Letters in Applied Microbiology 2000, **31**, 319–322

Cellular morphology of rough forms of *Listeria monocytogenes* isolated from clinical and food samples

N.J. Rowan, J.G. Anderson and A.A.G. Candlish

Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow, UK

172/2000: received 14 April 2000, revised 23 June 2000 and accepted 27 June 2000

N.J. ROWAN, J.G. ANDERSON AND A.A.G. CANDLISH. 2000. Transmission electron microscopy (TEM) studies revealed that rough cell-forms of *L. monocytogenes* (designated FR variants), isolated from clinical and food samples (and under conditions of sublethal heat stress), consist of either single or paired long-filaments. These FR variants markedly contrast in cell morphology from other previously described avirulent rough-mutants of *L. monocytogenes* that form long chains consisting of multiple cells of similar size (designated MCR variants). The identity of these *Listeria* isolates was determined using a commercially available, anti-*Listeria* polyclonal KPL antibody and by the API *Listeria* biochemical gallery. This study shows that filamentous rough-forms of *L. monocytogenes* may occur in clinical and food samples that are of undetermined pathogenicity.

INTRODUCTION

Listeria monocytogenes is a facultative intracellular bacterial pathogen responsible for serious disease in immunocompromised individuals and pregnant women (McLauchlin 1997). Epidemiological observations and electron microscopic studies of tissues of infected guinea pigs (Racz *et al.* 1972) provided evidence that the gastrointestinal tract is an important route of infection and that the epithelial cells of the intestine may be the primary site of entry for these bacteria.

Spontaneously occurring, rough mutants of *L. monocytogenes* secreting greatly reduced levels of a 60-kDa major extracellular housekeeping-protein (termed p60) and forming long chains of cells were previously described (designated MCR variants in this study) (Kathariou *et al.* 1987; Kuhn and Goebel 1989). This p60 protein is required for normal cell division (Bubert *et al.* 1992), and is transcribed independently of the central virulence regulator PrfA (Chakraborty *et al.* 1992). Although septum formation still occurs, separation of the divided cells does not take place (Wuenscher *et al.* 1993).

MCR variants were shown to have reduced virulence in the mouse model of infection and did not efficiently invade mouse 3T6-fibroblasts (Kuhn and Goebel 1989). Treatment of MCR variants with partially purified cell-free p60 led to disaggregation of cell chains to normal-sized single bacteria with restored invasiveness (Kathariou *et al.* 1987; Kuhn and Goebel 1989). Thus, for these MCR variants, cell-free p60 not only causes decay of cell chains but participates actively in the invasion process. We recently described atypical rough cell forms of *L. monocytogenes* from clinical and food samples that showed wild-type levels of adherence, invasion and cytotoxicity to human epithelial HEp-2 and HeLa cells (Rowan *et al.* 1999). Here, we show that these invasive rough forms of *L. monocytogenes* consist of single or paired long-filaments (designated FR variants).

Correspondence to: N.J. Rowan, Department of Bioscience and Biotechnology, University of Strathclyde, Royal College Bldng, 204 George St, Glasgow G1 1XW, UK (e-mail: n.j.rowan@strath.ac.uk).

MATERIALS AND METHODS

Bacterial strains

The *L. monocytogenes* strains used in the study were, if not otherwise indicated, derived or obtained from the Special *Listeria* Culture Collection [SLCC] of H. P. R. Seeliger, Würzburg, Germany, or from the National Collection of Type Cultures [NCTC], Public Health Laboratory Service [PHLS], Central Public Health Laboratory, Colindale, London, UK. (Table 1). Two auto-agglutinable blood culture and food isolates of *L. monocytogenes* exhibiting a rough phenotype were obtained from Dr Jim McLauchlin, Food Safety Microbiology Laboratory, PHLS, Colindale, London, UK. The clinical strains PHLRIII and PHLRIV were blood-culture isolates from a 76 and 72 years-old female and male, respectively; both individuals had sepsis and pyrexia. The spontaneously rough variants *L. monocytogenes* RI, RII and RIII were kindly supplied by Dr Andreas Bubert, Microbiological Analytics, Merck KGaA, 64271 Darmstadt, Germany. Where the rough variants RI and RII were previously derived from *L. monocytogenes*

Table 1 *L. monocytogenes* strains used

Strain	Serotype	Reference No.	Origin	Cell morphology		ELISA (A_{492})¶ KPL pAb
				Form	Designation	
S1	4b	NCTC 11994	Adult meningitis	Single, paired cells	WT smooth	0·56 ± 0·12
S2	4b	NCTC 9863	Infantile meningitis	Single, paired cells	WT smooth	0·82 ± 0·16
RI‡	1/2a	SLCC 5764	Kathariou *et al.* (1987)	Long cell chains	MCR variant	0·64 ± 0·05
RII§	1/2a		Kathariou *et al.* (1987)	Long cell chains	MCR variant	0·78 ± 0·12
RIII	1/2a	SLCC 5779	J. Potel (see text)	Long cell chains	MCR variant	0·39 ± 0·06
PHLRII		L7071	Dried custard powder	Long cell chains	MCR variant	1·23 ± 0·16
SURI*	4b		Rowan and Anderson (1998)	Long filaments	FR variant	0·62 ± 0·06
SURII†	4b		Rowan and Anderson (1998)	Long filaments	FR variant	0·93 ± 0·15
PHLRI		L6705	Dried custard powder	Long filaments	FR variant	1·05 ± 0·14
PHLRIII		L7346	Blood culture	Long filaments	FR variant	1·23 ± 0·16
PHLRIV		L1342	Blood culture	Long filaments	FR variant	1·16 ± 0·19

*Derived from *L. monocytogenes* NCTC 11994.
†Derived from *L. monocytogenes* NCTC 9863.
‡Derived from *L. monocytogenes* Mackaness (SLCC 5764).
§Derived from *L. monocytogenes* EGD
¶O.D.$_{492}$ values greater than 0·1 were considered a positive result. BHI broth controls gave a value of 0·03 ± 0·01.

Mackaness and EGD, respectively (Kuhn and Goebel 1989). Strain RII (SLCC 5779), was originally obtained from J. Potel (Institute for Medical Microbiology, Medical Academy, Hannover, Germany). *L. monocytogenes* RIII, another rough mutant derived from a smooth strain of serovar 1/2a, was obtained from J. Potel (Institute for Medical Microbiology, Medical Academy, Hannover, Germany). The rough variants SURI and SURII were derived from parent S1 and S2 strains, respectively, under conditions of heat stress (Rowan and Anderson 1998). Stored bacteria were kept at −70 °C in phosphate-buffered saline (PBS) with 20% glycerol (v/v) until used.

Biochemical and physiological methods

Catalase production was determined by applying a drop of 30% H_2O_2 to the colonies and observing the occurrence of O_2 bubbles, as described elsewhere (Bubert *et al.* 1997). The CAMP test was performed using standard procedures by streaking out bacteria perpendicular to *Staphylococcus aureus* on 5% sheep blood agar plates and observing zones of augmented haemolysis, as described elsewhere (Bubert *et al.* 1997). Characteristic blue-green sheen from colonies by obliquely transmitted light and tumbling motility of *Listeria* cells were determined as described elsewhere (Rowan and Anderson 1998). The commercial biochemical API *Listeria* test (bioMerieux, Marcy l'Etoile, France) was used according to the manufactures instructions.

Indirect ELISA

Detection of heat stable antigens in cell-free supernatants was achieved by indirect ELISA using an anti-*Listeria* affinity-purified polyclonal antibody that was obtained from Kirkegaard and Perry Laboratories (KPL, Gaithersburg, Maryland) in a lyophilized form. Preparation of antigen from overnight cultures involved centrifugation followed by the addition of 100 μl of supernatant per well of microtitre plates and incubation for 2 h at 37 °C. Antigen-coated plates were washed three times with wash buffer and the KPL-antibody was added at a dilution of 1/1000 (v/v) in wash buffer and incubated overnight for 1 h at room temperature. Unbound antibody was removed by washing three times with wash buffer, and rabbit antigoat horseradish peroxidase conjugate (Sigma) was added at 100 μl/well with a dilution of 1/1000 (v/v) in wash buffer and incubated for 1 h at room temperature. Excess conjugate was washed five times with wash buffer and the substrate, Sigma FAST^{TM} OPD tablets (Sigma), were added at 100 μl/well with 0·5 h incubation at room temperature. The A_{492nm} was measured after the addition of 50 μl per well 3 mol l^{-1} H_2SO_4.

Transmission electron microscopy (TEM)

Cells were grown to mid-log phase in brain heart infusion broth, washed twice with PBS and resuspended in sterile-distilled water before application to formvar-coated grids.

After the grid was dried, one drop of a solution containing 3% v/v tungstophosphoric acid and 0·3% v/v sucrose (pH 6·8–7·4) was added. The solution was removed after 30–60 s, and the grid was dried and examined on a Zeiss 902 transmission electron microscope.

RESULTS AND DISCUSSION

Due to the severity of listeriosis in predisposed-individuals, the identification of atypical rough cell-forms of *L. monocytogenes* in clinical or food samples (which are of undetermined pathogenicity) is of clinical relevance (McLauchlin 1997; Rowan 1999). All of the bacterial strains described in Table 1 were previously identified as *L. monocytogenes* by establishing characteristic morphological, physiological and biochemical properties associated with this bacterial pathogen and by analysing secretions of p60 in culture supernatants by indirect ELISA using a *L. monocytogenes*-specific antip60 monoclonal antibody (Rowan *et al*. 1999). The use of this *L. monocytogenes* p60-specific mAb for the unequivocal identification of this species has been previously demonstrated (Bubert *et al*. 1997). In this study, we have shown that these rough isolates of *L. monocytogenes* can also be identified by indirect ELISA using the commercially available anti-*Listeria* KPL pAb (Table 1) and with the API *Listeria* biochemical galleries.

Unlike typical wild-type smooth (S) *L. monocytogenes* strains that are characteristically 'coccobacillus' in cell appearance (approximately 2 μm in length), cell types associated with the rough or R variants were previously shown to be atypically long, measuring up to approximately 100 μm in length (Rowan *et al*. 1999). In the present study, we have shown that some R variants consisted of unseptated or paired-filaments (designated FR variants), whereas others formed long chains that consisted of multiple cells of similar size (designated MCR variants) (Table 1). Rough variants isolated from clinical specimens and food samples, or derived under conditions of heat stress predominately showed a FR-filamentous phenotype (Fig. 1a). Whereas spontaneously occurring *L. monocytogenes* RI, RII and RIII, and the food sample isolate PHLRII, exhibited a MCR-cell phenotype (Fig. 1b), which confirmed previous observations (Kuhn and Goebel 1989). MCR and FR variants were previously shown to be incapable of characteristic tumbling motility, and formed irregular or rough colonies that no longer produced a blue-green sheen upon oblique illumination (Rowan *et al*. 1999).

Here, we have shown that FR variants of *L. monocytogenes* differ from the wild-type smooth or S form of *L. monocytogenes* by forming single or paired filaments (Fig. 1a). Unlike MCR variants, the filamentous forms are not impaired in the synthesis of the major extracellular protein p60 (Rowan *et al*. 1999) which is required for a late step in cell division (Kuhn and Goebel 1989). We have previously shown that exposure of wild-type smooth forms of *L. monocytogenes* to environmental stress conditions, such as heat shock and growth at above-optimal temperatures, resulted in the generation of atypical cell forms of *Listeria* with a FR phenotype (Rowan and Anderson 1998; Rowan 1999). It has also been observed that long filamentous forms of *L. monocytogenes* with a rough phenotype similar

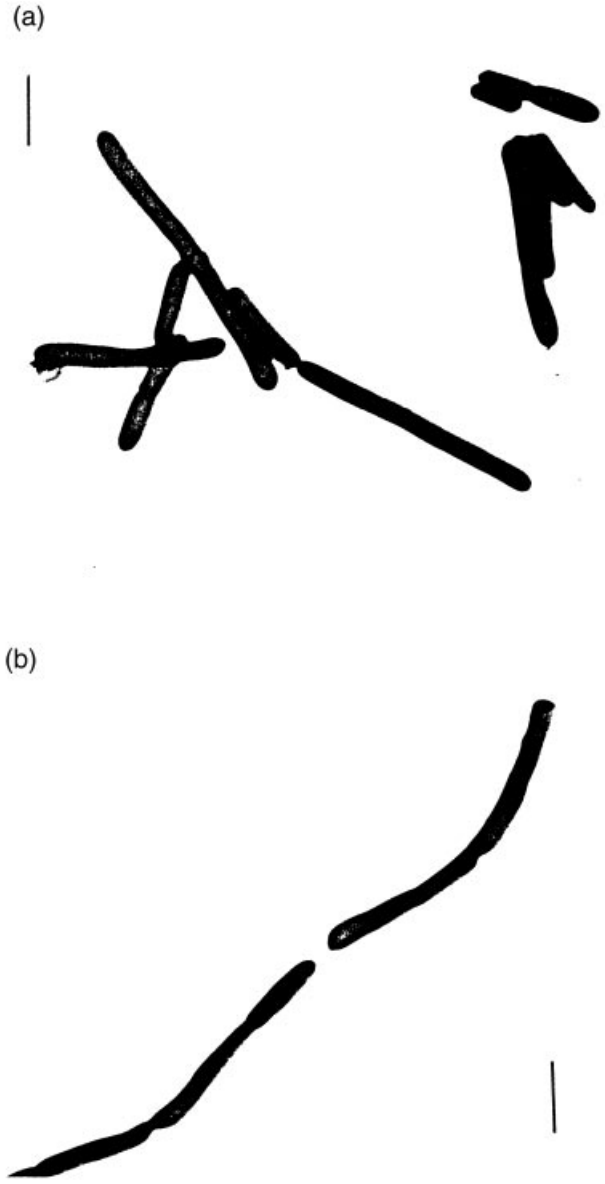

Fig. 1 Transmission electron micrographs of FR variant *L. monocytogenes* PHLRI (a) and MCR variant *L. monocytogenes* RI (b). Bar, 4 μm.

to that of lactobacilli or filamentous forms with a smooth phenotype can appear, in the latter case under the influence of suboptimal antibiotic concentrations (cited in Bubert *et al*. 1997).

In conclusion, rough cell-forms of *L. monocytogenes*, obtained from clinical and food samples and demonstrating wild-type levels of invasiveness in HEp-2 and HeLa epithelial cell lines (Rowan *et al*. 1999), were shown to consist of atypical paired or single filaments by TEM. While these rough forms of *L. monocytogenes* possess some unusual physiological and morphological properties, these variants can be identified by conventional biochemical and serological tests.

ACKNOWLEDGMENTS

We thank the University of Strathclyde for funding this research. We also thank Dr Jim McLauchlin (Food Safety Microbiology Laboratory, Central Public Health Laboratory Service, Colindale, London, UK) and Dr Andreas Bubert (Microbiological Analytics, Merck KGaA, 64271 Darmstadt, Germany) for the kind gift of rough *L. monocytogenes* strains.

REFERENCES

Bubert, A., Kuhn, M., Goebel, W. and Köhler, S. (1992) Structural and functional properties of the p60 proteins from different *Listeria* species. *Journal of Bacteriology* **174**, 8166–8171.

Bubert, A., Riebe, J., Schnitzler, N., Schönberg, A., Goebel, W. and Schubert, P. (1997) Isolation of catalase-negative *Listeria monocytogenes* strains from listeriosis patients and their rapid identification by anti-p60 antibodies and/or PCR. *Journal of Clinical Microbiology* **35**, 179–183.

Chakraborty, T., Leimeister-Wächter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein, T. and Notermans, S. (1992) Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *Journal of Bacteriology* **174**, 568–574.

Kathariou, S., Hacker, J., Hof, H., Then, I., Wagner, W., Kuhn, M. and Goebel, W. (1987) Bacterial cytotoxins – extracellular proteins and virulence factors. In *Molecular Basis of Viral and Microbial Pathogenesis* ed. Rott, R. and Goebel, W. pp. 141–150. Berlin: Springer-Verlag.

Kuhn, M. and 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.

McLauchlin, J. (1997) The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Reviews in Medical Microbiology* **8**, 1–14.

Racz, P., Tenner, K. and Mero, E. (1972) Experimental *Listeria* enteritis. 1. An electron microscopic study of the epithelial phase in experimental *Listeria* infection. *Laboratory Investigations* **26**, 694–700.

Rowan, N.J. (1999) Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. *Trends in Food Science and Technology* **10**, 261–270.

Rowan, N.J. and Anderson, J.G. (1998) Effects of above-optimum growth temperature and cell morphology on thermotolerance of *Listeria monocytogenes* cells suspended in bovine milk. *Applied and Environmental Microbiology* **64**, 2065–2071.

Rowan, N.J., Bubert, A. and McLauchlin, J. (1999) Characterisation of virulent atypical cell forms of *Listeria monocytogenes* from the clinical environment secreting wild-type levels of p60. *Federation of Infection Societies, 6th International Conference*, Manchester, 1–3 December, p.45.

Wuenscher, M.D., Köhler, S., Bubert, A., Gerike, U. and Goebel, W. (1993) The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. *Journal of Bacteriology* **175**, 3491–3501.