Genetic analysis of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus*

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The genes encoding the production of acidocin B, a bacteriocin produced by *Lactobacillus acidophilus* strain M46 which is active against *Listeria monocytogenes*, *Clostridium sporogenes*, *Brochothrix thermosphacta*, *Lactobacillus fermentum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, but inactive against most other *Lactobacillus* species, were previously localized on a 4 kb *Xba*I–*Hind*III fragment of plasmid pCV461. In the present work, DNA sequence analysis revealed the presence of three consecutive ORFs, which potentially code for hydrophobic peptides composed of 60, 91 and 114 amino acids, respectively, and a fourth ORF of opposite polarity which could potentially encode a peptide of 59 amino acids. The middle ORF (ORF-2; *acdB*) was identified as the gene encoding acidocin B by comparing the amino acid composition of highly purified acidocin B with the deduced amino acid sequence of ORF-2. Our results suggest that acidocin B is synthesized as a precursor which is processed at a site which conforms to the '–3, –1' rules of von Heijne to yield active acidocin B (59 amino acids). The presence of an immunity-protein-encoding gene on the 4 kb *Xba*I–*Bam*HI fragment was deduced from the capacity of a plasmid vector harbouring this fragment to confer immunity upon transformation of *L. fermentum* NCK127. One of the three non-assigned ORFs may encode this immunity protein.

Keywords: *Lactobacillus acidophilus*, bacteriocin, acidocin B

INTRODUCTION

Lactic acid bacteria (LAB) are traditionally used to preserve food and feed. The metabolic activities of LAB play an important role in this respect (Gilliland, 1985). The formation of copious amounts of acidic end-products, primarily lactic acid, from the fermentation of carbohydrate-rich substrates such as grain, silage, milk and vegetables is largely responsible for creating an environment unfavourable for the growth of spoilage organisms. Some strains of LAB also produce bacteriocins, proteins with bactericidal activity against closely related bacteria (Tagg *et al.*, 1976). The majority of bacteriocins produced by LAB can be classified into four distinct groups based on biochemical and genetic characteristics: (i) small

(< 10 kDa), heat-stable membrane-active peptides, (ii) large (> 30 kDa), heat-labile proteins, (iii) lantibiotics, small (< 5 kDa) proteins containing the unusual amino acid lanthionine, and (iv) complex bacteriocins comprising other chemical moieties besides protein (Klaenhammer, 1993).

During recent years a number of peptide bacteriocins have been isolated, purified and studied biochemically and genetically. Mature, class I bacteriocins are hydrophobic peptides, some of which require the presence of a second peptide for activity (Nissen-Meyer *et al.*, 1992). The two peptides are presumed to oligomerize and form membrane pores and ion channels (Nissen-Meyer *et al.*, 1992). Class I bacteriocins are synthesized as precursor molecules that are cleaved after a characteristic Gly–Gly doublet, invariably present at positions –2 and –1 with respect to the mature protein. Genetic characterization of bacteriocin production has shown the presence of operon structures, comprising one or two genes encoding the

Abbreviation: LAB, lactic acid bacteria.

The EMBL accession number for the sequence reported in this paper is Z34920.

bacteriocin, an immunity-protein-encoding gene, and genes implicated in secretion and maturation of the bacteriocin (Belkum *et al.*, 1992; Fremaux *et al.*, 1993).

In a screening programme of *Lactobacillus* strains set up to select for those producing bacteriocins that are inhibitory towards micro-organisms outside the genus *Lactobacillus*, we have isolated a strain of *Lactobacillus acidophilus* (M46) producing a potent bacteriocin which inhibits growth of *Listeria monocytogenes*, *Clostridium sporogenes* and *Brochothrix thermosphacta*, but which affects only a few *Lactobacillus* strains (Vossen *et al.*, 1994). The bacteriocin, acidocin B, is a protein and has an estimated size of 2.4 kDa. Acidocin B production, which is plasmid-encoded, has been transferred to a non-producing *L. plantarum* strain (Vossen *et al.*, 1994).

In this paper we present the nucleotide sequence of three clustered genes, at least one of which is involved in the production of acidocin B. From a comparison of the amino acid composition of purified acidocin B with the deduced amino acid sequences, one of the genes was identified as the gene encoding acidocin B. Our results also suggest that one of the other two genes encodes the immunity protein.

METHODS

Materials. All enzymes for molecular cloning were purchased from Boehringer or BRL and were used according to the recommendations of the suppliers.

Bacterial strains, plasmids and media. *L. plantarum* strain 80 (Scheirlinck *et al.*, 1989) and *L. fermentum* NCK127 (Barefoot & Klaenhammer, 1983) were maintained as frozen glycerol stocks at -20°C . *Lactobacilli* were cultivated in MRS broth (Difco) at 37°C . For plating, MRS was solidified with 1.5% (w/v) agar (Daishin). Erythromycin was used at a final concentration of $10\text{ }\mu\text{g ml}^{-1}$. For cloning in *Escherichia coli* the strain JM109 was used as the host. Plasmid pCV461 is a 14 kb plasmid from *L. acidophilus* M46 containing the genes for acidocin B production (Vossen *et al.*, 1994). Plasmids pGKV21, a shuttle vector that can be propagated in Gram-positive and Gram-negative bacteria (Vossen *et al.*, 1985), and pEI2, a derivative of the *E. coli* vector pUC19 (Posno *et al.*, 1991), were used for subcloning DNA fragments. Plasmid pLPE24M* (* denotes a spontaneous high-copy-number mutant) is a derivative of pLPE24M, which was obtained by introduction of a multicloning site polylinker into pLPE323 (Pouwels & Leer, 1993).

Isolation of plasmid DNA, bacterial transformation and DNA techniques. Isolation of *Lactobacillus* plasmid DNA, transformation of *Lactobacillus* and *E. coli* JM109, and DNA manipulations were all carried out as described previously (Posno *et al.*, 1991; Leer *et al.*, 1992). Nucleotide sequences were determined by the chain-termination method using plasmid DNA as the template and sequence-specific primers (Sanger *et al.*, 1977).

Assay of bactericidal activity of acidocin B. This was carried out as described previously (Brink *et al.*, 1994). In short, bacteria were cultivated overnight in MRS broth containing 2% (w/v) glucose and 3% (w/v) calcium carbonate. After centrifugation, the supernatant solution was filter-sterilized and the pH was adjusted to 7.0. Bacteriocin activity was measured with *C.*

sporogenes as the target organism by pipetting 20 μl into wells punched in a nutrient agar plate. After 20 h incubation the formation of inhibition zones around the wells was examined.

Production and purification of acidocin B. *L. acidophilus* M46 was cultivated in MRS broth overnight at 37°C . Cells were removed by centrifugation and the pH of the supernatant was adjusted to 7.7 with sodium hydroxide. Sodium chloride was added to saturation and the solution was extracted with 2-propanol. The 2-propanol phase was collected and concentrated by evaporation. The concentrated residue was taken up in 10 ml water followed by extraction with 1-butanol. The butanol extract was concentrated by evaporation and the residue was resuspended in 0.1% trifluoroacetic acid (TFA). This sample was further purified by reversed-phase HPLC on a C3 column, using a gradient of 16% to 51% (v/v) 2-propanol in acetonitrile acidified with TFA (0.1%). Buffer A was made up as 0.1% TFA in water. Buffer B was composed of 80% (v/v) 2-propanol in acetonitrile acidified with TFA (0.1%). The gradient consisted of a 20 min linear gradient from 80%A/20%B to 35%A/65%B. The A_{210} of the effluent was monitored. Fractions were evaporated, resuspended in water and assayed for bactericidal activity. Fractions showing activity were combined and subjected to a second HPLC purification cycle. After evaporation, the resuspended fractions were again assayed for activity.

Amino acid analysis. Purified acidocin B was hydrolysed under HCl vapour at 116°C for 24 h and derivatized with phenyl isothiocyanate. Quantification of the phenylthiocarbamyl amino acids obtained was carried out using reversed-phase HPLC (Noort *et al.*, 1991).

RESULTS AND DISCUSSION

Cloning of acidocin-B-encoding genes

We have previously shown that the 14 kb plasmid pCV461 from *L. acidophilus* M46 harbours the genetic information for production of acidocin B. This information could be transferred to a non-producing *L. plantarum* strain (Vossen *et al.*, 1994). To determine which parts of the plasmid are involved in bacteriocin production, pCV461 was digested with restriction enzymes, and the fragments obtained were subcloned in pGKV21 and used to transform *L. plantarum* 80. The culture medium from transformants cultivated in MRS broth containing erythromycin was assayed for the presence of acidocin B using *C. sporogenes* as indicator strain in a plate assay. The results (Fig. 1) show that the smallest fragment that confers the capacity to produce acidocin B is contained within a 4 kb *Xba*I–*Bam*HI fragment (pCV461-3). When the unique *Pst*I site present within the *Xba*I–*Bam*HI fragment was destroyed by removal of the protruding ends with Klenow enzyme and re-ligation, the resulting plasmid (pCV461-6) was no longer able to produce active acidocin B. Apparently, the *Pst*I site is located in a region which is essential for acidocin B production.

DNA sequence analysis

The nucleotide sequence of a 2.2 kb region surrounding the *Pst*I site was determined (Fig. 2). Three consecutive ORFs could be identified which potentially encoded

Fig. 1. Restriction-endonuclease-derived fragments from plasmid pCV461 cloned into pGKV21. The 7 kb *SalI*-*BglII* fragment corresponds with the left circle segment of pCV461 as published by Vossen *et al.* (1994). Supernatants of *L. plantarum* transformants (erythromycin-resistant) containing the different cloned fragments were assayed for acidocin B activity. The eliminated *PstI* site is indicated by X. The DNA sequence of the region surrounding the *PstI* site (↔) was determined.

polypeptides composed of 60, 91 and 114 amino acids, respectively. A fourth ORF (not shown) was found with a polarity opposite to that of the others. It overlaps the second ORF and could encode a polypeptide of 59 amino acids. The predicted *Pst*I site is located in ORF-2, implying that this ORF is essential for production of acidocin B.

Each of the ORFs is preceded by a typical translation initiation region comprising a Shine–Dalgarno element at a distance of 6–10 nucleotides before the translation start codon. The intergenic regions between ORFs 1–3 are 108 and 85 nucleotides long. In the intergenic region between ORF-2 and ORF-3 a palindromic sequence (nt 741–768; see Fig. 2) was found, which can form a stable stem-loop structure [$\Delta G = -16.1 \text{ kcal mol}^{-1}$ ($-67.4 \text{ kJ mol}^{-1}$)] followed by a series of T residues, resembling rho-independent transcription terminator sequences (Platt, 1986). If this sequence indeed functions as a transcription terminator, it would imply the presence of a promoter located between the putative terminator and the start of ORF-3 (nt 788). Alternatively, the promoter might overlap with the terminator, allowing a fraction of RNA polymerase molecules, which pause at this site, to read through the terminator sequence. A sequence (TAGACT – 21 nt – TATTAAT) showing resemblance to σ^{70} promoter elements is present 30 nt upstream of the translation start of ORF-3, partially overlapping the hypothetical terminator sequence. Upstream of ORF-1 and downstream from ORF-3, sequences were found (not shown), 800 and 350 nt in length, which do not seem to code for a polypeptide. These sequences show no particular

AAGCTTGGAGAAGAAATACCGATAAGGCCCTTATTGATTACACGATGGCTATTCTTTGTT 60

GTCAATTCTATCCTCCGGATATTTTTCACITTTTAGGAAGGTAGCATGTAGGTAAATAATCT 120

rbs

TTAAGGAGACAAATCAGCGCATGGATAAGAAAAACAAAATATTATTTGAAGTATTATACAT 180

M D K K T K I L F E V L Y I

CATCTGTATAATAGGCCCTCAATTITATATTATTGTGACTGCAAAAAACAAATATGTATCA 240

I C I I G P Q F I L F V T A K N N M Y Q

GTGGTGGGTTCGTTTGTGGAATAGTAGTGGTTTTTCGTATATTTTTTGGTATATTTTTT 300

L V G S F V G I V W F S Y I F W Y I F F

CAAAACAACATAAAAAATGTAGTATACCACTGTGTTTATTGATTATTAAAGGCGCTATAAT 360

K Q H K K M *

rbs

AATGGTGAATATTACTACAAAAAGGTACGTACACTCGTAATGGATTTCCTATGGGAGG 420

AAGATACTATGGTTACTAAGTACGGACGTAATTTAGGTTTGACGAAGGTAGACTTGTTCG 480

M V T K Y G R N L G L S K V E L F A

CAATTTGGGCGGTTTTAGTAGTTGCTCTTTTATTGGCCACAGCGAACATTATTGGATTG 540

I W A V L V V A L L L A T A N I Y W I A

CTGATCAATTCGGGATCTATTAGCGATGGAACGCCCGTAAGTTATTAGATGCGACTGG 600

D Q F G I H L A T G T A R K L L D L A V A

CTTCGGTGCCCTCATTGGGAACTGCCITTTGCTGCTATTTTGGGGCTGACATTACCTGCAT 660

S G A S L G T A F A A I L G V T L P A W

PstI

GGGCTTTGGCAGCTGCAGGAGCATTTGGGAGCGACTGCGGGCTAGTGATTATAAAGCTTTA 720

A L A A A G A L G A T A A

<-----> rbs

TGAATAGACTATTCAAAAAAGGCTAATAGGTATTAATACCTATTAGCCTTTTCTTAGGA 780

GACAGATTGTGATTAGAATTAATAACGTAAGATACTAATCCATTAGTCCTGTTTATTGG 840

L I R I N N V K I L I P L V L F I G

AATACTCGTTGGTGCCGGATATGAATACTATAGTTACGAAATTTTCAAGTTGAGATGAT 900

I L V G A G Y E Y Y S S G N F Q V E M M

GAAGGAAGTCAATATAAATCAATTAATGGCTCTATCTAGCATTAATCTAAAAAATACTGC 960

K E V N I N Q L M A L S S I I L K N T A

CGCGTTCTATTACTGGGTTTAACATTGATTGTTGGAAAATGGTTCATAATACTATTCTT 1020

A F L L L G L T L I V G K W F I I L F F

TTTAATAAATGGATTTAAACATAGGAATGTTTCAGTGTCATTTATCAATAATGAGTTTAT 1080

L I N G F N I G M F S V H L S I I E F I

AGTATTGTTTTTACCACATGGAATTTTTCAGTATGTAGTATTCTATTATTGAGAGTAAAG 1140

V L F L P H G I F E Y V V F Y Y *

TTTTCATAATAGATTAGATGGATCGCCTAGTAAAGCTT

Fig. 2. DNA sequence surrounding the *Pst*I site. Putative ribosome-binding sites (rbs) are indicated. A potential transcription terminator sequence is shown by horizontal arrows. The putative processing site in ORF-2 is indicated by a vertical arrow. The nucleotide sequence of both strands has been determined.

features, such as promoter sequences, terminator elements or palindromic structures capable of binding DNA-binding proteins (Sauer & Pablo, 1984).

Identification of ORF-2 as the acidocin-B-encoding gene

Acidocin B was purified in an attempt to determine part of its amino acid sequence. The compound could be purified to apparent homogeneity by taking advantage of its hydrophobic nature. More than 95% of the bactericidal activity could be recovered in the organic phase after extraction of the supernatant with 2-propanol. Subsequent purification of the protein by repeated reversed-phase HPLC resulted in a highly purified preparation

Fig. 3. Purification of acidocin B by HPLC. Arrows indicate peak fractions containing bactericidal activity in a *C. sporogenes* plate assay. Elution profiles (a) and (b) represent first and second purification cycles, respectively.

Table 1. Amino acid composition of acidocin B

Amino acid	Sample	No. of residues (deduced from DNA sequence)
Asx	2	2
Glx	1–2	1
Ser	3	3
Gly	8	7
His	1	1
Arg	1	1
Thr	5	5
Ala	15–16	18
Pro	1–2	1
Tyr	0–1	1
Val	2–3	2
Met	–	–
Cys	*	–
Ile	4	4
Leu	8	8
Phe	2	2
Lys	1	1
Trp	–	2

* Oxidation products detected.

(Fig. 3b) which displayed bactericidal activity against *C. sporogenes*. The activity of the purified polypeptide against other test bacteria has not yet been determined.

The presence of a single protein fraction with acidocin B activity suggests that acidocin B is a single peptide. This conclusion must, however, be regarded with some reservation, as the purified peptide had lost most of its activity. Individual peptides of two-component bacteriocins such as lactacin F exhibit a low intrinsic activity. The activity and specificity can be altered by complementation with the missing peptide (Allison *et al.*, 1994). We therefore cannot rule out the possibility that acidocin B is

composed of two peptides, and that the purification procedure resulted in separation of the two constituents.

Attempts to determine the N-terminal amino acid sequence of the purified peptide have been unsuccessful, suggesting that the N-terminal amino acid is blocked. The amino acid composition of the purified peptide indicates that it may contain as many as 59–62 residues (Table 1). Comparison of the amino acid composition of the purified peptide with the deduced amino acid sequences of the four ORFs indicates that acidocin B is encoded by ORF-2. An almost perfect correlation between the amino acid composition and the deduced amino acid sequence was found, assuming that acidocin B is synthesized as a precursor of 91 amino acids which is processed between amino acids 32 and 33 (Fig. 4) to yield mature acidocin B (59 amino acids). The gene coding for acidocin B has been designated *acdB*.

Acidocin B shows features typical for class I type peptide bacteriocins but lacks a Gly–Gly processing site

Acidocin B shows features that are characteristic of class I type peptide bacteriocins. The peptide is heat-stable and has a size similar to that of other members of this class. Acidocin B has a high Ala content (30%), and together with Leu, Ile, Val, Phe, Trp and Gly, the percentage of non-polar residues is 70 (Table 1). The extreme hydrophobic nature of acidocin B explains its aberrant migration behaviour in SDS-polyacrylamide gels (Vossen *et al.*, 1994) and its behaviour during purification. Acidocin B and the putative polypeptides encoded by ORFs 1 and 3 contain transmembrane helices (although they do not show any amphipathic properties) as observed in lactococcin G (Nissen-Meyer *et al.*, 1992). Acidocin B lacks cysteine residues, which rules out the possibility that the peptide is a lantibiotic (Kellner & Jung, 1989). No sequence homology was observed between acidocin B and bacteriocins from other LAB.

Inspection of the amino acid sequence of the putative leader peptide of acidocin B shows the presence of two cationic amino acids near the N-terminus, a high percentage of hydrophobic amino acids (13 Leu, Val and Ile) in the central region, and two small, neutral amino acids at positions –3 and –1 relative to the cleavage site (Fig. 2). The putative secretory signal peptide could form an α -helical structure ending immediately after the cleavage site. Such a sequence conforms to the rules of von Heijne (1983).

No Gly–Gly doublet, a characteristic feature of processing sites in class I bacteriocins (Kok *et al.*, 1993), is found immediately upstream of the predicted cleavage site, or in its vicinity. Apparently, secretion and processing of acidocin B does not take place by a signal-peptide-independent pathway with a dedicated peptidase recognizing the Gly–Gly motif. In addition acidocin B does not appear to be exported by a signal-sequence-independent pathway requiring the products of *lcnC* and *lcnD* as was observed for lactococcin A (Belkum *et al.*, 1992). In the *lcnA* operon *lcnC* and *lcnD* are located immediately

Fig. 4. Amino acid sequence of part of the N-terminal region derived from ORF-2 (a) and a hypothetical ancestor sequence from which it could have been derived (b). Putative processing sites are indicated by vertical arrows. Conserved amino acids in N-terminal sequences of class I bacteriocins are underlined. The DNA regions where a hypothetical deletion (d) and insertion (i) have occurred are given between horizontal arrows. Nucleotide 485 is given as N since deletion of any nucleotide at this position will result in an Ile codon.

upstream of the bacteriocin-encoding gene, *lcnA*. The size of the remaining unidentified sequences of the *Xba*I–*Bam*HI fragment of pCV461-3 is insufficient to carry genes of this size. Although the possibility that LcnC- and LcnD-like proteins are chromosomally encoded cannot be excluded it seems unlikely that acidocin B is exported by this route, as processing through this pathway also relies on the presence of a Gly–Gly doublet. From these data we tentatively conclude that the acidocin B precursor is processed by the general signal-sequence-dependent peptidase.

The apparent paradox that acidocin B belongs to class I bacteriocins as judged from its (physico)chemical and biological properties, but is not exported and processed by a Gly–Gly-dependent protease, may be resolved by assuming that acidocin B has evolved from an ancestor protein by means of two frameshift mutations (–1 and +1) which have changed the reading frame in the region between the mutations. By deletion of a nucleotide between coordinates 429 and 487 in the ancestor protein, the ancestral sequence TTG.GGC.GGT.TTT (Leu–Gly–Gly–Phe) would read in AcdB as TGG.GCG.GTT.TTA (Trp–Ala–Val–Leu). By insertion of a nucleotide in the ancestor protein between coordinates 497 and 523, which generates the consensus sequence for a signal-peptide-dependent protease, the original reading frame would be restored. As a result of the two mutations the mature bacteriocin would have been truncated by 10 amino acids. It should be noted that hydrophobic amino acids are found at positions –4 (I), –7 (L) and 12 (L), and charged amino acids at –8 (E) and –10 (K), and a Ser residue at –11 with respect to the presumed Gly–Gly cleavage site (Fig. 4). In other class I LAB bacteriocins these positions are occupied by the same amino acids (Fremaux *et al.*, 1993). If we assume that these residues were conserved to ensure the functionality of this part of the protein, this would mean that the region in which the nucleotide was deleted can be narrowed down to the sequence between nucleotides 485 and 488. The similarity of the N-terminal amino acid sequence of the acidocin B

precursor with that of class I bacteriocins, but the presence of the signal peptide cleavage site would suggest that terminal sequences of Gly-Gly-dependent proteases and signal sequences may be interchanged.

Features of other ORFs

ORFs 1 and 3 code for highly hydrophobic polypeptides (58 and 64% hydrophobicity), as was observed for bacteriocins and immunity proteins of other organisms (Belkum *et al.*, 1992; Nissen-Meyer *et al.*, 1992; Klaenhammer, 1993; Kok *et al.*, 1993), whereas ORF-4 codes for a peptide of 40 % hydrophobicity. A comparison of the deduced amino acid sequence or nucleotide sequence of ORFs 1, 3 and 4 with that of bacteriocins and immunity proteins from other organisms showed no obvious similarity. ORF-1 codes for a protein with a highly charged C-terminus (KQHKKM). Similar cationic sites are present at the C-terminal end of other peptide bacteriocins like lactococcins A and G (Holo *et al.*, 1991), lactacin F (Mariana & Klaenhammer, 1991) and pediocin PA-1 (Marugg *et al.*, 1992). No Gly-Gly doublets were found in ORFs 1, 3 and 4.

The location of the acidocin-B-encoding gene between two genes which could code for small, hydrophobic peptides strongly resembles the organization of bacteriocin operons found in other organisms. The two genes coding for the two peptides constituting lactacin F form an operon, together with a downstream ORF which could code for a hydrophobic polypeptide of 124 amino acids believed to be the precursor of the immunity protein (Fremaux *et al.*, 1993). Similarly, in the lactococcin A, B and M operons, and in the pediococcin PA-1 and leucocin A-UAL 187 operons, the gene coding for the bacteriocin is flanked at the 3' end by a gene varying in size between 300 and 460 nucleotides, and coding for a small immunity protein (Klaenhammer, 1993; Kok *et al.*, 1993). From the position of the gene relative to *acdB*, and the similarity in length and hydrophobicity with other immunity-protein-encoding genes, it is tempting to speculate that ORF-3 codes for the immunity protein.

Fig. 5. Inhibition of *C. sporogenes* by culture supernatants (neutralized) of *Lactobacillus* in an agar well diffusion assay. Wells: A, culture supernatant of *L. acidophilus* M46; B and C, supernatant of *L. plantarum* transformants containing the 4 kb *XbaI*–*Bam*HI fragment (Fig. 1) cloned in pGKV21 and pLPE24*, respectively.

Expression of acidocin B in heterologous hosts

The 4 kb *XbaI*–*Bam*HI fragment of pCV461 was cloned in the high-copy-number *Lactobacillus* vector pLPE24M* (~80 copies per bacterium) and in the low-copy-number vector pGKV21 (~20 copies per bacterium). Both vectors were introduced into *L. plantarum* 80. Acidocin B production by *L. plantarum* transformants was visualized using a plate assay. Fig. 5 shows that the level of acidocin B production of *L. plantarum* transformants increases as the copy number of the cloned acidocin-B-encoding genes increases. It appears that expression and secretion of acidocin B in *L. plantarum* is solely dependent on gene dosage and is not limited by host factors.

After introduction of pCV461-3 into *L. fermentum* NCK127, transformants were obtained that expressed acidocin B (not shown). Since this organism is sensitive to acidocin B, these results strongly suggest that the immunity-protein-encoding gene is present within the 4 kb DNA fragment, consistent with the above proposal that the immunity protein is encoded by ORF-3.

If we are correct in our assumption that acidocin B is processed by a signal-sequence-dependent peptidase, an enzyme which is ubiquitously present in bacteria, then acidocin B can, in principle, be synthesized in any bacterial species, unlike other non-lantibiotic and lantibiotic bacteriocins (Simonen & Palva, 1993; Smith *et al.*, 1987, 1988). Considering that at least some strains of *Lactobacillus* are sensitive to acidocin B, as demonstrated for *L. fermentum* NCK127, the acidocin-B-encoding genes may be useful for the development of food-grade vectors (Vossen *et al.*, 1994).

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