CURRENT MICROBIOLOGY Vol. 46 (2003), pp. 180–183
DOI: 10.1007/s00284-002-3844-0

Current Microbiology
An International Journal

Differential Roles of the Two-Component Peptides of Lactocin 705 in Antimicrobial Activity

Sergio A. Cuozzo, Patricia Castellano, Fernando J.M. Sesma, Graciela M. Vignolo, Raul R. Raya

Centro de Referencia para Lactobacilos (CERELA), CONICET, Chacabuco 145, 4000 Tucumán, Argentina

Received: 12 April 2002 / Accepted: 24 May 2002

Abstract. *Lactobacillus casei* CRL705 produces a class IIb bacteriocin, lactocin 705, which relies on the complementary action of two components, Lac705α and Lac705β. These peptides exert a bactericidal effect on the indicator strain *Lactobacillus plantarum* CRL691, with an optimal Lac705α/Lac705β peptide ratio of 1 to 4. Electron microscopy studies showed that treated CRL691 cells have their cell wall severely damaged, with mesosome-like membranous formations protruding into their cytoplasm. Although less pronounced, a similar effect was also observed with the Lac705β peptide alone. Furthermore, Lac705β increased the inhibitory action of a diluted supernatant of *L. casei* CRL705, while Lac705α protected CRL691 cells from inhibition. Both peptides were required to dissipate the proton motive force ($\Delta\psi$ and ΔpH) of CRL691 cells. These data suggested that of the two components of lactocin 705, the Lac705α peptide is responsible for receptor recognition, and the Lac705β peptide is the active component on the cell membrane of CRL691 cells.

Lactic acid bacteria (LAB) produce three main classes of ribosomally synthetized antimicrobial peptides (bacteriocins) [11]: (i) Class I: lantibiotics, small peptides (<5 kDa) which contains lanthionine and/or β-methyl-lanthionine residues. Some lantibiotics rely on the complementary action of two components; i.e., lactacin 3157 [10]; (ii) Class II: nonlantibiotic, low-molecular-weight (<10 kDa), heat stable peptides; and (iii) Class III: nonlantibiotic, large heat labile peptides (>30 kDa). Most of the bacteriocins produced by LAB belong to the Class II that can be subdivided into (IIa) *Listeria*-active peptides, (IIb) two peptide bacteriocins, (IIc) Sec dependent bacteriocins, and (IId) class II bacteriocins that do not belong to the other subgroups.

In general, most LAB bacteriocins appear to dissipate the proton motive force (PMF) of target cells through the formation of pores in the cell membrane releasing intracellular ions [1, 4, 11, 12, 14]. However, the mechanisms through which they achieve this appear to differ among the different bacteriocins; ultrastructural studies of treated sensitive cells indicate different mechanisms of membrane destabilization and cell death [8]. In the Class IIB bacteriocins, although complementary peptides are at least 1,000 times more active when they are combined than when they are present individually, most of them are required in a 1-to-1 peptide ratio for optimal bactericidal effect [2, 3, 7, 9, 13, 14]. Lactocin 705 is a class IIb nonlantibiotic bacteriocin, whose activity depends upon the complementation of two peptides (named Lac705α and Lac705β) of 33 amino acid residues each [5]. Castellano and Vignolo (submitted for publication) have recently shown that lactocin 705 also appears to exert its bactericidal action by destabilizing the cytoplasmic membrane of sensitive *Lactobacillus* CRL691 cells. Both components of the PMF (the membrane potential and the pH gradient) were affected on lactocin705-treated CRL691 cells.

In this paper we present evidence that Lac705β is the active component of lactocin 705 on cell membrane, while the peptide Lac705α is involved in receptor recognition on the sensitive cells. A Lac705α/Lac705β peptide ratio of 1-to-4 was required for optimal bactericidal effect.

Materials and Methods

Bacterial strains and culture conditions. The bacteriocin producer strain *L. casei* CRL705 and the indicator strain *L. plantarum* CRL691 were isolated from Argentinean dry-cured sausages, at the Centro de

Correspondence to: R.R. Raya; *email:* rraya@cerela.org.ar

Referencia para Lactobacilos (CRL collection, CERELA) [16]. Bacterial cells were grown in MRS [6] broth at 30°C and maintained at −20°C in MRS containing 10% glycerol. When needed, agar was added to broth medium to make solid media.

Bacteriocin assays and synthesis. Lactocin 705 purification and activity assays were performed as already described by Palacios et al. [15]. The peptides Lac705α and Lac705β (GMSGYIQGIPDFLKGYLHGISAANKHKKGRLGY and (GFWGGLGYIAGRVGAAYGHAQASANNHHSPING, respectively [5]), were synthesized by Bio-synthesis Inc. (Lewisville, Texas, USA). The 50% inhibitory concentration of the synthetic peptides was determined by adding variable concentrations of Lac705α and Lac705β to 10^6 CFU/ml of *L. plantarum* CRL691 (in 5 ml of MRS pH 6.5, at 30°C), and defined as the lowest concentration of these peptides resulting in 50% inhibition of visible growth (OD_{600}) after 6 h.

The bactericidal or bacteriolytic effect of lactocin 705 extract and Lac705α and Lac705β peptides on *L. plantarum* CRL691 cells was evaluated as follows: sensitive cells were grown to an OD_{540} nm of 0.6, harvested, washed, and suspended to approximately 10^6 CFU/ml in 5 ml of MRS pH 6.5. At this point lactocin 705 extract (256 AU/ml) and Lac705α plus Lac705β (180 nM) were added to the cells and incubated at 30°C. Samples were taken at appropriate times to determine viable cell counts and OD_{540}. A control without bacteriocin addition was also included.

Transmission electron microscopy studies. Ultrathin silver gray sections of untreated and 3-h-treated exponentially growing CRL691 cells were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 transmission electron microscope.

Measurements of the proton motive force. The transmembrane electrical potential ($\Delta\psi$) and the transmembrane pH gradient (ΔpH) were determined in intact cells from the distribution of the fluorescent probe 3,3′-dipropylthia dicarbocyanine iodide [$DiSC_3(5)$] and by loading the cells with the fluorescent pH indicator 3′, 7′-bis- (2-carboxyethil)-5 (and-6)-carboxyfluorescein (BCECF), respectively ([17], Castellano and Vignolo, submitted).

Results and Discussion

Lactobacillus casei CRL705 produces a bacteriocin (lactocin 705) whose activity relies on the complementary action of two peptides (Lac705α and Lac705β) [5]. Some lactic acid bacteriocins induce cell lysis on sensitive cells. A previous study [16] showed that the supernatant of a *L. casei* CRL705 culture ("lactocin 705 extract") induced lysis of exponential growing CRL691 cells. To evaluate if this bacteriolytic effect was due to lactocin 705, growth of sensitive CRL691 cells (cell viability and OD_{600}) was assayed in the presence of lactocin 705 extract or synthetic Lac705α and Lac705β peptides. The number of viable CRL691 cells dropped from 6.3 to approximately 4.9−4.6 log CFU/ml after one hour of incubation in the presence of lactocin 705 extract (256 AU/ml) as well as in the presence of Lac705α plus Lac705β (180 nM each). Cell lysis, however, was only observed with lactocin 705 extract (data not shown). These results indicate that lactocin 705 has a bactericidal effect on CRL691 cells and suggest that following treatment with lactocin 705 extract, autolytic enzymes of CRL691 cells are activated by other(s) factor present in the supernatant of CRL705 cells.

Figure 1 shows that each synthetic peptide did not display bacteriocin activity by itself. However, the antimicrobial activity of a diluted lactocin 705 extract (32 AU/ml), which showed an intermediate level of inhibition on the growth of *L. plantarum* CRL691, was stimulated with the supplementation of Lac705β (180 nM). On the contrary, the Lac705α peptide prevented the inhibitory action of the diluted supernatant. These data suggested that of the two components of lactocin 705, the Lac705α peptide is responsible for receptor recognition, and the Lac705β peptide is the active component of the bacteriocin. The lowest concentration of these peptides that produced a 50% inhibition of CRL691 cell growth, after 6 h of incubation at 30°C, were that of 5.6 and 22.5 nM of Lac705α and Lac705β, respectively, given a Lac705α/Lac705β ratio of 1 to 4.

Electron-microscopic studies (Fig. 2) showed that addition of a lactocin 705 extract or a mixture of the Lac705α and Lac705β peptides induced a dramatic change in sensitive cells. Exponentially growing cells of *L. plantarum* CRL691 had the typical structure of Gram-positive bacteria, with a thick, uniform wall to which the cytoplasmic membrane tightly adhered; mesosomes or other intracytoplasmic membrane formations were not observed. After 3 h of exposure to the bacteriocin, an electron-transparent layer between the plasma membrane and the outer wall layer as well as huge mesosome-like membranous formations protruding into the cytoplasm were observed, indicating that the cytoplasmic membrane was severely affected by the bacteriocin. Furthermore, treated cells seemed to be multiseptated. These morphological changes were only observed when both peptides were present simultaneously. However, some sensitive cells treated only with the Lac705β peptide also showed their cell membrane was affected (Fig. 2C), while in the presence of Lac705α, a multi-septum formation was stimulated in some cells (Fig. 2B). Again, both peptides were also required to dissipate the proton motriz force of CRL691 sensitive cells. The addition of 180 nM of Lac705α plus Lac705β to energized cells of *L. plantarum* CRL691 dissipated both components of the PFM ($\Delta\psi$ and ΔpH). No changes in $\Delta\psi$ or ΔpH were observed in the presence of Lac705α or Lac705β alone (data not shown).

Amino acid analysis of Lac705α (pI 9.87) and Lac705β (pI 8.61) indicated that these 33-amino acid peptides are cationic, rich in glycine (21.2 and 24.2%, respectively), and do not contain cystein. Computer analysis (http://bioinf.cs.ucl.ac.uk/cgi-bin/psipred) showed that they might form amphiphilic α-helices, a structural

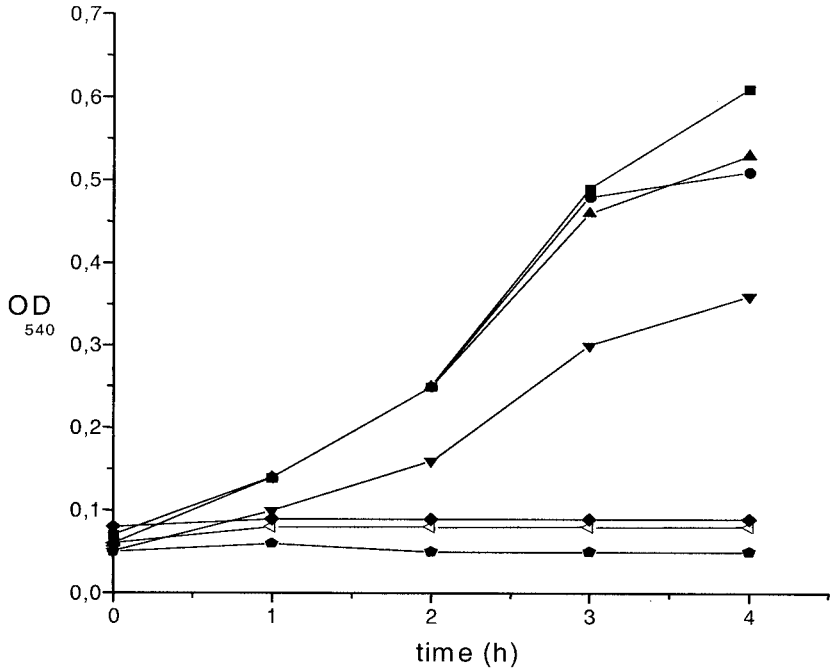

Fig. 1. Effect of lactocin 705 extract, Lac705α, and Lac705β on cell growth of *L. plantarum* CRL691. Symbols: (■) control, (▲) plus Lac705α, (●) plus Lac705β, (▼) lactocin 705 extract plus Lac705α, (◆) lactocin 705 extract plus Lac705β, (△) Lac705α plus Lac705β, and (⬟) lactocin 705 extract.

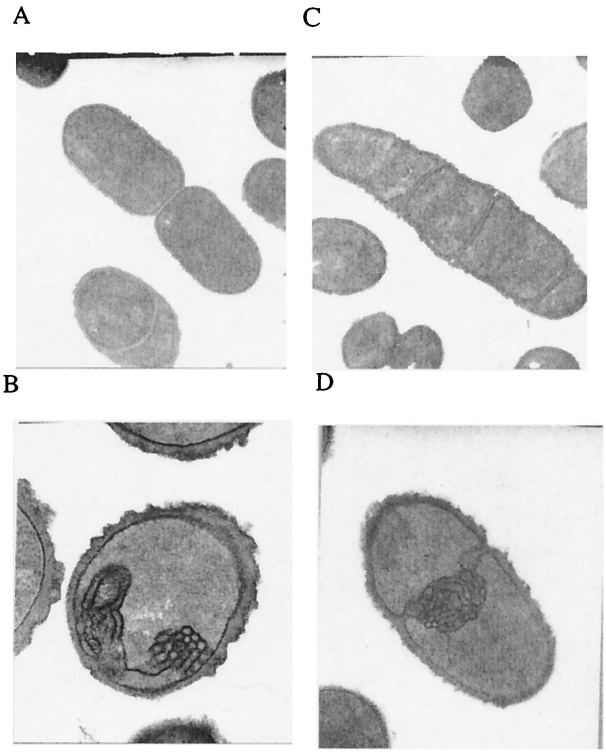

Fig. 2. Electron micrographs that show the effect of the two-peptide bacteriocin, lactocin 705 on sensitive *L. plantarum* CRL691 cells. (A) Untreated cells, (B) cells treated with 180 nM of Lac705α plus Lac705β, (C) cells treated with the Lac705α peptide (180 nM), and (D) cells treated with Lac705β (180 nM).

characteristic that may allow the peptides to oligomerize into membrane-spanning pores. Lactocin 705 shows a limited host range. Taken together, it is suggested that lactocin 705 acts as a complex of Lac705α and Lac705β (ratio 1:4) peptides and exert its action through interaction with cell wall-associated or membrane-associated binding sites in the sensitive cells. Our results suggest that the specificity of this porin complex is given by the Lac705α peptide; its net positive charge at the 14 C-terminal amino acids (5+ at pH values lower than 6) would neutralize the negative charge of teichoic and lipoteichoic acids in the cell wall of CRL691 cells, and interact specifically and competitively with some target cell entity, allowing the Lac705β peptide to form membrane pores, which increase membrane permeabilization and cause cell death.

ACKNOWLEDGMENTS

This work was supported by grants PICT97 N° 01-00132-02293 and PICT98 N° 09-04632 from Agencia Nacional de Promoción Científica y Técnica; by CIUNT, Universidad Nacional de Tucuman; and CAPES-SEPCIT (Proyecto BR/A99-SIX/23, 1325/99).

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