

Summary

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 awakened 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 IS1. In addition, biological assays demonstrated that pRK100 also encodes two colicins, ColV and Colla, 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 III₅₃₆ 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.