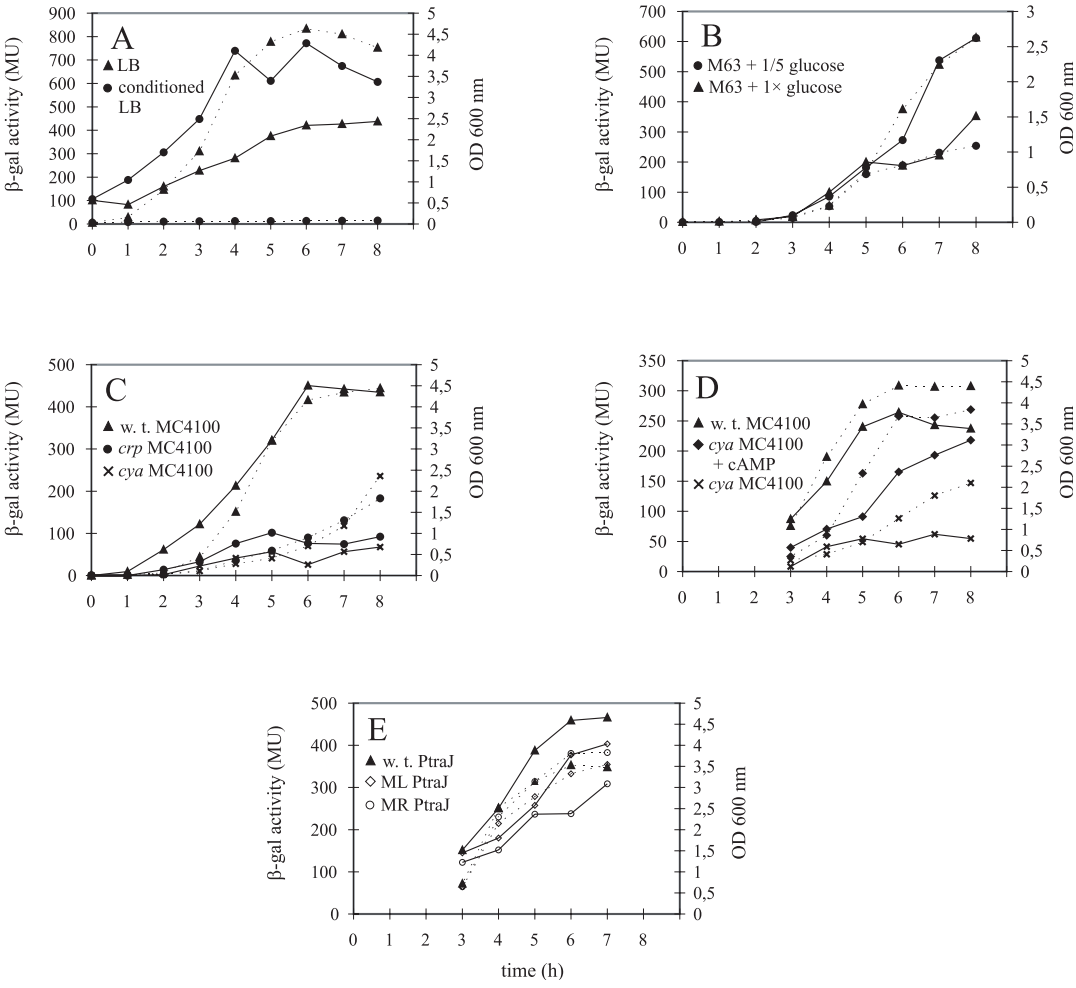

Fig. 2. **β-Galactosidase activity of the *traJ-lacZ* fusion**

At various time points (0-8 h), the optical density (OD_{600}) of the culture was measured (dotted lines) and aliquots were assayed for β-galactosidase activity (expressed in Miller Units, MU) (solid lines).

(A) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 grown in LB (▲) and in conditioned LB medium (●).

(B) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 grown in M63 medium with 1× glucose (▲) and with 1/5 glucose (●).

(C) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 (▲), and in the MC4100 *cya* (×) and *crp* mutant strains (●) grown in LB medium

(D) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 grown in LB medium (▲), and in MC4100 *cya* grown in LB medium with (◆) and without (×) 10 mM cAMP.

(E) β-Galactosidase activity in strain MC4100 carrying the wild type *traJ-lacZ* fusion (w. t. PtraJ, ▲) or the *traJ-lacZ* fusion in which the left (ML PtraJ, ◇) or right (MR PtraJ, ○) part of the CRP binding domain has been altered.

Experiments were carried out 3-4 times and representative results are shown.

The cAMP-CRP complex regulates the level of the *traJ* transcript

To ascertain that the cAMP-CRP complex influences the level of the *traJ* transcript, we compared the amounts of *traJ-lacZ* mRNA in the *cya* and *crp* mutant with that in the parent strain after 2 h of growth in LB medium using a competitive RT-PCR (12). The strategy used for quantification involved co-amplification of a competitive template, competing for the same oligonucleotide primers as the target RNA, but which, after amplification, could be distinguished from the target by a difference in size. Since the FinOP system reduced *traJ* mRNA levels below the level of the reliable detection by RT-PCR (data not shown), we used pTJ1 derived *traJ-lacZ* mRNA as the target and the Δ*lacZ* RNA as a competitive template. In these experiments semi-quantitative results were obtained by comparing the intensity of the DNA bands due to *traJ-lacZ* mRNA and the Δ*lacZ* RNA that were added to the sample (Fig. 3).

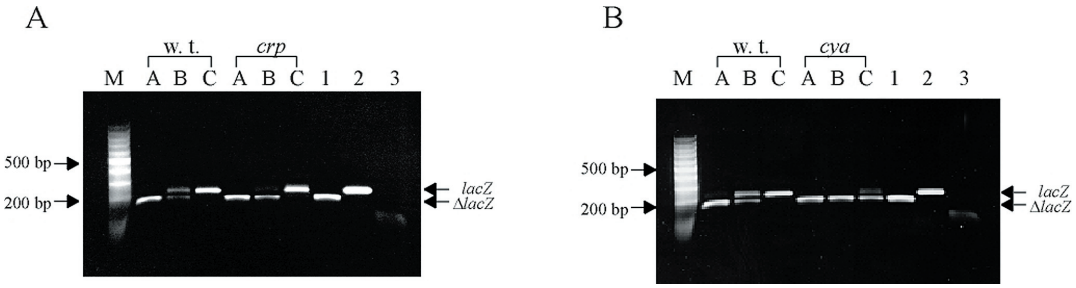

***Fig. 3.* Semi-quantitative determination of *traJ-lacZ* mRNA in the wild type and *crp* and *cya* mutant strains with competitive RT-PCR**

RT-PCR was performed on RNA isolated from the wild type MC4100 (w. t.) and *crp* mutant (*crp*) (panel A) and from the wild type MC4100 (w. t.) and *cya* mutant (*cya*) (panel B) in the presence of 50 pg (lanes A), 5 pg (lanes B) or 0.5 pg (lanes C) of competitive Δ*lacZ* RNA. Plasmid pINP2 carrying Δ*lacZ* (lane 1) and plasmid pTJ1 (lane 2) carrying the intact *lacZ* fragment, served as positive controls; RT-PCR with water instead of reverse transcriptase served as negative control (lane 3). M – 100 bp DNA ladder (MBI Fermentas).

Application of this approach to the wild type, *cya* and *crp* mutant strains carrying pTJ1 revealed that in the mutant strains much less *traJ-lacZ* mRNA was present than in the wild type strain (Fig. 3 panel A – lane B, Fig. 3 panel B - lanes B and C). This finding explains the observed reduced β-galactosidase activity in the mutant strains and suggests that this activity is due to the cAMP-CRP complex-dependent stimulation of transcription initiation.

Purified CRP protein binds to the *traJ* promoter region

To establish whether CRP regulates the level of *traJ* transcript by binding to the *traJ* promoter region electrophoretic mobility shift assays (EMSA) were performed. EMSA with a 226 bp PCR amplified and gel purified *ptraJ* fragment (containing the *traJ* promoter) and various concentrations of purified CRP showed that 5 nM of CRP was sufficient to cause a retardation of the migration of the *traJ* fragment (Fig. 4A).

The specificity of the CRP binding was confirmed in a competitive agarose gel shift assay with the DNA of a 100 bp ladder as a control. These experiments indicated that the addition of 0.5 μg CRP (1.1 μM) was already sufficient to cause a shift in the 226 bp *ptraJ* fragment, while much higher concentrations were needed to alter the migration of the "random" DNA of a 100 bp ladder (Fig. 4B). These results strongly suggest that CRP specifically bound to the *traJ* promoter region.

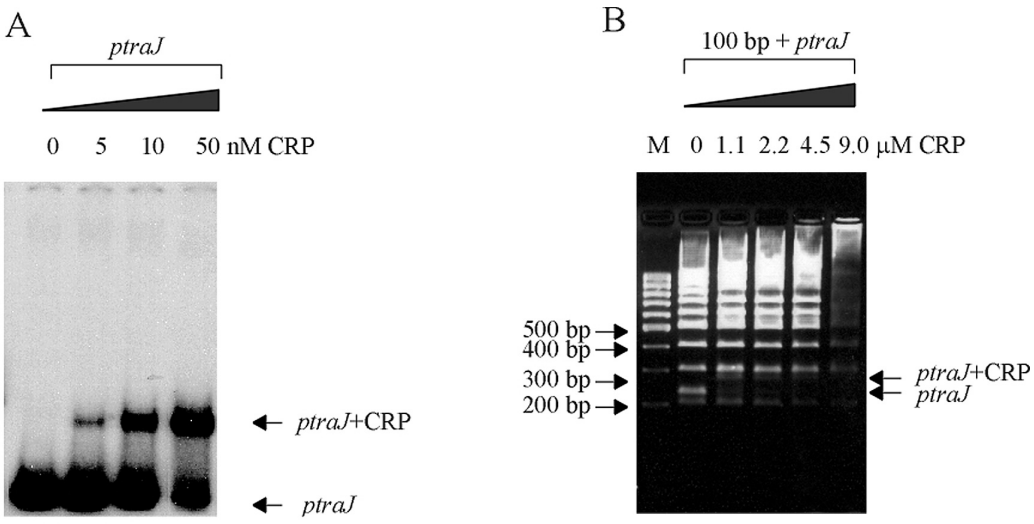

Fig. 4. **EMSA demonstrating the binding of purified CRP to the *traJ* promoter region**
(A) Migration of the ^{32}P-end labelled 226 bp PCR *traJ* promoter fragment (*ptraJ*) in the absence and presence of the indicated concentrations of purified CRP as determined by PAGE and autoradiography. The arrows indicate the *traJ* region and the shifted protein-DNA complex.
(B) Migration in an agarose gel of the 226 bp PCR *traJ* promoter fragment (*ptraJ*) after the addition of the indicated concentrations of CRP in the presence of 100 bp DNA ladder. Note the specificity of the shift of the *ptraJ* fragment (arrow) in the presence of CRP. M – 100 bp DNA marker.

Mapping of the CRP binding domain by DNaseI footprint experiments

In order to locate the CRP binding site on the *traJ* promoter region, we performed DNaseI protection assays. In these experiments, purified CRP was incubated with a radiolabelled *traJ* promoter fragment, which was then digested by DNaseI and subjected to gel electrophoresis and autoradiography. As shown in Fig. 5, CRP protected a distinct DNA region against DNaseI digestion in a dose-dependent fashion. Comparison with the nucleotide sequence of the pRK100 *traJ* regulatory region, run in parallel, indicated that the protected region extended from the position –57 to –78, relative to the transcription site. The corresponding nucleotide sequence 5'-aaaTTTGAct-tcgtTCAAAtat-3' strongly resembled the CRP consensus binding site 5'-aaaTGTGAtctagaTCACAttt-3' (31) (Fig. 6).

Mutagenesis of the identified CRP binding site

To unequivocally demonstrate that the identified CRP binding sequence acts in the regulation of the *traJ* promoter, we performed site-directed mutagenesis. Mutations in the left (TTTGA → GTC-GA) and right (TCAAA → ATCGA) part of the identified CRP-binding site (Fig. 6) were introduced by PCR. The mutated *traJ-lacZ* fusions were cloned onto pCB267, which was then transferred to *E. coli* strain MC4100. As illustrated in Fig. 2E, mutagenesis of each of the domains of the CRP binding site led to a less profound increase in enzyme activity upon prolonged growth than observed for the *traJ-lacZ* fusion carrying the intact CRP binding site.

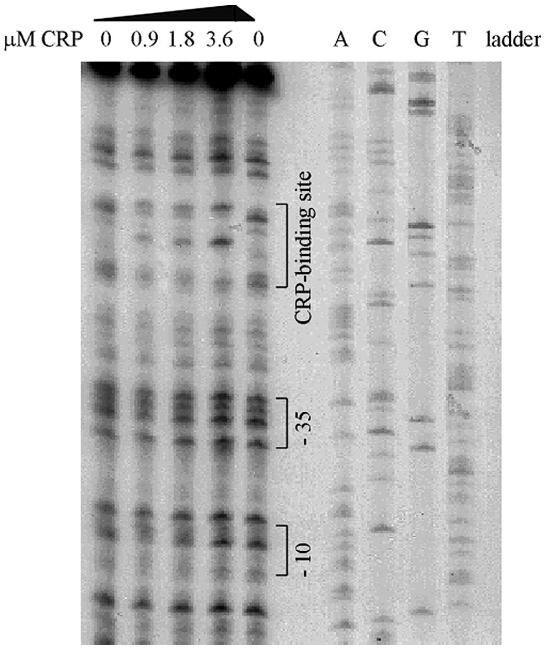

Fig. 5. **Mapping of the CRP binding site in the *traJ* promoter region as determined by DNaseI protection assay**
A 226 bp PCR ^{32}P labelled *traJ* promoter fragment was incubated with the indicated amounts of purified CRP, treated with DNaseI, and separated on 6% sequence gels. A nucleotide (A, C, G and T) sequencing reaction was run in parallel. The CRP-binding site, and the –10 and –35 promoter region are indicated.

Mutations in *cya* and *crp* inhibit conjugal transfer of pRK100

In an attempt to position our findings in a more natural setting, we investigated the apparent regulatory role of the cAMP-CRP complex in *traJ* transcription by measuring the efficiency of conjugal transfer of pRK100 for the wild type and *cya* and *crp* mutant strains. Conjugation experiments in which *E. coli* DH5α served as recipient strain demonstrated that the wild type strain MC4100 mated up to 100-fold more efficiently than the *cya* and *crp* mutant (Table 2). Addition of cAMP (10 mM) to the conjugative mixture considerably increased the mating efficiency of the *cya* mutant (Table 2) fully in line with the results with the *traJ-lacZ* fusion. Although the control of conjugation of F-like plasmids is very complex, these data support the idea that the cAMP-CRP complex influences *traJ* transcription in the natural pRK100 environment.

Table 2. **Conjugal transfer frequencies of plasmid pRK100**
Conjugal transfer of pRK100 was performed from the wild type (w. t.), *crp* and *cya* mutant strains into *E. coli* strain DH5α (2 h of conjugation) in the absence and presence of 10 mM cAMP.

Donor strain	Conjugal frequency (transconjugants/donor cells)		Conjugal frequency (transconjugants/recipient cells)	
	– cAMP	+ cAMP	– cAMP	+ cAMP
MC4100 w. t.	3.0×10^{-5}	3.6×10^{-5}	1.1×10^{-3}	1.3×10^{-3}
MC4100 *cya*	2.9×10^{-7}	2.2×10^{-6}	4.1×10^{-6}	3.1×10^{-5}
MC4100 *crp*	7.6×10^{-7}	7.7×10^{-7}	5.6×10^{-6}	5.7×10^{-6}

4. DISCUSSION

Conjugation is an important bacterial mechanism to transfer genetic material to other microorganisms. This process is facilitated via a large array of genes that encode the sophisticated conjugation system. Synthesis of the conjugative apparatus represents a heavy metabolic load and, due to the presence of receptors on the surface of conjugative pili, makes the bacterium vulnerable to bacteriophage infection. Conjugation of F-like plasmids is therefore a tightly regulated process resulting in only few bacteria synthesising a conjugative apparatus. It has been well-documented that the TraJ protein is the central positive regulator of F-like plasmid conjugal transfer. The expression of this protein is normally suppressed by the effects of the FinP antisense RNA and the FinO protein (7, 10). In this work, we provide direct evidence that the global regulator CRP acts as a positive transcriptional regulator of *traJ* expression via direct binding to the *traJ* promoter region.

The first indication that CRP positively influences *traJ* promoter activity was the increase in β-galactosidase activity in bacteria carrying a *traJ-lacZ* fusion during a prolonged period of growth and, more specifically, in conditioned medium in the absence of bacterial growth. This pointed to a depletion of nutrients as a signal for enhanced *traJ-lacZ* activity. The finding that glucose was an important factor in the regulation led us to hypothesise that CRP may act as a putative positive regulator molecule. Glucose starvation results in a rapid stimulation of cAMP synthesis, which in turn leads to the formation of high intracellular levels of the cAMP-CRP complex. The significant lower β-galactosidase activity in a *cya* and *crp* mutant carrying the *traJ-lacZ* fusion, the partial restoration of the defect in the *cya* mutant by the addition of exogenous cAMP, and the reduced *traJ-lacZ* mRNA levels detected by competitive RT-PCR collectively indicated the involvement of cAMP-CRP complex in the regulation of *traJ-lacZ* activity. The proposed function of CRP may form the basis of the observed association between intracellular levels of cAMP and the expression of transfer-related activities from F-like plasmids (14, 19).

Direct evidence that CRP regulates *traJ* promoter activity via specific recognition of a nucleotide sequence in the *traJ* promoter region was obtained in series of experiments: (a) mobility shift experiments demonstrated direct binding of CRP to the *traJ* promoter region, (b) DNaseI footprinting experiments showed that binding of purified CRP to a fragment carrying the *traJ* promoter region provided protection against DNAse I activity, and (c) targeted mutagenesis of the putative CRP binding sites in the *traJ* promoter region reduced the activation of TraJ under conditions of starvation. Binding of CRP to target promoter sequences is known to regulate gene expression. On interaction with cAMP, CRP undergoes a conformational change that enables binding of the cAMP-CRP complex to a distinct 22 bp DNA consensus sequence. This binding and the protein-protein interaction between CRP and RNA polymerase enhance the intrinsic promoter activity (17).

Mapping of the CRP binding domain in the *traJ* promoter region by DNaseI footprinting and confirmed by targeted mutagenesis, revealed that the binding site was centred around position –67.5 upstream of the assumed transcriptional start site of *traJ* and that it had strong similarity with the CRP consensus sequence (31). The obtained location at –67.5 differs from the one that has been putatively assigned for the F-plasmid in the database (Genbank Accession number U01159). This possible CRP site, which does not constitute a CRP consensus sequence (31), was proposed to be located in the first short stem-loop in the *traJ* mRNA. Comparative sequence analysis indicated that the identified CRP binding site in the *traJ* promoter region of pRK100 is conserved among other F-like plasmids (Fig. 6).

F-like plasmid	Sequence *traJ* promoter region
pRK100	gaataaaag-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
pColV-K30	gaataaaag-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
pSU316	gaataaaag-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
F	gaataaacg-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
pSU233	gaataaagggaaata-**aa**t**TTTGA**ct**t**t**g**t**TCAAAt**at**cagacttttt**
ColB4	gaataaatgaaaatg-**aa**g**TTTGA**gt**t**g**g**t**TCAAAt**at**aagaattttt**
P307	gaataaagggaaata-**aa**t**TTTGA**ct**t**t**g**t**TCAAAt**at**caggtttttt**
R100	gaataaaaaagaatta**aaa**GT**TGAt**t**t**act**TCAAAt**aa**caggattttg**
R1-19	gaataaaaaagaatta**aaa**GT**TGAt**t**t**gct**TCAAAt**aa**caggattttg**
CRP consensus	**aaaTGTGAtctagaTCACAttt**

***Fig. 6.* Sequence alignment of *traJ* promoter regions of different F-like plasmids**
Nucleotides that are identical to the CRP binding consensus sequence (31) are indicated in bold. The more conserved nucleotides are capitalised. Deviations from the CRP consensus sequence are grey-shaded.

Promoters activated by CRP have been grouped into three classes according to the location of the bound CRP. At class I CRP-dependent promoters the CRP binding sites are located at sites near positions –61.5, –71.5, –81.5 or –91.5 with respect to the transcription startpoint, whereas at class II CRP-dependent promoters the CRP-binding site is centred near position –41.5. In these cases, CRP and RNA polymerase are located at the same face of the DNA and CRP is thought to exert its effect by direct contact with the RNA polymerase (6, 23). Class III CRP-dependent promoters have the CRP-binding site situated further upstream and they require, besides the cAMP-CRP complex, also an additional regulator. Assuming that the transcription startpoint in the pRK100 *traJ* promoter is the same as in the related F-plasmid, the CRP binding site on pRK100 is located at position –67.5, which implies that CRP and the RNA polymerase bind at different faces of the DNA. This unusual position of a CRP binding site might be of particular interest, as it may indicate that besides the CRP-cAMP complex, an additional regulator binds to the DNA and assists CRP to establish contact with RNA polymerase. The pRK100 *traJ* promoter has many characteristics of a strong promoter. The putative –35 and –10 sequences exhibit strong similarity to the consensus sequence of *E. coli* promoters, and the distance between the –35 and –10 hexamers is 17 bp, which is optimal for promoter activity. Furthermore, a putative UP element is present upstream of the –35 region (Fig. 1) (26). This element is known to increase promoter activity. The regulation of the *traJ* promoter activity by the cAMP-CRP complex demonstrated in the present study, suggests that the initiation of transcription due to –35 and –10 sequences and the UP element may not be sufficient to increase *traJ* mRNA levels above those of FinP antisense RNA. The positive effect of cAMP-CRP complex on *traJ* transcription may be needed to derepress the transcription of the *tra* genes and to enable synthesis of the conjugation machinery. This scenario is particularly attractive as it relates conjugation activity to distinct environmental conditions (such as the limited availability of glucose) encountered by the bacteria. In combination with the strong negative regulation by the FinOP system, the effect of cAMP-CRP complex and perhaps additional regulatory intermediates, may enable fine tuning of the conjugation process, which is necessary to balance the efficient DNA transfer and the exposure of the required structural machinery to the environment. However, it should be noted that the control of conjugation is extremely complex and that much more work with intact conjugative plasmid is needed to firmly establish the role of the cAMP-CRP complex beyond the level of *traJ* transcription.

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Chapter 5

H-NS and Lrp Are Positive Modulators of Conjugal Transfer of the *Escherichia coli* Plasmid pRK100

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ABSTRACT

Conjugative transfer of F-like plasmids is a tightly regulated process. The TraJ protein is the main positive activator of the *tra* operon, which encodes products required for conjugal transfer of F-like plasmids. Nucleotide sequence analysis revealed potential Lrp and H-NS binding sites in the *traJ* regulatory region. Expression of a *traJ-lacZ* fusion in *hns* and *lrp* mutant strains showed that both are positive modulators of *traJ*. Competitive RT-PCR demonstrated that H-NS and Lrp exert their effect at the transcriptional level. Electrophoretic mobility-shift assays showed that H-NS and Lrp protein bind to the *traJ* promoter. Conjugal transfer of pRK100 was decreased 540- and 4-fold in *hns* and *lrp* mutant strains, respectively. Together, the results indicate H-NS and Lrp act as activators of *traJ* transcription.

Key words: TraJ, Lrp, H-NS, conjugation, F-like plasmids

1. INTRODUCTION

Conjugation is a process, which promotes DNA transfer from a donor to a recipient by forming direct cell-cell contact. In F-like plasmids the genes responsible for conjugative transfer are located in an approximately 33 kb long *tra* region. The *tra* region contains approximately 40 genes that are organised into three tightly regulated operons (Fig. 1). Two monocistronic operons encoding the *traM* and *traJ* genes are located immediately downstream of the origin of transfer (*oriT*) (14). TraM seems to link the control of transfer gene expression and the initiation of DNA transfer (27), while TraJ activates the transcription of the third multicistronic *tra* operon (31). This third operon is transcribed from the P_{traY} promoter and encodes genes involved in F pilus synthesis, pilus assembly as well as DNA unwinding and nicking (14).

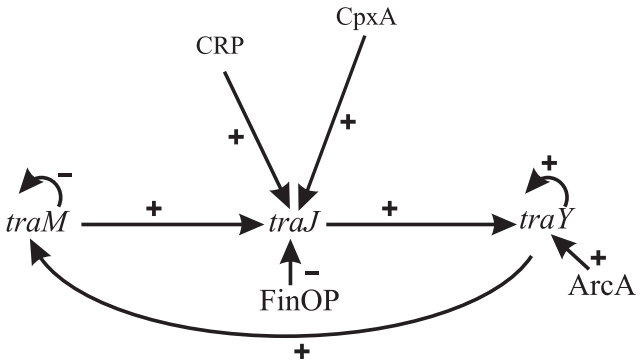

Fig. 1. **A general model for the regulation of expression of the *traM*, *traJ* and *traY* genes of F-like plasmids**
+ indicates a positive effect and – indicates a negative effect.

The expression of the *traJ* gene is subject to positive and negative regulation. We recently demonstrated that the cAMP-CRP complex activates transcription from the *traJ* promoter (Starčič M, Žgur-Bertok D, Jordi BJAM, Wösten MMSM, Gaastra W, van Putten JPM, in press) of pRK100, a conjugative ~145 kb F-like plasmid (1). Previously, CpxA of the two component signal transduction system CpxA/CpxR, which is activated by stress to the bacterial envelope (28), was reported to be required for efficient *traJ* expression (32). Negative regulation of TraJ expression occurs through the fertility inhibition FinOP system. FinP is an antisense RNA molecule complementary to part of the 5' untranslated region of *traJ* mRNA. FinO blocks FinP antisense RNA decay by promoting the formation of the *traJ*-FinP duplex. This duplex is subsequently degraded by RNase III preventing the synthesis of the TraJ protein (18). Repression of F plasmid transfer is also imposed by Dam methylation, which is required to sustain high levels of FinP RNA synthesis (37).

Increased expression of *traJ* stimulates $P_{traY,}$ the *traY* promoter. Full activation of the P_{traY} promoter requires, besides the TraJ protein, ArcA, which is part of the ArcA/B two component system sensing oxygen pressure (35). The TraY protein subsequently stimulates $P_{traY,}$ increasing the concentration of the *tra* operon proteins and enhancing expression of *traM*. TraY also induces DNA bending and stimulates nicking at the origin of transfer in co-operation with the integration host factor, IHF (24). Eventually, positive control of expression is interrupted by TraM autorepression. However, even though the *tra* regions of F-like plasmids have a common organisation, subtle differences in the regulation of transfer are evident (9, 27). Plasmid pRK100, used in our studies as a model system, was

isolated from a uropathogenic *Escherichia coli* strain. Partial nucleotide sequencing of the pRK100 *tra* region (*traM, finP, traJ, traY, traD,* and *finO*) showed highest similarity to F plasmid genes (14).

Despite TraJ's role as a central positive regulator of the transfer region, the mechanisms that control the expression of the *traJ* gene itself are not well understood. Assembly of the transfer apparatus and transfer of DNA is energetically demanding therefore, global regulators co-ordinating cellular metabolism in response to environmental signals could be involved in regulation of TraJ synthesis. Analysis of the pRK100 *traJ* promoter region sequences revealed several potential binding sites for global regulators, namely Lrp and H-NS (Fig. 2).

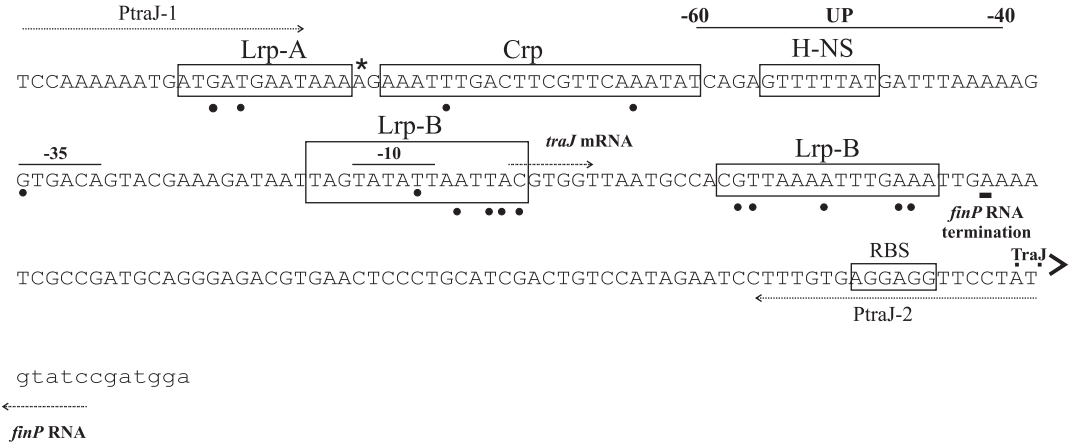

***Fig. 2.* Nucleotide sequence and locations of putative Lrp and H-NS regulatory sites in the *traJ* promoter region of plasmid pRK100**

The –35 and –10 promoter regions, the positions of transcription initiation and termination, the ribosomal binding site (RBS) as well as the initiation of the TraJ translation are indicated. Binding sites of the oligonucleotide primers PtraJ-1 and PtraJ-2 are marked. Putative binding sites for regulatory proteins Lrp and H-NS and for the CRP protein are indicated by boxes. The Lrp-A site is based on the consensus sequence described by Calvo and Matthews (5) and the Lrp-B sites are based on the consensus sequence described by Cui, *et al.* (8). The H-NS site is a putative DNA bending site as predicted by Lasergene PC, Dnastar, Inc. Madison, Wisconsin, USA. PC. The CRP site is based on results obtained with DNaseI footprinting and site-directed mutagenesis experiments (Starčič, *et al.,* in press). The nucleotides that differ from the known consensus binding sites are marked with dots. The Upstream element (UP) from –40 till –60 is also depicted. The indicated sequence of the pRK100 plasmid only differs at one base (indicated by an asterisk) compared with the published sequence of the same region of the F plasmid (accession number U01159).

The leucine-responsive regulatory protein (Lrp) is a host encoded regulator of *Escherichia coli* metabolism. Lrp influences expression of genes, whose protein products are involved in amino acid biosynthesis and degradation, nitrogen metabolism, carbohydrate degradation, synthesis of fimbriae and transport (25). In some cases leucine is required as an effector (21) while in others leucine has no effect *in vivo* (11). The histone-like nucleoid-structuring (H-NS) protein controls expression of many genes regulated by environmental signals (2). In the presented work we investigated the possible role of the global regulators Lrp and H-NS in *traJ* expression. The effect of the regulators was assessed using *in vitro* methods, such as a *lacZ* reporter system, RT-PCR, DNA binding assays, and also *in vivo* by conjugal transfer experiments. Our results demonstrate that H-NS and Lrp act as activators of pRK100 *traJ* transcription.

2. MATERIAL AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Table 1. **Bacterial strains and plasmids**

Strains	Genotype	Source or reference*
MC4100	*ara*D139 Δ(*argF-lac*)U169 *rps*L150 *rel*A1 *flb*B5301 *pts*F25 *deo*C1	7
GE3653	MC 4100 *lrp*-201::Tn*10*	10
MC-HNS	MC 4100 *hns*::*kan*	40
TR8	MC4100 *cpxA*::*cam*	T. J. Silhavy[1]
TR51	MC4100 *cpxR*::*spc*	T. J. Silhavy
GE2897	MC4100 Δ*82*(*himA*)::*tet*	10
RH90	MC4100 *rpoS*::Tn*10*	R. Hengge-Aronis[2]
RH98	MC4100 *relA251*::*kan spoT207*::*cat*	20

Plasmids	Properties / Vector	Insert	Source or reference
pRK100	F-like plasmid		1
pCB267	*lacZ* promoter probe vector		30
pTJ1	pCB267	226 bp *traJ* promoter	This study
pUC19	Multi-purpose cloning vector		41
pINP2	pGEM-T Easy	158 bp Δ*lacZ*	This study
pBE18	*gltBDF* promoter		39
PSJ4	*proU* promoter		17

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Media

Strains were grown in Luria-Bertani (LB) medium or M63 medium, which consists of M63 salts (26) supplemented with 0.2% glucose, 0.1% casamino acids and 100 μg/ml thiamine with aeration at 37 °C, unless stated otherwise. When appropriate, ampicillin (100 μg/ml), kanamycin (30 μg/ml), chloramphenicol (20 μg/ml), spectinomycin (20 μg/ml), and tetracycline (10 μg/ml) were added to the medium.

DNA manipulation techniques

General DNA manipulation techniques, as DNA isolation, ligation, and transformation experiments were performed using standard methods (29). Restriction endonuclease digestions were carried out as specified by the manufacturer (Promega, Boehringer). DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen). DNA sequencing was performed using a dye rhodamine terminator cycling reaction and an ABI PRISM™ 310 Genetic Analyzer automated sequencer and ABI PRISM™ software.

Construction of the *traJ-lacZ* fusion

PCR was performed to amplify a 210 bp fragment of pRK100 containing 208 nucleotides upstream of the *traJ* translation initiation site and the first two nucleotides of the *traJ* start codon ATG. For this purpose two primers, PtraJ-1 (5'-CGGGATCC-TCCAAAAAATGATGATGAAT-3') and PtraJ-2 (5'-GCTCTAGA-ATAGGAACCTCCTCACAAAG-3'), were used (Fig. 2). *Bam*HI and *Xba*I restriction sites in primers PtraJ-1 and PtraJ-2 facilitated cloning into the promoter probe plasmid pCB267 (30), generating pTJ1. Double-stranded nucleotide sequencing with PtraJ-1 and PtraJ-2 oligonucleotides as primers was carried out to confirm that no base changes had occurred while generating pTJ1.

β-Galactosidase assays

Overnight cell cultures of relevant strains were diluted 1:500 into fresh LB medium and grown with aeration at 37 °C to an OD_{600} of 1. Subsequently, cells were again diluted 1:500 into fresh LB medium and samples were periodically removed and assayed for β-galactosidase activity. β-Galactosidase assays were performed essentially as described (23). *o*-Nitrophenyl-β-D-galacto-pyranoside (ONPG) was used as a substrate in the β-galactosidase assays of cells treated with sodium dodecyl sulfate (SDS)-chloroform and washed with Z buffer (23). Enzyme activity is defined in units of optical density at 420 nm (OD_{420}) per minute per unit of OD_{600}, (23). At least two different colonies were tested in independent experiments.

Generating Δ*lacZ* RNA as competitive template for RT-PCR

PCR was performed to generate a 215 bp fragment of the *lacZ* gene by using plasmid pTJ1 as a template and primers lacZ-1 (5'- ACGATGCGCCCATCTACACC-3') and lacZ-2 (5'- ACGACTGTCCTGGCCGTAAC-3'). The obtained DNA fragment was digested with *Mse*I and the restriction fragments were ligated with T4-ligase (Amersham Pharmacia Biotech). This DNA was then used as a template in a PCR with primers lacZ-1 and lacZ-2. The different PCR products were separated onto a 4% NuSieve 3:1 agarose gel (BMA, BioWhittaker Molecular Applications) and a 158 bp Δ*lacZ* product with a deletion of the *Mse*I restriction fragment was purified from the gel. This fragment was cloned into the pGEM-T Easy vector (Promega), generating plasmid pINP2. The nucleotide sequence of the insert of pINP2 was determined to confirm, that apart from the deletion, no other base changes had been introduced. To obtain the RNA Δ*lacZ* competitive template for RT-PCR, pINP2, cut with *Pst*I, was used as the template in the Riboprobe In Vitro Transcription System (Promega).

RNA isolation

Overnight cell cultures were diluted 1:500 into fresh LB medium and grown with aeration at 37 °C to an OD_{600} of 1. The cell cultures were again diluted 1:500 into fresh LB medium and grown for 2 hours. For each sample, RNA was isolated from the same amount of cells with RNAzol™B (Campro Scientific) following the instructions of the manufacturer. After isopropanol precipitation, RNA was dissolved in 40 µl of H_2O, and stored at −70 °C. Prior to analysis of the *lacZ* mRNA in the competitive RT-PCR, samples were checked for DNA contamination by PCR using oligonucleotide primers lacZ-1 and lacZ-2. If DNA contamination was detected, samples were treated with RQ1 RNase-Free DNase (Promega).

Competitive RT-PCR

1 µl of DNA-free RNA sample of the relevant strain and 1 µl of the Δ*lacZ* competitive template, were used together with the lacZ-1 and lacZ-2 oligonucleotide primers in the competitive RT-PCR (16) performed with the Access RT-PCR System kit (Promega). The RT-PCR program was as follows: reverse transcription – synthesis of first cDNA strand at 48 °C for 45 min, AMV RT inactivation

and RNA/cDNA/oligonucleotide primer denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds, and one final extension for 10 min at 72 °C. The RT-PCR products were separated on 2% agarose gels. Competitive RT-PCR was performed twice on two different samples and similar results were obtained in all tests.

EMSA

The electrophoretic mobility shift assays (EMSA) were based on the method described in "Current Protocols in Molecular Biology" (4). The 226 bp PCR product, *ptraJ*, obtained with oligonucleotide primers PtraJ-1 and PtraJ-2 (see above), was labelled with [α-^{32}P] at the *Bam*HI site using the Klenow enzyme (USB). 20 ng of the labelled DNA fragment were used in a volume of 20 µl. Lrp binding reactions were performed essentially as described by Stauffer and Stauffer (34). However, there was no 5 min incubation before addition of purified Lrp and binding was allowed to proceed 30 min at 37 °C. The 480 bp *Eco*RI–*Pst*I fragment of pBE18 carrying the *gltBDF* promoter, ^{32}P-end labelled at the *Eco*RI site with the Klenow enzyme (USB), was used as a positive control.

H-NS binding reactions were performed essentially as described by Jordi, *et al*. (19) with protein binding proceeding for 15 min at 37 °C. The 317 bp *Bam*HI–*Pst*I fragment of pSJ4 (17) carrying the *proU* promoter was used as a positive control. After the binding reactions 1 µl of loading buffer (0.1% brom-phenol blue and 50% glycerol in water) was added to samples prior to loading onto a 5% PAGE gel, which was run at constant voltage (240V). The gels were subsequently transferred to Whatman 3MM paper, dried, and autoradiographed. The amounts of DNA and protein that were used are indicated in the Figures.

Competitive mobility shift assay using agarose gels

The mobility shift assay using agarose gels was based on the method described (19). However, the reaction volume was 20 µl and the incubation was performed for 15 min at 37 °C. The 100 bp DNA ladder (MBI Fermentas) was used as competitor DNA.

Mating assay

Conjugation experiments were performed essentially as described by Franklin and Möllby (13). Overnight cultures of donor and recipient strains were diluted 100-fold and incubated for 2 hours with aeration at 37 °C. A mating mixture consisting of 0.5 ml of the donor and 4.5 ml of the recipient culture was incubated for 2 hours at 37 °C. Transconjugants were selected on LB media supplemented with the appropriate antibiotics. Conjugal transfer frequencies were calculated per donor cells.

3. RESULTS

***traJ* expression is positively affected by Lrp and H-NS**

DNA sequence analysis of the *traJ* promoter region in plasmid pRK100 revealed putative H-NS and Lrp binding sites (Fig. 2). To test whether these two global regulators, as well as some other regulators (CpxA/R, ppGpp, IHF, σ^S), influence *traJ* expression, β-galactosidase activity of the *traJ-lacZ* transcriptional fusion was assayed in mutant strains defective for the above mentioned global regulators.

A significant decrease in β-galactosidase activity of the *traJ-lacZ* fusion was observed in the *hns* and *lrp* mutant strains throughout the growth cycle. The maximal difference between the wild type

strain and the *lrp* and *hns* mutants was observed in the early exponential phase, when activities were 6.5-fold and 5-fold lower, respectively, than in the wild type strain (Fig. 3A and B). Since Lrp in some cases requires leucine as an effector, β-galactosidase activity of the *traJ-lacZ* fusion was also tested in minimal medium with and without 50 mM leucine. Expression of the fusion was not affected by the addition of leucine (data not shown).

In contrast to the apparent positive effects of H-NS and Lrp on *traJ* expression, mutation in *cpxA* had only a small effect while mutations in *cpxR*, *relA spoT*, *himA* and *rpoS* had no significant effect on the β-galactosidase activity of the *traJ-lacZ* fusion (data not shown).

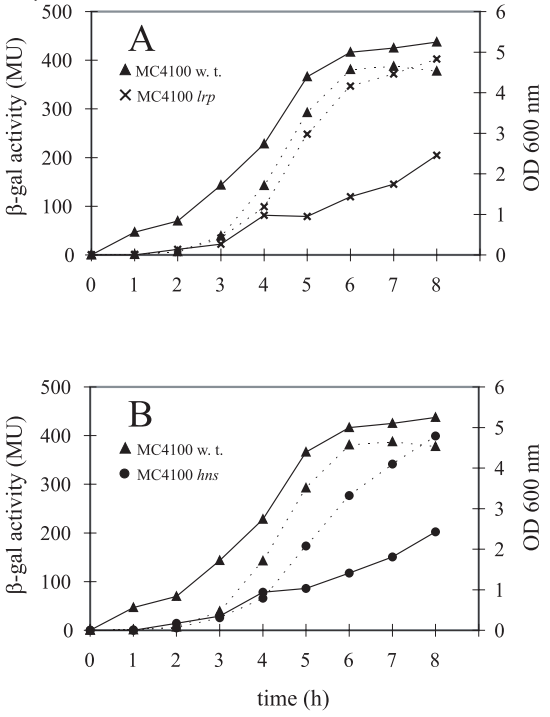

Fig. 3. **β-Galactosidase activity of the *traJ-lacZ* fusion**
At various time points (0-8 h), the optical density (OD600) of the culture was measured (dotted lines) and aliquots were assayed for β-galactosidase activity (expressed in Miller Units, MU) (solid lines).
(A) β-Galactosidase activity of the *traJ-lacZ* fusion in the MC4100 w. t. strain (▲) and in the MC4100 *lrp* mutant strain (×).
(B) β-Galactosidase activity of the *traJ-lacZ* fusion in the MC4100 w. t. strain (▲) and in the MC4100 *hns* mutant strain (●).
The experiments were performed in duplicate and a representative result is shown.

Competitive RT-PCR reveals lower mRNA levels in *hns* and *lrp* mutants

To ascertain whether, H-NS and Lrp act at the level of transcription of *traJ*, comparative analysis of mRNA in corresponding mutant strains was performed using competitive RT-PCR. Competitive RT-PCR is based on co-amplification of a competitive template, competing for the same oligonucleotide primers as the target RNA. The amplified products can be distinguished by a difference in size (16). The FinOP system reduced *traJ* mRNA levels below the level of "quantifiable" RT-PCR detection (data not shown). pTJ1 *lacZ* mRNA was therefore used as the target and the Δ*lacZ* RNA as a competi-

tive template. By comparing the intensity of the band due to *lacZ* mRNA with the band due to the defined amounts of the Δ*lacZ* RNA that were added to the sample, semi-quantitative results were obtained. Analysis of the RT-PCR results demonstrated that the Δ*lacZ* band was much more intense for both mutant strains than for the wild type strain (Fig. 4), indicating reduced levels of *lacZ* mRNA. These data, which are consistent with the observed reduction in β-galactosidase activity in the mutant strains, suggest that H-NS and Lrp likely exert their effect at the transcriptional level.

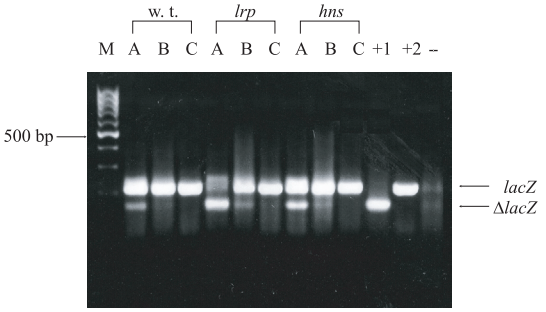

Fig. 4. **Semi-quantitative determination of *traJ-lacZ* mRNA in the wild type, *lrp* and *hns* mutant strains with competitive RT-PCR**

RT-PCR was performed on mRNA templates isolated from the wild type MC4100 (w. t.), *lrp* (*lrp*) and *hns* mutant (*hns*) in the presence of 50 pg (lanes A), 5 pg (lanes B) or 0.5 pg (lanes C) of competitive Δ*lacZ* RNA . +1 and +2 are positive controls, plasmid pINP2 with Δ*lacZ* and pTJ1 with the intact *lacZ* fragment, respectively. – is the negative control consisting of RT-PCR with water instead of mRNA. M – 100 bp DNA ladder (MBI Fermentas).

Note the change in *lacZ*-Δ*lacZ* ratio indicating a reduction in *traJ* mRNA in the *lrp* and *hns* mutant strains.

Lrp and H-NS proteins bind to the *traJ* promoter region

To further unravel the mechanism of regulation of *traJ-lacZ* expression by Lrp and H-NS, we assessed the abilities of the proteins to bind to the *traJ* promoter region. Mobility shift DNA-binding experiments were performed with pure Lrp and H-NS proteins and a 226 bp DNA fragment corresponding to the *traJ* promoter. As shown in Fig. 5, a 5 nM concentration of the Lrp protein was sufficient to promote significant retardation in the electrophoretic mobility of the 226 bp fragment. The fragment was also retarded by H-NS when the concentration of H-NS reached 500 nM. Shifts at similar concentrations of protein were observed for the respective positive controls, binding of Lrp to the *gltBDF* promoter and H-NS to the *proU* promoter (data not shown).

A competitive agarose gel shift assay was performed to determine the specificity of Lrp and H-NS protein binding to the *traJ* promoter. At concentrations needed to retard the *ptraJ* fragment both proteins also retarded some, but not other "random" DNA fragments of a 100 bp ladder used as competitive DNA (data not shown).

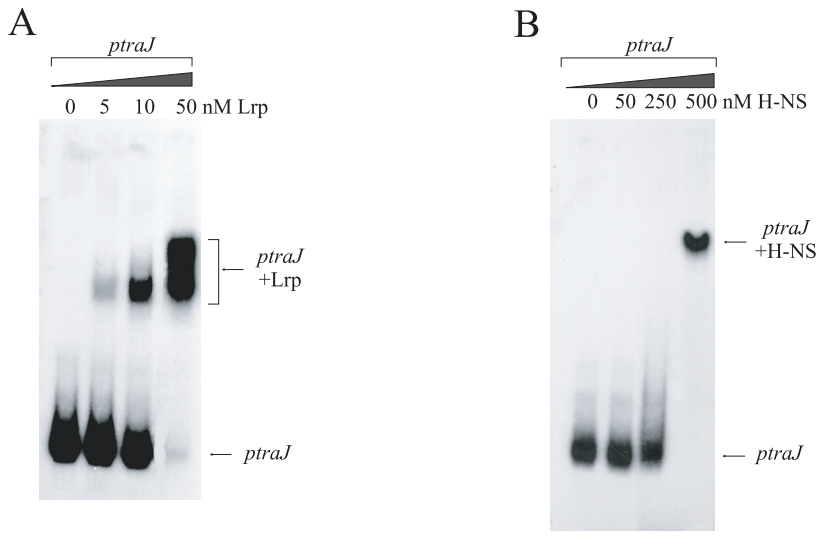

***Fig. 5*. Lrp and H-NS bind to the *traJ* promoter region**
Electrophoretic mobility shift assay with purified Lrp (A) and H-NS (B) and a 226 bp DNA fragment encompassing the P_{traJ} regulatory region (*ptraJ)*. The arrows indicate the ^{32}P-end labelled *ptraJ* fragment and the shifted protein-DNA complex. The amount of added protein is indicated.

Mutations in *hns* and *lrp* reduce conjugal transfer frequencies of pRK100

To confirm that Lrp and H-NS are activators of pRK100 conjugal transfer, mating experiments were performed and transfer frequencies from *hns, lrp* and the isogenic wild type strain MC4100 were compared (Table 2).

***Table 2.* Conjugal transfer frequencies of plasmid pRK100 from the MC4100 w. t. and mutant strains**

Strain	Conjugal frequency[a]	Strain	Conjugal frequency	Strain	Conjugal frequency
Serial A		**Serial B**		**Serial C**	
MC4100 w. t.	1.5×10^{-4}	MC4100 w. t.	5.1×10^{-4}	MC4100 w. t.	3.9×10^{-4}
MC4100 *lrp*	4.1×10^{-5}	MC4100 *hns*	9.4×10^{-7}	MC4100 *himA*	3.3×10^{-4}
MC4100 *rpoS*	2.2×10^{-4}			MC4100 *relA spoT*	5.0×10^{-6}

[a] Plasmid transfer frequencies were calculated per donor cells; experiments were performed in duplicate and a representative experiment is presented.

From both *hns* and *lrp* mutant strains conjugal transfer frequencies were significantly reduced, compared with transfer from the wild type MC4100 strain. Transfer frequencies, calculated per donor cell, were approximately 540-fold lower from the *hns* mutant and 4-fold lower from the *lrp* mutant, compared with the wild type MC4100 strain. Mutations in *cpxA*, *cpxR*, *himA*, *rpoS* and *sdiA* had no significant affect on conjugal transfer, but transfer was significantly reduced in a *relA spoT* mutant strain, producing no ppGpp. Together, these results are consistent with H-NS and Lrp being activators of conjugal transfer of plasmid pRK100.

4. DISCUSSION

Synthesis of the conjugative apparatus as well as DNA transfer itself represent a heavy metabolic burden to bacteria and the presence of receptors on the surface of conjugative pili bears the risk of bacteriophage infection. These factors demand conjugation to be a tightly regulated process. Regulation of F-like transfer has been extensively studied and has been demonstrated to be affected by environmental stimuli as well as plasmid and host factors (12). Yet, the molecular mechanisms underlying the regulation through environmental signals and host factors are still poorly understood. Recently, we demonstrated that the cAMP-CRP complex enhances *traJ* promoter activity in the F-like plasmid pRK100 (Starčič, *et al*., in press). The TraJ protein is the central positive activator of F-like *tra* genes activating the P_{traY} promoter (Fig. 1). In the present study, we identified two additional host encoded regulators of *traJ* promoter activity. Nucleotide sequence analysis revealed potential Lrp and H-NS binding sites in the *traJ* promoter region. Comparison of β-galactosidase activity of a transcriptional fusion of the pRK100 *traJ* promoter and the *lacZ* gene in the wild type with that in *lrp* and *hns* mutant strains indicated that both regulators positively affect *traJ* expression. Competitive RT-PCR suggested that the regulatory proteins enhanced *traJ* transcription.

Both H-NS and Lrp are global regulators of gene expression as well as nucleoid-structuring proteins. Lrp has been shown to be a sequence specific DNA binding protein while H-NS is a sequence-independent DNA-binding protein with preference for curved DNA (36, 38). Because of their function as global regulators, theoretically Lrp and H-NS may exert their effect on *traJ* transcription through direct binding to the *traJ* promoter region or indirectly by affecting genes that control *traJ* promoter activity. The results of our mobility shift assays indicate that both proteins directly bind to the *traJ* promoter region. It should be noted that the specificity of binding was not high, as particularly H-NS was found to bind to some fragments used as competitor DNA in the assay. This result is not surprising, considering the sequence-independent binding properties of H-NS. Both Lrp and H-NS proteins have been shown to have multiple binding sites around promoters and form nucleoprotein structures, which influence transcription (3, 33). Lrp and H-NS may form a complex that influences *traJ* transcription. The relevance of the *in vitro* data indicative of positive regulatory effects of Lrp and H-NS on *traJ* transcription was demonstrated by the mating experiments that plasmid pRK100 conjugal transfer was considerably reduced from the *lrp* and *hns* mutants (Table 2).

Recently, Lrp has been shown to be an activator of conjugation by promoting *traJ* transcription of the *Salmonella enterica* F-like virulence plasmid pSLT. The Lrp binding site described by Camacho and Casadesús (6), is immediately upstream of the region studied in our investigation, and is also conserved in plasmid pRK100 (Fig. 6). Nucleotide sequence analysis of the pRK100 *traJ* regulatory region revealed three additional potential Lrp binding sites (Fig. 2). Comparative nucleotide sequence analysis shows that the potential Lrp and H-NS binding sites described in this work are conserved in other F-like plasmids (Fig. 6).

From the other potential regulatory genes that we investigated, only *cpxA* showed a minor effect on *traJ-lacZ* transcription. This result seems at variance with work that showed that several *cpx* mutations affected the accumulation of TraJ (32). At this point however, it should be noted that this work was performed with plasmid F, while we adopted the plasmid pRK100 as a model system. Even though the *tra* regions of F-like plasmids have a common organisation, subtle differences in the regulation of transfer appear to exist. For example in R1 and R100 but not in F, *traJ* expression has been shown to be linked to *traM* (9, 27). Thus, caution is warranted in comparing the regulatory effects in different F-like plasmids.

A second noteworthy observation from the experiments with the other regulator mutants was the apparent absence of a regulatory effect of *relA spoT* on *traJ* transcription. Mutation of *relA spoT*,

which prevents the production of the stress alarmone ppGpp, has been demonstrated to reduce conjugal transfer in pRK100 (Table 2). Apparently this effect is not caused via regulation of *traJ* promoter activity. This finding once more illustrates the complexity of the regulation of the conjugation machinery in which TraJ is an important, but not the only factor, that is being regulated. How ppGpp affects conjugation awaits further investigation.

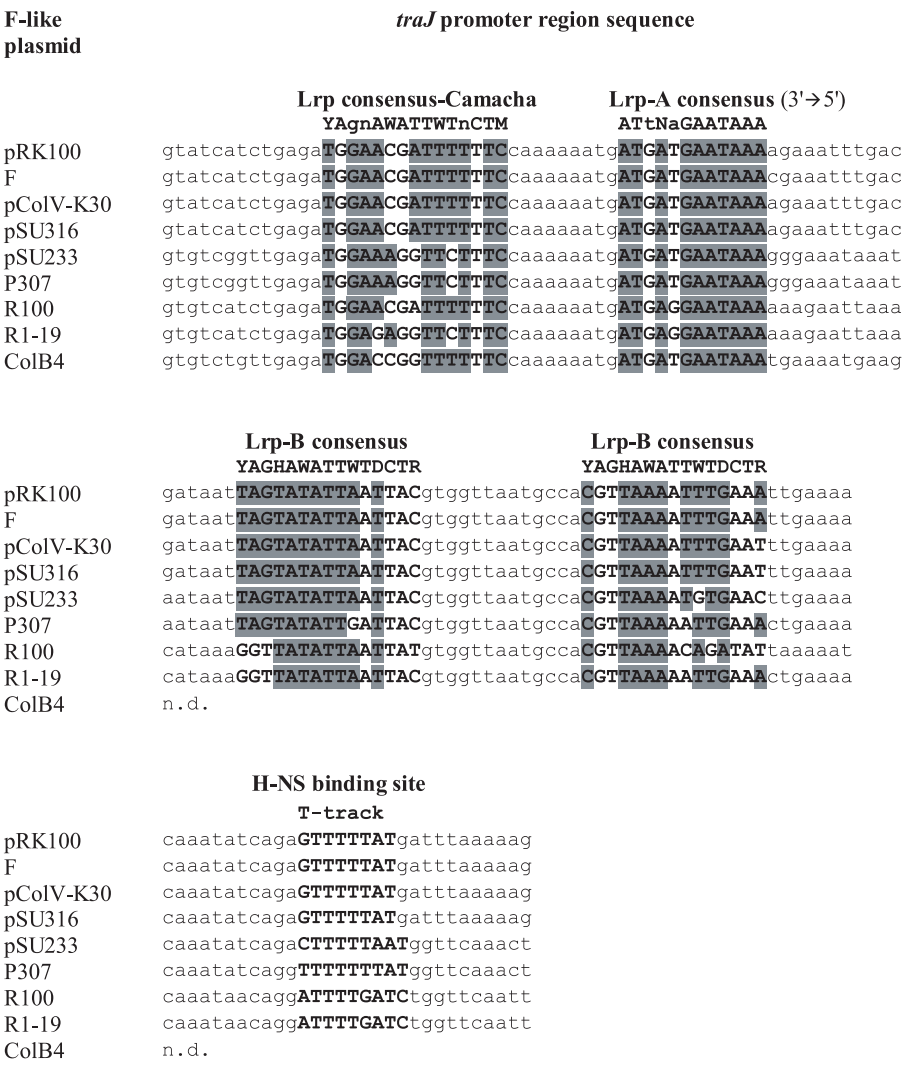

Fig. 6. **Sequences of the *traJ* regulatory region of several F-like plasmids, which are similar to the consensus Lrp and H-NS binding sites**

The Lrp-Camacho site was described by Camacho and Casadesús (6). The Lrp-A site is based on the consensus sequence described by Calvo and Matthews (5) and the Lrp-B sites are based on the consensus sequence described by Cui, *et al.*(8).

D = A or G or T; H = A or C or T; K = G or T; M = A or C; N = A or C or G or T; R = A or G; S = C or G; W = A or T and Y = C or T.

n.d. not determined

The mechanism by which Lrp and H-NS enhance *traJ* promoter activity remains to be defined. In a previous study we demonstrated that the cAMP-CRP complex enhances *traJ* promoter activity and that the CRP binding site is centred around position –67.5 upstream of the *traJ* transcription start site in pRK100 (Starčič, *et al*., in press). A cAMP-CRP binding site centred at –68.5, has also been demonstrated for *csiD*, a stationary phase inducible σ^S-dependent gene in *E. coli* (22). In a recent publication Germer, *et al*. (15) observed that, for the *csiD* promoter, the location of the activator site at –68.5 is a factor contributing to the pronounced $E\sigma^S$ selectivity at the *csiD* promoter. On the basis of β-galactosidase activity of the *traJ-lacZ* fusion and results of mating experiments we can conclude that, *traJ* expression in pRK100 is independent of σ^S and thus that positioning of the CRP binding site in the *traJ* regulatory region is not connected with σ factor selectivity. Despite this difference, it is interesting to note that cAMP-CRP, H-NS and Lrp are involved in the expression of *csiD* with H-NS and Lrp modulating, probably directly, activation of *csiD* by cAMP-CRP (15). Thus, it can be imagined that, in pRK100, binding of H-NS and Lrp to the DNA causes a conformational change in the *traJ* promoter, which facilitates binding of the cAMP-CRP complex to the CRP binding site centred at –67.5. As comparative sequence analysis indicates that the CRP binding site and the consensus Lrp and H-NS sequences are conserved among a number of F-like plasmids, this mechanism may also operate in other F-like plasmids.

Gene exchange in bacteria promotes adaptation to environmental challenges and conjugation is one of the main mechanisms responsible for horizontal gene transfer. Incorporation of Lrp, H-NS, cAMP-CRP, and positioning of the *traJ* CRP binding site at –67.5, into regulation of *tra* function in pRK100 ensures fine tuning of conjugation to specific environmental conditions. As F-like plasmids frequently encode antibiotic resistances and virulence factors, the identification of new regulatory factors as well as variations in regulation of conjugal transfer, is crucial to develop approaches to prevent dissemination of plasmid-encoded genes.

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Chapter 6

General Discussion

Horizontal gene transfer among bacteria is increasingly recognised as a major factor in the evolution of bacterial genetic diversity, the evolvement of emerging infectious diseases and in the spread of virulence traits and resistances among bacterial pathogens. One major mechanism of horizontal gene transfer is bacterial conjugation. This form of bacterial sex enables the transfer of bacterial DNA from a donor to a recipient strain. The machinery needed for their conjugal transfer is located on self-transmissible plasmids. These plasmids are also able to pick up and transfer genetic information from the chromosome, transposons, or other plasmids and thus seem to act as a DNA distribution system. In the present work, the large natural conjugative plasmid pRK100 was adopted as a model system to study the architecture, diversity and evolution of large natural plasmids, and the molecular mechanisms that regulate the conjugation event.

General architecture of pRK100

Awareness is growing that plasmids constitute an important part of the bacterial genetic pool. Plasmids generally encode products needed for their persistence (e.g. replication proteins and the controlling elements) as well as factors that contribute to host survival (e.g. resistance towards antibiotics or heavy metals). The experimental approach of constructing a plasmid map of pRK100 on the basis of DNA hybridisation results (Chapter 2), in conjunction with targeted PCR and sequence analysis of potentially interesting regions of the plasmid (Chapter 3), demonstrated that the approximately 145 kb plasmid indeed contains a wide variety of elements. Beside replication regions, the plasmid carries a *tra* region, colicin encoding genes and genes seemingly involved in iron acquisition and modulation of gene expression. In addition, pRK100 was found to contain several insertion sequences and a transposon, Tn*5431*, that codes for ampicillin and tetracycline resistance. Even though pRK100 appears to be a very stable plasmid, special plasmid maintenance systems were not discovered on it, at least with the techniques that were employed. It is tempting to speculate that the role of these systems is taken over by the colicins (post-segregational killing) and the Tn*5431* transposase (multimer resolution system). Definite evidence that pRK100 does not contain specific plasmid stability systems however, awaits determination of the entire pRK100 nucleotide sequence.

Replication of pRK100

Initial hybridisation experiments suggested that pRK100 carried two replication regions, one similar to the RepFIB and the other to the RepFIC replication region. Functional analysis of the regions via the construction of minireplicons in *E. coli* followed by sequence analysis (Chapter 3), demonstrated that the original assignment (based on hybridisation results) was premature and that pRK100 carried a RepFIB and a RepFIIA replication region. By construction of minireplicons, able to replicate in *E. coli,* it was demonstrated that both replication regions were functional and thus potentially could contribute to the replication of pRK100. At this time, it remains to be defined, which of the identified replication regions is responsible for replication of pRK100.

The occurrence of multiple replication regions on a single plasmid is not without precedent (19) and likely evolves from recombination between different plasmids (4). The mechanism that determines the establishment of the dominant replication region and the importance and possible advantage of the existence of multiple replication regions, are still under debate. It can be imagined that both replication origins are active but under different environmental conditions or in different hosts. Alternatively, it has been speculated that the co-existence of multiple replicons might be advantageous in escaping incompatibility, insertional inactivation and mutation (1). At this point it should be noted that the identified RepFIB and RepFIIA replication regions are located in different regions of pRK100 and that they have different replication control systems, iterons and ctRNA, respectively.

RepFIB and RepFIIA replication regions are widely distributed among plasmids. The most prominent plasmids harbouring RepFIB are the F plasmid (15), the enterotoxin plasmid P307 (1, 19), and the pO157 and pSFO157 from EHEC. RepFIIA, which was first found on the *Shigella flexneri* plasmid R100 (17, 22), is also present on prominent plasmids such as the pWR100 and pCP301 from *Shigella flexneri* and on pO157 and pB171 found in EHEC and EPEC strains, respectively. The finding that the large plasmids pO157 and pB171 in EHEC and EPEC strains carry similar type of replication regions (RepFIB and RepFIIA) as pRK100 may indicate that this combination of replication regions may be favourable to the persistence of these plasmids in pathogenic *E. coli* strains.

Mosaic and dynamic structure of pRK100

The identification of multiple replication origins is only one of the indications that pRK100 has evolved from different origins. The hybridisation and "shot-gun" sequencing experiments, described in Chapter 3, clearly demonstrate that pRK100 is composed of elements that can be found on the chromosome (e.g. the enterochelin uptake system) as well as, in different combinations, on a number of other plasmids. A similar mosaicism is emerging from the various large natural plasmid nucleotide sequencing projects. These plasmids often show the presence of sequences coding for non-essential plasmid functions that are interspersed among sequences required for plasmid replication and stability. Because of the mosaic and dynamic nature of conjugative plasmids, which are known for their high rates of recombination and plasmid transfer, it is difficult to reconstruct plasmid evolution and to determine which elements were acquired first. Nevertheless, juxtaposition and co-ordinate regulation of related survival functions may provide a higher level of organisation that represents a stabilising force. This may explain the similarities in the genomic maps of many different bacterial plasmids (26).

Because of the mosaic structure of pRK100, illustrated in Figure 2 in Chapter 3, it was difficult to classify the plasmid to a distinct group. Typically, plasmids are classified according to their similarity with other well-known plasmids, for example F-like, R100-like, or on the basis of their incompatibility with other plasmids and their replication regions (6). pRK100 contained both a number of pColV-K30-like and more F-like elements, and multiple replication regions. A solution to this dilemma provides the pragmatic approach to speak about pRK100 as a pColV-K30-like plasmid when colicin production and the aerobactin iron uptake systems are debated and to speak about a F-like plasmid when *tra* regions are studied. The designation of pRK100's RepFIIA replication region was difficult due to huge mosaicism in the broad RepFIIA family of replication regions caused by frequent recombination events (6, 18) (see Fig.1). The established designations of the replication regions are not adapted to the mosaicism, and are therefore misleading. The most prominent example of this is the replication region RepFIC, which was originally found on the plasmid P307 (19) and later proposed to be incompletely present also on plasmid F (23). However, comparison of the known plasmid F nucleotide sequence with those of other known plasmid nucleotide sequences clearly showed that only the *inc* region of plasmid F is similar to P307 RepFIC, while the Tn*1000* interrupted *repA1* gene for the Rep protein is unique. Another problem of the classification according the plasmid's replication region originates in the simple fact that large plasmids can have several functional replication regions and hence they can be differently grouped. Again pRK100, having a RepFIB and a RepFIIA replication region, is a good example of this.

The conjugative machinery of pRK100

An important feature of the large conjugative plasmids is their ability to transfer DNA through conjugation. pRK100 carries the complete, approximately 30 kb long, *tra* region that encodes the conjugative machinery and enables the conjugal transfer of DNA to recipient bacteria. The functionality of this system is demonstrated in Chapter 2. Nucleotide sequence analysis indicated that pRK100 *tra* region is the most similar to the F plasmid *tra* region (Chapter 3).

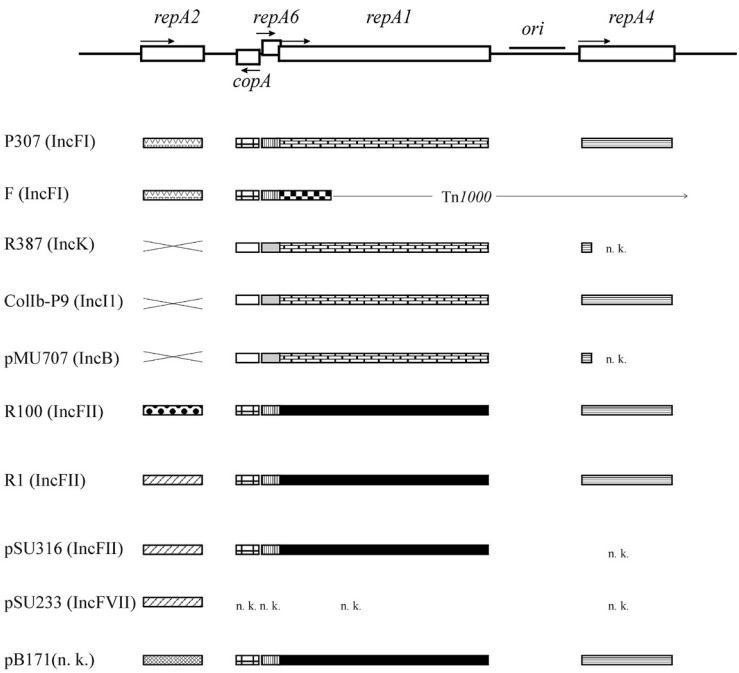

***Fig. 1*. Mosaicism in the broad RepFIIA family**
The organisation of IncFII-like replicons is shown at the top. Relationships between replicons of the IncFII-like family are presented using various patterns of shading. Note that in these mosaic replicons the *rep* region is closely related to one group while the *inc* replication control region, containing *copA*, as well as *repA2,* are related to other groups of replicons. "n. k." stands for "not known yet".

The process of conjugation is energy-consuming and, because of the required sex pili, it makes the bacterium susceptible to phage attack. Therefore, conjugation is a tightly controlled process with sophisticated regulation exerted on several levels. One of the key regulatory molecules of conjugation is the TraJ protein, the main positive transcriptional regulator of *tra* operon. It has been well documented that the amount of TraJ protein is negatively regulated by the fertility inhibition FinOP system (2, 10, 14, 16). The work presented in this thesis (Chapters 4 and 5) indicates that there also exist regulatory mechanisms that positively control the level of TraJ or at least the level of *traJ* transcript. The main positive activator of *traJ* gene expression was the cAMP-CRP complex, but also the regulatory proteins Lrp and H-NS positively influenced the level of *traJ* transcript. In these studies we applied a *traJ-lacZ* reporter system to monitor changes in *traJ* promoter activity. This system allowed to draw conclusions about the level of *traJ* transcript without the strong negative influence of the FinOP system, which regulates the translation of the transcript into TraJ protein. Additional advantages of the use of the transcriptional fusion were that promoter activity could be directly measured and that there was no need to construct a plasmid with a FinO mutation to derepress TraJ protein synthesis. The *in vivo* relevance of the data obtained with the reporter system was demonstrated by the results of the mating assays with bacteria that carried the intact plasmid and that showed that *cya* and *crp* mutants exhibited reduced levels of conjugation that could be partially restored by the addition of exogenous cAMP.

A hypothetical model for TraJ as target of regulation

Based on our findings, the following regulatory cascade in the conjugation process in pRK100 can be envisioned. Under conditions of glucose starvation, the intracellular levels of cAMP rise which results in high levels of the cAMP-CRP complex. This complex binds the DNA at position −67.5 from the assumed transcriptional startpoint of the *traJ* promoter. As this position is situated on the opposite site of the DNA helix than the RNA polymerase binding site, activation likely occurs only with the help of additional DNA binding proteins. Our results (described in Chapter 5) suggest that the global regulators Lrp and H-NS may perform this function and change the helical DNA conformation to enable the contact between CRP and the RNA polymerase. This will cause an increase in the level of TraJ protein, which in turn will activate the major *tra* promoter P_{traY}, and thus stimulate the synthesis of the conjugation machinery.

Although this scenario on the role of TraJ as a positive regulator of conjugation is plausible, several questions remain to be answered. One of them is if and how the various positive effectors interact to establish the effect on *traJ* transcription. Experiments with *crp lrp* and *crp hns* double knock-outs and DNA footprinting experiments with RNA polymerase, Lrp and H-NS proteins may provide further information on this point.

A second point that remains to be addressed is the seemingly mysterious influence of the Cpx proteins on the amount of TraJ protein. We (Chapter 5, data not shown) and others (11, 24) demonstrated that the two component system CpxA-CpxR (8, 28) that senses and responds to the cell envelope stress in *E. coli* (20), influences *traJ* expression. RT-PCR experiments however (data not shown), showed no changes in the amount of *traJ* mRNA between the wild type strain and a *cpxA* mutant, suggesting that the effect was not exerted at the transcriptional level. Gubbins, *et al.* (11) using the F plasmid obtained similar results with Northern blots and they were able to demonstrate that the Cpx dependent reduction in the amount of TraJ was due to a posttranscriptional event. However, they were not able to find the cause of this reduction. It is tempting to speculate that some of the genes with unknown function that have been located in the *tra* region of F-like plasmids, including pRK100, might play a role and thus that research on these putative genes would lead to the discovery of the missing effector.

Potential role of pRK100 in bacterial survival

Besides genes necessary for plasmid replication, stability and for conjugal transfer, pRK100 carries several sets of genes that likely contribute to bacterial survival. Our results indicate that two sets of genes of pRK100 likely code for two different iron uptake systems, the aerobactin and enterochelin uptake system. As bacteria require iron for growth in fairly large amounts and pathogenic microorganisms must be able to compete successfully for the limited amounts of free iron that are available in the host (25, 29), the pRK100 encoded iron uptake systems may have an important function and contribute to bacterial virulence.

Another group of genes that may impact on bacterial survival are the ampicillin and tetracycline resistance genes. Bacteria have developed different mechanisms that confer resistances to antibiotics. They are able to either prevent the intake of the antibiotic or actively transport it out, they have developed mechanisms to enzymatically inactivate the antibiotic, or modificate the target (5). These traits are often acquired by mutation or by horizontal gene transfer via conjugative plasmids, bacteriophages or conjugal transposons (25). The presence of resistance genes on pRK100 enlarges the survival potential of the host bacterium and, after spreading, even of the entire bacterial population.

A third group of factors encoded by pRK100 that contributes to bacterial survival are the colicins. These extracellular bacterial toxic proteins are often produced by different types of *Eubacteria* and also by *Archaebacteria* (21). The mechanism of action of these compounds involves their adsorption to specific receptors located on the external surface of sensitive bacteria, followed by killing via one of

three primary mechanisms: the formation of channels in the cytoplasmic membrane, the degradation of cellular DNA or the inhibition of protein synthesis (21). Bacteriocins usually kill only strains of the same or closely related species (7). A relatively high frequency of colicin encoding plasmids is found in isolates of pathogenic *E. coli* (27). Since pRK100 harbours two colicins, ColV and ColIa, it can be expected to provide the host bacterial cell a selective advantage in the intense competition between bacteria and thus may add to the pathogenic potential of the bacterial cell.

Many bacterial pathogens carry on their chromosome distinct regions that are rich in virulence-associated genes. These so-called pathogenicity islands (PAI) (3, 12) are often flanked by repeat structures and carry many fragments of other mobile and accessory genetic elements, such as bacteriophages, plasmids and insertion sequence elements. Therefore, PAIs are often considered to have evolved from mobile genetic elements by horizontal gene transfer. Since PAIs are frequently associated with *att* sites for temperate bacteriophages, bacteriophages are assumed to be the primary vehicles in the horizontal transfer of PAIs. Others have suggested that certain PAIs originate from plasmids that became integrated into chromosomal sites and subsequently lost their replication function (3, 13). Consistent with this hypothesis, some PAIs still carry mobilisation genes in their flanking regions and an *oriT* like sequence, which enables them to become mobilised and transferred to other cells (9). A third possibility is that PAIs were originally chromosomal elements that have been acquired by plasmids. This event may have involved the integration of the plasmid into the host chromosome at the site of the insertion sequences that flank the PAI, followed by incorrect excision of the plasmid from the chromosome and incorporation of (part of) the PAI into the plasmid. The enterochelin sequence harboured by pRK100, which has a high similarity with the enterochelin sequence of a PAI, is probably such an example.

Overall, the genetic organisation, plasticity, and plasmid-encoded functions strongly suggest that the presence of part of PAIs on conjugative plasmids may contribute to bacterial virulence and the spread of virulence genes to other bacteria.

Future perspectives

Since it will be extremely difficult to develop strategies to stop the process of horizontal gene transfer, we will have to accept that plasmids will continue to sequester and transfer foreign DNA and that one day, virulence and resistance genes will not be associated anymore with distinct bacterial species, but might be widely distributed throughout different bacterial populations. At that time, it would be wise for us, human beings, to have developed methods to rapidly detect and neutralise virulence factors and to buy us that way sufficient time to enable our immune system to do its job. However, to achieve this goal we need to know the complete repertoire of virulence factors and to develop rapid methods to identify and neutralise them. The sequencing of entire plasmids and whole bacterial genomes as well as functional analysis of the identified genes will be of great importance in this respect. Rapid screening for virulence factors may be achieved by novel detection methods such as DNA microarrays, but the tools to neutralise the action of virulence factors are still in their infancy. The discovery and application of this type of methods will be one of our major future challenges.

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SUMMARY

Plasmids are extrachromosomal DNA elements that can be found in prokaryotic as well as in eukaryotic cells. They can vary in size and genetic make-up. Some plasmids are very small (around 2 kb or even smaller), others are up to 300 kb or even larger. Even though the genes located on plasmids are highly diverse, they can be arranged into functional groups. One functional group consists of genes that are essential for plasmid existence, such as genes needed for plasmid replication, stability, and maintenance. Genes encoding elements that are not directly needed for plasmid existence, but may be beneficial to the plasmid's host cell, such as resistances to antibiotics, enzymes that enable alternative metabolic pathways or virulence factors, form another group. Plasmids benefit from these elements indirectly as they may facilitate bacterial adaptation to and survival in different environmental niches. In the third functional group of plasmid-encoded genes are those that enable plasmid transfer to other host cells. Some plasmids, the conjugative plasmids, carry all the genetic information that is needed for conjugal transfer, such as pilus synthesis, mating-pair formation and DNA transfer. Other plasmids, the mobilisable plasmids, carry only information for the transfer of DNA.

Plasmids awaked the interest of scientists already more than 50 years ago. This research led to many great discoveries. One of the most appreciated applications of plasmids is their use as vectors in molecular cloning, but also the use of plasmids as models for studies on the mechanisms of DNA replication, evolution, and gene transfer have been instrumental. Lately, due to highly efficient nucleotide sequencing strategies, determination of the entire nucleotide sequence of plasmids has further boosted our understanding of the role of plasmids as important vehicles in the transfer of DNA and distinct genetic traits among organisms.

The plasmid pRK100, which is the study subject of this thesis, is a large (145 kb) natural conjugative plasmid, which was isolated from a uropathogenic *E. coli* strain. Large natural conjugative plasmids are increasingly considered to play a major role in the rapid spread of virulence genes and other genetic traits among organisms. The work on the plasmid pRK100, described in this thesis, was designed to increase our understanding of the evolution of large natural plasmids and to unravel important aspects of the genetic regulation of the conjugal transfer event with emphasis on the regulation of the main positive activator of the conjugation, the TraJ protein.

Genetic characterisation of pRK100 using DNA hybridisation with probes, derived from a variety of known regions in other plasmids, revealed that pRK100 likely carries two replication regions, an aerobactin iron uptake system and the insertion sequence IS*1*. In addition, biological assays demonstrated that pRK100 also encodes two colicins, ColV and ColIa, and ampicillin and tetracycline resistance genes (Chapter 2). It was also established that pRK100 is a very stable plasmid and that it can be transferred by conjugation to other *E. coli* and *Klebsiella pneumoniae*. On the basis of the data obtained, a rough map of the genetic organisation of pRK100 was constructed. Since the hybridisation experiments showed that the plasmid had many similarities with the ColV plasmids, it was assumed that it belongs to the ColV group of plasmids. However, the hybridisation results for the *tra* region indicated that the pRK100 *tra* region is very similar to the F plasmid *tra* region, and hence may have been acquired from a F-like plasmid.

To further investigate the origin and evolution of pRK100 and to determine the pRK100's backbone, targeted nucleotide sequencing of hybridising as well as seemingly unique regions of pRK100 was performed (Chapter 3). Comparative sequence analysis confirmed that the *tra* region of pRK100 is the most similar to the F-plasmid *tra* region, while the aerobactin and ColV regions are the most similar to pColV-K30. Furthermore, a gene most similar to the plasmid R100 *rmoA* gene was identified as well as an additional iron uptake system, the enterochelin. The obtained pRK100 enterochelin sequences were the most similar with the enterochelin encoded by a chromosomal pathogenicity

island, the PAI III_{536}, of the uropathogenic *E. coli* strain 536. Together, these data led us to suggest that pRK100 has a truly mosaic structure with genetic elements that have apparently been acquired from several other plasmids as well as from the chromosome.

To further assess the origin of pRK100, we studied the replication regions of pRK100 in more detail (Chapter 3). Construction of minireplicons from plasmid fragments of pRK100 confirmed that the plasmid carried two replication regions and revealed that both were functional in an *E. coli* background. When the complete nucleotide sequences of both replication regions were obtained and compared with sequences of other known replication regions, it was demonstrated that one of them was the most similar to the F plasmid RepFIB replication region and that the other resembled the RepFIIA replication region in R1 plasmid, further illustrating the mosaic structure of pRK100.

The conjugal transfer of F and F-like plasmids has been studied already for more than 50 years and it is considered to be the paradigm and best studied system of plasmid conjugal transfer. During the research of F plasmid conjugal transfer it was established that the main positive regulator of conjugation encoded by plasmid is the TraJ protein. Further it was discovered that TraJ is regulated by the fertility inhibition system, which consists of two elements: the protein FinO and the antisense RNA molecule FinP. The combined action of these two elements precludes the translation of the *traJ* mRNA and thus prevents activation of the conjugation machinery and DNA transfer.

In our work, we postulated that apart from the regulation at the translational level by the FinOP system, the amount of TraJ in the cell may vary dependent of the activity of the *traJ* promoter. As positive regulation of *traJ* promoter activity had not been described, we initially focussed on the activity of the promoter under various environmental conditions using a *traJ-LacZ* reporter system. These experiments (described in Chapter 4) indicated that the level of glucose in the medium affects the expression of the *traJ-lacZ* gene fusion and further β-galactosidase assays with *cya* and *crp* mutant strains demonstrated that the formation of the cAMP-CRP complex is needed for a full transcription from the *traJ* promoter. This finding was confirmed in competitive RT-PCR assays with *cya* and *crp* mutants. DNA-protein binding assays (EMSA) showed that the CRP protein bound to the *traJ* promoter region. Additional DNAseI protection assays (DNA footprinting) showed that the CRP binding site was centred around −67.5 from the putative transcriptional *traJ* start site. Targeted mutagenesis of the identified CRP binding site influenced the activity of the *traJ* promoter further demonstrating the importance of the regulatory effect of cAMP-CRP on the transcription of *traJ*. To corroborate the relevance of these *in vitro* data, mating assays were performed with *crp* and *cya* mutants and the parent strain. These experiments demonstrated a clear decrease in the mating efficiency in the mutants compared to the wild type strain, strongly suggesting that the identified global regulator CRP indeed acts as an activator of conjugation.

The location of the CRP binding site centred around position −67.5, which is predicted to be at the opposite site of the DNA helix, compared to the binding site of the RNA polymerase at the −10 and −35 promoter sequences, led us to postulate that other DNA regulatory proteins have to be involved in the regulation of *traJ* transcription in addition to the cAMP-CRP complex. This hypothesis was further investigated by assessing the *traJ* promoter activity in a number of strains with defects in distinct known DNA regulatory proteins. Results obtained with *lrp*, *hns*, *cpx*, *himA*, *rpoS*, *relA spoT* mutants in the *traJ-lacZ* reporter assays and the competitive RT-PCR (presented in Chapter 5) indicated that both, H-NS and Lrp protein, were positive modulators of *traJ* transcription. EMSA showed that H-NS and Lrp protein bind to the *traJ* promoter, but also that this binding is less specific than the binding of the CRP protein. Mating tests with *hns* and *lrp* mutants demonstrated that H-NS and LRP were important for conjugation *in vivo*. Comparative analysis of the CRP, H-NS and Lrp binding sites on pRK100 with plasmid sequences in the database indicated similar sequences in the *traJ* promoter regions of other F-like plasmids. These data may indicate that the identified global regulators CRP,

Lrp and H-NS likely act as regulators of conjugal transfer not only in pRK100, but also in other F-like plasmids.

In conclusion, the work described in this thesis has revealed that during its evolution the large natural plasmid pRK100 sequestered elements from many different sources and evolved into a large plasmid with a mosaic structure consisting of the F plasmid backbone interrupted with genetic parts from other plasmids and even chromosomes. The highly mosaic structure of pRK100 makes the plasmid an example "par excellence" of the dynamic structure of plasmids and supports the assumed role of these plasmids in the spread of genetic information among bacterial populations. The identification of factors that regulate the conjugal transfer through modulation of the main regulator TraJ may be of importance in the development of novel strategies aimed at limitation of the spread of virulence factors encoded by these plasmids.

SAMENVATTING

Plasmiden zijn extrachromosomale DNA elementen met een grootte die kan variëren van 2 kb tot meer dan 300 kb en die zowel in prokaryote als eukaryote cellen kunnen voorkomen. Ofschoon de genen die op plasmiden kunnen voorkomen zeer divers van aard zijn, kunnen ze op basis van hun functionele eigenschappen in verschillende groepen worden ingedeeld. Eén functionele groep bestaat uit genen die essentieel zijn voor het voortbestaan van het plasmide zoals genen die nodig zijn voor plasmide replicatie en stabiliteit. Een tweede groep vormen de genen die op zich niet essentieel zijn voor het zich kunnen handhaven van het plasmide maar die indirect bijdragen aan hun voortbestaan door een gunstig effect te hebben op de overlevingsmogelijkheden van de gastheercel, bijvoorbeeld door hun gevoeligheid voor antibiotica en andere toxische stoffen te verminderen. Een derde groep bestaat uit genen die bijdragen aan de overdracht van het plasmide naar andere gastheercellen. Sommige plasmiden, de conjugatieve plasmiden, bezitten alle genetische informatie die nodig is voor de overdracht van erfelijk materiaal via conjugatie. Deze informatie is gelegen in de zgn. *tra* regio.

Onderzoek naar de structuur en het functioneren van plasmiden heeft geleid tot vele belangrijke ontdekkingen en toepassingen. Een van de meest bekende toepassingen van plasmiden is hun gebruik in de moleculaire biologie als cloneringsvector, maar ook bij het ontrafelen van de mechanismen van DNA replicatie, de overdracht van DNA en de verspeiding van genetische eigenschappen spelen plasmiden een belangrijke rol. De evolutionaire betekenis van deze processen wordt in toenemende mate in volle omvang duidelijk met het bekend worden van de complete DNA volgorde van plasmiden en van die van het volledige genoom van een reeks van micro-organismen.

Het plasmide pRK100, het onderwerp van studie in dit proefschrift, is een groot (145 kb) natuurlijk voorkomend conjugatief plasmide dat voor het eerst werd geisoleerd uit een uropathogene *E. coli* stam. Grote, natuurlijke conjugatieve plasmiden worden in toenemende mate herkend als belangrijke elementen in de snelle verspreiding onder bacteriële populaties van specifieke kenmerken, met name van virulentiegenen en resistentie-inducerende genen. Het in dit proefschrift beschreven onderzoek had als doel om aan de hand van pRK100 de evolutie van grote natuurlijke plasmiden beter te kunen begrijpen en om belangrijke stappen in de regulatie van het proces van conjugatie, essentieel voor de verspreiding van het plasmide, te leren kennen. Hierbij werd in het bijzonder aandacht besteed aan de regulatie van de expressie van het TraJ eiwit dat een centrale rol vervuld in het initiëren van het conjugatieproces.

Karakterisering van pRK100 door middel van DNA hybridisatie met probes gebaseerd op DNA sequenties die voorkomen in andere plasmiden toonde aan dat pRK100 waarschijnlijk twee DNA replicatie regio's bezit en daarnaast genen bevat die coderen voor een aerobactine ijzeropname systeem. Bovendien, bleek uit biologische assays dat op pRK100 waarschijnlijk ook genen aanwezig zijn die coderen voor twee two colicines, ColV and ColIa, en voor ampicilline en tetracycline resistentie (Hoofdstuk 2). Ook kon worden vastgesteld dat pRK100 een zeer stabiel plasmide is dat door middel van conjugatie overgedragen kan worden op andere *E. coli* stammen en op *Klebsiella pneumoniae*. Op basis van de verkregen gegevens was het mogelijk een plasmidekaart te construeren van de genetisch opbouw van pRK100 (Hoofdstuk 2). Op grond van de grote overeenkomsten in hybridisatieresultaten met pRK100 en het ColV plasmide, werd pRK100 vooralsnog als een plasmide behorende de ColV groep van plasmiden aangemerkt. Een belangrijke verschil met deze groep van plasmiden leek echter de *tra* regio te zijn. In pRK100 bleek deze groep van genen het meest verwant met soortgelijke genen gelegen op een ander natuurlijk conjugatief plasmide, het F plasmide.

Teneinde de oorsprong en evolutie van pRK100 nader te kunnen vaststellen werd, aan de hand van de hybridisatieresultaten, van delen van het plasmide de DNA sequentie bepaald (Hoofdstuk 3). Vergelijkende sequentieanalyse bevestigde dat de *tra* regio van pRK100 inderdaad de meest

overeenkomsten vertoont met de *tra* regio van het F plasmide, terwijl de aerobactine en ColV regio's de meeste verwantschap vertoonden met pColV-K30. Bij nader onderzoek van delen van pRK100 die bij hybridisatie schijnbaar uniek voor dit plasmide leken, werd een gen ontdekt dat het meest verwant was met het *rmoA* gen van plasmide R100 en ook een tweede ijzeropname systeem. Dit (enterocholine) systeem vertoonde qua DNA volgorde de meeste overeenkomst met genen gelegen op het pathogeniteitseiland, PAI III_{536}, dat aanwezig is op het chromosoom van de uropathogene *E. coli* stam 536. Het geheel van deze bevindingen wees erop dat pRK100 een echte mozaiekstructuur heeft met genetische elementen die afkomstig zijn van diverse andere plasmiden, maar ook van het chromosoom.

De oorsprong van pRK100 werd tevens onderzocht door een gedetailleerde bestudering van de replicatie genen van pRK100 (Hoofdstuk 3). Constructie van minireplicons bestaande uit fragmenten van pRK100 bevestigde dat het plasmide twee replicatie regio's bezit en dat beide regio's functioneel zijn in *E. coli*. Nadat de volledige DNA sequentie van beide replicatie regio's was verkregen en werd vergeleken met die van andere plasmiden bleek dat, anders dan de hybridisatie resultaten deden vermoeden, één van de replicatie regio's sterk verwant aan de RepFIB regio van plasmid F en de andere aan de RepFIIA regio van plasmid R1. De aanwezigheid van twee replicatie regio's schijnbaar afkomstig van twee verschillende plasmiden bevestigde het mozaiekkarakter van pRK100.

De overdracht via conjugatie van plasmid F and F-gelijkende plasmiden naar andere micro-organismen is al gedurende meer dan 50 jaar onderwerp van studie. In deze periode werd ontdekt dat het TraJ eiwit de belangrijkste positieve regulator is bij het op gang komen van het conjugatieproces. Het TraJ eiwit wordt gereguleerd door het zgn. fertiliteits inhibitie systeem, dat uit twee elementen bestaat: het eiwit FinO en het antisense RNA molecule FinP. Interactie van deze twee elementen voorkomt de translatie van *traJ* mRNA en aldus de vorming van het conjugatieapparaat en de overdracht van plasmide.

Gezien de centrale positie van het TraJ eiwit in de regulatie van conjugatie werd in het onderzoek beschreven in dit proefschrift de mogelijkheid onderzocht of er naast de reeds bekende regulatie op translatie niveau door het FinOP systeem wellicht een tweede vorm van regulatie van conjugatie zou kunnen bestaan, en wel door het reguleren van de activiteit van de *traJ* promoter. Aangezien regulatie van de *traJ* promoter nog nooit eerder was beschreven, werd eerst de activiteit van de promoter onder verschillende omgevingsomstandigheiden bepaald met behulp van een *traJ-LacZ* reporter systeem. Deze experimenten (beschreven in Hoofdstuk 4) wezen erop dat de hoeveelheid glucose in het groeimedium van invloed is op de mate van expressie van de *traJ-lacZ* genfusie. Daaropvolgende β-galactosidase assays met *cya* and *crp* mutanten toonden aan dat de vorming van het cAMP-CRP complex noodzakelijk is voor een sterke transcriptie vanaf de *traJ* promoter. Deze bevinding werd bevestigd met een competitive RT-PCR. Bovendien kon met mobility shift assays (EMSA) worden aangetoond dat het eiwit CRP, onderdeel van het cAMP-CRP complex, aan de *traJ* promoter regio bindt. DNAseI protectie assays (DNA footprinting) toonde aan dat CRP bond aan het DNA rondom positie −67.5 van de plaats waar waarschijnlijk de transcriptie van *traJ* begint. Gerichte mutagenese van de gevonden CRP bindingsplaats bevestigde dat deze sequentie van invloed is op de activiteit van de *traJ* promoter. De waarde van deze *in vitro* bevindingen werd verder onderzocht in conjugatie-experimenten. In deze experimenten bleken stammen met een defect in het *crp* and *cya* gen, en dus in de vorming van het CRP-cAMP complex, een duidelijk verminderd conjugatievermogen te hebben vergeleken met de wild-type stam (Hoofdstuk 4).

De aanwezigheid van de CRP bindingsplaats rondom positie −67.5 was opvallend aangezien deze positie wordt voorspeld zich aan de andere kant van de DNA helix te bevinden dan de −10 en −35 promoter sequenties. Dit bracht ons er toe te postuleren dat er naast het cAMP-CRP complex wellicht andere DNA regulator eiwitten betrokken waren bij de regulatie van de *traJ* transcriptie. Deze hypo-

these werd nader onderzocht door het meten van de *traJ* promoter activiteit in een reeks stammen met mutaties in bekende DNA regulerende eiwitten. De resultaten verkregen met *lrp*, *hns*, *cpx*, *himA*, *rpoS*, *relA spoT* mutanten in *traJ-lacZ* reporter assays en in de competitive RT-PCR (beschreven in Hoofdstuk 5) wezen erop dat zowel H-NS and Lrp als positieve modulatoren van de *traJ* transcriptie kunnen fungeren. EMSA toonde aan dat H-NS and Lrp binden aan de *traJ* promoter regio, maar ook dat deze binding veel minder specifiek is dan de binding van CRP. Conjugatie experimenten met *hns* and *lrp* mutanten bevestigden dat H-NS and Lrp van belang zijn voor conjugatie *in vivo*. Een vergelijking van de CRP, H-NS and Lrp bindingsplaatsen in de *traJ* promoter regio van pRK100 met plasmid sequenties in de database gaf aan dat vergelijkbare bindingsmotieven aanwezig waren in de *traJ* promoter regio's van ander F-gelijkende plasmids. Dit zou erop kunnen wijzen dat CRP, H-NS en Lrp niet alleen in pRK100, maar ook in andere F-like plasmiden een regulerend effect op conjugatie hebben.

Concluderend kan worden gezegd dat het onderzoek beschreven in dit proefschrift heeft aangetoond dat het grote natuurlijke plasmide pRK100 tijdens zijn evolutie DNA elementen van diverse bronnen heeft verworven wat heeft geleid tot een groot plasmide met een sterke mozaiekstructuur. Deze mozaiekstructuur maakt pRK100 een voorbeeld bij uitstek van de dynamische structuur van plasmiden en ondersteunt de veronderstelde rol van deze plasmiden in de verspreiding van genetische informatie onder bacterie populaties. De ontdekking van een nieuw mechanisme van regulatie van de belangrijke initiator van conjugatie, TraJ, geeft inzicht in de mechanismen waarlangs omgevingsfactoren de overdracht van erfelijke materiaal tussen bacteriën kunnen beinvloeden. Deze kennis zou kunnen bijdragen aan het ontwikkelen van methoden gericht op beperking van de verspreiding van de virulentie- en antibiotica-resistentiegenen die op deze plasmiden liggen.

POVZETEK

Plazmidi so izvenkromosomske molekule DNA, ki jih lahko najdemo tako v prokartiontskih kot evkariontskih celicah. Plazmidi se med seboj razlikujejo po velikosti, kot tudi po genetski sestavljenosti. Nekateri plazmidi so zelo majhni (okoli 2 kb ali še manj), drugi plazmidi so lahko veliki 300 kb ali celo večji. Čeprav so geni, zapisani na plazmidih, zelo raznoliki, jih lahko razporedimo v nekaj skupin. Eno skupino sestavljajo geni, ki so bistveni za obstoj plazmida. To so geni potrebni za podvojevanje, stabilnost in vzdrževanje plazmida v celici. Geni z zapisi, ki niso neposredno povezani z obstojem plazmida v celici, ampak nudijo gostiteljevi celici določeno prednost, tvorijo drugo skupino. Primeri takšnih genov so geni za odpornost proti antibiotikom, geni z zapisi encimov alternativnih metabolnih poti, geni za virulenčne dejavnike. Tretjo skupino genov predstavljajo geni, katerih produkti sodelujejo pri razširjanju plazmidov med bakterijskimi celicami. Nekateri plazmidi, t. i. konjugativni plazmidi, so sposobni prenosa v drugo bakterijsko celico zaradi lastnih zapisov, za sintezo pilov, vzpostavitev konjugacijskega para in prenos DNA, skratka imajo zapisano vso informacijo potrebno za konjugacijo, medtem ko imajo nekateri drugi plazmidi samo informacijo za mobilizacijo prenosa svoje DNA s pomočjo drugega konjugativnega plazmida.

Že pred več kot 50 leti so plazmidi pritegnili pozornost znanstvenikov. Raziskave na plazmidih so privedle do mnogo velikih odkritij in uporabe plazmidov kot vektorjev v molekularnem kloniranju. Plazmide se uporablja kot modele v raziskavah mehanizmov podvojevanja DNA, evolucije, prenosa genske informacije. V zadnjem času se je, zaradi možnosti učinkovitega sekveniranja in s tem povezanega določanja celotnih nukleotidnih zaporedij tudi velikih plazmidov, zanimanje za plazmide še povečalo, saj se plazmidi razkrivajo kot pomembni prenašalci genske informacije med organizmi.

Plazmid pRK100, ki je opisan v tej doktorski disertaciji, je velik (145 kb) naravni konjugativni plazmid, ki smo ga izolirali iz uropatogenega seva *E. coli*. Veliki naravni plazmidi so pomembni razširjevalci genov za virulenčne dejavnike in tudi druge genske informacije med bakterijami. Namen raziskav plazmida pRK100, ki so predstavljene v tej doktorski disertaciji, je doprinesti k razumevanju nastanka velikih naravnih plazmidov in odkriti pomembne vidike genskega uravnavanja prenosa DNA s konjugacijo, predvsem uravnavanja izražanja gena glavnega pozitivnega regulatorja konjugacije, proteina TraJ.

Genetska označitev plazmida pRK100 s hibridizacijami s sondami, ki so izvirale iz različnih znanih plazmidov, so razkrile, da ima pRK100 verjetno dve replikacijski regiji, aerobaktinski sistem za privzem železa in insercijsko zaporedje IS*1*. Biološki poskusi so pokazali, da ima pRK100 zapisa za dva kolicina, ColV in ColIa, in zapisa za odpornost proti antibiotikoma ampicilin in tetraciklin (Poglavje 2). Ugotovili smo tudi, da je pRK100 zelo obstojen plazmid in da se lahko s konjugacijo prenese v druge celice bakterij *E. coli* in *Klebsiella pneumoniae*. Na podlagi dobljenih podatkov smo pripravili prvo mapo, ki je prikazovala razporeditev genskih zapisov plazmida pRK100. Že hibridizacijski poskusi so nakazovali, da je plazmid pRK100 sestavljen plazmid, saj smo na njem našli tako zapise zelo podobne ali celo enake zapisom plazmidov iz skupine ColV kot tudi zapise zelo podobne ali celo enake zapisom plazmidov podobnih plazmidu F.

Da bi osvetlili izvor in evolucijo plazmida pRK100, smo določili nukleotidna zaporedja določenih predelov pRK100, ki so s hibridizacijami nakazovala, da so bodisi edinstvena ali pa podobna drugim plazmidom (Poglavje 3). S primerjavo dobljenih nukleotidnih zaporedij, smo potrdili, da je regija *tra* plazmida pRK100 najbolj podobna regiji *tra* plazmida F, medtem ko sta aerobaktinski sistem privzema železa in ColV najbolj podobna plazmidu pColV-K30. Dalje, smo na plazmidu pRK100 našli gen *rmoA*, ki je najbolj podoben genu *rmoA* plazmida R100, kot tudi še enterohelinski sistem za privzem železa, katerega nukleotidno zaporedje je bilo najbolj podobno enterohelinu zapisanem na kromosomskem otoku patogenosti, PAI III_{536}, uropatogenega seva 536 bakterije *E. coli*. Vsi dobljeni podatki

so pokazali, da je pRK100 resnično mozaično sestavljen plazmid iz elementov, dobljenih iz različnih drugih plazmidov in tudi kromosoma.

V poglavju 3 so prikazani tudi rezultati dobljeni z raziskavami replikacijskih regij. S pripravo minireplikonov iz fragmentov plazmida pRK100 smo potrdili, da ima pRK100 dve delujoči replikacijski regiji. S primerjavo nukleotidnega zaporedja obeh replikacijskih regij z nukleotidnimi zaporedji, deponirami v genski banki, smo ugotovili, da je ena replikacijska regija pRK100 najbolj podobna replikacijski regiji RepFIB plazmida F, druga replikacijska regija pRK100 pa je najbolj podobna replikacijski regiji RepFIIA plazmida R1. Ti rezultati so še enkrat pokazali mozaičnost plazmida pRK100.

Konjugacija plazmida F in plazmidu F podobnih plazmidov se proučuje že več kot 50 let in je najbolje preučen konjugacijski prenos, ki velja za paradigmo. Odkrili so, da je glavni pozitivni regulator konjugacije, ki je zapisan na konjugativnem plazmidu, protein TraJ. Nadalje so tudi odkrili, da je TraJ uravnan s sistemom FinOP, ki ga sestavljata dva elementa: protein FinO in protiprepisna molekula RNA, FinP. Sistem FinOP deluje tako, da prepreči prepisovanje mRNA *traJ* v protein TraJ in posledično ne pride do aktivacije konjugacijskega prenosa.

Ker smo predvidevali, da mora biti TraJ uravnan tudi še kako drugače in ne samo s sistemom FinOP, smo pripravili transkripcijsko gensko fuzijo *traJ-lacZ* in ugotavljali aktivnost te fuzije v različnih pogojih (Poglavje 4). Ti poskusi so pokazali, da raven glukoze v gojišču vpliva na izražanje genske fuzije *traJ-lacZ* in nadaljnji β-galaktozidazni poskusi z mutantami v genu *cya* oziroma *crp* so razkrili, da kompleks cAMP-CRP na prepisovanje promotorja *traJ* pozitivno vpliva. Pozitivni vpliv kompleksa cAMP-CRP smo potrdili tudi s kompetitivnim RT-PCR. Vezavni poskusi DNA-protein so pokazali, da se protein CRP veže na promotor *traJ*. Poskusi z DNazoI so razkrili, da je vezavno mesto za CRP na promotorju *traJ* razporejeno okoli mesta –67.5, glede na predviden začetek prepisovanja mRNA za *traJ*. Da je najdeno vezavno mesto za CRP dejansko pomembno za izražanje gena *traJ*, smo preverili z β-galaktozidaznimi testi, s katerimi smo merili aktivnost promotorja *traJ* z mestno specifično mutiranim vezavnim mestom za CRP. Vsi ti poskusi *in vitro* so torej nakazovali vpliv kompleksa cAMP-CRP na izražanje gena *traJ*. Da bi preverili ali kompleks cAMP-CRP tudi resnično vpliva na konjugacijo, smo izvedli poskuse tudi *in vivo*. Ugotavljali smo frekvence konjugacijskega prenosa plazmida pRK100 iz seva divjega tipa in iz mutant *crp* oziroma *cya*. Glede na dobljene rezultate, frekvence konjugacijskega prenosa pRK100 iz mutant so namreč nižje od frekvenc prenosa pRK100 iz seva divjega tipa, lahko sklepamo, da kompleks cAMP-CRP vpliva na konjugacijo.

Ker ima CRP nenavadno pozicijo vezavnega mesta, –67.5, na promotorju *traJ*; in se zato verjetno CRP veže na nasprotno stran DNA kot DNA-polimeraza, ki se veže na promotorska zaporedja –10 in –35, smo predvidevali, da pri uravnavanju izražanja *traJ* sodelujejo tudi še drugi regulatorni proteini. To hipotezo smo preverili (Poglavje 5) z β-galaktozidaznimi poskusi, s katerimi smo merili aktivnost genske fuzije *traJ-lacZ* v sevih z mutacijami v genih za različne globalne regulatorje. Rezultati dobljeni s sevi z mutacijami v *lrp*, *hns*, *cpx*, *himA*, *rpoS*, *relA spoT* so pokazali, da Lrp in H-NS pozitivno vplivata na izražanje *traJ*, kar so potrdili tudi rezultati dobljeni s kompetitivnim RT-PCR. Promotor *traJ* ima nukleotidna zaporedja, ki se skladajo s konsenzusnimi vezavnimi zaporedji Lrp in H-NS, in vezavni poskusi DNA-protein so pokazali, da se oba proteina, Lrp in H-NS, vežeta na promotor *traJ*, a da je njuna vezava manj specifična kot vezava CRP. Poskusi konjugacijskega prenosa plazmida pRK100 iz sevov z mutacijami *lrp* in *hns* so potrdili, da sta Lrp in H-NS tudi pomembna za konjugacijo in ne samo za izražanje *traJ*. Primerjava nukleotidnega zaporedja z vezavnimi mesti za CRP, Lrp in H-NS promotorja *traJ* plazmida pRK100 z nukleotidnimi zaporedji promotorjev *traJ* drugih plazmidu F podobnih plazmidov, je pokazala, da imajo tudi ti plazmidi v promotorju *traJ* vezavna mesta za CRP, Lrp in H-NS, kar nakazuje, da so ti trije proteini pomembni za konjugacijo tudi pri drugih plazmidu F podobnih plazmidih.

Delo predstavljeno v tej doktorski disertaciji je pokazalo, da je plazmid pRK100 v svoji evoluciji pridobil elemente iz različnih virov in se tako razvil v velik, mozaično sestavljen plazmid, ki je v svoji osnovi podoben plazmidu F, a ima zapise privzete tudi od drugih plazmidov in celo kromosoma. Zaradi izrazite mozaičnosti je plazmid pRK100 zelo lep primer dinamične zgradbe plazmidov in podpira vlogo plazmidov pri razširjanju genske informacije v in med bakterijskimi populacijami. Razkritje dejavnikov, ki vplivajo na konjugacijski prenos preko uravnavanja izražanja *traJ*, je lahko pomembno za razvoj novih strategij, ki so usmerjene v omejevanje razširjanja virulenčnih dejavnikov.

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Curriculum Vitae

Publications

CURRICULUM VITAE

Marjanca Starčič Erjavec was born on September 25th, 1971 in Maribor, Slovenia.

In 1990 she started to study Biology (orientation Molecular Biology) at the Department of Biology, Biotechnical Faculty in Ljubljana. She graduated in July 1995 on the subject: "*Escherichia coli* strain KS533 with plasmid pRK100. Possibilities of integration into its chromosome and the production of four different colicins". The mentor was Dr. Darja Žgur-Bertok. This work was awarded the Prešern's Faculty award. The average note of exams was 9.6 out of 10 and therefore she was awarded with Jesenko's student award.

In October 1995 she began studying the graduate studies of Molecular biology and Biochemistry at the Medical Faculty in Ljubljana and graduated in October 1997 with an average note 9.8 out of 10. The subject of the Master of Science thesis was: "Virulence determinants of *Escherichia coli* strains isolated form diarrheic dogs". The mentors were Dr. Darja Žgur-Bertok from University of Ljubljana and Dr. Wim Gaastra from the Faculty of Veterinary Medicine in Utrecht.

After obtaining her M.Sc. Marjanca Starčič Erjavec started the scientific work described in this thesis. The research was performed at the Department of Biology, University of Ljubljana, Slovenia as well as at the Division of Bacteriology of the Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands.

Since November 1995 she is fully employed at the Department of Biology of University in Ljubljana as an assistant for Molecular Biology.

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