

Generation of Polyclonal Antibodies against Nisin: Immunization Strategies and Immunoassay Development

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Murine polyclonal antibodies reactive to the lantibiotic bacteriocin nisin A (nisA) have been produced by immunization with nisA-cholera toxin and nisA-keyhole limpet hemocyanin (nisA-KLH) conjugates. Mice immunized with nisA-cholera toxin developed nisA-specific antibodies with low relative affinities and poor sensitivities, while the immunization of mice with nisA-KLH conjugates resulted in the production of nisA-specific antibodies with high relative affinities and much-increased sensitivities. nisA antibodies could also be readily mass produced in less than 8 weeks in ascites fluid by using the nisA-KLH conjugate. A competitive direct enzyme-linked immunosorbent assay (ELISA) whereby nisA-horseradish peroxidase and free nisA competed for antibody binding was devised. The detection limit for nisA in the competitive direct ELISA with the nisA-KLH-generated antibodies was from 5 to 100 ng/ml, while the amount of free nisA required for 50% antibody binding inhibition ranged from 0.3 to 5 µg/ml. Both antisera and ascites polyclonal antibodies cross-reacted with nisZ either in the supernatant of a producer strain or with the pure lantibiotic but did not cross-react at all with non-lantibiotic-type bacteriocins. These polyclonal antibodies should find a wide usage for nisA ELISA analysis in foods and other matrices.

Nisin A (nisA) is a lantibiotic, or lanthionine-containing peptide, with antimicrobial activity produced by several strains of *Lactococcus lactis* subsp. *lactis*. This compound is the most extensively characterized bacteriocin produced by lactic acid bacteria (LAB). Currently, nisA is still the only bacteriocin utilized by the food industry as a food preservative and its use is permitted in more than 50 countries.

The traditional agar diffusion test (13, 25) is still the most commonly used analytical tool for determination of nisA and other bacteriocins. This technique is of undeniable importance, but it also has drawbacks such as its unspecificity and limited sensitivity. Highly specific immunochemistry-based methods have been developed and routinely used as analytical tools in many research areas since the 1970s. Surprisingly, the impact of these techniques in the bacteriocin research field has been marginal. The information related to this subject is limited, and the results reported have been far from satisfactory (5, 6, 12, 24). The scarcity of immunological methods is likely due to the difficulties encountered in raising specific antibodies. Among those hurdles, the most important are the lack of purified bacteriocins and the low molecular masses (generally <5,000 Da) of these compounds, which make them poorly immunogenic or nonimmunogenic. Thus, bacteriocins, like other haptens, need to be conjugated to carrier proteins to become immunogenic. In addition, peculiar characteristics of the bacteriocin molecules, such as their hydrophobicity and, in the case of lantibiotics, the presence of modified amino acids that form intrachain disulfur rings, might also interfere with immunoassay development when proper formats are not used.

The objective of this work was to devise optimal immunological procedures to raise specific antibodies against bacteriocins to further enable the development of sensitive immunoassays. As a model for bacteriocin studies, nisA has been used

in this work. Here, we report novel immunization procedures in the bacteriocin research field that resulted in the rapid generation of murine nisA serum and ascites polyclonal antibodies. The immunization strategies devised involved using as immunogens conjugates of nisA with the carrier protein keyhole limpet hemocyanin (KLH) and with cholera toxin (CT), a protein with dual carrier-adjutant properties that has been proven useful in the past with other haptenic compounds (1, 3, 9). This paper also describes the development of sensitive competitive immunoassays for nisA analysis.

MATERIALS AND METHODS

Materials. Pure nisA (30,000 U/mg) was from NBS Biologicals (Hartfield, United Kingdom), and nisZ was kindly provided by Oscar Kuipers (NIZO, Ede, The Netherlands). Ovalbumin (OA) (grade III and fraction VII), CT, horseradish peroxidase (HRP) (fraction VI), Tween 20, 2,2'-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS), glutaraldehyde, Freund's adjuvants, and pristane were obtained from Sigma Chemical Co., St. Louis, Mo. KLH was purchased from Pierce Chemical Company, Rockford, Ill. Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase was obtained from Cappel Laboratories, West Chester, Pa. The myeloma cell line P3X63-Ag8.653 was kindly given by the Instituto Llorente (Madrid, Spain). Mice (BALB/c) were a gift obtained from the in-house colonies of the Facultad de Medicina de la Universidad Autonoma, Madrid, Spain.

Preparation of immunoconjugates and immunization. nisA was conjugated by the glutaraldehyde method (2, 8) to CT (nisA-CT) (50:1, mol/mol) and to KLH (nisA-KLH) (1:2, wt/wt) for use as immunogens and to OA (fraction VII) (nisA-OA) (10:1, mol/mol) for use as a solid-phase antigen. nisA was conjugated to HRP (nisA-HRP) (1:5, wt/wt) by the periodate method (19) for use in the competitive direct enzyme-linked immunosorbent assay (CD-ELISA).

Female BALB/c mice (6 to 8 weeks of age) were immunized by either the intraperitoneal (i.p.) or subcutaneous (s.c.) route. nisA-CT (10 µg in 0.2 ml of 0.01 M phosphate-buffered saline [PBS] [pH 7.2 to 7.4]) was administered i.p. following the procedure described by Azcona-Olivera et al. (3). The time intervals were 10 days between the first two doses and 6 days between other doses. Mice immunized with the nisA-KLH conjugate received three 25-µg doses of immunogen at 2-week intervals. The first injection consisted of 0.3 ml of conjugate in a 1:1 ratio of saline and Freund's complete adjuvant. The second dose consisted of 0.3 ml of conjugate in saline and Freund's incomplete adjuvant (1:1), and the third injection was presented in saline (0.2 ml). Ether-anesthetized mice were bled from the retrobulbar plexus, and serum was obtained after overnight incubation of blood at 4°C and centrifugation at 1,000 × g for 15 min.

Production of mouse polyclonal antibodies in ascites fluid. For large-scale generation of polyclonal antibodies, mice were immunized by a modified version

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TABLE 1. Reactivities of serum polyclonal antibodies against culture supernatants of LAB as determined by CD-ELISA

| Microorganism (bacteriocin) ^a | Source ^b | Cross-reactivity (%) ^c |
|---|---------------------|-----------------------------------|
| <i>Lactococcus lactis</i> G18 (nisA) | Our collection | 100 |
| <i>Lactococcus lactis</i> BB24 (nisA) | Our collection | 98 |
| <i>Lactococcus lactis</i> CNRZ 150 (nisZ) | INRA | 99 |
| <i>Lactococcus lactis</i> MG1614 (none) | IFR | NR |
| <i>Lactobacillus sake</i> 148 (lactocin S) | Our collection | NR |
| <i>Pediococcus acidilactici</i> 347 (pediocin PA-1) | Our collection | NR |
| <i>Pediococcus acidilactici</i> L50 (pediocin L50) | Our collection | NR |

^a The bacteriocin produced is shown in parentheses.

^b INRA, Institut National de la Recherche Agronomique, Station de Recherches Laitières; IFR, Institute of Food Research, Norwich Laboratory, Norwich, United Kingdom.

^c Cross-reactivity is defined here as [(antibody binding inhibition produced by a culture supernatant/antibody binding inhibition produced by supernatant of *Lactococcus lactis* G18) × 100]. NR, no reactivity.

(3) of the method of Kurpisz et al. (16). Mice ($n = 4$) were given three i.p. 25- μ g doses of nisA-KLH immunogen prepared as described before. Immunization proceeded with the following schedule. The animals received 0.3 ml of immunogen i.p. on days 0 and 14 and 0.3 ml of pristane on days 3 and 17; on day 21 blood samples were taken for assessing the course of immunization. At day 28, the mice were given a third i.p. dose of immunogen, and on day 31, they were injected i.p. with 6×10^6 cells of a nonsecreting myeloma (P3X63-Ag8.653) cell line. On day 42, ascites fluid was collected and pooled and immunoglobulins were purified by 50% saturation with ammonium sulfate (15).

ELISA. For antiserum titration, wells of polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight (4°C) with 100 μ l of nisA-OA (5 μ g/ml) in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6. The plates were washed four times with 300 μ l of washing solution (0.02% Tween 20 in PBS). The wells were blocked for 30 min at 37°C with 300 μ l of 1% (wt/vol) OA (grade III) in PBS (OA-PBS) and then washed four times. Next, 50 μ l of serially diluted serum was added to each well and incubated for 1 h at 37°C. Unbound antibody was removed by washing four times, and 100 μ l of goat anti-mouse immunoglobulin G-peroxidase conjugate (diluted 1:500 in OA-PBS) was added to each well. The plates were incubated for 30 min at 37°C and washed eight times, and the amount of bound peroxidase was determined with ABTS substrate as described previously (20). The A_{405} was read with an iEMS reader with a built in software package for data analysis (Labsystems, Helsinki, Finland). The titer of each serum was arbitrarily designated as the maximum dilution that yielded at least twice the absorbance of the same dilution of nonimmune control serum.

A competitive indirect ELISA (3) was initially used to assess the presence of specific nisA antibodies in mouse serum. Microtiter plates were coated and blocked as described for the antiserum titration procedure. Next, 50 μ l of nisA dissolved in PBS (or another vehicle) was simultaneously incubated with 50 μ l of antiserum (diluted properly in PBS) over the nisA-OA solid phase for 1 h at 37°C. The amount of bound antibody was determined by the addition of anti-mouse immunoglobulin G-peroxidase conjugate as described above. Relative antibody affinity was arbitrarily designated as the bacteriocin concentration required to inhibit antibody binding by 50%.

A CD-ELISA (4) to assess the presence of specific nisA antibodies in mouse serum and ascites fluid was also developed. Plates were coated overnight by air drying at 40°C with 125 μ l of nisA antiserum or ascites fluid (properly diluted in coating buffer). After washing and blocking were performed, to each well were added consecutively 50 μ l of nisA standard (or sample) and 50 μ l of nisA-HRP (diluted 1:500 in OA-PBS). After 1 h of incubation at 37°C, the plates were washed and the amount of bound peroxidase was determined as described before.

Microorganisms, media, and bacteriocin assay. The bacteriocin-producing LAB used to test antibody cross-reactivity are listed in Table 1. Microorganisms were propagated overnight in MRS broth (Oxoid Ltd., Basingstoke, United Kingdom) at 37°C. *Lactococcus lactis* BB24 counts were made in 1.5% MRS agar. Culture supernatants were obtained by centrifugation at 12,000 \times g for 10 min at 4°C. The pH of the supernatants was adjusted to 6.1 with 1 N NaOH, next they were filtered through 0.22- μ m-pore-size filters (Millipore Corp., Bedford, Mass.), and finally they were stored at -20°C until required. Bacteriocin activity assessment was done by the agar diffusion test as described by Rodríguez et al. (21), with *Lactobacillus helveticus* CH-1 (Christian Hansen's Laboratories, A/S, Copenhagen, Denmark) as the indicator strain.

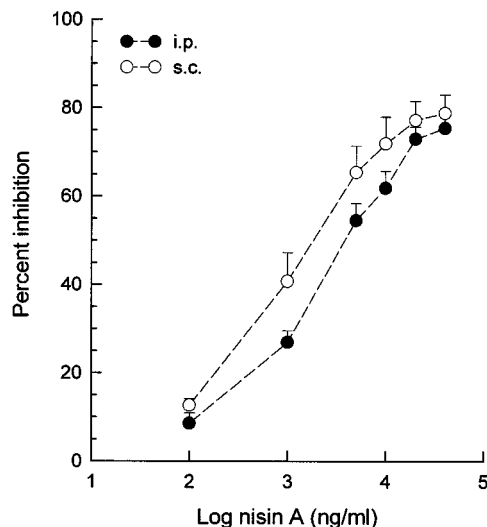


FIG. 1. Results of CD-ELISA for nisA with serum polyclonal antibodies produced after three i.p. and s.c. injections with nisA-KLH. The data are mean values \pm standard errors of the means (error bars) ($n = 4$). Serum dilutions of 1/1,000 and 1/2,500 were used for i.p. and s.c. groups, respectively.

RESULTS

Firstly, mice were immunized i.p. with multiple doses of nisA-CT. On the 15th day of the immunization process, and after two doses of the immunogen had been administered, the animals had apparent titers in serum of 1,600 to 3,200 that slightly increased, up to 6,400, with two more booster doses. The presence in serum of nisA-specific antibodies with low relative affinities was detected for all the animals by a competitive indirect ELISA. The detection limit of this assay was 1 to 5 μ g of nisA per ml, while the amount of free nisA required for a 50% binding inhibition was over 40 μ g/ml. No sensitivity increase or improvement in the relative affinity was observed when the nisA-CT-generated antibodies were assayed in a CD-ELISA. Animals immunized i.p. and s.c. with nisA-KLH (25- μ g dose) had apparent titers in serum ranging from 12,800 to 25,600; however, the presence of nisA-specific antibodies could not be demonstrated when those antisera were tested in a competitive indirect ELISA. In contrast, when nisA-KLH-derived antisera were assayed in a CD-ELISA, the presence of nisA-specific antibodies with high relative affinities could be demonstrated (Fig. 1). The average detection limit of the CD-ELISA with the nisA-KLH antiserum was 0.1 μ g/ml of nisA, while the amount of free nisA required for a 50% binding inhibition ranged from 0.5 to 5 μ g/ml. From this stage through the rest of our research, the CD-ELISA with the nisA-KLH-generated antibodies was used.

Competition ELISA curves of nisA in PBS, MRS broth, and nisaplin (Applin & Barret Ltd., Beaminster, United Kingdom) are shown in Fig. 2. The response range for PBS and MRS curves was from 100 to 10,000 ng/ml, and that for nisaplin (prepared from the commercial product by dilution in PBS) was from 156 to 25,000 ng/ml. While the ELISA competition curve of nisA in nisaplin resembled that in PBS, the MRS curve showed notably higher inhibition values, suggesting that the sensitivity of the immunoassay could be improved with the culture media as a vehicle. That observation was confirmed in a follow-up experiment comparing the sensitivity of the CD-ELISA using the serum antibodies with that of the classical agar diffusion test (Fig. 3). When nisA estimations were made in MRS, the detection limit of the immunoassay was improved

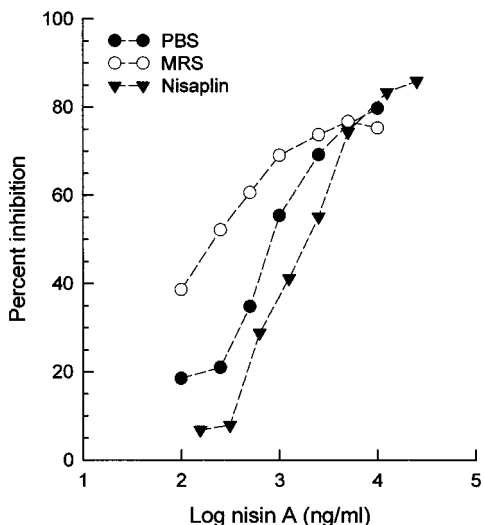


FIG. 2. nisa detection in PBS, MRS, and nisaplin by CD-ELISA using serum polyclonal antibodies. Each datum point represents the average value of triplicate determinations in a single microtiter plate.

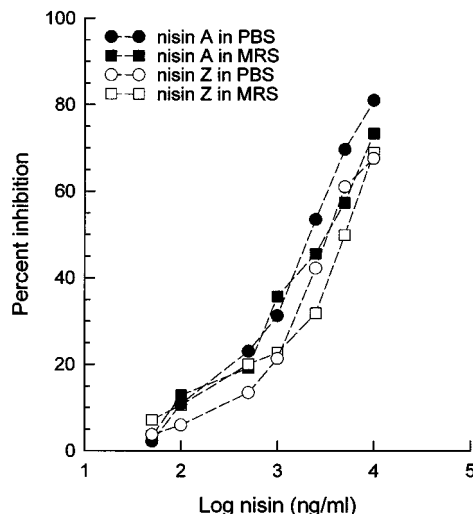


FIG. 4. Standard curves for nisA and nisZ in PBS and MRS as determined by CD-ELISA using purified ascites polyclonal antibodies. Each datum point represents the average value of triplicate determinations in a single microtiter plate.

to 5 to 10 ng/ml and the 50% binding inhibition value was lowered to 300 ng/ml (Fig. 3). Additionally, ELISA sensitivity compared favorably with that of the bacteriocin activity test.

The specificities of the serum polyclonal antibodies in supernatants of 16-h MRS cultures of various LAB were evaluated by CD-ELISA (Table 1). The immunoassay strongly reacted with the supernatants of *Lactococcus lactis* BB24 and G18, both nisA producer strains (21), and with the supernatant of *Lactococcus lactis* CNRZ 150 (Institut National de la Recherche Agronomique, Jouy-en-Josas, France), a nisZ producer strain, but did not react with the supernatant of the plasmid-free non-nisin-producing *Lactococcus lactis* MG1614 (14). No reactivity was observed with the supernatants of *Lactobacillus sake* 148, *Pediococcus acidilactici* 347, and *P. acidilactici* L50, strains which produce lactocin S (22), pediocin PA-1 (17), and pediocin L50 (10), respectively.

Anti-nisA antibodies were also readily mass produced in

ascites fluid with the nisA-KLH conjugate. All animals developing ascites fluid yielded nisA-specific antibodies. The reactivity of these antibodies with LAB culture supernatants was identical to that described for the serum antibodies. Direct ELISA competition curves for nisA and nisZ, generated with the purified ascites antibodies, are shown in Fig. 4. Curve patterns of both nisin variants were very similar, with response ranges from 50 to 10,000 ng/ml. The amounts of nisA and nisZ required for 50% binding inhibition were 1,900 and 3,000 ng, respectively, thus giving a cross-reactivity for nisZ of 65% [cross-reactivity in this case was defined as (nanograms of nisA per milliliter required for 50% inhibition)/(nanograms of analog per milliliter required for 50% inhibition) × 100]. Finally, nisA production of *Lactococcus lactis* BB24 in MRS broth was assessed by a CD-ELISA with the ascites antibodies and by the agar diffusion test (Fig. 5). Significant nisA levels were detected as early as 6 h by the immunoassay and at 8 h by the

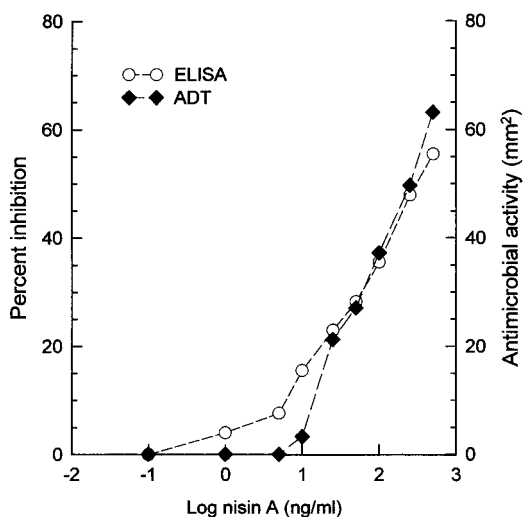


FIG. 3. Standard curves for nisA in MRS as determined by the agar diffusion test (ADT) and by CD-ELISA using serum polyclonal antibodies.

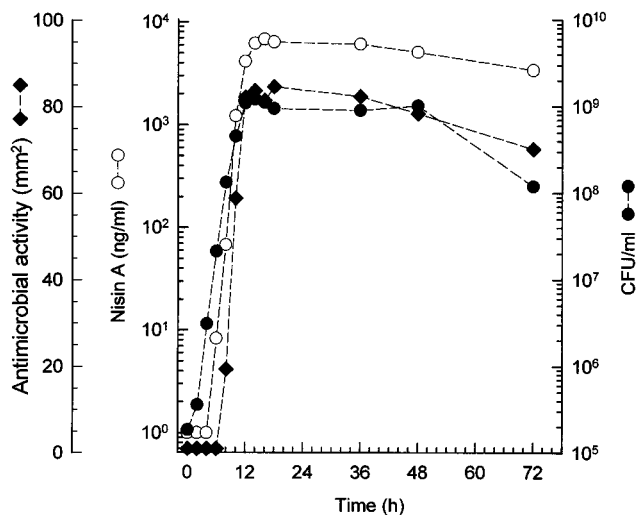


FIG. 5. Growth curve of *Lactococcus lactis* BB24 in MRS broth and nisA production by the agar diffusion test and CD-ELISA using ascites polyclonal antibodies.

bioassay. The maximal production occurred at 14 to 18 h, coinciding with the beginning of the stationary phase. From that point thereafter nisA concentration in the culture supernatants decreased.

DISCUSSION

The production of antibodies against bacteriocins requires the conjugation of the bacteriocins to large carrier proteins to render them immunogenic. Attempts to generate specific antibodies against nisA (24), pediocin AcH (7), and pediocin RS2 (5) have failed or rendered unsatisfactory results when these bacteriocins were injected alone. Pediocin AcH conjugated to bovine serum albumin (7) or pediocin RS2 bound to heat-killed *Lactobacillus plantarum* (5) also failed to induce an immunoresponse. Thus, the selection of carrier proteins, conjugation sites and procedures, and immunoassay formats should be carefully done in a case-by-case situation in accordance to the peculiarities of each bacteriocin.

The presence of various lysine residues together with the amino-terminal group in the nisA molecule makes feasible its rapid and simple conjugation to protein carriers by the classical glutaraldehyde method. Using conjugates of nisA-CT and nisA-KLH prepared by this coupling strategy, we have been able to generate nisA-specific antibodies with different relative affinities. CT has been proven as an effective carrier-adjutant in the generation of antibodies against other hapten molecules (1, 3, 9); however, in the present study, although all the animals immunized with nisA-CT produced nisA-specific antibodies, the relative affinity and sensitivity of these antibodies were low. In contrast, the nisA-KLH-generated antibodies showed good sensitivity and much-improved relative affinity. Similarly, Usleber et al. (26) described an increase in sensitivity by two orders of magnitude in the generation of antibodies for the mycotoxin fumonisin B₁, using a KLH conjugate, in comparison with that for another fumonisin B₁ antibody, generated with a CT conjugate (3, 4). The differences in relative antibody affinity could be attributed to the smaller primary immunization performed with the nisA-CT immunogen, to the carrier protein itself, or to the immunization procedures involved in the immunogen presentation.

The importance of the selection of a proper immunoassay format needs to be emphasized. In this study, the presence of nisA-specific antibodies in the nisA-CT antisera could be demonstrated by both competitive ELISA formats while the presence of nisA-specific antibodies on the nisA-KLH antisera could be demonstrated by only CD-ELISA. This observation might suggest that the antibodies generated with both immunogens recognized different epitopes. The failure of the competitive indirect ELISA in recognizing the presence of nisA-specific antibodies could be due to several reasons: (i) a steric hindrance problem derived from the immobilization of the nisA-OA immunoreagent occurred, (ii) the conjugation site of nisA-OA occurred in the area of the nisA molecule that conforms to the antibody epitope, and (iii) the nisA-KLH antisera might include other antibodies of stronger affinity that recognize the bacteriocin carrier bridge or a conjugation by-product and interfere in the competition between the free nisA and the nisA-OA conjugate for antibody binding.

To our knowledge, the only other nisA-specific antibody described until now has been generated by immunization of a sheep with a conjugate of nisA-egg albumen (12). The detection limit of the sheep polyclonal antibodies in a sandwich-type ELISA was 0.019 IU/ml (or 1.9 arbitrary units [AU]), which is slightly better than the 5 to 10 ng/ml (equivalent to 0.15 to 0.3 IU or 15 to 30 AU) of our immunoassays (but in a comparable

range). Still, both polyclonal antibody-derived immunoassays are considerably more sensitive than the monoclonal antibody-based immunoassay for pediocin RS2 developed by Bhunia (5), which had a detection limit of 32,000 AU. Besides the difference in immunoassay format, there are several differences between the immunological procedures that we devised and the ones described by Falahee and coworkers (12). Firstly, the latter authors obtained the specific antisera through a long immunization process (9 months), in contrast to our procedure that generated specific antibodies in less than 8 weeks. Secondly, the sheep antibodies were rendered nisA specific by affinity purification with a nisA matrix, while our system bypassed that step since antibody specificity was achieved by the selection resulting from the competitive immunoassay. Finally, bacteriocin needs were much smaller in our system since the affinity purification step was circumvented and obviously because the mouse immunization required less antigen (10 to 25 µg per dose for mice; 0.5 to 1 mg per dose for large animals). The previously stated differences probably will make our antibody production approach more suitable and realistic than that of Falahee's group for cases in which the production of antibodies against other bacteriocins is attempted.

Competition curves of nisA contained in the nisaplin and nisA in PBS were very similar, while the curve generated for nisA in MRS broth differed notably, showing greater binding inhibition values (Fig. 2). nisA solubility is known to be strongly pH dependent, being maximal at low pH values while it decreases continuously with increasing pH; a 10-fold reduction in solubility is produced when the pH is raised from 3 to 7 (23). The remarkable binding differences in the competition curves could be due to the different pHs of the vehicles, 6.1 to 6.2 for the MRS and 7.2 to 7.4 for the PBS and the nisaplin (since it was diluted in PBS). The pH could also determine a better or worse antigen (nisin)-antibody interaction, thus affecting the sensitivity of the immunoassay. That might explain why the sensitivity of our immunoassay was better when nisA was assayed in the MRS broth than when it was assayed in PBS and nisaplin (Fig. 2 and 3).

The antibodies produced in this study strongly recognized nisA production in supernatants of producer strains grown in MRS (Table 1). They also reacted extensively with the supernatant of a nisZ producer strain and with pure nisZ (Fig. 4), which could be expected since nisA and nisZ differ in only a single amino acid, at position 27 (18). The antibodies did not react to any degree with supernatants of LAB producing other nonantibiotics. The sheep anti-nisin antibodies produced by Falahee et al. (12) cross-reacted with the lantibiotic subtilin (133%) but did not cross-react with three other lantibiotics (gallidermin, Pep 5, and RO 09-0198) or with any of the eight nonlantibiotic bacteriocins analyzed (11). On the basis of the latter results, it is possible that our antibodies might cross-react with at least other type-A lantibiotics, especially subtilin, since its structure is very similar to that of nisA.

In summary, in the present work we report reliable immunization procedures for the rapid and efficient production of serum-specific polyclonal antibodies to nisA which required only very small amounts of immunogens. We also report, to our knowledge for the first time in the bacteriocin research field, the development of very sensitive immunoassays of the competitive type. An alternative and effective method for the production of moderately large amounts of murine polyclonal antibodies in ascites fluid is also presented. All the methodologies we describe can be used as models of immunization strategy and immunoassay development for other lantibiotic and nonlantibiotic bacteriocins. Potential specific applications of these nisA antibodies will include analysis by ELISA of nisin in

foods, the rapid identification and isolation of nisin (A and Z) producer strains, and use as a tool for regulation and mechanistic studies. Finally the antibodies could be utilized for the preparation of immunoaffinity chromatography columns for purification purposes.

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