Replication and maintenance of plasmids in lactococcus lactis

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Chapter VII

Summary and general discussion

Whereas the majority of plasmids present in lactic acid bacteria, including those of lactococci, are cryptic, several (industrially) important traits are plasmid-encoded and, therefore, can be lost due to plasmid instability. Since plasmid stability is intrinsically linked to the replication of the plasmid, it is clear that a more detailed knowledge of plasmid replication functions is needed for a better understanding of plasmid instability. An overview of plasmid replication in bacteria is presented in Chapter I, with emphasis on replicons endogenous to lactococci. With respect to replication, three groups of plasmids can be distinguished: (i), plasmids using the theta mode of replication which is characterised by the formation intermediates resembling a Θ figure; (ii), plasmids using rolling-circle (RC) replication, a process which generates single-stranded DNA as intermediate; (iii), plasmids that use the linear mode of replication. The currently known plasmids from *Lactococcus lactis* use either of the first two mechanisms. Apart from their mode of replication, RC plasmids can be distinguished from theta plasmids by their small size (usually less than 12 kb), and their relatively high copy numbers. RC plasmids have been isolated primarily from Gram-positive bacteria. All RC plasmids have a similar structural and functional organisation. In their natural host, the high copy numbers are normally sufficient to guarantee stable maintenance of the plasmid via random segregation. Especially when propagated outside in a non-native host, RC plasmids are frequently segregationally instable, however. In comparision to theta plasmids, RC plasmids are frequently also characterised by high levels of structural instability.

Theta plasmids can be as large as several hundred kilobases and these plasmids can initiate replication in a number of different ways. Theta plasmids are often maintained at copy numbers that are too low to ensure their stable inheritance via random segregation. In addition to replication functions, a number of other plasmid-located functions have been identified, mainly in plasmids isolated from Gram-negative bacteria, which increase plasmid maintenance.

The majority of the cloning vectors currently available for *Lactococcus* species are based on the RC plasmids endogenous to these bacteria, such as pWV01, or on the non-native, broad-host-range theta plasmid pAMß1. Since at the start of the work carried out for this thesis no data were available on the segregational stability of these vector systems in *Lactococcus*, especially with respect to the effect of the insertion of heterologous DNA fragments, this aspect was studied here and the results are described in Chapter II. DNA inserts, varying in size from approximately 2.5 kb to 19 kb were introduced in pWV01 and in a deletion derivative of the theta

plasmid pAMß1. These two plasmids have similar copy numbers. The pWV01-derived vectors showed segregational instability if the total plasmid size exceeded about 12 kb. This happens to be the approximate size limit of natural RC plasmids. The instability was attributed to the generation of linear high-molecular-weight plasmid DNA, and a decrease in the number of monomeric plasmid forms. No effects on plasmid stability were observed with the pAMß1-derived vectors indicating that, as a cloning vector, pAMß1 is superior to pWV01. However, plasmid pAMß1 and its relatives have a broad host-range, sustaining replication in many Gram-positive bacteria. This is an undesirable property for applications in, for instance, food production.

The remainder of this thesis (Chapters III to VI) is focused on plasmids endogenous to one particular strain, *L. lactis* subsp. *cremoris* Wg2. Plasmid pWV01 is also derived from this strain. In Chapter III the isolation of pWV02, a 3.8 kb, segregationally stable plasmid that was shown to replicate via the theta mechanism, is described. The complete nucleotide sequence of this plasmid was determined. The minimal replicon of this plasmid appeared to consist of a non-coding region of about 250 bp which is located upstream of an open reading frame, called *repB*, which could encode a protein of 385 amino acid residues. In Chapter VI the nucleotide sequences of the replication regions of the 19 kb cryptic plasmid pWV04, the 27 kb proteinase plasmid pWV05, and the phage- resistance plasmid pIL7, are described. The replication regions of these plasmids showed a substantial level of sequence homology to the minimal replicon of pWV02. In addition, the nucleotide sequences of the replication region of a number of other lactococcal plasmids, not belonging to the RC-type, showed a high sequence similarity to the pWV02 family of replicons. The structural organisation of these replicons resembles that of the class A-type of plasmids, which have been studied in Gram-negative bacteria. These plasmids probably all replicate via the theta mode. The newly characterised plasmids exhibit a narrow host-range. The 250 bp non-coding sequence upstream of *repB* contains the origin of replication which is active only in *cis*. The protein encoded by the *repB* gene, RepB, is capable to act *in trans*. The observation that different members of the pWV02 family of plasmids show 60 to 70% homology in their replication fuctions both atthe DNA and protein levels, raises the question how these plasmids can co-exist within the same strain. For instance, pWV02, pWV04, and pWV05 are jointly present in *L. lactis* subsp. *cremoris* Wg2. Probably, the differeces in the replication fuctions underlie their compatibility in the same host.

An important tool for obtaining insight in plasmid replication is the isolation and characterisation of copy number mutants. In Chapter V, a copy-up mutant of pWV04

is described. Although the mutant plasmid had a twenty-fold increased copy number it was, in contrast to the parental plasmid, segregationally unstable. The mutant phenotype was caused by the transposition of an insertion sequence (IS) element. The complete nucleotide sequence of the region containing the IS element was determined. This revealed a novel IS element, which has homology with several other IS elements such as the lactococcal IS*981* element. The IS element in the pWV04 copy-up mutant appeared to have integrated into a direct repeat that surrounds the RNA polymerase binding site of *repB*. ß-Galactosidase gene fusion assays indicated that the copy number mutant possessed an increased *repB* promoter activity.

Two major systems can be used to introduce new traits in *L. lactis*: via plasmids or via integration of the genes of interest into the chromosome. If the former option is chosen, and if use in food production is the goal, the plasmid has to fulfill a number of criteria. It should be stably maintained and, preferably it should have a narrow host-range. In addition, it should only contain DNA from organisms which are "Generally Regarded As Save" (GRAS). In Chapter VI, the development of the theta plasmid pWV02 for such a food-grade vector/host system is described. The plasmid was provided with the sucrose genes from a GRAS bacterium, *Pediococcus pentosaseus*. A low level of segregational instability was observed with the initially developed vectors in media containing sucrose, but with the final expression/secretion vectors, no sucrose-induced instability occurred. However, to achive completly stability, further research concerning stability determinants that, upon cell division, improve the random distribution of plasmid copies or elements that ensure a better than random distribution of plasmids copies to daughter cells, will be necessary.