Developments and use of plasmid integration systems for lactococci

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CHAPTER IX

SUMMARY AND GENERAL DISCUSSION

Various traits of *Lactococcus lactis* important for dairying are encoded by unstable plasmids. The use of large scale dairy product fermentations necessitates the availability of genetically stable strains with defined properties. This thesis deals with efforts to develop plasmid integration systems which can be used to meet these requirements.

In Chapter I, two unstable plasmid-encoded traits essential for dairying are highlighted and chromosomal integration systems are discussed which are being used, or are potentially useful for, the stabilization of such traits, with the emphasis on plasmid integration systems, the main subject of this thesis.

Part A of tis thesis describes integrations of various heterologous plasmids by Campbell-type or replacement recombination. The stability of single - and multiple copy integrations were examined.

Chapter II describes the use of integration vectors based on plasmid pHV60, essentially a pBR322 replicon carrying the chloramphenicol resistance (Cm^r) gene of pC194, in which 1.3-kilobase (kb) chromosomal fragments of *L. lactis* MG1363 had been inserted. Three constructs were used. It was shown that the vectors integrated via Campbell-type recombination, and that amplification to approximately 15 copies per chromosome occurred after selection of the transformants on a low concentation of chloramphenicol. Although the amplification was gradually lost during nonselective growth conditions with a frequency of approximately 9 x 10^{-3} per generation, one copy remained stably integrated in the chromosome.

Chapter III describes the use of integration plasmids based on the replicons of pBR322, pUB110, pSC101, and pTB19, each containing the lactococcal chromosomal fragments A or B, also used in Chapter II. However, in this case the integration vectors carried the erythromycin resistance (Em^r) gene of pE194 as a selectable marker. All vectors were integrated via a Campbell-like mechanism into the chromosomal location for which the homology was provided. The pBR322 derivatives gave rise to transformants containing head to tail arrangements of 2 to 5 plasmid copies, whereas the pSC101 and pTB19-derived integration vectors produced single copy integrations. All of these integrations were stably maintained during nonselective growth; the frequency of plasmid loss being less than 10^{-4} per generation. Transformants obtained using the pUB110 derivatives resulted in amplified plasmid copies in the *L. lactis* chromosome. The amplifications were completely lost during nonselective growth with frequencies which varied between 8 and 9 x 10^{-3} per generation. Experimental data suggested that residual replicative activity of the pUB110 replicon destabilized the amplified structures in *L. lactis*.

In Chapter IV the plasmid-located proteinase genes *prtP* and *prtM* of *L. lactis* Wg2 were integrated by a Campbell-type mechanism into the *L. lactis* MG1363 chromosome, using the insertion vector pKLG610. This vector is a derivative of the pTB19-based integration plasmid used in Chapter III. The two transformants obtained carried a variable number of amplified plasmid copies in a head-to-tail arrangement on their chromosomes, strain MG610 containing approximately 2 copies and strain MG611 about 8 copies. The amplifications were stably maintained during growth in milk in the absence of anti-biotics, the frequency of loss of the Em^r and Prt^+ phenotype being less than 10^{-4} per generation. The proteolytic activity of strain MG611 was approximately 11-fold

higher than that of strain MG610, which produced only small amounts of proteinase. Nevertheless, both strains showed rapid growth in milk with concomitant rapid production of acid. The results indicated that a limited number of copies of the proteinase genes *prtP* and *prtM* per genome is sufficient for good growth in milk.

Chapter V describes replacement recombination in the *L. lactis* chromosome. Covalently closed circular DNA of the pUC18-derived integration plasmid pML336, carrying a 5.3-kb lactococcal chromosomal fragment containing the 2.3-kb *pepXP* gene, inactivated by the insertion of an Em^r gene, was used for this purpose. In 2% of the Em^r transformants the *pepXP* gene was inactivated by gene replacement recombination between pML336 and the *L. lactis* chromosome. In the other transformants pML336 had integrated by a Campbell-like mechanism which, owing to the particular structure of pML336, did not inactivate the *pepXP* gene. Recombination between 1.6-kb nontandem repeats which had been generated in the transformants carrying a Campbell-like integrated copy of pML336, occurred with low frequencies varying between less than 2.8×10^{-6} and 8.5×10^{-6} per generation and resulted in strains with chromosomal structures resembling those obtained after gene replacement recombination.

The integrations described in Part A of the thesis resulted in strains containing heterologous plasmid DNA and antibiotic resistance markers. The presence of such DNA in the chromosomes of the genetically engineered strains might present an obstacle for their use in dairy practice. Part B of the thesis describes the first steps towards the development of plasmid integration systems which can be used to construct food-grade strains.

In Chapter VI a cotransformation procedure with pGK1 as a replicating indicator plasmid and pML336 (Chapter V) as a 'nonselectable' integration plasmid, was used to integrate pML336 sequences into the *pepXP* gene by replacement recombination without selection for this event. Integrated pML336 sequences were present in 0.56% of the colonies containing pGK1. Two percent of these integrations appeared to have resulted from replacement recombination. The data indicated that by using cotransformation, any nonselectable gene can potentially be integrated by replacement recombination in any non-essential region of the *L. lactis* chromosome.

Since the integration of plasmids via a Campbell-type mechanism results in the incorporation of the complete plasmid, one of the prerequisites for integrations using this mechanism with the aim to construct food-grade strains is the availability of lactococcal plasmid-derived integration vectors. To this purpose the attention was focussed on the lactococcal broad-host-range plasmid pWVO1. Chapter VII describes the sequence and characterization of pWVO1. The plasmid appeared to replicate via the rolling-circle mode of replication which produces single-strand DNA intermediates. The replication initiator protein RepA encoded by pWVO1 was shown to act *in trans* on the plus origins of pWVO1-deletion derivatives lacking *repA*.

Chapter VIII describes the construction of a *Bacillus subtilis* strain (8G5::*repA*), which contains the *repA* gene of pWVO1 in its chromosome under transcriptional control of the lactococcal promoter P23. This strain providing the RepA protein *in trans*, enabled the construction of the pWVO1-based integration vector pINT1 which lacked the *repA* gene (Chapter VII). Plasmid pINT1 contained the Em^r gene of pE194, which was used as selection marker, and one of the same lactococcal chromosomal fragments as used in Chapters II and III. No replication of pINT1 was observed in *L. lactis* MG1363. Instead, the plasmid integrated by a Campbell-type mechanism via the homology provided. The transformants contained between 1 and 4 plasmid copies in tandem arrangements in the chromosome. During nonselective growth of these transformants the integrated plasmid copies were stably maintained. Loss of the Em^r phenotype by a mechanism of

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The results presented in this thesis clearly show that the techniques of integration of plasmid DNA into the chromosome by a Campbell-type - and replacement recombination are feasible in *L. lactis* (Chapters II, III and V). Integrations via a Campbell-like mechanism resulted either in single - or multiple plasmid copies which had a tandem arrangement on the chromosome. Amplified structures were found even after selection of the transformants on low concentrations of antibiotics. Using pHV60-derived integration plasmids it was made plausible that only one copy of the Cm^r gene was insufficient to confer Cm^r to the transformants in the presence of low concentrations of chloramphenicol. This suggests that the amplification was crucial for the survival of the transformants under these conditions (Chapter II). As to how amplifications were generated using other integration plasmids remains a matter of speculation. One possibility might be that plasmid multimers were integrated (Chapters III, IV and VIII) or that subsequent integration of several plasmid monomers present in the cells after the electrotransformation procedure resulted in the amplified structures (Chapter IV).

The single copy integrations and amplified structures had a high degree of stability during growth in the absence of antibiotics. The frequencies of loss of the antibiotic resistance markers varied between less than 2.8 x 10^{-6} and 3 x 10^{-4} per generation (Chapters III, IV, V and VIII). Integrated plasmids which had residual replicative activity were lost with higher frequencies: approximately 8 x 10^{-3} per generation (Chapter III). The presence of multiple copies of the Cm^r gene of pC194 in the integrated structures also had a negative effect on the stability of the integrations, the Cm^r phenotype being lost with a frequency of approximately 9 x 10^{-3} per generation (Chapter II). Integration plasmids derived from the same replicon, but carrying an Em^r gene as selectable marker, showed no loss of the Em^r phenotype under the same conditions (Chapter III). The instability of multiple copies of integrated plasmids carrying the Cm^r gene is unclear. Possibly, this relates to the expression of multiple copies of the Cm^r gene, because the presence of a single integrated copy of the Cm^r gene did not have an adverse negative effect on the stability (Chapters II and III).

The plasmid integration techniques described here can be used to mutate chromosomal genes (Chapter V), which can be helpful in the functional analysis of such genes, as well as for the stabilization of genes important for dairing in the *L. lactis* chromosome, thus contributing to strain improvement (Chapter IV). By Campbell-like integrations of pKLG610, strains were constructed which differed considerably with respect to the amount of proteinase produced. The overproduction of proteinases did not increase the growth rate of *L. lactis* in milk. Apparently, the amino acid pool present in milk is sufficient for optimal initial growth and can not be stimulated by increasing the amino acid and peptide pool as the result of increased breakdown of casein. The effect of the overproduction of proteinase on cheese ripening time and taste development was not determined. The construction of food-grade strains which produce less, or of strains which overproduce proteinase will enable to examine such effects. The integration systems and strategies described in Chapters VI and VIII can be potentially used for the construction of food-grade strains either by integration of a single copy of the gene of interest via gene replacement recombination, or by integration via Campbell-type recombinations which may give rise to stable multiple copies. To render the Campbell-like integration system based on a pWVO1-derivative (pINT1) completely homologous, the Em^r gene in pINT1 should be replaced by a selectable marker of lactococcal origin.