

Studies on the multiple bacteriocin production and biosynthesis of a circular bacteriocin produced by *Enterococcus faecium* NKR-5-3

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(*Enterococcus faecium* NKR-5-3が生産する多成分バクテリオシンの
生産挙動と環状バクテリオシンの生合成機構に関する研究)

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論 文 内 容 の 要 旨

Bacteriocins from lactic acid bacteria (LAB) have recently gained an increasing interest among a number of research groups due to its huge application potential both in food and pharmaceutical industries. In the food industry, bacteriocins have long been proposed as the possible solution to the problems of food spoilage and food-borne infections. In fact, nisin A, the most extensively studied bacteriocin, has been used as a food preservative in more than 50 countries. On the other hand, the emergence of antibiotic resistant pathogens has cast some doubt on the usefulness of antibiotics in the twenty-first century. Due to the very strong potency of some bacteriocins against clinical pathogens, including antibiotic resistant strains, bacteriocins have been considered as a viable alternative to antibiotics.

While most bacteriocin-producing LAB strains synthesize only single bacteriocin, some LAB strains have the unique ability to synthesize multiple bacteriocins. Multiple-bacteriocin producing LAB have a great industrial application potential as antimicrobial starter cultures. By understanding its multiple-bacteriocin regulation mechanisms, appropriate bacteriocin production can be controlled depending on the demand of the industrial processes. On the other hand, among the family of bacteriocins, a group of bacteriocins has recently been gaining much attention due to its unique structural feature characterized by the head-to-tail cyclization of their backbone, the circular bacteriocins. The circular nature of these peptides provides them better resistance against many proteases and higher stability to thermal stress than linear-type bacteriocins, making them an ideal candidate as highly stable antimicrobial agents for use as human therapeutics or as stable scaffolds for drug delivery agents. Much like any bacteriocins, understanding the molecular mechanism of the biosynthesis of circular bacteriocins would materialize its great application potential.

This thesis manuscript describes the studies done on a very promising LAB strain, *Enterococcus faecium* NKR-5-3, which can synthesize multiple antimicrobial peptides, termed enterocins NKR-5-3A, B, C, D, and Z (Ent53A, B, C, D, and Z). Ent53A and Z make up a novel two-peptide (class IIb) bacteriocin that shows synergistic activity with each other for its antimicrobial activity. Ent53B is a novel circular bacteriocin that showed a broad spectrum of antibacterial activity. Ent53C, a pediocin-like (class IIa) bacteriocin, although showed a relatively moderate spectrum of antibacterial activity, possesses a very strong anti-listerial activity, which could be a viable solution to control the highly pathogenic food-borne bacteria *Listeria monocytogenes*. Ent53D on the other hand, although showed relatively weak antimicrobial activity, has been shown to induce the production of other NKR-5-3 enterocins. However, in order to maximize the application potential of strain NKR-5-3 and its bacteriocins, understanding the biology of these bacteriocins, in particular, to elucidate its structure-function relationships, production, immunity, regulation and mode of antimicrobial action is essential.

In order to study the complex regulation mechanism of multiple bacteriocin production of strain NKR-5-3, an ESI-LC/MS-based quantification system that can simultaneously monitor the production profiles of NKR-5-3 enterocins as influenced by different fermentation conditions was developed. The novel circular bacteriocin, Ent53B, was found out to have independent production regulation system from the other NKR-5-3 enterocins. Ent53B production was not as variable compared to the other NKR-5-3 enterocins when strain NKR-5-3 was cultivated in different cultivation media. Production of Ent53B was also found to have a wider optimum incubation temperature (20 – 30°C) than Ent53A, C and D (25°C). Furthermore, Ent53D can induce the production of NKR-5-3 enterocins except Ent53B at a threshold concentration of 2 nM.

The genes involved in the production, maturation, secretion, and self-immunity of the novel circular bacteriocin, Ent53B, were identified by successfully establishing a heterologous expression system of Ent53B. Mature (circular) Ent53B was successfully produced when the five putative genes encompassing the region *ent53B* through *ent53B4* (gene products Ent53B1234) was cloned into different host strains (*Enterococcus* and *Lactococcus* strains). In order to gain further insights on the details of the Ent53B biosynthesis, several mutagenesis on the *ent53B* structural gene were done that lead to new perspectives on its cyclization. It is very likely that a hydrophobic interaction-driven reaction governs the interaction between the Ent53B precursor and its biosynthetic enzymes Ent53B1234. Only mutations with helix-structure-promoting hydrophobic residues (Ala, Ile, Val, or Phe) at the Leu1 position lead to the production of the mature Ent53B derivatives. It is also likely that the leader peptide in the Ent53B precursor peptide functions to ensure a certain fold that is essential to its interaction with the biosynthetic enzymes. Any truncations of the leader peptide moiety interfered the processing of the precursor peptide, yet when propeptides of other circular bacteriocins (circularin A, leucocyclicin Q, or lactocyclicin Q) were cloned at the C-terminus of the leader peptide, the enzyme complex Ent53B1234 cannot process them to yield a mature bacteriocin.

Furthermore, it was also found out that there are two independent system of self-protection that is working against the antimicrobial action of Ent53B. Recombinant strains expressing *ent53B4* and/or combination of *ent53B1* and *ent53B3* acquired high immunity against the inhibitory action of Ent53B.

In conclusion, results described in this thesis manuscript on the unique regulation mechanism of multiple bacteriocin production of strain NKR-5-3 could be deemed very helpful in the future for the industrial application of this strain as an antimicrobial starter culture in many food fermentations. Furthermore, the new perspectives on the possible mechanism of biosynthesis of Ent53B described in this thesis manuscript could serve as a stepping-stone for further research in order to unravel the mystery surrounding the biosynthetic mechanism of circular bacteriocins and in the process would open up the door for the application of circular bacteriocins not only as food preservative in the food industry but as a therapeutic agent for medical applications as well.