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Conjugal Transfer of Plasmid-Encoded Determinants for Bacteriocin Production and Immunity in *Lactobacillus acidophilus* 88†

PETER M. MURIANA AND TODD R. KLAENHAMMER*

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27695-7624

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***Lactobacillus acidophilus* 88 produced a bacteriocin, designated lactacin F, that demonstrated inhibitory activity toward *L. acidophilus* 6032, *L. lactis* 970, *L. helveticus* 87, *L. bulgaricus* 1489, *L. leichmanii* 4797, *L. fermentum* 1750, and *Streptococcus faecalis* 19433. Production of lactacin F was pH dependent and could be maximized in MRS broth cultures maintained at pH 7.0. Lactacin F was heat stable and sensitive to ficin, proteinase K, trypsin, and *Bacillus subtilis* protease. *L. acidophilus* 88 harbored plasmids of 4 and 27 megadaltons. Variants of *L. acidophilus* 88 which were deficient in lactacin F production (Laf^+) and lactacin F immunity (Laf^r) retained the two resident plasmids. A Laf^- Laf^s derivative, *L. acidophilus* 89, was used as a recipient in agar surface mating experiments with *L. acidophilus* 88 (Laf^+ Laf^r). Two types of Laf^+ Laf^r transconjugants were recovered. One type (T-E) had acquired two plasmids of 68 (pPM68) and 52 (pPM52) megadaltons that were not detected in either the conjugal donor or the other type of Laf^+ Laf^r transconjugants (T-89). Laf^+ and Laf^r were unstable in the plasmid-bearing transconjugant. Plasmid analysis of Laf^- Laf^s variants revealed that pPM52 and pPM68 were cured with loss of Laf^+ and Laf^r. Bacteriocin production and immunity phenotypes were genetically stable in Laf^+ Laf^r transconjugants not harboring pPM52 and pPM68, suggesting chromosomal integration of the transferred determinants. The data demonstrated intragenic conjugation in *L. acidophilus* and provided direct evidence for involvement of transient plasmid determinants in Laf^+ and Laf^r.**

Lactobacillus acidophilus is an important natural inhabitant of the intestinal tract of humans and other animals. In dairy product applications, *L. acidophilus* is notably used in acidophilus milk products as a source of dietary lactobacilli (24). Strain improvement of such commercially important lactobacilli has relied almost exclusively on natural selection and mutagenesis. Improvement of *Lactobacillus* cultures through genetic modification provides exciting opportunities, but potential developments are hampered by the paucity of genetic transfer systems and information on native plasmid-encoded traits in these bacteria.

In recent years, efforts to accumulate genetic information and define mechanisms of gene transfer for lactobacilli have increased. However, breakthroughs have been slow and sporadic. Transduction has yet to be identified in this genera despite a wide distribution of lysogenic phages (25). Procedures have been reported for effective protoplast formation and regeneration protocols (14, 31) and for liposome-mediated transfection of *L. casei* (23). These developments combined with initial reports for transformation (15) and protoplast fusion (7, 11) should accelerate further progress in definition of fusion and transformation systems for lactobacilli. In contrast, conjugal transfer events have been widely observed in numerous *Lactobacillus* species, using the broad-host-range conjugative plasmid pAMβ1 (9, 30, 34). In a preliminary report, Chassy and Rokaw described conjugal transfer of plasmid-associated lactose-fermenting ability in *L. casei* (6). These reports affirm that conjugal transfer events occur within the *Lactobacillaceae*. However, with the single exception of *L. casei*, native conjugal transfer systems and processes remained unidentified.

Bacteriocin production and resistance determinants should serve as suitable markers to study and develop genetic systems in lactobacilli. Bacteriocins are proteinaceous and demonstrate bactericidal activity against sensitive cells (26). Native bacteriocin phenotypes and genetic determinants would be of particular interest for use in gene transfer and cloning systems in lactobacilli targeted for food fermentations or other applications. Bacteriocins are widely produced by lactobacilli, particularly *L. acidophilus* strains. DeKlerk and Coetzee (8) reported bacteriocin production by 11 strains of *L. acidophilus*, while Barefoot and Klaenhammer (4) observed bacteriocin production by 42 of 52 strains of *L. acidophilus* examined.

Plasmid-encoded bacteriocin production and host cell immunity have been routinely described for many gram-negative (3, 33) and gram-positive (20, 32) bacteria. Numerous strains of *L. acidophilus* harbor plasmid DNA (16, 28, 29) and production of bacteriocins by members of this species is widespread (4, 8). However, previous attempts to correlate bacteriocin production and immunity to plasmid DNA in *Lactobacillus* species have been unsuccessful (4, 12, 19, 27). In this study we report genetic transfer of plasmids encoding bacteriocin production and host cell immunity in *L. acidophilus* 88 by a conjugationlike process. The bacteriocin, designated lactacin F, was heat stable and proteinaceous and exhibited a narrow inhibitory spectrum. Use of lactacin F as a selective agent in genetic transfer experiments allowed detection of conjugal events and implicated plasmid determinants for lactacin F production and immunity.

* Corresponding author.

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TABLE 1. Bacterial strains

Strain	Phenotype	Relevant plasmids (MDa)	Reference
L. acidophilus 6032 (neotype)	Lab$^+$ Lab$^{r a}$		13
L. acidophilus 88[b]	Laf$^+$ Laf^r Lac$^+$	4, 27	13
L. acidophilus 88-B	Laf$^-$ Laf^s Lac$^+$	4, 27	This study
L. acidophilus 88-C	Laf$^-$ Laf^r Lac$^+$	4, 27	This study
L. acidophilus 88-4	Laf$^-$ Laf^s Lac$^-$	4, 27	This study
L. acidophilus 88-5	Laf$^-$ Laf^r	4, 27	This study
L. acidophilus 89	Laf$^-$ Laf^s Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus 90	Laf$^-$ Laf^s Lac$^-$ Spc^r Gn^r	4, 27	This study
L. acidophilus T89	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus T90	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus T91	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus T92	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus T93	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus T94	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus T-E	Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27, 52, 68	This study
L. acidophilus T-E1	Laf$^-$ Laf^s Lac$^-$ Sm^r Rif^r	4	This study
L. acidophilus 89-M	Laf$^-$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus 89-R	Laf$^-$ Laf^r Lac$^-$ Sm^r Rif^r	4, 27	This study
L. acidophilus C-7		40	This study
L. bulgaricus 1489 (neotype)	Laf$^-$ Laf^s		4
L. lactis 970	Laf$^-$ Laf^s		4
L. fermentum 1750 (neotype)	Laf$^-$ Laf^s		4
L. leichmannii 4797 (neotype)	Laf$^-$ Laf^s		4
L. helveticus 87	Laf$^-$ Laf^s		4
E. coli V517		35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4	17
E. coli K-12 (sublines J5, J53, C600)		34, 23, 5.5	22
S. lactis ML3-C145		33, 60	2

[a] Lab, Lactacin B.

[b] Previously listed as 11088 by Johnson et al. (13). Ferments galactose, glucose, lactose, maltose, mannose, sucrose, and trehalose. Resistant to 100 ng of nisin per ml.

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MATERIALS AND METHODS

Bacterial cultures and media. Bacterial strains and derivatives used in this study are shown in Table 1. Cultures were maintained as frozen stock cultures held at −20°C in their appropriate growth medium plus 10% glycerin (Fisher Scientific Co., Raleigh, N.C.). *Escherichia coli* and *Streptococcus faecalis* strains were propagated in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.). *Streptococcus lactis* was propagated in Elliker broth (Difco Laboratories, Detroit, Mich.). *Lactobacillus* cultures were inoculated into MRS broth (Difco) at the 1% level and propagated twice before use in experiments. MRS agar was prepared by addition of 1.5% granulated agar (BBL) to the broth medium. Overlay agar was prepared with 0.75% granulated agar. Media containing lactacin F were prepared by addition of spent MRS culture supernatants of *L. acidophilus* 88, neutralized to pH 7.0, to MRS broth or agar before autoclaving.

Bacteriocin detection and assay. Bacteriocin production by *L. acidophilus* 88 was detected by adaptations of direct (18) and deferred antagonism described previously (4). Indicator cultures evaluated were described previously (5) and included *L. leichmannii* 4797, *L. lactis* 970, *L. bulgaricus* 1489, *L. helveticus* 87, *L. fermentum* 1750, *L. acidophilus* 6032, and *S. faecalis* 19433. A modification (12) of the critical dilution assay (18) was used to assign activity units (AU) per milliliter to filter-sterilized MRS broth containing bacteriocin. For lactacin F assays, indicator lawns of *L. leichmannii* 4797 and *L. fermentum* 1750 were prepared by propagating cultures to an optical density (590 nm) of 0.4 and adding 100 μl of the cell suspension to 3.5 ml of overlay MRS agar.

Bacteriocin production. The influence of agar pH and incubation time on production of lactacin F by colonies of *L. acidophilus* 88 was evaluated. MRS agar was adjusted with HCl or NH_4OH to an initial pH level of 5.5, 6.6, or 7.6. The plates were inoculated with an overnight culture of *L. acidophilus* 88 appropriately diluted in 0.1% nonfat dry milk. The plates were immediately overlaid with 6 ml of MRS agar (1.5%) and incubated anaerobically for either 24 or 72 h before application of indicator lawns. Indicator lawns were prepared by addition of 100 μl of overnight cultures of *L. leichmannii* 4797 or *L. fermentum* 1750 to 3.5 ml of MRS overlay agar.

Fermentor conditions for production of lactacin F by *L. acidophilus* 88 were evaluated at 37°C as described previously (4) in MRS broth (500 ml) maintained at a pH of 5.0, 6.0, 7.0, or 7.5. Lactacin F culture filtrates used in experiments were obtained from fermentor cultures held at pH 7.0 for 10.5 h. The cells were removed by centrifugation and the supernatant fraction was filter sterilized. This material was frozen at −20°C and designated crude lactacin F.

Inhibitory effects due to lactic acid or hydrogen peroxide. Potential inhibitory actions of *L. acidophilus* 88, which may be unrelated to bacteriocin-mediated antagonism, were examined. Uninoculated MRS broths at pH 6.7, or acidified with HCl or lactic acid to pH 4.2, and MRS broth culture filtrates of *L. acidophilus* 88 (pH 4.2) were tested for inhibitory activity by direct antagonism against *L. leichmannii* 4797. Suspensions of crude lactacin F were also evaluated for inhibitory responses due to hydrogen peroxide. Solutions (10 μl) of 3% H_2O_2, 3% H_2O_2 plus catalase (4,000 U/ml; Sigma Chemical Co., St. Louis, Mo.), catalase (4,000 U/ml), and crude lactacin F plus catalase (4,000 U/ml) were spotted

directly onto lawns of *L. leichmannii* 4797 and examined for inhibition after 24 h of incubation at 37°C under anaerobic conditions.

Sensitivity to heat and proteolytic enzymes. Crude lactacin F (1 ml at 12,800 AU) was subjected to 99°C in a boiling-water bath for 5, 10, 15, and 20 min. Lactacin F activity was assayed against *L. leichmannii* 4797 before and after heat treatment.

Crude lactacin F was treated with ficin, proteinase K, *B. subtilis* protease, and trypsin. The enzymes and buffer suspensions used were described previously (12). Crude lactacin F was treated for 2 h at 37°C with each protease at a final concentration of 1 mg/ml. Protease activity was stopped by heating at 100°C for 5 min. Lactacin F activity remaining after protease treatment was assayed against *L. leichmannii* 4797. Controls consisted of enzyme plus buffer, lactacin F plus buffer, and heat-inactivated enzyme plus lactacin F.

Mutant screening and isolation. Single colonies of *L. acidophilus* 88 were examined by deferred antagonism for loss of lactacin F activity. Loss of bacteriocin activity was evaluated after propagation in MRS broth at 37 or 43°C and after treatment with plasmid-curing agents as follows: ethidium bromide (2 μg/ml), acridine orange (20 μg/ml), acriflavine (20 μg/ml), novobiocin (0.1 μg/ml), and novobiocin (0.1 μg/ml) plus nalidixic acid (1.0 μg/ml).

Colonies that did not produce lactacin F (Laf^-) were isolated directly from deferred antagonism plates. The agar was first flipped from the base plate into the cover. Mutants were picked with a sterile needle through the sterile basal layer and streaked onto MRS agar plus 0.15% Oxgall bile salts (Difco). *L. acidophilus* 88 was resistant to Oxgall, whereas *L. leichmannii* 4797 indicator would not form colonies on this medium. This purification step circumvented the misidentity of any 4797 colonies as Laf^- isolates of *L. acidophilus*. Loss of host cell immunity (Laf^r) in Laf^- isolates was evaluated by direct antagonism, using crude lactacin F to challenge Laf^- isolates.

Antibiotic-resistant derivatives of Laf^- isolates were isolated for use in genetic experiments. Spontaneous mutants resistant to streptomycin (Sm; 2 mg/ml) and rifamycin (Rif; 100 μg/ml) or gentamicin (Gn; 500 μg/ml) and spectinomycin (Spc; 50 μg/ml) were isolated by sequential selection on MRS agar containing increasing antibiotic concentrations.

Phenotypic and genetic variants of *L. acidophilus* 88 were examined for carbohydrate fermentation patterns and loss of nisin resistance. Carbohydrate solutions (4%, wt/vol) included amygdalin, arabinose, cellobiose, galactose, glucose, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Cultures to be tested were harvested from overnight growth on MRS agar, using sterile cotton swabs. Cells were suspended in double-strength MRS basal broth (MRS broth without beef extract or carbohydrate) to equal the turbidity of a no. 5 McFarland standard. Equal volumes of carbohydrate solutions and cell suspensions were mixed in microtiter wells and incubated anaerobically for 48 h at 37°C. Acid production (yellow color) was evaluated in each well after addition of 50 μl of 0.04% bromocresol purple, pH 7.2.

Plasmid isolation. Small-scale plasmid isolations were performed as described previously (16). Large-scale plasmid isolations were performed by the method of Anderson and McKay (1), with the following modifications: incubations with lysozyme were performed at 0°C for 1 h followed by 15 min at 37°C; incubation was continued at 37°C for 15 min after addition of lysis solution; lysate shearing was omitted; following phenol extraction, the aqueous phase was extracted with an equal volume of chloroform; DNA was precipitated with 2 volumes of cold ethanol and held overnight at −20°C. After centrifugation, plasmid DNA from large-scale preparations was purified in cesium chloride-ethidium bromide gradients.

Conjugation experiments. Conjugation experiments were conducted between *L. acidophilus* 88 (Laf^+ Laf^r Lac^+ Sm^s Rif^s) and *L. acidophilus* 89 (Laf^- Laf^s Lac^- Sm^r Rif^r). Overnight cultures in MRS broth were transferred to fresh broth and grown to an optical density at 590 nm of 0.5. The cultures were then diluted 1:10 with 0.1% nonfat dry milk. Donor (100 μl) and recipient (300 μl) cells were mixed and immediately centrifuged in 1.5-ml Eppendorf tubes for 5 min. A 300-μl aliquot of the supernatant was discarded. The pellet was suspended in the remaining 100 μl of supernatant which was then surface plated directly onto MRS agar at pH 5.5. In donor-only control matings, 100 μl of donor cells was plated directly. In recipient-only control matings, 300 μl of recipient cells was centrifuged, suspended in 100 μl, and plated. Mating plates were incubated anaerobically for 24 h at 37°C. A 1-ml portion of 0.1% nonfat dry milk was added to the surface of the plates, and the cells were suspended with a sterile glass rod. Cell suspensions were harvested, diluted with 0.1% nonfat dry milk, and plated onto selective MRS agar containing lactacin F (1,600 AU/ml), streptomycin (2 mg/ml), and rifamycin (100 μg/ml). The level of lactacin F used in selection plates was determined by the highest concentration which did not inhibit donor (Laf^+ Laf^r) cells. Selective plates were incubated anaerobically for 48 h at 37°C and scored for Laf^r Sm^r Rif^r colonies. Laf^r Sm^r Rif^r colonies were replica plated to MRS agar, pH 7.5, and examined for lactacin F production by the deferred method of bacteriocin detection. Laf^+ transconjugants were scored by the presence of an inhibition zone against the *L. leichmanii* 4797 indicator.

Conjugation controls used nonviable donor cells, DNase I-treated donor cells, and cell-free spent broth from donor cells. Donor cells were killed by mixing equal volumes of cell suspension with chloroform. The mixture was vortexed for 30 s at high speed and allowed to separate into aqueous and organic phases. The cell suspension was drawn from the upper phase and used as the donor contribution to mating experiments. A 200-μl aliquot was plated directly onto MRS agar to assure the absence of viable donor cells. In the DNase I control, donor cells were diluted 1:10 with a solution of DNase I (100 μg/ml in 0.75% NaCl). Donor-recipient cell pellets were also suspended with the DNase solution prior to mating. As a transduction control, spent broth from donor cells was filter sterilized and used in matings with recipient cells. A 200-μl aliquot of donor broth filtrate was plated directly onto MRS agar to assure proper filter sterilization.

RESULTS

Inhibitory spectrum. *L. acidophilus* 88 demonstrated inhibitory activity against *L. leichmannii* 4797, *L. fermentum* 1750, *L. lactis* 970, *L. helveticus* 87, *L. bulgaricus* 1489, *L. acidophilus* 6032, and *S. faecalis* 19433 by both direct and deferred antagonism. These data confirmed previous observations (4) noting antagonistic action of *L. acidophilus* 88 towards members of the *Lactobacillaceae* and a member of the enterococci. The inhibitory activity produced by *L. acidophilus* 88 was designated lactacin F.

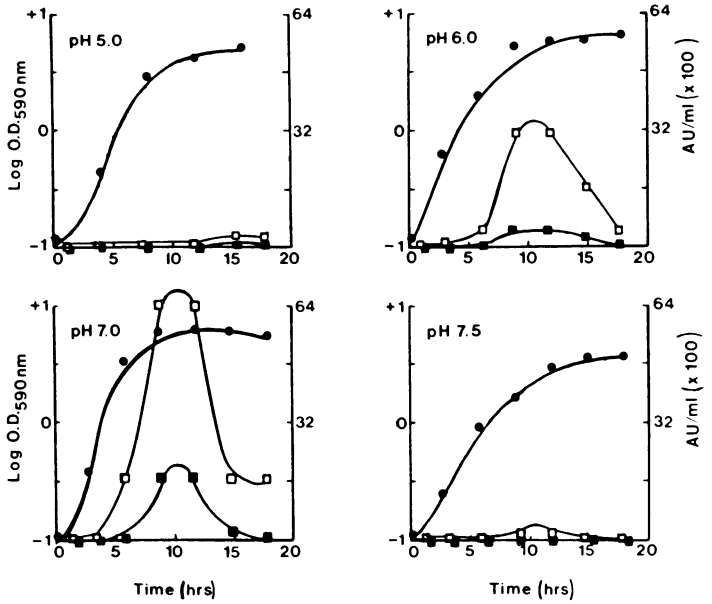

FIG. 1. Production of lactacin F by *L. acidophilus* 88 maintained at constant pH in MRS broth. Symbols: Log optical density at 590 nm (●); lactacin F activity assayed against *L. leichmannii* 4797 (□) or *L. fermentum* 1750 (■).

Attributing the inhibitory activity of *L. acidophilus* 88 to bacteriocinlike substances required elimination of other inhibitory factors produced by lactobacilli. MRS broth or MRS broth acidified to pH 4.2 with HCl or lactic acid showed no inhibition of *L. leichmannii* 4797 cell lawns. However, MRS broth culture filtrates of *L. acidophilus* 88 (at pH 4.2) spotted onto *L. leichmannii* 4797 lawns formed distinct and clear zones of inhibition. Catalase was effective in eliminating the inhibitory activity of a 3% solution of hydrogen peroxide, but failed to eliminate the lactacin F activity. Lactacin F activity could not be propagated from inhibition zones to fresh indicator lawns and showed no individual plaques when the activity was diluted to extinction. These observations ruled out any contribution of a phage in the inhibitory reactions.

Production of lactacin F in broth and agar. Lactacin F production by *L. acidophilus* 88 was examined in pH-controlled fermentors, using the most sensitive (*L. leichmannii* 4797) and least sensitive (*L. fermentum* 1750) indicator strains to monitor production (Fig. 1). Negligible lactacin F activity was observed against *L. leichmannii* 4797 when *L. acidophilus* 88 was grown in MRS broth maintained at pH 5.0 or 7.5. At pH 6.0, lactacin F activity reached 3,200 AU/ml in 10.5 h, but decreased to 400 AU/ml by 18 h. The highest titers of lactacin F were observed in MRS broth cultures at pH 7.0. Lactacin F activity in culture filtrates

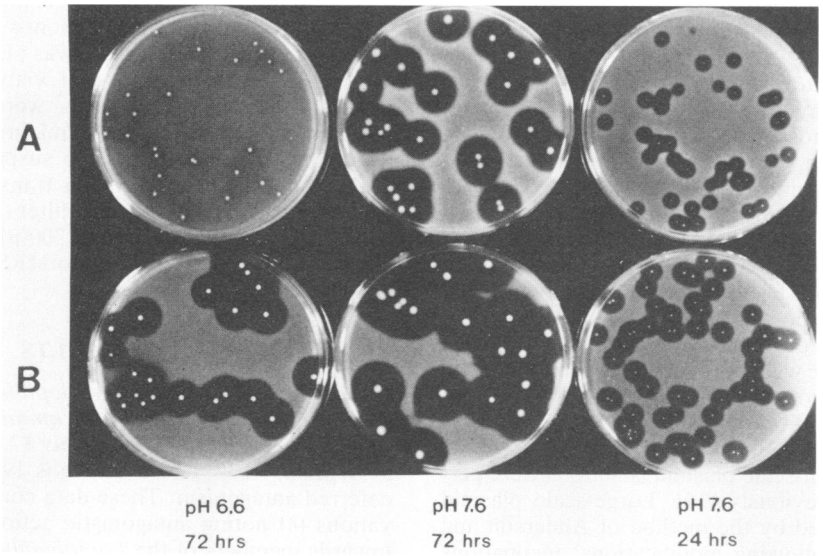

FIG. 2. Effect of pH and incubation time on production of lactacin F by colonies of *L. acidophilus* 88 on MRS agar. Row A, *L. fermentum* 1750 indicator lawn; row B, *L. leichmannii* 4797 indicator lawn.

reached 6,400 AU/ml in 10.5 h, but again, decreased to 1,600 AU/ml by 18 h. The time course of lactacin F production detected with *L. fermentum* 1750 paralleled that of *L. leichmannii* 4797, albeit at lower activity levels.

Since pH caused significant differences in the amount of inhibitory activity produced in broth, studies were conducted to identify any pH effects on agar (Fig. 2). On MRS agar at pH 6.6 (72-h colonies), no inhibitory zones were detected against *L. fermentum* 1750 indicator cell lawns. However, clear inhibition of *L. leichmannii* 4797 confirmed the production of an inhibitory substance by *L. acidophilus* 88 colonies under identical conditions. At pH 7.6 (72 h), distinct inhibitory zones were observed for both *L. fermentum* 1750 and *L. leichmannii* 4797 indicator cell lawns. No inhibitory zones were detected against either indicator strain when *L. acidophilus* 88 colonies were formed on MRS agar at pH 5.5 (data not shown). The zone size could be reduced by decreasing producer colony incubation time (24 h; Fig. 2). Routinely, 24-h colonies formed on MRS agar at pH 7.6 were used to screen variants in lactacin F production because a larger number of colonies could be examined.

Sensitivity to heat and proteolytic enzymes. Lactacin F activity was unaffected after 20 min at 99°C or after autoclaving at 121°C for 15 min at 15 lb/in^2 (data not shown). Lactacin F activity was not detected after treatment with ficin, proteinase K, trypsin, and *B. subtilis* protease, while full lactacin F activity remained in samples treated with heat-inactivated proteases (Table 2). The data showed that lactacin F was proteinaceous and heat stable.

Laf$^-$/Laf^s variants and plasmid involvement. *L. acidophilus* 88 was screened for the occurrence of Laf$^-$ variants following repetitive transfers at 37 and 43°C and in the presence of plasmid-curing dyes. No Laf$^-$ variants were detected among 10,319 colonies examined after growth of the 88 culture in the presence of ethidium bromide, acriflavine, acridine orange, novobiocin, or novobiocin plus nalidixic acid. In contrast, four Laf$^-$ variants were isolated (3,848 colonies examined) following several transfers of *L. acidophilus* 88 in MRS broth at 37 and 43°C. Of the four colonies that did not form zones of inhibition in *L. leichmannii* 4797 lawns, two isolates (88-B and 88-4) were also sensitive to lactacin F. Laf$^-$ isolates 88-C and 88-5 retained the Laf^r phenotype. The four Laf$^-$ variants were examined for other differences in phenotypic properties exhibited by *L. acidophilus* 88. Resistance to nisin and all carbohydrate fermentation capabilities were retained in the variants, except in 88-4 in which lactose-fermenting ability (Lac) was also lost.

L. acidophilus 88 harbored two plasmids of 4 and 27 megadaltons (MDa), designated pPM4 and pPM27, respectively (Fig. 3). The band at the 8-MDa position was identified as an open circular form of pPM4 (data not shown). None of the Laf$^-$ Laf^s or Laf$^-$ Laf^r derivatives showed detectable differences in plasmid composition from the parent strain (Fig. 3, lanes B, C, and D). These data provided evidence that the resident plasmids of *L. acidophilus* 88 (pPM4 or pPM27) were not involved in lactacin F production, immunity, or lactose-fermenting ability.

Conjugal transfer of lactacin F. The availability of a Laf$^-$ Laf^s derivative of *L. acidophilus* 88 provided opportunities to perform genetic transfer experiments within an homologous host background. An antibiotic-resistant derivative of *L. acidophilus* 88-4 (Laf$^-$ Laf^s Lac$^-$), designated 89 (Laf$^-$ Laf^s Lac$^-$ Sm^r Rif^r), was selected and used as a conjugal recipient in surface matings with *L. acidophilus* 88 (Laf$^+$ Laf^r Lac$^+$ Sm^s Rif^s) on MRS agar, pH 5.5.

Laf$^+$ transconjugants (Laf$^+$ Laf^r Lac$^-$ Sm^r Rif^r) were generated when viable cells of *L. acidophilus* 88 and 89 were mated in three separate experiments (Table 3). No colonies were obtained on selective media with donor-only controls. Likewise, in recipient-only controls, no Laf$^+$ colonies were detected when spontaneous Laf^r mutants of *L. acidophilus* 89 were replica plated and overlaid with indicator cells. Spontaneous Laf^r mutants of *L. acidophilus* 89 arose at frequencies approximating 10^{-6} to 10^{-7} per postmating recipient. Laf$^+$ Laf^r transconjugants were detected at frequencies approximating 10^{-8} to 10^{-9} per postmating recipient. The identity of Laf^r transconjugants as genetic recombinants of 89 was further confirmed by analysis of the Lac$^-$ phenotype. In two conjugation experiments, Laf$^+$ transconjugants were also obtained when donor and recipient cells were mated in the presence of DNase. Use of nonviable donor cells or cell-free donor filtrates in matings with 89 did not result in Laf$^+$ transconjugants. These data demonstrated the operation of a conjugationlike process for transfer of lactacin F production.

TABLE 2. Effects of proteases on lactacin F activity

Enzyme[a]	Inhibitory activity (AU/ml)[b]			
	Enzyme	Lactacin F	Enzyme + lactacin F	Heat-inactivated enzyme + lactacin F
Ficin	0	3,200	0	3,200
Proteinase K	0	3,200	0	3,200
Bacterial protease	0	1,600	0	1,600
Trypsin	0	3,200	0	3,200

[a] 1 mg of enzyme per ml, reaction concentration.
[b] Assayed against *L. leichmannii* 4797.

Plasmid and phenotypic analysis of Laf$^+$ transconjugants. The plasmid composition of Laf$^+$ transconjugants was examined and compared with profiles of the donor, recipient, and spontaneous Laf$^-$ Laf^r mutants selected from recipient-only (strain 89-R) and mating (strain 89-M) plates. Two types of plasmid profiles were observed for the Laf$^+$ transconjugants (Fig. 3, lanes M and N). In one case, represented by Laf$^+$ Laf^r strain T-89, no change from the recipient's plasmid profile was detected in three of six transconjugants examined. For the remaining transconjugants, represented by Laf$^+$ Laf^r strain T-E, two new plasmid bands approximating 68 (pPM68) and 52 (pPM52) MDa were detected in addition to the two resident plasmids (4 and 27 MDa) harbored by *L. acidophilus* 89. Since Laf$^-$ Laf^r spontaneous mutants 89-M and 89-R (Fig. 3, lanes F and G) failed to show pPM52 and pPM68, a mutational event from Laf^s to Laf^r could not account for the appearance of these large plasmids. These data strongly implicated pPM52 and pPM68 in conjugal transfer of Laf$^+$.

Genetic stability of the Laf$^+$ Laf^r phenotypes were compared between Laf$^+$ transconjugants T-89 and T-E. After a single transfer in MRS broth, approximately 50% of T-E colonies were Laf$^-$. In contrast, no genetic instability of Laf$^+$ was detected from strain T-89 propagated under identical conditions (100% Laf$^+$, Fig. 4). Propagation of strain T-E under selective conditions in MRS broth plus lactacin F (1,600 AU/ml) stabilized the Laf$^+$ character, suggesting a close genetic association between Laf^r and Laf$^+$ in the transconjugant bearing pPM52 and pPM68. Further examination of 20 spontaneous Laf$^-$ variants isolated from T-E, after growth in MRS broth without lactacin F selection, demonstrated that all were also Laf^s. Plasmid analysis of a spontaneous Laf$^-$ Laf^s T-E derivative (*L. acidophilus* T-E1)

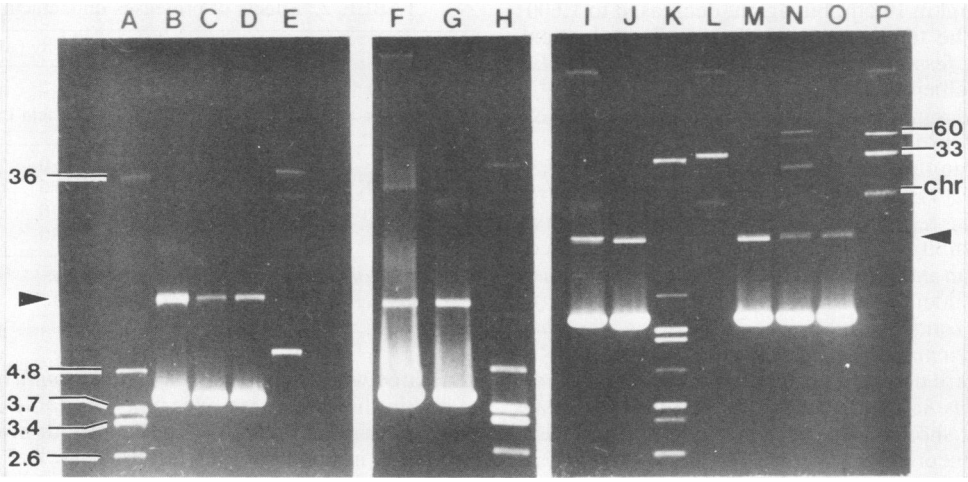

FIG. 3. Plasmid DNA profiles of *L. acidophilus* 88 variants and transconjugants. Lanes A, H, and K, *E. coli* V517; lanes B and I, *L. acidophilus* 88 (Laf^+ Laf^r donor); lane C, 88-B (Laf^- Laf^s variant); lane D, 88-C (Laf^- Laf^r variant); lane E, *E. coli* K-12 (34, 23, 5.5 MDa); lane F, 89-M (Laf^- Laf^r mutant); lane G, 89-R (Laf^- Laf^r mutant); lane J, 89 (Laf^- Laf^s Lac^- recipient); lane L, *L. acidophilus* C-7 (40 MDa); lane M, T-89 (Laf^+ Laf^r transconjugant); lane N, T-E (Laf^+ Laf^r transconjugant); lane O, T-E1 (Laf^- Laf^s variant); lane P, *S. lactis* ML3-C145 (33 and 60 MDa). Lanes A, E, H, K, L, and P harbor reference plasmids for molecular weight comparisons. "chr" denotes position of chromosomal DNA. Arrows denote position of the open circular form of pPM4. Numbers to the left and right denote the molecular mass of reference plasmids in megadaltons.

demonstrated loss of the 68- and 52-MDa plasmids, as well as the 27-MDa resident plasmid (Fig. 3, lane O). These data confirmed that pPM52 and pPM68 encoded determinants for Laf^+ and Laf^r, but failed to differentiate between the two plasmids and their association with either phenotype.

Subsequent attempts to segregate the Laf^+ and Laf^r phenotypes in transconjugant T-E were unsuccessful. No Laf^- colonies were detected when 2,071 colonies were examined after growth of T-E in MRS broth plus lactacin F (3,200 AU/ml). After growth of T-E in MRS broth without lactacin F selection, 639 of 1,182 colonies examined (54%) exhibited concurrent loss of both Laf^+ and Laf^r.

Matings were conducted between *L. acidophilus* T-E (Laf^+ Laf^r Lac^- Sm^r Rif^r) and strain 90 (Laf^- Laf^s Lac^- Gn^r Spc^r) to determine if Laf^+ transfer would occur from the pPM52- and pPM68-bearing transconjugant. Five T-E exconjugants were isolated (Table 3) and examined for plasmid composition. Neither pPM52 nor pPM68 was detected in the exconjugants (data not shown). After progagation in MRS broth, the exconjugants exhibited instability of Laf^+ at levels of 1 to 3%. This level of instability appeared consistent with the absence of pPM52 and pPM68 in the exconjugants, but was substantially greater than had been detected previously in either *L. acidophilus* 88 or the Laf^+ Laf^r transconjugant T-89 in which pPM52 or pPM68 are also not detected.

Carbohydrate fermentation patterns were compared among strains 89, T-E, and T-E1 to examine for other potential phenotype-plasmid linkages. No changes in fermentation ability were detected upon acquisition of pPM52 and pPM68 or with loss of the 68-, 52-, and 27-MDa plasmids.

DISCUSSION

Antagonism by lactobacilli, due to metabolic end products such as acid and hydrogen peroxide, can be erroneously attributed to the production of bacteriocinlike compounds (4). After possible inhibitory effects due to acid, hydrogen peroxide, or bacteriophage were eliminated, *L. acidophilus* 88 was found to produce a heat-stable, proteinaceous compound that was inhibitory to *L. acidophilus* 6032, *L. bulgaricus* 1489, *L. helveticus* 87, *L. lactis* 970, *L. fermentum* 1750, *L. leichmannii* 4797, and *S. faecalis* 19433. Phenotypic and genetic properties examined during the course of this study clearly established lactacin F as a bacteriocin produced by *L. acidophilus* 88.

Lactacin F appeared similar to lactacin B described previously (4, 5). Both bacteriocins were heat stable and proteinaceous and exhibited similar production and stability properties in pH-controlled fermentations. Although the host range for both bacteriocins was identical for members of the *Lactobacillaceae*, it is notable that lactacin F extends this host range to include a member of the group D streptococci. Extensive biochemical characterization of lactacin F has not been conducted so that direct comparisons of physical, structural, or compositional characteristics with lactacin B (5) are not possible at the present time.

TABLE 3. Conjugal transfer of Laf^+ and Laf^r from *L. acidophilus* 88 to *L. acidophilus* 89

Donor[a]	Recipient	No. of Laf^+ transconjugants per ml[b]			
		Expt 1	Expt 2	Expt 3	Avg
88[c]	89[d]	56	75	18	50
88		0	0	0	0
	89	0	0	0	0
88 + chloroform	89	0	ND[e]	ND	0
88 + DNase I	89	5	38	ND	23
88 filtrate	89	0	ND	ND	0
T-E[f]	90[g]	5	ND	ND	5

[a] Donor/recipient cell ratios were approximately 1:3.
[b] Scored transconjugants were Laf^+ Laf^r Lac^- Sm^r Rif^r.
[c] *L. acidophilus* 88 was Laf^+ Laf^r Lac^+ Sm^s Rif^s.
[d] *L. acidophilus* 89 was Laf^- Laf^s Lac^- Sm^r Rif^r.
[e] ND, Not determined.
[f] *L. acidophilus* T-E was Laf^+ Laf^r Lac^- Sm^r Rif^r.
[g] *L. acidophilus* 90 was Laf^- Laf^s Lac^- Gn^r Spc^r and transconjugants scored as Laf^+ Laf^r Lac^- Gn^r Spc^r.

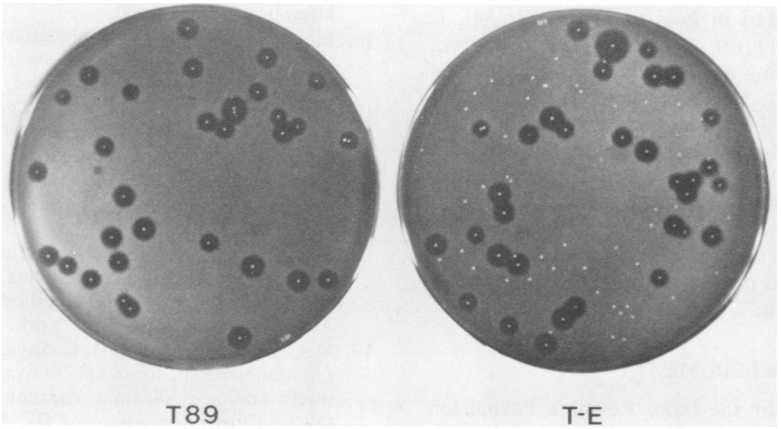

FIG. 4. Inhibition of an *L. leichmannii* 4797 indicator lawn by colonies of *L. acidophilus* T-89 and *L. acidophilus* T-E. T-89 and T-E cultures were propagated once through MRS broth at 37°C, in the absence of lactacin F selection, and plated for isolated colonies.

Tagg et al. (26) noted the predominance of plasmid-encoded bacteriocins throughout the bacterial genera. Consequently, they included the involvement of plasmid-borne determinants for bacteriocins as a low-priority criterion for defining bacteriocins. Previous studies addressing *Lactobacillus* bacteriocins have attempted to use plasmid-curing agents to destabilize bacteriocin phenotypes and identify plasmid-encoded determinants within this genus (4, 12, 19). Failure to isolate bacteriocin-deficient clones after treatment with plasmid-curing agents led investigators to implicate chromosomal determinants for bacteriocins produced by lactobacilli (4, 12, 19). In the present study, Laf^- Laf^s variants of *L. acidophilus* 88 were obtained at low frequency ($\sim 10^{-3}$) following repetitive transfers in broth. Use of plasmid-curing agents was ineffective and did not accentuate the appearance of variants. Laf^- Laf^s variants demonstrated no detectable difference in native plasmid composition when compared with the parent culture. These data are supportive of chromosomal determinants for lactacin production and immunity in *L. acidophilus* 88. However, a remote possibility remains that resident plasmids harboring determinants for Laf^+ and Laf^r were simply not detected in the parental culture.

Genetic transfer of both Laf^+ and Laf^r by a conjugation-like process was demonstrated with *L. acidophilus* 88 and an isogenic Laf^- Laf^s derivative. The design of the mating and selection conditions was based on the following observations. First, *L. acidophilus* 88 did not produce lactacin F on MRS agar plates at pH 5.5. Therefore, a lactacin F-producing donor could be mated with a Laf^s recipient without antagonism occurring during growth of the mating mixtures. Second, use of lactacin F in the selection media allowed detection of transconjugants at relatively low frequencies. Laf^r colonies that appeared were assumed to be of two types, either spontaneous Laf^r mutants or true transconjugants. Examination of all Laf^r colonies for concurrent acquisition of Laf^+ differentiated true transconjugants from spontaneous Laf^r mutants. In confirmed Laf^+ transconjugants harboring pPM52 and pPM68, Laf^r was genetically unstable and correlated with plasmid DNA. Therefore, cotransfer of Laf^+ and Laf^r was demonstrated. However, by this approach it could not be determined whether or not Laf^r was subject to transfer in the absence of Laf^+.

Laf^+ Laf^r transconjugants generated in matings between *L. acidophilus* 88 and 89 were of two distinct types. In the Laf^+ Laf^r transconjugant T-E, transfer of Laf^+ and Laf^r was accompanied by the appearance of two large plasmids, pPM52 and pPM68, that were not detected in the donor. Evidence for the involvement of pPM52 and pPM68 in Laf^+ and Laf^r accumulated from experiments demonstrating direct correlations between plasmid transfer and concomitant loss of both plasmids upon destabilization of both phenotypes. Segregation of either the Laf^+ Laf^r phenotypes or plasmids was not detected in either curing or genetic transfer experiments with T-E. Therefore, the specific functions and phenotypes associated with either plasmid remain unclear.

In the second type of Laf^+ Laf^r transconjugant (T-89), pPM52 and pPM68 were not detected in the plasmid profile. Laf^+ and Laf^r were genetically stable in the absence of selective pressure in strain T-89, suggesting that chromosomal integration of the mobilized elements may have occurred following conjugal transfer. Considering that pPM52 and pPM68 encoding Laf^+ and Laf^r were maintained tenuously in the extrachromosomal state, genetically stable transconjugants such as T-89 may be representative of a native conjugal event. Determinants mobilized from the donor chromosome are reintegrated into the chromosome of the recipient following transfer. The autonomous state of pPM52 and pPM68 may, therefore, reflect a mobilization event. Plasmids pPM52 and pPM68 were not detected in Laf^+ Laf^r exconjugants of T-E. Whether or not the plasmids were simply not detected or had integrated into the chromosome is not clear. Genetic instability in the exconjugants (1 to 3%) was atypical of curing levels associated with pPM52 and pPM68 (50%). This instability was substantially greater than detected with either *L. acidophilus* 88 or the Laf^+ Laf^r transconjugant T-89, in which pPM52 and pPM68 are not detected and Laf^+ Laf^r determinants are suspected to be chromosomally associated. The level of instability suggests plasmid involvement; however, an unstable chromosomal integration event in the T-E exconjugant remains possible.

Transfer of genetic determinants encoding lactacin F supports characterization of this inhibitory agent as a bacteriocin. Criteria suggested by Hopwood (10) to define a plasmid-encoded antibiotic function were satisfied during this study and included (i) genetic transfer, (ii) correlation of synthesis with the presence of a particular plasmid, and (iii) phenotype instability correlated with loss of the plasmid species. More importantly, definition of a native conjugation system in *Lactobacillus* species extends a previous report by Chassy and Rokaw (6) noting a conjugal transfer process for lactose-fermenting ability in *L. casei*. Conjugation in

lactobacilli has been reported in *L. plantarum* (21, 34), *L. casei* (9), *Lactobacillus reuteri*, *Lactobacillus salivarius*, and *L. acidophilus* (30), using the conjugative streptococcal plasmid pAMB1. Although these studies clearly demonstrated conjugative potential for lactobacilli, inherent conjugative processes and determinants subject to mobilization remain largely unknown. Availability of native conjugation systems in lactobacilli, promoted by *Lactobacillus* conjugative plasmids, will undoubtedly provide new and expanded opportunities to study genetic processes and define mechanisms for gene delivery and mobilization in this industrially significant group of bacteria.

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