Association of the lactococcin A immunity factor with the cell membrane: purification and characterization of the immunity factor

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The physicochemical characteristics of the lactococcin A immunity protein, as deduced from its gene sequence, were used to devise a procedure for its purification. The protein was purified from cell extracts by cation-exchange and reverse-phase chromatography. As judged from the amino acid composition and amino acid sequencing, the immunity protein is not post-translationally processed by cleavage at its N- or C-terminus. Consequently, the absorption coefficient at 280 nm, the isoelectric point, and the molecular mass of the immunity protein may be calculated to be, respectively, $8.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, 10.2 and 11 163 Da from the amino acid sequence predicted from the nucleotide sequence. The immunity protein is a major cell protein component – one cell may contain (to an order of magnitude) 10^5 molecules – and it is in part associated with the cell membrane, as judged by immunoblot analysis of membrane vesicle-associated proteins. Exposing lactococcin-A-sensitive cells to an excess of the immunity protein did not affect the lactococcin-A-induced killing of the cells, indicating that the immunity protein does not protect cells by simply binding to lactococcin A, nor to externally exposed domains on the cell surface. Exposing immune-positive cells to antiserum against the immune protein did not sensitize the cells to lactococcin A, suggesting that the immunity protein in fact does not act extracellularly.

Introduction

Bacteriocins are bacteria-produced polypeptides which have a bactericidal and/or bacteriostatic effect, usually directed towards bacteria which are closely related to the bacteriocin-producing species (Tagg *et al.*, 1976). In order to protect themselves against the toxic effect of their own bacteriocin, bacteriocin-producing cells generally also produce an immunity protein (Konisky, 1982). This protein renders a cell resistant to its own bacteriocin, but not necessarily to other unrelated bacteriocins.

There has been considerable interest in lactic acid bacteria (LAB)-produced bacteriocins, due to their potential use as antimicrobial agents. Nisin was for a long time the only thoroughly studied LAB bacteriocin (Buchman *et al.*, 1988; Dodd *et al.*, 1990; Gross & Morell, 1971; Hurst 1981; Kaletta & Entian, 1989; Sahl *et al.*, 1987). Recently, however, several new LAB bacteriocins have been identified, purified and

characterized (Hastings *et al.*, 1991; Henderson *et al.*, 1992; Holo *et al.*, 1991; Mørtvedt *et al.*, 1991; Muriana & Klaenhammer, 1991*a,b*; Nieto Lozano *et al.*, 1992; Nissen-Meyer *et al.*, 1992; Piard *et al.*, 1992; Stoffels *et al.*, 1992; Tichaczek *et al.*, 1992; van Belkum *et al.*, 1991*a*). Lactococcin A, produced by *Lactococcus lactis* subsp. *cremoris*, is perhaps the most studied among these new LAB bacteriocins. It is an unmodified polypeptide, 54 amino acid residues long, which does not share any apparent amino acid sequence homology with other isolated bacteriocins (Holo *et al.*, 1991). Lactococcin A appears to induce cell death by permeabilizing the membrane of susceptible cells (van Belkum *et al.*, 1991*b*). Evidence for the existence of a lactococcin A immunity protein is based on the identification of a gene whose presence in a functional form in a cell renders the cell resistant to lactococcin A (Holo *et al.*, 1991; van Belkum *et al.*, 1991*a*). Upon cloning and sequencing the lactococcin A gene, the gene encoding the lactococcin A immunity protein was identified and sequenced (Holo *et al.*, 1991; van Belkum *et al.*, 1991*a*). The presence of the immunity gene in a functional form is sufficient to render a cell resistant to lactococcin A. Judging from the

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Abbreviation: LAB, lactic acid bacteria.

nucleotide sequence of this gene, it should encode a cationic protein with a molecular mass of approximately 11000 Da.

It is not known how immunity proteins render cells resistant to the toxic effect of LAB bacteriocins. This lack of knowledge may be partly due to inability to isolate and thereby thoroughly characterize LAB immunity proteins. One major hindrance to purifying an immunity protein is the lack of a functional assay. However, some of the physicochemical characteristics of an immunity protein may be deduced from its gene sequence and these characteristics may then be used to devise a procedure for purification of the protein. In this study we used this strategy to develop a simple three-step procedure for the purification of the lactococcin A immunity protein to homogeneity, and we show that a fraction of the protein is associated with the cell membrane. It is to our knowledge the first purification of a LAB bacteriocin immunity protein. The procedure used should be applicable to the purification of other LAB bacteriocin immunity proteins whose genes have been identified and sequenced.

Methods

Bacterial strains and media. The *Lactococcus lactis* subsp. *cremoris* strains LMG 2130 and LMG 2131, and the subsp. *lactis* strains IL 1403 and IL 1403(pON7) used in this study have been described previously (Holo *et al.*, 1991). Strain LMG 2130 is the lactococcin A producer from which lactococcin A was originally identified and isolated (Holo *et al.*, 1991). In addition to the lactococcin A gene, this strain contains the gene encoding the lactococcin A immunity protein. Strain LMG 2131 was derived from LMG 2130 after exposing the latter to plasmid curing, resulting in the loss of both the lactococcin A gene and the immunity gene in LMG 2131 (Holo *et al.*, 1991). Strain IL 1403 is a lactococcin-A-sensitive strain which lacks the immunity gene, and this strain has been used as indicator strain in the bacteriocin assay when measuring lactococcin A activity (Holo *et al.*, 1991). Strain IL 1403(pON7) was derived by transforming IL 1403 with the genes encoding lactococcin A and its immunity protein (Holo *et al.*, 1991). IL 1403(pON7) has as a consequence become resistant to lactococcin A, and it produces a small amount of the bacteriocin (Holo *et al.*, 1991). All strains were grown and maintained in M17 broth as described earlier (Holo *et al.*, 1991).

Preparation of bacterial extracts. Bacterial cultures (2 litres) were grown to the late exponential/early stationary phase. The cells were collected by centrifugation at 4000 g for 15 min at 4 °C, and washed three times in 20 mM-phosphate buffer, pH 7 (buffer A). The cells were then resuspended in 20 ml buffer A and incubated for 40 min at 37 °C in the presence of 2 mg lysozyme ml⁻¹. Triton X-100 was then added to a final concentration of 1% (v/v) and the preparation was incubated for 40 min at 4 °C, after which the preparation was centrifuged at 15000 g for 20 min. The resulting supernatant (bacterial extract, fraction I) was used for purifying the immunity protein when prepared from strain LMG 2130 or IL 1403(pON7), both of which contain the immunity gene.

Purification of the immunity protein. All the purification steps were performed at room temperature; the chromatographic equipment was obtained from Pharmacia-LKB Biotechnology. Bacterial extract

(fraction I; 20 ml) was applied at a flow rate of 1–2 ml min⁻¹ to a 3 ml S-Sepharose Fast Flow cation-exchange column equilibrated with buffer A. After subsequently washing the column with 15 ml buffer A, the immunity protein was eluted from the column with 7 ml 0.5 M-NaCl in buffer A (fraction II). Fraction II was diluted to 50 ml with buffer A, after which the fraction was applied at a flow rate of about 1.5 ml min⁻¹ to a Mono S HR 5/5 cation-exchange column equilibrated with buffer A. The proteins which bound to the column were eluted with a linear gradient of NaCl in buffer A. The immunity protein eluted at a NaCl concentration between 320 and 380 mM (fraction III). Fraction III (about 4 ml) was applied to a C₂/C₁₈ reverse-phase column, PepRPC HR 5/5, equilibrated with H₂O containing 0.1% trifluoroacetic acid (TFA). The immunity protein was eluted with a linear gradient of 35–55% 2-propanol containing 0.1% TFA (fraction IV).

SDS-PAGE of the immunity protein. SDS-PAGE was carried out using PhastGel high-density gels, PhastGel SDS buffer strips, and the PhastSystem (Pharmacia-LKB Biotechnology) according to the manufacturer's recommendations. The molecular mass standards (Pharmacia-LKB) consisted of five peptides with molecular masses between 2500 and 17000 Da.

Isolation of membrane vesicles and assay for lactate dehydrogenase. The procedure for preparing membrane vesicles was – with some modifications – similar to that described by Hough *et al.* (1986). Cells from 200 ml cultures in the late exponential or early stationary phase of growth were pelleted, washed, and resuspended in 4 ml 50 mM-phosphate buffer, pH 7, containing 7 mM-MgCl₂ (buffer B). This preparation was incubated for 1 h at 37 °C in the presence of 2 mg lysozyme ml⁻¹. The lysozyme-treated cells were then lysed by a single passage through a French pressure cell at 15000 p.s.i. (103 MPa). The lysed-cell suspension was centrifuged twice for 15 min at 8000 g. Membrane vesicles in the resulting supernatant fraction were then pelleted by layering 3 ml of the supernatant on an 8 ml sucrose cushion [10% (w/v) sucrose in buffer B] and centrifuging for 1.5 h at about 100000 g using a swinging-bucket rotor. The pellet containing membrane vesicles was resuspended in 3 ml buffer A and frozen at –20 °C (membrane fraction). The supernatant (3 ml), which contains proteins not associated with membranes, was also collected and frozen (cytosol fraction). The protein concentrations in the membrane and cytosol fractions were, respectively, about 0.7 and 3.5 mg ml⁻¹. Protein concentrations were determined by the method of Bradford (1976), using the Bio-Rad Protein Assay Kit.

Lactate dehydrogenase in the membrane and cytosol fractions was assayed by spectrophotometrically monitoring the conversion of NAD⁺ to NADH using pyruvate as substrate. The Boehringer Mannheim LD/LDH assay kit was used according to the manufacturer's recommendations, except that fructose 1,6-bisphosphate was included (1 mM final concn) in the reaction mixture, and the reaction was carried out at 20 °C. One unit of lactate dehydrogenase activity was arbitrarily defined as the level of enzyme which in 1 min changed the absorbance at 340 nm by 0.001 in a 1 ml reaction mixture.

Antiserum against the lactococcin A immunity protein. A rabbit was injected subcutaneously three times at 3 week intervals with about 20 µg purified immunity protein. The first injection was given with 50% (v/v) Freund's complete adjuvant, whereas the second and third injections were given with 50% Freund's incomplete adjuvant.

Immunoblot analysis of membrane-vesicle-associated proteins. For the immunoblot analysis, SDS-PAGE was performed essentially as described by Laemmli (1970), using 17% (w/v) gels. After SDS-PAGE, proteins were transferred electrophoretically (60 V overnight) to a nitrocellulose membrane. Non-specific protein binding sites on the nitrocellulose membrane were blocked prior to adding antiserum by incubating the membrane for 30 min with a 3% (w/v) dry milk suspension in phosphate-buffered saline (PBS). The membrane was then incubated for 1 h in 50 ml PBS/Tween [0.05% (v/v)] containing

both antiserum (1:1000 dilution) against the immunity protein and crude cell extract prepared from a 0.5 l culture of an immunity-negative lactococcal strain. The crude extract was included to further reduce non-specific binding of antibodies to the membrane. For preparation of this crude extract, cells were lysed using a French press, and cell debris removed from the extract by centrifugation at 5000 *g* for 10 min. The nitrocellulose membrane blots were incubated for 1 h with alkaline-phosphatase-conjugated goat anti-rabbit antibody (1:2500) (Bio-Rad) followed by washing and incubation in 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Amino acid composition and sequencing. The purified immunity protein (fraction IV) was hydrolysed and analysed on an amino acid analyser as described earlier (Nieto Lozano *et al.*, 1992). The amino acid sequence was determined by Edman degradation using an Applied Biosystems 477A automatic sequencer with an on-line 120A phenylthiohydantoin amino acid analyser (Nieto Lozano *et al.*, 1992).

Results and Discussion

Purification of the lactococcin A immunity protein

The lactococcin A immunity gene should encode a cationic protein (isoelectric point estimated to be 10.2) with a molecular mass of about 11 000 Da (or less if it is cleaved post-translationally). Due to the lack of a functional assay for the immunity protein, we used these characteristics as a starting point for purifying the protein.

Although indirect evidence suggests that the immunity protein might be linked to the cell membrane (van Belkum *et al.*, 1991*b*), it is not certain where in the cell the immunity protein is localized. Consequently, crude whole cell extracts were prepared rather than extracts from subcellular fractions, in order to increase the chance of obtaining extracts which contain the immunity protein. Extracts (fraction I) prepared from the lactococcal strains LMG 2130, LMG 2131, IL 1403, and IL 1403(pON7) were applied to S-Sepharose cation-exchange columns at neutral pH. The cationic proteins in the extracts, which all should bind to this column at neutral pH, were then eluted with 0.5 M-NaCl (fraction II). Upon SDS-PAGE analysis of fraction II obtained from strains LMG 2130 and LMG 2131, more than 30 distinct protein bands could be discerned for each strain (data not shown). The band patterns obtained for the two strains were nearly identical, with the exception that fraction II from LMG 2130 contained a protein which migrated with an apparent molecular mass of 8000–9000 Da which was not detected in fraction II from LMG 2131. The stained protein was barely visible, but it was stained relatively much more strongly by Coomassie staining than by silver staining. This protein was also found in fraction II from strain 1403(pON7), but not from strain IL 1403. The fact that the protein was only present in strains [LMG 2130, IL 1403(pON7)] which contained and expressed the immunity gene suggested that this might be the immunity protein. Similar amounts of this putative immunity protein were produced by

Fig. 1. Chromatography on a Mono S cation-exchange column of fraction II obtained from (a) strain IL 1403(pON7) and (b) strain IL 1403. The flow rate was 1.5 ml min⁻¹ and the fractions applied to the column were from 2 litres culture. The bar indicates the fraction (fraction III) containing the immunity protein. —, A_{280} ; ---, NaCl concn.

strains LMG 2130 and IL 1403(pON7) as judged by the staining intensity of the putative immunity protein band obtained upon SDS-PAGE. However, extracts prepared from LMG 2130 contained greater amounts of contaminating proteins than corresponding extracts from IL 1403(pON7). Extracts prepared from IL 1403(pON7) rather than LMG 2130 were, consequently, used for further purification of the putative immunity protein, using SDS-PAGE analysis as an assay for the protein.

Fraction II from IL 1403(pON7) was applied to a Mono S cation-exchange column and the proteins which bound to the column were eluted with a linear NaCl gradient (Fig. 1*a*). Upon SDS-PAGE analysis of the column fractions, the relatively broad A_{280} peak eluting between 320 and 380 mM-NaCl (Fig. 1*a*) was found to be due to the putative immunity protein (fraction III). This broad peak was not detected when fraction II from IL 1403 was chromatographed on the Mono S column (Fig. 1*b*), consistent with the fact that IL 1403 lacks the immunity gene. The A_{280} peak was detected when fraction II from LMG 2130 was chromatographed on the Mono S column, but not when fraction II from LMG 2131 was chromatographed (data not shown), again consistent with the fact that LMG 2130 contains the immunity gene, whereas LMG 2131 does not. The putative immunity protein eluted from the Mono S column as a broad peak

Fig. 2. Reverse-phase chromatography of fraction III prepared from strain IL 1403(pON7). The amount applied on the column represents that obtained from 2 litres of culture. The immunity protein was eluted from the column by increasing the amount of 2-propanol in H₂O containing 0.1% trifluoroacetic acid. —, A_{280} ; ----, 2-propanol.

Fig. 3. SDS-PAGE analysis of the immunity protein in fraction IV obtained from strain IL 1403(pON7). Lane 1, peptide molecular mass standards (Pharmacia-LKB); lane 2, the immunity protein (im). SDS-PAGE was carried out using PhastGel high-density, PhastGel-SDS buffer strips, and the PhastSystem (Pharmacia-LKB) according to the manufacturer's recommendations.

with a characteristic tailing (Fig. 1*a*). In addition to the salt concentration at which it eluted from the Mono S column, the putative immunity protein could, consequently, be identified by the characteristic shape of this broad peak.

Table 1. *Purification scheme for the lactococcin A immunity protein*

Purification stage	Volume (ml)	Total A_{280} *	Total A_{254} *
Cell culture	2000		
Cell extract (fraction I)	20	200	900
Binding to S-Sepharose (fraction II)	7	2.1	1.7
Chromatography on Mono S column (fraction III)	4	0.16	0.08
Chromatography on reverse-phase column (fraction IV)	1	0.12	0.05

* Total A_{280} and A_{254} is the absorbance at the indicated wavelength multiplied by the volume in ml.

Reverse-phase chromatography of fraction III from the Mono S column revealed only one major A_{280} peak eluting at about 43% 2-propanol (fraction IV) (Fig. 2), and this peak was due to the putative immunity protein as judged by SDS-PAGE analysis (Fig. 3). The putative immunity protein was, consequently, already the dominant protein in fraction III obtained after chromatography on the Mono S column, consistent with the fact that it was the only major protein in fraction III detected by SDS-PAGE (data not shown). The A_{280} profile obtained in the last reverse-phase chromatography purification step (Fig. 2) and analysis of the protein(s) eluting from the reverse-phase column (fraction IV) by SDS-PAGE (Fig. 3), indicated that the putative immunity protein was at this stage pure. Amino acid sequence analysis of fraction IV confirmed the purity, and showed that the purified protein was in fact the lactococcin A immunity protein (see below).

The purification protocol is summarized in Table 1. The total A_{280} was 200 in fraction I, and this was reduced about 10³-fold in fraction IV, where the total A_{280} was 0.12 (Table 1). There was little decrease in the total A_{280} going from fraction III to IV (Table 1), consistent with the observation that the immunity protein was already the dominant protein in fraction III, after chromatography on the Mono S column. Overall, this procedure for purification of the lactococcin A immunity protein is simple and rapid; and it is likely that immunity proteins for other bacteriocins may be purified by a basically similar procedure if they have a molecular mass and isoelectric point similar to those of the lactococcin A immunity protein.

Characteristics of the lactococcin A immunity protein

The sequence of the first 17 amino acid residues at the N-terminus of the immunity protein was determined to be Met-Lys-Lys-Lys-Gln-Ile-Glu-Phe-Glu-Asn-Glu-

Table 2. Amino acid composition of the immunity protein

Amino acid	nmol*	Calculated residues per molecule†	Predicted residues per molecule‡
Ala	6.6	8	8
Arg	4.1	5	5
Asp/Asn	9.9	12	12
Glu/Gln	10.0	12	11
Gly	4.0	5	4
His	0.7	1	1
Ile§	5.9	7	8
Leu	13.2	16	16
Lys	8.1	10	10
Met	2.2	3	3
Phe	2.4	3	3
Pro	2.0	2	2
Ser	6.2	7-8	8
Thr	3.1	4	4
Trp	ND	—	1
Tyr	1.8	2	2
Val	0.3	0	0
Total no. of amino acid residues		97-98	98

ND, Not determined.

* nmol measured of the indicated amino acid residue: gives the relative amount of the indicated amino acid residue in one immunity protein molecule.

† Calculated from the relative amount of the indicated amino acid residue, assuming the presence of 97 amino acid (not including Trp, which was not measured in the composition analysis) residues per immunity protein molecule.

‡ The number of residues per immunity protein molecule as predicted by the immunity protein gene sequence (Holo *et al.*, 1991; van Belkum *et al.*, 1991a), assuming that the immunity protein is not post-translationally cleaved.

§ The immunity protein contains an Ile-Ile bond which may require more than the 24 h hydrolysis time used in order to obtain complete hydrolysis.

Leu-Arg-Ser-Met-Leu-Ala-. This sequence is identical to that predicted by the immunity gene sequence (Holo *et al.*, 1991; van Belkum *et al.*, 1991a), proving that the isolated protein was in fact the immunity protein. Moreover, the N-terminal amino acid sequence shows that the immunity protein is not post-translationally processed by cleavage at the N-terminal end. This is in contrast to the bacteriocin itself, in which the 21-amino-acid-residue N-terminal region is missing in the extra-cellular and active lactococcin A molecule (Holo *et al.*, 1991).

The C-terminal sequence was determined to be ?-Trp-Gly-?-Leu-Phe after cyanogen bromide cleavage of the immunity protein at the methionine residue at position 92. This sequence is also consistent with that predicted by the immunity gene sequence (Holo *et al.*, 1991; van Belkum *et al.*, 1991a), showing that the protein is not post-translationally processed by cleavage at the C-terminal end. The amino acid composition of the immunity protein was also largely as one would expect if

the immunity protein is not post-translationally cleaved (Table 2). The major differences in the determined composition and that expected from the gene sequence are that the composition obtained suggests the presence of 12, 5 and 7 residues, respectively, of glutamine/glutamic acid, glycine and isoleucine, whereas the gene sequence predicts, respectively, 11, 4 and 8 residues (Table 2). The discrepancy seen for isoleucine is probably due to the Ile-Ile peptide bond at position 48-49 which may not have been completely hydrolysed.

Since the immunity protein was not post-translationally cleaved, its absorption coefficient, isoelectric point and molecular mass may be determined from the predicted amino acid sequence. The absorption coefficient at 280 nm was calculated to be $8.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and the isoelectric point 10.2. The molecular mass was calculated to be 11 163 Da, somewhat more than the 8000-9000 Da suggested by SDS-PAGE. SDS-PAGE, however, may not always give accurate molecular mass estimates for peptides with a molecular mass less than 20000 Da. From the absorption coefficient, molecular mass and the amount purified from 2 litres of culture (Table 1), one may estimate (to an order of magnitude) that 10^5 immunity protein molecules are present per cell, assuming that the recovery of the protein upon purification is between 20 and 2%. Thus the immunity protein appears to be a major cell protein component.

Exposing the lactococcin-A-sensitive strain IL 1403 to an excess of purified or partially purified immunity protein before exposure to lactococcin A did not affect the lactococcin A-induced killing of the cells, and neither did exposing lactococcin A to an excess of the immunity protein prior to addition of the cells (data not shown). This suggests that the immunity protein does not simply protect cells from lactococcin A by directly binding to and thereby inactivating the bacteriocin, nor by directly binding to externally exposed domains on the cell surface, such as a putative bacteriocin receptor. Exposing IL 1403(pON7) cells, which contain the immunity gene and are thus resistant to lactococcin A, to antiserum against the immune protein did not sensitize the cells to lactococcin A, even when the cells had been pretreated with lysozyme (data not shown). This again suggests that, although the immunity protein is associated with the cell membrane (see below) it does not act outside the cell.

Association of the immunity protein with the cell membrane

Immunoblot analysis of membrane-associated proteins from the immune strain LMG 2130 indicated that the immunity protein is associated with the cell membrane (Fig. 4). Almost 50% of the immunity protein detected

Fig. 4. Immunoblot analysis of cytosol-associated and membrane-vesicle-associated proteins of strains LMG 2131 (lanes 1 and 2) and LMG 2130 (lanes 3–8) using antiserum raised against the purified immunity protein. The final volume of the membrane and cytosol fractions prepared from 200 ml cell cultures was 3 ml, and the following volumes of these fractions were loaded on the gel: 25 µl on lanes 1, 2, 7 and 8; 8 µl on lanes 5 and 6; 2.5 µl on lanes 3 and 4. Cytosol fractions were loaded on lanes 1, 3, 5 and 7, and membrane fractions on lanes 2, 4, 6 and 8. Positions of molecular mass standard proteins are shown on the right.

in cells was associated with the membrane fraction (Fig. 4), and this fraction was relatively free of cytosolic enzymes. There was 140 times more activity of lactate dehydrogenase, a cytosolic enzyme, in the cytosolic fraction (70 000 units ml⁻¹) than in the membrane fraction (500 units ml⁻¹). Adding Triton X-100 [0.4 and 1% (v/v)] to disrupt membrane vesicles did not affect the amount of lactate dehydrogenase measured, indicating that the relatively low amount of this enzyme in the membrane fraction was not simply due to entrapment of the enzyme within membrane vesicles. The same distribution of the immunity protein between the membrane and cytosolic fractions was also found in the immune strain IL 1430(pON7) (data not shown).

The immunity protein was also found in the cytosolic fraction (Fig. 4). Its presence in this fraction may have been due to: (i) the existence of both a membrane-associated and a cytosolic pool of the immunity protein, (ii) the presence of small membrane vesicles in the cytosolic fraction, and/or (iii) the release of membrane-associated proteins during fractionation of the membrane vesicles – the immunity protein is probably a peripheral membrane protein (see below) and might, consequently, be loosely associated with the membrane. The immunity protein band detected in Fig. 4 migrated identically with purified immunity protein (data not shown). No immunity protein was detected in the immune-negative strains, LMG 2131 (Fig. 4, lanes 1 and 2) and IL 1403 (data not shown), indicating that the antiserum was specific.

Judging from its amino acid sequence, the immunity protein is relatively hydrophilic and thus it is not a

typical integral membrane protein. It is more likely to be a peripheral membrane protein, perhaps associated with an integral membrane protein on the inside of the cell through electrostatic and hydrogen bonding interactions. By interacting with an integral membrane protein, the immunity protein might possibly prevent binding of lactococcin A to cells and/or lactococcin-A-induced membrane pore formation. The evidence that the immunity protein is associated with the cell membrane is consistent with earlier studies which showed that lactococcin A inhibits leucine uptake in membrane vesicles of sensitive strains, but not in membrane vesicles of resistant strains carrying the immunity gene (van Belkum *et al.*, 1991b).

The purification of the immunity protein and the availability of antiserum raised against the purified protein will permit further characterization of how immunity functions: for instance by studying how the incorporation of the immunity protein into membrane vesicles affects leucine uptake upon exposing the vesicles to lactococcin A.

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