Development and use of host-vector systems for the characterization of lactococcal expression signals

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SUMMARY AND GENERAL CONCLUSIONS

This thesis describes investigations on a number of aspects of the genetics of lactococci, organisms which are widely used in dairy fermentations processes.

In chapter I a general introduction is given concerning the present state of knowledge of the genetics of lactococci, including recent developments which have made these organisms amenable to genetic manipulations.

Chapter II of this thesis describes the construction of the promoter screening vectors pGKV110 and pGKV210, which contain the promoter-less chloramphenicol acetyl transferase, *cat*-86, gene derived from *Bacillus pumilus*. This promoter-less gene and an upstream multiple cloning site, were subsequently introduced in the pWVO1 derived vector pGK3. This chapter also describes the construction of a vector, pGKV11, which can be used for the isolation of transcription termination signals. The suitability of this vector was demonstrated by using the transcription terminator of the *Bacillus licheniformis* penicillinase gene in *B. subtilis*. This terminator also functioned in the lactococcal strain MG1363.

The promoter screening vector pGKV110 was used to isolate DNA fragments from *Lactococcus lactis* subsp. *cremoris* Wg2 containing promoter activity in *B. subtilis*. Isolation of promoter containing fragments directly in *L. lactis* subsp. *lactis* MG1363 failed because the protoplast transformation efficiency was too low to transform strain MG1363 with DNA ligation mixtures. For this reason only promoter containing recombinant plasmids could be introduced in strain MG1363 via *B. subtilis* as an intermediate host. Analysis of the promoter strengths in both hosts showed that the isolated promoters expressed the *cat*-86 gene well in *B. subtilis* but at a reduced level in the lactococcal host.

Since it is conceivable that certain classes of promoters would go undetected, when lactococcal promoters are selected via *B. subtilis* as intermediate host, attempts were made to increase the protoplast transformation efficiency of *L. lactis* subsp. *lactis* to such an extend that promoters could be isolated in a direct way.

Chapter III of this thesis describes the enhancement of the transformation efficiency by including liposomes in the transformation mixture. Liposomes containing phosphatidyl cholin and cardiolipin in a molar ratio of 6 to 1, respectively, at a final concentration of 50 μg/ml lipids in the transformation mixture, appeared to be giving the best results. Under

these conditions 3.2 x 10^5 transfomansts per μg pGK12 DNA were obtained, which is 1600 times more efficient than in the absence of liposomes. This chapter also describes intergeneric protoplast fusion between *B. subtilis* and lactococcal protoplasts which obviates the isolation of plasmid DNA for the transfer of the pGKV shuttle vectors from the one host to the other.

In a direct assay, using the improved protoplast transformation protocol, 34 chloramphenicol resistant strain IL1403 transformants per μg DNA were obtained when *Mbo*1 fragments of strain Wg2 were inserted into the *Bam*H1 site upstream the promoter-less *cat*-86 gene of the promoter screening vector pGKV210. This chapter describes the characterization of five randomly isolated promoters of various strenghts, partly isolated directly in strain IL1403, and partly via precloning in *B. subtilis*, by sequence analysis and S1 nuclease mapping. The promoters on these fragments, located 5 to 9 base-pairs upstream the start site of transcription, corresponded closely to the consensus promoters of *E. coli* and to σ43 promoters of *B. subtilis*. The spacing between the -35 hexanucleotide (TTGACA) and the -10 hexanucleotide (TATAAT) was 17 to 18 base pairs. The stronger lactococcal transcription initiation signals contained a TG sequence upstream of and separated by one nucleotide from the -10 hexanucleotide. This TG pair appears to be strongly conserved in promoters of Gram-positive bacteria.

Analysis of the promoter strengths indicated that the higher chloramphenicol resistances in both strain IL1403 and *B. subtilis* were observed with those promoters which have a higher degree of similarity with the canonical -35 hexanucleotide sequence. This agreed with the observation that in *E. coli* most nucleotide substitutions towards the consensus promoter sequence resulted in increased promoter activity.

Ribosome binding sites were observed upstream an open reading frame starting with the ATG initiation codon on three of the promoter fragments obtained. These ribosome binding sites showed a high degree of complementarity to the 3'end of the 16S rRNA of *L. lactis* subsp. *lactis*, UCUUUCCUCCA. The free energy of base pairing ranged from -14.4 to-16.2 kcal/mole, which corresponded well with those reported for Gram-positive bacteria. The functionality of these RBS sequences was tested in *E. coli* using *lac*Z fusions.

In chapter V the five randomly isolated promoter containing fragments referred to in chapter IV have been compared with the oppositely directed promoters of the *prt*M and *prt*P genes of the proteolytic system of strain Wg2. The functionality of these promoters was assessed by determining the transcriptional starts of the *prt*P and *prt*M genes.

Both promoters of the *prt* genes are less efficient in expressing the *cat*-86 gene in *L. lactis* subsp. *lactis* than the five promoters referred to

earlier. In an attempt to increase the lactococcal proteolytic activity both the *prt*M and *prt*P genes were expressed under the control of the stronger promoter P32. To accomplish this the *prt*P and *prt*M genes were first organized in an operon-like structure by recombinant DNA technologies. This was done because the expression of both genes was required for proteolytic activity. In strain MG1363 containing pGKV832 in which the *prt* genes are expressed by P32, a moderate increase in proteolytic activity was observed. This chapter also describes the use of a new transcription terminator screening vector, pGKV259, in which the *cat*-86 gene is expressed by the strong promoter P59. This vector was used to characterize the putative transcription terminator downstream the *prt*P gene of strain Wg2. This structure, an inverted repeat followed by four T's still gave approximately 30% read through both in *B. subtilis* and the lactococcal strain MG1363. Evidence for the involvement of this structure in transcription termination was shown by deleting part of the stem-loop structure, which resulted in a significant drop of the terminating activity.