SUMMARY AND GENERAL DISCUSSION.

Lactic acid bacteria produce a variety of antimicrobial substances which are important in food fermentation and preservation. Apart from metabolic end products such as hydrogen peroxide, diacetyl and organic acids, some species secrete bactericidal substances of proteinaceous nature, which are termed bacteriocins. Although bacteriocins of lactic acid bacteria have been the subject of many studies, relatively little is known about their chemical structure, their mode of action, and their genetic determinants. A better insight into the molecular aspects of bacteriocins could direct the improvement of bacteriocin-producing strains in their use against food-spoilage. Furthermore, the genetic determinants of bacteriocins are interesting from the point of view of the development of 'food-grade' gene cloning and expression vectors, first because bacteriocin-producing strains of lactic acid bacteria are commonly used in food fermentations and second because they are selectively retained in the bacterial population.

In Chapter I a brief survey of bacteriocins is given. This chapter shows that bacteriocins are quite heterogeneous with respect to their mode of action, biochemical characteristics, optimal conditions of activity, and the location of the genetic determinants for production and immunity. Those bacteriocins are emphasized the genetic determinants of which have been cloned and characterized in recent years.

In *Lactococcus lactis* subsp. cremoris 9B4, the genetic determinants for bacteriocin production and immunity are located on the 60-kb plasmid p9B4-6. This plasmid was the starting material for the cloning of the genes specifying the antagonistic activity. Chapter II descibes the cloning of the genetic determinants of two bacteriocins from p9B4-6. A restriction enzyme map of p9B4-6 was made and several fragments of this plasmid were cloned in *Escherichia coli* and subsequently transferred to *L. lactis* to screen for antagonistic activity. Two distinct regions on p9B4-6 were identified which specified inhibitory activity on *L. lactis* indicator strains: a 7.9-kb fragment specifying low antagonistic activity and a fragment of 15 kb specifying high antagonistic activity. The inhibitory substances produced by both clones were sensitive to proteolysis, confirming their proteinaceous nature. Inhibition studies showed that the two bacteriocins had different specificities. By deletion analyses, the determinants for high and low antagonistic activities could be further confined to subfragments of 1.3 and 1.8 kb, respectively, and these were sequenced completely.

The nucleotide sequences of both fragments are presented in Chapter III. The 1.8-kb fragment contained one operon, consisting of three genes. Two of these could encode polypeptides of 69 and 77 amino acids, and were involved in bacteriocin activity. The third gene contained 154 codons and was required for immunity. The bacteriocin specified by the 1.8-kb fragment was designated lactococcin M and the two genes specifying bacteriocin activity *lcnMa* and *lcnMb*. The corresponing immunity gene was designated *lciM*. On the 1.3-kb fragment an operon containing two genes of 75 and 98 codons was present. The first gene, *lcnA*, specified bacteriocin production, the second encoded immunity (*lciA*). The bacteriocin specified by *lcnA* was termed lactococcin A. The nucleotide sequences upstream of *lcnMa* and *lcnA* as well as the first 20 bp of both genes appeared to be identical. Using primer extension analyses, a promoter upstream of *lcnMa* and *lcnA* was identified. Lactococcin A from the supernatant of *L. lactis* migrated to a position corresponding to a molecular size of approximately 3.4 kDa on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, indicating that lactococcin A had an aberrant

mobility and/or had been subject to proteolytic processing.

Downstream of the lactococcin A operon, a third bacteriocin determinant was identified. The gene specifying the production of this bacteriocin and the corresponding immunity gene were analyzed in detail and the results are presented in Chapter IV. Also in this case, the two genes appeared to be organized in an operon. The first gene, *lcnB*, contained 68 codons and was the structural gene for lactococcin B. The second gene, *lciB*, contained 91 codons and was responsible for lactococcin B immunity. The specificity of lactococcin B was different from that of lactococcin A and M. Also in this case, a nucleotide sequence upstream of, and encompassing the 5' part of the bacteriocin structural gene, showed similarity to the equivalent sequences of the other two bacteriocin operons. The similarity of these regions may suggest that recombinational events assembled these genes on one plasmid.

Recently, a bacteriocin was purified to homogeneity from *L. lactis* subsp. *cremoris* which appeared to be encoded by a gene identical to *lcnA* (Holo et al. J. Bacteriol. **173**:3879-3887). This bacteriocin, lactococcin A, is a polypeptide of 54 amino acids. The mature lactococcin A sequence is located in the C-terminus of the 75 amino acid polypeptide encoded by *lcnA*. Apparently, *lcnA* encodes a precursor of lactococcin A from which an N-terminal extension of 21 amino acids is proteolytically removed. Comparison of the *lcnA*, *lcnB*, and *lcnMa* primary translation products revealed that their 21 N-terminal amino acids are nearly identical, suggesting that the latter two are also produced as precursors and cleaved at equivalent sites. Cleavage of the precursor of lactococcin A, and probably also those of lactococcin B and M, occurs at the C-terminal side of two adjacent glycine residues. Identical cleavage sites have also been identified in the precursors of two other bacteriocins, namely lactacin F from *Lactobacillus acidophilus* 11088 (Muriana and Klaenhammer. 1991. J. Bacteriol. **173**:1779-1788) and pediocin PA-1 from *Pediococcus acidilactici* PAC1.0 (Marugg. J., pers. comm.). These observations suggest that the bacteriocins are processed by a mechanism common to lactic acid bacteria. Using the T7 RNA polymerase specific promoter to express the lactococcin B operon in *E. coli*, it was possible to detect antagonistic activity in extracts of this host. However, the rate of migration in an SDS-polyacrylamide gel of the polypeptide produced by *E. coli* was less than that of the mature lactococcin B, suggesting that *E. coli* is not able to process the bacteriocin precursor.

Chapter V describes experiments with purified lactococcin A to study its mode of action. Lactococcin A increased the permeability of the cytoplasmic membrane of sensitive *L. lactis* cells, thereby dissipating the membrane potential. A significantly higher concentration of lactococcin A was needed to dissipate the membrane potential of an immune strain of *L. lactis*. Lactococcin A at low concentrations inhibited the uptake of amino acids in and induced leakage of accumulated amino acids from sensitive cells. The activity of lactococcin A on whole cells was proton motive force-independent. The same concentration of lactococcin A did not affect immune cells. Lactococcin A also inhibited proton motive force-driven leucine uptake and leucine counterflow in membrane vesicles of the sensitive strain, but not in membrane vesicles of the immune strain. This result indicates that lactococcin A also increased the permeability of membrane vesicles in the absence of a proton motive force. Moreover, this result shows that the immunity protein is associated with the cytoplasmic membrane. Membrane vesicles of *Clostridium acetobutylicum*, *Bacillus subtilis* and *E. coli* were not affected by lactococcin A, nor were liposomes derived from phospholipids of *L. lactis*. These results reflect the narrow spectrum of activity of lactococcin A and indicate that a receptor protein associated with the cytoplasmic membrane is required for lactococcin A to be effective.

As is described in Chapter II and IV, the immunity proteins of lactococcin M, A, and B are

specific: immunity towards one of the lactococcins does not result in immunity towards the other two lactococcins. *L. lactis* IL1403 cells selected for tolerance to lactococcin A or B appeared to be tolerant to both bacteriocins, but not to lactococcin M (unpublished results). The simultaneous acquisition (presence) of tolerance to lactococcin A and B was also observed in natural isolates of *L. lactis* (unpublished results). Apparently, lactococcin A and B share a common target or receptor which is different from that of lactococcin M.