Cell-surface location of *Listeria*-specific protein p60 – detection of *Listeria* cells by indirect immunofluorescence

GERHARD J. RUHLAND,¹ MANUELA HELLWIG,¹ GERHARD WANNER² and FRANZ FIEDLER^{1*}

¹ Institut für Genetik und Mikrobiologie der Universität München, Maria-Ward-Str. 1a, 8000 München 19, FRG

² Botanisches Institut der Universität München, Menzinger Strasse 67, 8000 München 19, FRG

(Received 18 September 1992; revised 23 October 1992; accepted 26 October 1992)

A specific polyclonal antiserum was prepared against a gel-purified 60 kDa extracellular protein of *Listeria monocytogenes* ATCC 19111 corresponding to protein p60 previously detected in culture broths of *L. monocytogenes* strains Mackaness and EGD [Kuhn, M. & Goebel, W. (1989), *Infection and Immunity* 57, 55–61]. Indirect immunogold labelling combined with transmission electron microscopy and high-resolution scanning electron microscopy were used to investigate the location and distribution of p60 on the bacterial cell surface. In bacteria grown to the early stationary phase about 25 % of the extracellular protein was estimated to be associated with the cell surface. The anti-p60 antiserum proved to be *Listeria*-specific. In an indirect immunofluorescence test the antiserum reacted with *Listeria* strains representing all species and different serotypes, except *L. seeligeri*, *L. welshimeri*, *L. grayi* and *L. murrayi*. No immunological cross-reactions were observed with 27 strains of bacteria from 16 other genera. The value of the anti-p60 antiserum in developing a diagnostic assay for *Listeria* cells in environmental samples and foods is discussed.

Introduction

Listeria monocytogenes is an opportunistic pathogen in humans and various animal species; *L. ivanovii* affects only the latter. The other species of the genus *Listeria* are avirulent. As a facultative intracellular parasite *L. monocytogenes* can penetrate and replicate within professional and nonprofessional phagocytic cells (Cossart & Mengaud, 1989).

Cell surface components and extracellular proteins of *L. monocytogenes* are known to be involved in pathogenicity, e.g. by mediating adherence and penetration of the host cell. The cell surface of *Listeria* is composed of peptidoglycan, cell wall teichoic acids, lipoteichoic acids, and proteins (Fiedler *et al.*, 1984; Fiedler & Ruhland, 1987; Tabouret *et al.*, 1992). Cowart *et al.* (1990) proposed that the attachment of a virulent *Listeria* strain to eukaryotic cells is mediated by the interaction of α -D-galactose residues exposed at the microbial cell surface with a eukaryotic galactose receptor. Lipoteichoic acids anchored to the outside of the cytoplasmic membrane were found to carry α -D-galactosyl substituents probably involved in this process (Ruhland & Fiedler, 1987). The presence of adhesins at the cell surface of *L. mono-*

cytogenes was demonstrated using direct agglutination with neoglycoproteins (Cottin *et al.*, 1990). Gaillard *et al.* (1991) identified an 80 kDa cell surface protein (internalin) that mediates the entry of *L. monocytogenes* into nonprofessional phagocytic cells. A 60 kDa protein (p60) detected in the culture fluid of *L. monocytogenes* probably participates in this process (Kuhn & Goebel, 1989). Noninvasive rough mutants of *L. monocytogenes* forming long cell chains were found to be impaired in the synthesis of p60. Treatment of rough mutants with partially purified p60 led to disaggregation of cell chains and restored invasiveness. Listeriolysin O, an SH-activated cytolysin secreted by *L. monocytogenes*, enables the bacterium to escape from the phagocytic vacuole of the infected cell (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Tilney & Portnoy, 1989). In the cytoplasm of the host cell *L. monocytogenes* interacts with the microfilament system, promoting intracellular movement and cell-to-cell spread (Tilney & Portnoy, 1989; Dabiri *et al.*, 1990). A 90 kDa cell-surface protein was identified as a key component of this process (Kocks *et al.*, 1992; Domann *et al.*, 1992).

Listeria strains are widely distributed in nature, and *Listeria*-contaminated food (e.g. vegetables, dairy and meat products) was identified as the main source of infection in several outbreaks of listeriosis. Time-consuming selective enrichment culture methods and

* Author for correspondence. Tel. 89 1791 98 52; fax 89 1791 98 49.

physiological tests are currently used in the identification of *Listeria* (Hof, 1990). Recent outbreaks of listeriosis emphasize the need for more rapid and reliable detection procedures. The main interest has focused on genetic and immunoassay methods (Butman *et al.*, 1988; McLaughlin *et al.*, 1989; Köhler *et al.*, 1990; Siragusa & Johnson, 1990; Wang *et al.*, 1991).

We have prepared a specific antiserum against the 60 kDa extracellular protein (p60) of *L. monocytogenes* ATCC 19111 serotype 1/2a. Immunogold labelling combined with electron microscopy and an indirect immunofluorescence test were used to investigate whether p60 is a cell surface antigen which can be used for a rapid identification of *Listeria* cells.

Methods

Bacterial strains and growth conditions. The organisms used and their sources are listed in Table 1. Strains of *Listeria* spp., *Jonesia denitrificans* and *Erysipelothrix rhusiopathiae* were grown in brain/heart infusion broth (BHI, Oxoid) at 37 °C unless otherwise stated. Lactobacilli were cultured in MRS broth [10 g peptone (Oxoid), 5 g glucose, 4 g yeast extract (Oxoid), 2 g KH₂PO₄, 5 g sodium acetate, 2 g diammonium hydrogen citrate, 0.2 g MgSO₄·7H₂O, 0.05 g MnSO₄·4H₂O, and 1 ml Tween 80 per litre, pH 6.3–6.5] at 30 °C, and *Enterococcus faecalis* in antibiotic medium 3 (Oxoid) at 37 °C. Coryneform organisms were grown in a medium composed of (l⁻¹): 17 g casein peptone (Oxoid), 3 g soya peptone (Oxoid), 5 g yeast extract (Oxoid), 2.5 g glucose, 5 g NaCl, 2.5 g K₂HPO₄ and 1 ml Tween 80 (pH 7.3) at 30 °C. The remaining organisms were grown in nutrient broth containing (l⁻¹): 5 g glucose, 5 g yeast extract (Oxoid), 10 g tryptone (Oxoid) and 5 g NaCl, pH 7.5. The growth temperature was 37 °C for the *Staphylococcus* spp., *Escherichia coli*, *Salmonella typhimurium* and *Streptococcus thermophilus* and 30 °C for the other organisms. *Er. rhusiopathiae*, *Lactobacillus* spp., *Ent. faecalis* and *Strep. thermophilus* were grown without aeration, the other strains were cultured aerobically with shaking.

Preparation of extracellular proteins. BHI broth was filtered in an Amicon ultrafiltration cell through a YM 10 membrane to remove high-molecular-mass compounds (e.g. large polypeptides). Strains of *Listeria* spp. were grown in the filtrate to the early stationary phase; the optical density at 580 nm (OD₅₈₀) corresponded to approximately 2.0. Bacteria were harvested by centrifugation (14000 g for 30 min, 4 °C). The pellet was heated at 100 °C for 15 min, washed twice with PBS (0.02 M-sodium phosphate, pH 7.2, 0.9% NaCl), lyophilized, and weighed. The culture supernatant was filter-sterilized (0.2 µm pore diameter, Sartorius) and subsequently concentrated 100-fold in an Amicon ultrafiltration cell using a YM 30 membrane. The filtered supernatant was diluted twice (1/10) in the cell with double-distilled water and concentrated again. All the treatments were performed at 4 °C.

Preparation of antisera. An antiserum was raised in a female rabbit against gel-purified samples of a 60 kDa exoprotein of *L. monocytogenes* ATCC 19111 serogroup 1/2a. The identity of the 60 kDa protein with p60 was established by immunoblotting using an antiserum against p60 derived from the non-haemolytic mutant *L. monocytogenes* M20 (Köhler *et al.*, 1990). The culture supernatant proteins were separated by SDS-PAGE (6%, w/v, polyacrylamide) and visualized in 1 M-KCl (4 °C, 16 h). The protein was recovered from carefully excised bands by electroelution using a Biotrap electro-separation system (Schleicher & Schüll). Prior to immunization, the rabbit was examined

for the absence of antibodies reacting with extracellular proteins of *L. monocytogenes* ATCC 19111 by immunoblotting. The protein preparation for primary injection was emulsified in complete Freund's adjuvant. Subsequent booster injections, with incomplete Freund's

Table 1. *Bacterial strains*

Species	Strain*	Serotype
<i>Listeria monocytogenes</i>	ATCC19111	1/2a
<i>L. monocytogenes</i>	SLCC 5782 ^a	1/2a
<i>L. monocytogenes</i>	ATCC 19113	3a
<i>L. monocytogenes</i>	ATCC 19115	4b
<i>L. monocytogenes</i>	SLCC 5778 ^b	4b
<i>L. monocytogenes</i>	SLCC 2482	7
<i>L. ivanovii</i>	ATCC 19119	5
<i>L. innocua</i>	ATCC 33090	6a
<i>L. innocua</i>	ATCC 33091	6b
<i>L. seeligeri</i>	SLCC 3954	1/2b
<i>L. welshimeri</i>	SLCC 5334	6a
<i>L. grayi</i>	ATCC 25401	
<i>L. murrayi</i>	ATCC 19120	
<i>Bacillus subtilis</i> †		
<i>Brevibacterium linens</i>	ATCC 19391	
<i>Bb. linens</i>	Ac 831 ^c	
<i>Bb. linens</i>	Ac 577 ^c	
<i>Bb. linens</i>	Ac 480 ^c	
<i>Brochothrix thermosphacta</i>	DSM 20171	
Coryneform organism	CNRZ 925 ^d	
Coryneform organism	CNRZ 926 ^d	
<i>Enterobacter aerogenes</i> †		
<i>Enterococcus faecalis</i>	DS16 ^e	
<i>Enterococcus faecalis</i>	OG1S ^e	
<i>Erysipelothrix rhusiopathiae</i>	NCTC 8163	
<i>Escherichia coli</i> †		
<i>Jonesia denitrificans</i>	ATCC 14870	
<i>Lactobacillus casei</i>	DSM 20011	
<i>Lb. curvatus</i>	DSM 20019	
<i>Lb. plantarum</i>	DSM 20174	
<i>Micrococcus luteus</i>	DSM 20030	
<i>Pseudomonas aeruginosa</i>	ATCC 27853	
<i>Salmonella typhimurium</i>	LT2 ^f	
<i>Serratia marcescens</i>	DSM 30121	
<i>Staphylococcus aureus</i> H†		
<i>Staph. aureus</i> Copenhagen	DSM 20232	
<i>Staph. saprophyticus</i>	DSM 20229	
<i>Staph. epidermidis</i>	DSM 20044	
<i>Staph. xylosus</i>	DSM 20266	
<i>Streptococcus thermophilus</i>	NCDO 573	

* ATCC, American Type Culture Collection, Rockville, Md, USA; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG; SLCC, Special *Listeria* Culture Collection, Würzburg, FRG; NCDO, National Collection of Dairy Organisms, Reading, UK; NCTC, National Collection of Type Cultures, London, UK; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France. ^{a,b} Vaccine strains from Prof. Dr J. Potel, Hannover, FRG (rough mutants forming long cell chains; derived from serotype 1/2a, original no. 32/46J, and serotype 4b, original no. 1345, respectively). ^c From Dr W. H. J. Crombach, Microbiology Laboratory, Agricultural University of Wageningen, The Netherlands. ^d Isolates from the surface of French cheese, provided by Dr J. P. Accolas (Station Centrale des Recherches Laitières et de Technologie des Produits Animaux, Jouy-en-Josas, France). ^e From Dr R. Wirth, Institut für Genetik und Mikrobiologie, Universität München, FRG. ^f From Prof. Dr H. Schmiegler, Institute für Genetik und Mikrobiologie, Universität München, FRG.

† Culture collection of the Institut für Genetik und Mikrobiologie, Universität München, FRG.

adjuvant, were given 4 and 6 weeks after the first immunization. The rabbit was injected intradermally at multiple sites with approximately 60 µg protein per immunization. The animal was bled, and antiserum prepared, 12 d after the third injection. For control experiments an anti-listeriolysin O antiserum was prepared in the same way.

Immunogold labelling of cells and electron microscopy. Cells corresponding to 1 ml of an overnight culture in BHI broth were washed with 1.5 ml PBS immediately after harvesting. For indirect immunogold labelling the cells were resuspended in 1 ml PBS containing 1 mM-sodium azide and the anti-p60 antiserum (dilution: 1/250). The suspension was incubated for 2 h at room temperature with gentle agitation. Cells were then washed twice with 1.5 ml PBS and resuspended in 1 ml of the same buffer. After the addition of 300 µl Auro Probe EM (gold-labelled protein A with a mean particle size of 10 nm, Janssen Biotech) the cell/immunogold suspension was mixed carefully for 3 h at room temperature. The cells were separated by centrifugation (8000 g, 10 min), washed twice with 1.5 ml PBS, and fixed for electron microscopy (Wanner *et al.*, 1989). Transmission and high-resolution scanning electron microscopy were performed as described by Wanner *et al.* (1989).

Indirect immunofluorescence antibody test. This was carried out as described by Walker *et al.* (1971). Cells were grown overnight in BHI broth, heat killed (100 °C, 15 min) and washed once with double-distilled water. The cells were resuspended in double-distilled water, applied to slides, air-dried, and heat-fixed. The samples were incubated with the anti-p60 antiserum, diluted in Tris-buffered saline (TBS; 0.1 M-Tris/HCl, pH 7.4, 0.9% NaCl) as indicated in Table 2, for 1 h at room temperature in a humid chamber. The slides were washed six times with TBS and dried with filter paper. The fixed cells were labelled with anti-rabbit IgG-fluorescein isothiocyanate conjugate (FITC, Sigma) at a 1/20 dilution in TBS and incubated for 30 min at room temperature in a humid chamber. The slides were washed again six times with TBS and dried with filter paper. The samples were mounted under a coverslip in 0.5 M-sodium carbonate-buffered glycerol (1:9, v/v), pH 9.0, and examined by fluorescence microscopy. Controls were treated with pre-immune serum or TBS buffer instead of the anti-p60 antiserum.

SDS-PAGE and immunoblotting. SDS-PAGE was performed in stacking gels with 4% (w/v) and separating gels with 6 or 10% (w/v) polyacrylamide according to Laemmli (1970). Gels (1 mm thickness) were prepared using a minigel apparatus (8 × 8 cm, Biometra). Cell-associated protein was analysed by boiling lyophilized and PBS-washed cells in SDS-sample buffer for 5 min. Cells were removed by centrifugation (8000 g, 10 min) and the supernatant (SDS extract) was applied to the gel. Protein bands on the gels were visualized with Coomassie Brilliant Blue R250. Electrophoretic transfer of proteins to nitrocellulose membranes was carried out as described by Towbin *et al.* (1979). Proteins reacting with the anti-p60 antiserum were visualized by subsequent incubation of the filter with anti-rabbit IgG-peroxidase conjugate (Sigma) and diaminobenzidine/H₂O₂. Alternatively, ¹²⁵I-protein A (Amersham Buchler) and autoradiography on Kodak X-ray film were used to detect reacting proteins. For quantification, the immunoreactive bands on nitrocellulose filters were excised and measured in Rotiszint 11 (Carl Roth) using a liquid scintillation counter (Kontron MR 300).

Results

Immunoblot analysis of culture supernatants

The extracellular protein pattern of *L. monocytogenes* ATCC 19111 serotype 1/2a obtained by SDS-PAGE is shown in Fig. 1(a) (lane 2). The most prominent bands

Fig. 1. SDS-PAGE and immunoblot analysis of extracellular proteins from *L. monocytogenes* serotype 1/2a grown at 37 °C (a). Coomassie-stained 10% SDS-PAGE gel of culture supernatants from *L. monocytogenes* ATCC 19111 (lane 2) and *L. monocytogenes* SLCC 5782 (lane 3). Lane 1 contains molecular mass standards (from top to bottom, 205, 116, 97.4, 66, 45, and 29 kDa). (b). Immunoblot analysis of extracellular proteins of *L. monocytogenes* ATCC 19111 (lane 1) and *L. monocytogenes* SLCC 5782 (lane 2) with anti-p60 antiserum diluted 1/200 000. Immunoreacting proteins were visualized on nitrocellulose membranes using anti-rabbit IgG-peroxidase conjugate. Extracellular proteins corresponding to 1 mg dry cells were applied in (a) and (b).

Fig. 2. Immunoblot analysis of culture supernatants from *L. monocytogenes* ATCC 19111 grown at 37 °C (lane 1) and 26 °C (lane 2) with anti-p60 antiserum. Antibody binding was visualized using anti-rabbit IgG-peroxidase conjugate. Extracellular proteins corresponding to 1 mg dry cells were applied to each lane.

correspond to listeriolysin (58 kDa) and protein p60, as shown by Kuhn & Goebel (1989) for other haemolytic *L. monocytogenes* strains.

Immunoblot analysis of culture supernatant of *L. monocytogenes* ATCC 19111 revealed that the anti-p60 antiserum reacted strongly with protein p60 (Fig. 1b, lane 1). However, an additional weakly reacting protein with an apparent molecular mass of about 110 kDa was visible. This band was also detected when p60 excised from a gel was reanalysed by SDS-PAGE and subsequent immunoblotting. This indicated that the 110 kDa protein band was derived from p60, most probably due to aggregation. In the culture supernatant of the rough

Fig. 3. High-resolution scanning electron micrographs (*a-c*) and transmission electron micrograph of an ultrathin section (*d*) of *L. monocytogenes* ATCC 19111. Prior to immunogold labelling (diameter of gold particles 10 nm), cells were incubated with anti-p60 antiserum (*a*, *b* and *d*) or pre-immune serum (*c*). Note that the immunogold label is uniformly distributed over the cell surface in (*a*), (*b*) and (*d*) but totally absent in the control (*c*). Bars, 1 μ m in (*a*); 100 nm in (*b*)-(*d*).

Fig. 4. High-resolution scanning electron micrographs of *L. monocytogenes* SLCC 5782. Prior to immunogold labelling, cells were incubated with anti-p60 antiserum (a) or pre-immune serum (b). The cell surface is uniformly labelled in (a). Immunogold labelling is absent in the control (b). Bars, 100 nm.

mutant, *L. monocytogenes* SLCC 5782, p60 was detected with the anti-p60 antiserum (Fig. 1b, lane 2); however, the concentration of the protein was too low to be visualized in Coomassie-stained SDS-polyacrylamide gels (Fig. 1a, lane 3).

Culture supernatants from various strains of *Listeria* spp. (Table 1) were analysed by immunoblotting with anti-p60 antiserum. Proteins immunologically related to p60 with a similar molecular mass were detected in all strains except *L. grayi* and *L. murrayi* (data not shown). In the case of *L. seeligeri* and *L. welshimeri*, however, only a weak reaction was observed.

Production of p60 by *L. monocytogenes* ATCC 19111 was examined at 26 and 37 °C. Immunoblot analysis of supernatants prepared from early stationary phase cultures indicated that cells grown at 26 °C produced much less p60 than those grown at 37 °C (Fig. 2).

Cell surface location of protein p60

L. monocytogenes ATCC 19111 and the rough mutant *L. monocytogenes* SLCC 5782 were investigated for cell surface association of protein p60. High-resolution scanning electron microscopy revealed that cells of both the wild-type and the mutant strain exhibited immunogold label after successive treatment with the

anti-p60 antiserum and protein A-gold complex (Figs 3a,b and 4a). The immunogold particles were not restricted to particular areas, but were distributed uniformly over the cell surface. This was confirmed by transmission electron microscopy of an ultrathin section of immunogold-labelled cells (Fig. 3d). The maximum density of gold grains on the cell surface was estimated to be about 300 per μm^2 for both ATCC 19111 and the rough mutant SLCC 5782. The specificity of immunogold labelling was established by control experiments in which the anti-p60 antiserum was replaced by either pre-immune serum or the anti-listeriolysin antiserum. High-resolution scanning electron micrographs demonstrated that these cells were totally free of gold particles (Figs 3c and 4b).

These findings clearly indicated that protein p60, at first detected in the culture supernatant, was also located at the cell surface, whereas listeriolysin did not appear to be associated with the cell.

Quantification of cell-associated and cell-free p60

Cell surface associated protein p60 was not released from the cells by boiling for 10 min, washing with PBS, or extraction with 5 M-LiCl (data not shown). However, the protein was removed when PBS-washed cells were

Table 2. Reactivity of the anti-p60 antiserum in the indirect immunofluorescence test

Bacterial strain	Dilution of antiserum					
	1/32	1/64	1/128	1/256	1/512	1/1024
<i>Listeria monocytogenes</i> ATCC 19111	+	+	+	+	+	—
<i>L. monocytogenes</i> SLCC 5782*	+	+	+	—	—	—
<i>L. monocytogenes</i> ATCC 19113	+	+	+	+	—	—
<i>L. monocytogenes</i> ATCC 19115	+	+	—	—	—	—
<i>L. monocytogenes</i> SLCC 5778*	+	+	+	—	—	—
<i>L. monocytogenes</i> SLCC 2482	+	+	—	—	—	—
<i>L. ivanovii</i> ATCC 19119	+	+	—	—	—	—
<i>L. innocua</i> ATCC 33090	+	+	+	—	—	—
<i>L. innocua</i> ATCC 33091	+	—	—	—	—	—
<i>L. seeligeri</i> SLCC 3954	—	—	—	—	—	—
<i>L. welshimeri</i> SLCC 5334	—	—	—	—	—	—
<i>L. grayi</i> ATCC 25401	—	—	—	—	—	—
<i>L. murrayi</i> ATCC 19120	—	—	—	—	—	—
<i>Staphylococcus aureus</i> H	+	+	+	+	+	+
<i>Staph. aureus</i> Copenhagen	+	+	+	+	+	+
Other bacterial strains†	—	—	—	—	—	—

* Rough mutants.

† See Table 1.

Fig. 5. Immunoblot analysis of *L. monocytogenes* ATCC 19111 culture supernatants (lanes 1 and 2) and SDS extracts of whole cells (lanes 3 and 4) with anti-p60 antiserum. Immunoreacting proteins were visualized with ^{125}I -protein A and autoradiography. Samples corresponding to 1 mg (lanes 1 and 3) or 2 mg (lanes 2 and 4) dry cells were applied. For quantification of the immunoreaction, radioactive bands were excised from the nitrocellulose filter and their radioactivity measured.

boiled in SDS-sample buffer. This SDS extract and the culture supernatant were analysed by immunoblotting using the anti-p60 antiserum and ^{125}I -protein A (Fig. 5). The radioactivity of the immunoreactive bands was measured and the ratio of cell-bound (Fig. 5, lanes 3 and 4) to free protein (Fig. 5, lanes 1 and 2) was calculated to be 0.24 and 0.23, respectively. This would mean that about 25% of p60 was located at the cell surface.

Specificity of the anti-p60 antiserum

The antiserum raised against protein p60 of *L. monocytogenes* ATC 19111 was examined in an indirect immunofluorescence assay with 13 strains of *Listeria*,

representing all species and different serotypes, as well as two rough mutants of *L. monocytogenes*. Twenty seven strains from 16 other genera were also investigated (Table 1). The anti-p60 antiserum (dilution > 1/32) reacted with all *Listeria* strains tested, except *L. seeligeri*, *L. welshimeri*, *L. grayi* and *L. murrayi* (Table 2). The reaction of the rough mutants was quite similar to that of the wild-type strains. With cells of *L. monocytogenes* grown at 26 °C instead of 37 °C a positive, but less intense, reaction was obtained (data not shown). None of the strains from the other genera, except the *Staph. aureus* strains, were labelled in the indirect immunofluorescence assay with anti-p60 antiserum (Table 2).

In a further experiment, cells of *L. monocytogenes* ATCC 19111 harvested in the early (OD_{580} 0.42), middle (OD_{580} 0.95), and late exponential growth phase (OD_{580} 1.90) were examined in the indirect immunofluorescence test. A positive reaction with cells was observed in all cases, although p60 was not detected in culture supernatants of the early and middle exponential growth phase (data not shown). The antiserum prepared against listeriolysin O, however, did not react when tested with *L. monocytogenes* ATCC 19111, although this protein is secreted in large amounts in comparison to p60 (Fig. 1a).

Discussion

Protein p60 was originally detected in the culture supernatant of *L. monocytogenes* by Kuhn & Goebel (1989). Our immunological data provide evidence that a portion of p60 is associated with the cell surface of *L. monocytogenes*. Since the antiserum was elicited with the purified protein together with Freund's complete

adjuvant it could also contain antibodies against fragments of the mycobacterial cell wall causing an unspecific reactivity. This, however, could be excluded because an antiserum raised against purified listeriolysin O, also with Freund's complete adjuvant, did not react with the cell surface of *L. monocytogenes*.

In an early stationary phase culture of *L. monocytogenes* ATCC 19111, about 25% of p60 was cell-associated. Interestingly, the rough mutant *L. monocytogenes* SLCC 5782, which is impaired in the secretion of p60, was found to carry the protein uniformly distributed over the cell surface. Assuming that the quantity of gold particles counted approximately represents the actual number of protein molecules, the maximum density of molecules was identical for both the wild-type strain and the rough variant. Detailed experiments to determine how p60 is attached to the cell surface were not performed. The observation that the protein was not extracted with 5 M-LiCl may suggest that hydrophobic rather than ionic interactions play a prominent role in the attachment.

Kuhn & Goebel (1989) showed that p60 participates in the invasion of nonprofessional phagocytic cells by *L. monocytogenes*. Treatment of a noninvasive rough mutant of *L. monocytogenes* serotype 1/2a with partially purified p60 resulted in restoration of invasiveness. Since the rough mutant and the wild-type strain of *L. monocytogenes* did not differ in the amount of cell-surface-bound p60, the secreted form of the protein seems to be necessary in the invasion of nonprofessional phagocytes.

Recently, Köhler *et al.* (1990, 1991) analysed expression of the gene encoding p60 of *L. monocytogenes* 1/2a EGD and the rough variant *L. monocytogenes* RIII. They found equal amounts of p60-specific mRNA in both the wild-type and the mutant strain, indicating control at the post-transcriptional level by a specific factor that is defective in the mutant strain. According to our data, the reduced expression of p60 in rough mutants only affected the amount of secreted, but not cell surface associated p60. Leimeister-Wächter *et al.* (1992) reported that expression of listeriolysin O and other virulence factors is repressed at lower growth temperatures. We found that the concentration of p60 in the culture supernatant of *L. monocytogenes* ATCC 19111 was lower at 26 °C than at 37 °C, implying thermoregulated expression. Cells grown at the lower temperature also exhibited a less intense reaction with the anti-p60 antiserum, indicating a reduced amount of cell surface associated p60.

Hybridization experiments with the gene encoding protein p60 indicated the presence of homologous DNA sequences in *Listeria* species except *L. grayi* and *L. murrayi* (Köhler *et al.*, 1990; Flamm *et al.*, 1989). Our

indirect immunofluorescence data provide evidence that the immunologically cross-reactive proteins are also exposed at the cell surface. The antiserum reacted significantly with all strains of *L. monocytogenes*, *L. ivanovii* and *L. innocua* investigated but not with the species *L. seeligeri*, *L. welshimeri*, *L. grayi* and *L. murrayi*. These data correlate with the results obtained by immunoblot analysis of the culture supernatants, where *L. grayi* and *L. murrayi* showed no reaction, and *L. seeligeri* and *L. welshimeri* only a very weak reaction. No cross-reactivity of the antiserum was observed with 27 non-*Listeria* strains representing 16 different genera, except *Staph. aureus* strains. The positive reaction of *Staph. aureus* strains, however, is most probably not immunospecific but may be explained by the presence of F_c receptors (protein A) on the cell surface (Forsgren & Forsum, 1970). The anti-p60 antiserum reacted with cells of *L. monocytogenes* grown at both 37 °C and 26 °C, and with bacteria obtained from different growth phases in batch culture. Hence protein p60 seems to be a stably expressed cell surface antigen.

In the last few years, preparations of various *Listeria*-specific monoclonal antibodies have been reported (Butman *et al.*, 1988; McLauchlin *et al.*, 1989; Siragusa & Johnson, 1990), although little is known about the corresponding antigens. However, a monoclonal antibody recognizing a 66 kDa cells surface antigen of *L. monocytogenes* was reported by Bhunia & Johnson (1992). We have now defined protein p60 as a *Listeria*-specific cell-wall-associated antigen. The reaction of the anti-p60 antiserum seems to be restricted to strains belonging to the human and animal pathogenic species *L. monocytogenes* and *L. ivanovii*, except in the case of the avirulent *L. innocua* strains.

We suggest that the antiserum prepared against protein p60 of *L. monocytogenes* is suitable for developing a specific immunoassay (e.g. immunofluorescence test) for detecting *Listeria* strains. Since it is well known that the cell surface composition of bacteria may change with culture conditions (Brown & Williams, 1985), it is necessary to determine whether the anti-p60 antiserum also reacts with *Listeria* strains obtained from environmental samples or foods.

We thank Drs J. Kreft and S. Köhler (Institut für Genetik und Mikrobiologie, Universität Würzburg, FRG) for performing the control immunoblot experiment with culture supernatant of *L. monocytogenes* ATCC 19111 and anti-p60 antiserum derived from the non-haemolytic mutant *L. monocytogenes* M20.

References

- BROWN, M. R. W. & WILLIAMS, P. (1985). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology* **39**, 527-556.
- BUHIA, A. K. & JOHNSON, M. G. (1992). Monoclonal antibody specific for *Listeria monocytogenes* associated with a 66-kilodalton cell

- surface antigen. *Applied and Environmental Microbiology* **58**, 1924–1929.
- BUTMAN, B. T., PLANK, M. C., DURHAM, R. J. & MATTINGLY, J. A. (1988). Monoclonal antibodies which identify a genus-specific *Listeria* antigen. *Applied and Environmental Microbiology* **54**, 1564–1569.
- COSSART, P. & MENGAUD, J. (1989). *Listeria monocytogenes* – a model system for the molecular study of intracellular parasitism. *Molecular Biology and Medicine* **6**, 463–474.
- COTTIN, J., LOISEAU, O., ROBERT, R., MAHAZA, C., CARBONNELLE, B. & SENET, J. M. (1990). Surface *Listeria monocytogenes* carbohydrate-binding components revealed by agglutination with neoglycoproteins. *FEMS Microbiology Letters* **68**, 301–306.
- COWART, R. E., LASHMET, J., MCINTOSH, M. E. & ADAMS, T. J. (1990). Adherence of a virulent strain of *Listeria monocytogenes* to the surface of a hepatocarcinoma cell line via lectin–substrate interaction. *Archives of Microbiology* **153**, 282–286.
- DABIRI, G. A., SANGER, J. M., PORTNOY, D. A. & SOUTHWICK, F. S. (1990). *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6068–6072.
- DOMANN, E., WEHLAND, J., ROHDE, M., PISTOR, S., HARTL, M., GOEBEL, W., LEIMEISTER-WÄCHTER, M., WUENSCHER, M. & CHAKRABORTY, T. (1992). A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO Journal* **11**, 1981–1990.
- FIEDLER, F. & RUHLAND, G. J. (1987). Structure of *Listeria monocytogenes* cell walls. *Bulletin Institute Pasteur* **85**, 287–300.
- FIEDLER, F., SEGER, J., SCHRETTENBRUNNER, A. & SEELIGER, H. P. R. (1984). The biochemistry of murein and cell wall teichoic acids in the genus *Listeria*. *Systematic and Applied Microbiology* **5**, 360–376.
- FLAMM, R. K., HINRICHS, D. J. & THOMASHOW, M. F. (1989). Cloning of a gene encoding a major secreted polypeptide of *Listeria monocytogenes* and its potential use as a species-specific probe. *Applied and Environmental Microbiology* **55**, 2251–2256.
- FORSQREN, A. & FORSUM, U. (1970). Role of protein A in nonspecific immunofluorescence of *Staphylococcus aureus*. *Infection and Immunity* **2**, 387–391.
- GAILLARD, J. L., BERCHE, P. & SANSONETTI, P. (1986). Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. *Infection and Immunity* **52**, 50–55.
- GAILLARD, J.-L., BERCHE, P., FREHEL, C., GOUIN, E. & COSSART, P. (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* **65**, 1127–1141.
- HOF, H. (1990). Listerien – eine Herausforderung für die Diagnostik. *Immunität und Infektion* **18**, 35–39.
- KATHARIOU, S., METZ, P., HOF, H. & GOEBEL, W. (1987) Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *Journal of Bacteriology* **169**, 1291–1297.
- KOCKS, C., GOUIN, E., TABOURET, M., BERCHE, P., OHAYON, H. & COSSART, P. (1992). *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. *Cell* **68**, 521–531.
- KÖHLER, S., LEIMEISTER-WÄCHTER, M., CHAKRABORTY, T., LOTTSPEICH, 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.
- KÖHLER, S., BUBERT, A., VOGEL, M. & GOEBEL, W. (1991). Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. *Journal of Bacteriology* **173**, 4668–4674.
- KUHN, M. & GOEBEL, W. (1989). Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. *Infection and Immunity* **57**, 55–61.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEIMEISTER-WÄCHTER, M., DOMANN, E. & CHAKRABORTY, T. (1992). The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. *Journal of Bacteriology* **174**, 947–952.
- MCLAUCHLIN, J., RIDLEY, A. M. & TAYLOR, A. G. (1989). The use of monoclonal antibodies against *Listeria monocytogenes* in a direct immunofluorescence technique for the rapid presumptive identification and direct demonstration of *Listeria* in food. *Acta Microbiologica Hungarica* **36**, 467–471.
- RUHLAND, G. J. & FIEDLER, F. (1987). Occurrence and biochemistry of lipoteichoic acids in the genus *Listeria*. *Systematic and Applied Microbiology* **9**, 40–46.
- 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. & DUBRAY, G. (1992). Analysis of surface proteins of *Listeria* in relation to species, serovar and pathogenicity. *Journal of General Microbiology* **138**, 743–753.
- TILNEY, L. G. & PORTNOY, D. A. (1989). Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *Journal of Cell Biology* **109**, 1597–1608.
- TOWBIN, H., STAEBELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- WALKER, P. D., BATTY, I. & THOMSON, R. O. (1971). The localization of bacterial antigens by the use of the fluorescent and ferritin labelled antibody techniques. *Methods in Microbiology* **5A**, 219–254.
- WANG, R.-F., CAO, W.-W. & JOHNSON, M. G. (1991). Development of a 16S rRNA-based oligomer probe specific for *Listeria monocytogenes*. *Applied and Environmental Microbiology* **57**, 3666–3670.
- WANNER, G., FORMANEK, H., GALLI, D. & WIRTH, R. (1989). Localization of aggregation substances of *Enterococcus faecalis* after induction by sex pheromones. *Archives of Microbiology* **151**, 491–497.