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Functional Analysis of the Gene Encoding Immunity to Lactacin F, *lafI*, and Its Use as a *Lactobacillus*-Specific, Food-Grade Genetic Marker†

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Lactacin F is a two-component class II bacteriocin produced by *Lactobacillus johnsonii* VPI 11088. The *laf* operon is composed of the bacteriocin structural genes, *lafA* and *lafX*, and a third open reading frame, ORFZ. Two strategies were employed to study the function of ORFZ. This gene was disrupted in the chromosome of NCK64, a *lafA729 lafX* ORFZ derivative of VPI 11088. A disruption cassette consisting of ORFZ interrupted with a *cat* gene was cloned into pSA3 and introduced into NCK64. Manipulation of growth temperatures and antibiotic selection resulted in homologous recombination which disrupted the chromosomal copy of ORFZ with the *cat* gene. This ORFZ mutation resulted in loss of immunity to lactacin F but had little effect on production of LafX, which is not bactericidal without LafA. Expression of ORFZ in this ORFZ$^-$ background rescued the immune phenotype. Expression of ORFZ in a bacteriocin-sensitive derivative of VPI 11088 also reestablished immunity. These data indicate that ORFZ, renamed *lafI*, encodes the immunity factor for the lactacin F system. The sensitivity of various *Lactobacillus* strains to lactacin F was further evaluated. Lactacin F inhibited 11 strains including several members of the A1, A2, A3, A4, B1, and B2 *L. acidophilus* homology groups. Expression of *lafI* in bacteriocin-sensitive strains *L. acidophilus* ATCC 4356, *L. acidophilus* NCFM/N2, *L. fermentum* NCDO1750, *L. gasseri* ATCC 33323, and *L. johnsonii* ATCC 33200 provided immunity to lactacin F. Furthermore, it was shown that lactacin F production by VPI 11088 could be used to select for *L. fermentum* NCDO1750 transformants containing the recombinant plasmid encoding LafI. The data demonstrate that *lafI* is functional in heterologous hosts, suggesting that it may be a suitable food-grade genetic marker for use in *Lactobacillus* species.

Bacteriocins are proteinaceous antimicrobial compounds produced by several bacterial species (30). Bacteriocins of lactic acid bacteria have been of particular interest over the past decade because of their existing and potential applications as natural preservatives in foods (15, 28, 50). More recently, there has been increased interest in the use of bacteriocin markers in food-grade cloning and expression systems (45, 50, 67).

Bacteriocins have been categorized by their biochemical properties, and the following four groups are currently recognized: class I, lantibiotics; class II, small, heat-stable peptides; class III, large, heat-labile proteins; and class IV, complex bacteriocins composed of protein and carbohydrate or lipid moieties (30). Although the biological characteristics of these peptides may vary, many of the class I and II peptides act through the formation of pores or ion channels in the membranes of sensitive cells (reviewed in references 1 and 13). In contrast, little is known about the mechanism of immunity that protects the producer from its own bacteriocin. The genes encoding the immunity factors for several of the class I and II bacteriocins have been identified. The corresponding proteins are usually small and cationic, but there is little homology between them. The immunity proteins of lactococcin A (LciA) (60), carnobacteriocin B2 (CbiB2) (47), and various lantibiotics (reviewed in references 1 and 52) have been studied in detail. The data suggest that there may be at least three different mechanisms of immunity (reviewed in references 1 and 52): the immunity factor may interact with the bacteriocin receptor, the immunity factor may inhibit and/or block formation of a pore or channel, or the bacteriocin may be imported and inactivated in the cell.

Lactacin F is a class II bacteriocin produced by *Lactobacillus johnsonii* VPI 11088. Lactacin F is a two-component bacteriocin composed of LafA and LafX peptides (4). The two peptides combine to form a pore in the membrane of sensitive cells, leading to the efflux of intracellular ions and eventual death (2). Together, the LafA and LafX peptides define the inhibitory spectrum of lactacin F, which includes several strains of lactobacilli, *Enterococcus faecalis* ATCC 19433, and *Carnobacterium divergens* LV13 (3, 38). The LafA peptide alone is active against only *Lactobacillus helveticus* NCDO87, which is also sensitive to the lactacin F complex. The structural genes for the lactacin F peptides are located on a 2.3-kb *Eco*RI chromosomal fragment in *L. johnsonii* VPI 11088. Open reading frame (ORF) ORFZ is located downstream of the bacteriocin genes and encodes a hydrophobic protein that is predicted to contain four transmembrane helices (23). The three genes *lafA*, *lafX*, and ORFZ compose the *laf* operon characterized by a promoter (P_{laf}) upstream of *lafA* and a *rho*-independent terminator downstream of ORFZ (23). Several other bacteriocin systems have a similar genetic organization. In most cases, the bacteriocin structural gene(s) and the gene encoding the immunity protein are located adjacent to one another. The function of ORFZ in the operon has not been demonstrated, but ORFZ has been proposed to encode the immunity factor for the lactacin F system (23).

In this study, two different genetic strategies were used to define the function of ORFZ. The analysis confirmed that

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ORFZ does encode the immunity factor for the lactacin F system, and it is therefore designated *lafI*.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. All cultures were maintained and stored at −20°C in 20% glycerol. Lactobacilli were cultured at 37°C (unless otherwise specified) in MRS broth (Difco Laboratories, Detroit, Mich.) or MRS broth containing either erythromycin (5 or 7.5 μg/ml [MRS Em5 or Em7.5 broth, respectively]) or chloramphenicol (5 μg/ml [MRS Cm5 broth]). When lactobacilli were propagated on agar plates, incubation was conducted in anaerobic jars (Difco Laboratories) under anaerobic gas (85% N_2, 10% CO_2, 5% H_2) at the temperatures indicated. Rifampin-resistant *L. johnsonii* VPI 11088 was generated by plating 200 μl of a stationary-phase culture on MRS agar containing 100 μg of rifampin per ml. Colonies were evident after 24 to 48 h of incubation. *Escherichia coli* DH5α (GibcoBRL, Gaithersburg, Md.), SURE (Stratagene, La Jolla, Calif.), MC1061 (Stratagene), and JM110 (Stratagene) were propagated at 37°C in LB (51). SURE strains were grown in the presence of 10 μg of tetracycline per ml. When appropriate for clonal selection, ampicillin, erythromycin, and chloramphenicol were used at concentrations of 100, 150, and 15 μg/ml, respectively. Agar plates contained 1.5% agar (BBL Microbiology Systems, Cockeysville, Md.), and overlays contained either 0.75 or 1.2% agar. The following plasmids were used in this study: pTRK160, pBluescript::2.3-kb *Eco*RI fragment from the VPI 11088 genome which contains the *laf* operon (23); pGK12, a *Lactobacillus-E. coli* shuttle vector encoding erythromycin resistance (Em^r) and chloramphenicol resistance (Cm^r) (32); pGKV210, promoter-probe vector, Em^r (Cm^r dependent on the presence of a promoter upstream of the *cat-86* gene) (58); pTRK205, pGKV210::0.7-kb *Dra*I fragment containing P_{laf} *lafA lafX* cloned in opposite orientation to the *cat-86* gene, Em^r (23); pBV5030, a pGKV210 derivative that has the regulatory region of *cat-86* removed (12); pLA6, pBV5030::474-bp P6 promoter from *Lactobacillus acidophilus* ATCC 4356, Em^r Cm^r (18); and pSA3, a *Lactobacillus-E. coli* shuttle vector, Tet^r Cm^r Em^r in *E. coli* and Em^r in *Lactobacillus* spp. (14).

Determination of bacteriocin activity. Activity units (AU) of lactacin F (culture supernatant or concentrate) per milliliter were determined by using a critical-dilution assay described previously (36). Briefly, the bacteriocin was serially diluted twofold with equal volumes of MRS broth, and 10 μl of each dilution was spotted onto MRS plates and overlaid with the test strain. The activity was determined by taking the reciprocal of the last dilution showing inhibition and recorded as AU per milliliter. The lactacin F producer, NCK64(pTRK205), and the lactacin F nonproducer, NCK89(pGKV210) (23), were inoculated from frozen stocks and grown for 18 to 24 h in MRS Em5 broth. NCK64(pTRK205) was used because of its consistent bacteriocin-producing phenotype. Cells were pelleted at 7,000 × *g* for 15 min, and the culture supernatant was removed. The activity of the NCK64(pTRK205) supernatant was 25,600 AU/ml against the indicator strain *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797, whereas that for the bacteriocin-negative control, NCK89(pGKV210), was <100 AU/ml. (NCK89 is a *str-6 rif-7* derivative of NCK65. See Table 1 for the genotype and phenotype of NCK65). Lactacin F activity was concentrated by mixing equal volumes of culture supernatant with 20% trichloroacetic acid (TCA) and incubating the mixture on ice for 60 min prior to centrifugation at 8,000 × *g* for 30 min. The supernatant was removed, and the precipitate was washed with 70% ethanol and air dried. The precipitate was then resuspended in 1/10 the original volume of 0.1 M Tris-HCl (pH 8.0). The activity of the TCA-concentrated lactacin F was determined as outlined above. All cultures tested for lactacin F sensitivity or immunity were inoculated from frozen stock into MRS broth alone or MRS broth plus erythromycin and subcultured at least once prior to testing. MRS soft agar (0.75%) was inoculated (1%) with the test strain and overlaid onto the agar plates spotted with lactacin F. The overlaid plates were incubated anaerobically for 12 to 18 h before the results were read. Complementation assays were conducted as outlined by Allison et al. (4), with the following modifications: 5 μl of an overnight culture was spotted onto MRS agar, and cultures were incubated anaerobically for 24 h prior to being overlaid with indicator.

DNA isolation. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Sambrook et al. (51) and from lactobacilli by the procedure of Walker and Klaenhammer (64). For transformation of lactobacilli, plasmids were obtained on a preparative scale from *E. coli* cultures (50 to 100 ml) by using a Qiagen tip 100 and reagents as recommended by the manufacturer (Qiagen, Chatsworth, Calif.). Chromosomal DNAs from *L. johnsonii* NCK64 and variants thereof were obtained by using the protocol of Joerger and Klaenhammer (27).

Molecular cloning and transformation. All enzymes were used according to the recommendations of the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). When necessary, DNA restriction fragments were purified from agarose gels with a GENECLEAN II kit (Bio 101, La Jolla, Calif.). For cloning of PCR-generated fragments containing ORFZ, chromosomal template DNA was extracted from *L. johnsonii* VPI 11088 as outlined above. The following primers were used for amplification: 5′-TATTGTCGACTTCATAGA TTCATTAGTAGG-3′ (upstream primer; *Sal*I site underlined) and 5′-ATAACTGCAGAGCTAGAATTAGTGTTATCC-3′ (downstream primer; *Pst*I site underlined). The reagents and amounts used were as recommended in the PCR core kit (Boehringer Mannheim Biochemicals), and the PCR was conducted under the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 1 min, through 40 cycles. The PCR mixtures were purified with a GENECLEAN II kit (Bio 101). The PCR products were cloned into pGEM-T under conditions recommended by the manufacturer (Promega, Madison, Wis.).

E. coli cells were prepared for electrotransformation as outlined by Dower et al. (20). The following strains of *Lactobacillus* were prepared for transformation as indicated: *L. johnsonii* VPI 11088 (ATCC 11506) derivatives, *L. johnsonii* ATCC 33200, *L. acidophilus* NCFM/N2, *Lactobacillus gasseri* ATCC 33323, and *Lactobacillus fermentum* NCDO1750 were transformed by using the protocol of Raya et al. (48); *L. johnsonii* NCK64 was transformed with pSA3 by using the protocol of Holo and Nes (26) as modified by Walker and Klaenhammer (64); and *L. acidophilus* ATCC 4356 was transformed by using the protocol of Bhowmik and Steele (10) as modified by Walker et al. (63). Cells were electroporated in 0.2-cm cuvettes (0.4-cm cuvettes were used for transformation of *L. acidophilus* ATCC 4356) with a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) set at 25 μF, 200 Ω, and 2.5 kV for all strains. After electroporation, the cells were incubated in appropriate nonselective media for the following times prior to plating: 1 h for all *E. coli* strains, 2 to 4 h for lactobacilli transformed with pBV5030 derivatives, and 12 h for lactobacilli transformed with pSA3 derivatives.

Southern hybridization. Chromosomal DNA (10 μg) was digested with *Eco*RI (Boehringer Mannheim Biochemicals) and electrophoresed on a 0.75% (wt/vol) agarose gel (1× Tris-acetate-EDTA buffer, pH 8.0). Southern transfer of DNA from agarose gels to MagnaCharge nylon membranes (Micron Separations Inc., Westboro, Mass.) was done as outlined by Le Bourgeois et al. (33). [^{32}α-P]dCTP-labelled probes were prepared with a Multiprime DNA labelling kit (Amersham, Arlington Heights, Ill.). Hybridization conditions were as outlined by Robbins Scientific Corporation (49).

Chromosomal gene disruption. The protocol of Bhowmik et al. (9) was applied with the following modifications. Briefly, NCK64 (*lafA729 lafX* $ORFZ^+$) was transformed with pTRK418 (pSA3::ORFZ-*cat* cassette) as indicated above, and transformants were selected on MRS Em7.5 agar at 37°C. The transformants were then transferred (1% inoculum) twice in MRS Em7.5 broth at 37°C before the growth temperature of freshly inoculated (1%) cultures was shifted to 45°C. The cultures were transferred three times (approximately 18 generations) in MRS Em7.5 broth at 45°C prior to being plated on MRS Em7.5 agar. MRS Em7.5 broth was inoculated with individual colonies and propagated at 45°C before the freshly inoculated cultures were shifted to 37°C in MRS broth (no antibiotic added). The cultures were transferred five times (approximately 30 generations) under these conditions and then plated on MRS Cm5 agar and incubated at 37°C. Individual colonies were then replica plated on MRS Cm5 agar and MRS Em5 agar and incubated at the same temperature. In all cases, the cultures were incubated for 18 to 24 h between transfers.

Utilization of lactacin F as a selectable marker. MRS broth was inoculated with a rifampin-resistant derivative of *L. johnsonii* VPI 11088 from frozen stock, and the culture was allowed to grow overnight. The stationary-phase culture was diluted 10^{-6}, plated on MRS agar, and incubated anaerobically for 36 to 48 h (~9×10^8 CFU/ml in original culture, 900 CFU per plate). The plates were subjected to 360 mJ of UV light (UV Stratalinker 1800; Stratagene) to kill VPI 11088, overlaid with 7 ml of MRS agar (1.2%), and incubated at 37°C for 45 min. *L. fermentum* NCDO1750 was transformed with pTRK434 ($LafI^+$) and pTRK435 ($LafI^-$) by the transformation protocol of Raya et al. (48). After transformation, cells (200 μl) were resuspended in 1 ml of MRS broth and incubated at 37°C for 1.5 h prior to being plated (10 μl of cell suspension) on the lactacin F-containing plates and MRS Em5 agar (control).

Nucleotide sequence accession number. The DNA sequence information for the 2.3-kb *Eco*RI fragment encoding lactacin F has been deposited in GenBank under accession no. M57961 (23).

RESULTS

Chromosomal disruption of ORFZ. One strategy used in the functional analysis of ORFZ was to create an $ORFZ^-$ variant of *L. johnsonii* by disrupting this gene in the chromosome of NCK64. NCK64 is an isogenic variant of VPI 11088 that has a frameshift mutation in the *lafA* gene but still produces LafX (4). LafX alone is not toxic to strains sensitive to the lactacin F complex (4); therefore, a mutation in the immunity gene of this $LafA^-$ $LafX^+$ strain was predicted to be nonlethal. A disruption cassette was constructed by placing the gene encoding chloramphenicol acetyltransferase (*cat*) from pGK12 (32) within ORFZ and cloning this cassette into pSA3 as outlined in Fig. 1. Regions upstream of ORFZ were deleted from pTRK160 (pBluescript::2.3-kb *Eco*RI fragment from VPI 11088) to create pTRK416. pTRK416 was digested with *Bgl*II, a unique site located in the middle of ORFZ, and ligated to the 1.4-kb *Mbo*I fragment of pGK12 containing *cat* to create

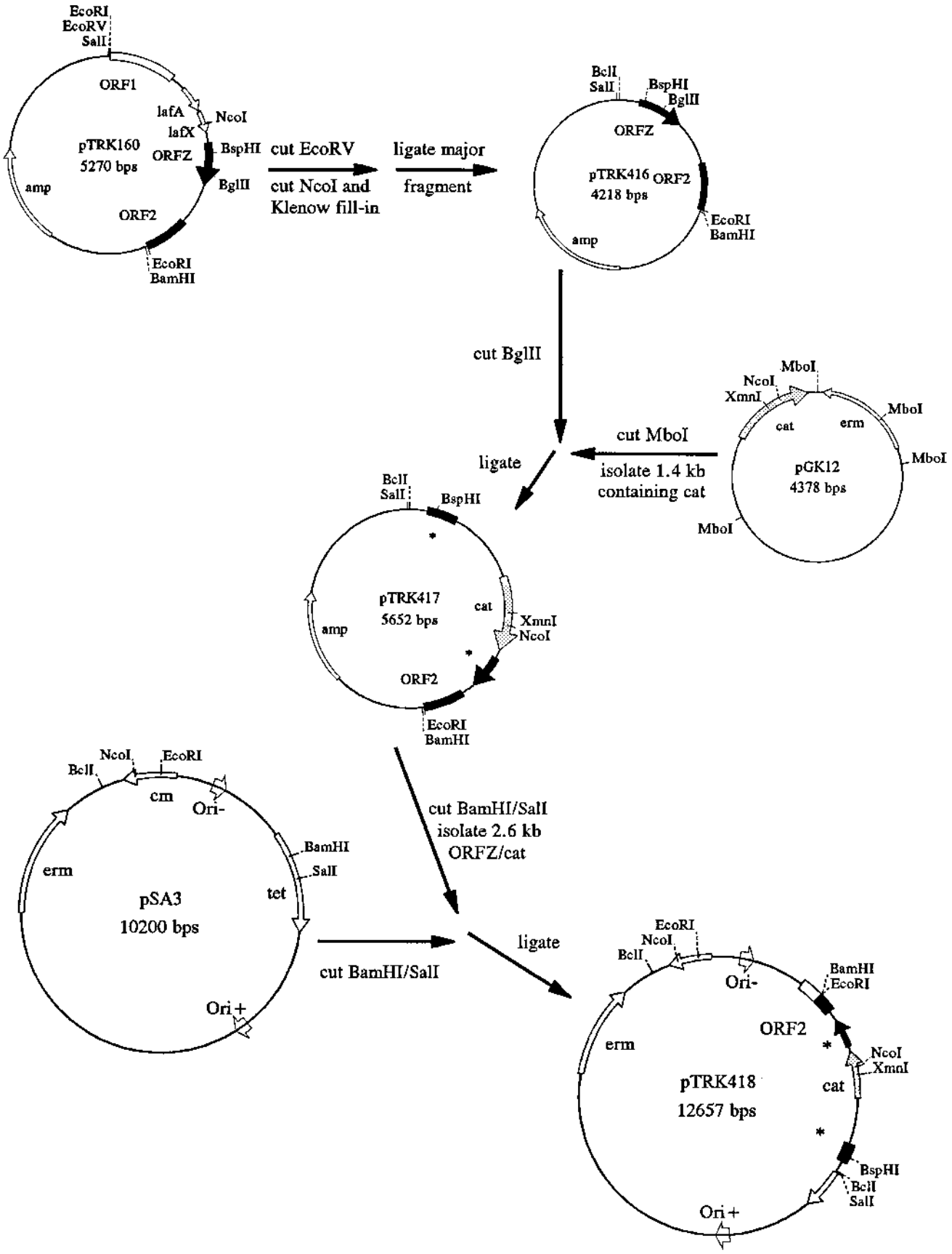

FIG. 1. Construction of the temperature-sensitive integration vector pTRK418. *, remaining portions of the ORFZ gene disrupted with *cat* (*cat* gene from pGK12); *amp*, pBluescript (Stratagene) gene conferring ampicillin resistance; *erm*, pGK12 and pSA3 genes conferring erythromycin resistance; *tet*, pSA3 gene conferring tetracycline resistance in *E. coli*; Ori−, gram-negative origin of replication; Ori+, gram-positive origin of replication; pTRK160, pBluescript::2.3-kb *Eco*RI fragment of VPI 11088 containing the *laf* operon (GenBank accession no. M57961) (23); pSA3, shuttle vector (14) (*cm* encodes chloramphenicol resistance only in *E. coli* and was used to select for pTRK418 in this background); pGK12, broad-host-range plasmid (32). The *Bcl*I site in pTRK416 was generated during cloning. Plasmids are not drawn to scale.

pTRK417. This ORFZ-*cat* disruption cassette, containing 400 bp of homology upstream and 800 bp of homology downstream of the *cat* gene, was then cloned into pSA3 to create pTRK418 (Fig. 1). pSA3 is a temperature-sensitive replicon in gram-positive bacteria that has been successfully used in gene replacement and disruption experiments, particularly in lactobacilli (9). pTRK418 was introduced into NCK64, and transformants were selected on MRS Em7.5 agar plates at 37°C, the permissive temperature for pSA3 replication. The temperature was then shifted to 45°C, the nonpermissive temperature for pSA3 replication. Total DNA from 11 isolates propagated at 45°C was extracted and digested with *Eco*RI. Total digested and undigested DNAs, as well as digested and undigested pTRK418 (controls), were subjected to agarose gel electrophoresis. A Southern transfer was prepared and hybridized with a 1.2-kb probe consisting of ORFZ and ORF2 (Fig. 2). This probe was used because hybridization of total DNA with a 372-bp ORFZ probe did not yield a strong enough signal, since this is a single-copy chromosomal gene (data not shown). In the undigested controls, the probe hybridized only to high-molecular-weight fragments which did not correspond to supercoiled pTRK418 (data not shown), indicating that pTRK418 was integrated in the chromosome of all 11 isolates. The 1.2-kb ORFZ-ORF2 probe hybridized to *Eco*RI fragments of 1.8, 2.3, and 3.0 kb in the NCK64 chromosome (Fig. 2). ORF2 alone hybridizes to all three fragments; ORFZ hybridizes only to the 2.3-kb fragment which contains the *laf* operon (data not shown). The *Eco*RI digests of the 11 isolates yielded two hybridization patterns relative to NCK64. In seven of the isolates, the probe hybridized to a fragment of 10.7 kb (corresponding to the larger of two *Eco*RI fragments of pTRK418; Fig. 1) in addition to hybridizing to the same three

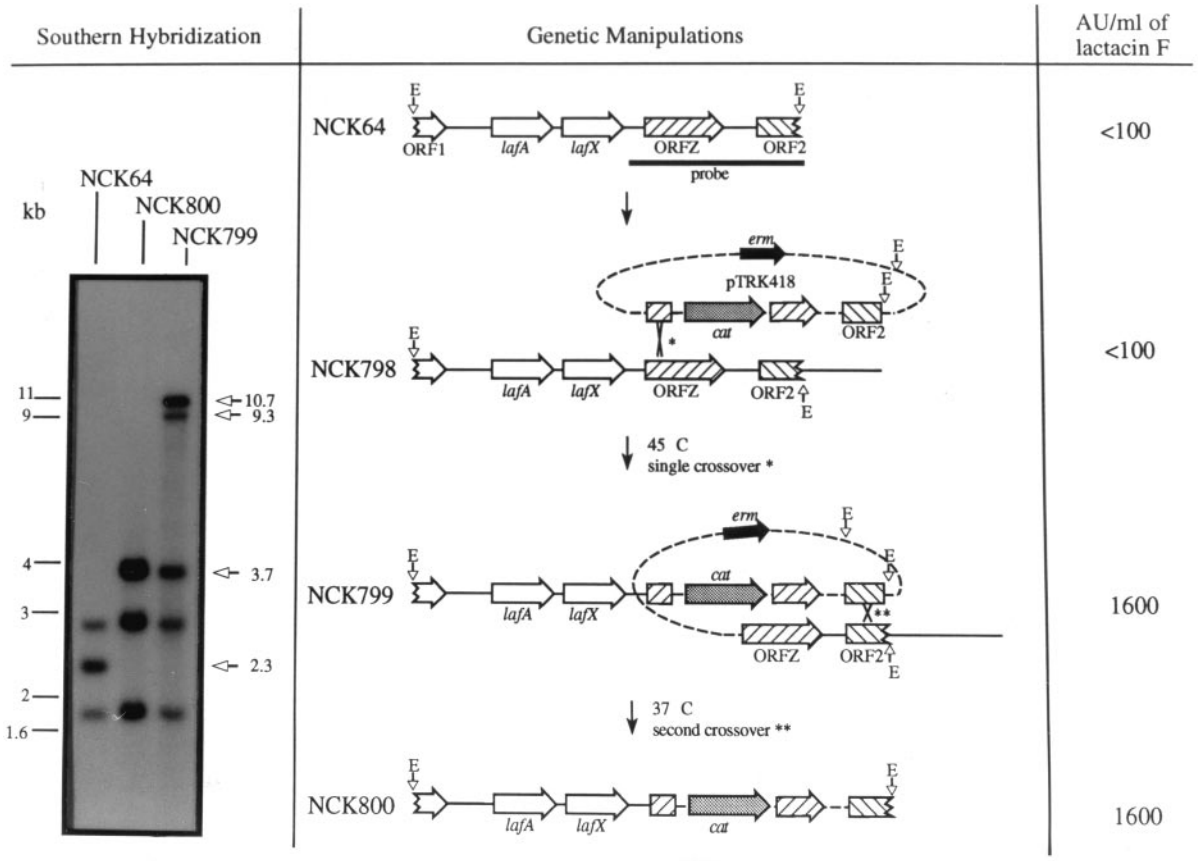

FIG. 2. Construction and characterization of an ORFZ⁻ derivative of NCK64. The proposed mechanism of integration and deletion of pTRK418 which disrupted ORFZ and created the ORFZ⁻ variant, NCK800, is outlined (middle panel; not drawn to scale). E ↓, *Eco*RI restriction site; dotted lines, pTRK418 DNA; *cat*, pGK12 gene; and *erm*, pSA3 gene conferring erythromycin resistance. The corresponding results of Southern hybridization conducted with *Eco*RI digests of total DNA from NCK64, NCK799, and NCK800 are shown on the left. The composition of the 1.2-kb ORFZ-ORF2 probe used is indicated (solid black line under NCK64, middle panel). Size markers (in kilobases) (left of gel) and the sizes of relevant fragments hybridizing to the 1.2-kb probe as described in the text (right of gel) are indicated. The sensitivities of the four strains (described in the middle panel) to TCA-concentrated lactacin F are shown on the right. None of the strains were inhibited by TCA-concentrated supernatant from NCK89(pGKV210) (negative control).

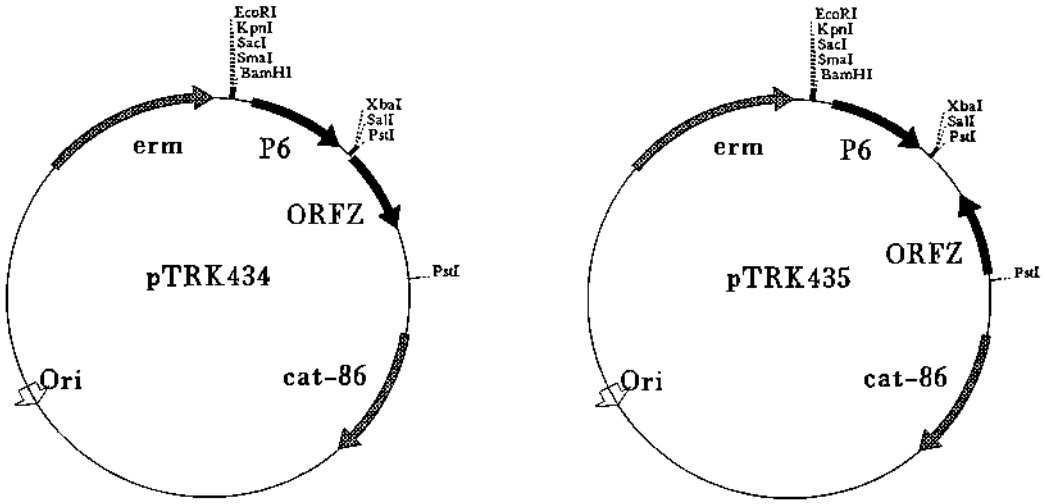

FIG. 3. Schematic representation of ORFZ$^+$ (pTRK434) and ORFZ$^-$ (pTRK435) plasmids. Details of cloning are outlined in the text. P6, promoter of pLA6 (18); Ori, pWVO1 origin of replication; *erm* and *cat-86*, genes conferring erythromycin and chloramphenicol resistance, respectively.

fragments of NCK64 (data not shown). These data indicated that the initial crossover event had occurred in the 800-bp region of homology downstream of the *cat* gene in the ORFZ-*cat* cassette of pTRK418. In four of the isolates, the probe hybridized to fragments of 10.7, 9.3 (the largest *Eco*RI fragment of pTRK418 minus the 1.4-kb fragment containing *cat*), and 3.7 kb (the 2.3-kb fragment containing the *laf* operon plus the 1.4-kb fragment containing *cat*), in addition to the 1.8- and 3.0-kb fragments of NCK64 (Fig. 2). This pattern of hybridization indicated that an initial single crossover event occurred in the 400-bp of homology upstream of the *cat* gene in the ORFZ-*cat* cassette of pTRK418 to create the 9.3- and 3.7-kb junction fragments as indicated in Fig. 2. The intensity of the 10.7-kb fragment corresponding to pTRK418 suggests that amplification may have occurred, a phenomenon that is frequently observed in homologous recombination experiments (9, 11).

The lactacin F sensitivity of NCK64, NCK64(pTRK418) (NCK798), and one of the four integrants described above, NCK799, was analyzed (Fig. 2). As expected, NCK64 and NCK64(pTRK418) were not sensitive to lactacin F (<100 AU/ml; Fig. 2). However, NCK799 was sensitive to lactacin F at a level of 1,600 AU/ml (Fig. 2). Although homologous recombination would result in the reconstruction of a complete copy of ORFZ (Fig. 2), these data suggest that this copy is not functional, presumably because of the absence of an upstream promoter. Therefore, replacement of ORFZ by the ORFZ-*cat* disruption cassette eliminated the immunity phenotype.

To totally remove the complete copy of ORFZ from the NCK799 chromosome, a second crossover was attempted. The NCK799 culture was transferred successively in the absence of antibiotic at 37°C (permissive temperature for pSA3) and plated on MRS Cm5 agar. Of the 400 colonies examined, one was Cm^r and Em^s, suggesting that pSA3 had been deleted as proposed in Fig. 2. To confirm this, the isolate, designated NCK800, was propagated in MRS Cm5 broth at 37°C and total DNA was extracted and digested with *Eco*RI for Southern hybridization. In addition to the background fragments, the ORFZ-ORF2 probe hybridized to a 3.7-kb fragment corresponding to the *laf* operon plus an insertion of 1.4 kb, concurring with the *cat*-containing fragment cloned from pGK12 (Fig. 2). The probe did not hybridize to fragments of 9.3 and 10.7 kb, indicating that the pSA3 replicon had been deleted at the second crossover site as proposed in Fig. 2.

The lactacin F sensitivity of NCK800 was also analyzed. NCK800 was sensitive to lactacin F at a level of 1,600 AU/ml (Fig. 2), similar to NCK799 and at least 16 times more sensitive than NCK64. Therefore, disruption of ORFZ in the chromosome of NCK64 resulted in a loss of immunity to the bacteriocin. These data indicate that ORFZ is essential for immunity to lactacin F. Further analysis of NCK800 also showed that this strain had lost two resident plasmids, pPM4 and pPM27, which are present in both VPI 11088 and NCK64.

Cloning and expression of ORFZ. A recombinant plasmid encoding ORFZ was constructed and used to transform NCK800 (plasmidless, ORFZ$^-$) and NCK65. NCK65 is a variant of VPI 11088 that has a ca. 10-kb chromosomal deletion that includes the *laf* operon (4). The gene encoding ORFZ was amplified from total DNA of VPI 11088 by PCR. Primers used in the PCR were designed to amplify from nucleotides 1178 to 1800 (622 bp) of the 2,312-bp *Eco*RI fragment which includes the native ribosomal binding site of ORFZ and the downstream *rho*-independent terminator which flanks the *laf* operon (23). *Sal*I and *Pst*I sites were included on either end of the PCR primers (see Materials and Methods). The PCR fragment was cloned directly into pGEM-T, and the orientation of the fragment was determined. One of the recombinant pGEM-T::ORFZ plasmids released the 622-bp ORFZ-containing fragment when digested with *Pst*I. This fragment was cloned into the *Pst*I site of pLA6, which is composed of the 474-bp P6 promoter from *L. acidophilus* ATCC 4356 (18) cloned into pBV5030. Two recombinant plasmids were characterized (Fig. 3): pTRK434 contained ORFZ in the correct orientation relative to the promoter (ORFZ$^+$), and pTRK435 contained ORFZ in the incorrect orientation relative to the promoter (ORFZ$^-$; negative control). These plasmids were introduced into NCK800 and NCK65, and the bacteriocin sensitivity of the transformants was evaluated. NCK65, NCK65(pTRK435) (ORFZ$^-$), NCK800, and NCK800(pTRK435) (ORFZ$^-$) were all sensitive to concentrated lactacin F (1,600 AU/ml; Table 1). In contrast, NCK65(pTRK434) (ORFZ$^+$) and NCK800 (pTRK434) (ORFZ$^+$) were 16 times less sensitive to lactacin F than the ORFZ$^-$ controls (<100 versus 1,600 AU/ml; Table 1). The wild-type producer VPI 11088 and NCK64 are considered immune to lactacin F and were not inhibited by the bacteriocin (Table 1 and Fig. 2). Therefore, cloning and expression of ORFZ in the lactacin F-sensitive variants, NCK800

TABLE 1. Lactacin F sensitivity of VPI 11088, NCK64, and NCK65 and variants thereof

L. johnsonii strain	Relevant characteristics[a]	Lactacin F AU/ml[b]
VPI 11088 (ATCC 11506)	Wild-type lactacin F producer; Laf^+ Laf^{Imm}	<100
NCK64	VPI11088 derivative; *lafA729 lafX* $ORFZ^+$ $LafX^+$ Laf^{Imm}	<100
NCK800	NCK64 derivative; chromosomal ORFZ disrupted with *cat* gene of pGK12; $ORFZ^-$ Cm^r	1,600
NCK800(pTRK435)	$ORFZ^-$ Cm^r Em^r	1,600
NCK800(pTRK434)	$ORFZ^+$ Cm^r Em^r	<100
NCK65	VPI 11088 derivative; Δ*laf* operon; Laf^- Laf^S	1,600
NCK65(pTRK435)	$ORFZ^-$ Em^r	1,600
NCK65(pTRK434)	$ORFZ^+$ Em^r	<100

[a] Laf^+ or $LafX^+$, lactacin F or LafX producer, respectively; Laf^-, lactacin F nonproducer; Laf^{Imm}, immune to lactacin F; Laf^S, lactacin F sensitive; $ORFZ^+$, functional ORFZ gene; $ORFZ^-$, nonfunctional ORFZ gene present; Em^r, erythromycin resistant (conferred by pTRK434 or pTRK435); and Cm^r, chloramphenicol resistant (conferred by the *cat* gene of pGK12 integrated into the chromosome).

[b] TCA-concentrated culture supernatant from the lactacin F producer NCK64(pTRK205) was used. TCA-concentrated culture supernatant from NCK89(pGKV210), a bacteriocin-negative control, did not inhibit any of the strains tested.

and NCK65, rescued immunity to a level comparable to that of the wild-type producer. The data show that ORFZ encodes the immunity protein for the lactacin F system. Furthermore, ORFZ appears to be the sole determinant for the immunity phenotype, since disruption of this gene leads to a dramatic increase in sensitivity, and expression of ORFZ recovered the phenotype. Consequently, ORFZ is designated *lafI* in compliance with current bacteriocin nomenclature.

Effects of mutated *lafI* on bacteriocin production. The ability of the NCK64 variants to produce LafX was also studied to determine if mutations in the immunity gene exerted any pleiotropic effects on expression of this bacteriocinogenic peptide. To detect LafX production, a complementation assay between NCK65(pTRK203) ($LafA^+$) (4, 23) and the following strains was conducted: NCK64 (*lafA729 lafX* $LafI^+$), NCK798 (*lafA729 lafX* $LafI^+$), NCK799 (*lafA729 lafX* $LafI^-$), and NCK800 (*lafA729 lafX* $LafI^-$). *L. delbrueckii* subsp. *lactis* ATCC 4797 (lactacin F sensitive only) was used as the indicator. In the intervening region between NCK65(pTRK203) and all the NCK64 variants, a zone of inhibition against strain ATCC 4797 occurred, indicating a positive reaction between the LafA and LafX peptides (Fig. 4). Therefore, NCK800 produced LafX regardless of the mutated immunity gene. The amount of LafX produced by NCK800 appeared to be less than that of the other NCK64 variants, but the assay used was not a quantitative measurement of LafX. These experiments also demonstrated that production of the LafX peptide alone, in the absence of LafA and LafI, is not inhibitory towards the producer.

Lactacin F activity across the *Lactobacillus* genus. With increasing interest in genetic engineering of lactobacilli to improve and expand their applications (22, 31, 44, 65), appropriate food-grade selection markers are necessary. Consequently, we reexamined the inhibitory effect of lactacin F on various *Lactobacillus* strains and species to determine the potential use of *lafI* as a selection marker. Of the 16 strains tested (Table 2), the most sensitive were those originally reported as bacteriocin indicators (38), which include the following: *L. helveticus* NCDO87, *L. delbrueckii* subsp. *lactis* ATCC 4797, *Lactobacillus lactis* NCDO970, *Lactobacillus bulgaricus* NCDO1489, and *L. fermentum* NCDO1750. *L. amylovorus* ATCC 33620 was as sensitive to lactacin F as *L. fermentum* NCDO1750 (6,400 AU/ml; Table 2). Other strains of lactobacilli exhibiting moderate sensitivity included *L. acidophilus* (NCFM/N2 and ATCC 4356), *L. johnsonii* ATCC 33200, *L. gallinarum* ATCC 33199, *L. gasseri* ATCC 33323, and *Lactobacillus reuteri* 1063. Strains exhibiting low-level sensitivity to lactacin F included *L. gasseri* ATCC 19992, *Lactobacillus crispatus* ATCC 33820, and *L. reuteri* ATCC 23272. Of all the strains tested, only *L. plantarum* C-11 was not affected by lactacin F. Although sensitivity varies, lactacin F was inhibitory against 11 different strains. The data suggest that a marker conferring immunity to lactacin F may be appropriate for use in a variety of lactobacilli.

Heterologous expression of *lafI*. Several lactobacilli were electrotransformed with plasmids pTRK434 ($LafI^+$) and pTRK435 ($LafI^-$) (Table 3). The presence of the respective plasmids in the transformants was confirmed (data not shown), and the sensitivity to lactacin F was determined (Table 3). In all cases, transformants containing pTRK435 ($LafI^-$; negative control) exhibited levels of sensitivity similar to those of their background controls (Table 3). However, transformants containing pTRK434 ($LafI^+$) showed a significant decrease in sensitivity to lactacin F. The sensitivity of *L. fermentum* NCDO1750 to the bacteriocin was decreased at least 64-fold

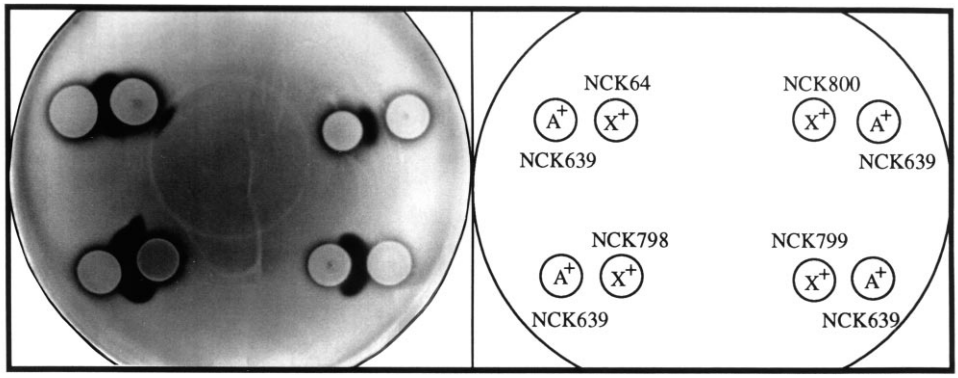

FIG. 4. Production of LafX by *L. johnsonii* NCK64 and variants. Cultures were plated and overlaid as outlined in Materials and Methods. The bacteriocin-sensitive indicator strain was *L. delbrueckii* subsp. *lactis* ATCC 4797. NCK639 [NCK65(pTRK203)], $LafA^+$; NCK64, *lafA729* $LafX^+$; NCK798, NCK64(pTRK418); NCK799, pTRK418 integrant; and NCK800, $LafI^-$ variant of NCK64. A^+, $LafA^+$; X^+, $LafX^+$.

TABLE 2. Lactacin F sensitivity of lactobacilli

Strain[a]	Relevant characteristics	Lactacin F AU/ml[b]
L. helveticus NCDO87	LafA- and lactacin F-sensitive indicator[c,d]	102,400
L. delbrueckii subsp. *lactis* ATCC 4797	Lactacin F-sensitive indicator[c]	51,200
L. lactis NCDO970	Lactacin F-sensitive indicator[c]	51,200
L. bulgaricus NCDO1489	Lactacin F-sensitive indicator[c]	12,800
L. fermentum NCDO1750	Lactacin F-sensitive indicator[c]	6,400
L. acidophilus		
ATCC 4356	Neotype of homology group A1	800
NCFM/N2	Lactacin B producer[e]; industrial strain	1,600
L. crispatus ATCC 33820	Neotype of homology group A2	100
L. amylovorus ATCC 33620	Neotype of homology group A3	6,400
L. gallinarum ATCC 33199	Neotype of homology group A4	800
L. gasseri		
ATCC 33323	Neotype of homology group B1	800
ATCC 19992 (VPI 6033)		400
L. johnsonii ATCC 33200 (VPI 7960)	Neotype of homology group B2	1,600
L. plantarum C-11[f]	Plantaricin A producer	<100
L. reuteri		
ATCC 23272	Neotype	100
1063[g]	Reuterin producer[g]	1,600

[a] VPI, Virginia Polytechnic Institute and State University, Blacksburg (from J. L. Johnson); NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.
[b] TCA-concentrated culture supernatant from the lactacin F producer NCK64(pTRK205) was used. TCA-concentrated culture supernatant from NCK89(pGKV210), the negative control, did not inhibit any of the strains tested.
[c] Muriana and Klaenhammer (38).
[d] Allison et al. (4).
[e] Barefoot and Klaenhammer (8).
[f] U.S. Department of Agriculture Fermentation Laboratory, North Carolina State University.
[g] W. J. Dobrogosz, Department of Microbiology, North Carolina State University (see reference 6).

when *lafI* was introduced (6,400 versus <100 AU/ml; Table 3). The lactacin F sensitivity of all other strains tested decreased 8- to 16-fold upon heterologous expression of *lafI* (Table 3). Therefore, *lafI* functions in several *Lactobacillus* backgrounds, where it appears to confer a level of immunity which is at least equivalent to that of the wild-type producer, *L. johnsonii* VPI 11088.

Utilization of lactacin F as a selective agent. To evaluate whether lactacin F could be used to select for LafI$^+$ transformants from sensitive lactobacilli, a simple assay using the native bacteriocin producer was developed (see Materials and Methods). Briefly, a rifampin-resistant derivative of *L. johnsonii* VPI 11088 was plated so that approximately 900 individual colonies were present on each MRS plate. The plates were incubated for 36 to 48 h to allow the producer to grow and express lactacin F. The producer cells were then killed by exposure to UV light, and fresh MRS agar was layered over the plate. *L. fermentum* NCDO1750, freshly electroporated with pTRK434 or pTRK435 (LafI$^-$ control), was plated onto the lactacin F-containing agar and incubated at 37°C for approximately 24 h. Areas on the plate devoid of the producer colonies, which resulted from uneven spreading, supported growth of a confluent lawn of NCDO1750 in both pTRK434 and pTRK435 transformations. However, individual pTRK434 (LafI$^+$) transformants of *L. fermentum* NCDO1750 had grown on top of the producer colonies, where the concentration of lactacin F was the highest. Ten of these individual colonies were picked from the lactacin F plates, and all grew in MRS Em5 broth. The presence of pTRK434 in five of the isolates was confirmed. In the pTRK435 (LafI$^-$) transformation, there were no colonies present on top of the producer colonies. In areas of the plate where there were few producer colonies, i.e., where the concentration of lactacin F was very low, a few individual colonies were present. MRS Em5 broth was inoculated with five of these colonies from the lactacin F-containing plates. None of these isolates grew, indicating that pTRK435 was not present. As an additional control, the original pTRK434 and pTRK435 transformation mixes of *L. fermentum* NCDO1750 were plated on MRS Em5 agar at the same time as they were plated on the lactacin F-containing plates. After 36 to 48 h, colonies from both transformations were evident. When the total numbers of pTRK434 transformants on lactacin F-containing plates versus MRS Em5 plates were compared, approximately two to three times more transformants were obtained when lactacin F was used as the selective agent (data not shown). Therefore, lactacin F served as a food-grade selective agent that can be used to select for *L. fermentum* NCDO1750, and potentially other lactacin F-sensitive lactobacilli, transformed with a recombinant plasmid encoding LafI. It was noted, however, that the degree of selectivity fluctuated between experiments because of the inherent variability in the

TABLE 3. Expression of *lafI* by heterologous lactobacilli and evaluation of lactacin F sensitivity

Strain	Lactacin F AU/ml[a]		
	Wild type	pTRK435 (*lafI*)	pTRK434 (*lafI*$^+$)
L. fermentum NCDO1750	6,400	3,200	<100
L. johnsonii ATCC 33200	1,600	1,600	<100
L. acidophilus			
NCFM/N2	1,600	1,600	<100
ATCC 4356	800	ND[b]	<100
L. gasseri ATCC 33323	800	800	<100

[a] TCA-concentrated culture supernatant from the lactacin F producer NCK64(pTRK205) was used. TCA-concentrated culture supernatant from NCK89(pGKV210), a bacteriocin-negative control, did not inhibit any of the strains tested.
[b] ND, not determined.

TABLE 4. Immunity factors of class II bacteriocins

Bacteriocin system	Immunity gene	Immunity protein		Reference(s)
		Size (amino acids)	pI	
Lactacin F	*lafI*	124	9.8	This study
Carnobacteriocin B2	*cbiB2*	111	9.3	46, 47
Divergicin A	*dviA*	56	10.3	67
Lactococcin A	*lciA*	98	10.2	40, 55, 60
Lactococcin B	*lciB*	91	9.8	54, 59
Lactococcin M/N	*lciM*	154	10.1	53
Leucocin A	*lcaB*	113	9.5	56
Pediocin PA-1	*pedB*	112	7.4	61
Sakacin A	*saiA*	90	10.1	5

number of producer cells and their potential to produce lactacin F.

DISCUSSION

The genetic analyses presented in this paper demonstrate that ORFZ (*lafI*) encodes the immunity factor for the lactacin F system. Disruption of *lafI* resulted in sensitivity to lactacin F. Cloning and expression of *lafI* in lactacin F-sensitive strains, NCK65 and NCK800, restored immunity to the bacteriocin. Furthermore, *lafI* was successfully expressed in heterologous strains of lactobacilli, in which it conferred immunity to lactacin F, and LafI$^+$ transformants of *L. fermentum* were selected for by using lactacin F. Consequently, *lafI* provides a potential food-grade marker for genetic experiments with some lactobacilli.

While numerous studies of the mode of action of class II bacteriocins have been conducted, little is known about the mechanism of immunity (reviewed in reference 1). One common feature among bacteriocin producers is that immunity is frequently encoded adjacent to the bacteriocin structural gene(s). Table 4 outlines class II bacteriocins for which the gene encoding the immunity protein has been identified through genetic experiments. These proteins are small and cationic except for PedB (Table 4). However, there is little similarity among the amino acid sequences of these proteins, and, when compared with data banks, there are no other proteins with significant homology, except in three cases. LcaB (Table 4) and MesI, the putative immunity protein for the mesentaricin Y105 system, are 74.5% identical (the two bacteriocins differ by only 2 amino acids) (24); LcaB (Table 4) and the protein encoded by ORF2 of the enterocin A system are 44.5% identical (enterocin A and leucocin A are 46% identical) (7); and SaiA (Table 4) and the protein encoded by ORFα2, the putative immunity factor for carnobacteriocin BM1 (46), are 49% identical (the two bacteriocins differ by 13 amino acids) (5). Leucocin A, mesentaricin Y105, enterocin A, sakacin A, and carnobacteriocin BM1 are all class IIa, *Listeria*-active peptides (30). To our knowledge, cross immunity among these systems, particularly that of leucocin and mesentaricin, have not been reported. Recently, van der Vossen et al. (57) reported that the acidocin A immunity factor provides cross protection to plantaricin F. To our knowledge, this is the only report of this kind suggesting that the majority of bacteriocins each require their own specific immunity factor.

Two of the class II immunity proteins, CbnB2 (47) and LciA (40, 60), have been studied in detail. The cellular locations of both immunity proteins have been determined: CbiB2, a minor cellular protein, is located mainly in the cytoplasm (92%), with a minor amount (8%) present in the membrane fraction of *Carnobacterium piscicola* LV17B, and LciA, a major cellular protein, is present in equal amounts in the cytoplasm, membrane, and membrane-associated fractions of *L. lactis*. LciA is predicted to have a transmembrane helix (amino acids 29 to 47), whereas CbiB2 is not. Given the cellular location of CbiB2, Quadri et al. (47) have proposed that it may interfere with CbnB2 pore formation or may block a functional pore from the cytoplasmic side, thus preventing efflux of intracellular components. Venema et al. (60) have proposed that the membrane-bound form of LciA is the active form. Their data also suggest that LciA interacts with the lactococcin A receptor, thus preventing a productive receptor-bacteriocin interaction.

LafI is similar to other class II immunity factors in size and charge (Table 4). On the basis of structural characteristics, however, LafI is most similar to LciM, the immunity protein for the lactococcin M/N system (53). LciM and LafI share the following characteristics (Fig. 5): the primary amino acid sequence is predicted to have four transmembrane helices, approximately half of the amino acids located between the second and third helices are charged, and the C terminus contains several lysine residues. The similarities in the putative structures of these two proteins suggest that the immunity mecha-

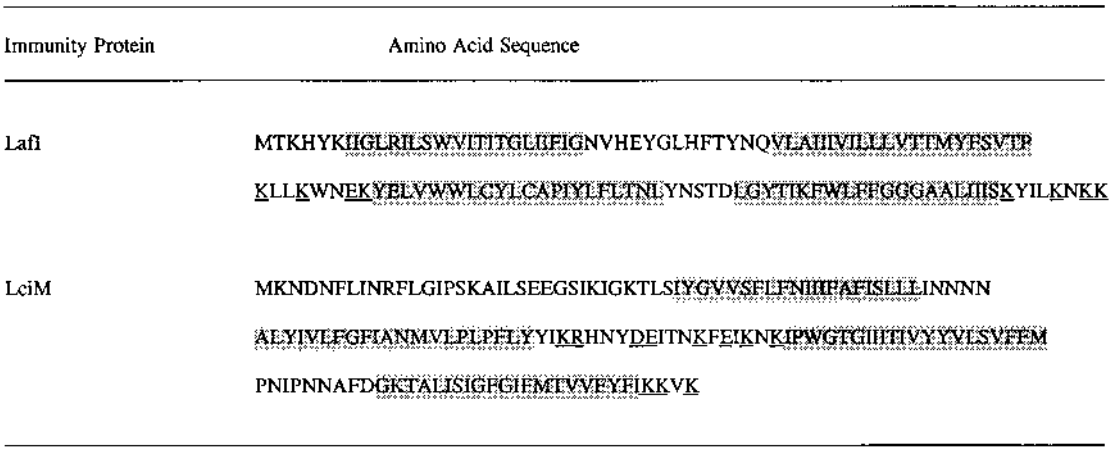

FIG. 5. Comparison of the amino acid sequences of LafI and LciM. Transmembrane helices (shaded), predicted by the method of Eisenberg et al. (21), and charged residues (underlined) are indicated.

nisms may be conserved. Several other two-component bacteriocin systems that have been characterized genetically include the following: lactococcin G produced by *Lactococcus lactis* LMG 2081 (37, 39, 41); plantaricins A and T, produced by *L. plantarum* (42, 66); and thermophilin 13, produced by *Streptococcus thermophilus* Sfi13 (35). The plantaricin A structural gene is located proximal to genes encoding a histidine kinase and response regulatory proteins (17), and recent data have suggested that the plantaricin A peptides are inducing factors for bacteriocin production (16). Like lactacin F and lactococcin M/N, the lactococcin G and thermophilin 13 systems also encode an ORF directly downstream of the two bacteriocin structural genes. The protein encoded by this ORF in the lactococcin G system, LagC (103 amino acids), is also predicted to have four transmembrane segments (39). The proteins encoded by the third ORF are presumed to be the immunity factors, but the function has not been characterized. While it appears that there is a conserved genetic organization among two-component bacteriocins, it remains to be determined whether there is a conserved mechanism of immunity.

Heterologous expression of *lafI* by numerous lactobacilli conferred immunity to lactacin F. Reports of heterologous expression of other class II immunity factors include the following: *lciA* expression by *L. lactis* IL1403 (60), *lciB* expression by *L. lactis* SK112 and IL1403 (59), *pedB* expression by *Pediococcus pentosaceus* PPE1.2 (61), and *cbiB2* expression by *C. piscicola* LV17C and UAL26 (47). Quadri et al. (47) reported that the level of immunity of the CbiB2-expressing strains LV17C and UAL26 was not as great as that of the wild-type strain. They proposed that either the pMG36e promoter was not efficient in these backgrounds or an additional factor(s) was required to confer full immunity. Heterologous expression of *lciA*, *lciB*, and *pedB* conferred wild-type immunity to the respective bacteriocins. Both lactococcin A and lactococcin B are proposed to interact with membrane-bound receptors (59, 60). If these receptors are conserved among lactococci, expression of the immunity proteins in heterologous lactococcal hosts would lead to a functional interaction between the immunity factor and receptor, thus providing protection (60). It may be possible that lactacin F interacts with a receptor molecule that is conserved among lactobacilli and therefore also leads to protection across the genera. If this is the case, variations in sensitivity to lactacin F could be due to differences in the number of receptor molecules present in each strain. It may be possible, therefore, that more LafI would be needed to protect highly sensitive strains, such as *L. helveticus* NCDO87 and *L. delbrueckii* subsp. *lactis* ATCC 4797. Whether expression of LafI by pTRK434 would be sufficient to protect these strains is unknown, since attempts to transform them have been unsuccessful. Analysis of *L. helveticus* NCDO87 would be of particular interest, since it is sensitive to both the LafA-LafX complex and LafA alone. Heterologous immunity could also be conferred if LafI is the sole determinant that directly interacts with the formation of and/or functionality of the LafA-LafX pore, as is proposed for CbiB2 (47).

Expression of *lafI* in other genera, however, may not confer immunity. When pTRK434 was introduced into lactacin F-sensitive strains *C. divergens* LV13 and *E. faecalis* ATCC 19433, the transformants remained sensitive to the bacteriocin (data not shown). It has been reported that the P6 promoter is active in these backgrounds (19); however, the transcriptional and translational efficiencies of LafI are unknown, and the observed lack of protection could reflect failure in expression. The immunity-negative phenotype in these strains could also indicate that lactacin F interacts with different genera in different ways. For example, enterococci and carnobacteria may have different receptors for lactacin F which do not interact with LafI. Therefore, *lafI* may confer lactacin F immunity specifically in lactobacilli, an important consideration in using this gene as a food-grade marker.

Lactacin F produced by VPI 11088 was used to select for transformants of *L. fermentum* NCDO1750 containing the LafI-encoding plasmid, pTRK434. Incorporation of the lactacin F producer into the selective media was more effective than the addition of lactacin F (supernatant or TCA-concentrated supernatant) to agar. The latter method was unsuccessful because of the inability to harvest large quantities of supernatant containing high-activity lactacin F. There are a number of limitations, however, in using lactacin F as a food-grade marker. The system is limited to *Lactobacillus* strains that are sensitive to lactacin F, and conditions must be manipulated and optimized for each strain. Bacteriocin production by VPI 11088 can be variable, making standardization of conditions difficult. Background due to spontaneous resistance to the bacteriocin may be a consideration, although this was not a factor in our experiments. To our knowledge, nisin is the only other bacteriocin that has been used as a primary selective agent. Froseth and McKay (25) and von Wright et al. (62) successfully used the nisin resistance (Nis^r) determinant to directly select for nisin-sensitive strains transformed with a recombinant plasmid encoding Nis^r. Other reports of heterologous genes, originating from lactobacilli, that could potentially serve as food-grade markers include the following (reviewed in reference 31): xylose utilization encoded by *Lactobacillus pentosus* has been expressed in *Lactobacillus casei* and *L. plantarum*, α-amylase from *L. amylovorus* has been expressed in *L. plantarum*, β-galactosidase of *Lactobacillus bulgaricus* has been expressed by *L. helveticus*, acidocin A production and immunity of *L. acidophilus* have been expressed by *L. casei* (29), and acidocin B production and immunity of *L. acidophilus* have been expressed by *L. plantarum* and *L. fermentum* (34). Proteins from other genera expressed by *Lactobacillus* spp. include cellulase, xylanase, endoglucanase, β-glucanase, chitinase, and others (reviewed in reference 31). To our knowledge, the current data and those of Posno et al. (43) are the only reports of utilizing food-grade agents for the direct selection of *Lactobacillus* transformants. In the latter case, D-xylose was used to select for lactobacilli encoding proteins involved in xylose utilization (see above). Other agents showing potential for direct selection of transformants are starch and inulin, which could be used as the primary carbohydrate sources for lactobacilli producing amylase and levanase (22, 65), respectively.

Over the past decade, the ability to manipulate lactobacilli has become a reality which is leading to increasing interest in improving the characteristics of these strains through genetic engineering. However, there are few food-grade vectors and markers available for use in this genus. The interest in using bacteriocins as selectable markers in lactic acid bacteria and lactobacilli has increased recently (34, 45, 50, 67). Development of such tools will likely be important for public acceptance by legislative bodies (45). The use of native *Lactobacillus* genes and expression signals as markers should promote efficient expression and stability. Furthermore, most genes and expression signals from one *Lactobacillus* species are usually expressed among heterologous species (44), whereas expression of genes from other genera can produce variable results (31). The *lafI* gene from *L. johnsonii* VPI 11088 was expressed by *L. acidophilus* NCFM/N2 and ATCC 4356, *L. fermentum* NCDO1750, *L. gasseri* ATCC 33323, and *L. johnsonii* ATCC 33200. In all cases, the lactacin F sensitivity of the LafI-expressing transformants decreased more than 8- to 64-fold, depending on the strain. Transformation of strain ATCC 4356

has been impossible until recently (63). Furthermore, strains ATCC 4356, NCFM/N2, and ATCC 33323 are human isolates. Efficient expression of a selectable marker by these strains and other intestinal isolates may facilitate colonization studies and the construction of food-grade vectors for manipulation of intestinal and industrial lactobacilli.

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