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Production of Thiol-dependent Haemolysins by *Listeria monocytogenes* and Related Species

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Twenty-six strains belonging to the five main species of the genus *Listeria* were examined for production of thiol-dependent exotoxins. All strains of *L. monocytogenes* cultured in charcoal-treated broth secreted a haemolytic factor at a level ranging from 200 to 800 haemolytic units (HU) ml^{-1}, except for the strain EGD (1500 HU ml^{-1}) and the type strain CIP 82110T (10 HU ml^{-1}). The haemolytic activity reached a maximum level by 8–10 h and then rapidly declined as soon as bacterial exponential growth ceased. The titres of haemolytic activity were markedly reduced when bacteria were grown in charcoal-untreated broth. The haemolytic factor produced by *L. monocytogenes* strains was characterized as listeriolysin O (M_r about 60000), a member of the group of thiol-dependent exotoxins. Strains of *Listeria ivanovii* also produced high levels of thiol-dependent exotoxin (about 2500 HU ml^{-1}), in both charcoal-treated and untreated broth. Small amounts of haemolytic factor (about 9–30 HU ml^{-1}) were also produced by *Listeria seeligeri* in charcoal-treated broth. The haemolysin produced by *L. seeligeri* was identified for the first time as a thiol-dependent exotoxin of M_r about 60000, antigenically related to listeriolysin O. As expected, we failed to detect thiol-dependent exotoxin in the two nonhaemolytic species, *Listeria innocua* and *Listeria welshimeri*.

INTRODUCTION

Recent experimental evidence suggests that the production of an extracellular haemolysin constitutes a major mechanism promoting intracellular growth of *Listeria monocytogenes* (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Portnoy *et al.*, 1988), in both nonprofessional and professional phagocytes (Gaillard *et al.*, 1987, Kuhn *et al.*, 1988). The haemolytic factor from the virulent strain EGD has been purified and characterized as a 60 kDa thiol-activated exotoxin, designated listeriolysin O (Geoffroy *et al.*, 1987), antigenically related to streptolysin O (SLO) and fully active at pH 5·5. Its structural gene has been cloned by Vicente *et al.* (1985) and recently sequenced by Mengaud *et al.* (1988). Listeriolysin O is a polypeptide of 529 amino acids (Mengaud *et al.*, 1988), sharing strong homologies with SLO and pneumolysin (Kehoe & Timmis, 1984; Mengaud *et al.*, 1987; Walker *et al.*, 1987). However, if this haemolytic factor is really of crucial importance for virulence, all virulent strains of *L. monocytogenes* should produce it. This prerequisite is apparently challenged by the finding of Parrisius *et al.* (1986) that only 2 out of 26 strains of *L. monocytogenes* secreted SLO-like haemolysin of M_r 55000–60000, termed α-listeriolysin. This was found by using a rabbit immune serum raised against partially purified SLO-like haemolysin obtained from *Listeria ivanovii* culture supernate. Consequently, it appears important to determine whether or not *L. monocytogenes* strains secrete a thiol-activated

Abbreviations: HU, haemolytic unit; SLO, streptolysin O; SRBC, sheep red blood cell.

exotoxin. On the other hand, although *L. ivanovii* produces an SLO-like haemolysin (Parrisius *et al.*, 1986), the nature of the haemolytic factor secreted by *Listeria seeligeri* remains unknown. The aim of this work was therefore to study the presence of thiol-activated exotoxins in the five main species of the genus *Listeria*.

METHODS

Bacterial strains and culture media. The 26 strains of *Listeria* used in this work are listed in Table 1. These strains originated from culture collections, except for five clinical isolates (Hôpital Necker, Paris, France) obtained from blood or spinal fluid and subcultured only once or twice before storage at −80 °C. Bacteria were grown in brain heart infusion broth (Diagnostics Pasteur), harvested in exponential-phase growth (about 10^8 bacteria ml^{-1}), and stored in 1 ml portions at −80 °C until required. Viable bacteria were determined by plating 0·1 ml of doubling dilutions on tryptic soy agar (Diagnostics Pasteur). Colony forming units (c.f.u.) were counted after 48 h of incubation at 37 °C.

Titration of haemolytic activity during bacterial growth. For the titration of haemolytic activity in culture supernates, bacteria were grown either in proteose-peptone broth [proteose-peptone no. 3 (Difco), 20 g; yeast extract (Difco), 5 g; $Na_2HPO_4.12H_2O$, 8·3 g; KH_2PO_4, 0·7 g; quartz-distilled water to 1000 ml] or in charcoal-treated broth prepared as follows. A tenfold concentrated proteose-peptone broth (as described above) was supplemented with charcoal (Vegetable Activated Charcoal, Prolabo) at 0·2% final concentration and the medium was stirred (100 r.p.m.) for 1 h at room temperature (Incubator Shaker, model G 25, New Brunswick Scientific). The charcoal-treated concentrate was further diluted in quartz-distilled water (1 in 10), adjusted to pH 7·5 and autoclaved at 115 °C for 20 min. Sterile glucose to a final concentration of 1% was added before inoculation. The amount of iron in the medium was assessed at 6–8 μM by atomic absorption spectroscopy (Perkin-Elmer 403 apparatus). Preliminary assays showed that charcoal-treated medium gave the same results as the Chelex-treated medium previously described (Geoffroy *et al.*, 1987), and it was used for convenience.

Bacteria were grown overnight in proteose-peptone broth or charcoal-treated broth, then 2·5 ml volumes of the cultures were inoculated into 100 ml of the same medium and incubated at 37 °C without shaking. Bacterial growth was measured by optical density at 600 nm (OD_{600}) in a Beckman M25 spectrophotometer, after brief centrifugation (1 min at 485 ***g***) of culture samples to remove charcoal particles. Samples (1 ml) of cultures were then centrifuged at 10000 ***g*** for 10 min at 4 °C, and the supernatant fluid was assayed for haemolytic activity by the method of Alouf *et al.* (1965). Briefly, 0·5 ml of 2·25% sheep red blood cell (SRBC) suspension (6×10^8 ml^{-1}) in phosphate buffered saline (0·075 M-NaH_2PO_4, 0·075 M-Na_2HPO_4, 0·075 M-NaCl; PBS), pH 6·0, were added to 1·0 ml volumes of varying dilutions of supernates supplemented with cysteine (20 mM final concentration) in the same buffer supplemented with 0·1 % bovine albumin (Sigma). After 45 min at 37 °C the tubes were centrifuged at 1000 ***g*** for 30 s at 4 °C and haemoglobin in the supernate was measured by absorbance at 541 nm in a spectrophotometer (Beckman M25). One haemolytic unit (HU) is defined as the amount of toxin required to release half the haemoglobin (50% lysis) of the erythrocytes. It is estimated graphically by plotting percentage lysis versus toxin volume on a log-probit graph (Alouf *et al.*, 1965).

SDS-polyacrylamide gel electrophoresis and immunoblotting. Samples (1 ml) of culture supernates from strains of *L. monocytogenes* and *L. ivanovii* grown in charcoal-treated broth (containing 200–500 HU), supplemented with cysteine (20 mM), were incubated for 30 min at 37 °C with 0·5 ml of SRBC suspension (6×10^8 ml^{-1}) in PBS pH 6·0. For strain CIP 82110^T and strains of *L. seeligeri*, the same procedure was applied after ultrafiltration at 4 °C in an Amicon cell apparatus, using an Amicon PM30 membrane. After complete lysis, the ghosts were sedimented at 12000 ***g*** for 15 min at 4 °C, and the supernate discarded. Cell pellets were then washed twice in PBS pH 6·0, and dissolved in 50 μl of 10% SDS in water. Membrane-associated proteins (about 100 μg per well) were then analysed by SDS-PAGE, as described by Laemmli (1970). Samples of 50 μl were added to 50 μl of 2% SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0·002% bromophenol blue in 0·1 M-Tris/HCl buffer (pH 6·8). This mixture was boiled for 90 s and electrophoresis was performed in a linear gradient of 7·5–25% (w/v) acrylamide at 5 mA for 15 h. The proteins were electrophoretically transferred to nitrocellulose sheets (BA 85, Schleicher & Schüll) as described by Kyhse-Andersen (1984). The sheets were incubated for 1 h at room temperature with shaking in 50 mM-Tris, 150 mM-NaCl solution (pH 8·0) containing 5% (w/v) dried skim milk (Régilait) prior to 1 h incubation in anti-listeriolysin O serum diluted (1 in 20) in the same buffer. The sheets were then washed eight times in the same milk buffer before addition of 20 ml milk buffer containing 1 μCi (37 kBq) of ^{125}I-protein-A kindly prepared by R. Predeleanu (Institut Pasteur, Paris). Shaking was continued for an additional hour and then the filters were washed six times in the same buffer supplemented with 0·1% Triton X-100. The filters were dried at 80 °C and then autoradiographed using Kodak X-O-Mat (SO-282) film (Eastman Kodak).

Anti-listeriolysin O serum and anti-SLO sera. Female albino rabbits (3 kg), supplied by Iffa-Credo, were immunized by injecting subcutaneously 75 μg of highly purified listeriolysin O prepared as previously described (Geoffroy *et al.*, 1987), emulsified in complete Freund's adjuvant, on days 0, 7 and 14, and in incomplete Freund's

Table 1. *Haemolysin production by Listeria strains*

Strain*	Serovar	Haemolytic phenotype on blood agar†	Haemolytic activity in untreated broth‡	Haemolytic activity in charcoal-treated broth‡
L. monocytogenes				
EGD	1/2a	+++	220	1500
N 3636	1/2a	++	30	400
N 62262	1/2a	++	25	300
N 8018	4b	++	20	200
N 74217	1/2b	++	25	250
N 2661	4b	++	30	250
L 028	1/2c	++	30	800
SLCC 4324	4b	++	20	400
SLCC 5156	1/2a	++	25	220
SLCC 3551	4b	++	25	350
SLCC 5132	1/2a	++	30	350
CIP 82110^T	1/2a	−	0	10
L. ivanovii				
SLCC 4121	5	+++	2500	2500
CIP 7842^T	5	+++	2750	2500
L. seeligeri				
CIP 100100^T	1/2	+	0	22
SLCC 3503	1/2	+	0	30
SLCC 4152	1/2	+	0	25
SLCC 3990	1/2	+	0	9
SLCC 3502	1/2	+	0	10
L. innocua				
CIP 8011^T	6	−	0	0
SLCC 6462	ND	−	0	0
SLCC 4202	ND	−	0	0
SLCC 4213	ND	−	0	0
SLCC 6466	ND	−	0	0
L. welshimeri				
SLCC 5334^T	6	−	0	0
SLCC 5871	6	−	0	0

ND, Not determined.

* Source of strains: EGD, Trudeau Institute, NY, USA; N, Hôpital Necker, Paris, France; L 028, Ramon y Cajal Collection, Madrid, Spain; SLCC, Special *Listeria* Culture Collection, Würzburg, Federal Republic of Germany; CIP, Collection de l'Institut Pasteur, Paris, France.

† Haemolytic phenotypes on 5% horse blood-tryptic soy agar: hyperhaemolytic (+++), haemolytic (++), weakly haemolytic (+), nonhaemolytic (−).

‡ The haemolytic activity (HU ml^{-1}) was estimated at the peak production. Four to five determinations were made. The results of a representative experiment are shown here.

adjuvant on day 21. Blood was collected 2 weeks after the last injection. The anti-SLO serum (no. 525) was a hyperimmune serum previously described by Alouf *et al.* (1965).

Inhibition of haemolytic activity. Culture supernates were treated according to several procedures prior to haemolysin titration. Samples (1 ml) of the supernates diluted in PBS, pH 6·0, to contain 30 HU were incubated for 30 min: (i) at 37 °C with 10 µl of various dilutions of cholesterol (1 mg ml^{-1}) in ethanol; (ii) at 22 °C with the thiol-group reagent $HgCl_2$ (Sigma), at a final concentration of 10 mM in PBS, pH 6·0; (iii) at 37 °C with hyperimmune horse anti-SLO serum (no. 525) or nonimmune horse serum (Gibco) diluted to 1 in 10. The haemolytic activity was then measured as described above.

RESULTS AND DISCUSSION

The 12 strains of *L. monocytogenes* tested produced haemolytic factor when cultured in charcoal-treated broth (Table 1). The peak of haemolytic titres in the culture supernates ranged from 200 to 800 HU ml^{-1}, except for strain EGD (about 1500 HU ml^{-1}), and the type strain ATCC 82110^T (about 10 HU ml^{-1}). These levels corresponded to the haemolytic phenotypes on

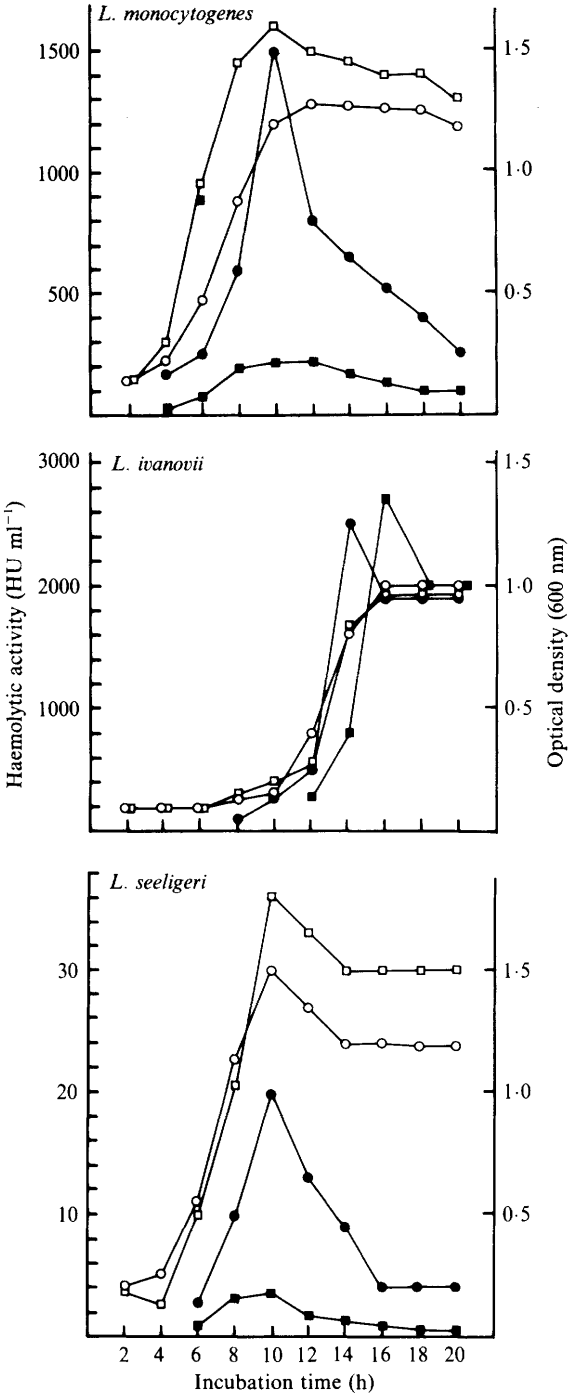

Fig. 1. Kinetics of haemolysin production *in vitro*. *L. monocytogenes* (strain EGD), *L. ivanovii* (strain SLCC 7842^T) and *L. seeligeri* (strain CIP 100100^T) were cultured in proteose-peptone broth (□, ■) or in charcoal-treated broth (○, ●). Bacterial growth was followed by optical density at 600 nm (open symbols) and haemolytic activity was titrated in supernates (solid symbols). The kinetics of haemolysin production was similar for the other strains of *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*. The peak of haemolytic activity was reached by 8–10 h for *L. monocytogenes* and *L. seeligeri*, and by 14–16 h for *L. ivanovii*. Three to four determinations were made for each strain. The results of a representative experiment are shown here.

blood agar since strain EGD was hyperhaemolytic and the type strain ATCC 82110^T was nonhaemolytic, whereas the other strains were similarly haemolytic in this medium (Table 1). The haemolytic activity was strongly enhanced in charcoal-treated broth as compared with untreated broth (Table 1). The increase of haemolytic activity in charcoal-treated broth cannot be attributed to a difference in bacterial growth, since bacterial yield was better in untreated broth. Indeed, for all *L. monocytogenes* strains, the titres of viable bacteria after an 18 h incubation at 37 °C ranged from 5×10^7 c.f.u. ml^{-1} to 5×10^8 c.f.u. ml^{-1} in charcoal-treated medium and from 5×10^8 c.f.u. ml^{-1} to 1×10^9 c.f.u. ml^{-1} in untreated broth. By studying the kinetics of haemolysin production for *L. monocytogenes* EGD (Fig. 1), it was further established that the peak of haemolytic activity in culture supernates was reached by 8–10 h at the end of the exponential phase of bacterial growth. Then a sharp drop of the haemolytic activity was observed, declining to a significantly lower level after 20 h incubation.

L. ivanovii strains produced high levels of haemolytic activity when cultured in either charcoal-treated or untreated broth (Table 1). *L. ivanovii* (strain CIP 7842^T) behaved differently from *L. monocytogenes* with respect to the kinetics of haemolysin production (Fig. 1). The haemolytic activity was optimal at 14–16 h and then declined slowly at the end of the exponential phase of growth. For *L. seeligeri* strains, low levels of haemolytic activity (9–30 HU ml^{-1}) were detected in the culture supernates of bacteria grown in charcoal-treated broth (Table 1). These values correlated well with the weakly haemolytic phenotypes on blood agar (Table 1). As shown above for *L. monocytogenes*, the haemolytic activity for *L. seeligeri* (strain CIP 100100^T) reached a maximum titre by 8–10 h and then rapidly declined as soon as bacterial exponential growth ceased (Fig. 1). Finally, no haemolytic activity was detected in the supernates of five strains of *L. innocua* or two strains of *L. welshimeri* cultured in untreated or charcoal-treated broth (Table 1).

Since it is known that the EGD strain of *L. monocytogenes* produces a thiol-dependent exotoxin, listeriolysin O (Geoffroy *et al.*, 1987), supernates obtained from other strains of *L. monocytogenes*, and from those of *L. ivanovii* and *L. seeligeri*, were examined for the presence of thiol-dependent haemolysin. The haemolytic activity of all culture supernates was totally abolished by the following treatments: (i) incubation (30 min at 37 °C) with cholesterol (10 ng cholesterol HU^{-1}); (ii) incubation (30 min at 22 °C) with a thiol-group reagent, $HgCl_2$ (1 mM), with restoration of haemolytic activity by further incubation with 2 mM-cysteine; (iii) incubation (30 min at 37 °C) with anti-SLO (1 in 10). These results indicate that the haemolytic factors produded by *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* share the classical properties of thiol-dependent toxins (Alouf & Geoffroy, 1984). The production by *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* of an exotoxin antigenically related to listeriolysin O was confirmed by Western-blot analysis using a rabbit immune serum raised against highly purified listeriolysin O. It was shown that all strains of *L. monocytogenes*, including the type-strain CIP 82110^T, produced a 60 kDa protein recognized by this immune serum (Fig. 2*a*). This also holds true for *L. ivanovii* and *L. seeligeri* strains (Fig. 2*b*).

The production of a 60 kDa thiol-dependent toxin, listeriolysin O, by all strains of *L. monocytogenes* is at a variance with the results of Parrisius *et al.* (1986). These authors failed to demonstrate SLO-like haemolysin in most strains of *L. monocytogenes*, including four strains used here (SLCC 4324, SLCC 5156, SLCC 3551, SLCC 5132). Our results show the influence of culture conditions upon the level of toxin produced, as already reported for strain EGD (Geoffroy *et al.*, 1987), and this may explain this discrepancy. The low amount of iron may influence the expression of the gene(s) encoding for listeriolysin O, as suggested by previous observations (Cowart & Foster, 1981; Cowart & Foster, 1985; Sword, 1966). However, the actual reasons for the increase of haemolytic activity when bacteria are grown in charcoal-treated broth remain unknown. Our results are in full agreement with those reported by Mengaud *et al.* (1988), who showed, by using a DNA probe under stringent conditions, that the structural gene of listeriolysin O is present in all *L. monocytogenes* strains tested. The data presented here support the view that listeriolysin O is the major factor responsible for the haemolytic phenotype of colonies on blood agar, as is strongly suggested by the observation that mutants derived by transposon insertion in the structural gene of listeriolysin O appear

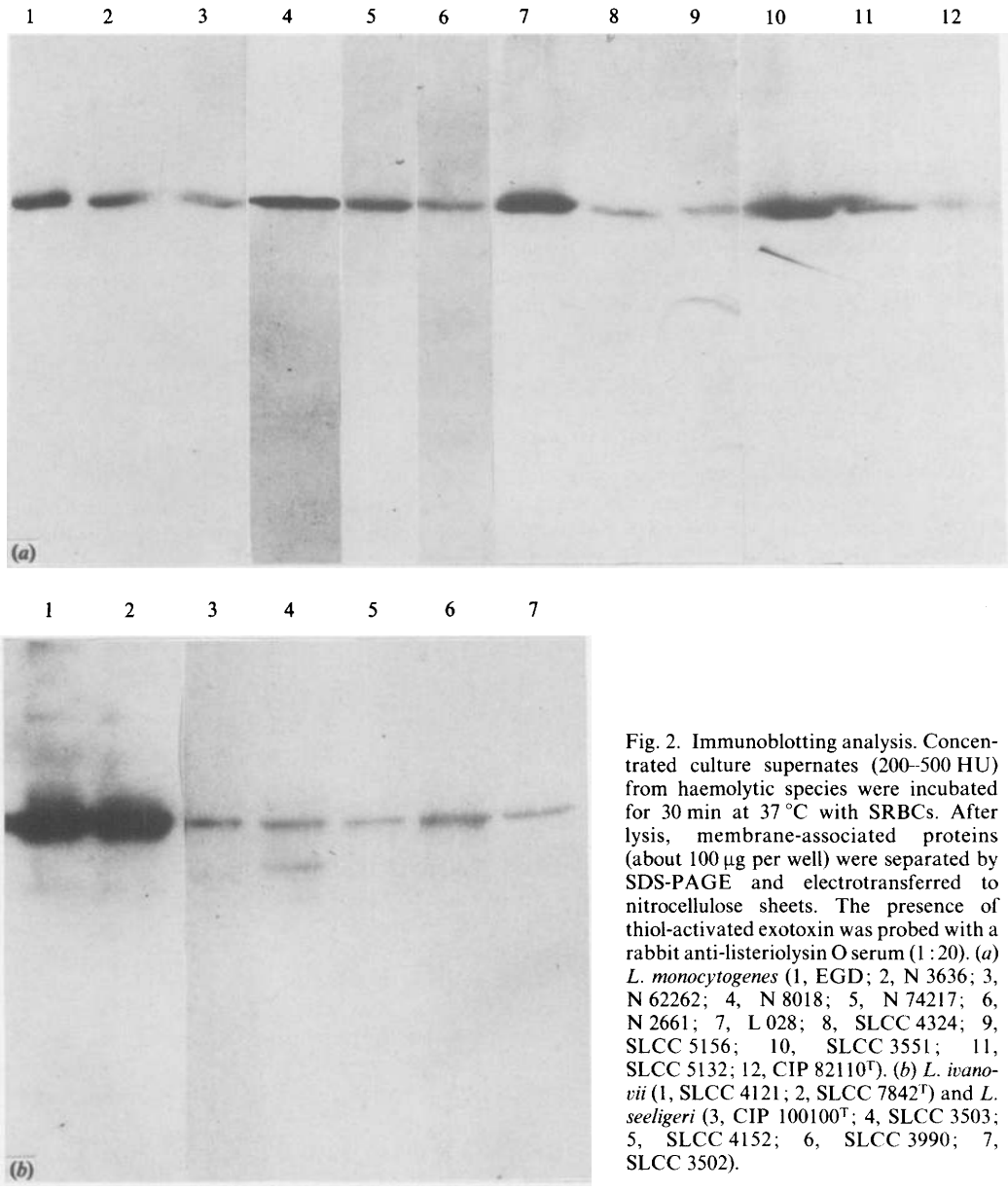

Fig. 2. Immunoblotting analysis. Concentrated culture supernates (200–500 HU) from haemolytic species were incubated for 30 min at 37 °C with SRBCs. After lysis, membrane-associated proteins (about 100 μg per well) were separated by SDS-PAGE and electrotransferred to nitrocellulose sheets. The presence of thiol-activated exotoxin was probed with a rabbit anti-listeriolysin O serum (1 : 20). (*a*) *L. monocytogenes* (1, EGD; 2, N 3636; 3, N 62262; 4, N 8018; 5, N 74217; 6, N 2661; 7, L 028; 8, SLCC 4324; 9, SLCC 5156; 10, SLCC 3551; 11, SLCC 5132; 12, CIP 82110^T). (*b*) *L. ivanovii* (1, SLCC 4121; 2, SLCC 7842^T) and *L. seeligeri* (3, CIP 100100^T; 4, SLCC 3503; 5, SLCC 4152; 6, SLCC 3990; 7, SLCC 3502).

nonhaemolytic on blood agar (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Portnoy *et al.*, 1988).

With respect to the other haemolytic species, we found that *L. ivanovii* produces high levels of a thiol-dependent exotoxin as previously described (Parrisius *et al.*, 1986). Strains of *L. seeligeri* produced low levels of haemolytic activity (9–30 HU ml^{-1}) in charcoal-treated broth. We were able to identify for the first time the nature of this haemolytic factor also as a thiol-activated exotoxin with an M_r of about 60000, antigenically related to listeriolysin O and SLO (Fig. 2). Using a DNA probe in stringent conditions, Mengaud *et al.* (1988) detected the listeriolysin O gene only in *L. monocytogenes*, indicating that there exists noticeable divergence between the genes encoding for thiol-dependent exotoxins in the three haemolytic species of the genus *Listeria*. On the basis of our results showing the existence of thiol-dependent toxins in *L. ivanovii* and *L. seeligeri*, we propose to designate these two toxins as listeriolysin O var. ivanovii and

listeriolysin O var. seeligeri, according to the nomenclature recommended by Bernheimer (1976).

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