

Short Communication

Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes

Dongyou Liu,¹ A. Jerald Ainsworth,¹ Frank W. Austin² and Mark L. Lawrence¹

Correspondence
Mark L. Lawrence
lawrence@cvm.msstate.edu

Department of Basic Sciences¹ and Department of Pathobiology and Population Medicine², College of Veterinary Medicine, Mississippi State University, Mississippi State, MS, 39762-6100, USA

Listeria monocytogenes is an opportunistic bacterial pathogen that is an important cause of human food-borne illness worldwide. However, *L. monocytogenes* strains demonstrate considerable variation in pathogenic potential. In this report, virulent and avirulent *L. monocytogenes* isolates were compared by using a comparative screening strategy. Two clones were identified that contained DNA that was only present in virulent *L. monocytogenes* strains. PCR primers were designed for three genes from these clones and for five other selected *L. monocytogenes* genes. All eight primer sets predominantly detected virulent *L. monocytogenes* isolates, as determined by a mouse virulence assay; one of the putative internalin genes, *lmo2821*, was detected in all strains that were considered to be virulent. Primers from these eight genes were then tested by PCR against a larger panel of bacterial strains; each of the genes was detected predominantly in clinical or food *L. monocytogenes* isolates, rather than environmental isolates. The findings from this study suggest that virulent *L. monocytogenes* strains may possess genes that are not present in avirulent isolates, which could serve as markers for PCR assessment of *L. monocytogenes* virulence.

Received 20 June 2003

Accepted 8 September 2003

Introduction

Listeria monocytogenes is an important food-borne pathogen that can cause septicaemia, encephalitis, meningitis and gastroenteritis, particularly in children, the elderly and immunosuppressed individuals; it also causes miscarriage in pregnant women (Robinson *et al.*, 2000). Despite being pathogenic at the species level, *L. monocytogenes* in fact comprises a diversity of strains with varying virulence. Whilst many *L. monocytogenes* strains have pathogenic potential and can result in disease and/or mortality, others have limited capability to establish infection and are relatively avirulent (Barbour *et al.*, 1996, 2001; Erdenlig *et al.*, 2000; Olier *et al.*, 2002; Roche *et al.*, 2003). The ability to distinguish virulent from avirulent strains of *L. monocytogenes* could potentially reduce unnecessary recalls of food products and help to prevent disease outbreaks.

Methods that have been developed for *L. monocytogenes* virulence assessment include mouse virulence assays and *in vitro* culture techniques. The mouse virulence assay provides an *in vivo* measurement of virulence and often serves as a reference standard for other methods (Pine *et al.*, 1991;

Nishibori *et al.*, 1995; Erdenlig *et al.*, 2000; Roche *et al.*, 2001). *In vitro* culture techniques measure the ability of *L. monocytogenes* to cause cytopathogenic effects in the enterocyte-like cell line Caco-2 (Pine *et al.*, 1991), to form plaques in the human adenocarcinoma cell line HT-29 (Roche *et al.*, 2001) or to cause death in chicken embryos (Olier *et al.*, 2002). Although they are somewhat slow and technically demanding, these methods have good predictive value.

Unfortunately, no reliable molecular method for prediction of *L. monocytogenes* virulence has been developed. PCR detection of known *L. monocytogenes* virulence genes, such as *inlA*, *inlB*, *actA*, *hlyA*, *plcA* and *plcB*, has not proven suitable for differentiation of virulent and avirulent isolates (Nishibori *et al.*, 1995), as these genes are consistently found in *L. monocytogenes* (Jaradat *et al.*, 2002). Genetic lineage analysis has some predictive value for *L. monocytogenes* pathogenicity; however, the correlation between genetic lineages and pathogenicity is not consistent for all strains (Piffaretti *et al.*, 1989; Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997).

In the current study, we tested PCR primers that were derived from a panel of eight *L. monocytogenes* genes on 12 strains with known virulence and on a larger panel that included clinical, food and environmental isolates. We report evidence

Abbreviations: ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

Table 1. Bacterial strains used in this study

Strain	Serovar	Source
<i>Listeria monocytogenes</i> :		
ATCC 19111	1	Poultry
ATCC 19112	2	Human
ATCC 19113	3	Human
ATCC 19114	4a	Human
ATCC 19115	4b	Human
ATCC 19116	4c	Chicken
ATCC 19117	4d	Sheep
ATCC 19118	4e	Chicken
ATCC 15313 ^T	1	Rabbit
EGD (NCTC 7973)	1/2a	Guinea pig
HCC7	1	Catfish brain
HCC8	1	Catfish brain
HCC12	4	Catfish brain
HCC13	4	Catfish kidney
HCC16	4	Catfish brain
HCC17	4	Catfish brain
HCC18	4	Catfish spleen
HCC19	4	Catfish spleen
HCC23	4	Catfish brain
HCC24	4	Catfish spleen
HCC25	4	Catfish kidney
168		Aborted calf fetus
180		Human outbreak
418		Freezer study
742		Ground beef
874		Cow brain
1002		Pork sausage
1084		Chicken
1400		Jalisco outbreak
<i>Listeria innocua</i> :		
ATCC 33090 ^T	6a	Cow brain
415		Turkey burger
416		Veal/beef patty
417		Beef steak
662		Raw milk
1419		Ground cheese
1425		Pecorino romano
1720		Chicken
1944		Ground turkey
<i>Listeria grayi</i> :		
ATCC 19120 ^T		Chinchilla faeces
ATCC 25400		Corn leaves/stalks
ATCC 25401		Corn leaves/stalks
<i>Listeria ivanovii</i> :		
ATCC 19119 ^T		Sheep
3325		Cheese
<i>Listeria seeligeri</i> :		
ATCC 35967 ^T		Soil
3008		Soil
3321		Cheese
<i>Listeria welshimeri</i> :		
ATCC 35897 ^T		Plant
ATCC 43550	1/2b	Soil

ATCC 43551	6a	Soil
CCF4		Catfish brain
1471		Environment
<i>Aeromonas hydrophila</i> ATCC 35654		
<i>Clostridium perfringens</i>		
<i>Enterococcus faecalis</i> ATCC 29212		
<i>Escherichia coli</i> ATCC 25922		
<i>Flavobacterium indologenes</i>		
<i>Klebsiella pneumoniae</i> ATCC 13883 ^T		
<i>Proteus vulgaris</i> ATCC 13315 ^T		
<i>Pseudomonas aeruginosa</i> ATCC 27853		
<i>Salmonella typhimurium</i> ATCC 14028		
<i>Serratia marcescens</i> ATCC 8100		
<i>Staphylococcus aureus</i> ATCC 25923		
<i>Streptococcus pneumoniae</i>		
<i>Streptococcus pyogenes</i> ATCC 19615		
<i>Vibrio cholerae</i>		
<i>Yersinia pseudotuberculosis</i>		

that virulent *L. monocytogenes* isolates contain genes that could potentially be used to differentiate virulent and avirulent isolates.

Methods

Bacterial strains and genomic DNA extraction. In total, 52 *Listeria* strains and 15 other common Gram-positive and -negative species were examined (Table 1). *Listeria* strains were cultivated on tryptic soy agar plates with 5 % sheep blood or brain heart infusion (BHI) agar plates. Batch cultures were cultivated in BHI broth at 37 °C with rotary aeration. Genomic DNA was isolated from *L. monocytogenes* and other species by using a previously described technique (Liu *et al.*, 2003).

Dot-blot hybridization. A recombinant genomic DNA library of *L. monocytogenes* strain EGD was constructed in pGEM-3Zf(+) (Promega) as described previously (Liu & Yong, 1993; Liu, 1994). Cloned inserts were retrieved from the plasmid vector by using appropriate restriction enzymes (e.g. *EcoRI* and *PstI* or *SmaI* and *HindIII*) for use as probes. Each insert was screened against a panel of six *L. monocytogenes* strains, which consisted of three virulent strains (ATCC 19115, EGD and HCC7) and three avirulent strains (ATCC 15313^T, HCC23 and HCC25) by dot-blot hybridization (Liu, 1994; Liu & Yong, 1993). Approximately 300 clones were screened and clones that showed preferential binding to *L. monocytogenes* virulent strains were selected. Plasmid DNA from the selected clones was purified by using a QIAprep Spin Miniprep kit (Qiagen); DNA sequences of the inserts were determined by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Mouse virulence assay. The virulence of 12 *L. monocytogenes* strains was assessed by using a mouse virulence assay (Erdenlig *et al.*, 2000). Female, 6–8-week-old A/J mice (Jackson Laboratory) were housed (five mice per cage) and allowed to acclimatize for 1 week. For each *L. monocytogenes* strain, four groups of mice were inoculated intraperitoneally with 0·1 ml aliquots of appropriate dilutions of bacteria. One group of five mice was injected with 0·1 ml sterile saline and one group of three mice was not injected. On the fifteenth day after inoculation, all surviving mice were euthanized and one mouse per group was necropsied and cultured from the spleen for *L. monocytogenes*. The LD₅₀ for each strain was then calculated (Reed & Muench, 1938).

PCR. Each reaction was performed in a 25 µl volume of reaction mixture that consisted of 0.5 U *Taq* DNA polymerase (Fisher Scientific), 50 µM dNTPs, 25 pmol each primer and 10 ng DNA. Cycling conditions consisted of 94 °C for 2 min, 25 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 45 s, and finally 72 °C for 2 min.

Results and Discussion

Identification of genes present in virulent *L. monocytogenes*

As a result of comparative screening against a panel of six *L. monocytogenes* strains of known virulence, two clones (lmo2–28 and lmo2–432) that hybridized predominantly with virulent isolates were selected from the recombinant DNA library of *L. monocytogenes* EGD (data not shown).

Clone lmo2–28 contained an insert of approximately 600 bp. DNA sequencing revealed that the first 458 bp corresponded to the genome sequence of *L. monocytogenes* EGD-e (serovar 1/2a) (GenBank accession no. AL591976) at positions 224 432–224 889. Therefore, the clone contained portions of two genes from the *L. monocytogenes* EGD-e chromosome: one encodes a protein similar to transcriptional regulators (*lmo0833*; bp 223 784–224 730) and the other encodes a protein of unknown function (*lmo0834*; bp 224 810–225 537) (Glaser *et al.*, 2001) (Table 2). Neither of these genes has an orthologue in the genome of *Listeria innocua* strain CLIP 11262 (serovar 6a) (Glaser *et al.*, 2001). PCR primers were designed to both genes.

The insert from clone lmo2–432 was approximately 900 bp in length. The first 420 bp of this insert was sequenced; it correlated to the genome sequence of *L. monocytogenes* EGD-e (GenBank accession no. AL591978) at positions 54 835–55 247. Therefore, this clone contained portions of a gene that encodes an unknown protein (*lmo1188*; bp 53 636–55 087) and a gene that encodes a putative transcriptional regulator (*lmo1189*; bp 55 085–55 679). *lmo1188* has no orthologue in the *L. innocua* CLIP 11262 genome; on the other hand, *lmo1189* does possess such an orthologue (Glaser *et al.*, 2001). Therefore, primers were designed to *lmo1188*.

These findings suggest that transcriptional regulator genes might serve as useful virulence markers for *L. monocytogenes*. Therefore, nucleotide–nucleotide BLAST searches were conducted to compare the gene sequences of *L. monocytogenes* transcriptional regulators against the genome sequence of *L. innocua* (Glaser *et al.*, 2001); three genes (*lmo2672*, *lmo1116* and *lmo1134*), which had the lowest nucleotide identity with any sequences from the *L. innocua* genome, were selected from the published *L. monocytogenes* EGD-e transcriptional regulator list. In addition, as internalins play important roles in listerial internalization and virulence (Gaillard *et al.*, 1991; Lingnau *et al.*, 1995; Parida *et al.*, 1998), we selected two genes (*lmo2470* and *lmo2821*) that encode internalin-like proteins from the *L. monocytogenes* EGD-e genome sequence, which do not have orthologues in the *L. innocua* genome (Glaser *et al.*, 2001).

Table 2. List of *L. monocytogenes* genes and primers used in the PCR assay

Gene	Genome segment/location	Putative function	Size (aa)	Primer sequences (5'→3')	Primer position	Size of PCR product (bp)
<i>lmo0833</i>	4/223 784–224 730	Transcriptional regulator	296	GGCTATTCCTTACGGGAGGA AGTAGCGCGAGGGATTGTGA	223 996–224 015 224 633–224 613	638
<i>lmo1188</i>	6/53 621–55 087	Unknown protein	483	TTTCGCCGTTAGAAAATACGA TTCGGACAAAAATTGGAATGG	54 027–54 047 54 689–54 668	663
<i>lmo0834</i>	4/224 810–225 537	Unknown protein	237	AACTTCGCATTGTATGTGTAC TCACTGACCATTCCTCCAAA	224 940–224 963 225 533–225 513	594
<i>lmo1116</i>	5/262 997–263 783	Transcriptional regulator	257	GGGAACGATGAAAAACGAAGA TGGCTTATCGCACAAAGCTAAT	263 006–263 025 263 593–263 573	591
<i>lmo2672</i>	12/25 985–26 804	Transcriptional regulator	268	CGGCACACTGGATTCTCAT AGGGCTAGTGACGGATGCTA	26 117–26 136 26 597–26 578	481
<i>lmo1134</i>	6/8 009–8 368	Transcriptional regulator	115	ACCCGATAGCAAGGAGGAAC AACTCTCTCGATACCCATCCA	7 998–8 017 8 364–8 343	367
<i>lmo2470</i>	11/149 254–150 433	Internalin	388	TGATTCCATGCAATTACTAGAACG AGGATTCTAAACTAGTAAAGTTGGTG	149 527–149 550 150 071–150 046	545
<i>lmo2821</i>	12/188 153–190 708	Internalin	851	TGTAACCCCGCTTACACAGTT TTACGGCTGGATTGTCTGTG	188 989–189 009 189 599–189 580	611

Mouse virulence assay

Previous mouse virulence testing had shown that strains EGD, ATCC 19115 and HCC7 are virulent, but HCC23 and ATCC 15313^T are avirulent (Erdenlig *et al.*, 2000). In the current study, three of these strains were tested as controls (EGD, ATCC 19115 and ATCC 15313^T) and nine additional strains were assessed (Table 3). Strains EGD and 874 were the most virulent of the strains tested (LD₅₀ < 10⁸); ATCC 19115, ATCC 19116, ATCC 19117, HCC8 and 1002 were the next most virulent strains (LD₅₀ < 10⁹). Strains ATCC 19112 and ATCC 19118 had intermediate virulence (LD₅₀ < 10¹⁰) and strains ATCC 19114, HCC25 and ATCC 15313^T were considered to be avirulent (LD₅₀ > 10¹⁰).

Spleens from mice that died during the challenge contained viable *L. monocytogenes* that was recovered on BHI agar and confirmed by PCR. On the other hand, spleens from mice that survived the *L. monocytogenes* challenge by day 15 had no viable *L. monocytogenes* detectable on BHI agar (data not shown).

PCR

Results of PCR with oligonucleotide primers that were designed from the assembled panel of eight potential virulence genes (Table 2) correlated well with the mouse virulence results: avirulent strains ATCC 19114 and HCC25 (LD₅₀ > 10¹⁰) were non-reactive by PCR, whereas the other strains (LD₅₀ < 10¹⁰) were all PCR-positive for at least two of the primer sets tested (Table 4). In particular, primers derived from *lmo2821* permitted identification of all virulent *L. monocytogenes* strains in our panel. PCR results also correlated with two previously tested strains (Erdenlig *et al.*, 2000): HCC7, which is virulent by mouse assay, was PCR-positive for all primer sets tested, and HCC23, which is avirulent by mouse assay, was PCR-negative for all primer sets tested.

One notable exception was ATCC 15313^T, which was posi-

tive for seven of the eight genes tested by PCR, but was avirulent by mouse virulence assay. Strain ATCC 15313^T was originally isolated from an infected rabbit during an outbreak of listeriosis, but it later became avirulent after successive laboratory subculturing (Kathariou & Pine, 1991). It has been demonstrated that ATCC 15313^T does not express listeriolysin, a well-known virulence factor (Gaillard *et al.*, 1986), and it has been proposed that this occurred spontaneously after its original isolation (Kathariou & Pine, 1991). Therefore, it would be logical that ATCC 15313^T, which is derived from a virulent *L. monocytogenes* isolate, would retain many of its virulence properties. In support of this, ATCC 15313^T has maintained its ability to cause cytopathogenic effects in Caco-2 monolayers (Kathariou & Pine, 1991).

The eight primer sets were then evaluated by PCR against a larger collection of bacterial strains (52 *Listeria* strains and 15 common Gram-positive and -negative species; Table 4). At least two PCR primer sets were positive for 19 of the 29 *L. monocytogenes* strains tested. The remaining 10 *L. monocytogenes* strains were not detected by any of the primers tested. The 19 isolates that were recognized were primarily clinical or food isolates, whereas the negative strains were primarily catfish isolates. The catfish isolates were obtained from healthy channel catfish during routine screening for potential food-borne pathogens. Therefore, they are considered to be environmental isolates that could be isolated from normal catfish as they enter the processing plant. The one PCR-negative strain that was not isolated from catfish was ATCC 19114, which is a human isolate. However, mouse assay confirmed that this strain is avirulent. All eight primer sets generated no PCR products with genomic DNA from other *Listeria* species or the other 15 species tested.

Putative internalin genes were detected in most *L. monocytogenes* strains in our panel, with *lmo2821* being detected in 19 of 29 strains tested and *lmo2470* being present in 18 of 29 strains. In particular, strain 874 was highly virulent in mice, yet it was PCR-positive for the internalin genes and negative for all transcriptional regulator genes in our panel. The putative transcriptional regulator genes *lmo2672* and *lmo1134* were detected in 17 of 29 strains. Genes *lmo1116*, *lmo0834*, *lmo0833* and *lmo1188* were detected in 15, 15, 14 and 13 of 29 strains, respectively.

In summary, our results provide evidence that virulent *L. monocytogenes* isolates contain genes that are not present in avirulent *L. monocytogenes*, and that detection of these genes has the potential to provide an alternative method for distinguishing virulent from avirulent isolates. Further testing on a larger panel of *L. monocytogenes* strains with known virulence will determine whether this technique has the potential to serve as a diagnostic assay.

Acknowledgements

This research was supported by Agricultural Research Service/US Department of Agriculture Agreement no. 58-6202-5-083. We thank Dr Catherine Donnelly of the Department of Nutrition and Food

Table 3. Mouse virulence trial results

<i>L. monocytogenes</i> strain	Serovar	LD ₅₀
ATCC 19112	2	1·6 × 10 ⁹
ATCC 19114	4a	1·9 × 10 ¹⁰
ATCC 19115	4b	6·0 × 10 ⁸
ATCC 19116	4c	2·6 × 10 ⁸
ATCC 19117	4d	8·8 × 10 ⁸
ATCC 19118	4e	7·8 × 10 ⁹
ATCC 15313 ^T	1	> 1·2 × 10 ¹¹
EGD	1/2a	< 1·1 × 10 ⁷
HCC8	1	< 7·0 × 10 ⁸
HCC25	4	3·5 × 10 ¹⁰
874	ND	< 8·0 × 10 ⁷
1002	ND	5·2 × 10 ⁸

ND, Not determined.

Table 4. PCR results using primers derived from eight putative *L. monocytogenes* transcriptional regulator and internalin genesResults shown are for *L. monocytogenes* strains only; all other *Listeria* strains and other bacterial species were negative for all eight primer pairs.

Strain	<i>lmo0833/lmo1188</i> *	<i>lmo0834</i>	<i>lmo1116</i>	<i>lmo2672/lmo1134</i> †	<i>lmo2470</i>	<i>lmo2821</i>
ATCC 19111	+	+	+	+	+	+
ATCC 19112	+	+	+	+	+	+
ATCC 19113	+	+	+	+	+	+
ATCC 19114	—	—	—	—	—	—
ATCC 19115	—	+	—	+	+	+
ATCC 19116	—	—	+	—	—	+
ATCC 19117	—	+	+	+	+	+
ATCC 19118	—	—	+	+	+	+
ATCC 15313 ^T	+	—	+	+	+	+
EGD (NCTC 7973)	+	+	+	+	+	+
HCC7	+	+	+	+	+	+
HCC8	+	+	+	+	+	+
HCC12	—	—	—	—	—	—
HCC13	—	—	—	—	—	—
HCC16	—	—	—	—	—	—
HCC17	—	—	—	—	—	—
HCC18	—	—	—	—	—	—
HCC19	—	—	—	—	—	—
HCC23	—	—	—	—	—	—
HCC24	—	—	—	—	—	—
HCC25	—	—	—	—	—	—
168	+	+	+	+	+	+
180	+/-	+	—	+	+	+
418	+	+	—	+	+	+
742	+	+	+	+	+	+
874	—	—	—	—	+	+
1002	+	+	+	+	+	+
1084	+	+	+	+	+	+
1400	+	+	+	+	+	+

**lmo0833* and *lmo1188* yielded identical PCR results except for *L. monocytogenes* 168, which was positive for *lmo0833* and negative for *lmo1188*.†*lmo2672* and *lmo1134* yielded identical PCR results.

Sciences, University of Connecticut, and Dr Robert Mandrell of USDA-ARS, for providing *Listeria* isolates for this study. We also thank Drs Robert Read, Daniel Scruggs and Shane Burgess for their critical review of the manuscript. This paper is MAFES publication J10364.

References

- Barbour, A. H., Rampling, A. & Hormaeche, C. E. (1996). Comparison of the infectivity of isolates of *Listeria monocytogenes* following intragastric and intravenous inoculation in mice. *Microb Pathog* **20**, 247–253.
- Barbour, A. H., Rampling, A. & Hormaeche, C. E. (2001). Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice. *Infect Immun* **69**, 4657–4660.
- Erdenlig, S., Ainsworth, A. J. & Austin, F. W. (2000). Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. *J Food Prot* **63**, 613–619.
- 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*. *Infect Immun* **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.
- Glaser, P., Frangeul, L., Buchrieser, C. & 52 other authors (2001). Comparative genomics of *Listeria* species. *Science* **294**, 849–852.
- Jaradat, Z. W., Schutze, G. E. & Bhunia, A. K. (2002). Genetic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. *Int J Food Microbiol* **76**, 1–10.
- Kathariou, S. & Pine, L. (1991). The type strain(s) of *Listeria monocytogenes*: a source of continuing difficulties. *Int J Syst Bacteriol* **41**, 328–330.
- Lingnau, A., Domann, E., Hudel, M., Bock, M., Nichterlein, T., Wehland, J. & Chakraborty, T. (1995). Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. *Infect Immun* **63**, 3896–3903.

- Liu, D. (1994). Development of gene probes of *Dichelobacter nodosus* for differentiating strains causing virulent, intermediate or benign ovine footrot. *Br Vet J* **150**, 451–462.
- Liu, D. & Yong, W. K. (1993). *Dichelobacter nodosus*: differentiation of virulent and benign strains by gene probe based dot blot hybridisation. *Vet Microbiol* **38**, 71–79.
- Liu, D., Ainsworth, A. J., Austin, F. W. & Lawrence, M. L. (2003). Identification of *Listeria innocua* by PCR targeting a putative transcriptional regulator gene. *FEMS Microbiol Lett* **223**, 205–210.
- Nishibori, T., Cooray, K., Xiong, H., Kawamura, I., Fujita, M. & Mitsuyama, M. (1995). Correlation between the presence of virulence-associated genes as determined by PCR and actual virulence to mice in various strains of *Listeria* spp. *Microbiol Immunol* **39**, 343–349.
- Olier, M., Pierre, F., Lemaître, J.-P., Divies, C., Rousset, A. & Guzzo, J. (2002). Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. *Microbiology* **148**, 1855–1862.
- Parida, S. K., Domann, E., Rohde, M., Müller, S., Darji, A., Hain, T., Wehland, J. & Chakraborty, T. (1998). Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells. *Mol Microbiol* **28**, 81–93.
- 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. *Proc Natl Acad Sci U S A* **86**, 3818–3822.
- Pine, L., Kathariou, S., Quinn, F., George, V., Wenger, J. D. & Weaver, R. E. (1991). Cytopathogenic effects in enterocytelike Caco-2 cells differentiate virulent from avirulent *Listeria* strains. *J Clin Microbiol* **29**, 990–996.
- Rasmussen, O. F., Skouboe, P., Dons, L., Rossen, L. & Olsen, J. E. (1995). *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology* **141**, 2053–2061.
- Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.
- Robinson, R. K., Batt, C. A. & Patel, P. D. (editors) (2000). *Encyclopedia of Food Microbiology*. San Diego, CA: Academic Press.
- Roche, S. M., Velge, P., Bottreau, E., Durier, C., Marquet-van der Mee, N. & Pardon, P. (2001). Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. *Int J Food Microbiol* **68**, 33–44.
- Roche, S. M., Gracieux, P., Albert, I., Gouali, M., Jacquet, C., Martin, P. M. V. & Velge, P. (2003). Experimental validation of low virulence in field strains of *Listeria monocytogenes*. *Infect Immun* **71**, 3429–3436.
- Wiedmann, M., Bruce, J. L., Keating, C., Johnson, A. E., McDonough, P. L. & Batt, C. A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect Immun* **65**, 2707–2716.