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HETEROLOGOUS EXPRESSION OF PISCICOLIN 61, A BACTERIOCIN FROM *CARNOBACTERIUM PISCICOLA* LV61, IN *LACTOBACILLUS SAKE* LB706-X

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SUMMARY

Strains of lactic acid bacteria producing heterologous bacteriocins may be of use to the food fermentation industry. Genetic studies have shown that a number of genes are involved in regulation and secretion of bacteriocin expression. The genes involved in expression of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706, have been identified. These genes have been employed in the expression of a heterologous bacteriocin, piscicolin 61, from *Carnobacterium piscicola* LV61 in *L.sake.*

INTRODUCTION

Bacteriocins are proteinaceous compounds produced by bacteria. They usually inhibit the growth of closely related species. In addition, they may also inhibit the growth of food-borne pathogens and food spoilage bacteria. Bacteriocins and bacteriocin producing strains may thus have some potential for enhancement of the microbiological safety and prolonged shelf-life and acceptance of foods.
Genetic studies have shown that a number of genes are involved in regulation and secretion of bacteriocin expression. In order to construct strains that overproduce bacteriocins, the structure and regulation of these genes should be elucidated. Different bacteriocins inhibit the growth of different sensitive strains. Construction of strains that produce several bacteriocins could widen the inhibitory spectrum of a producing strain and thus render it more useful in food fermentations. In cases where regulation and secretion of bacteriocin production has been optimized for one bacteriocin, heterologous bacteriocin expression may be a rapid way of obtaining large amounts of other bacteriocins.
We have previously analyzed the genes involved in production, regulation and secretion of the bacteriocin sakacin A from *Lactobacillus sake* Lb706 (1). This bacteriocin inhibits the growth of *Listeria monocytogenes.* We have also purified piscicolin 61, a bacteriocin from *Carnobacterium piscicola* LV61, which inhibits strains of *Carnobacterium, L.sake and Enterococcus* (2). Here we report the heterologous expression of piscicolin 61 in *L.sake* employing the genes necessary for the expression of sakacin A.

MATERIALS AND METHODS

Strains, plasmids and growth conditions. *C.piscicola* LV61 Psc^+Imm^+, the cured variant *C.piscicola* LV61 A and the piscicolin 61 sensitive indicator strain *C.divergens* L66 were grown in modified MRS medium (2). *L.sake* Lb706 Sap^+Imm^+ (harbouring the sakacin A gene cluster on a 60 kb plasmid) and a plasmid free variant *L.sake* Lb706-X Sap^-Imm^- was grown in MRS broth at 30 °C. Transformed *Escherichia coli* TG1 was grown on SOB agar containing 30 μg/ml chloramphenicol. pSAK27 is a derivative of the broad host range plasmid pVSB1 harbouring a10.4 kb fragment containing the genes necessary for regulation, production and secretion of sakacin A. pSAK20 is a derivative of pSAK27 containing the genes for regulation and secretion of sakacin A on a 7.5 kb fragment (1). pSAK17B is a derivative of pLPV111 containing the structural gene and the immunity gene encoding sakacin A and immunity to sakacin A, respectively, on a 3.0 kb fragment. pLPV111 is a derivative of pGEM-7Zf(+) containing an erythromycin resistance gene and a replicon from a *L.plantarum* strain(1). pSAK20 and pSAK17B are compatible when present in the same cells.
Bacteriocin production and immunity. Colonies were tested for bacteriocin production and immunity by a deferred agar plate assay. In short, colonies were allowed to grow on suitable agar plates to a colony diameter of approx 1-2 mm. Indicator cells mixed with 5 ml of melted agar was then poured over the colonies, and the agar plates were incubated over night. Clear zones around the single colonies indicated bacteriocin production.
General molecular cloning techniques. Standard procedures for molecular cloning were as described by Sambrook et al.(3). Transformation of different strains with plasmid DNA was done by electroporation. DNA was sequenced by the dideoxy-chain termination method by use of a primer walking strategy and an erase-a-base strategy.

RESULTS AND DISCUSSION

The DNA sequence of an 8,668 bp *Pac*I-*Sph*I fragment was determined. This fragment contained all the information needed for heterologous expression of sakacin A in *L.sake.* The gene cluster was organized in two divergent operons, one containing the structural gene, *sapA,* and the immunity gene, *saiA,* and the other containing the two *agr*-like genes, *sapKR*, involved in regulation of bacteriocin expression, and two genes, *sapTE,* involved in secretion of the bacteriocin (Fig.1). An insertion element, IS*1163*, is present between the two operons, but appear not to be involved in expression. In addition, several other small open reading frames are present. The two operons may be present on different plasmids in the same cell conferring bacteriocin production and immunity. Bacteriocins are synthesized as precursor molecules with a leader sequence of approx 20 amino acids. This leader is involved in recognition of the dedicated secretory system SapTE and is absent from the mature bacteriocin. Bacteriocins may differ strongly in their amino acid sequences, yet share similar features in their leader sequences.

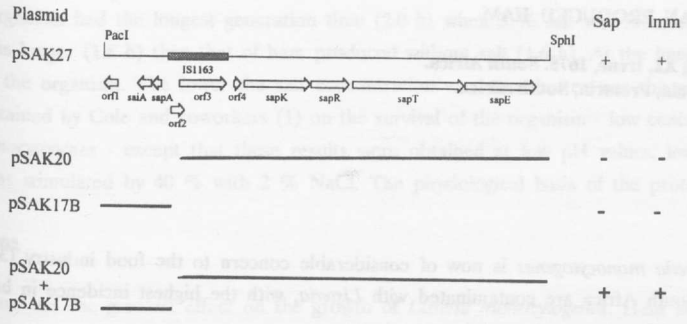

FIG. 1. Linear map of the sequenced region encompassing the sakacin A gene cluster and structure of derived constructs. The organisation and direction of the 10 ORFs are indicated by arrows. Plasmid names are shown on the left, and the phenotypes when the plasmids were introduced into L.sake strains are shown on the right.

When the leader sequences of pre-sakacin A was compared to that of piscicolin 61, a striking sequence similarity was observed (Fig.2) even though the sequences of the mature bacteriocins shared no significant similarities.

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Piscicolin 61          MNNVKELSIKEMQQVTGG
                       ********: *:* :***
Sakacin A              MNNVKELSMTELQTITGG
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FIG.2. Comparison of the leader sequences of pre-piscicolin 61 and pre-sakacin A. Asterisks denote identical amino acids while colons denote conservative amino acid changes. The arrow after the two glycine residues shows where the leader sequence is cut to yield the mature bacteriocins.

The structural gene encoding piscicolin 61 residing on a 2.0 kb *Eco*RI fragment was ligated into the unique *Eco*RI site of pSAK20 and transformed into *E.coli* TG1 (Fig.3). Plasmid DNA was purified from transformants and cloned into *L.sake* Lb706-X. Transformants harbouring the new plasmid construct pSAK20-P61 were analyzed for bacteriocin production and immunity (Fig.4). The transformants produced piscicolin 61 and were also immune to this bacteriocin.

CONCLUSIONS

Strains of lactic acid bacteria producing heterologous bacteriocins may be of use to the food fermentation industry. Several bacteriocins may be exported through the same export system. The export system appear to recognize primarily the leader sequences of the pre-bacteriocins.

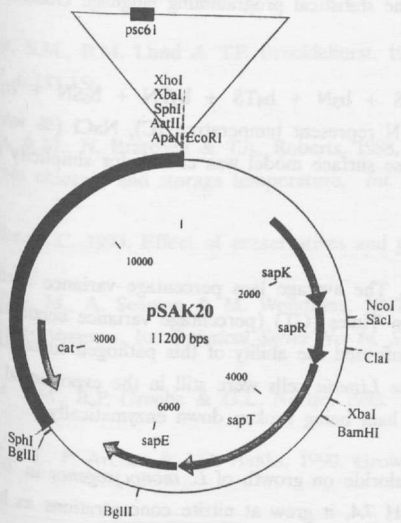

FIG.3. The structural gene gene psc61 was cloned in plasmid pSAK20 containing the genes necessary for sakacin A expression.

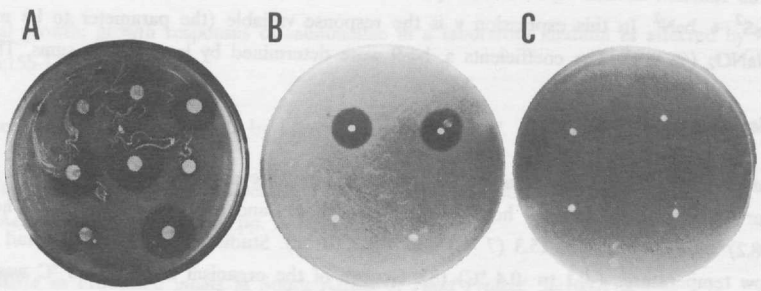

FIG. 4. Heterologous production of the bacteriocin piscicolin 61. A : L.sake Lb706-X(pSAK20-P61) producing inhibition zones. The indicator organism is C. divergens L66. *Non-producing colonies are controls. B: Inhibition zones showing the sensitivity of L.sake Lb706-X to piscicolin 61. Bacteriocin producing colonies are C.piscicola LV61, non producing colonies are the correspondent cured variant C.piscicola LV61 A. Indicator organism is L.sake Lb706-X. C: Colonies as in B, indicator organism is L.sake Lb706-X(pSAK20-P61)*

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