Analysis of surface proteins of *Listeria* in relation to species, serovar and pathogenicity

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SDS extracts of whole bacteria, representing five species and 15 serovars of *Listeria*, were analysed by SDS-PAGE and by immunoblotting with serum directed against whole formalin-treated *L. monocytogenes*. Profiles of *L. monocytogenes* were very different from those of other species of *Listeria* (i.e. *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*). This low degree of similarity between species was found even in the case of common serovars. Within the species *L. monocytogenes*, protein patterns were characterized, on the one hand, by a high degree of homogeneity between all strains of the same serovar and, on the other hand, by large differences between serovars, especially between sv. 1/2 and 4b. Thus we have identified major, surface-located protein antigens, specific for *L. monocytogenes*, either common to all serovars (64 and 68 kDa) or characteristic of certain serovars: 98 kDa for sv. 1/2 and 3; 76 and 78 kDa for sv. 4b, 4d and 4e; and 80 and 100 kDa for sv. 4a and 4c. Moreover, some of these bands (68 and 98 kDa) might be related to virulence, since differences were noticed between the profiles of haemolytic *L. monocytogenes* sv. 1/2a differing only in their virulence for immunocompromised mice. All these results confirmed, for the first time, the classification of *Listeria* obtained previously by genomic studies. They should help in the identification of new virulence factors and the development of easier and more specific methods of detection and identification.

Introduction

Bacteria of the genus *Listeria* are widely distributed in nature (Welshimer, 1968) and may be isolated from the intestinal tract of healthy humans (Gray & Killinger, 1966). Among the five species of the genus *Listeria* (Seeliger & Jones, 1986), i.e. *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua* and *L. monocytogenes*, defined by the genomic studies of Rocourt *et al.* (1982), only *L. monocytogenes* is commonly pathogenic for humans.

The implication of food products in large outbreaks of listeriosis has shown the need for systematic control of the presence of *L. monocytogenes* in products at risk, such as dairy products. The detection of *Listeria*, currently achieved by enrichment procedures in selective broth, is time-consuming. Species identification requires further biochemical tests such as acid production from L-rhamnose, D-xylose and methyl α -D-mannoside, and haemolysin-production (Rocourt *et al.*, 1983). Clearly then, there is a need for new methods of rapid detection for *L. monocytogenes* in food products. Moreover, the differences in the level of virulence of *L. monocytogenes*

strains, as suggested by epidemiological studies (McLauchlin, 1990) and confirmed in an immunocompromised mouse model of infection (Tabouret *et al.*, 1991), imply that future methods of diagnosis should be specific for pathogenic *L. monocytogenes*. The most-suitable rapid detection systems are based on detection of marker genes using nucleic acid probes or on immunological detection (Pickup, 1991). More and more nucleic acid probes are being described for the detection of *L. monocytogenes* but they are not always suited to large-scale use. Development of specific antibodies seems more difficult (Siragusa & Johnson, 1990; Bhunia *et al.*, 1991) and available immunoassays remain specific for the genus *Listeria* but not for the species *L. monocytogenes* (Mattingly *et al.*, 1988; Kerr *et al.*, 1990).

The development of more-specific immunological tools requires a better knowledge of the antigenic structure of *Listeria*, particularly cell-surface proteins, which have been poorly characterized so far, but which could be involved in the virulence of the organism (Chakraborty & Goebel, 1988). In order to define specific antigens of *L. monocytogenes*, we have compared electrophoretic patterns of cell-surface components,

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firstly from different species of the genus *Listeria*, and secondly within the species *L. monocytogenes* for strains of different serovars with different levels of virulence.

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Methods

Organisms. Forty-six *Listeria* isolates were analysed (Table 1) including one *L. welshimeri* strain, two *L. seeligeri* strains, three *L. ivanovii* strains, three *L. innocua* strains and 37 *L. monocytogenes* strains. Within the species *L. monocytogenes*, 12 serovars were analysed: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7. Some isolates were from human outbreaks from France (Carbonnelle *et al.*, 1988), Canada

Table 1. List and characteristics of *Listeria* strains used

ATCC, American Type Culture Collection; CHUT, Centre Hospitalier Universitaire de Tours, France; CNL, Centre National des *Listeria*, Nantes, France; SLCC, Special *Listeria* Culture Collection, Würzburg, Germany.

Strain	Other designation	Species	Serovar	Virulence*	Haemolytic titre†	Origin
1	ATCC 19115	<i>L. monocytogenes</i>	4b	+	180	Human, Germany
5	CHUT 83008	<i>L. monocytogenes</i>	4b	+	300	Human, Angers, France
10	CHUT 83336	<i>L. monocytogenes</i>	4b	+	110	Human, Boston, USA
11	CHUT 83337	<i>L. monocytogenes</i>	4b	+	100	Human, Boston, USA
13	CHUT 83340	<i>L. monocytogenes</i>	4b	+	120	Human, Nova Scotia, Canada
14	CHUT 83364	<i>L. monocytogenes</i>	4b	+	220	Human, Boston, USA
15	CHUT 83416	<i>L. monocytogenes</i>	4b	+	220	Human, Nova Scotia, Canada
18	CHUT 850203	<i>L. monocytogenes</i>	4b	+	300	Human, Los Angeles, USA
76		<i>L. monocytogenes</i>	4b	+	100	Human, Switzerland
23	CNL 895788	<i>L. monocytogenes</i>	1/2a	+	490	Raw milk, France
33	ATCC 19111	<i>L. monocytogenes</i>	1/2a	+	45	Poultry, UK
34	CNL 895791	<i>L. monocytogenes</i>	1/2a	+	160	Cheese, Germany
35	CNL 895792	<i>L. monocytogenes</i>	1/2a	—	5	Cheese, France
36	CNL 895793	<i>L. monocytogenes</i>	1/2a	—	6	Cheese, France
37	CNL 861001	<i>L. monocytogenes</i>	1/2a	+	110	Cheese, France
39	CHUT 861175	<i>L. monocytogenes</i>	1/2a	+	100	Raw milk, France
41	CNL 895795	<i>L. monocytogenes</i>	1/2a	—	600	Cheese, France
47	CNL 895801	<i>L. monocytogenes</i>	1/2a	+	300	Raw milk, France
49	CNL 895803	<i>L. monocytogenes</i>	1/2a	—	5	Cheese, France
50	CNL 895804	<i>L. monocytogenes</i>	1/2a	—	6	Cheese, France
56	EGD	<i>L. monocytogenes</i>	1/2a	+	270	
60	CNL 895805	<i>L. monocytogenes</i>	1/2a	+	140	Sheep, France
61	CNL 895806	<i>L. monocytogenes</i>	1/2a	—	8	Dairy product, France
62	CNL 895807	<i>L. monocytogenes</i>	1/2a	—	500	Dairy product, France
64	CNL 895809	<i>L. monocytogenes</i>	1/2a	—	8	Dairy product, France
44	CNL 895798	<i>L. monocytogenes</i>	1/2b	+	90	Cheese, France
51	CNL 880203	<i>L. monocytogenes</i>	1/2b	+	90	Human, France
42	CNL 895796	<i>L. monocytogenes</i>	1/2c	+	110	Cheese, France
89	LO28	<i>L. monocytogenes</i>	1/2c	+	ND	
78		<i>L. monocytogenes</i>	4a	+	170	
79		<i>L. monocytogenes</i>	4c	+	100	
80		<i>L. monocytogenes</i>	4d	+	180	
81		<i>L. monocytogenes</i>	4e	+	280	
82		<i>L. monocytogenes</i>	7	+	150	
83		<i>L. monocytogenes</i>	3b	+	110	
83		<i>L. monocytogenes</i>	3c	+	300	
85		<i>L. monocytogenes</i>	3a	—	250	
2	CNL 895787	<i>L. seeligeri</i>	1/2b	—	2	Silage, France
31	SLCC 3954	<i>L. seeligeri</i>	1/2a	—	4	Soil, Germany
29	ATCC 33090	<i>L. innocua</i>	6a	—	0	Cow, Germany
67	CHUT 880919	<i>L. innocua</i>	ND	—	0	Cheese, France
86		<i>L. innocua</i>	4ab		0	
30	SLCC 5334	<i>L. welshimeri</i>	6a	—	0	Vegetation, USA
32	CHUT 860479	<i>L. ivanovii</i>	5	—	2000	Sheep
65	CHUT 880414	<i>L. ivanovii</i>	5	—	2500	Raw milk, France
75	ATCC 19119	<i>L. ivanovii</i>	5	—	1600	Sheep, Bulgaria

ND, Not determined.

* Determined on immunocompromised mice with carrageenan (Tabouret *et al.*, 1991)

† Assayed in culture supernatants of bacteria grown in proteose-peptone broth (Tabouret *et al.*, 1991)

(Schlech *et al.*, 1983), USA (Fleming *et al.*, 1985; Linnan *et al.*, 1988) and Switzerland (Bille & Glauser, 1988). Most of the *Listeria* strains ($n=34$) were from the collection of A. Audurier (Centre Hospitalier Universitaire de Tours, France). The other strains were kindly provided by P. Berche (Faculté de Médecine Necker, Paris, France) (strain 56), J. Bille (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland) (strain 76), E. P. Espaze (Centre Hospitalier Universitaire de Nantes, France) (strains 78 to 86) and J. Rocourt (Institut Pasteur, Paris, France) (strain 75).

Production of bacterial extracts. Bacterial isolates were grown in brain heart infusion (BHI) broth for 18 h at 37 °C with shaking (160 r.p.m.) on a G10 gyratory shaker (New Brunswick Scientific). Cells were collected by centrifugation (5000 g for 20 min) and washed three times in phosphate-buffered saline (PBS: NaCl, 8 g; KCl, 0.2 g; CaCl₂·2H₂O, 0.1 g; MgCl₂·H₂O, 0.1 g; KH₂PO₄, 0.2 g; Na₂HPO₄·12H₂O, 2.9 g; distilled water to 1000 ml). The resulting pellet was resuspended in PBS (2% of the initial volume) and supplemented with 1% (w/v) SDS. After 5 min shaking at room temperature, bacteria were centrifuged (15000 g for 10 min) and the supernatant was concentrated by ultrafiltration in microconcentrators (Centricon 30, Amicon). The protein concentration of the extracts was determined by using the bicinchoninic acid method (BCA) (Pierce) and bovine serum albumin as the standard protein. Extracts were stored at -20 °C.

Electrophoresis. Electrophoresis was performed in 7.5% (w/v, polyacrylamide) resolving gels and 4% stacking gels by the method of Laemmli (1970) using 0.75 mm thick slab gels and Tris/HCl buffer (pH 6.8). Samples were diluted in an equal volume of sample buffer (2%, w/v, SDS, 10%, v/v, glycerol, 5%, v/v, β -mercaptoethanol, 0.002% bromophenol blue, 0.02 M-Tris/HCl) and boiled for 3 min at 100 °C. After electrophoresis (120 min, 60 mA), gels were stained with silver nitrate (Heukeshoven & Dernick, 1985) or with Coomassie blue. For gels stained with silver nitrate, 3 μ g of proteins were applied to the wells compared to 80 μ g for gels stained with Coomassie blue or transferred to nitrocellulose. A molecular mass standard mixture (SDS-6H, Sigma), including carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β -galactosidase (116 kDa) and myosin (205 kDa), was used in each electrophoresis.

The action of proteinase K was studied to confirm the protein nature of revealed bands. Proteinase K (3 μ g; Boehringer) was added to 150 μ g of SDS-extracted proteins diluted (1/10, v/v) in Tris/HCl (0.125 M, pH 6.8). After incubation (2 h at 50 °C and overnight at 20 °C), treated extracts were analysed by SDS-PAGE as described above.

Comparison of protein patterns. The average similarity, S , between two strains was assessed by the Coincidence Index of Dice (Lamont *et al.*, 1986), whereby average similarity = (number of matching bands $\times$ 2)/total number of bands in both strains. This calculation was based on a visual comparison of bands above 40 kDa. It was performed only between patterns on the same gel because visual comparison did not allow the correction of gel-to-gel variations. All strains were run several times to confirm results. Strains were then clustered by the method of unweighted pair-group with arithmetic averages (UPGMA) (Johnson & Wichern, 1982).

Production of antisera. Antisera were produced in New Zealand White rabbits against killed, whole, washed *L. monocytogenes* sv. 1/2a (strain 60) or 4b (strain 15). Bacteria were grown in BHI broth for 18 h at 37 °C with shaking, washed three times by centrifugation (5000 g for 20 min) in saline (0.85% NaCl) and harvested in saline containing 0.3% formaldehyde (5×10^8 bacteria ml⁻¹) to kill the bacteria. Preimmune sera were collected and rabbits were immunized intravenously at 4 d intervals with the following dosés: 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml (Seeliger & Höhne, 1979).

Immunoblotting. After electrophoresis, proteins were transferred to nitrocellulose sheet (BA85, Schleicher and Schuell) using a semi-dry fast electroblotter (Biometra). The buffer was 48 mM-Tris, 39 mM-glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol, and a constant

current of 0.8 mA cm⁻² was applied for 1 h at 20 °C. The nitrocellulose was then saturated overnight with 0.5% gelatin and incubated for 90 min in rabbit antiserum diluted in PBS containing Tween 20 (0.1%) and gelatin (0.5%). After three washings in PBS containing 0.1% Tween 20 the nitrocellulose paper was incubated for 60 min with goat anti-rabbit immunoglobulin G linked to horseradish peroxidase (Nordic) diluted 1 in 1000 in PBS/Tween/gelatine. After two washes in PBS/Tween and two in PBS, immunoreactive bands were revealed by using 4-chloro-1-naphthol (HRP colour development reagent, Bio-Rad; 0.5 mg ml⁻¹) and H₂O₂ (0.03% v/v).

Biotinylation of cell-surface proteins of *Listeria*. To check that proteins extracted with SDS were cell-surface proteins, cells were labelled with biotin for a short time just before extraction with SDS. Briefly, after harvesting and washing the bacteria, the pellet was resuspended in PBS (pH 8.0, 4 °C). Cells were then treated with sulphy-NHS-biotin (Pierce; final concentration 0.5 mg ml⁻¹) (Lisanti *et al.*, 1988) for 2 min with gentle shaking. Bacteria were washed three times in PBS (pH 7.4). Extraction with SDS was performed as described above and extracts were analysed by SDS-PAGE and Western blotting. Detection of biotinylated protein on nitrocellulose membrane was performed, after saturation, using peroxidase-conjugated streptavidin (Jackson) diluted (1 in 1500) in PBS/Tween/gelatine (90 min). Reactive bands were revealed as described above with 4-chloro-1-naphthol and H₂O₂.

Results

General features of SDS extracts and PAGE patterns

SDS-PAGE of SDS extracts showed between 20 to 50 bands with molecular masses in the range 29–120 kDa. The differences between the heavily stained bands were more numerous in the 40–100 kDa region. The reproducibility of SDS extracts was very high: studied for four *L. monocytogenes* strains (sv. 1/2 and 4b), similarities between patterns, as assessed by the Dice Coincidence Index of independently prepared extracts of the same strain, were always larger than 95%.

The protein nature of major bands was assessed firstly by their colouration with Coomassie blue, secondly by their degradation with proteinase K and lastly by their capacity to fix biotin (not shown).

The surface location of the proteins was suggested by the absence of lysis of *Listeria* during extraction and was assessed, using two *L. monocytogenes* strains (sv. 1/2a and 4b), by labelling surface proteins with biotin just before extraction with SDS. All major bands in PAGE patterns were labelled with biotin in under 2 min of contact with bacteria (not shown). This very short time confirmed the surface location of extracted major proteins.

Comparison of the patterns of different *Listeria* species

SDS-extracted protein patterns were strictly specific for species (Fig. 1). Only a few major bands appeared common to all species and they were mostly located in the lower part of the gel (42 and 50 kDa). Large differences could be seen between protein patterns of *L.*

Fig. 1. SDS-PAGE analysis of SDS extracts of various *Listeria* species. Each track contained 3 µg of proteins and the gel was stained with silver nitrate. The molecular size of notable bands is indicated on the sides of the figure. Lane 1, *L. monocytogenes* sv. 4b (strain 1); lane 2, *L. monocytogenes* sv. 4b (strain 76); lane 3, *L. monocytogenes* sv. 4b (strain 11); lane 4, *L. innocua* sv. 6a (strain 29); lane 5, *L. innocua* (strain 67); lane 6, *L. seeligeri* sv. 1/2b (strain 2); lane 7, *L. seeligeri* sv. 1/2a (strain 31); lane 8, *L. ivanovii* sv. 5 (strain 32); lane 9, *L. ivanovii* sv. 5 (strain 75); lane 10, *L. welshimeri* sv. 6a (strain 30); lane 11, *L. monocytogenes* sv. 1/2a (strain 23); lane 12, *L. monocytogenes* sv. 1/2b (strain 47); lane 13, *L. monocytogenes* sv. 1/2c (strain 89).

monocytogenes, *L. innocua*, *L. seeligeri*, *L. ivanovii* and *L. welshimeri* and, interestingly, these differences between species were also found in the case of common serovars (for instance *L. seeligeri* and *L. monocytogenes* sv. 1/2a or 1/2b). The representation of these differences was assessed by the calculation of the average similarity, *S*, with visual comparison. In spite of the visual comparison, good reproducibility was obtained because of the high resolution of gels. Analysis of clusters confirmed the clear distinction between *L. monocytogenes* and the other species (*S* = 51%) (Fig. 2).

The specificity of protein patterns was even more marked in immunoblotting where only immunoreactive bands were selected. Only a few bands were revealed in non-*L. monocytogenes* species (Figs 3 and 4), especially with serum against *L. monocytogenes* sv. 1/2a. By comparison, preimmune sera (diluted 1 in 100) did not reveal any bands in SDS extracts of the various species of *Listeria*. Therefore, results of immunoblotting, as with those of SDS-PAGE, showed that numerous bands seemed to be specific for *L. monocytogenes* with, however, large variation between serovars.

Comparison of SDS-PAGE patterns from various serovars of *L. monocytogenes*.

Within the species *L. monocytogenes*, protein patterns appeared strictly related to serovar. As a first step, we studied in detail sv. 1/2 (12 pathogenic isolates) and 4b (nine pathogenic isolates), which constitute from 93 to 98.5% of *L. monocytogenes* strains isolated from the environment and from clinical cases (McLauchlin, 1990; Espaze *et al.*, 1990). Strains of sv. 4b constitute a very homogeneous group (*S* = 86%) (Fig. 2), whose profiles were characterized by the association of two major characteristic bands at 76 and 78 kDa (Fig. 1). Apart from some common bands (42, 50, 66 and 68 kDa), patterns of strains of sv. 4b were clearly different from those of strains of sv. 1/2, characterized by the association of three major bands at 60, 64 and 98 kDa (Fig. 1). The strains of sv. 1/2 constitute a less homogeneous cluster (*S* = 68%) without clear distinct patterns for sv. 1/2a, 1/2b and 1/2c.

In spite of the weak similarity in SDS patterns, immunoblotting showed a large antigenic community

Fig. 2. Dendrogram of the cluster analysis based on protein patterns (above 40 kDa) of SDS extracts of *Listeria* strains. The horizontal axis indicates the percentage similarity, S , as determined by the Coincidence Index of Dice and UGMA clustering. Virulence was determined on immunocompromised mice as previously described (Tabouret *et al.*, 1991).

between sv. 4b and 1/2 (Figs 3 and 4): the 4b antiserum reacted in the profiles of sv. 4b with three major bands (56, 76 and 78 kDa), but also with the two major bands at 60 and 98 kDa in the profiles of *L. monocytogenes* sv. 1/2. This same serum showed that the 64 kDa major band of *L. monocytogenes* sv. 1/2 was also present in extracts of sv. 4b (Fig. 3). The 1/2 antiserum revealed mainly the 98 kDa band in the profiles of sv. 1/2, as well as two bands at 64 and 68 kDa which seemed common to sv. 1/2 and 4b (Fig. 4).

L. monocytogenes strains from less frequent serovars could be divided into four distinct groups. Firstly, sv. 4d and 4e ($S = 90\%$) showed high similarity with sv. 4b ($S = 88\%$); they presented, in particular, two bands at 78 and 76 kDa, albeit of the lowest intensity (Fig. 5). Secondly, sv. 3 (3a, 3b and 3c) had a high degree of similarity with sv. 1/2 ($75\% < S < 83\%$), in particular the characteristic band at 98 kDa (although expressed weakly in sv. 3c). Thirdly, the pattern of sv. 7 was

intermediate between the patterns of sv. 1/2 ($S = 79\%$) and sv. 4b ($S = 76\%$). Finally, sv. 4a and 4c ($S = 86\%$) constituted a distinct group with a similarity of 66% with sv. 1/2 and 4b. Strains of these two serovars were characterized by two major bands at 80 and 100 kDa.

Immunoblotting with sv. 4b antiserum (Fig. 6) and with sv. 1/2 antiserum (not shown) showed an antigenic community between all serovars of *L. monocytogenes*. The 64 and 68 kDa bands, which are difficult to see in SDS-PAGE of some strains (Fig. 5), appeared to be common to all serovars in immunoblotting with anti-1/2 serum. The four groups previously described were reduced to three, since in immunoblotting sv. 7 was not distinguishable from sv. 1/2 and 3, presenting in particular a weak band at 98 kDa (Fig. 6). The 100 kDa band of sv. 4a and 4c was not revealed with sv. 1/2 antiserum, showing its clear distinction from the 98 kDa antigen. By contrast, the 80 kDa band of these strains was strongly revealed by sv. 4b antiserum, suggesting its

Fig. 3. Immunoblotting of SDS extracts of various *Listeria* species after electrophoresis on a 7.5% polyacrylamide gel. The blot was developed with rabbit serum (diluted 1 in 100) against *L. monocytogenes* sv. 4b. Molecular mass markers and the molecular size of notable bands are indicated, respectively, on the left and on the right of the figure. Lane 1, *L. innocua* sv. 6a (strain 29); lane 2, *L. welshimeri* sv. 6a (strain 30); lane 3, *L. seeligeri* sv. 1/2a (strain 31); lane 4, *L. ivanovii* sv. 5 (strain 32); lane 5, *L. monocytogenes* sv. 4b (strain 18); lane 6, *L. monocytogenes* sv. 1/2b (strain 51); lane 7, *L. monocytogenes* sv. 1/2a (strain 62); lane 8, *L. monocytogenes* sv. 1/2a (strain 41); lane 9, *L. monocytogenes* sv. 1/2a (strain 56); lane 10, *L. monocytogenes* sv. 1/2a (strain 23); lane 11, *L. monocytogenes* sv. 1/2c (strain 42); lane 12, *L. monocytogenes* sv. 4b (strain 14).

Fig. 4. Immunoblotting of SDS extracts of various *Listeria* species after electrophoresis on a 7.5% acrylamide gel. The blot was developed with rabbit serum (diluted 1 in 100) against *L. monocytogenes* sv. 1/2a. Molecular mass markers and the molecular size of notable bands are indicated, respectively, on the left and on the right of the figure. Lane 1, *L. innocua* sv. 6a (strain 29); lane 2, *L. welshimeri* sv. 6a (strain 30); lane 3, *L. seeligeri* sv. 1/2a (strain 31); lane 4, *L. ivanovii* sv. 5 (strain 32); lane 5, *L. monocytogenes* sv. 4b (strain 18); lane 6, *L. monocytogenes* sv. 1/2c (strain 42); lane 7, *L. monocytogenes* sv. 1/2b (strain 51); lane 8, *L. monocytogenes* sv. 1/2a (strain 62); lane 9, *L. monocytogenes* sv. 1/2a (strain 33); lane 10, *L. monocytogenes* sv. 1/2a (strain 41); lane 11, *L. monocytogenes* sv. 1/2a (strain 56); lane 12, *L. monocytogenes* sv. 1/2a (strain 23); lane 13, *L. monocytogenes* sv. 4b (strain 14).

Fig. 5. SDS-PAGE examination of SDS extracts of *L. monocytogenes* isolates of various serovars. Each track contained 3 µg of proteins and the gel was stained with silver nitrate. Molecular mass markers and the molecular size of notable bands are indicated, respectively, on the left and on the right of the figure. Lane 1, *L. monocytogenes* sv. 4b (strain 10); lane 2, *L. monocytogenes* sv. 4e (strain 81); lane 3, *L. monocytogenes* sv. 4d (strain 80); lane 4, *L. monocytogenes* sv. 7 (strain 82); lane 5, *L. monocytogenes* sv. 4a (strain 78); lane 6, *L. monocytogenes* sv. 4c (strain 79); lane 7, *L. monocytogenes* sv. 3c (strain 84); lane 8, *L. monocytogenes* sv. 3b (strain 83); lane 9, *L. monocytogenes* sv. 3a (strain 85); lane 10, *L. monocytogenes* sv. 1/2c (strain 42); lane 11, *L. monocytogenes* sv. 1/2b (strain 44); lane 12, *L. monocytogenes* sv. 1/2a (strain 56).

Fig. 6. Immunoblotting of SDS extracts of *Listeria* isolates of various serovars after electrophoresis on a 7.5% acrylamide gel. The blot was developed with rabbit serum (diluted 1 in 200) against *L. monocytogenes* sv. 4b. Molecular mass markers and the molecular size of notable bands are indicated, respectively, on the left and on the right of the figure. Lane 1, *L. monocytogenes* sv. 1/2c (strain 42); lane 2, *L. monocytogenes* sv. 4b (strain 76); lane 3, *L. innocua* sv. 4ab (strain 86); lane 4, *L. monocytogenes* sv. 3a (strain 85); lane 5, *L. monocytogenes* sv. 3b (strain 83); lane 6, *L. monocytogenes* sv. 3c (strain 84); lane 7, *L. monocytogenes* sv. 7 (strain 82); lane 8, *L. monocytogenes* sv. 4e (strain 81); lane 9, *L. monocytogenes* sv. 4d (strain 80); lane 10, *L. monocytogenes* sv. 4c (strain 79); lane 11, *L. monocytogenes* sv. 4a (strain 78); lane 12, *L. monocytogenes* sv. 1/2a (strain 60); lane 13, *L. monocytogenes* sv. 1/2b (strain 51); lane 14, *L. seeligeri* sv. 1/2b (strain 2).

Fig. 7. Comparison by SDS-PAGE (stained with silver nitrate) of SDS extracts of pathogenic and non-pathogenic haemolytic *L. monocytogenes* sv. 1/2a. Molecular mass markers and the molecular size of notable bands are indicated, respectively, on the right and on the left of the figure. Each track contained 3 µg of proteins and the gel was stained with silver nitrate. Lane 1, strain 60 (pathogenic); lane 2, strain 62 (non-pathogenic); lane 3, strain 56 (pathogenic); lane 4, strain 41 (non-pathogenic).

homology with antigens of *L. monocytogenes* sv. 4b. Serovar 4a was characterized by several immunogenic bands above 100 kDa (Fig. 6).

Identification of proteins associated with virulence

To establish a possible relationship between protein pattern and virulence, we first compared profiles of *L. monocytogenes* sv. 1/2a producing listeriolysin O in similar quantity, differing only by their virulence in immunocompromised mice (Table 1) (Tabouret *et al.*, 1991). With a similarity with pathogenic strains of 72%, the two non-pathogenic strains made a separate group within *L. monocytogenes* 1/2a (Fig. 2). These strains were characterized mainly by the loss of two major bands (68 and 98 kDa) found in all of the eight pathogenic *L. monocytogenes* strains of the same serovar. This 98 kDa band was, in fact present in the lowest quantity, as shown by its slight appearance when the largest quantity of extracts was used (not shown). Immunoblotting confirmed this last observation (Fig. 3, lanes 7 and 8; Fig. 4,

lanes 8 and 10) and showed that the 68 kDa band was replaced by another band of greater molecular size (Fig. 4). Using the sv. 4b antiserum, immunoblotting showed also an additional band at 58 kDa in non-pathogenic strains (Fig. 3).

Profiles of non-pathogenic *L. monocytogenes* sv. 1/2a (six strains), which produced small quantities of haemolysin compared to their haemolytic counterparts, were similar.

Discussion

The viability of *Listeria* was already known to be unaltered by moderate treatment with SDS; indeed, this detergent was used, at concentrations similar to ours, to enhance the detection of *Listeria* in media heavily contaminated with other bacteria (Dominguez *et al.*, 1989). More recently, Bhunia *et al.* (1991) used SDS (2%) and β-mercaptoethanol (0.5%) to extract *Listeria* surface proteins and they showed that the bacteria were still motile after such treatment (30 min, 37 °C). In our study, extraction was performed under low stringency conditions (1% SDS, 5 min, 20 °C) and no lysis of bacteria was observed during the extraction, confirming previous observations (Lucas & Levin, 1989; Bhunia *et al.*, 1991). The surface location of all major components extracted by SDS was confirmed by their biotinylation in a very short time. Biotinylation, colouration with Coomassie blue and digestion with proteinase K of these major components demonstrated conclusively that they were proteins.

Electrophoretic analysis of the SDS extracts of whole *Listeria* revealed large differences between species and, within the species *L. monocytogenes*, between serovars. Compared to other species, i.e. *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*, *L. monocytogenes* isolates gave recognizable protein patterns. Only two major bands, at 42 and 50 kDa, appeared to be common to all species. The low number of common bands between *L. monocytogenes* and other species, also observed even in the case of common serovars, was highly relevant to our final purpose, which was the identification of proteins specific for *L. monocytogenes*. Within the species *L. monocytogenes*, SDS extract patterns appeared to be related to serovars. Large differences could be observed between serovars, particularly between sv. 1/2 and 4b. The comparison led to the identification of major proteins specific for some serovars of this species. The 76 and 78 kDa proteins were present only in sv. 4b, and to a lesser extent in sv. 4d and 4e. The 80 and 100 kDa bands were specific for patterns of *L. monocytogenes* sv. 4a and 4c. The 98 kDa band was present in sv. 1/2, 3 and to a lesser degree in sv. 7. Immunoblotting with sera directed

against *L. monocytogenes* simplified profiles and confirmed these observations. All serovar-related bands, described above, showed high reactions with rabbit sera. Moreover, immunoblotting revealed two highly immunogenic proteins common to all *L. monocytogenes* strains, at 64 and 68 kDa. Immunoblotting studies presented a number of interesting features; they allowed further distinction between bands migrating in two patterns at the same apparent level. Immunoblotting also indicated that global antigenic similarity was low between species of *Listeria* and high between serovars of *L. monocytogenes*. Furthermore, immunoblotting studies suggested the possibility of significant homology between antigens of different molecular masses. The use in immunoblotting of sera directed against other serovars or other species of *Listeria* could reveal other species- or serovar-related antigens.

All these differences, either between species or between serovars, contrasted with results obtained in previous studies on the components of *Listeria*. The analysis of peptidoglycan and lipoteichoic acids has shown a similar structure for all *Listeria* species (Uchikawa *et al.*, 1986; Fiedler & Ruhland, 1987). The analysis of somatic (O) and flagellar (H) antigens, using highly absorbed antisera, allowed the determination of 16 serovars but not the identification of strains, since different species can have the same O and H composition (Seeliger & Jones, 1986). Moreover, structural analysis of O antigen, represented by teichoic acid, did not reveal differences between species with identical serovar (Fiedler & Ruhland, 1987). In the same way, analysis of metaquinone, fatty acid and cytochrome composition did not facilitate *Listeria* species differentiation (Collins *et al.*, 1979; Feresu & Jones, 1988). Our results contrasted especially with those involving electrophoretic analysis of whole-cell proteins (Lamont *et al.*, 1986), of 'soluble surface proteins' (Paquet *et al.*, 1986) and of cell-wall proteins (Hof & Chatzipanagiotou, 1987), where no differences were revealed either between *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua* and *L. monocytogenes*, and/or between *L. monocytogenes* serovars. Only results obtained by McLauchlin *et al.* (1988), with monoclonal antibodies, were to some extent analogous with our observations. The reaction, revealed by immunofluorescence of whole bacteria, of a monoclonal antibody with both *L. monocytogenes* sv. 4b and 50% of *L. monocytogenes* sv. 4 but not 4b can be correlated with the antigens at 76 and 78 kDa found in sv. 4b, 4d and 4e. A second monoclonal antibody reacted with both *L. monocytogenes* sv. 1/2 and 3, two serovars for which we found high similarity and numerous common bands. Unfortunately, the first monoclonal antibody reacted with *L. innocua* sv. 6a and the second one with *L. seeligeri* sv. 1/2. Therefore the originality of our work is to have identified, for the

first time, structures that are apparently species-specific and rank above serovar.

For the first time with regard to structural components, the differences observed between SDS protein patterns of *Listeria* species were in agreement with DNA relatedness as determined by Rocourt *et al.* (1982). DNA relatedness studies allowed the division of the species *L. monocytogenes sensu lato* into five distinct genomic groups. As seen in the dendrogram (Fig. 2), the *L. monocytogenes* isolates in this study formed a cluster distinct from the other species, confirming the established classification. In the study by Rocourt *et al.* (1982) on DNA relatedness, differences among *L. monocytogenes* isolates were not examined. The analysis of SDS-extracted proteins in our study revealed a significant separation between serovars, especially between sv. 1/2 and 4b, which formed distinct clusters on the dendrogram. However, this clustering of *L. monocytogenes* was different from the one obtained using multilocus enzyme electrophoresis (MEE) studies. The use of MEE (Boerlin *et al.*, 1991) has permitted confirmation of the conclusions of DNA-DNA hybridization studies and the definition among *L. monocytogenes* strains of two distinct clusters, comprising on the one hand sv. 4b, 4a, 1/2b and 3b and on the other hand sv. 1/2a, 1/2c and 3a (Piffaretti *et al.*, 1989; Bibb *et al.*, 1990; Boerlin *et al.*, 1991).

The distribution of serovars of *L. monocytogenes* in the environment and clinical cases has indicated a possible association between serovar and virulence (McLauchlin, 1987, 1990). The surface proteins described here could contribute to the differences in virulence observed between serovars. Within *L. monocytogenes* sv. 1/2a, the study of profiles from highly haemolytic strains has related some protein (98 and 68 kDa) to virulence for immunocompromised mice. The presence of a weak band of 98 kDa in profiles of non-pathogenic strains could be explained, besides a simple quantitative problem, by the superposition of two antigens in the profiles of pathogenic strains, one of them being missed in non-pathogenic strains. This hypothesis could be confirmed by two-dimensional analysis. The other band at 68 kDa, which seemed to be absent on SDS-PAGE of non-pathogenic strains, and which was replaced by another band with the highest molecular mass in immunoblotting of these strains, was interesting since it was present in all serovars of *L. monocytogenes*. The possible association of these protein bands with virulence of *L. monocytogenes* deserves further study, in particular in relation to intracellular penetration and cell-to-cell spread, due to their surface location. Recently, the involvement of two proteins in intracellular penetration of *L. monocytogenes* has been described. The first was an extracellular protein of molecular mass

60 kDa (protein p60) (Kuhn & Goebel, 1989; Kölher *et al.*, 1990) and the second was a surface-located protein (internalin) of predicted molecular mass 80 kDa, coded by the gene *InlA* (Gaillard *et al.*, 1991). In our extracts, no difference between pathogenic and non-pathogenic strains could be seen at this molecular size. Lastly, the lack of difference noticed in profiles of non-pathogenic and weakly haemolytic *L. monocytogenes* strains confirmed that the limiting factor in the virulence of these strains was probably the quantity of secreted listeriolysin.

In conclusion, the SDS-extracted proteins pattern of *Listeria* allowed an unequivocal identification of species. The identified surface proteins of *L. monocytogenes*, specific for species, serovars or virulence, could be used now to develop simpler methods of serotyping, as well as specific methods of strain identification and serodiagnosis. Further preliminary studies would have to be carried out to examine possible homologies between antigens of different molecular mass present in different species and serovars of *Listeria*. Moreover, the use of these results in the development of new methods of diagnosis requires the study of cross-reactions with surface proteins from other Gram-positive species. The high resolution of our gels allows studies of the influence of growth conditions (temperature, media, *in vivo* and *in vitro* adaptation) on the expression of surface proteins. It could also be fruitful to examine the role of the extracted proteins in the virulence of *L. monocytogenes*.

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References

- BIBB, W. F., GELLIN, B. G., WEAVER, R., SCHWARTZ, B., PLIKAYTIS, B. D., REEVES, M. W., PINNER, R. W. & BROOME, C. V. (1990). Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. *Applied and Environmental Microbiology* **56**, 2133–2141.
- BILLE, J. & GLAUSER, M.-P. (1988). Listériose en Suisse. *Bulletin de l'Office fédéral de la santé publique* **3**, 28–29.
- BHUNIA, A. K., BALL, P. H., FUAD A. T., KURZ B. W., EMERSON J. W., & JOHNSON, M. G. (1991). Development and characterization of a monoclonal antibody specific for *Listeria monocytogenes* and *L. innocua*. *Infection and Immunity* **59**, 3176–3184.
- BOERLIN, P., ROCOURT, J. & PIFFARETTI, J.-C. (1991). Taxonomy of the genus *Listeria* by using multilocus enzyme electrophoresis. *International Journal of Systematic Bacteriology* **41**, 59–64.
- CARBONNELLE, B., COTTIN, J., ACHARD, J. & GROSIEUX, P. (1988). *Listeria monocytogenes* et listérioses en Anjou de 1975 à 1987. *Semaine des Hôpitaux de Paris* **64**, 2211–2215.
- CHAKRABORTY, T. & GOEBEL, W. (1988). Recent developments in the study of virulence in *Listeria monocytogenes*. *Current Topics in Microbiology and Immunology* **139**, 41–58.
- COLLINS, M. D., JONES, D., GOODFELLOW M. & MINNIKIN D. E. (1979). Isoprenoid quinone composition as a guide to the classification of *Listeria*, *Brochothrix*, *Erysipelothrix*, and *Caryophanon*. *Journal of General Microbiology* **111**, 453–457.
- DOMINGUEZ, L., BRIONES, V., BLANCO, M., FERNANDEZ-GARAYZABAL, J. F., BLANCO, J. L., VAZQUEZ, J. A. & SUAREZ, G. (1989). Direct isolation (without enrichment) of *Listeria monocytogenes*. *Acta Microbiologica Hungarica* **2–3**, 297–301.
- ESPAZE, E. P., ROCOURT, J. & COURTIEU, A. L. (1990). La listériose en France en 1988: étude à partir des souches adressées au centre national de référence. *Bulletin Epidemiologique Hebdomadaire* **1**, 1–2.
- FERESU, S. B. & JONES, D. (1988). Taxonomic studies on *Brochothrix*, *Erysipelothrix*, *Listeria* and atypical *Lactobacilli*. *Journal of General Microbiology* **134**, 1165–1183.
- FIEDLER, F. & RUHLAND, G. L. (1987). Structure of *Listeria monocytogenes* cell walls. *Bulletin de l'Institut Pasteur* **85**, 287–300.
- FLEMING, D. W., COCHI, S. L., MACDONALD, K. L., BRONDUM, J., HAYES, P. S., PLIKAYTIS, B. D. & HOLMES, M. B. (1985). Pasteurized milk as a vehicle of infection in an outbreak of Listeriosis. *New England Journal of Medicine* **312**, 404–407.
- GAILLARD, J.-L., BERCHE, P., FREHEL, C., GOVIN, E. & COSSART, P. (1991). Entry of *L. monocytogenes* into cells is mediated by a repeat protein analogous to surface antigens from Gram-positive, extra-cellular pathogens. *Cell* **65**, 1–20.
- GRAY, M. L. & KILLINGER, A. H. (1966). *Listeria monocytogenes* and listeric infections. *Bacteriological Reviews* **30**, 309–382.
- HEUKESHOVEN, J. & DERNICK, R. (1985). Simplified method for silver staining of protein in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6**, 103–112.
- HOF, H. & CHATZIPANAGIOTOU, S. (1987). The role of surface structure of *Listeria* spp. for pathogenicity. *Annales de l'Institut Pasteur/Microbiologie* **138**, 268–273.
- JOHNSON, R. A. & WICHERN, D. W. (1982). Classification and grouping techniques. In *Applied Multivariate Statistical Analysis*, pp. 459–478. Edited by R. A. Johnson & D. W. Wichern. London: Prentice-Hall.
- KERR, K. G., ROTOWA, N. A., HAWKEY, P. M. & LACEY, R. W. (1990). Incidence of *Listeria* spp. in precooked, chilled chicken products as determined by culture and enzyme-linked immunoassay (ELISA). *Journal of Food Protection* **43**, 606–607.
- KÖHLER, S., LEIMEISTER-WÄCHTER, M., CHAKRABORTY, T., LOTT-SPEICH, F. & GOEBEL, W. (1990). The gene coding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes*. *Infection and Immunity* **58**, 1943–1950.
- KUHN, M. & GOEBEL, W. (1989). Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in extracellular uptake by mammalian cells. *Infection and Immunity* **57**, 55–61.
- LAEMMLI, U. K. (1970). Cleavage of a structural protein during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAMONT, R. J., PETRIE, D. T., MELVIN, W. T. & POSTLETHWAITE, R. (1986). An investigation of the taxonomy of *Listeria* species by comparison of electrophoretic patterns. In *Listériose, Listeria, Listeriosis 1985–86*, pp. 41–46. Edited by A. L. Courtieu, E. P. Espaze & A. E. Reynaud. Nantes: Université de Nantes.
- LINNAN, M. J., MASCOLA, M., XIAO, D. L., GOULET, V., MAY, S., SALMINEN, C., HIRD, D. W., YONEKURA, M. L., HAYES, P., WEAVER, R., AUDURIER, A., PLIKAYTIS, B. D., FANNIN, S. L., KLEKS, A. & BROOME, C. V. (1988). Epidemic listeriosis associated with Mexican-style cheese. *New England Journal of Medicine* **319**, 823–828.
- LISANTI, M. P., SARGIACOMO, M., GRAEVE, L., SALTIEL, A. R. & RODRIGUEZ-BOULAN, E. (1988). Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 9557–9561.
- LUCAS, R. D. & LEVIN, R. E. (1989). Genetic transformation between strains of *Listeria monocytogenes*. *Letters in Applied Microbiology* **9**, 215–218.
- MATTINGLY, J. A., BUTMAN, B. T., PLANK, M. C. & DURHAM, R. J. (1988). Rapid monoclonal antibody-based enzyme linked immunosorbent assay for detection of *Listeria monocytogenes* in food products. *Journal of the Association of Official Analytical Chemists* **71**, 679–681.

- McLAUCHLIN, J. (1987). *Listeria monocytogenes*, recent advances in the taxonomy and epidemiology of listeriosis in humans. *Journal of Applied Bacteriology* **63**, 1–11.
- McLAUCHLIN, J. (1990). Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. *European Journal of Microbiology and Infectious Disease* **9**, 210–213.
- McLAUCHLIN, J., BLACK, A., GREEN, H. T., NASH, J. Q. & TAYLOR, A. G. (1988). Monoclonal antibodies show *Listeria monocytogenes* in necropsy tissue samples. *Journal of Clinical Pathology* **41**, 983–988.
- PAQUET, N., BITUTSI, F., ESPAZE, E. P. & PECHERE, J. C. (1986). Western blot analysis of surface proteins. In *Listeriose, Listeria, Listeriosis 1985–86*, pp. 89–91. Edited by A. L. Courtieu, E. P. Espaze & A. E. Reynaud. Nantes: Université de Nantes.
- PICKUP, R. W. (1991). Development of molecular methods for the detection of specific bacteria in the environment. *Journal of General Microbiology* **137**, 1009–1019.
- PIFFARETTI, J. C., KRESSEBUCH, H., AESCHBACHER, M., BILLE, J., BANNERMAN, E., MUSSER, J. M., SELANDER, R. K. & ROCOURT, J. (1989). Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3818–3822.
- ROCOURT, J., GRIMONT, F., GRIMONT, P. A. D. & SEELIGER, H. R. P. (1982). DNA relatedness among *Listeria monocytogenes* (sensu lato) serovars. *Current Microbiology* **7**, 383–388.
- ROCOURT, J., SCHRETTENBRUNNER, A. & SEELIGER, H. P. R. (1983). Différenciation biochimique des groupes génomiques de *Listeria monocytogenes*. *Annales de Microbiologie* **134A**, 65–71.
- SCHLECH, W. F., LAVIGNE, P. M., BORTOLUSSI, R. A., ALLEN, A. C., HALDANE, E. V., WORT, A. J., HIGHTOWER, A. W., JOHNSON, S. E., KING, S. H., NICHOLLS, E. S. & BROOME, C. V. (1983). Epidemic listeriosis – evidence of transmission by food. *New England Journal of Medicine* **308**, 203–206.
- SEELIGER, H. P. R. & HÖHNE, K. (1979). Serotyping of *Listeria monocytogenes* and related species. *Methods in Microbiology* **13**, 31–49.
- SEELIGER, H. P. R. & JONES, D. (1986). Genus *Listeria* Pirie (1940). In *Bergey's Manual of Systematic Bacteriology*, pp. 1235–1245. Edited by P. H. A. Sneath, S. N. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- SIRAGUSA, G. R. & JOHNSON, M. G. (1990). Monoclonal antibody specific for *Listeria monocytogenes*, *Listeria innocua*, and *Listeria welshimeri*. *Applied and Environmental Microbiology* **56**, 1897–1904.
- TABOURET, M., DE RYCKE, J., AUDURIER, A. & POUTREL, B. (1991). Pathogenicity of *Listeria monocytogenes* isolates in immunocompromised mice in relation to production of listeriolysin. *Journal of Medical Microbiology* **34**, 13–18.
- UCHIKAWA, K. I., SEKIKAWA, I. & AZUMA, I. (1986). Structural studies on lipoteichoic acids from four *Listeria* strains. *Journal of Bacteriology* **168**, 115–122.
- WELSHIMER, H. J. (1968). Isolation of *Listeria monocytogenes* in nature. *Journal of Bacteriology* **95**, 300–303.