

MICROBIAL PATHOGENICITY

Cloning, sequencing and characterisation of a *Listeria monocytogenes* gene encoding a fibronectin-binding protein

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Listeria monocytogenes is a gram-positive, non-sporulating food-borne pathogen of man and animals that is able to invade many eukaryotic cells. *L. monocytogenes* possesses several proteins that bind fibronectin. In this study, an *L. monocytogenes* DNA library in pUC19 was screened with fibronectin and a gene encoding a 24.6-kDa fibronectin-binding protein (Fbp) was isolated and sequenced. Transcripts of the *fbp* gene were found in wild-type, in $\Delta prfA$, and PrfA-S183A strains, despite the presence of a 'PrfA-like' box around its ribosome-binding site. The *fbp* gene was found to be present in all tested isolates of the species *L. monocytogenes* and a homologous DNA fragment was amplified in *L. welshimeri*. No homologies between the *fbp* gene and its translation product with any other DNA or proteins deposited in databanks were found. Restriction endonuclease-PCR (RE-PCR) showed that the *fbp* gene displays a degree of allelic variation among isolates of *L. monocytogenes*, whereas the corresponding amplified fragment of *L. welshimeri* seems to be monomorphic among isolates of this species. RE-PCR with *Hha* I, *Dde* I or *Taq* I produced DNA banding profiles specific for each of these two species, allowing their identification.

Introduction

Listeria monocytogenes is a gram-positive, non-sporulating, food-borne pathogen of man and animals. This facultative intracellular organism is widely distributed in the environment and is responsible for severe human infections. Human disease due to *L. monocytogenes* usually occurs in pregnant women, the newborn, the elderly and immunocompromised patients. Clinical manifestations range from mild flu-like symptoms and gastro-enteritis to septicaemia, central nervous system infections and feto-maternal infections with abortion, premature labour or birth of an infected child. Despite the low incidence of listeriosis, the disease is associated with a high mortality rate (20–40%) [1, 2]. While all other species of the genus *Listeria* (*L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. murrayi*) are also widely distributed in the environment, only *L. monocytogenes* is considered pathogenic to man. Nevertheless, occasional human infections caused

by *L. ivanovii* and *L. seeligeri* have been reported [3, 4].

In recent years a number of outbreaks and sporadic cases of listeriosis have been linked to *L. monocytogenes*, emphasising the continued threat to public health posed by listerial contamination of foods [1, 2, 5]. Elimination of this pathogen from foods is very difficult because of its ubiquitous distribution and its ability to grow at refrigeration temperature. Thus, the complete understanding of the molecular mechanism governing the infectious process of *L. monocytogenes*, as well as active surveillance of foodstuffs and food-processing equipment, are particularly important in the control of listeriosis.

Adhesion of *L. monocytogenes* to host tissues is an essential event for invasion leading to infection. In recent years, several listerial components (InlA, InlB, p60, p104, ActA, α -D-galactose, lipoteichoic acid, *N*-acetylneuraminic acid) were shown to interact with eukaryotic cells and tissues indicating that, as in other bacterial pathogens, adherence is a very complex and multifactorial mechanism [6–13]. An earlier study reported that *L. monocytogenes* binds to human fibronectin, a 450-kDa dimeric glycoprotein found in body fluids, on the surface of eukaryotic cells and in an insoluble component

Received 10 Dec 1999; revised version received 10 March 2000; accepted 20 March 2000.

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of the extracellular matrix. The binding of fibronectin to *L. monocytogenes* appeared to be saturable and dependent on proteinaceous receptors. Several of these fibronectin-binding proteins were identified in cell lysates and cell-wall extracts of the bacterium [14].

In this study, an *L. monocytogenes* gene encoding a 24.6-kDa protein that binds human fibronectin has been cloned, sequenced and characterised.

Materials and methods

Bacterial strains, plasmid and growth conditions

L. monocytogenes strain 90/636 was used in all experiments. This strain, serovar 1/2a-esterase type IB, was isolated in Belgium from a 57-year-old man suffering from meningo-encephalitis. The LD50 values of the strain for conventional and carrageenan-treated NMRI/Han white mice were 9.2×10^2 cfu and <11 cfu, respectively [15]. The following *L. monocytogenes* strains were also used in some experiments: 62-3-90 (sv 1/2a-esterase type IC, cheese isolate), 92-066-01 (sv 1/2a-esterase type ID, cheese isolate), 90-266 (sv 1/2a-esterase type IE, clinical isolate), 90-110 (sv 1/2a-esterase type IF, clinical isolate), 92-782 (sv 1/2a-esterase type IG, clinical isolate), 92-598 (sv 1/2a-esterase type IH, clinical isolate), 91-089 (sv 1/2a-esterase type II, clinical isolate), 90-326 (sv 1/2a-esterase type IJ, clinical isolate), 265-62-90 (sv 3a-esterase type IIA, cheese isolate), 91-371 (sv 1/2a-esterase type IIG, clinical isolate), 91-008 (sv 4b-esterase type IIH, clinical isolate), ATCC 51772, ATCC 51776, ATCC 51777, ATCC 51778, ATCC 51779, ATCC 51780, ATCC 51781, ATCC 51782, LO28 [16], BUG 802 [17] and BUG 1236 [18]. Apart from the last three strains, which were obtained from the Unité des Interactions Bactéries-Cellules (Pasteur Institute, Paris, France), strains were from the collection of the Belgian Reference Centre for listeriosis, Institute of Hygiene and Epidemiology (IHE), Brussels, Belgium. Other *Listeria* spp. used were *L. innocua* serovar 6a, *L. innocua* serovar 6b, *L. innocua* 95-013, 15 strains of *L. welshimeri*, *L. ivanovii*, *L. seeligeri* 024-20, *L. seeligeri* 1140-09-03, *L. seeligeri* 89-59-06 (all from IHE) and *L. grayi* CLIP 640, CLIP 14014, CLIP 73019 (Centre de Référence des *Listeria*, Pasteur Institute, Paris, France) [19].

Plasmids pUC19 and *Escherichia coli* strain DH5 α were used for cloning experiments [20].

Unless otherwise stated, *E. coli* and *Listeria* spp. strains were grown overnight at 37°C (with shaking) in Luria Bertani (LB) medium and in Brain-Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA), respectively. For *E. coli* containing pUC19, or derivatives, ampicillin was added to a final concentration of 100 μ g/ml.

Preparation of bacterial extracts

For preparation of *E. coli* sonicates, bacteria were grown overnight in 10 ml of LB at 37°C (with shaking). The bacterial suspension was centrifuged at 10 000 *g* for 10 min at 4°C; cells were washed three times in phosphate-buffered saline, pH 7.2 (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄·2H₂O) and resuspended in 1 ml of this buffer containing 10 mM phenylmethanesulphonyl fluoride (PMSF) and 1 mM EDTA. Cells were lysed by sonication (Sonics and Materials, Danbury, CT, USA) with ice-bath cooling. The protein concentration of the resulting sonicate was determined with Folin reagent (Sigma) with bovine serum albumin as the standard. Reagents were added to total sonicates to obtain the final concentration of a classic SDS-PAGE loading buffer (SDS 5% w/v, β -mercaptoethanol 10% v/v, glycerol 20% v/v, bromophenol blue 0.05% w/v, 0.125 M Tris-HCl, (pH 6.8) before storing at -20°C, until used.

Separation of bacterial extracts (SDS-PAGE) and Western blot analysis

Bacterial extracts were fractionated by SDS-PAGE (4 and 14% polyacrylamide for stacking and separating gels, respectively) under denaturing conditions. Electrophoresed components were then transferred from the polyacrylamide gel to polyvinylidene difluoride (PVDF) membranes by the use of a Transblot Unit (217 Multiphor 2, LKB, Bromma, Sweden). Transblotted membranes were incubated successively with a solution of human fibronectin (catalogue no. 688851; Boehringer, Mannheim, Germany) 80 μ g/ml, with peroxidase-labelled rabbit anti-human fibronectin immunoglobulins (Dako, Copenhagen, Denmark) and were finally revealed by the addition of α -chloronaphol and hydrogen peroxide, as described previously [14]. Reference samples of transblotted proteins and molecular mass markers were visualised by colloidal gold staining as indicated by the manufacturer (Aurodyne forte, Amersham).

Recombinant DNA techniques

Unless otherwise stated all DNA cloning techniques were standard methods [21]. Plasmid DNA was purified with a Qiagen plasmid purification kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). For preparation of *L. monocytogenes* DNA, bacteria from a 3-ml overnight culture were washed in 1 mM EDTA, 0.1 M NaCl, 50 mM Tris-HCl (pH 8.0) solution, suspended in 1 ml of this solution containing lysozyme 2.5 mg and incubated for 1 h at 37°C. Then 25 μ l of a self-digested pronase 20 mg/ml solution and 50 μ l of an SDS 10% solution were added, followed by incubation for 2 h at 37°C. The solution was extracted three times with an equal volume of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and traces of phenol

were removed with ether. Five μ l of a solution of boiled RNAase A 10 mg/ml, 15 mM NaCl, 10 mM Tris-HCl (pH 7.5) were added and incubation was continued for 1 h at 37°C. The mixture was again extracted three times with the phenol-chloroform-isoamyl alcohol solution and then with ether. The DNA was precipitated with ethanol and suspended in 200 μ l of 0.1 mM EDTA, 10 mM Tris-HCl (pH 7.4) buffer.

DNA sequencing

The sequence of the *L. monocytogenes* DNA cloned in pUC19-106 Δ 1 was determined by fluorescent dye-primer cycle sequencing of both strands of the insert with Texas red-labelled primers, the Thermo Sequenase pre-mixed cycle sequencing kit from Amersham and the Vistra DNA Sequencer 725 apparatus (Amersham).

PCR-restriction endonuclease analysis

PCR amplifications from chromosomal DNA of *Listeria* spp. were performed with primers 5'-CGGGATCCTGAAAGAGTTTATCGAGCCATACC-3' (Fig. 2, nucleotides 173–196) and 5'-GGAATTCTTATTTACGTTTCTTAACAACCTC-3' (Fig. 2, nucleotides 819–796) and Taq DNA polymerase (Promega) according to the manufacturer's instructions. Amplifications were performed in a Perkin-Elmer thermocycler (Gene Amp^R PCR system 24000) through the following temperature programme: one cycle of 45 s at 95°C, then 35 cycles of 45 s at 95°C, 60 s at 55°C, 90 s at 72°C and finally one cycle of 10 min at 72°C. After precipitation, 2 μ g of the amplified DNA fragment were digested with one of the following restriction endonucleases: *Rsa*I, *Hha*I, *Dde*I, *Taq*I, *Mse*I (*Tru*9I) or *Sac*I, following the manufacturer's instructions (Promega). Restriction fragments were separated by agarose 2 or 4% gel electrophoresis, stained with ethidium bromide and visualised by transillumination under UV light.

RNA isolation

For extraction of total RNA from *L. monocytogenes*, the cells from 10 ml of an overnight culture in BHI (37°C) were harvested (5000 g, 10 min, 4°C) and treated as described by Bashyam and Tyagi [22]. Purified RNA dissolved in 100 μ l DEPC-treated water was stored at –70°C until used.

RT-PCR

For RT-PCR, 20 μ g of total RNA from *L. monocytogenes* were first incubated for 20 min at 25°C in a buffer containing amplification grade RNAase-free DNAase I (Gibco) 3 U, 2 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl, pH 8.4. DNAase I was inactivated by addition of EDTA (2.5 mM final concentration) and incubation at 65°C for 10 min. Reverse transcription of mRNA encoding the 24.6-kDa protein was performed

for 45 min (37°C) in 50 μ l of a solution containing 4 μ g of DNA-free RNA, 10 pmol primer 5'-GGAATTCTTATTTACGTTTCTTAACAACCTC-3' (Fig. 2, nucleotides 819–796), 1 mM dNTP, 10 mM DTT, RNasin (Promega) 50 U, Moloney Murine Leukaemia Virus Reverse Transcriptase (Gibco) 200 U, 3 mM MgCl₂, 75 mM KCl, 50 mM Tris-HCl, pH 8.3. The reverse transcriptase was then inactivated (incubation for 10 min at 65°C). Twenty μ l of the reverse transcription reaction mixture were used for PCR amplification of the cDNA with the above primer plus primer 5'-CGGGATCCTGAAAGAGTTTATC GAGCCATACC-3' (Fig. 2, nucleotides 173–196), and Taq DNA polymerase, according to the manufacturer's instructions (Promega). Amplification was carried out in a Perkin-Elmer thermocycler (Gene Amp PCR system 24000) as described for the PCR-restriction analysis. Control RT-PCR reactions, omitting reverse transcriptase, were performed to check for DNA contamination of the RNA preparations.

Nucleotide sequence accession number

The sequence of the *fbp* gene has been deposited in the EMBL database under accession number AJ132543.

Results

Cloning of a fibronectin-binding protein gene

To isolate gene(s) encoding *L. monocytogenes* protein(s) implicated in the binding of fibronectin, a DNA library was constructed in pUC19. Chromosomal DNA of strain 90/636 was partially digested with *Sau*3AI, ligated into the *Bam*HI site of plasmid pUC19 and used to transform *E. coli* DH5 α . Pools of 10 *E. coli* recombinants were then grown overnight in the presence of IPTG. After sonication, *E. coli* proteins were separated by electrophoresis on an SDS-14% polyacrylamide gel and transblotted to PVDF membranes, which were incubated with human fibronectin. Membranes were then compared for fibronectin binding to components not present in lysates of *E. coli* DH5 α (pUC19), used as a control. A positive pool was found and screened for the clone expressing the listerial fibronectin-binding protein. Clone 106 was identified. This clone expresses a *L. monocytogenes* protein of c. 25 kDa, which avidly binds human fibronectin (Fig. 1B, lane 2). The binding is not non-specific binding to an overexpressed protein, as several proteins overexpressed by other clones failed to bind fibronectin (e.g., clone 128, Fig. 1B, lane 3).

Restriction endonuclease analysis of the pUC19-106 recombinant plasmid indicated that the listerial DNA insert is 2500 bp long and contains two internal *Eco*RI sites (results not shown). As the length of the coding frame necessary to encode a 25-kDa protein is c. 680 bp, it was decided to truncate the cloned fragment before sequencing. pUC19-106 was digested with

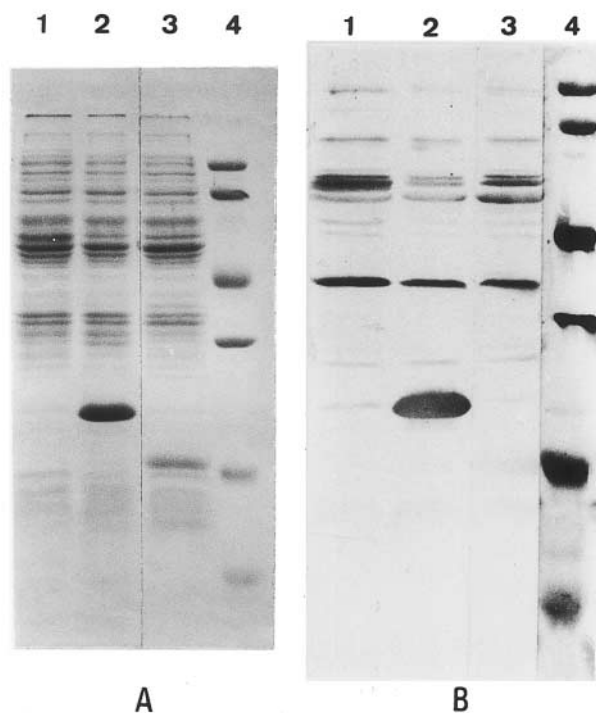


Fig. 1. Expression in *E. coli* of a *L. monocytogenes* fibronectin-binding protein. *E. coli* DH5 α clone 106 (lanes 2) and clone 128 (3), isolated from a genomic library of *L. monocytogenes* strain 90/636 in pUC19 were grown overnight in the presence of ampicillin 100 μ g/ml and 10 mM IPTG. *E. coli* DH5 α transformed by pUC19 was treated in a similar manner and used as control (lanes 1). Total sonicates of the bacteria (50 μ g of protein) were fractionated by SDS-14% PAGE and either (A) stained with Coomassie Blue or (B) transferred to a PVDF membrane which was incubated with human fibronectin. Membrane-bound fibronectin was revealed by peroxidase-labelled rabbit anti-human fibronectin immunoglobulins and α -chloronaphthol as the substrate. Lanes 4, molecular mass markers (94.0, 67.0, 43.0, 30.0, 20.1 and 14.2 kDa from top to bottom, respectively) were stained with either (A) Coomassie Blue or (B) colloidal gold.

*Eco*RI, generating a 3650-bp DNA fragment (containing pUC19 and a 950-bp listerial DNA fragment), and a 900-bp listerial DNA fragment and a 600-bp listerial DNA fragment containing part of the pUC19 multiple cloning site (from the *Bam*HI to the *Eco*RI sites). The 3650-bp fragment was self-ligated and used to transform *E. coli* DH5 α , generating clone 106 Δ 1. Clone 106 Δ 1 was shown to overexpress a 25-kDa *L. monocytogenes* protein which binds human fibronectin (results not shown).

Expression of the 25-kDa protein was not influenced by IPTG (data not shown) in either clone 106 or 106 Δ 1, suggesting that the 25-kDa protein gene is transcribed in *E. coli* from its own promoter.

Sequencing of the gene (*fbp*) encoding the *L. monocytogenes* 25-kDa protein

The sequence of the *L. monocytogenes* DNA fragment inserted in clone 106 Δ 1 was determined on both

strands (Fig. 2). The 951-bp listerial DNA insert has a G+C content of 37.6 mol%, which is in the 37–39 mol% range of the G+C content of all *L. monocytogenes* chromosomal DNA [23]. The presence of four stop codons, in frame with the pUC19 α -peptide, in the first 160 nucleotides of the sequence excludes the expression of an α -peptide-fibronectin-binding fusion protein in *E. coli*. The cloned DNA contains only one large open reading frame extending from position 172 to 819. This 648-bp open reading frame (ORF) encodes a polypeptide of 215 amino acids (calculated mol. wt of 24.6 kDa). The initiation codon of the 648-bp ORF is an ATG and is preceded by the sequence TAAAGGAGA (nucleotides 157–165) which is complementary to the 3' end of the 16S rRNA of both *L. monocytogenes* and *E. coli* [24–26]. Therefore, this sequence, located six bases upstream of the start codon, may represent a ribosome-binding site for the mRNA encoded by the *fbp* gene in both *E. coli* and *L. monocytogenes*. The ORF is terminated by a TAA stop codon, followed immediately by a 26-bp inverted repeat (with only two mismatches) which could form a secondary structure with a free energy (ΔG at 25°C) of -26.0 kcal/mol [27], and therefore function as a *rho*-independent transcription terminator. A palindromic sequence (convergent dotted arrows in Fig. 2) with homology to the 14-bp canonical binding box of PrfA, a regulator of the expression of several listerial virulence genes [28–38, EMBL X97014], was detected 20 bp upstream of the initiation codon ATG. This PrfA-like box (boxed in Fig. 2) has three mismatches compared to the perfect palindrome (TTAACANNTGT TAA). Despite the presence of this 'PrfA-like' box, RNA transcripts of the *fbp* gene were found in wild-type *L. monocytogenes* strains (90/636 and LO28), in a Δ *prfA* strain (BUG 802) and in a strain (BUG 1236) overexpressing a mutated PrfA protein possessing a higher affinity for PrfA boxes than its wild-type analogue (PrfA-S183A) (Fig. 3).

The predicted 215 amino-acids sequence has a calculated iso-electric point of 7.63 and is rich in lysine (11.2%) residues, distributed throughout the molecule. Apart from these characteristics, other notable features are a repeated amino-acid block (EFIE) at the ends of the protein (positions 3–6 and 206–209) and another (AKTK) in the middle of the protein (positions 99–102 and 170–173). A cytochrome C family haem-binding signature (Prosite PDOC00169) was also present at positions 156–161. Protein and nucleotide database searches (BLAST P and BLAST N programs) found no significant similarity with any other known protein or nucleic acid sequences.

Detection of sequences homologous to the *fbp* gene among *Listeria* species

To study the occurrence of the *fbp* gene in strains of *L. monocytogenes*, PCR-amplification of the complete

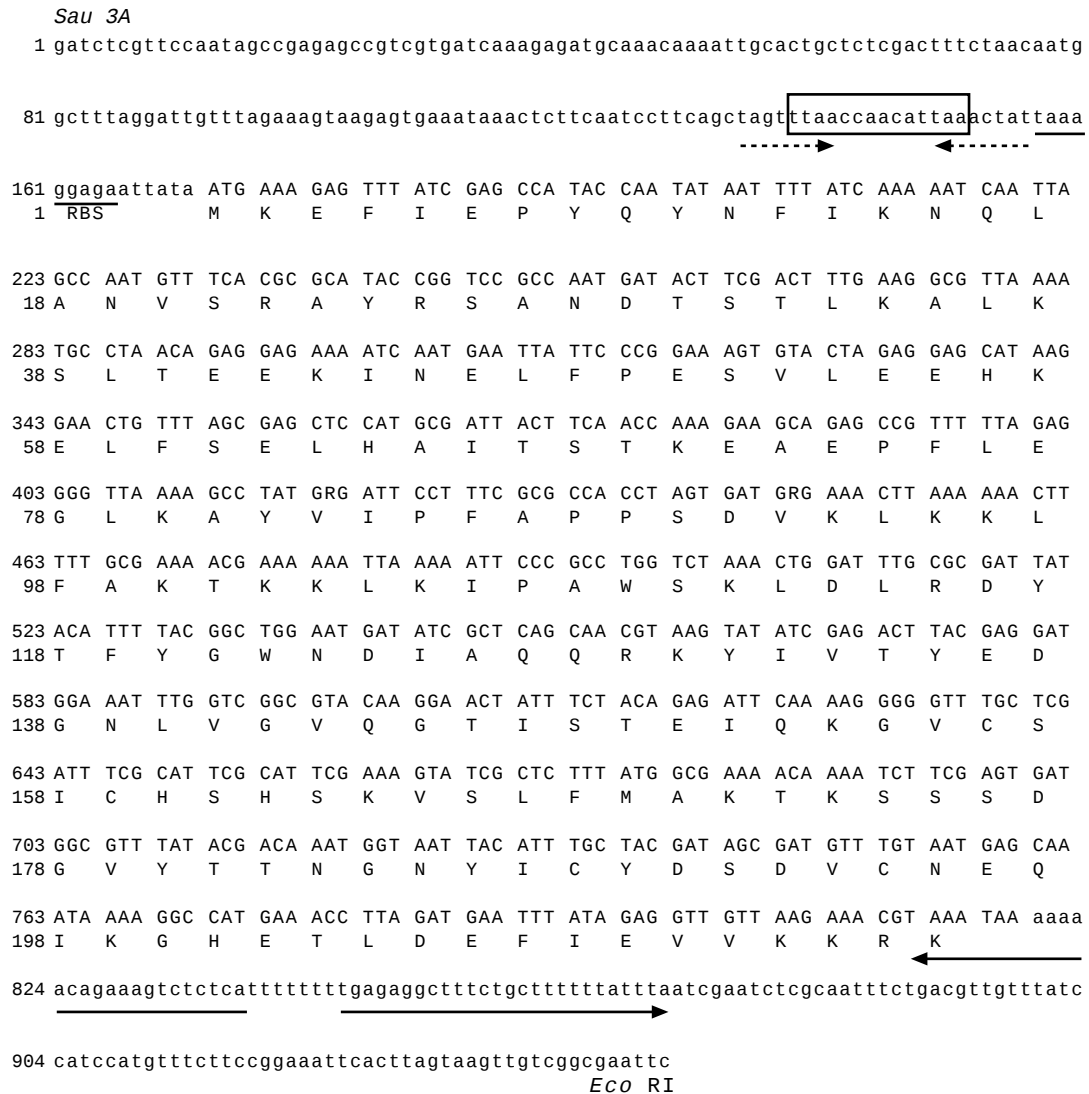


Fig. 2. Nucleotide sequence of the region containing the gene encoding the Fbp of *L. monocytogenes*. The sequence of the gene and of its translation product (single-letter amino-acid code) are shown. The potential ribosome binding site (RBS) is underlined. A palindromic sequence (dotted convergent arrows) extending from a boxed PrfA-like binding site is indicated. Large divergent arrows at the end of the ORF indicate a predicted *rho*-independent transcription terminator.

648 bp ORF was performed with chromosomal DNA purified from 20 strains of non-clonal origin. Each of these strains belongs to one of the 20 different esterase types detected during a previously made analysis of *L. monocytogenes* populations isolated from foodstuffs and from human patients with listeriosis in Belgium (see *Materials and methods*) [15, 39]. In all the tested strains, a 648-bp DNA fragment was amplified, indicating a broad distribution of the *fbp* gene in this species (results not shown). The occurrence of the *fbp* gene in other species of the genus *Listeria* was also tested by PCR. No DNA amplification was observed with chromosomal DNA purified from three strains of *L. grayi*, three strains of *L. seeligeri*, three strains of *L. innocua* or one strain of *L. ivanovii*; but a DNA fragment of similar size was amplified from the chromosomal DNA of 15 strains of *L. welshimeri* (Fig. 4 and results not shown).

The PCR-amplified *fbp* gene of *L. monocytogenes* strain 90/636 used as a probe was then hybridised under high stringency to *ScaI*-digested DNA from several strains of the five different *Listeria* species. This probe hybridised to a c. 7500-bp fragment of DNA from *L. monocytogenes*, but not to DNA from the other *Listeria* species analysed, including *L. welshimeri* (results not shown).

The above results suggest that only some regions of the 648-bp DNA fragment amplified in *L. welshimeri* and in *L. monocytogenes* are homologous. This hypothesis was tested by restriction endonuclease analysis of the molecules amplified from the DNA of the 20 *L. monocytogenes* strains and the 15 *L. welshimeri* strains described above. Six restriction endonucleases (*RsaI*, *SacI*, *HhaI*, *MseI*, *DdeI* and *TaqI*) cutting at sites covering the entire Fbp ORF of

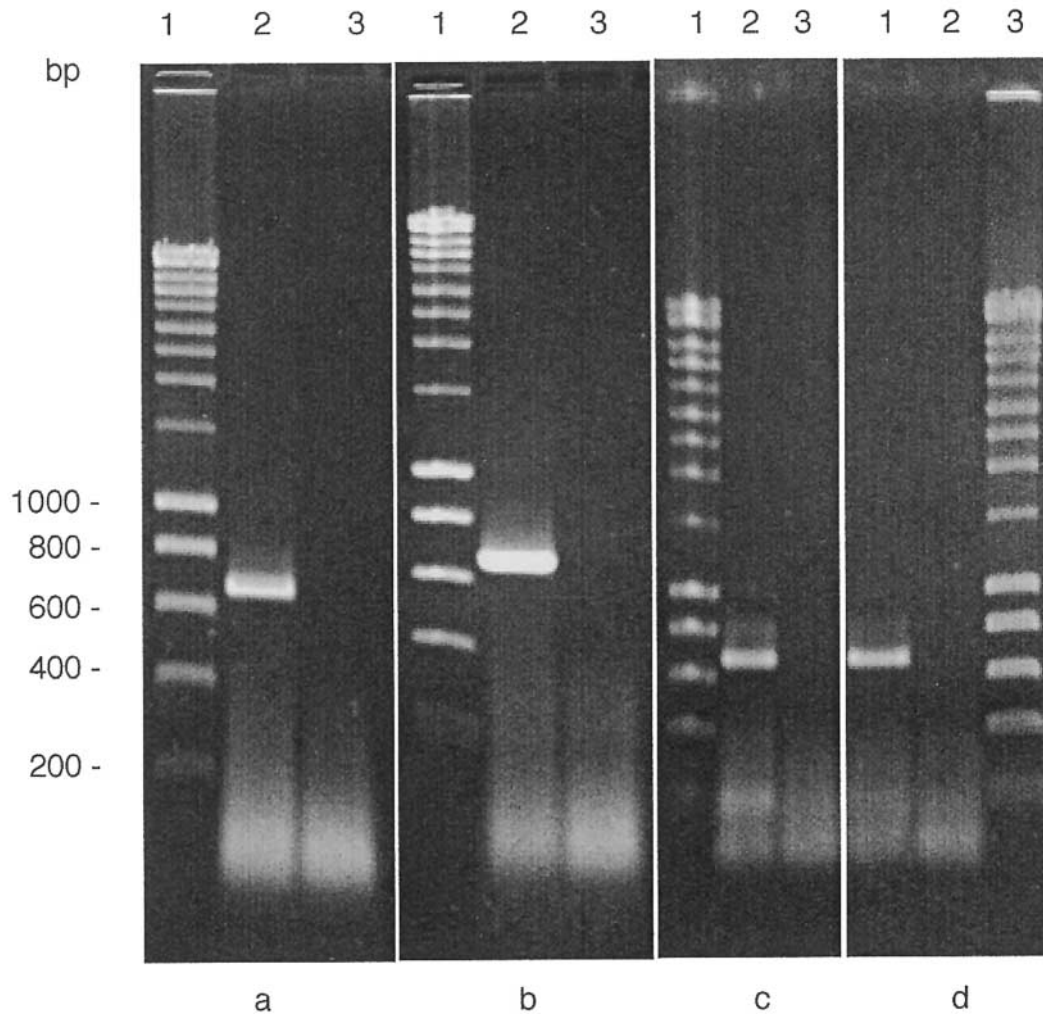


Fig. 3. Transcription of the *fbp* gene in wild-type, $\Delta prfA$ and PrfA-S183A strains of *L. monocytogenes*. *L. monocytogenes* wild-type strain 90/636 (a), wild-type strain LO28 (c), strain LO28 $\Delta prfA$ (BUG 802) (b) and strain LO28 PrfA-S183A (BUG 1236) (d) total RNA were extracted. Reverse transcriptions of mRNA encoding the FBP protein were performed and the cDNAs obtained were used for PCR amplifications (lanes a2, b2, c2 and d1). Control RT-PCR reactions made in the absence of reverse transcriptase were performed to check for DNA contamination (lanes a3, b3, c3 and d2). Mol. wt markers (Smart DNA ladder, Eurogentec) of the indicated size are in lanes a1, b1, c1 and d3.

L. monocytogenes strain 90/636 were chosen (Fig. 5b). These experiments confirmed the nucleotide sequences of the *fbp* gene of strain 90/636 and showed that this gene displays a low degree of allelic variation among isolates: all the strains hydrolysed with *Mse*I and with *Hha*I have the same restriction profiles (Fig. 5a; lanes 1 and 12, respectively), and only two different profiles were observed with each of the four other enzymes tested (Fig. 5a; lanes 4 and 5 for *Rsa*I, lanes 8 and 9 for *Sac*I, lanes 15 and 16 for *Dde*I, and lanes 19 and 20 for *Taq*I). In contrast, the DNA fragments amplified from the *L. welshimeri* strains are completely monomorphic (Fig. 5a; lanes 2, 6, 10, 13, 17 and 21). Interestingly, the enzymes *Hha*I, *Dde*I and *Taq*I produced banding profiles of the *L. welshimeri* amplified DNA fragment different from those observed for the *L. monocytogenes* Fbp ORF, allowing a specific identification of each species (Fig. 5a).

Discussion

In this study the gene for a 24.6-kDa protein of *L. monocytogenes* that binds human fibronectin was cloned and sequenced. No homology was found between the listerial Fbp and known fibronectin-binding domains of other prokaryotic or eukaryotic proteins, including the RGD motif of integrins. The biological relevance and putative role of the Fbp during listerial infection is thus speculative. Nevertheless, it is worth noting that, whereas some homologies have been found between streptococcal and staphylococcal fibronectin-binding domains, these binding sites are completely different from those of mycobacterial fibronectin-binding protein [40–46]. This indicates that pathogens have evolved several different mechanisms for binding to fibronectin and that the one used by *L. monocytogenes* could be original to this species.

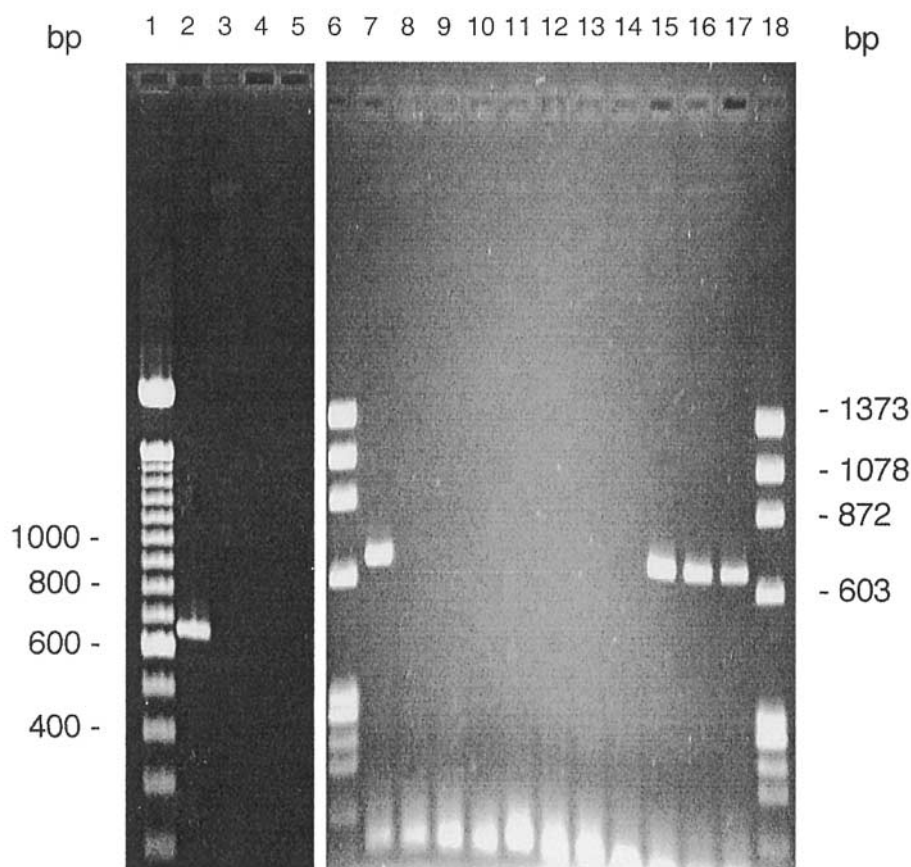


Fig. 4. Search for DNA sequences homologous to the *fbp* gene of *L. monocytogenes*. Chromosomal DNAs of *L. monocytogenes* 90/636 (lanes 2, 7), *L. grayi* CLIP 73019 (3), *L. grayi* CLIP 640 (4), *L. grayi* CLIP 14014 (5), *L. innocua* sv 6a (8), *L. innocua* sv 6b (9), *L. innocua* 95/013 (10), *L. seeligeri* 024/20 (11), *L. seeligeri* 89/59/06 (12), *L. seeligeri* 1140/09/03 (13), *L. ivanovii* (14), *L. welshimeri* P.P. (15), *L. welshimeri* 90/060/01 (16) and *L. welshimeri* 90/060/02 (17) were purified. The DNAs were used for the PCR-amplification of the Fbp ORF. Amplification products were separated on an agarose 1.5% gel and visualised under UV light. Mol. wt markers of the indicated size are in lanes 1 (100-bp DNA ladder) and 18 (ϕ X174 DNA cut by *Hae*III).

The presence of a putative *prfA* box upstream of the Shine-Dalgarno sequence of the *fbp* gene is puzzling, as such a palindromic sequence at this position would represent an ideal repressor site. PrfA is mainly known as an activator of several virulence *Listeria* genes. PrfA-controlled genes are preceded by conserved 14-bp long DNA sequences (PrfA boxes), centred near position -40 [28–38, EMBL X97014]. PrfA-dependent genes are differently regulated: promoters with perfect palindromes are transcribed more efficiently than those having mismatches and a 'PrfA-site hierarchy' model was proposed to explain these findings. Recently 'pseudo PrfA boxes' also located between the transcriptional start site and the translational start triplet were described. These boxes resemble a conventional PrfA box, but have larger loop structures. These PrfA boxes were also claimed to be important for the expression of their downstream genes [32, 36]. The complexity of regulation by PrfA has been reinforced by observations made on motility genes and on the stress response mediator ClpC, indicating that PrfA is responsible for downregulation of these genes by an as yet unknown mechanism [47, 48]. Despite the presence of a putative PrfA box near the RBS on the *fbp* gene,

RNA transcripts of this gene were found in wild-type strains, in a strain deleted of *prfA* and in a strain overexpressing a PrfA protein with a high affinity for PrfA boxes. This indicates that the identified 'PrfA-like' box is not implicated in an absolute 'switch on-switch off' mechanism of regulation of the *fbp* gene transcription. However, as far as the RT-PCR used could be considered as being quantitative, it seems that PrfA could somewhat downregulate the transcription of the *fbp* gene as more transcripts were revealed in the $\Delta prfA$ strain (Fig. 3). Other explanations for the putative role of this palindromic box would be its use as a PrfA binding site for the regulation of a gene transcribed in the direction opposite to the *fbp* gene or as a binding site of another not yet identified regulator.

The PCR based on the *fbp* gene was found to be specific for *L. monocytogenes* and *L. welshimeri* strains (Fig. 4). As parts of the amplified sequences are dissimilar in these two species, digestion of the amplified products with the enzymes *Hha*I, *Dde*I or *Taq*I were able to produce specific banding patterns for *L. monocytogenes* and for *L. welshimeri* strains (Fig.

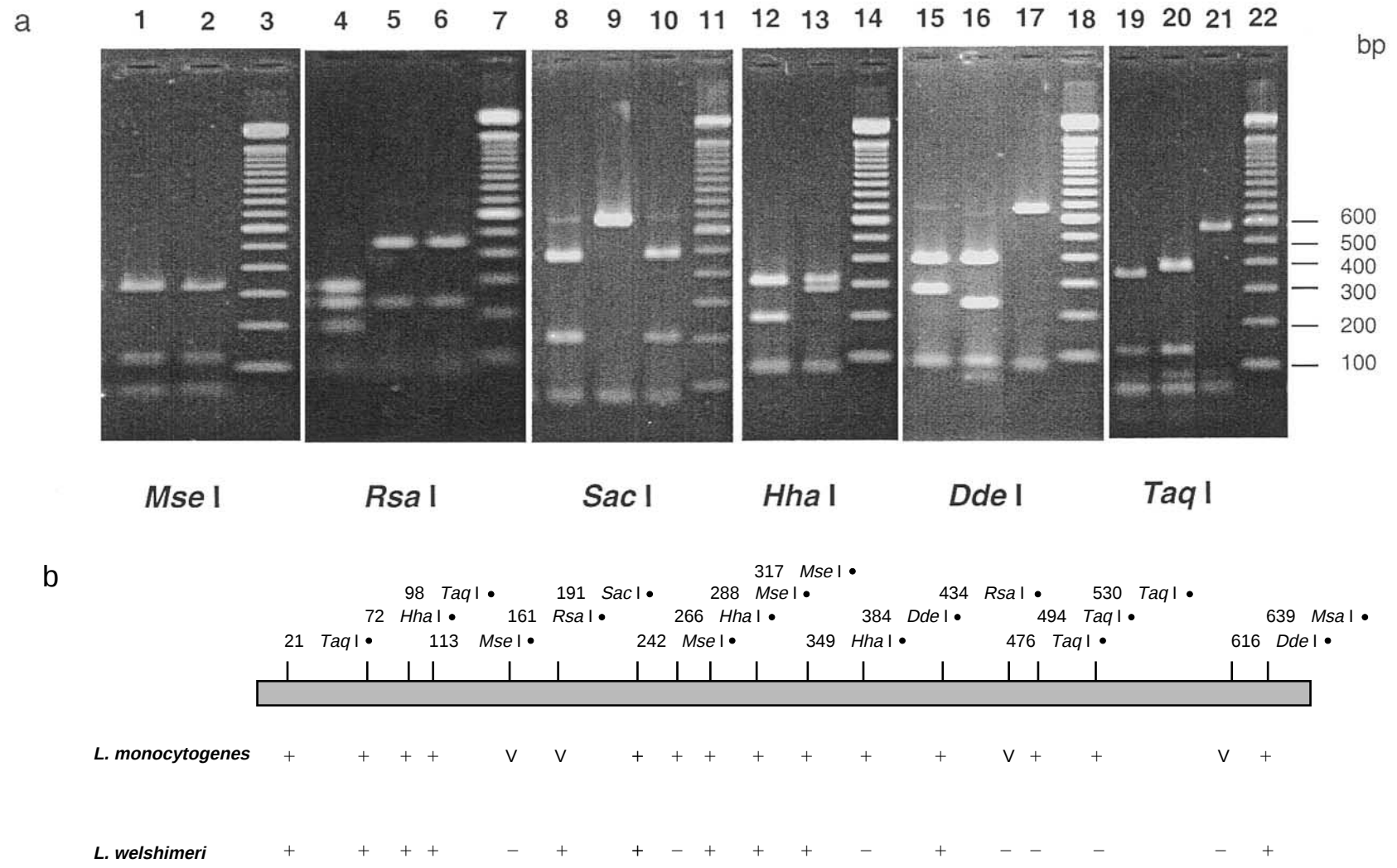


Fig. 5. Restriction polymorphism in the *fbp* gene of *L. monocytogenes* and in the homologous DNA fragment of *L. welshimeri*. (a) Chromosomal DNA from different strains of *L. monocytogenes* and *L. welshimeri* was used for the PCR-amplification of the *fbp* gene. The amplified fragments were then digested with *Mse*I, *Rsa*I, *Sac*I, *Hha*I, *Dde*I or *Taq*I and electrophoresed on an agarose 2% gel. Examples of each of the different restriction types obtained after analysis of 20 *L. monocytogenes* strains (lanes 1, 4, 5, 8, 9, 12, 15, 16, 19 and 20) and of 15 *L. welshimeri* strains (lanes 2, 6, 10, 13, 17 and 21) of different origins are shown. Mol. wt markers of the indicated sizes are in lanes 3, 7, 11, 14, 18 and 22. (b) A restriction map based on the sequence of the *fbp* gene of *L. monocytogenes* strain 90/636 is shown. The presence (+) of absence (-) of each of the indicated restriction enzyme sites in all or in some (V) of the amplified fragments from the tested strains of *L. monocytogenes* and *L. welshimeri* are indicated.

5). Therefore this RE-PCR technique could be used as a new tool for identification of *L. monocytogenes* and *L. welshimeri* among species of the genus *Listeria*.

We are grateful to P. Cossart (Pasteur Institute, Paris, France), A. Genicot (Institute of Hygiene and Epidemiology, Brussels, Belgium), C. Jacquet (Pasteur Institute, Paris) and J. Rocourt (Pasteur Institute, Paris) for providing strains of *Listeria* spp., and to M. Braibant for critical reading of the manuscript. This work was part of an application that received the award of the Eugene Yourassowski Foundation (Brussels).

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