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).

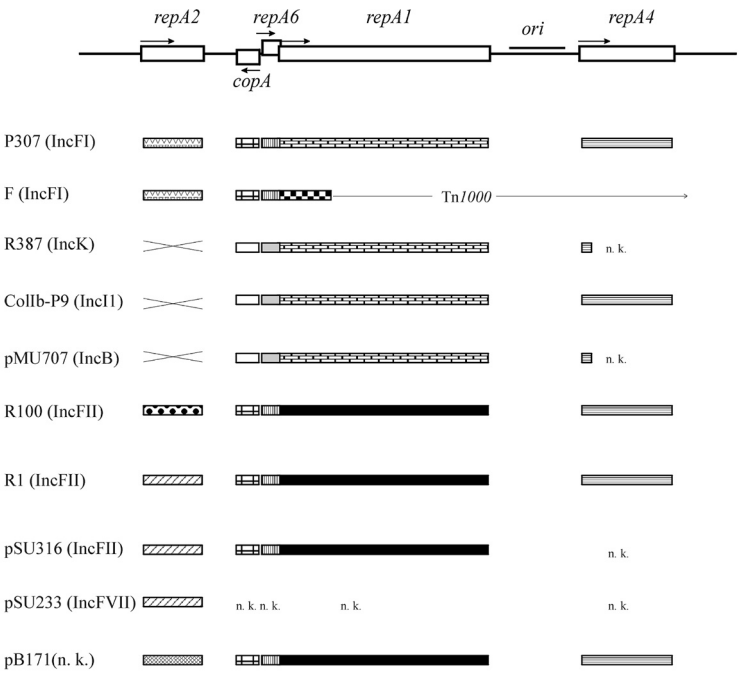

***Fig. 1*. Mosaicism in the broad RepFIIA family**
The organisation of IncFII-like replicons is shown at the top. Relationships between replicons of the IncFII-like family are presented using various patterns of shading. Note that in these mosaic replicons the *rep* region is closely related to one 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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