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Heterologous gene expression in lactococcus lactis.

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SUMMARY AND GENERAL DISCUSSION

Lactic acid bacteria are of major economic importance, as they occupy a key position in the manufacture of fermented foods. Fermentation serves the preservation of the foods and, moreover, adds to the development of flavour and texture of the products. A considerable amount of research is currently being devoted to the development of lactic acid bacterial strains with improved characteristics, that may be used to make fermentations pass of more efficiently, or to make new applications possible. Therefore, and because the lactococci are designated "GRAS" organisms (generally regarded as safe) which may be used for safe production of foreign proteins, detailed knowledge of homologous and heterologous gene expression in these organisms is desired. Expression of heterologous genes may add to both improvement of fermentation qualities and the extension of the applicability of the lactococci.

In chapter I an overview is given of what is known about gene expression in *Lactococcus lactis*. Not surprising, a general picture of gene expression signals in *L.lactis* emerges that shows considerable similarity to those observed in *Escherichia coli* and *Bacillus subtilis*. This feature allowed the expression of a number of *L.lactis*-derived genes in the latter bacterial species. Several studies have indicated, however, that in spite of the similarities, the expression signals from *E.coli, B.subtilis*, and *L.lactis* are not equally efficient in these three organisms. As it remains obscure which features of the gene expression signals determine their efficiency in *L.lactis*, the most straight forward approach to heterologous gene expression in this bacterium makes use of *L.lactis*-specific gene expression signals.

Chapter II describes the construction of a lactococcal expression vector based on this principle. This vector contains *L.lactis*-derived gene expression signals preceding a short 3'-truncated open reading frame (ORF32) and a multiple cloning site. In chapter II and subsequent chapters, the use of this vector in the expression of several heterologous genes (i.e. cDNA encoding mature hen egg white lysozyme (HEL), *E.coli lacZ*, *E.coli* bacteriophage T4 *e*, *E.coli* bacteriophage λ *R*, and *B.subtilis nprE*) in *L.lactis* is described. Configurations are described in which in-frame fusions were made between the lactococcal ORF32 and the heterologous genes, resulting in the production of fusion proteins. Alternatively, the heterologous coding sequences were directly fused to the gene expression signals preceding ORF32, resulting in the production of unfused heterologous proteins, under control of *L.lactis*-specific transcription and translation-initiation signals. In addition to these translational fusions, transcriptional fusions have been made, in which the heterologous genes were transcribed under control of the *L.lactis*-derived transcription initiation signals, and translated under control of the translation initiation signals of the heterologous genes.

Chapter III describes how in the case of a transcriptional fusion the expression of a heterologous gene (*E.coli lacZ*) may be improved by coupling its translation to that of the preceding lactococcal ORF32. This study showed that the result of translational coupling, which in this case most likely occurred via a translational restart mechanism, may critically depend on the configuration in which the two open reading frames involved are juxtaposed. Only configurations in which the stop codon of the upstream ORF32 and the start codon of the downstream *lacZ* were contiguous or partially overlapping, resulted in translational coupling. The best result was obtained with an AUGA configuration, in which UGA represents the stop codon of the upstream ORF32, and AUG the start codon of *lacZ*. It was shown that translational coupling could be used to enhance

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the expression of *lacZ* as compared to the expression of this gene in the absence of ORF32.

As discussed in chapters III and IV, this observation may be (partly) explained by assuming that the same translation initiation region upstream of the lactococcal ORF32 effected a more efficient initiation of translation than when it was placed upstream of *lacZ*. Differential efficiencies of translation initiation may also contribute to the reduced efficiency of expression of several genes (i.e. *lacZ* and HEL) as compared to the expression of the corresponding ORF32-fused genes (i.e. ORF32-*lacZ* and ORF32-HEL).

In chapter IV the possible contribution of mRNA secondary structure to the efficiency of translation initiation in *L.lactis* is discussed. This chapter describes how in the mRNA the translation initiation region upstream of the lactococcal ORF32 may give rise to a translationally more favourable secondary structure in combination with ORF32, than in combination with the heterologous ORFs studied (i.e. *lacZ* and HEL). Since the lactococcal ORF32 and the preceding expression signals were originally isolated as one unit from the *L.lactis* subsp. *cremoris* chromosome, these results are in support of the hypothesis that an ORF and the preceding expression signals need to be adjusted to each other for efficient expression to occur. In addition to mRNA secondary structure, the nucleotide sequence of the (5'-part of the) coding region per se may be important in this respect. Therefore, coupling the translation of a heterologous gene to that of an efficiently expressed (3'-truncated) homologous gene may be the most universally applicable method to accomplish efficient expression of the heterologous gene.

In chapter V, the application of this method to enhance the expression of the *E.coli* bacteriophage T4 *e* gene, encoding the T4 lysozyme, in *L.lactis* is described.

Chapter VI describes the functional expression of the lysozyme-encoding *E.coli* bacteriophage λ *R* gene in *L.lactis*. In this case, translational coupling of ORF32 and the λ *R* gene occurred in a configuration in which the stop codon of the former and the start codon of the latter were separated by three nucleotides, presumably because translation of the upstream ORF32 served to remove secondary structures in the mRNA that occluded the ribosome binding site of the λ *R* gene in the absence of translation of ORF32. Unfortunately, the plasmid in which the λ *R* gene was directly fused to the expression signals preceding ORF32 suffered from instability in *L.lactis*. Therefore, it was impossible to examine whether the λ *R* gene was expressed more efficiently through translational coupling to ORF32 than in the absence of ORF32.

In chapter VII the production and secretion of the *B.subtilis* neutral protease by *L.lactis* is described. Although the promoter of the *nprE* gene very much resembles the promoters generally observed in *L.lactis*, no detectable expression was obtained under the control of this promoter in *L.lactis*. In contrast, the gene was readily expressed under the control of the lactococcal promoter 32 present in the expression vector described in chapter II. The signal peptide of the neutral protease served to direct the secretion of the protein, which most likely was correctly processed to give a biologically active mature protease.

As pointed out at the start of this summary, heterologous genes may be expressed in lactic acid bacteria to serve a more efficient course of fermentation. The *B.subtilis* neutral protease has been reported to be successfully employed to achieve accelerated cheese ripening. Therefore, this enzyme may be the first heterologous enzyme produced in *L.lactis* with a future application in the dairy industry.