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## Mode of Action of Lactococcin B, a Thiol-Activated Bacteriocin from *Lactococcus lactis*

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**Lactococcin B (LcnB) is a small, hydrophobic, positively charged bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* 9B4. Purified LcnB has a bactericidal effect on sensitive *L. lactis* cells by dissipating the proton motive force and causing leakage of intracellular substrates. The activity of LcnB depends on the reduced state of the Cys-24 residue. Uptake and efflux studies of different solutes suggest that LcnB forms pores in the cytoplasmic membrane of sensitive *L. lactis* cells in the absence of a proton motive force. At low concentrations of LcnB, efflux of those ions and amino acids which are taken up by proton motive force-driven systems was observed. However, a 150-fold higher LcnB concentration was required for efflux of glutamate, previously taken up via a unidirectional ATP-driven transport system. Strains carrying the genetic information for the immunity protein against LcnB were not affected by LcnB. The proton motive force of immune cells was not dissipated, and no leakage of intracellular substrates could be detected.**

Lactic acid bacteria (LAB) are of eminent economic importance because of their widespread use in food and feed fermentations. Several LAB produce a number of different substances with antimicrobial activity which are of potential interest for food preservation. In a number of instances, the inhibitory activity could be attributed to metabolic end products such as hydrogen peroxide, diacetyl, and organic acids (9). In addition, various LAB strains secrete proteinaceous substances, usually of low molecular weight (bacteriocins), that are often active against closely related strains.

Bacteriocins of LAB can be divided into two subclasses: (i) lantibiotics, small bacteriocins containing unusual amino acids such as dehydroalanine, lanthionine, and  $\beta$ -methyllanthionine (a well-known example is nisin [20], a 3.4-kDa cationic peptide), and (ii) nonlantibiotics, a group of size-variable bacteriocins (2.5 to 37 kDa) containing regular amino acids (this group encompasses the lactococcins, which are small [5- to 10-kDa] cationic peptides). In several cases it has been demonstrated that bacteriocin production is associated with plasmid DNA (1, 10, 13, 38), whereas in other cases production was linked to chromosomal DNA (22). In recent years, a large number of different bacteriocins from LAB have been purified (2, 21, 31; for reviews, see references 9, 25, 26, and 43) and some of the corresponding genes have been cloned and sequenced (6, 46, 47; for a review, see reference 26). However, the mode of action has been described in detail for lactococcin A and nisin only (28, 34, 48).

The lactococcins belong to a broader group of antimicrobial cationic peptides (35) including some of completely different origins: Pep5 from *Staphylococcus aureus* (27), epidermin from *Staphylococcus epidermidis* (19), subtilin from *Bacillus subtilis* (28), gallidermin from *Staphylococcus gallinarum* (24), and the eukaryotic nonbacteriocin peptides cecropins (8), alamethicin (16), melittin (17, 52), and magai-

nins (23, 53). In spite of their different sources, these peptides have some properties in common, i.e., a molecular mass of 3 to 6 kDa and a high isoelectric point. The peptides also differ in certain aspects; some act voltage dependently (3, 27, 36, 39), and others act voltage independently (48). Some act on liposomes or planar lipid bilayers (8, 14, 15, 23, 52), whereas LcnA has no effect on liposomes and needs, in all likelihood, a protein receptor for activity (48). However, a common feature of these peptides seems to be their capacity to form pores in the cytoplasmic membrane, the pore channel being composed of several molecules of the same peptide (8, 16, 37).

*L. lactis* subsp. *cremoris* 9B4 produces at least three bacteriocins, named lactococcins A, B, and M. The structural genes encoding the bacteriocins and their corresponding immunity proteins are carried by plasmid p9B4 and have been cloned and characterized (46, 47). *lcnA* from *L. lactis* subsp. *cremoris* LMG2130 (18) and from *L. lactis* subsp. *lactis* biovar diacetylactis WM4 (42) has also been cloned and characterized. LcnA has been purified previously (18), and its mode of action has been described elsewhere (48). An active LcnM molecule consists of two complementary gene

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant properties	Source or reference
Bacteria		
<i>L. lactis</i> subsp. <i>lactis</i> IL1403	Plasmid free, LcnB <sup>s</sup>	7
<i>L. lactis</i> subsp. <i>cremoris</i> SK112	LcnB <sup>s</sup>	41
Plasmids		
pMB580	Em <sup>r</sup> pGKV210 derivative containing the lactococcin B operon ( <i>lcnB lciB</i> )	47
pMB583	Em <sup>r</sup> pMB580 derivative containing only the immunity gene for lactococcin B ( <i>lciB</i> )	47

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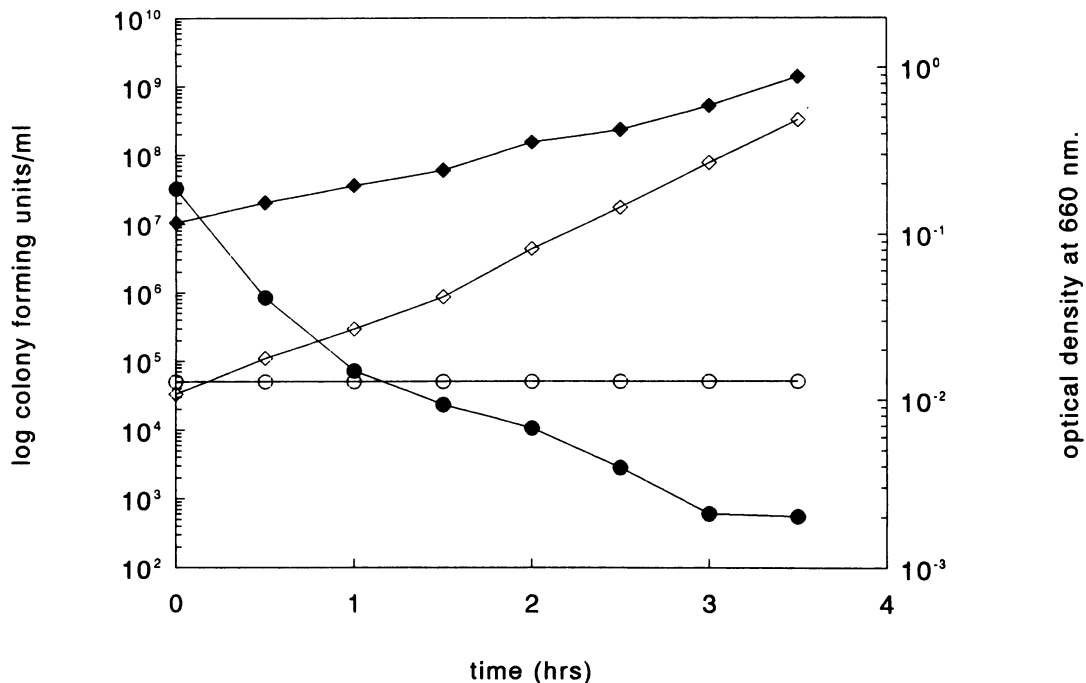


FIG. 1. Effect of incubation with LcnB ( $1.7 \times 10^2$  AU/mg of protein) on the viability and optical density of *L. lactis* SK112 and *L. lactis* SK112(pMB583). ● and ○, SK112 viability and optical density, respectively; ◆ and ◇, SK112(pMB583) viability and optical density, respectively. Counts of viable lactococcal cells are expressed as CFU per milliliter.

products (49). We purified LcnB (50) and report here its effect on the membrane potential, pH gradient, and uptake and efflux of amino acids in *L. lactis* cells.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown in M17 (44) broth supplemented with 0.5% glucose (GM17) or in MRS (11) broth containing 0.5% glucose at 30°C. Erythromycin was used at a final concentration of 5  $\mu$ g/ml unless indicated otherwise.

**Analytical methods.** Protein concentrations were determined by the method of Bradford (5). Bovine serum albumin was used as a standard.

**Lactococcin B isolation.** LcnB was purified from the supernatant of *L. lactis* IL1403(pMB580) by ethanol precipitation and preparative isoelectric focusing as described elsewhere (50). The activity in the preparation was determined by serial twofold dilution spot tests on a GM17 agar plate seeded with the indicator strain *L. lactis* SK112 (50). The highest dilution which gave a distinct zone of inhibition after 16 h was defined as 1 arbitrary unit (AU). By this method a 0.4-ml LcnB solution ( $10^6$  AU/ml) was obtained from 1 liter of culture.

**Effect of lactococcin B on sensitive cells and immune cells.** Overnight cultures of *L. lactis* SK112 and *L. lactis* SK112(pMB583), the strain carrying the gene for the immunity protein for LcnB (*lciB*), were diluted to approximately  $10^7$  cells per ml in GM17. After addition of LcnB ( $1.7 \times 10^2$  AU/mg of cell protein), samples were taken at 30-min intervals to determine (i) the viable cell count by plating onto GM17 agar and (ii) the optical density at 660 nm.

**Transport assays.** Cells of strain *L. lactis* IL1403 grown

overnight in MRS broth were diluted 100-fold in fresh medium and grown to an optical density at 600 nm of 0.6. Cells were harvested by centrifugation, washed once with KMES buffer [50 mM potassium 2-(*N*-morpholino)ethanesulfonic acid (MES), 50 mM KCl, and 2 mM MgSO<sub>4</sub>, pH 6.0], and resuspended in 1/50 of the original volume in KMES buffer. Cells were stored on ice. Transport assays were performed at 30°C. To 1.9 ml of KMES buffer containing 0.4% glucose as an energy source, 0.1 ml of cell suspension was added. Cell suspensions were incubated for 2 min. Uptake was initiated by the addition of [<sup>14</sup>C]2- $\alpha$ -amino isobutyric acid (AIB) (Amersham, Buckinghamshire, United Kingdom) (59 mCi/mmol) or of L-[U-<sup>14</sup>C]glutamate (Amersham) (285 mCi/mmol) to final concentrations of 8.5 and 1.75  $\mu$ M, respectively. Samples (0.1 ml) were taken from the incubation mixture, diluted in 2 ml of 0.1 M LiCl to stop uptake, and filtered with a 0.45- $\mu$ m-pore-size cellulose nitrate filter (Schleicher & Schuell, Dassel, Germany). Filters were washed once with 2 ml of 0.1 M LiCl and air dried, and the radioactivity was measured by liquid scintillation spectrometry in 2 ml of scintillator emulsifier 299 (Packard, Downers Grove, Ill.) by using a Packard 460CD liquid scintillation analyzer.

**Measurement of the membrane potential.** The change in membrane potential of the cells was monitored by the distribution of the lipophilic cation tetraphenylphosphonium (TPP<sup>+</sup>) by using a TPP<sup>+</sup>-selective electrode (40). Cells were washed and resuspended in KMES buffer as described above. The incubation mixture was composed of 0.1 ml of cell suspension and 1.9 ml of KMES buffer containing 0.4% glucose and 4  $\mu$ M TPP<sup>+</sup>. Upon addition of nigericin (1.0  $\mu$ M) (Sigma Chemical Co., St. Louis, Mo.), the interconversion of the pH gradient resulted in an increase in the membrane potential (12).

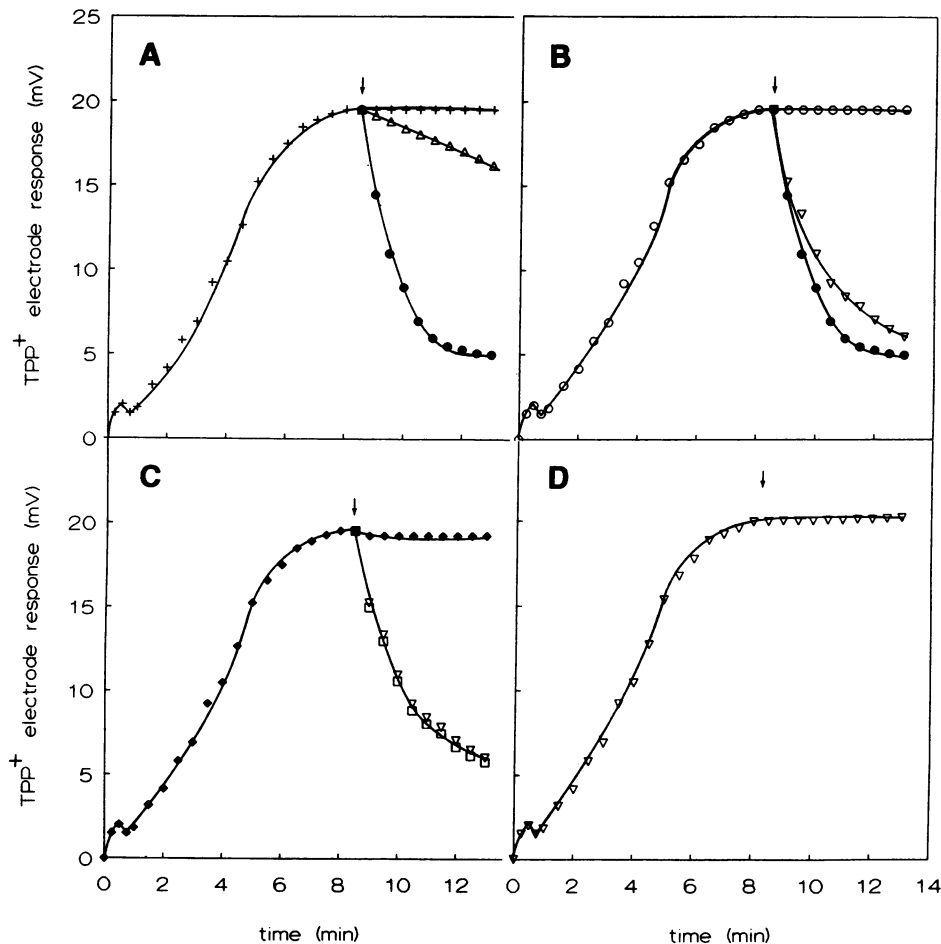


FIG. 2. (A) Effect of LcnB (3.3 AU/mg of protein) on the membrane potential of energized cells of *L. lactis* IL1403. +, no addition;  $\Delta$ , addition of non-DTT-treated LcnB;  $\bullet$ , addition of valinomycin (1  $\mu$ M). (B) Effect of DTT (5 mM) on LcnB (3.3 AU/mg of protein) activity.  $\nabla$ , addition of DTT-treated LcnB;  $\circ$ , addition of 5 mM DTT;  $\bullet$ , addition of valinomycin (1  $\mu$ M). (C) Effect of oxidation by  $\text{HgCl}_2$  (10 nM) on LcnB (3.3 AU/mg of protein) activity.  $\blacklozenge$ , addition of  $\text{HgCl}_2$ -treated LcnB;  $\square$ , addition of  $\text{HgCl}_2$ - and DTT-treated LcnB;  $\nabla$ , addition of DTT-treated LcnB. (D) Effect of DTT-treated LcnB (3.3 AU/mg of protein) on the membrane potential of energized cells of *L. lactis* subsp. *lactis* IL1403(pMB583). The additions were made at the time indicated by the arrows.

**Measurement of intracellular pH.** Cells of *L. lactis* IL1403 and *L. lactis* IL1403(pMB583), grown in MRS broth, were loaded with 2',7'-bis-(2-carboxyethyl)-5[and 6]-carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, Oreg.) by using an acid shock as described elsewhere (29). For both cell suspensions, 3  $\mu$ l of 0.5 M HCl gave optimal loading. Cells were energized with 0.5% glucose. Fluorescence was measured as described previously (29) on a Perkin-Elmer LS50 spectrofluorimeter with computer-controlled data acquisition and storage. Experiments were done at pH 6.0 and 30°C. Data were corrected for ATP-driven efflux of the fluorescent probe (30).

## RESULTS

**Lactococcin B is bactericidal.** To investigate whether the mode of action of LcnB on sensitive cells was bactericidal, bacteriostatic, or bacteriolytic, purified bacteriocin was added to sensitive cells and the viable count and optical density changes were determined as a function of time.

Strains *L. lactis* SK112 and *L. lactis* SK112 carrying the

immunity gene *lciB* on plasmid pMB583 were incubated with LcnB ( $1.7 \times 10^2$  AU/mg of protein). Cells of strain *L. lactis* SK112(pMB583) showed no growth inhibition or decrease in viability. In contrast, 99% of the cells of the indicator strain *L. lactis* SK112 treated with LcnB lost their colony-forming ability after 1 h (Fig. 1). After 3 h of treatment only 0.001% of the cells survived. However, prolonged incubation did not result in further killing. The optical density of the treated culture did not change during this experiment (Fig. 1). These results indicate that LcnB acts bactericidally rather than bacteriolytically on sensitive cells.

**Reduced lactococcin B dissipates the membrane potential of sensitive cells but not of immune cells.** To examine whether LcnB was capable of permeabilizing the cytoplasmic membrane of sensitive cells, its effect on the membrane potential was determined by monitoring the distribution of the lipophilic cation  $\text{TPP}^+$  in energized cells.

The results are presented in Fig. 2 and show that the membrane potential of the sensitive *L. lactis* IL1403 cells was dissipated by an LcnB concentration of 3.3 AU/mg of protein. However, the rate of dissipation caused by the

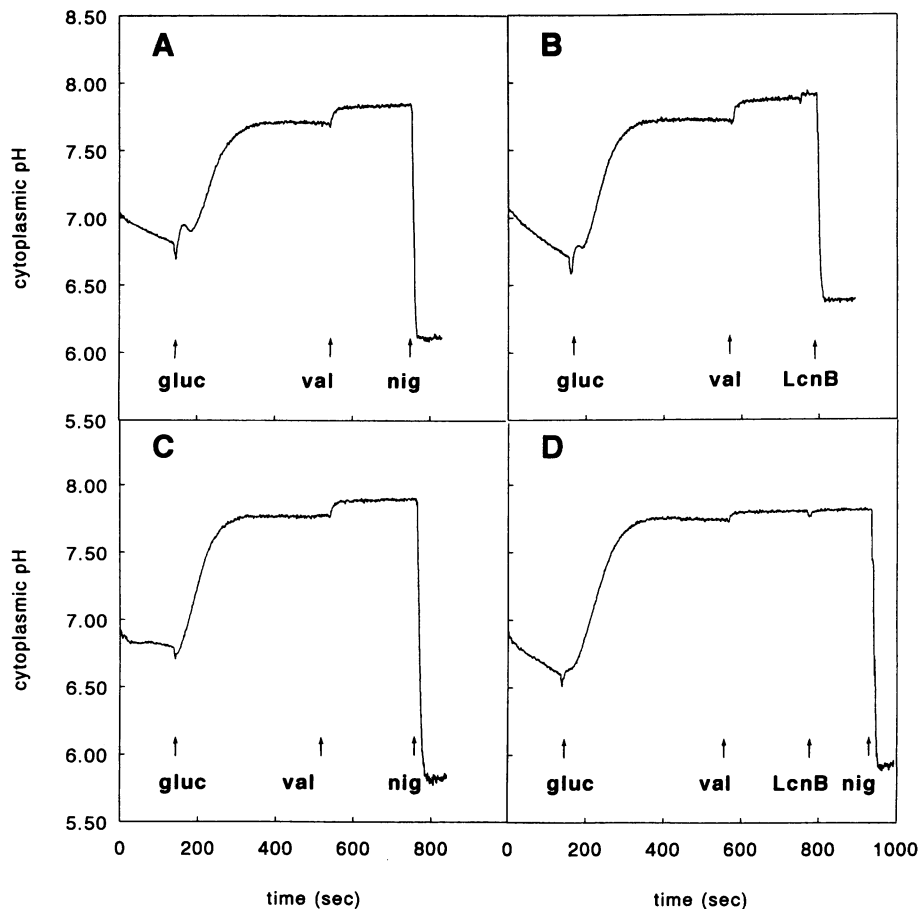


FIG. 3. Effect of LcnB (6.7 AU/mg of protein) on the pH gradient of energized cells of *L. lactis* IL1403 (B) and *L. lactis* IL1403(pMB583) (D). As a control, nigericin was added to cells of *L. lactis* IL1403 (A) and *L. lactis* IL1403(pMB583) (C). The additions were made at the times indicated by the arrows. Abbreviations: gluc, glucose; val, valinomycin; nig, nigericin.

addition of 1  $\mu\text{M}$  (final concentration) valinomycin, a  $\text{K}^+$  ionophore which dissipates the membrane potential if  $\text{K}^+$  ions are present, was much higher (Fig. 2A).

LcnB contains one cysteine residue at position 24 (47), which might be oxidized during purification, thus affecting the activity of LcnB. This possibility was examined by comparing the effects of reduced and oxidized LcnB on the membrane potential. LcnB was reduced by treatment with 5 mM dithiothreitol (DTT). Subsequently the effect on the membrane potential of sensitive IL1403 cells by the DTT-treated preparation was assayed. As shown in Fig. 2B, this resulted in the dissipation of the membrane potential at a rate similar to that observed after the addition of 1  $\mu\text{M}$  valinomycin. As documented in the same figure, DTT alone had no effect on the membrane potential. Treatment of LcnB with DTT also increased the bactericidal activity of the bacteriocin; in a spot test the activity of LcnB increased twofold after treatment with DTT (data not shown). Addition of twice the amount of non-DTT-treated LcnB to the cells did not increase the dissipation rate of the membrane potential in a  $\text{TPP}^+$  experiment (data not shown). These results indicate that LcnB activity requires the Cys-24 residue in a reduced state. To corroborate this conclusion, the Cys-24 residue in LcnB, fully activated by DTT treatment, was oxidized by treatment with 10 nM  $\text{HgCl}_2$ . The  $\text{HgCl}_2$ -LcnB mixture was added to sensitive *L. lactis* IL1403 cells. As shown in Fig.

2C, oxidized LcnB was not capable of dissipating the membrane potential. Subsequent addition of DTT to the  $\text{HgCl}_2$ -LcnB mixture fully restored activity (Fig. 2C). Pretreatment of sensitive cells with  $\text{HgCl}_2$  did not affect their sensitivity towards LcnB (data not shown).

Strain *L. lactis* IL1403(pMB583) carries the *lciB* gene (42). This gene codes for the immunity protein against LcnB. In this strain the membrane potential did not dissipate after addition of reduced LcnB (Fig. 2D), not even at concentrations 100 times higher than those used for sensitive cells (data not shown).

From these results we conclude that LcnB affects the permeability of the cytoplasmic membrane of *L. lactis* IL1403 for protons, thereby dissipating the membrane potential, and that LcnB activity critically depends on the reduced state of the Cys-24 residue. In strains carrying the immunity protein, LcnB is inactive, even at high concentrations.

**Lactococcin B dissipates the pH gradient of sensitive cells but not of immune cells.** To examine the effect of LcnB on the dissipation of the pH gradient, *L. lactis* IL1403 cells were loaded with BCECF and diluted in 50 mM potassium-phosphate buffer (KP buffer), pH 6.0, to build up a pH gradient upon energization with 0.5% glucose (Fig. 3A). Upon addition of 1  $\mu\text{M}$  valinomycin, the interconversion of the membrane potential resulted in an increase in the pH

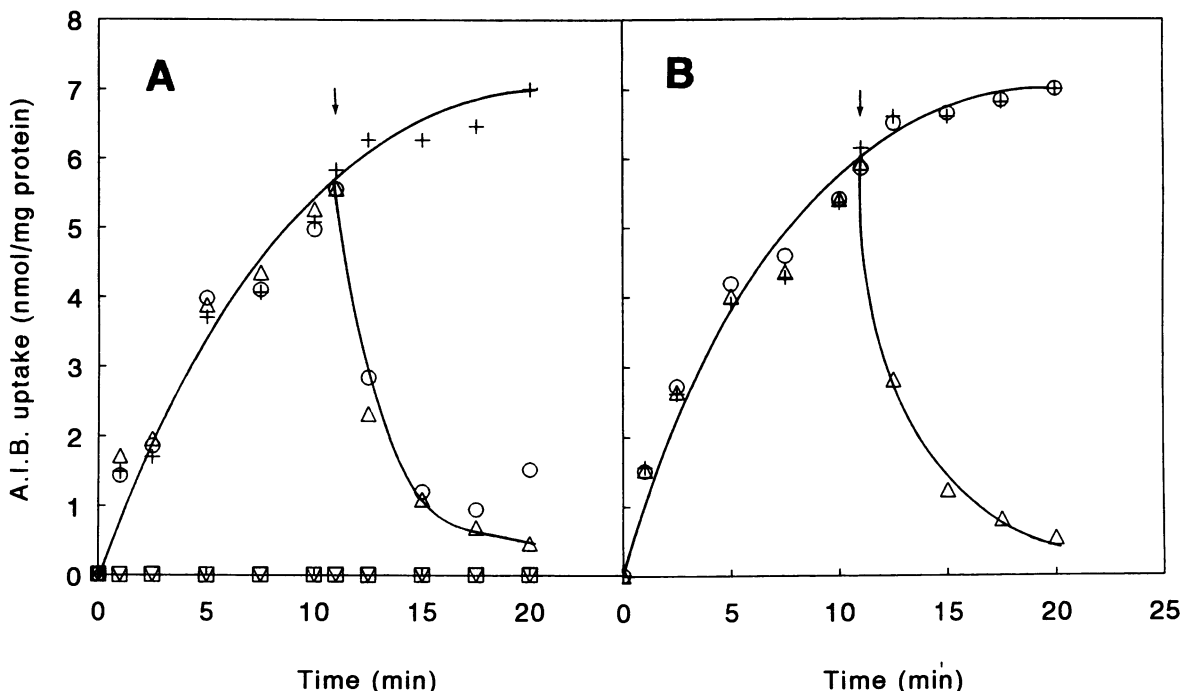


FIG. 4. Effect of LcnB on uptake and efflux of AIB in energized cells of *L. lactis* IL1403 (A) and *L. lactis* IL1403(pMB583) (B). Labeled AIB was added at  $t = 0$ . At the time indicated by the arrows either valinomycin ( $1 \mu\text{M}$ ) plus nigericin ( $1 \mu\text{M}$ ) ( $\Delta$ ) or reduced LcnB ( $6.7 \text{ AU/mg}$  of protein) ( $\circ$ ) was added. No additions were made to the control (+).  $\square$  and  $\nabla$ , AIB uptake after preincubation (3 min) with valinomycin plus nigericin or with LcnB, respectively.

gradient. Upon addition of  $1 \mu\text{M}$  nigericin, a  $\text{K}^+/\text{H}^+$  ionophore, the pH gradient was dissipated and the pH of the cytoplasm became equal to that of the KP buffer (Fig. 3A). Reduced LcnB ( $6.7 \text{ AU/mg}$  of protein) also dissipated the pH gradient, at a rate similar to that caused by nigericin (Fig. 3B).

*L. lactis* IL1403(pMB583) cells were not affected by LcnB ( $6.7 \text{ AU/mg}$  of protein) (Fig. 3D). As expected, the pH gradient was dissipated by nigericin (Fig. 3C and D). These results indicate that LcnB dissipates the pH gradient in energized sensitive *L. lactis* cells but not in immune cells.

**Lactococcin B destroys the PMF.** The results presented above clearly demonstrate that LcnB causes a collapse of both the membrane potential and the pH gradient in sensitive cells but not in immune cells, indicating that LcnB makes the membrane permeable for protons. The resulting collapse of the proton motive force (PMF) should therefore also affect PMF-dependent processes. To evaluate the effect of LcnB on PMF-dependent amino acid transport, two sets of experiments were carried out. The effects of reduced LcnB on (i) the uptake of AIB (a nonmetabolizable analog of alanine [45]) and (ii) the efflux of preaccumulated AIB were studied. Figure 4A shows that, similar to the ionophore combination valinomycin and nigericin ( $1 \mu\text{M}$  each), LcnB (at  $6.7 \text{ AU/mg}$  of protein) blocked uptake of AIB and caused efflux of preaccumulated AIB. Cells of strain IL1403(pMB583) showed no leakage of AIB at LcnB concentrations of  $6.7$  and  $1,000 \text{ AU/mg}$  of protein (Fig. 4B), whereas efflux did occur upon addition of valinomycin and nigericin, as expected.

**Lactococcin B induces pore formation in the cytoplasmic membrane.** To investigate whether LcnB also permeabilizes the membrane for solutes larger than protons, use was made of the fact that glutamate is taken up by *L. lactis* by a

unidirectional ATP-driven transport system (33). This system is further characterized by being active in the absence of a PMF in cells kept at an alkaline pH but by being inactive in cells at pH 6.0. At the latter pH the ATP-driven transport system can function only in the presence of a pH gradient. However, because the uptake system is unidirectional, efflux of glutamate is PMF independent. Figure 5A shows that preincubation of *L. lactis* IL1403 cells at pH 6.0 with LcnB completely blocked uptake of glutamate, which is in agreement with the observation that the pH gradient is dissipated by LcnB (see previous section).

Addition of LcnB at a concentration of  $6.7 \text{ AU/mg}$  of protein had, similar to valinomycin and nigericin, no effect on efflux of glutamate. However, addition of LcnB at a 150 times higher concentration ( $1,000 \text{ AU/mg}$  of protein) caused efflux of glutamate (Fig. 5A). In contrast, no efflux of glutamate was observed in *L. lactis* IL1403(pMB583), not even at this high a concentration of LcnB. These results indicate that at high concentrations of LcnB the cytoplasmic membrane of *L. lactis* IL1403 becomes permeable for solutes as large as glutamate.

Several bacteriocins have been described as having a voltage-dependent pore-forming ability (32, 36, 39), whereas LcnA increases the permeability of the cytoplasmic membrane in a manner independent of the PMF (48). To investigate the PMF dependency of LcnB, cells of strain *L. lactis* IL1403 were allowed to accumulate glutamate. Subsequently, the preloaded cells were treated with the uncouplers valinomycin and nigericin to dissipate the membrane potential and the pH gradient, after which LcnB was added. As documented in Fig. 5A, a high concentration of LcnB ( $1,000 \text{ AU/mg}$  of protein) caused efflux of glutamate in the

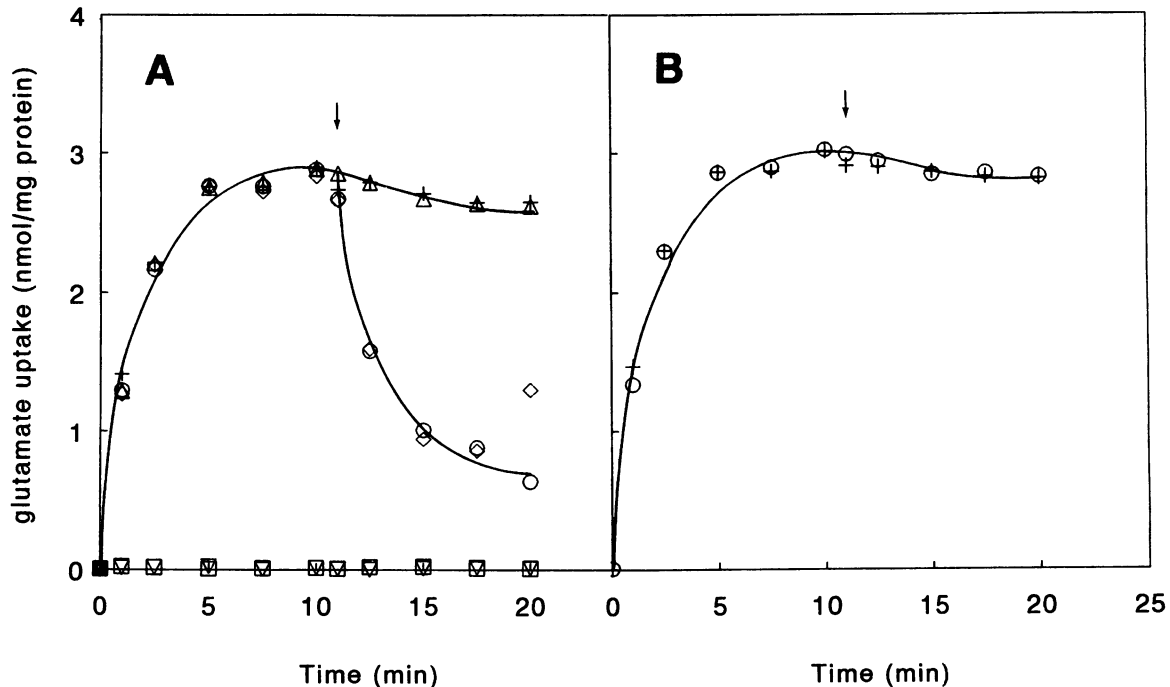


FIG. 5. Effect of LcnB on uptake and efflux of glutamate in energized cells of *L. lactis* IL1403 (A) and *L. lactis* IL1403(pMB583) (B). Labeled glutamate was added at  $t = 0$ . At the time indicated by the arrows either valinomycin (1  $\mu$ M) plus nigericin (1  $\mu$ M) ( $\Delta$ ) or reduced LcnB (1,000 AU/mg of protein) ( $\circ$ ) was added. No additions were made to the control (+).  $\diamond$ , addition of first valinomycin plus nigericin followed by LcnB;  $\square$  and  $\nabla$ , glutamate uptake after preincubation (3 min) with valinomycin plus nigericin or with LcnB, respectively.

absence of a PMF. This result suggests that LcnB is active in the absence of a PMF.

## DISCUSSION

LcnB is one of the three bacteriocins produced by *L. lactis* subsp. *cremoris* 9B4. In this work we determined the effect of LcnB on cell viability and lysis of cell cultures of a sensitive strain. Furthermore, we investigated the influence of LcnB on sensitive and immune cells with respect to the membrane potential, pH gradient, and amino acid uptake and efflux.

Apparently the activity of LcnB is bactericidal, causing a decrease in CFU of a sensitive culture of over 99% in 3 h. LcnB causes an increase in membrane permeability in the absence of a membrane potential, similar to LcnA (48). However, LcnB differs from LcnA with respect to its requirement for a reducing environment for activity. It is conceivable that the reduced state of the Cys-24 residue, the only cysteine present in the molecule, is uniquely required for receptor recognition. Alternatively, the oxidized state of Cys-24 may affect the structure of the bacteriocin in such a way that it is unable to insert itself in the membrane or is unable to participate in pore formation. In this regard LcnB would resemble a group of thiol-activated toxins (4) in which the reduced state of the cysteine residue appears to be essential for the generation of functional lesions in toxin-treated membranes (4).

The bactericidal effect of LcnB on sensitive *L. lactis* cells appears to be due to dissipation of the membrane potential and pH gradient caused by leakage of ions. At low concentrations of LcnB, efflux of AIB from sensitive cells occurs; only in the presence of a high concentration of LcnB (1,000

AU/mg of protein) was glutamate efflux from sensitive cells observed. The requirement for a high concentration of the bacteriocin to allow the passage of glutamate may be explained on the basis of the assumption that several LcnB molecules form a multi-peptide complex with an internal channel through which low-molecular-weight solutes such as ions and amino acids flow. Apparently LcnB forms these pores in a PMF-independent manner, since depolarization of the membrane with valinomycin and nigericin prior to addition of LcnB does not influence the effect of LcnB on the efflux of glutamate. The quantity of LcnB added in this experiment (1,000 AU/mg of protein) corresponds to approximately  $1.3 \times 10^4$  molecules per cell. A twofold reduction in added LcnB (500 AU/mg of protein or  $6.6 \times 10^3$  molecules per cell) did not affect the efflux of accumulated glutamate (data not shown). However, at this and lower concentrations (6.7 AU/mg of protein or 88 molecules per cell), efflux of ions and AIB was observed. This suggests that at low concentrations a multi-peptide complex of LcnB molecules forms a pore which is too narrow for the passage of amino acids but allows the passage of protons and possibly other small solutes.

Liposomes are insensitive to lactococcins (48), including LcnB (51), suggesting that for the action of lactococcins a membrane-associated receptor protein is necessary.

The present results clearly indicate that cells carrying the immunity gene *lciB* are insensitive to LcnB. The immunity proteins are probably located in the cytoplasmic membrane, since vesicles derived from immune cells are also immune to the action of the bacteriocin LcnA (48). Several possibilities can be entertained to explain the insensitivity of immune cells to the action of the bacteriocin: (i) the immunity protein shields the receptor protein, such that the bacteriocin cannot

recognize its target, (ii) the immunity protein interacts with the bacteriocin, such that the bacteriocin molecules are unable to form a pore in the membrane, or (iii) the immunity protein closes the pores formed by the bacteriocin molecules. In all of these possible mechanisms to explain immunity it might be expected that an excess of bacteriocin molecules over immunity protein molecules would result in breakdown of immunity, which actually has been shown to be the case for LcnA (48).

An important finding of this study is that bacteriocins can apparently exist in an oxidized, inactive state. In view of this conclusion, it is recommended that screening programs of antimicrobial agents assay possible candidates under aerobic as well as under anaerobic conditions, because otherwise interesting bacteriocins might escape detection.

#### ACKNOWLEDGMENTS

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