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# Requirement of Autolytic Activity for Bacteriocin-Induced Lysis

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**The bacteriocin produced by *Lactococcus lactis* IFPL105 is bactericidal against several *Lactococcus* and *Lactobacillus* strains. Addition of the bacteriocin to exponential-growth-phase cells resulted in all cases in bacteriolysis. The bacteriolytic response of the strains was not related to differences in sensitivity to the bacteriocin and was strongly reduced in the presence of autolysin inhibitors ($Co^{2+}$ and sodium dodecyl sulfate). When *L. lactis* MG1363 and its derivative deficient in the production of the major autolysin AcmA (MG1363*acmA*Δ*1*) were incubated with the bacteriocin, the latter did not lyse and no intracellular proteins were released into the medium. Incubation of cell wall fragments of *L. lactis* MG1363, or of *L. lactis* MG1363*acmA*Δ*1* to which extracellular AcmA was added, in the presence or absence of the bacteriocin had no effect on the speed of cell wall degradation. This result indicates that the bacteriocin does not degrade cell walls, nor does it directly activate the autolysin AcmA. The autolysin was also responsible for the observed lysis of *L. lactis* MG1363 cells during incubation with nisin or the mixture of lactococcins A, B, and M. The results presented here show that lysis of *L. lactis* after addition of the bacteriocins is caused by the resulting cell damage, which promotes uncontrolled degradation of the cell walls by AcmA.**

Bacteriocins are antimicrobial polypeptides synthesized ribosomally by bacteria (34). Most bacteriocins from lactic acid bacteria exert their antibacterial effect by permeabilizing the target cell membrane, whereby the cells lose their viability (1, 5, 29). Apart from damaging cell membranes, some bacteriocins have also been reported to cause bacteriolysis. Bierbaum and Sahl (4) were among the first to show the involvement of autolysins in the bacteriolytic effect of a bacteriocin. Autolysins are peptidoglycan hydrolases that are capable of causing bacterial autolysis (39). The authors showed that the bacteriocins Pep5, produced by *Staphylococcus epidermidis*, and nisin, produced by *Lactococcus lactis*, activate an *N*-acetylmuramoyl-L-alanine amidase and an β-*N*-acetylglucosaminidase of *S. simulans* (4). Plantaricin C has been shown to be bacteriolytic for *Lactobacillus fermentum* LM 13554 and *L. delbrueckii* subsp. *bulgaricus* LMG 13551, while no reduction of the optical densities (ODs) of mid-exponential-phase cultures of *L. sake* CECT 906, *L. helveticus* LMG13555, or *Leuconostoc mesenteroides* was observed (14, 15). Microscopic analysis of *L. fermentum* cells treated with plantaricin C showed that changes had taken place in the cell wall. The authors suggested that cell lysis could be a secondary effect of the bacteriocin caused by a deregulation of the autolytic system of the sensitive cells resulting in destruction of the peptidoglycan layer. While no lysis of *Lc. mesenteroides* cells treated with plantaricin C was seen, a clear reduction of the OD was observed when these cells were incubated with pediocin AcH (3). This effect was not observed with *Lactobacillus plantarum*, although intracellular components were released. Transmission electron micrographic analysis of cells of both bacterial species revealed the presence of lysed ghost cells upon treatment with pediocin AcH (18). The action of nisin against *Listeria monocytogenes* Scott A cells resulted in the loss of cellular material following lysis, as shown by electron microscopic analysis (10). The antibacterial cyclic peptide AS-48 produced by *Enterococcus faecalis* S-48 also has bactericidal and bacteriolytic activity against several *L. monocytogenes* strains (28). These authors show that cells adapted to AS-48 have a changed fatty acid composition of their cytoplasmic membrane and a thicker cell wall and become more resistant to autolysins. For *L. monocytogenes* and *E. faecalis* growing cells, it was observed that loss of viability was much more rapid than the observed reduction of the OD. *L. monocytogenes* growing cells also lysed upon addition of pediocin PA-1, while the amount of bacteriocin activity added did not have a great influence on the degree of reduction of the OD (37).

A bacteriolytic effect of bacteriocins on lactococci was first reported by Kok et al. (21), who described that treatment of lactococcal cells with lactococcin A (LcnA) resulted in the release of UV-absorbing material. Using the same bacteriocin, Morgan et al. (30) obtained bacteriolysis and subsequent release of an intracellular enzyme from sensitive lactococcal cells only when LcnA acted in concert with the lactococcins B and M. Another bacteriocin which has been shown to cause lysis of sensitive *L. lactis* cells is the bacteriocin produced by *L. lactis* IFPL105 (previously identified as *Lactobacillus curvatus* IFPL105) (9). This secreted broad-spectrum bacteriocin has been shown to cause lysis of logarithmically growing *L. lactis* and *Lactobacillus casei* (26). The importance of the lytic effect of this bacteriocin in accelerating cheese proteolysis has been demonstrated in cheese curd slurries manufactured with sensitive strains as starter and bacteriocin-producing adjuncts (27). Increase of starter cell lysis and free amino acid concentration in Cheddar cheese have been described by Morgan et al. (31) after using as starter adjunct a lactococcal strain producing lactococcins A, B, and M.

The major autolysin activity described for lactococci and lactobacilli is that of an *N*-acetylmuramidase (24, 32). The gene (*acmA*) encoding the enzyme in *L. lactis* has been cloned and sequenced (6). The construction of an *acmA* deletion mutant

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TABLE 1. Cell viability and lysis after 3 h of incubation at 37°C in 20 mM sodium phosphate buffer (pH 6.8) of suspensions of log-phase cells of *Lactococcus* and *Lactobacillus* strains with or without bacteriocin

| Strain | Initial counts[a] (CFU/ml) | Incubation in buffer | | Incubation with bacteriocin (150 AU/ml) | |
|---|---|---|---|---|---|
| | | Counts (CFU/ml) | Lysis[b] (%; mean ± SE) | Counts (CFU/ml) | Lysis (%; mean ± SE) |
| *Lactococcus lactis* IFPL359 | $3.3 \times 10^9$ | $1.0 \times 10^8$ | 32.5 ± 2.3 | $4.8 \times 10^3$ | 51.3 ± 1.2 |
| *L. lactis* T1 | $1.5 \times 10^9$ | $5.0 \times 10^7$ | 22.4 ± 2.8 | $3.8 \times 10^3$ | 56.4 ± 3.5 |
| *L. lactis* IFPL22 | $2.0 \times 10^9$ | $5.0 \times 10^7$ | 15.6 ± 1.3 | $3.6 \times 10^3$ | 28.4 ± 0.9 |
| *L. lactis* IFPL1053 | ND[c] | ND | 4.3 ± 1.1 | ND | 29.8 ± 1.3 |
| *Lactobacillus casei* IFPL731 | $1.0 \times 10^9$ | $1.0 \times 10^8$ | 14.9 ± 1.5 | $8.7 \times 10^3$ | 25.7 ± 0.8 |
| *L. casei* JCL1227 | $5.9 \times 10^8$ | $1.0 \times 10^8$ | 16.3 ± 1.6 | $1.1 \times 10^3$ | 30.2 ± 0.8 |
| *L. plantarum* IFPL935 | $9.9 \times 10^8$ | $1.0 \times 10^8$ | 15.2 ± 4.8 | $2.3 \times 10^5$ | 74.5 ± 4.4 |
| *L. rhamnosus* JCL1211 | $6.8 \times 10^8$ | $1.0 \times 10^8$ | 15.6 ± 1.3 | $3.0 \times 10^5$ | 74.5 ± 3.7 |

[a] Cell viability expressed as the number of CFU after plating two appropriate dilutions of the cell suspensions in M-17 agar (lactococci) and MRS-agar (lactobacilli).
[b] Percent lysis = $100 - (A_1/A_2 \times 100)$, where $A_1$ is the lowest and $A_2$ is the highest value of the $OD_{600}$ measured during incubation of the cell suspensions.
[c] ND, not determined.

by replacement recombination has allowed to demonstrate that AcmA is required for cell separation and autolysis of cells during stationary growth phase (6, 7). Several factors, such as starvation for a carbon source, reagents which cause depletion of either the electrical or pH gradients of cellular membranes or which cause disruption of these membranes, as well as proteolytic degradation, have been shown to influence the autolytic behavior of cells (8, 11, 19, 20, 24).

The object of this work was to investigate whether bacteriolysis by the bacteriocin produced by *L. lactis* IFPL105 on different strains of lactococci and lactobacilli was a direct or indirect effect of the bacteriocin. The results show that bacteriolysis is observed only when active autolysins are present in the sensitive cells. The bacteriocin does not activate the autolysin AcmA of *L. lactis*. Rather, depletion of cellular energy causes an imbalance in the control of the action of the autolysin, resulting in cell wall degradation and, thus, lysis of cells.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacteriocin producer *L. lactis* IFPL105 and its mutant *L. lactis* IFPL1053 ($Bac^-$ $Imm^-$) (9) are from the Culture Collection of the Instituto del Frío, Madrid, Spain. The bacteriocin-sensitive microorganisms used in this study are listed in Table 1. *L. casei* JCL1227 and *L. rhamnosus* JCL1211 were kindly provided by Juan Jimeno (FAM Sektion Biochemie, Liebefeld CH-3003, Bern, Switzerland). Other lactococcal strains used were *L. lactis* subsp. *cremoris* MG1363 (12), its derivative MG1363*acmA*Δ*1* (6), and *L. lactis* subsp. *cremoris* 9B4 (41), which produces lactococcins A, B, and M. Culture media were M-17 broth (Adsa-Micro, Pharmafaster SA, Barcelona, Spain) containing lactose or glucose (5 g/liter) for lactococci and MRS broth (Adsa-Micro) for lactobacilli. The incubation temperature for all strains was 30°C. The microorganisms were stored at −80°C in reconstituted skim milk (100 g/liter).

**Isolation of bacteriocins from the culture supernatant.** The bacteriocin was isolated from a 1-liter culture of *L. lactis* IFPL105 grown to the late exponential-early stationary phase. Cells were removed by centrifugation at 6,000 × *g* for 15 min at 4°C, and 400 g of ammonium sulfate was added to the culture supernatant. The protein precipitate was pelleted by centrifugation at 8,000 × *g* for 20 min at 4°C, solubilized in 15 ml of 20 mM phosphate buffer (pH 6.8), and loaded onto $C_{18}$ Sep-Pak cartridges (Millipore Co., Bedford, Mass.). Cartridges were washed with 25% 2-propanol in 0.1% trifluoroacetic acid (TFA), and bacteriocin activity was eluted with 40% 2-propanol in 0.1% TFA. Lactococcins A, B, and M were concentrated by ammonium sulfate precipitation from a 1-liter culture supernatant of *L. lactis* 9B4 as described above. The solubilized precipitate in phosphate buffer of the lactococcins A, B, and M and the bacteriocin from *L. lactis* IFPL105 (crude bacteriocin) was autoclaved (121°C, 10 min) to avoid residual autolysin AcmA activity. Nisin was purchased from Sigma Chemical Co., St. Louis, Mo.).

The titer of bacteriocin activity (arbitrary units [AU]) was assayed by a serial twofold dilution test as described previously (9), using *L. lactis* IFPL359 or MG1363 as the indicator strain.

**Analysis of the bacteriocin effect by plate counting and by measuring $OD_{600}$ reduction and the release of peptidase activity.** Exponentially growing cultures (OD at 600 nm [$OD_{600}$] of 0.7) in M-17 or MRS broth were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Pellets were washed and suspended in 20 mM sodium phosphate buffer (pH 6.8) containing 150 AU of bacteriocin per ml. Lysis was monitored during 3 h of incubation at 37°C by recording the decrease in $OD_{600}$ using a Spectronic 20 D (Milton Roy Co., Rochester, N.Y.). Percentage of lysis was determined as $100 - (A_1/A_2 \times 100)$, where $A_1$ is the lowest and $A_2$ is the highest value of the $OD_{600}$ measured during incubation (23). Cell lysis was also analyzed after direct addition of the bacteriocin (500 AU/ml) to logarithmically growing cultures ($OD_{600}$ of 0.7) at 30°C in M-17 or MRS broth, by monitoring the decrease in $OD_{600}$ during further growth. Sensitivity of the strains to the bacteriocin was examined at regular intervals by plate counting on M-17 or MRS agar plates.

Controls for components other than the bacteriocin included culture supernatant from *L. lactis* IFPL1053 ($Bac^-$) precipitated with 40% ammonium sulfate, loaded on $C_{18}$ cartridges, and eluted with 40% 2-propanol in 0.1% TFA as described for the parental strain.

Incubations were performed in triplicate, and results were statistically compared by using one-way analysis of variance to determine significant differences ($P < 0.05$) in percentage of lysis among incubation conditions and strains.

The lytic effect of the bacteriocin produced by *L. lactis* IFPL105 was also tested by the addition of the autoclaved crude bacteriocin (300 AU/ml) to exponential-phase cultures ($OD_{600}$ of 0.7) of *L. lactis* MG1363 or MG1363*acmA*Δ*1*. Lysis was followed during incubation at 30°C by monitoring the $OD_{600}$ decrease and the release of intracellular X-prolyl dipeptidyl aminopeptidase (PepX) activity as described before (8). PepX activity was measured in culture supernatants (100 μl) filtered through a 0.22-μm-pore-size filter (Millipore Co.), using as substrate 100 μl of 1 mM Gly-Pro-*p*-nitroanilide (Sigma) solution in 50 mM phosphate buffer (pH 7.0). The total volume of the reaction mixture was brought to 1 ml with phosphate buffer, and incubation was at 37°C using a Peltier CPS-240A temperature controller in a model UV-1601 spectrophotometer (Shimadzu Inc., Columbia, Md.). Release of *p*-nitroaniline was measured as the increase in absorbance at 410 nm ($E_{410}$ = 8,800), and PepX activity was expressed as units of supernatant per milliliter.

The effects of the bacteriocin produced by *L. lactis* IFPL105, the mixture of lactococcins A, B, and M, and nisin were compared after addition of each of the bacteriocin samples (300 AU/ml) to cell suspensions of *L. lactis* MG1363 or MG1363*acmA*Δ*1* in the supernatant fraction of an overnight culture of *L. lactis* MG1363*acmA*Δ*1* ($OD_{600}$ of 2) filtered through a 0.22-μm-pore-size filter. Cell suspensions were obtained by centrifugation (10,000 × *g* for 10 min) of exponential-phase cultures ($OD_{600}$ of 0.7) of the two strains. Lysis was examined after 3 h of incubation at 37°C by monitoring the $OD_{600}$ decrease and the release of PepX activity.

**Effect of metal ions and chemical reagents on bacteriolysis.** The effect of $Co^{2+}$ on the action of the bacteriocin of *L. lactis* IFPL105 was measured by adding the chloride salt (1 mM, final concentration) to *L. lactis* IFPL359 and *L. rhamnosus* JCL1211 cell suspensions in 20 mM phosphate buffer (pH 6.8), obtained as described above. Bacteriocin (500 AU/ml) was added to the cells, and the lysis was monitored during incubation at 37°C by recording the decrease in $OD_{600}$. The effects of sodium dodecyl sulfate (SDS; 0.40 mg/ml) and cardiolipin (0.04 mg/ml) were analyzed by their addition to exponentially growing cultures ($OD_{600}$ of 0.6 to 0.7) of the two strains incubated for 30 min with 500 AU of bacteriocin per ml. Results were expressed as percentage of decrease in cell density measured at 600 nm during the following incubation at 30°C.

**Detection and determination of cell wall hydrolytic activity.** Autolysin activity of *Lactobacillus* and *Lactococcus* strains was assayed after addition of bacteriocin (500 AU/ml) to cell suspensions in buffer or to growing cultures. Samples of 5 ml were obtained at different intervals and centrifuged at 10,000 × *g* for 5 min at 4°C. Portions (0.1 ml) of the supernatants were tested for cell wall hydrolytic activity, using as substrate 0.9 ml of 0.2% (wt/vol) autoclaved *Micrococcus lysodeikticus* cells (Sigma) for *L. lactis* and autoclaved cells of the tested strain for *Lactobacillus*, in 20 mM phosphate buffer, pH 6.8 or 7.5. The reaction mixture

was incubated at 30 or 37°C (depending on the strain) for 3 h. Activity was determined by the decrease in the $OD_{600}$ of the cell suspension per minute.

Lytic activities of the strains were also tested by renaturing SDS-polyacrylamide gel electrophoresis (PAGE) (zymograms) as described by Potvin et al. (36), using SDS–12.5% polyacrylamide gels containing 0.2% (wt/vol) autoclaved cells. *M. lysodeikticus* cells were used as substrate for *L. lactis* samples, while samples of the *Lactobacillus* strains were assayed on autoclaved cells of the tested strain. Samples (5 ml) were obtained at different intervals during incubation of cell suspensions in buffer or broth cultures, with or without bacteriocin, and centrifuged (10,000 × *g*, 5 min, 4°C). Before loading, the samples (cell pellets and lyophilized supernatants) were treated with Laemmli buffer (22) as described by Valence and Lortal (40). Electrophoresis was done in a Mini-Protean II cell unit (Bio-Rad Laboratories, Hercules, Calif.) at 180 V for 1 h. Gels were washed with distilled water, and proteins were renatured in 25 mM Tris-HCl (pH 7.0, 7.5, or 8.0, depending on the strain tested) containing 1% Triton X-100. The renatured cell wall hydrolytic activities appeared as clear bands on the opaque background. The contrast was enhanced by staining the gels with 1% methylene blue in 0.01% KOH and destaining in distilled water.

Effect of the bacteriocin produced by *L. lactis* IFPL105 on autolysin activity was also tested by mixing 300 AU of bacteriocin per ml with a lactococcal cell wall fraction derived from *L. lactis* MG1363 or MG1363*acmA*Δ*1* and suspended (to give a final $OD_{600}$ of 0.7) in the supernatant fraction of overnight cultures of the two strains (6). Native cell walls were obtained at 4°C from exponentially growing cells, harvested by centrifugation (8,000 × *g*, 15 min), suspended in 50 mM potassium phosphate buffer (pH 7.0), mixed (1:1, vol/wt) with glass beads (150 to 212 μm in diameter; Sigma), and disrupted for 16 min (four intervals of 4 min each) in a Mini Blend (Sunbeam-Oster Co. Inc., Miami, Fla.). Whole cells were removed by centrifugation at 1,000 × *g* for 15 min, and the cell walls were recovered from the supernatant by centrifugation at 14,000 × *g* for 15 min at 4°C. The cell wall fragments were suspended in 20 mM sodium phosphate buffer (pH 6.8) containing 0.02% sodium azide. Reduction of the $OD_{600}$ of the cell wall suspensions during incubation at 37°C was followed over time using a Shimadzu UV-1601 spectrophotometer.

**DNA analysis and manipulation.** Genomic DNA of lactococci and lactobacilli was obtained by the method of Anderson and McKay (2). Total DNA was restricted with *Eco*RI (Roche, Mannheim, Germany), separated in a 0.7% agarose gel, and blotted onto positively charged nylon membranes (Roche). A 1.1-kb DNA fragment from the *acmA* gene of *L. lactis* MG1363, amplified by PCR as described by Buist et al. (6), was used as the probe. Probe labeling, hybridization, and immunological detection were performed with the DIG High prime labeling and detection kit according to the instructions of the manufacturer (Roche).

## RESULTS

**Cell lysis and viability of various lactococci and lactobacilli in the presence of the bacteriocin of *L. lactis* IFPL105.** Lysis of cells of lactococci and lactobacilli during incubation in phosphate buffer with 150 AU of a 40% 2-propanol-eluted preparation of bacteriocin produced by *L. lactis* IFPL105 per ml was followed by measuring the percentage of decrease in $OD_{600}$ (Table 1). To ensure that the bacteriocin preparation contained no cell wall-degrading enzymes, it was subjected to SDS-PAGE in the presence of *M. lysodeikticus* autoclaved cells. No bands of clearing, indicative of the presence of cell wall hydrolases, were detected (data not shown).

The lytic response of the various strains to the bacteriocin was statistically different ($P < 0.05$). Addition of the supernatant fraction of *L. lactis* IFPL1053 ($Bac^-$) had no significant effect on the reduction of cell viability and lysis compared to the incubation of cells in phosphate buffer. Autolysis of the four lactococcal strains after 3 h of incubation in phosphate buffer differed considerably, ranging from 4 to 32%. Addition of the bacteriocin and incubation over the same period resulted in 25 to 30% lysis of *L. lactis* IFPL22 and IFPL1053 and 50 to 55% of lysis of the *L. lactis* IFPL359 and T1 strains, showing that the bacteriocin-induced lysis differs among different lactococcal strains. The extents of bacteriocin-induced lysis of the two *L. casei* strains were found to be similar, while the highest percentage of lysis was obtained for the *L. plantarum* and *L. rhamnosus* strains.

Cell viability of the different strains 3 h after addition of the bacteriocin (150 AU/ml) to suspensions of cells taken from the logarithmic phase of growth is also shown in Table 1. Interestingly, the two *Lactobacillus* strains showing the highest lytic response to the bacteriocin, *L. plantarum* IFPL935 and *L. rhamnosus* JCL1211, showed the least loss of viability, whereas *L. casei* IFPL731 and JCL1227 exhibited a loss of viability similar to that of the *Lactococcus* strains.

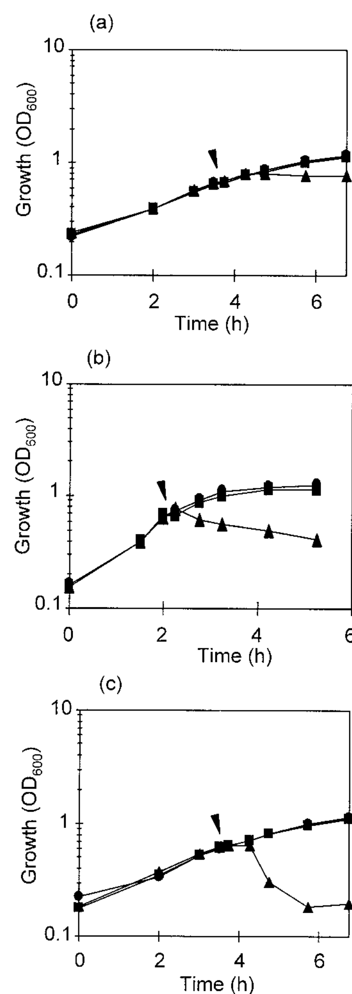


FIG. 1. Growth at 30°C of *L. casei* IFPL731 (a), *L. lactis* IFPL359 (b), and *L. rhamnosus* JCL1211 (c) in broth (●). At the time point indicated by arrowheads, bacteriocin (500 AU/ml) from *L. lactis* IFPL105 (▲) or the supernatant precipitate from *L. lactis* IFPL1053 ($Bac^-$) culture (■) was added.

In all strains studied, reduction of cell viability and $OD_{600}$ were not simultaneous. Figure 1 shows the decrease in $OD_{600}$ of three representative strains after addition of the bacteriocin to exponentially growing cultures. In the case of the lactobacilli, the cell density of the cultures hardly changed within 0.5 to 1 h after addition of the bacteriocin and then decreased rapidly in *L. rhamnosus* JCL1211 to reach 71.3% of lysis after 2 h of further incubation.

**Effect of the bacteriocin of *L. lactis* IFPL105 on autolysin activity.** The observation that loss of viability was not concurrent with cell lysis suggested that the latter phenomenon was not directly caused by the bacteriocin but, likely, was the result of activities of other enzymes such as cell wall-degrading enzymes. The involvement of the autolytic enzymes in cell lysis was studied by adding autolysin activity inhibitors. The results showed that 1 mM $Co^{2+}$ (Fig. 2) and 0.40 mg of SDS per ml (Fig. 3) strongly reduced the lytic response of *L. lactis* IFPL359 to the bacteriocin. Results of previous experiments showed

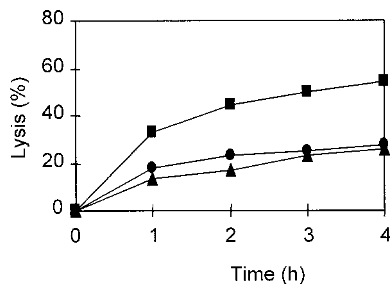


FIG. 2. Lysis during incubation of logarithmic-phase cells of *L. lactis* IFPL359 suspended in 20 mM sodium phosphate buffer (pH 6.8) (●), with 500 AU of bacteriocin from *L. lactis* IFPL105 per ml (■), and with 1 mM $Co^{2+}$ plus bacteriocin (500 AU/ml) (▲).

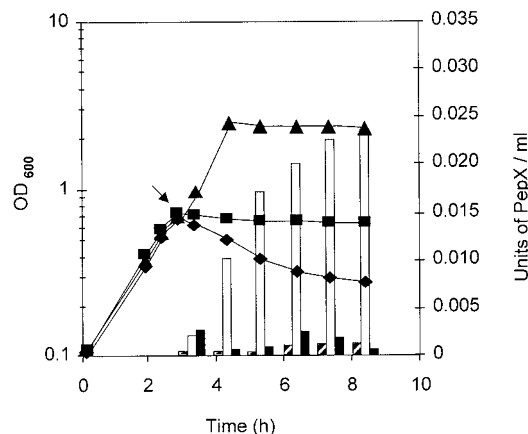


FIG. 4. Evolution of $OD_{600}$ (lines) and release of PepX (bars) during growth at 30°C of *L. lactis* MG1363 in M-17 broth (▲; striped bars) and during incubation with 300 AU of the bacteriocin of *L. lactis* IFPL105 per ml of logarithmic-phase cells of *L. lactis* MG1363 (◆; open bars) and *L. lactis* MG1363*acmA*Δ*1* (■; solid bars). The arrow indicates point of bacteriocin addition.

that autolysis of lactococci is severely reduced upon the addition of $Co^{2+}$ (35). No reduction of lysis was observed when these components were added to a mixture of *L. rhamnosus* JCL1211 cells and the bacteriocin. In this case, a 50% reduction of lysis was observed during the incubation with bacteriocin (500 AU/ml) in the presence of 0.04 mg of cardiolipin per ml.

**Detection of the cell wall hydrolytic activities.** Results of the analysis by renaturing SDS-PAGE of the cell wall hydrolytic activities present in the cell and supernatant fractions of all lactococcal strains showed a banding pattern similar to that of AcmA (6). No other activities could be detected (results not shown). Hybridization experiments using a PCR probe directed against *acmA* showed that the gene was present in all lactococcal strains. The cell wall hydrolytic activity patterns obtained for the three *Lactobacillus* strains were all different. A clearing band of 110 kDa obtained for the *L. plantarum* strain used was of the same size as that obtained for several strains of this species (25). The bands present in the samples of *L. rhamnosus* and *L. casei* were 35 and 70 kDa, respectively (data not shown).

Cell wall hydrolytic activity of the strains in the presence of the bacteriocin from *L. lactis* IFPL105 was analyzed spectrophotometrically and by renaturing SDS-PAGE using autoclaved *M. lysodeikticus* cells or autoclaved cells of the tested strain as a substrate for *L. lactis* and *Lactobacillus* strains, respectively. Total autolysin activity did not increase when the bacteriocin was present in the assays (results not shown).

**AcmA is responsible for bacteriolysis of lactococci.** The involvement of the autolysin AcmA in cell lysis after loss of viability was investigated by comparing the effects of the bacteriocin on lysis of *L. lactis* MG1363 and its mutant *L. lactis* MG1363*acmA*Δ*1*, which cannot produce autolysins. The addition of a crude extract of autoclaved bacteriocin of *L. lactis* IFPL105 (300 UA/ml) to logarithmic-phase cells of *L. lactis* MG1363 growing in broth resulted in a sharp decrease in OD (56% of lysis after 5 h of incubation with the bacteriocin [Fig. 4]). This lysis was concomitant with the release of intracellular material (0.022 U of PepX activity per ml). No lysis or release of PepX was observed after addition of a crude extract from the supernatant of *L. lactis* IFPL1053 ($Bac^-$) or after addition of the autoclaved bacteriocin preparation to logarithmic-phase cells of *L. lactis* MG1363*acmA*Δ*1* (Fig. 4). However, cell counts carried out over the same period resulted in a reduction of cell viability to $10^3$ CFU/ml for both *Lactococcus* strains (MG1363 and MG1363*acmA*Δ*1*) 3 h after addition of the bacteriocin.

**The bacteriocin of *L. lactis* IFPL105 does not activate AcmA activity.** To study whether the effect of the bacteriocin on cell lysis was caused by direct activation of the autolysin AcmA, autoclaved bacteriocin (300 AU/ml) was added to native cell walls of *L. lactis* MG1363 suspended in a supernatant of an overnight culture of *L. lactis* MG1363*acmA*Δ*1*. The results of cell wall degradation, as determined by decrease in $OD_{600}$ of the mixture after 4 h of incubation at 37°C, are shown in Table 2. Hydrolysis of *L. lactis* MG1363 cell walls was not influenced by the presence of the bacteriocin, nor was activation of AcmA found when native cell walls of *L. lactis* MG1363*acmA*Δ*1*, suspended in *L. lactis* MG1363 supernatants containing extracellular AcmA, were incubated with the bacteriocin (Table 2). No significant $OD_{600}$ reduction was observed after incubation

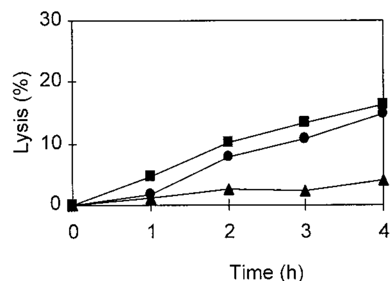


FIG. 3. Lysis during incubation of logarithmic-phase cells of *L. lactis* IFPL359 in M-17 broth with 500 AU of bacteriocin from *L. lactis* IFPL105 per ml (●) and after addition of cardiolipin (0.04 mg/ml) (■) or SDS (0.40 mg/ml) (▲).

TABLE 2. Degradation of *L. lactis* MG1363 and *L. lactis* MG1363*acmA*Δ*1* cell walls suspended in an overnight culture supernatant containing or lacking AcmA activity after 4 h of incubation at 37°C with or without bacteriocin

| Cell walls from: | AcmA (supernatant) | Cell wall degradation (%; mean ± SE) | |
|---|---|---|---|
| | | Control | Bacteriocin (300 AU/ml) |
| *L. lactis* MG1363 | − | 21.9 ± 0.1 | 23.1 ± 0.9 |
| *L. lactis* MG1363*acmA*Δ*1* | + | 22.8 ± 2.1 | 20.9 ± 0.5 |
| | − | 3.8 ± 0.8 | 3.8 ± 1.3 |

TABLE 3. Cell lysis and PepX activity of suspensions of exponentially growing *L. lactis* MG1363 or MG1363*acmA*Δ*1* after 3 h of incubation at 30°C in the presence of bacteriocin

| Strain | Bacteriocin added (300 AU/ml) | Lysis (%)[a] | PepX (U/ml)[b] |
|---|---|---|---|
| *L. lactis* MG1363 | None[c] | 1 | 0.001 |
| | Bacteriocin of *L. lactis* IFPL105 | 17.5 | 0.007 |
| | Lactococcins A, B, and M | 12 | 0.006 |
| | Nisin | 5 | 0.004 |
| *L. lactis* MG1363*acmA*Δ*1* | None | 0 | 0.001 |
| | Bacteriocin of *L. lactis* IFPL105 | 0 | 0.001 |
| | Lactococcins A, B, and M | 2.5 | 0.002 |
| | Nisin | 0 | 0.001 |

[a] Estimated by reduction of $OD_{600}$.
[b] 1 U = 1 μmol of *p*-nitroaniline released/min/ml.
[c] Cell suspensions in the supernatant fraction of an overnight culture of *L. lactis* MG1363*acmA*Δ*1* ($OD_{600}$ of 2) filtered through a 0.22-μm-pore-size filter, used as a control.

of native cell walls of *L. lactis* MG1363*acmA*Δ*1* in a supernatant without AcmA activity.

**AcmA is also responsible for bacteriolysis observed with other lactococcal bacteriocins.** The effect of the bacteriocin of *L. lactis* IFPL105 on lysis of sensitive lactococcal cells was compared with that of nisin and the mixture of lactococcins A, B, and M (Table 3). Cell lysis and the subsequent release of PepX was observed with all three bacteriocin preparations on exponential-phase cells of *L. lactis* MG1363. Lysis was absent when the bacteriocin of *L. lactis* IFPL105 or nisin was added to the *acmA* deletion mutant, while a very small reduction of the $OD_{600}$ was detected when the lactococcin mixture had been added. Also, the release of PepX from the AcmA-negative strain was much less than that obtained for the wild-type strain for all bacteriocins tested. These results indicate that the autolysin is also required for lysis of *L. lactis* MG1363 cells sensitive to bacteriocins other than the bacteriocin from *L. lactis* IFPL105.

## DISCUSSION

Addition of the bacteriocin produced by *L. lactis* IFPL105, nisin, or a mixture of the lactococcins A, B, and M to logarithmic-phase cultures causes effective lysis of *L. lactis* MG1363 cells but not in its autolysin-negative derivative *L. lactis* MG1363*acmA*Δ*1* (Fig. 4 and Table 3). Apparently, the bacteriocins themselves are not capable of lysing lactococcal cells. The results presented here clearly demonstrate that cell lysis induced by addition of lactococcal bacteriocins to bacteriocin-sensitive strains is, in fact, caused by the autolytic system of these strains.

The fact that cell lysis caused by addition of the bacteriocin of *L. lactis* IFPL105 to *Lactobacillus* and *Lactococcus* strains was not concurrent with loss of viability (Fig. 1 and Table 1) suggests that it involves two steps. First, viability is lost due to insertion of the bacteriocin into the membrane of the sensitive cell and depletion of cellular energy (38, 41, 42). Second, a gross imbalance between cell wall buildup and degradation caused in *L. lactis* by AcmA leads to the observed cell lysis. Autolysis as a secondary effect of bacteriocin action has been suggested previously, but the causative agent has never been definitely pinpointed. Some delay between the decrease in cell viability and cell lysis has been observed in the mechanism of action of other bacteriocins (3, 30). Morgan et al. (30) showed that more than 99% of the cells of a lactococcal culture were killed within 10 min upon treatment with a mixture of lactococcins A, B, and M. Ten hours after addition, only 57% of the total amount of the activity of an intracellular marker was released, indicating that bacteriolysis follows loss of viability.

Similarity in the autolytic activities present in the cell and supernatant fraction of the various lactococcal strains investigated was not consistent with their lytic responses to the bacteriocin, which differed considerably. One possible explanation for this observation could be that the cell walls of the different strains have different compositions. The difference in the lytic response of the *Lactobacillus* strains might also be the result of the expression of different cell wall hydrolytic activities. This could also explain the different effects of the autolysin inhibitors used on *L. lactis* and *L. rhamnosus* cells.

Topological regulation of autolytic enzymes by the electrochemical potential of the cell membrane, by cell wall lipoteichoic acids, or by extracellular proteinases has been shown for several species of gram-positive bacteria (4, 8, 13, 19, 20). Incubation of the bacteriocin of *L. lactis* IFPL105 with native cell wall fragments of *L. lactis* MG1363 and its AcmA-defective mutant had no effect on lysis of these cell walls, indicating that the bacteriocin does not activate AcmA, either bound to the cell wall or in supernatants. In light of these results, direct activation of AcmA, as postulated for the autolysin *N*-acetylmuramoyl-L-alanine amidase of *S. simulans* by its replacement from the teichoic acids by cationic bacteriocins (4), does not occur.

Bacteriocins are capable of causing lesions in the cytoplasmic membrane of sensitive cells based on their small size, high hydrophobicity, and hydrophobic regions predicted to form amphipathic α helices (16, 17, 33). This originates dissipation of the proton motive force (5, 29, 42), which has a direct effect on autolysis (19, 20). This effect is shared with other substances such as holin protein of bacteriophages (11, 43). The differences in decrease in OD and in release of the intracellular PepX activity upon addition of the different bacteriocins used might be caused by the difference in effectiveness of pore formation.

Apart from providing fundamental insights into bacteriocin action, the result of this work is also of practical interest. Since the bacteriocin producer *L. lactis* IFPL105 can be used as an adjunct in cheese manufacture (27) and the bacteriocin it produces has a broad spectrum of action, this strain and bacteriocin have both technological and preservative potentials.

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