Characterization of four plasmids harboured in a *Lactobacillus brevis* strain encoding a novel bacteriocin, brevicin 925A, and construction of a shuttle vector for lactic acid bacteria and *Escherichia coli*

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In this study we isolated over 250 lactic acid bacteria (LAB) candidates from fruit, flowers, vegetables and a fermented food to generate an LAB library. One strain, designated 925A, isolated from kimchi (a traditional Korean fermented dish made from Chinese cabbage) produced a novel type of bacteriocin, brevicin 925A, which is effective against certain LAB, including strains of *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Bacillus* and *Listeria*. Strain 925A, identified as *Lactobacillus brevis*, harboured at least four plasmids and we determined the entire nucleotide sequence of each one. The four plasmids were designated pLB925A01–04, and have molecular sizes of 1815, 3524, 8881 and 65 037 bp, respectively. We obtained bacteriocin non-producing derivatives by treatment of strain 925A with novobiocin. All of these derivatives, which were susceptible to their own antibacterial product, lost the largest plasmid, pLB925A04, suggesting that the genes for bacteriocin biosynthesis (*breB* and *breC*) and immunity (*breE*) are located on pLB925A04. The partial amino acid sequence of purified brevicin 925A and sequence analysis of pLB925A04 showed that *breB* is the structural gene for brevicin 925A. We constructed a shuttle vector (pLES003, 6134 bp) that can replicate in both *Escherichia coli* and LAB such as *Lactobacillus plantarum*, *Lb. brevis*, *Lactobacillus helveticus*, *Lactobacillus hilgardii* and *Enterococcus hirae*. To determine the function of gene *breE*, which displays no significant similarity to any other sequences in the BLAST search database, the gene was inserted into pLES003. A pLB925A04-cured derivative transformed with pLES003 carrying *breE* acquired immunity to brevicin 925A, suggesting that *breE* encodes an immunity protein.

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INTRODUCTION

Lactic acid bacteria (LAB) is the generic name given to bacteria that produce large amounts of lactic acid by fermentation of various sugars and other carbohydrates. LAB have been suggested to improve the gut microflora and are regarded as potentially beneficial bacteria with

therapeutic effects for the health of the host, such as anti-allergy properties and intestinal homeostasis (Adolfsson *et al.*, 2004; Cross *et al.*, 2001; Heyman, 2000; Meydani & Ha, 2000), and are often termed 'probiotics' (Guarner & Schaafsma, 1998).

LAB are roughly classified into two groups according to the source of isolation. One group comes from animal sources, such as fermented raw milk, cheese and intestines. The other group is isolated from plant sources, such as grasses, silage, vegetables and fruit (Lavermicocca *et al.*, 2005; Okada, 2002; van Hylckama Vlieg *et al.*, 2006). Although

Abbreviations: LAB, lactic acid bacteria; RCR, rolling-circle replication.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB370334, AB370335, AB370336, AB370337 and AB370338.

Lactococcus lactis is a primary constituent of starter cultures for many fermented dairy products, the same species can also be isolated from plant material. Our interest is in the molecular and phenotypic diversity found in animal- and plant-derived LAB.

In the present study, we isolated 250 LAB from fruit, flowers, vegetables and a fermented dish to generate an LAB library. In the plant-derived library, a strain numbered 925A, and identified as *Lactobacillus brevis*, was isolated from kimchi (a traditional Korean fermented dish made from Chinese cabbage). This strain produces a bacteriocin (brevicin 925A) which inhibits the growth of *Listeria monocytogenes* and *Streptococcus mutans*. Since *Lb. brevis* 925A harbours at least four plasmids, we determined whether the bacteriocin-biosynthetic cluster is located on any of these plasmids or the chromosome by analysing the entire nucleotide sequence of the four plasmids.

In addition, to develop one of these plasmids as a genetic tool for LAB, we created a shuttle vector carrying the replication origins of pLB925A03 and pUC19. The constructed vector, pLES003, was able to replicate in *Escherichia coli* and some LAB. Bacteriocin-susceptible derivatives transformed with pLES003 carrying *breE*, which displays no significant similarity to any other sequences in the BLAST search database, acquired immunity to brevicin 925A, suggesting that *breE* encodes an immunity protein.

METHODS

Media and growth conditions. MRS broth (Merck) was used for the growth of all LAB. *E. coli*, *Staphylococcus* and *Bacillus* strains were cultured in LB medium (Sambrook *et al.*, 1989), and the *Listeria* strains were cultured in tryptic soy broth medium supplemented with 0.3 % yeast extract (TSBYE). *E. coli* DH5 α and plasmid pUC19 were used for the cloning experiments. When required, ampicillin (100 $\mu\text{g ml}^{-1}$), erythromycin (300 $\mu\text{g ml}^{-1}$ for *E. coli*, and 5 $\mu\text{g ml}^{-1}$ for LAB) and 1.5 % agar were added.

Assay of antibacterial activity. The antibacterial activity of bacteriocins produced by LAB was determined by the agar diffusion method (Klaenhammer, 1988; van Reenen *et al.*, 1998). Bacteriocin-containing supernatant fluid, obtained by centrifugation of LAB cultures, was adjusted to pH 7.0, membrane-filtered and used for the antibacterial assay. The bacteria listed in Table 2 were used as test organisms to investigate the antibacterial spectra at a concentration of 10^6 cells ml^{-1} .

Isolation and identification of bacteriocin-producing bacteria. Each strain of LAB isolated from plants was inoculated into MRS broth and incubated anaerobically at 28 or 37 °C for 2–3 days. An aliquot of the culture was plated onto MRS broth supplemented with 1.5 % agar and incubated at the given temperature for 2–3 days. Each colony generated on the plate was re-spread onto fresh MRS agar medium for colony purification. We carried out Gram-staining, and organic acid- and catalase-production tests on the purified colonies prior to species identification.

To identify the LAB candidates, we analysed the sugar fermentation profile of each strain using an API 50 CHL kit (bioMérieux). Furthermore, the entire 16S rDNA sequence of the collected LAB was determined as described previously (Hiraishi, 1992; Lane, 1991;

Weisburg *et al.*, 1991) and compared with that of typical LAB obtained from the DNA Data Bank of Japan (DDBJ) website (www.ddbj.nig.ac.jp/Welcomes-e.html). LAB species were identified by analysis of the sequence alignment using the CLUSTAL W program (<http://clustalw.ddbj.nig.ac.jp/top-e.html>).

Purification of bacteriocin. To purify bacteriocin produced extracellularly by *Lb. brevis* 925A, the bacterium was grown in 1 l MRS broth at 28 °C for 24 h. After removal of the cells by centrifugation at 3000 g for 10 min, the resulting supernatant was applied to a Toyopearl SP-650S column (2.5 $\times$ 4.5 cm; Tosoh) equilibrated with 20 mM sodium phosphate buffer (pH 5.8), and the excess protein was eluted with a 15-bed volume of the same buffer. After the column was washed with a 5-bed volume of 20 mM sodium phosphate buffer containing 0.15 M NaCl, the antibacterial peptide was eluted with a 7-bed volume of the same buffer containing 1 M NaCl. Ammonium sulfate was added to the eluate until the concentration reached 0.42 M, and the resulting solution was applied to a Phenyl-Sepharose column (1.0 $\times$ 10 cm; Amersham Biosciences) equilibrated with 20 mM sodium phosphate buffer (pH 5.8) containing 1 M NaCl and 0.42 M ammonium sulfate. The column was then washed with a 6-bed volume of an equilibrating buffer. The antibacterial fraction was eluted with a 1–0 M NaCl and 0.42–0 M ammonium sulfate linear gradient in the same buffer and finally eluted with water containing 0.1 % trifluoroacetic acid. The resulting fractions were collected, and a portion of each fraction was tested for antibacterial activity. The active fractions were desalted with PD-10 (Amersham Biosciences) and dried *in vacuo*. The fraction containing antibacterial activity was stored at –20 °C until use. During these purification steps, the antibacterial activity contained in each fraction was evaluated using *Lactobacillus plantarum* NBRC 3070 as a test organism.

Amino acid sequencing of bacteriocin. The peptide was rehydrated, extracted from an SDS-PAGE gel, digested with trypsin and then concentrated in a centrifugal vacuum concentrator. After desalting of the concentrate using Zip-TipC18 tips (Millipore), the samples were eluted in 50 % acetonitrile/0.1 % trifluoroacetic acid, and a 1 μl portion of the eluate was mixed with a matrix solution (1 : 1 ratio) and applied on a MALDI target plate. Acquisition of MS data was performed on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). After acquisition and mass axis recalibration, database searches were performed using the MASCOT program (Matrix Science).

Production of bacteriocin non-producing derivatives. Bacteriocin non-producing derivatives from *Lb. brevis* 925A were isolated according to the method described by Chin *et al.* (2005) with a slight modification: an aliquot of the culture broth was seeded (approx. 10^5 c.f.u. ml^{-1}) in MRS broth containing 5, 10, 20, 40 and 50 $\mu\text{g novobiocin ml}^{-1}$, which is an inhibitor of bacterial DNA gyrase. After cultivation at 28 °C for 2–3 days, a portion of the cultures was plated onto fresh MRS agar and anaerobically incubated at the same temperature for 2–3 days. The bacteriocin-sensitive derivatives were selected from the colonies generated on the plate, and plasmid profiles of each colony were investigated. A bacteriocin-sensitive derivative from strain 925A, designated *Lb. brevis* 925A- Δ 02/03/04, which harbours plasmid pLB925A01 but not pLB925A02, pLB925A03 and pLB925A04, was used as a host for the plasmid introduction experiments.

DNA preparation and manipulation. A plasmid from *E. coli* was isolated by a standard method (Sambrook *et al.*, 1989). Chromosomal DNA and plasmids from *Lb. brevis* were isolated from cells grown at 28 °C for 24 h as described previously (Hiraishi, 1992) with slight modifications. Cells collected by centrifugation were washed with a glucose-EDTA buffer (50 mM glucose, 25 mM Tris/HCl, 10 mM

EDTA, pH 8.0) and resuspended in the same buffer containing 10 µg RNase ml⁻¹ (Roche), 0.4 mg achromopeptidase ml⁻¹ (Wako Pure Chemicals) and 4 mg lysozyme ml⁻¹ (Wako Pure Chemicals). After cell lysis by incubation at 37 °C for 1 h, protein denaturation and chloroform/isoamylalcohol extraction were carried out. The chromosomal and plasmid DNAs were concentrated by PEG precipitation. If necessary, the plasmid was purified by CsCl₂ density-gradient centrifugation.

DNA sequencing. Nucleotide sequencing was achieved with the ABI PRISM 310 genetic analyser using the BigDye Terminator v1.1 Cycle Sequencing kit according to the manufacturer's protocol (Applied Biosystems). Genetic analysis was performed using ATGC and GENETYX software (Genetyx Corporation). ORFs were predicted using an ORF finder tool at NCBI (www.ncbi.nlm.nih.gov/gorf/gorf.html). Homology searches were performed with the BLAST algorithm (Altschul *et al.*, 1990) utilizing the non-redundant database provided by NCBI.

Nucleotide sequence analysis of plasmids. pLB925A01 and pLB925A02 DNA, extracted from agarose gels, was digested with *NheI* and *EcoRI*, respectively, and subcloned into the same site of pUC19. In the case of pLB925A03, the *PstI*-digested 0.6 kb fragment was subcloned into pUC19. Another region of pLB925A03 was amplified by PCR using pLB925A03 as a template with sense (5'-CTTAT-GAAAAGTCAAAGGAAGTGCCTAT-3') and antisense (5'-TGATTT-TCTACGTGCGAAATTGTTGTGA-3') primers designed on the basis of the 0.6 kb *PstI*-digested sequence. The nucleotide sequence of the amplified 8.2 kb DNA was determined by the direct sequence method.

Because the size of pLB925A04 was expected to be quite large, the bacteriocin gene cluster region was first subcloned into pUC19. Restriction-digested pLB925A04 DNA was fractionated using 0.5% agarose gel electrophoresis and transferred to a Hybond-N+ membrane (GE Healthcare) using a standard protocol (Sambrook *et al.*, 1989). A DNA probe (222 bp) for cloning of the bacteriocin biosynthetic gene was PCR-amplified with sense (5'-ATGGA-GAAATTCGAGTGTT-3') and antisense (5'-TTATTTGTTATTT-AGGCAGC-3') primers, using the *Lb. brevis* 925A plasmid DNA as a template. These primers were designed on the basis of the nucleotide sequence of the gene encoding plantaricin 1.25γ (PRF accession no. 2607194A). PCR was conducted under the following conditions: 1 cycle of 5 min at 96 °C, 15 s at 58 °C and 30 s at 72 °C followed by 29 cycles of 1 min at 96 °C, 15 s at 58 °C and 30 s at 72 °C, and finally a 7 min extension period at 72 °C. Labelling of the probe and detection were performed using an AlkPhos direct labelling and detection kit (GE Healthcare), according to the manufacturer's instructions. The hybridized DNA fragment was extracted from the agarose gel and ligated into the same site of pUC19. The chimaeric plasmid was introduced into *E. coli* DH5α and screened using a colony hybridization technique (Sambrook *et al.*, 1989). After determination of the nucleotide sequence around the bacteriocin biosynthetic gene, the entire nucleotide sequence of pLB925A04 was determined using inverse PCR (Sambrook & Russell, 2001). For inverse PCR, *Lb. brevis* 925A plasmid DNA was digested with an appropriate single restriction enzyme and purified by the phenol/chloroform extraction method. The purified DNA fragments were self-ligated using a DNA Ligation kit (TaKaRa) at a concentration of about 1 ng µl⁻¹, and a 4 µl portion of the self-ligated mixture was used as a template for inverse PCR together with properly designed primer pairs. The nucleotide sequence of the PCR product was determined by the direct sequencing method.

Construction of the shuttle vector pLES003. The shuttle vector pLES003 contains the erythromycin resistance gene together with the replication origins of pLB925A03 and pUC19. The drug-resistance

gene from plasmid pH2515 (Shimizu-Kadota *et al.*, 1991), expressed in *Enterococcus*, was obtained by PCR amplification using sense (5'-CATATGTAGAAGCAAACCTTAAGAGTGTGTT-3') and antisense (5'-CATATGATAGACGGTTTTTCGCCCTTTGAC-3') primers (*NdeI* sites underlined). The amplified DNA was digested with *NdeI* and inserted into the same site of pUC19 to yield pre-pLES003. Next, the *ori* region of pLB925A03 was amplified by PCR using pLB925A03 as a template with sense (5'-GACGTCGCTGTGTCGTACATAATT-ATACGAA-3') and antisense (5'-GACGTCAGTCTGCCTATTCTCT-GCTTGAAAA-3') primers (*AatII* sites underlined). The PCR product was digested with *AatII* and inserted into *AatII*-digested pre-pLES003 to yield pLES003.

Construction of a plasmid carrying *breE*. The *breB* promoter region was amplified by PCR using sense (5'-GCTCTAGA-GGCTGTCTGTCCTTA-3'; *XbaI* site underlined) and antisense (5'-AAGGATCCCGCTCTCCCTCTTATTT-3'; *BamHI* site underlined) primers. The amplified DNA fragment was digested with *XbaI* and *BamHI*, and inserted into the same sites of pLES003. Next, the *breE* gene was amplified by PCR using sense (5'-AAGG-ATCCATGAAAGCTATTATGAAA-3'; *BamHI* site underlined) and antisense (5'-CCGAATTCTCAATAAATACGTTTGAT-3'; *EcoRI* site underlined) primers. The amplified DNA was digested with *BamHI* and *EcoRI* and subcloned into the same sites of pLES003 with the *breB* promoter to yield pLES003/*breE*.

Transformation of LAB. The preparation of competent cells from bacteriocin non-producing derivatives and other LAB was conducted according to the methods described by Alegre *et al.* (2004) and Dunny *et al.* (1991). Competent cells (0.1 ml) mixed with plasmid DNA were placed into a 0.2 cm electroporation cuvette from a Gene Pulser apparatus (Bio-Rad) and held on ice for 5 min. After pulsing under specific conditions (field strength, 12.5 kV cm⁻¹; capacitance, 25 µF; resistance, 400 Ω), the cell suspension was immediately diluted with 1 ml MRS broth and then incubated at 28 or 37 °C for 5 h. A portion of the cell suspension was placed on selective media and incubated anaerobically at 28 or 37 °C for 1–2 days.

RESULTS

Identification of bacteriocin-producing strains

In this study we isolated many LAB candidates from fruit, flowers and vegetables to generate an LAB library. Out of 328 candidate strains, 250 (about 76%) were classified as LAB on the basis of their 16S rDNA sequence (data not shown). Most of the LAB isolates from vegetables and flowers were classified in the genera *Enterococcus* and *Lactococcus*, respectively, whereas strains from fruit and other plants belonged to a wide range of genera (Table 1). We found that the culture broth of one strain, which was isolated from kimchi and designated 925A, possessed antibacterial activity. Carbohydrate utilization profiles, analysed with API 50 CHL, suggested that strain 925A is a strain of *Lb. brevis*. The entire 16S rDNA sequence also exhibited the highest level of similarity with the sequences of *Lb. brevis* R097 (accession no. AF515219, 99.67% identity), R066 (AF515220, 99.736%) and ATCC 367 (CP000416, 99.802%), confirming that strain 925A is indeed a strain of *Lb. brevis*.

As listed in Table 2, the antibacterial activity produced in the 925A liquid culture was effective against the strains of

Table 1. Origin of isolates of plant-associated LAB

Genus	No. of isolates from:				
	Fruit	Vegetables	Flowers	Fermented vegetables	Other plants
<i>Enterococcus</i>	29	46	1	0	6
<i>Lactobacillus</i>	27	0	0	12	4
<i>Lactococcus</i>	7	0	44	0	9
<i>Leuconostoc</i>	7	2	7	0	2
<i>Streptococcus</i>	14	1	2	0	0
<i>Pediococcus</i>	16	0	0	0	0
Other genera	8	4	0	0	2

Bacillus, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Listeria* and *Streptococcus*. Interestingly, it was effective against *Listeria monocytogenes*, a food pathogen, and an oral bacterium, *S. mutans*. In addition, the antibacterial activity

Table 2. Antibacterial activity of brevicin 925A

Species	Indicator strain*	Activity
Gram-positive bacteria		
<i>Bacillus coagulans</i>	NBRC 12583	++
<i>Bacillus cereus</i>	ATCC 11778	—
<i>Bacillus licheniformis</i>	NBRC 12195	—
<i>Bacillus sphaericus</i>	NBRC 15095	—
<i>Bacillus stearothermophilus</i>	NBRC 13737	—
<i>Bacillus subtilis</i>	ATCC 6633	—
<i>Enterococcus hirae</i>	NBRC 3128	+
<i>Enterococcus faecalis</i>	NBRC 12964	—
<i>Lactobacillus curvatus</i>	NBRC 15884	—
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	NBRC 3073	—
<i>Lactobacillus helveticus</i>	NBRC 15019	—
<i>Lactobacillus hilgardii</i>	NBRC 15886	+++
<i>Lactobacillus sakei</i>	NBRC 15893	++
<i>Lactobacillus plantarum</i>	NBRC 3070	++
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	NBRC 100676	++
<i>Listeria monocytogenes</i>	ATCC 7644	—
<i>Listeria monocytogenes</i>	ATCC 15313	+
<i>Pediococcus pavulus</i>	NBRC 100673	—
<i>Staphylococcus aureus</i>	NBRC 12732	—
<i>Staphylococcus aureus</i>	IID 1677 (MRSA)	—
<i>Staphylococcus epidermidis</i>	NBRC 12993	—
<i>Streptococcus mutans</i>	MT 8148R	+
<i>Streptococcus sobrinus</i>	ATCC 27607	—
<i>Streptococcus equinus</i>	NBRC 12553	—
Gram-negative bacteria		
<i>Escherichia coli</i>	NBRC 3972	—
<i>Salmonella enteritidis</i>	NBRC 3313	—
<i>Pseudomonas aeruginosa</i>	NBRC 13275	—

*ATCC, American Type Culture Collection, Manassas, VA, USA; IID, Laboratory Culture Collection, Institute of Medical Sciences, University of Tokyo, Japan; NBRC, Biological Resource Center, National Institute of Technology and Evaluation (NITE), Kisarazu-shi, Chiba, Japan; MRSA, methicillin-resistant *Staphylococcus aureus*.

was resistant to heat treatment at 100 °C for 10 min, but not to proteases such as proteinase K and trypsin (data not shown), suggesting that the antibacterial substance is a polypeptide of the bacteriocin type. In this study, the antibacterial polypeptide from strain 925A was designated brevicin 925A.

Purification and characterization of brevicin 925A

Brevicin 925A was purified from the supernatant fluid from an overnight culture of strain 925A by cation-exchange and hydrophobic chromatography. Since Phenyl-Sepharose chromatography yielded a single peak for antibacterial activity (Fig. 1), the amino acid sequence of brevicin 925A was determined by *de novo* sequence analysis after peptide mass fingerprint analysis and an MS/MS ion search were performed (data not shown). The partial amino acid sequence of the N-terminal region of brevicin 925A was KKKKKYTGPNYR.

Plasmid profile and curing experiments

The agarose gel electrophoresis profile suggests that *Lb. brevis* 925A has at least four plasmids (Fig. 2a). We named these four plasmids pLB925A01–04 in order of size from small to large. To determine whether the genes for bacteriocin biosynthesis and immunity to their own antibiotic product are located on the plasmids, plasmid-curing experiments were conducted on *Lb. brevis* 925A. We observed that bacteriocin non-producing derivatives lost one to four of the plasmids randomly, but all of the derivatives lost the largest plasmid pLB925A04 with similar frequency. In addition, these derivatives also lost immunity

Fig. 1. Hydrophobic chromatography of partially purified brevicin 925A recovered from cation-exchange chromatography. Each fraction obtained by chromatography was assayed for antibacterial activity. Antibacterial activity (growth inhibition, solid line) was measured using *Lb. plantarum* NBRC 3070 as a test organism. Dashed line, NaCl concentration; dotted line, $(\text{NH}_4)_2\text{SO}_4$ concentration.

Fig. 2. Profiles of plasmids harboured in *Lb. brevis* 925A. (a) Electrophoretic profile of plasmids separated on a 0.5% (w/v) agarose gel. Lanes: 1, λ HindIII molecular mass marker; 2, plasmids isolated from *Lb. brevis* 925A. Each plasmid band was confirmed by Southern blot analysis using a DNA fragment from each plasmid as a probe (data not shown). C, Chromosome. (b, c) Physical map and gene organization of plasmids pLB925A01–04. Gene organization in the putative brevicin 925A gene cluster is also indicated in (c). The filled black arrows indicate the transcriptional regulator.

to brevicin 925A, suggesting that the genes for bacteriocin production and immunity are on plasmid pLB925A04.

During the plasmid-curing trials, pLB925A04 was cured with high frequency (~55 %), but the smaller plasmids had a tendency to be maintained in *Lb. brevis* 925A.

Sequence analysis of pLB925A01–04

We determined the entire nucleotide sequence of the four plasmids maintained in the strain 925A. pLB925A01–04 have molecular sizes of 1815, 3524, 8881 and 65 037 bp, respectively. Fig. 2 shows the gene organization of each plasmid. The mean G + C contents of pLB925A01–04 are 37.3, 37.5, 42.2 and 40.8 mol%, respectively, whereas that of the *Lb. brevis* ATCC 367 chromosome is 46.2 % (Makarova *et al.*, 2006). All ORFs larger than 50 bp were compared against the protein database using the BLAST server as shown in Table 3.

The ORFs seen on each plasmid are annotated as follows.

(i) pLB925A01 and pLB925A02. Both plasmids may be replicated via a rolling-circle replication (RCR) mechanism. pLB925A01 has two genes, annotated *repA* and *copA*, which are involved in plasmid replication. The *repA* gene product exhibits high similarity with the Rep protein from *Lactobacillus helveticus* ATCC 15009 plasmid pLH2 (Pridmore *et al.*, 1994), and the *copA* gene product is similar to the RepA protein from *Lb. plantarum* WCFS1 (van Kranenburg *et al.*, 2005).

pLB925A02 has the sequence 5'-TTCTTCTTATCTTGAT-ACTA-3' located upstream of *repA*. The sequence is homologous to the double-stranded origin (*dso*) of the pC194/pUB110-type RCR plasmid (Bouia *et al.*, 1989; del Solar *et al.*, 1998; Leer *et al.*, 1992). The *repA* gene product is almost the same as the replication-associated protein from *Lactobacillus casei* A23 plasmid pSMA23 (accession no. DQ116709). Thus, pLB925A02 may belong to the pC194/pUB110 family, but the plasmid control system has never been identified (del Solar *et al.*, 1998).

(ii) pLB925A03. This plasmid contains eight ORFs, which consist of three genes (*repA*, *repB* and *repC*) for replication, three genes for mobilization and two unknown genes (Fig. 2b & Table 3). Although the *repC* gene product is similar to a hypothetical protein that controls plasmid copy number of *Lactobacillus paracasei* subsp. *paracasei* NFBC 338 plasmid pCD01 (Desmond *et al.*, 2005), it is truncated. While RepB also exhibits high similarity with the putative replication protein (ORF9) from pCD01 (Desmond *et al.*, 2005), the *ori* sequence for *repB* is absent. Therefore, the *repB* and *repC* gene products might not be functional. The *repA* product exhibits high similarity (88 %) to the replication protein of pLJ42 from *Lb. plantarum* (accession no. DQ099911). The upstream sequence of *repA* contains a 10 bp direct repeat and a 22 bp direct repeat, which suggests that it is similar to the

pUCL287-type replication origin (Desmond *et al.*, 2005) (Fig. 3a).

(iii) pLB925A04. pLB925A04 has a putative *ori* downstream of *repA* (Fig. 3b), and the iteron region of this plasmid is similar to that of pAM β -1-family plasmids (del Solar *et al.*, 1998).

This plasmid seems to contain several genes that may be involved in brevicin 925A biosynthesis (Fig. 2c & Table 3). As shown in Fig. 2(c), the predicted gene cluster contains the structural genes for bacteriocin biosynthesis (*breB* and *breC*), transcriptional regulators (*breD* and *breG*), an ABC-transporter (*breH*) and its accessory protein (*breA*). The deduced amino acid sequence of the N-terminal region of BreB is identical to the partial amino acid sequence KKKKKYTGPNYR from purified brevicin 925A, indicating that *breB* is the structural gene for brevicin 925A. The deduced amino acid sequence of BreB is identical to that of PlnC in *Lb. plantarum* TMW1.25 (Ehrmann *et al.*, 2000). The function of PlnC in *Lb. plantarum* TMW1.25 has not yet been elucidated. The deduced amino acid sequence of BreC was also identical to the amino acid sequence of the bacteriocin plantaricin 1.25 β , which is encoded by *plnB* of *Lb. plantarum* TMW1.25 (Ehrmann *et al.*, 2000; Remiger *et al.*, 1999). Like *breB*, which is located just upstream of *breC*, *plnC* is located just upstream of *plnB* in TMW1.25 (Ehrmann *et al.*, 2000). The organization of the structural genes for plantaricin biosynthesis (*plnC* and *plnB*) appears to be similar to that for brevicin (*breB* and *breC*); however, the genes for an ABC transporter and transcriptional regulators, which are evident in the brevicin 925A biosynthetic gene cluster, are absent in *Lb. plantarum* TMW1.25 (Ehrmann *et al.*, 2000). The gene products of *breD* and *breG* contain a helix–turn–helix DNA-binding motif (COG1476). *breE*, which displays no significant similarity in the BLAST search database, is co-transcribed with *breD*, a fact confirmed by RT-PCR analysis (data not shown).

Construction of a shuttle vector for LAB and *E. coli*.

To construct a shuttle vector for LAB and *E. coli*, which will be useful as a gene manipulation tool for LAB, we employed the replicon of pLB925A03. The shuttle vector, designated pLES003, consists of a replication origin from pLB925A03, *repA*, a ColE1 origin, the erythromycin resistance gene from vector pH2515 (Shimizu-Kadota *et al.*, 1991), and the multi-cloning site from pUC19 (Fig. 4). We confirmed that the vector could be replicated in cells of *Lb. brevis*, *Lb. plantarum*, *Lb. helveticus* and *Enterococcus hirae* (data not shown).

Characterization of the *breE* gene product.

A hydropathy plot of the *breE* gene product, constructed using the SOSUI program (<http://bp.nuap.nagoya-u.ac.jp/sosui>), suggests that the putative protein has four trans-

Table 3. Similarities of ORFs in plasmids pLB925A01–04

Plasmid and gene	Location*	Size (aa)	Best BLAST match (source)	Protein accession no.	Identity (% overlapping amino acids)
pLB925A01					
<i>copA</i>	845–1000	51	Copy-number control protein (<i>Lb. plantarum</i> WCFS1: pWCFS102)	YP_133711	68 (50)
<i>repA</i>	1073–1765	230	Plasmid replication protein (<i>Lb. helveticus</i> ATCC 15009: pLH2)	CAA57504	81 (230)
pLB925A02					
<i>mobA</i>	55–1140	361	Mobilization protein (<i>Pediococcus pentosaceus</i> R64: pRS4)	CAI92903	72 (365)
<i>repA</i>	1839–2795	318	Replication protein (<i>Lb. casei</i> A23: pSMA23)	AAZ30709	99 (318)
pLB925A03					
<i>repB</i>	555–16 C	179	Putative replication protein (<i>Lb. paracasei</i> subsp. <i>paracasei</i> NFBC 338: pCD01)	AAW81271	96 (179)
<i>orf2</i>	1263–760 C	167	Hypothetical protein (<i>Lb. paracasei</i> subsp. <i>paracasei</i> NFBC 338: pCD01)	AAW81289	99 (161)
<i>orf3</i>	3433–1283 C	716	YbfG (<i>Bacillus subtilis</i> subsp. <i>subtilis</i> 168)	CAB12014	38 (697)
<i>repA</i>	4250–5173	307	RepA (<i>Lb. plantarum</i> : pLJ42)	AAZ13604	85 (306)
<i>mobC</i>	5872–6210	112	MobC (<i>Lb. plantarum</i> : pLJ42)	AAZ13605	97 (112)
<i>mobA</i>	6189–7436	415	MobA (<i>Lb. plantarum</i> : pLJ42)	AAZ13606	74 (445)
<i>mobB</i>	7433–8101	222	MobB (<i>Lb. plantarum</i> : pLJ42)	AAZ13607	62 (229)
pLB925A04					
<i>topA</i>	81–2198	705	Hypothetical protein gbs0387 (<i>Streptococcus agalactiae</i> NEM316)	NP_734855	33 (588)
<i>orf2</i>	2200–2982	260	No hits	–	–
<i>clpA</i>	3003–4796	597	Hypothetical protein gbs0388 (<i>Streptococcus agalactiae</i> NEM316)	NP_734856	45 (586)
<i>orf4</i>	4862–5185	107	Hypothetical membrane-spanning protein (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536707	31 (98)
<i>orf5</i>	5256–7427	723	Hypothetical membrane associated protein (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536706	19 (741)
<i>srtA</i>	8220–9059	279	Sortase (<i>Lb. brevis</i> ATCC 367)	YP_794685	37 (162)
<i>orf7</i>	9085–9978	297	No hits	–	–
<i>orf8</i>	10027–12378	783	Hypothetical protein EfaeDRAFT_1901 (<i>Enterococcus faecium</i> DO)	ZP_00603526	26 (654)
<i>orf9</i>	12915–13769	284	Transposase, IS30 family (<i>Pediococcus pentosaceus</i> ATCC 25745)	YP_804033	91 (285)
<i>orf10</i>	13983–14675	230	Dihydrodipicolinate synthase/N-acetylneuraminate lyase (<i>Lb. casei</i> ATCC 334)	YP_805579	60 (217)
<i>orf11</i>	14808–15491	227	Transposase (<i>Lb. casei</i> ATCC 334)	YP_796441	69 (227)
<i>orf12</i>	15730–16431	233	Conserved hypothetical protein (<i>Enterococcus faecium</i> DO)	ZP_00603671	32 (202)
<i>orf13</i>	16989–17423	144	Hypothetical protein OEOE_1493 (<i>Oenococcus oeni</i> PSU-1)	YP_811017	44 (134)
<i>orf14</i>	19218–18379 C	279	Transposase, IS30 family (<i>Lb. brevis</i> ATCC 367: plasmid 2)	YP_796412	100 (279)
<i>orf15</i>	19640–20623	327	Hypothetical protein pRUM_p05 (<i>Enterococcus faecium</i> U37: pRUM_p05)	NP_863148	24 (302)
<i>orf16</i>	20939–21556	205	Recombinase (<i>Enterococcus faecalis</i> : pAM373)	NP_072000	49 (203)
<i>breA</i>	25140–24730 C	136	Transport accessory protein (<i>Brochothrix campestris</i> ATCC 43754)	AAC95141	31 (130)
<i>breB</i>	25717–25938	73	Plantaricin 1.25 γ (<i>Lb. plantarum</i> TMW1.25)	2607194A	100 (73)
<i>breC</i>	25970–26179	69	Plantaricin 1.25 β (<i>Lb. plantarum</i> TMW1.25)	CAA09476	100 (69)
<i>breD</i>	26436–26239 C	65	Helix–turn–helix motif (<i>Lb. reuteri</i> 100-23)	ZP_01273725	53 (58)
<i>breE</i>	26944–26447 C	178	Hypothetical protein BA5499 (<i>Bacillus anthracis</i> Ames)	NP_847659	29 (164)
<i>orf22</i>	28304–27375 C	309	Transposase, IS30 family (<i>Pediococcus pentosaceus</i> ATCC 25745)	YP_804033	98 (309)

Table 3. cont.

Plasmid and gene	Location*	Size (aa)	Best BLAST match (source)	Protein accession no.	Identity (% overlapping amino acids)
<i>breF</i>	29232–29375	47	No hits	–	–
<i>breG</i>	30929–31561	210	Transcriptional regulator (<i>Lb. plantarum</i> WCFS1)	NP_786546	40 (212)
<i>breH</i>	31628–33784	718	Lactacin F ABC transporter permease component (<i>Lb. johnsonii</i> NCC 533)	NP_964620	47 (712)
<i>orf26</i>	34833–33943 C	296	Integrase, catalytic region (<i>Lb. reuteri</i> F275)	YP_001271387	55 (298)
<i>traG</i>	35830–38544	904	Conjugation-related ATPase (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536705	36 (807)
<i>orf28</i>	38572–41091	839	Ser-Asp-rich hypothetical protein (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536704	26 (803)
<i>orf29</i>	41217–41564	115	Hypothetical protein LSL_1815 (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536703	30 (97)
<i>orf30</i>	41557–42207	216	Hypothetical protein LSL_1814 (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536702	31 (199)
<i>traE</i>	42220–44172	650	Putative conjugation-related ATPase (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536701	41 (652)
<i>orf32</i>	44187–45476	429	Hypothetical secreted protein (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536700	35 (442)
<i>orf33</i>	46069–46689	206	Hypothetical protein LEUM_1035 (<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC 8293)	YP_818508	22 (187)
<i>orf34</i>	46770–47795	341	Putative transposase (<i>Lb. delbrueckii</i> subsp. <i>lactis</i> ATCC 15808)	CAC79142	62 (341)
<i>orf35</i>	48393–47995 C	132	Hypothetical protein OEOE_0063 (<i>Oenococcus oeni</i> PSU-1)	YP_809732	42 (75)
<i>repB</i>	49214–48390 C	274	Replication-associated protein RepB (<i>Enterococcus faecalis</i> V583: pTEF3)	NP_817020	63 (265)
<i>repA</i>	50068–51588	506	Similar to plasmid replication protein (<i>Enterococcus faecium</i> DO)	ZP_00603338	35 (444)
<i>orf38</i>	52351–53343	330	Hypothetical protein SH2391 (<i>Staphylococcus haemolyticus</i> JCSC1435)	YP_254306	38 (312)
<i>orf39</i>	53990–54439	149	Thiol-disulfide isomerase and thioredoxin (<i>Lb. casei</i> ATCC 334)	YP_805832	26 (130)
<i>orf40</i>	54432–55355	307	Hypothetical protein LSL_1854 (<i>Lb. salivarius</i> subsp. <i>salivarius</i> UCC118: pMP118)	YP_536743	25 (324)
<i>orf41</i>	55580–55876	98	Hypothetical protein gbs0380 (<i>Streptococcus agalactiae</i> NEM316)	NP_734848	24 (99)
<i>orf42</i>	56837–58822	661	Hypothetical protein gbs0384 (<i>Streptococcus agalactiae</i> NEM316)	NP_734852	33 (273)
<i>orf43</i>	59064–60791	575	Hypothetical protein LEUM_1109 <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC 8293	YP_818582	27 (344)
<i>orf44</i>	60949–62250	433	Nucleotidyltransferase/DNA polymerase for DNA repair (<i>Lb. casei</i> ATCC 334)	YP_806285	58 (430)
<i>orf45</i>	62561–62956	131	Hypothetical protein of possible phage origin (<i>Lb. casei</i> ATCC 334)	YP_807053	39 (124)
<i>orf46</i>	63006–63188	60	Hypothetical protein BPSS0390 (<i>Burkholderia pseudomallei</i> K96243)	YP_110413	57 (59)
<i>orf47</i>	63218–63616	132	Hypothetical protein CTC02135 (<i>Clostridium tetani</i> E88 Massachusetts)	NP_782694	47 (131)
<i>orf48</i>	63772–65007	411	Hypothetical protein pLH1_p07 (<i>Lb. helveticus</i> ATCC 15009: pLH1)	NP_052195	56 (114)

*C, Complementary sequence

Fig. 3. Proposed *ori* regions of plasmids pLB925A03 and 04. Single arrows indicate direct repeats. (a) Iteron region of pLB925A03. (b) Predicted *ori*, iteron and *repA* region in pLB925A04. The nucleotide sequence of the iteron is also shown.

membrane domains. To speculate a role for the gene product, a DNA fragment containing *breE* was inserted into pLES003 and introduced into a pLB925A04 plasmid-cured derivative of *Lb. brevis* 925A, designated *Lb. brevis* 925A-Δ02/03/04. This strain, which is susceptible to brevicin 925A, harbours plasmid pLB925A01, but not pLB925A02–04. As shown in Fig. 5, the transformant acquired immunity to brevicin 925A, suggesting that the *breE* gene product is an immunity protein which functions as a self-resistant determinant in brevicin 925A-producing strains.

DISCUSSION

In this study we isolated a new bacteriocin from the culture broth of *Lb. brevis* 925A and determined the nucleotide sequence of four plasmids harboured in this strain.

A BLAST search for Rep proteins encoded by ORFs on plasmids harboured in *Lb. brevis* 925A suggests that two types of plasmid are present with regard to replication: pLB925A01 and pLB925A02 use an RCR-type replication mechanism, whereas pLB925A03 and pLB925A04 use theta replication. pLB925A01–03 may not contain any significant functional genes, except for their *cop*, *rep* and *mob* genes (Fig. 2b). As listed in Table 3, pLB925A04 carries many ORFs other than those that constitute the bacteriocin-biosynthesizing gene cluster. However, most of the ORFs cannot be annotated. Interestingly, the *srtA* gene product exhibits similarity with sortase (Pfam PF04203), which is necessary for adhesion and expected

to be a useful target for future antimicrobial agents (Mazmanian *et al.*, 1999; Scott & Zähler, 2006). However, comparative genomics of LAB carried out by Makarova *et al.* (2006) suggests that the sortase gene in *Lb. brevis* ATCC 367 is on the chromosome.

A new bacteriocin, brevicin 925A, was identified in *Lb. brevis* 925A. It showed bacteriocin activity against *Listeria monocytogenes* and *S. mutans*, which cause food poisoning and dental caries, respectively. This polypeptide may be useful in preventative dental medicine, in addition to applications in food sanitation as a biopreservative. Brevicin 925A is encoded on pLB925A04 – both the structural gene *breB* and the immunity gene *breE* were identified on the plasmid. The deduced amino acid sequence of BreB (=brevicin 925A) is identical to that of PlnC in *Lb. plantarum* TMW1.25 (Ehrmann *et al.*, 2000). The function of PlnC has not been elucidated. Strain TMW1.25 has been reported to produce two bacteriocins, designated plantaricin 1.25α and 1.25β, but only the biosynthetic gene *plnB* encoding plantaricin 1.25β has been cloned (Ehrmann *et al.*, 2000; Remiger *et al.*, 1999). *plnB* is located just downstream of *plnC* in TMW1.25 (Ehrmann *et al.*, 2000). Plantaricin 1.25β, which corresponds to the *breC* gene product (BreC) of strain 925A, has been purified from *Lb. plantarum* TMW1.25 (Remiger *et al.*, 1999). However, the *breC* gene product could not be detected in the strain 925A culture supernatant. Although the precise mode of action of BreC has not yet been elucidated, it might interact with BreB to express bacteriocin activity.

Fig. 4. Construction of shuttle vector pLES003. The *NdeI* and *AatII* sites were used to construct the shuttle vector.

Until now, it has not been confirmed whether the plantaricin biosynthetic gene cluster is located on a plasmid or on the *Lb. plantarum* chromosome. Since the brevicin 925A gene cluster appeared to be located on the largest plasmid, pLB925A04, from the plasmid-curing and sequencing analyses, we transformed *Lb. brevis* 925A-Δ02/03/04 with pLB925A04. The transformant acquired both production of brevicin 925A and immunity to the bacteriocin. This indicates that the brevicin 925A biosynthetic gene cluster is indeed located on pLB925A04.

We speculate that the brevicin 925A biosynthetic cluster is composed of eight ORFs (*breA–H*) on pLB925A04 (Fig. 2c).

Fig. 5. Function of the *breE* gene product. The pLB925A04-cured derivative of strain 925A transformed with pLES003 or pLES003/*breE* was grown on MRS-agar medium containing 5 μg erythromycin ml⁻¹. Growth of the plasmid-cured derivative and the same strain carrying pLES003 was inhibited by brevicin 925A, as shown by the inhibition zone. However, growth of the parental strain (WT) and the plasmid-cured derivative transformed with pLES003/*breE* were not inhibited by brevicin 925A.

As listed in Table 3, the *breA* gene product displays significant similarity with bacteriocin transport accessory proteins from *Lb. plantarum* (protein ID AAP83159, 28 %) and *Streptococcus pneumoniae* (EDT90159, 30 %). In general, an accessory protein, which is involved in the secretion of polypeptides, works in conjunction with a bacteriocin ABC transporter (Franke *et al.*, 1999). In addition to the ABC transporter conserved domain, the *breH* gene product contains the N-terminal peptidase domain (Pfam PF03412) which recognizes and cleaves the bacteriocin precursors at a double-glycine site. Most non-lantibiotic bacteriocins are synthesized as precursor peptides with N-terminal leader extensions that are cleaved off during maturation (Franke *et al.*, 1999). This motif is also found in the peptides predicted from *breB* and *breC*. Since the *breE* gene was not annotated by a BLAST search, we tried to introduce *breE* into a pLB925A04-deleted derivative, *Lb. brevis* 925A-Δ02/03/04. As a result, the transformant was observed to acquire immunity to brevicin 925A (Fig. 5). We confirmed by RT-PCR analysis that the expression of *breF* (not annotated by the BLAST search) is synchronized with the expression of seven other genes (data not shown). *breF* may therefore also play a significant role in bacteriocin biosynthesis.

It should be noted that to date, there has been no full characterization of the many genes essential for bacteriocin export, immunity and transcriptional regulation. Further functional analysis of the genes of the brevicin 925A biosynthetic gene cluster, including a mechanism that regulates the production of the bacteriocin and the synergistic effect of two bacteriocins, is ongoing.

To develop a plasmid vector as a tool for LAB gene manipulation and characterization of several genes in the brevicin 925A biosynthetic cluster, we generated a shuttle

vector that replicates in both *E. coli* and LAB. Since RCR plasmids generally exhibit broad host-range replication properties at high copy number (Pérez-Arellano *et al.*, 2001), at first we employed pLB925A01 for the construction of a shuttle vector. However, the resulting vector was not successfully maintained in *E. coli*. Next, we used another high-copy-number plasmid, pLB925A03, for vector construction (Fig. 4). The resulting vector, pLES003, replicated successfully in both *E. coli* and LAB such as *Lb. plantarum*, *Lb. brevis*, *Lb. helveticus*, *Lb. hilgardii* and *Enterococcus hirae*. *breE* inserted into pLES003 also conferred immunity to brevicin 925A in the bacteriocin-susceptible host cell (Fig. 5). This shuttle vector will be useful for functional analysis of ORFs located in the brevicin 925A biosynthetic gene cluster.

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