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Purification and Characterization of Two *Listeria ivanovii* Cytolysins, a Sphingomyelinase C and a Thiol-Activated Toxin (Ivanolysin O)

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The strong bizonal hemolysis on blood agar and the positive CAMP reaction with *Rhodococcus equi* denotes the production of two different cytolytic factors by *Listeria ivanovii*. One was characterized as a thiol-activated (SH) cytolysin of 61 kilodaltons and was termed ivanolysin O (ILO) since data suggested that it is different from listeriolysin O, the SH-cytolysin produced by *Listeria monocytogenes*. The other is a 27-kilodalton hemolytic sphingomyelinase C that was found to be the cytolytic factor responsible for the halo of incomplete hemolysis synergistically enhanced by *R. equi* exosubstances. When thiol-disulfide exchange affinity chromatography and gel filtration were applied to the purification of ILO from concentrated *L. ivanovii* culture supernatants, the copurification of the two cytolysins was observed. This phenomenon seems to be due to the formation of intermolecular disulfide bonds between ILO and the sphingomyelinase, since the latter was found to contain free SH groups, not essential for the activity. These SH groups could react with the single cysteine residue characteristically present in the SH-cytolysins, forming a dimeric cytolytic complex. The purification of ILO was achieved by a further gel filtration with a reducing agent (dithiothreitol) in the eluent. A method for the purification of the sphingomyelinase based on selective sequestration of ILO from the *L. ivanovii* concentrated culture supernatant by the SH cytolysin target molecule cholesterol and thiol-disulfide affinity chromatography is described.

The non-spore-forming gram-positive bacterium *Listeria ivanovii* was isolated for the first time in 1955 by I. Ivanov from abortions and perinatal infections of sheep (13). It is, together with *Listeria monocytogenes*, the causative agent of listeriosis. However, unlike *L. monocytogenes*, *L. ivanovii* predominantly infects animals (human infection is very rare), especially ovine species, in which it results in abortions (neonatal sepsis and enteritis have also been observed) but not meningitis or encephalitis (6, 13, 28–30). The bacterium has also been isolated from healthy carriers and from the environment (29). Until 1984, *L. ivanovii* was classified as "*L. monocytogenes* serovar 5" strains. However, some sharply distinctive phenotypic characters, i.e., the homogeneous O-antigenic composition patterns (all the strains described belong specifically to serovar 5) and the strong hemolytic activity, as well as intrageneric DNA relatedness studies, led to the establishment of an independent species for these strains (14, 26, 28–30).

As mentioned above, *L. ivanovii* displays a characteristic strong hemolysis when grown on sheep blood agar, in contrast to that produced by *L. monocytogenes*, which is so weak that it is frequently difficult to interpret (7). The hemolytic effect of *L. ivanovii* is often bizonal (5, 6, 28, 30, 31), with a zone of complete hemolysis around the colonies surrounded by a ring of incomplete hemolysis that develops with time (Fig. 1). In the vicinity of a culture of *Rhodococcus equi* the latter is totally hemolyzed (30, 31), giving a typical shovel-shaped lytic phenomenon (positive CAMP test with *R. equi*), which is considered a fundamental criterion for the identification of *L. ivanovii* (14, 27–30). These observations suggest that *L. ivanovii* produces at least two different cytolytic factors with different diffusibilities, probably depending on differences in molecular mass. However, only one cytolysin has been identified to date. Preliminary studies on the characterization of the hemolytic activity of *L. ivanovii* associated it with the secretion of a heat-labile protein of 67 kilodaltons (kDa) inhibited by cholesterol (30). Further studies on the cytolysin isolated from target erythrocyte membranes showed that it is a 55- to 60-kDa streptolysin O (SLO)-related toxin (thiol-activated [SH] cytolysin) (23). However, the secretion of this kind of cytolysin, in accordance with these known to date (1, 4, 20, 32), does not explain the bizonal hemolysis and the positive CAMP reaction with *R. equi*. These phenomena more likely associated with the production of other membrane-damaging substances, such as phospholipases (3, 20). This assumption correlates with the fact that *L. ivanovii* gives a positive reaction on egg yolk agar (25, 28, 30).

As a first step in research on virulence factors of *L. ivanovii*, we undertook the study of the cytolysin(s) of this bacterium. In this paper we show that *L. ivanovii*, like other gram-positive bacilli, e.g., *Clostridium perfringens* (33, 36) and *Bacillus cereus* (12), produces a thiol-activated toxin (ivanolysin O [ILO]) and a sphingomyelinase (involved in the CAMP phenomenon with *R. equi*).

MATERIALS AND METHODS

Bacterial strains. *L. ivanovii* (serovar 5) SLCC (Special *Listeria* Culture Collection, Institut für Hygiene und Mikrobiologie, University of Würzburg, Federal Republic of Germany) 2379 (ATCC 19119), *L. monocytogenes* (serovar 1/2a

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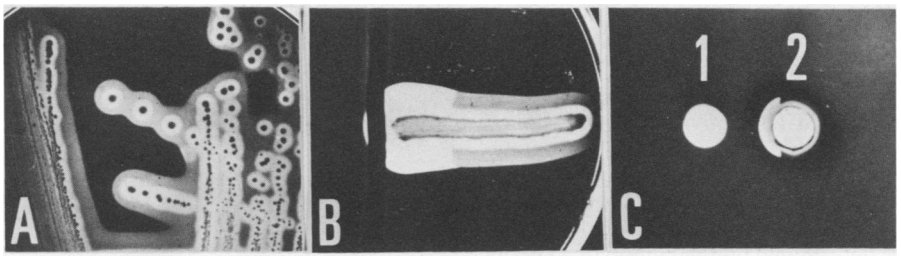

FIG. 1. (A) *L. ivanovii* SLCC 2379 (ATCC 19119) cultured (37°C for 48 h) on sheep blood agar. (B) The same strain as in panel A in the CAMP test with *R. equi* (vertical streak). Note the bizonal hemolytic effect. (C) Reproduction of the CAMP phenomenon by the purified *L. ivanovii* sphingomyelinase (well 2) confronted with a *R. equi* CCS (well 1) in a diffusion test on sheep erythrocyte agar (37°C for 24 h).

SLCC) 2371 (NCTC 7973), and *R. equi* CIP (Collection de l'Institut Pasteur, Paris, France) 5869 were used in the experiments.

Concentrated culture supernatants (CCS). *Listeria* strains were grown in 300 ml of brain heart infusion (Difco Laboratories) at 37°C for 18 h with shaking (100 rpm). These cultures were used to seed 9 liters of the same medium. After 18 h of incubation under the same conditions, the cells were harvested by centrifugation (12,000 × *g* for 30 min), and the supernatant was concentrated to 300 ml by ultrafiltration through 10-kDa exclusion limit membranes (Pellicon apparatus; Millipore Corp.). *R. equi* CCS (100 ml) was obtained by the same method, with different cultivation conditions (3 liters of brain heart infusion was inoculated with a 50-ml culture and incubated statically at 37°C for 48 h).

Purification of ivanolysin O (ILO). Selective purification of ILO from the *L. ivanovii* CCS (65 ml) by thiol-disulfide exchange affinity chromatography was performed on Thiopropyl-Sepharose 6B (Pharmacia) essentially as described previously (11). The starting buffer was 0.05 M phosphate-buffered saline (pH 6.5) containing 0.02% sodium azide (PBS). Selective elution of thiol-containing material was performed with 10 mM dithiothreitol (DTT) in PBS. The eluted hemolytic fractions were concentrated to 12 ml by ultrafiltration with a 10-kDa exclusion limit (Amicon stirred cell with YM-10 membrane), and after ultracentrifugation (90,000 × *g* for 60 min) they were gel filtered through 20- to 350-kDa fractionation range Ultrogel AcA 34 (LKB Instruments, Inc.) in PBS (100- by 2.6-cm column) with a flow rate of 15 to 20 ml/h. Fractions (5 ml) presenting high hemolytic activity (over 512 complete hemolysis units [CHU]) were pooled and concentrated, as mentioned above, to 4 ml, made to contain 5 mM DTT, and finally subjected to molecular sieving on a 5- to 250-kDa fractionation range Sephacryl S-200 Superfine column (Pharmacia) (60- by 1.6-cm column) with 2.5 mM DTT–PBS as the eluent and a flow rate of 10 ml/h. Fractions with high specific hemolytic activity were concentrated to 2.5 ml as described above. All steps were performed at 4°C.

SH-cytolysin selective sequestration by cholesterol and isolation of cholesterol-SH-cytolysin complexes (C/Cly). Selective sequestration of ILO (in general, SH-cytolysins) present in culture supernatants or aqueous solutions by the target molecule cholesterol (1, 4, 15, 32) leading to the formation of amphiphilic cholesterol-SH-cytolysin complexes (C/Cly) in homogeneous suspensions was performed by a method described elsewhere (J. A. Vázquez-Boland, L. Dominguez, E. F. Rodriguez-Ferri, J. F. Fernández-Garayzábal, and G. Suárez, FEMS Microbiol. Lett., in press). Various quantities of a cholesterol (Panreac, Spain) solution (10 mg/ml) in absolute ethanol were added to the sample containing the SH-cytolysin (in reduced or activated form) (final concentration of cholesterol ranged from 25 to 500 μg/ml, depending on the SH-cytolysin activity). The resultant suspension was incubated at 37°C for 30 min with shaking, after which C/Cly were pelleted by centrifugation (25,000 × g for 30 min at 4°C). The supernatant obtained lacked SH-cytolysin activity. The C/Cly were suspended in PBS and washed thrice, and the final suspension was stored by freezing it at −30°C.

Isolation and purification of the CAMP (*R. equi*) factor of *L. ivanovii* (sphingomyelinase C). For preliminary isolation of this cytolytic factor, 15 ml of the *L. ivanovii* CCS was subjected to molecular sieving in the Ultrogel AcA 34 column as described above. Fractions of 5 ml were collected and tested for hemolytic activity before and after exposure to 20 mM L-cysteine (Sigma Chemical Co.) for 15 min at 37°C. Fractions were also tested for synergistic hemolysis with *R. equi* exosubstances, and those showing increases of the hemolytic titer were pooled (50 ml), concentrated (3.5 ml) as described above, and tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of the *L. ivanovii* CAMP (*R. equi*) factor was carried out as follows: 5 ml of the *L. ivanovii* CCS was centrifuged (25,000 × *g* for 30 min) to eliminate insoluble material and exposed to 5 mM DTT, after which the SH-cytolysin cholesterol sequestration treatment was performed to eliminate ILO. The resultant ILO-devoid CCS (as determined by SDS-PAGE and Western [immuno-] blot [see Fig. 3, part 1, and 4]) was subjected to molecular sieving on a Sephacryl S-200 column as described above with 2.5 mM DTT in the eluent. Fractions of 3 ml were collected, and those showing hemolytic activity were pooled (25 ml), concentrated to 3.5 ml by ultrafiltration as mentioned above, diafiltered against PBS through 10-kDa exclusion limit membranes to eliminate excess DTT, and subjected to thiol-disulfide exchange affinity chromatography in a Thiopropyl-Sepharose 6B column (a Pasteur pipette filled with 3 ml of gel, corresponding to 0.8 g of freeze-dried powder). After elution with 10 mM DTT in PBS (25 ml), the SH-reactive fractions which showed the main hemolytic activity (synergistically enhanced by *R. equi* exosubstances) were concentrated to 1 ml and gel filtered on a Sephadex G-75 Superfine column.

Hemolytic activity determinations. Samples to be tested were diluted twofold in 50 μl of PBS in a V-form microtiter plate. To each dilution, 50 μl of a 1% suspension of washed sheep erythrocytes in the same buffer was added, and after 1 h of incubation at 37°C the titers were recorded as CHU (7). For the determination of the optimal pH for hemolytic activity, the sample was diluted twofold in 50 μl of 0.1 M PBS adjusted to the desired pH; to each dilution, 40 μl of the same PBS and 10 μl of a 5% erythrocyte suspension in 0.05

M PBS (pH 7) was added (to protect erythrocytes from extreme pH stress). Titration of the hemolytic activity with *R. equi* crude exosubstances was performed by using a 1% sheep erythrocytes suspension previously exposed (for 15 min at 37°C) to *R. equi* CCS (10 activity units per ml of erythrocyte suspension). Alternatively, synergistic hemolysis was also determined by adding 25 μl of *R. equi* CCS (25 activity units) to the titration mixtures. *R. equi* factor (the *R. equi* exosubstance responsible for synergistic hemolysis [3, 31]) activity units were determined by the hemolytic synergy with crude β-hemolysin from *Staphylococcus aureus* CIP 5710 as previously described (31).

Whole-cell extracts. Some of the *L. ivanovii* harvested cells (see above) were washed twice in PBS, mixed with glass beads (0.17 to 0.18 mm in diameter), and disrupted with a Braun type 853032 apparatus (10 min of discontinuous treatment at 0°C). The homogenate was extracted with 100 ml of PBS and centrifuged twice (25,000 × *g* for 30 min and 90,000 × *g* for 60 min) to eliminate cell debris. The supernatant was precipitated with ammonium sulfate to 65% saturation, and after centrifugation (25,000 × *g* for 30 min) the pellet was suspended in 10 ml PBS and dialyzed.

Phospholipase activity determinations. The phospholipase activity was determined by previously described methods (3). Substrates (sphingomyelin, phosphatidylcholine, and phosphatidylglycerol) (Sigma) solubilized in chloroform were dried and dispersed by sonication in 0.01 M Tris hydrochloride–0.145 M NaCl, pH 7.2 containing 10 mM $MgCl_2$ and 0.1% Triton X-100. Reaction mixtures consisted of 50 μl of the lipid dispersion (0.15 to 0.4 mg) and 0.4 CHU of the *L. ivanovii* CAMP (*R. equi*) factor in 50 μl of the same buffer. After 60 min of incubation at 37°C, reaction mixture samples (4 μl) were applied directly to high-performance thin-layer chromatography silica gel 60 plates (Merck) and developed with chloroform-methanol-NH_4OH (65:25:4) as the solvents. Spots in chromatoplates were visualized by exposure to iodine vapors.

Antisera. Specific *Listeria* SH-cytolysin antisera were produced as described elsewhere (Vázquez-Boland et al., in press) by immunizing rabbits with C/Cly from *L. monocytogenes* SLCC 2371 (NCTC 7973) and *L. ivanovii* SLCC 2379 (ATCC 19119). The C/Cly were obtained as described previously by adding 2 ml of the cholesterol solution to 20 ml of ultracentrifuged (90,000 × *g* for 60 min) CCS diluted in an equal volume of PBS containing 20 mM L-cysteine (final concentration of cholesterol, 500 μg/ml). The C/Cly were washed thrice in PBS, and the final pellet was suspended in 20 ml of PBS and tested by SDS-PAGE to determine the presence of the SH-cytolysin (see Fig. 6). New Zealand White female rabbits (2 months old) received three intravenous injections of 1 ml of the C/Cly suspension with 10-day intervals, and 7 days after the final inoculation they were bled. The sera were tested by SDS-PAGE and Western blotting, using as positive controls previously described (10, 11) hyperimmune horse anti-SLO sera (no. 94 and 525).

SDS-PAGE and Western blot analysis. SDS-PAGE was performed by the method of Laemmli (19), with 3% stacking and 15% resolution acrylamide slab gels. Proteins were detected with Coomassie brilliant blue R-250. After SDS-PAGE, the proteins were electrotransferred as described previously (35) from gels to nitrocellulose paper (Trans Blot 0.2-μm pore diameter; Bio-Rad Laboratories) at 500 mA for 2 h at 4°C. The blots were washed in PBS–0.3% Tween 20 and then saturated for 1 h with 5% skim milk in the same buffer, after which listeriolysin O (LLO) and ILO antisera were added (1/200). After overnight incubation at room temperature, the immunoblots were washed and peroxidase-conjugated second antibodies (swine anti-rabbit immunoglobulins; Dakopatts, Denmark) were added (1/500; 90-min incubation at room temperature). After being washed, the immunoblots were developed with a solution containing 50 mg of diaminobenzidine and 10 μl of H_2O_2 in 100 ml of PBS.

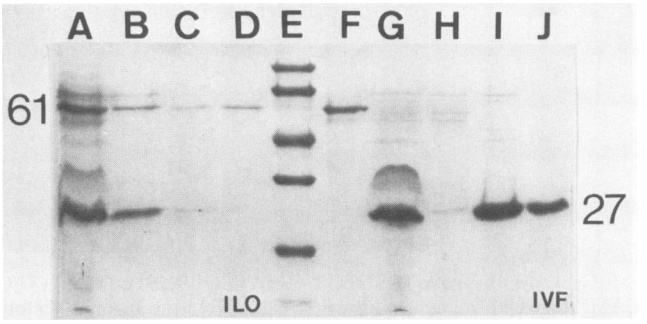

FIG. 2. SDS-PAGE of samples representing the purification steps of the two *L. ivanovii* cytolysins. Lanes: A, CCS; B, hemolytic fractions eluted with 10 mM DTT after thiol-disulfide exchange affinity chromatography of the contents of lane A; C, gel filtration of the contents of lane B (note the copurification of two proteins of 61 and 27 kDa, corresponding, respectively, to ILO and the sphingomyelinase); D, gel filtration of the contents of lane C with 2.5 mM DTT in the eluent (purified ILO); E, molecular mass standards (from top to bottom, 94, 67, 43, 30, 20, and 14.4 kDa); F, cholesterol-ILO complexes obtained from lane A; G, ILO-devoid contents of lane A, after gel filtration; H, thiol-disulfide exchange affinity chromatography of the contents of lane G (not retained fractions); I, thiol-disulfide exchange affinity chromatography of the contents of lane G (retained and subsequently 10 mM DTT-eluted hemolytic fractions); J, gel filtration of the contents of lane I (purified sphingomyelinase [the *L. ivanovii* factor (IVF)] involved in the CAMP phenomenon with *R. equi*). Numbers indicate known molecular masses in kilodaltons.

RESULTS AND DISCUSSION

Purification and characterization of ILO. A total of 8.52 × 10^6 CHU in 65 ml of *L. ivanovii* CCS with a specific activity of 27,300 CHU/mg was subjected to thiol-disulfide exchange affinity chromatography. Retained and selectively eluted (with 10 mM DTT) fractions were concentrated, ultracentrifuged (112,027 CHU/mg), and subjected to two gel filtration steps. The final concentrated fractions (2.5 ml) presented a total hemolytic activity of 0.65 × 10^6 CHU with a specific activity of 2.62 × 10^6 CHU/mg, a result similar to that obtained by other authors for LLO (11). Since the total hemolytic activity not retained after thiol-disulfide exchange affinity chromatography was 5.16 × 10^6 CHU, 19.34% recovery was obtained.

When analyzed by SDS-PAGE, the 10 mM DTT-eluted hemolytic fractions from the thiopropyl-Sepharose 6B column showed two main bands; one was a 61-kDa protein, and the other was a 27-kDa protein that appeared to be predominant. These bands corresponded to the two major proteins present in the *L. ivanovii* CCS (Fig. 2 and 3-1). Interestingly, the 27-kDa molecule was absent from the *L. monocytogenes* CCS (not shown), indicating that it is specifically produced by *L. ivanovii*. After the first gel-filtration step, the two proteins still copurified, suggesting some kind of molecular interaction in the absence of the denaturing conditions of the SDS-PAGE technique (i.e., SDS, β-mercaptoethanol, and heating) (Fig. 2). The same results were also obtained recently by other authors who tried to purify the *L. ivanovii*

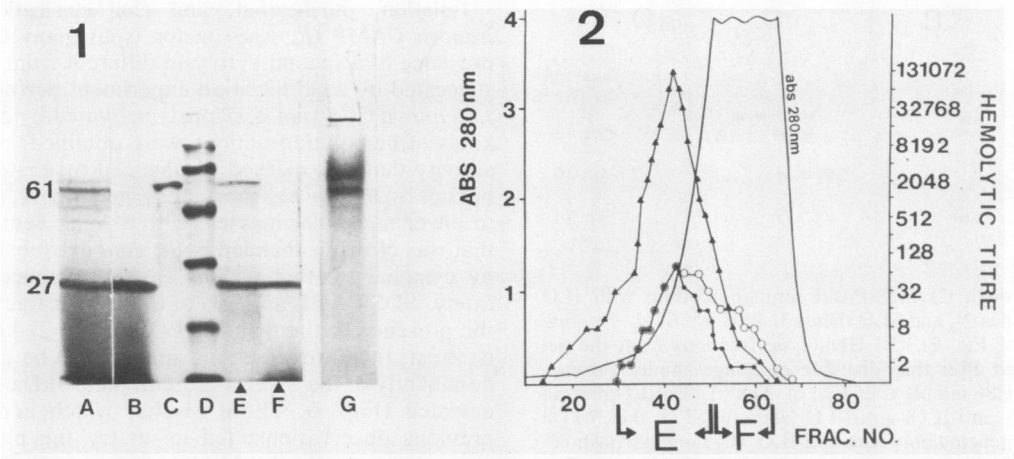

FIG. 3. Experiments indicating the presence of two different hemolytic activities in the *L. ivanovii* CCS. (1) Lanes: A through F, SDS-PAGE; A, CCS with 131,072 CHU of hemolytic activity showing two major bands of 61 (ILO) and 27 (sphingomyelinase) kDa; B, contents of lane A after SH-cytolysin sequestration by the cholesterol-binding method (the 61-kDa ILO was absent, but 8 to 16 CHU of hemolytic activity, synergistically enhanced by *R. equi* to 256 CHU, remained, together with the 27-kDa protein); C, cholesterol-ILO complexes showing the presence of the 61-kDa ILO and the absence of the 27-kDa sphingomyelinase; D, molecular mass reference standards, as described for Fig. 2; E, concentrated fraction E (see below) from the gel-filtration experiment showing the presence of ILO and the sphingomyelinase; F, concentrated fraction F (see below) from the gel-filtration experiment showing the presence of the sphingomyelinase and the absence of ILO; G, conventional PAGE of the concentrated fraction F. (2) Gel filtration of the CCS. Symbols: ●, hemolytic activity without cysteine activation treatment; ▲, hemolytic activity after activation with cysteine; ○, hemolytic activity with *R. equi* exosubstances present in the titration mixtures. Hemolytic fractions with the activity enhanced by cysteine and not by *R. equi* (30 to 50 [fraction E]) and those with the activity enhanced by *R. equi* and not by cysteine (52 to 62 [fraction F]) were concentrated separately.

SH-cytolysin by the same method, but they could not attribute any function to the 27-kDa molecule (or 24 kDa, as they described it; repeated determinations performed by us indicate that the molecular mass of this protein is indeed 27 kDa [26.92 ± 1.15]) and did not find any explanation for the copurification mechanism (18). These findings led us to think about the possible presence of free SH groups in the 27-kDa molecule that would react, forming disulfide bonds, with those of the thiolated gel or with the cysteine residue of the SH-cytolysin. Therefore, 2.5 mM DTT was added to the eluent in the second gel filtration step with Sephacryl S-200; in this case, the separation of the two proteins was achieved, and a single band of 61 kDa was obtained (Fig. 2).

The hemolytic activity of this purified fraction was suppressed by exposure to 10 mM $HgCl_2$ (37°C for 15 min), after which it was partially restored by treatment with 20 mM L-cysteine (37°C for 15 min). Irreversible inactivation followed exposure to cholesterol (20 μl/ml of the 10-mg/ml cholesterol solution in absolute ethanol; 37°C for 15 min). On the other hand, Western blot analysis revealed antigenic relationships between the *L. ivanovii* 61-kDa protein and other SH-cytolysins (LLO and SLO) (Fig. 4 and 5), as well as alveolysin (from *Bacillus alvei*) and perfringolysin O (from *C. perfringens*) (not shown). These results demonstrated that this protein is a SH-cytolysin, since they fulfilled all the reported characteristics of this kind of toxin (1, 4, 10, 32). As mentioned above, the *L. ivanovii* SH-cytolysin was partially purified and characterized by other authors (18), being described as a 58-kDa protein with an optimal pH for hemolytic activity of 5.5, the same reported for the toxin produced by *L. monocytogenes* (10); therefore, it was identified as LLO (18). However, several things suggested that this SH-cytolysin is related to but different from LLO: (i) no hybridization of *L. ivanovii* DNA was detected in a probe using an internal *Hin*dIII 0.65-kilobase fragment of the LLO cloned gene (21); (ii) the optimal pH for activity of the *L. ivanovii* SH-cytolysin, determined with the purified toxin obtained in our experiment (diluted to 10^3 CHU in reduced state), was found to be 6.5 instead of 5.5 (activity was expressed between pH 4 and 8.5), in contrast with the optimal pH and the pH range of activity of LLO (undetectable activity at pH 7) (10). Moreover, in spite of the fact that ILO and LLO present apparently the same mobility in SDS-PAGE with a 15% acrylamide separation gel, slight differences in the migration of the two cytolysins could be observed when the acrylamide concentration was reduced to 7.5% (LLO seems to be slightly lower [0.5 to 1 kDa] in molecular mass [Fig. 6]). Taking into consideration these facts and in accordance with the nomenclature adopted for SH-cytolysins (1, 4, 32), we have termed the toxin produced by *L. ivanovii* ILO.

SH-cytolysin-selective sequestration by cholesterol from culture supernatants as a method for demonstrating the presence of other cytolysins. After performing the SH-cytolysin cholesterol-sequestration treatment, hemolytic activity (initially 16,384 CHU after activation by cysteine) could not be detected in the *L. monocytogenes* CCS. However, in the CCS of *L. ivanovii* (131,072 CHU after activation), a weak hemolytic activity remained (8 to 16 CHU), in spite of the

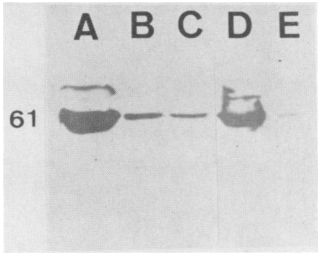

FIG. 4. SDS-PAGE Western blot with LLO antiserum. Lanes A, B, and C correspond to samples A, B, and C of Fig. 2. Lanes D and E correspond to samples C and B of Fig. 3, part 1. Note that anti-LLO does recognize the 27-kDa sphingomyelinase.

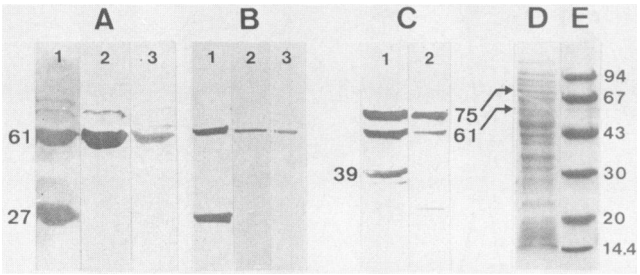

FIG. 5. (A through C) SDS-PAGE immunoblotting with ILO (lanes 1), LLO (lanes 2), and SLO (lanes 3) antisera. (A) *L. ivanovii* CCS (sample A of Fig. 2). (B) Hemolytic fractions from the gel filtration performed after thiol-disulfide exchange affinity chromatography of the CCS (sample C of Fig. 2). The 61-kDa ILO protein was recognized by anti-ILO, anti-LLO, and anti-SLO; the 27-kDa sphingomyelinase reacted only with anti-ILO. (C) *L. ivanovii* whole-cell extract showing the presence of a predominant 75-kDa anti-ILO and anti-LLO cross-reacting antigen, together with the 61-kDa ILO band; the 27-kDa sphingomyelinase was not recognized by anti-ILO, a 39-kDa antigen being detected instead. (D and E) SDS-PAGE of the *L. ivanovii* whole-cell extract and molecular mass standards, respectively. Positions of the 75- and 61-kDa antigens are indicated. Numbers correspond to known molecular masses in kilodaltons.

fact that the cholesterol treatment was repeated. This activity, which was expressed only after at least 30 min of incubation (unlike SH-cytolysin activity, which was expressed almost immediately), was enhanced (reaching 256 CHU) when *R. equi* exosubstances were present in hemolytic activity titrations and was not significantly affected by exposure to 10 mM $HgCl_2$. This result reflected the presence of a CAMP (*R. equi*)-reactive cytolytic factor different from ILO in the *L. ivanovii* CCS. SDS-PAGE and Western blot analysis demonstrated the absence of the SH-cytolysin band in the *L. ivanovii* (and *L. monocytogenes*) CCS after cholesterol treatment. These proteins were present in the C/Cly. In contrast, the 27-kDa specific second major protein of *L. ivanovii* remained unaffected in the CCS after cholesterol treatment and was not observed in the C/Cly (Fig. 2–4). This finding suggested that the 27-kDa protein might be the *L. ivanovii* cytolytic factor involved in the CAMP phenomenon with *R. equi*.

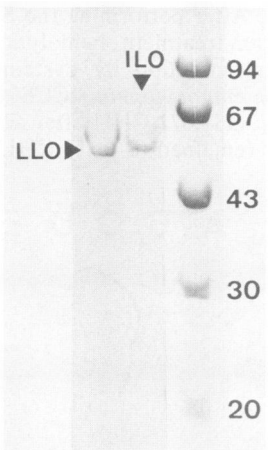

FIG. 6. SDS-PAGE (7.5% acrylamide) of ILO and LLO-C/Cly complexes obtained from *L. ivanovii* and *L. monocytogenes* CCS. These C/Cly were used for the production of ILO and LLO antisera. Note that ILO and LLO show a slightly different migration.

Isolation, purification, and characterization of the *L. ivanovii* CAMP (*R. equi*) factor (sphingomyelinase C). The presence of a second cytolysin different from ILO was also suggested by a gel filtration experiment performed with the *L. ivanovii* CCS (not previously activated by cysteine). Two kinds of hemolytic fractions were obtained: ones with high activity that was markedly enhanced by cysteine treatment but not by *R. equi* exosubstances, and others, corresponding to lower molecular masses, with a weak hemolytic activity that was clearly enhanced by *R. equi* exosubstances but not by cysteine treatment (Fig. 3). When concentrated separately, SDS-PAGE analysis of the former fractions revealed the presence of the 61-kDa ILO and the 27-kDa protein; in contrast, the latter fractions appeared to be composed predominantly of the 27-kDa protein, the 61-kDa ILO not being detected (Fig. 3). These results, which agreed with our previous observations, led us to try the purification and characterization of the 27-kDa protein.

Since the 27-kDa molecules did not attach to cholesterol, a first step in the purification procedure consisted of ILO extraction by the cholesterol-binding method. Afterwards, the ILO-devoid *L. ivanovii* CCS was gel filtered. The hemolytic fractions obtained, which showed enhancement of the activity with *R. equi* exosubstances, consisted mainly of the 27-kDa protein (Fig. 2). On the basis of the fact that this protein copurified with ILO by thiol-disulfide exchange affinity chromatography, presumably because of the presence of SH groups in the molecule, we chromatographed the hemolytic fractions in a thiopropyl-Sepharose 6B column. The 27-kDa protein was retained in the column and subsequently eluted with 10 mM DTT, this fact demonstrating that it contains reactive SH groups (Fig. 2). The observation that hemolytic fractions showing almost exclusively a 27-kDa protein by SDS-PAGE displayed, in contrast, two evident closely migrating bands by conventional (nondenaturing) PAGE (Fig. 3), a phenomenon that has been related to differences in the net charge of the same molecular species depending on the oxidized or reduced state of the sulfhydryl groups (32), is consistent with this result. This step led to the obtaining of a hemolytic fraction composed predominantly of the 27-kDa molecules, contaminated with a few other proteins (weak bands corresponding to molecular masses of over 40 kDa). The final purification step (gel filtration on Sephadex G-75) allowed us to obtain a hemolytic fraction showing a single band of 27 kDa (Fig. 2), which reproduced the CAMP synergistic hemolysis phenomenon when confronted with the *R. equi* CCS in a double diffusion assay on sheep erythrocyte agar (Fig. 1).

The cholesterol-independent character and the absence of antigenic relatedness with LLO (Fig. 4 and 5) indicated that the *L. ivanovii* 27-kDa protein responsible for the incomplete hemolysis produced by this species on blood agar and for the CAMP reaction with *R. equi* is a cytolysin functionally and structurally unrelated to SH-toxins. Taking into consideration previous reports indicating the presence of phospholipase C activity in *L. ivanovii* culture supernatants (E. Menciková, Abstr. 10th Int. Symp. Listeriosis, Pécs, Hungary, p. 46, 1988), we investigated the effect of this cytolytic factor on different phospholipids (sphingomyelin phosphatidylcholine, and phosphatidylglycerol). Our results demonstrated that it is a phospholipase with substrate specificity for sphingomyelin (0.4 CHU, measured without *R. equi* interaction, hydrolyzed to an undetectable level of 0.15 mg of sphingomyelin [~0.2 μmol] in 60 min at 37°C), and with C-type activity (reaction products migrated with the ceramide [Sigma] reference standard). With the experimental

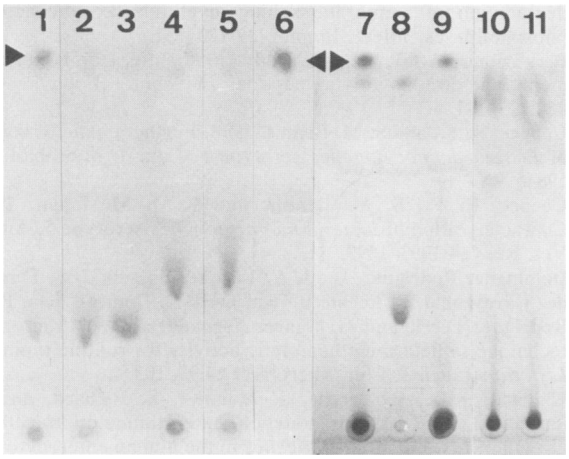

FIG. 7. Phospholipase (sphingomyelinase C) activity of the *L. ivanovii* 27-kDa cytolysin involved in the CAMP phenomenon with *R. equi* as determined by thin-layer chromatography. Lanes: 1 to 3, 0.4 mg sphingomyelin in the reaction mixtures; 7 to 11, 0.15 mg of sphingomyelin; 4 and 5, 0.5 and 0.3 mg of phosphatidylcholine, respectively. Lanes 1 and 7 show that 0.4 CHU of the *L. ivanovii* 27-kDa cytolysin split sphingomyelin (0.15 mg, totally [lane 7]; 0.4 mg, partially [lane 1]; substrate reference spots are shown in lanes 3 and 8), leading to the formation of hydrolysis products which migrated with the ceramide standard (lane 6) (arrowheads), this result indicating phospholipase C activity. Lane 2 shows the absence of sphingomyelinase activity in the *L. ivanovii* whole-cell extract. Lanes 4 and 5 show the absence of lecithinase activity in the 27-kDa cytolysin. Lanes 9 to 11 correspond to reaction mixtures with the cytolysin treated previously with 5 mM *N*-ethylmaleimide, iodoacetamide, and $HgCl_2$, respectively: sphingomyelinase activity was not affected (note absence of sphingomyelin spots). The solvent front is at the top of the figure.

conditions used, lecithinase activity was not detected (Fig. 7). Our results are in accordance with those recently reported by other authors which demonstrated a pronounced sphingomyelinase activity in *L. ivanovii* culture supernatants (18); these authors also described a weak and slow lecithinase activity (detectable only after 16 h of incubation) (18), an observation that is in accordance with the *L. ivanovii* long-term positive reaction on egg yolk agar (observed after 14 days of incubation [25] and not after 3 days [6]). The presence of a 27-kDa sphingomyelinase C in *L. ivanovii* agrees with the reported results on the molecular characterization of other bacterial phospholipases (22). In this sense, the *S. aureus* sphingomyelinase C (β-toxin) and the *B. cereus* sphingomyelinase have about the same molecular masses (12, 22, 34). On the other hand, the *C. perfringens* α-toxin, displaying both sphingomyelinase and lecithinase C activity, has a mass of about 43 kDa (33), nearly the sum of the reported sizes of the native phospholipase C and sphingomyelinase of the phylogenetically very close *B. cereus* (12).

The *L. ivanovii* sphingomyelinase is hemolytic, this fact indicating that it is able to interact with cell membrane sphingomyelin, resulting in membrane damage. As shown above, this damage is remarkably favored by the presence of *R. equi* exosubstances. Since the *R. equi* factor involved in this synergistic hemolysis is unknown, the mechanism of the cooperative action between the *L. ivanovii* sphingomyelinase and the *R. equi* CAMP factor remains to be determined. In this sense, it should be noted that *R. equi* produces a nonhemolytic phospholipase C and a cholesterol-oxidase that were found to be responsible for the synergistic hemolysis with *Corynebacterium ovis* (3, 20). These *R. equi* enzymes could be involved as well in the CAMP reaction with the *L. ivanovii* sphingomyelinase. This correlates with the fact that the nonhemolytic *C. ovis* sphingomyelinase D taking part in the synergistic hemolysis with *R. equi* also interacts with the *L. ivanovii* sphingomyelinase, inhibiting the partial hemolysis zone induced by it when previously incubated with erythrocytes (E. Menciková, 10th Int. Symp. Listeriosis). However, a nonenzymatic mechanism of the *R. equi* CAMP factor, like that of the CAMP protein of *Streptococcus agalactiae* or that of renalin from *Corynebacterium renale* (2), should not be discounted.

In order to determine the contribution of the SH groups detected in the *L. ivanovii* sphingomyelinase to the activity, reaction mixtures were prepared with the enzyme previously exposed (for 15 min at 37°C) to the SH-group reagents *N*-ethylmaleimide, iodoacetamide, and $HgCl_2$ (5 mM). The SH-blocking treatment did not impair the sphingomyelinase activity (Fig. 7), a result indicating that SH groups seem not to be essential for the function of this molecule. This suggests that the cysteine residues are not directly involved in the conformation of the functionally active sites of the sphingomyelinase (e.g., by the establishment of intramolecular disulfide bridges), being more likely present as a single cysteine in a region without functional significance. This point of view agrees with recently reported results, based on gene sequence data, showing that the alpha-toxin (phospholipase C) from *C. perfringens*, a species also producing an SH-cytolysin (perfringolysin O or theta-toxin) (36) and belonging to a genus closely related to *Listeria*, contains a unique cysteine residue (33). On the other hand, it has been demonstrated that the SH-cytolysins SLO (*Streptococcus pyogenes*), pneumolysin (*Streptococcus pneumoniae*), perfringolysin O, and LLO contain a unique cysteine residue located in a highly conserved undecapeptide at the carboxy-terminal region (17, 21, 36, 37), this fact allowing the assumption that this is the case for ILO, in accordance with the close structural and functional homologies of these toxins (1, 4, 10, 17, 21, 32, 36, 37). Therefore, taking into account the experimental evidence obtained, the copurification of the sphingomyelinase and ILO would most likely be due to the formation of sphingomyelinase-ILO dimers by the establishment of intermolecular disulfide bonds between their respective putative single cysteine residues. Molecular cloning and sequence determination studies of the 27-kDa *L. ivanovii* sphingomyelinase gene, which are in progress, will provide confirmation for these statements.

In conclusion, our results demonstrate that *L. ivanovii* secretes two cytolysins: a thiol-activated toxin (ILO) and a sphingomyelinase C. Taking into consideration the close relationship between hemolytic activity and pathogenicity in the genus *Listeria* (9, 16, 21, 28, 31), it can be speculated that ILO and the sphingomyelinase would play a crucial role in the virulence of *L. ivanovii*. In this sense, it was determined by a genetic approach that the SH-cytolysin produced by *L. monocytogenes* (LLO) seems to be the major virulence factor of this bacterium (9, 16, 21), promoting intracellular growth (8). On the other hand, phospholipases, such as the *C. perfringens* alpha-toxin, are also known to play a key role in the pathogenesis of infections (33). The exact contribution of ILO and the sphingomyelinase to the pathogenicity of *L. ivanovii*, a species which in spite of displaying a higher hemolytic activity (qualitatively and quantitatively) is less

virulent for mice than *L. monocytogenes* (29) and which seems to selectively affect pregnant ewes (6, 13, 29), is a question to be investigated.

Other results. Interestingly, anti-ILO recognized not only the 61-kDa ILO band but also that of the 27-kDa sphingomyelinase in *L. ivanovii* CCS and derived samples in Western blot analysis (Fig. 5). However, the sphingomyelinase appeared to be antigenically unrelated to SH-cytolysins, in accordance with the results obtained with anti-LLO immunoblots, which failed to recognize the 27-kDa protein (Fig. 4 and 5). These results are probably due to the presence of undetectable amounts of the sphingomyelinase in the C/Cly used for immunizations (carried indirectly by the cholesterol-bound ILO molecule by the same mechanism responsible for the copurification of these two proteins).

On the other hand, the anti-LLO Western blots of the *L. ivanovii* whole-cell extracts revealed the presence of two antigens: the 61-kDa ILO and a predominant band of 75 kDa. Traces of the latter were also detected by anti-LLO in the immunoblots of the *L. ivanovii* CCS, together with the 61-kDa ILO band. The same results were observed with anti-ILO (Fig. 4 and 5). These findings strongly suggest that the 75-kDa antigen is a precursor form of ILO which would be cleaved during the exportation process and/or after secretion, resulting in the 61-kDa secreted form of the toxin. This suggestion correlates with the fact that many excreted bacterial proteins are synthesized as precursors of higher molecular mass, with an amino-terminal signal peptide that is separated from the native protein during excretion (24). In this sense, it should also be noted that postsecretion proteolytic cleavage in SH-cytolysins (e.g., SLO) has been described (17). Furthermore, the 27-kDa sphingomyelinase was not detected by anti-ILO in the *L. ivanovii* cell extract immunoblots, a 39-kDa reactive band that was not observed in the CCS immunoblots being recognized instead (Fig. 5). These results suggest that the 39-kDa antigen might be an intracellular precursor of the 27-kDa extracellular form of the *L. ivanovii* sphingomyelinase that is inactive, in accordance with the lack of sphingomyelinase activity in the whole-cell extract (Fig. 7). Further studies on these aspects are in progress.

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LITERATURE CITED

1. **Bernheimer, A. W.** 1976. Sulfhydryl activated toxins, p. 86–97. *In* A. W. Bernheimer (ed.), Mechanisms in bacterial toxinology. John Wiley & Sons, Inc., New York.
2. **Bernheimer, A. W., and L. S. Avigad.** 1982. Mechanisms of hemolysis by renalin, a CAMP-like protein from *Corynebacterium renale*. Infect. Immun. **36**:1253–1256.
3. **Bernheimer, A. W., R. Linder, and L. S. Avigad.** 1980. Stepwise degradation of membrane sphingomyelin by corynebacterial phospholipases. Infect. Immun. **29**:123–131.
4. **Bernheimer, A. W., and B. Rudy.** 1986. Interactions between membranes and cytolytic peptides. Biochim. Biophys. Acta **864**:123–141.
5. **Cooper, R. F., and S. M. Dennis.** 1978. Further characterization of *Listeria monocytogenes* serotype 5. Can. J. Microbiol. **24**: 598–599.
6. **Cooper, R. F., S. M. Dennis, and K. J. McMahon.** 1973. Characterization of *Listeria monocytogenes* serotype 5. Am. J. Vet. Res. **34**:1093–1099.
7. **Dominguez Rodriguez, L., J. A. Vázquez-Boland, J. F. Fernández Garayzábal, P. Echalecu Tranchant, E. Gómez-Lucia, E. F. Rodriguez Ferri, and G. Suárez Fernández.** 1986. Microplate technique to determine hemolytic activity for routine typing of *Listeria* strains. J. Clin. Microbiol. **24**:99–103.
8. **Gaillard, J.-L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55**:2822–2829.
9. **Gaillard, J.-L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52**:50–55.
10. **Geoffroy, C., and J. E. Alouf.** 1984. Antigenic relationships between sulfhydryl-activated toxins, p. 241–243. *In* J. E. Alouf, F. J. Fehrenbach, J. H. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, Inc. (London) Ltd., London.
11. **Geoffroy, C., J.-L. Gaillard, J. E. Alouf, and P. Berche.** 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. **55**:1641–1646.
12. **Gilmore, M. S., A. L. Cruz-Rodz, M. Leimester-Wächter, J. Kreft, and W. Goebel.** 1989. A *Bacillus cereus* cytolytic determinant, Cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. J. Bacteriol. **171**:744–753.
13. **Ivanov, I.** 1962. Untersuchungen über die Listeriose der Schafe in Bulgarien. Monatsh. Veterinaermed. **17**:729–736.
14. **Ivanov, I.** 1975. Establishment of non-motile strains of *Listeria monocytogenes* type 5, p. 18–26. *In* M. Woodbine (ed.), Problems of listeriosis. Leicester University Press, Leicester, United Kingdom.
15. **Johnson, M. K., G. Geoffroy, and J. E. Alouf.** 1980. Binding of cholesterol by sulfhydryl-activated cytolysins. Infect. Immun. **27**:97–101.
16. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **169**:1291–1297.
17. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. Infect. Immun. **55**:3228–3232.
18. **Kreft, J., D. Funke, A. Haas, F. Lottspeich, and W. Goebel.** 1989. Production, purification and characterization of hemolysins from *Listeria ivanovii* and *Listeria monocytogenes* sv 4b. FEMS Microbiol. Lett. **57**:197–202.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Linder, R.** 1984. Alteration of mammalian membranes by the cooperative and antagonistic actions of bacterial proteins. Biochim. Biophys. Acta **779**:423–475.
21. **Mengaud, J., M.-F. Vicente, J. Chenevert, J. Moniz Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J.-C. Pérez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56**:766–772.
22. **Möllby, R., and M. Thelestam.** 1981. Bacterial phospholipases with special reference to staphylococcal β-hemolysin. *In* J. Jeljaszewicz (ed.), Staphylococci and staphylococcal infections. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Suppl. **10**:243–252.
23. **Parrisius, J., S. Bhakdi, M. Roth, J. Tranum-Jensen, W. Goebel,**

and H. P. R. Seeliger. 1986. Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. Infect. Immun. **51**:314–319.
24. **Randall, L. L., and S. J. S. Hardy.** 1984. Export of protein by bacteria. Microbiol. Rev. **48**:290–298.
25. **Rocourt, J., and B. Catimel.** 1985. Caractérisation biochimique des espèces du genre *Listeria*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **260**:221–231.
26. **Rocourt, J., F. Grimont, P. A. D. Grimont, and H. P. R. Seeliger.** 1982. DNA relatedness among serovars of *Listeria monocytogenes sensu Iato*. Curr. Microbiol. **7**:383–388.
27. **Rocourt, J., A. Schrettenbrunner, and H. P. R. Seeliger.** 1983. Différentiation biochimique des groupes génomiques de *Listeria monocytogenes* (sensu lato). Ann. Microbiol. (Paris) **134A**: 359–364.
28. **Seeliger, H. P. R., and D. Jones.** 1986. Genus *Listeria* Pirie 1940, 383^{AL}, p. 1235–1245. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt, Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
29. **Seeliger, H. P. R., J. Rocourt, A. Schrettenbrunner, P. A. D. Grimont, and D. Jones.** 1984. *Listeria ivanovii* sp. nov. Int. J. Syst. Bacteriol. **34**:336–337.
30. **Seeliger, H. P. R., A. Scherettenbrunner, G. Pongratz, and H. Hof.** 1982. Zur Sonderstellung stark hämolysierender Stäme der Gattung *Listeria*. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A **252**:176–190.
31. **Skalka, B., J. Smola, and K. Elischerová.** 1982. Routine test for in vitro differentiation of pathogenic and apathogenic *Listeria* strains. J. Clin. Microbiol. **15**:503–507.
32. **Smyth, J., and J. L. Duncan.** 1978. Thiol-activated (oxygen labile) cytolysins, p. 129–183. *In* J. Jeljaszewicz and T Wadström (ed.), Bacterial toxins and cell membranes. Academic Press, Inc. (London), Ltd., London.
33. **Titball, R. W., S. E. C. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson, and D. C. Kelly.** 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun. **57**:367–376.
34. **Tomita, M., R. Taguchi, and H. Ikezawa.** 1982. Molecular properties and kinetic studies on sphingomyelinase of *Bacillus cereus*. Biochim. Biophys. Acta **704**:90–99.
35. **Towbin, H., J. S. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
36. **Tweten, R. K.** 1988. Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with genes for streptolysin O and pneumolysin. Infect. Immun. **56**:3235–3240.
37. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55**:1184–1189.