Novel protein targets of the humoral immune response to *Listeria monocytogenes* infection in rabbits

Wei Ling Yu,^{1,2} Hanhong Dan¹ and Min Lin^{1,2}

Correspondence

Min Lin

linm@inspection.gc.ca

¹Animal Diseases Research Institute, Ottawa, ON K2H 8P9, Canada

²Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada

The role of the humoral immune response in protective immunity against listerial infection has been overlooked and is essentially unknown. This study aimed to discover the protein targets of *Listeria monocytogenes* that elicit an antibody response following infection in a rabbit model. A genomic expression library for *L. monocytogenes* was constructed and differentially screened to identify genes encoding proteins that reacted with antiserum from rabbits infected with live *L. monocytogenes* serotype 4b (R_zL), but not with that from animals immunized with heat-killed bacteria (R_zK). Thirty-one clones expressing proteins that reacted exclusively with R_zL were identified and sequenced. Sequence analysis, together with Western blot analysis of the proteins expressed from positive clones, led to the identification of eight *L. monocytogenes* proteins as targets of humoral immune responses during listerial infection: three internalin members (InlA, InlD and InlC2) and five novel proteins of unknown function (designated IspA, IspB, IspC, IspD and IspE, respectively). Exhibition of humoral immune responses to these proteins in actively infected rabbits but not in animals receiving heat-killed *L. monocytogenes* suggested that they were induced or significantly upregulated *in vivo* during infection and thus are important in *Listeria* pathogenesis. With the exception of antibodies to InlA, this is the first demonstration of antibodies to the other seven proteins in infected hosts. These immunogenic proteins may be useful candidates for elucidation of the role of antibodies in protective immunity in the context of listerial infection, as well as potential targets for serodiagnostic reagents and vaccine and drug development.

Received 29 September 2006

Accepted 9 March 2007

INTRODUCTION

Listeria monocytogenes is a Gram-positive, rod-shaped, facultatively anaerobic, intracellular bacterium that can infect humans through the consumption of contaminated food (Meier & Lopez, 2001), leading to the development of a severe food-borne illness referred to as listeriosis. Clinical symptoms include meningitis, meningoencephalitis, septicaemia and gastroenteritis. This pathogen causes significant disease in elderly people, immunocompromised patients, infants and pregnant women. Almost all human infections can be attributed to three serotypes, 4b, 1/2a and 1/2b (Lorber, 1997), with serotype 4b strains accounting for approximately 40% of the sporadic cases and many outbreaks (Rocourt & Bille, 1997).

Abbreviations: AP, alkaline phosphatase; Isp, immunogenic surface protein.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are EF409978–EF409984.

L. monocytogenes has the capacity to invade professional phagocytes (macrophages) and various types of non-phagocytic cells including epithelial cells, fibroblasts, hepatocytes, endothelial cells, dendritic cells and neurons through a complex process involving a number of protein factors (reviewed by Vazquez-Boland *et al.*, 2001). Internalization of the bacterium by induced phagocytosis is mediated through the interaction of the specific cell receptors with at least two internalins, InlA and InlB (Braun *et al.*, 2000; Mengaud *et al.*, 1996; Shen *et al.*, 2000). Escape from the primary phagosome to the cytosol requires lysis of the membrane by the pore-forming toxin listeriolysin O (LLO) and a phosphatidylinositol-specific phospholipase C (PI-PLC). The bacterial surface protein ActA promotes the polymerization of host-cell actin molecules, which propels the intracellular motility of the bacterium. LLO and a second phospholipase C (PC-PLC) with a broad spectrum of substrate specificity mediate the disruption of a double-membrane phagosome whose inner membrane originates from the previously infected cell.

Antibodies are generally considered to be ineffective in protection against *Listeria* infection, although the antibody response is normally observed during listerial infection, even in the absence of clinical symptoms. The protein antigens eliciting the antibody response include LLO, InlA, InlB, ActA, the internalin-related protein IrpA and the autolysin p60 (Berche *et al.*, 1990; Bhunia, 1997; Boerlin *et al.*, 2003; Gentshev *et al.*, 1992; Grenningloh *et al.*, 1997). Earlier studies by Mackaness (1962) and others demonstrated that serum taken from mice infected with a sublethal dose of *L. monocytogenes* provided no resistance against infection when passively transferred to naive mice. Focus was then placed on cellular immunity with almost no attention paid to the role of antibodies in immune defence against *Listeria* infection. This has led to the establishment of the crucial role of cell-mediated immunity in resistance to *Listeria* and intracellular pathogens (reviewed by Edelson & Unanue, 2000). The view that antibody-mediated protection is ineffective was challenged by two reports, which demonstrated a surprising role for a specific monoclonal anti-LLO antibody (Edelson *et al.*, 1999) and natural antibodies (Ochsenbein *et al.*, 1999). These studies suggest the necessity for a re-evaluation of the role of antibodies in protection against *L. monocytogenes* infection, which may require the identification of additional antigen targets of the humoral immune response.

In this study, a genomic expression library of *L. monocytogenes* serotype 4b was constructed and differentially screened using two different antisera, one from rabbits infected with live *L. monocytogenes* (RzL) and one from rabbits immunized with heat-killed bacterial cells (RzK). This led to the discovery of several novel proteins of unknown function that reacted exclusively with RzL. A similar strategy and a modified form of it referred to as *in vivo*-induced antigen technology (Handfield *et al.*, 2000; Rollins *et al.*, 2005) have been used successfully to identify the microbial proteins specifically induced during infection for several bacterial pathogens: *Borrelia burgdorferi* (Suk *et al.*, 1995), *Mycobacterium avium* subsp. *paratuberculosis* (Bannantine & Stabel, 2001), *Mycobacterium tuberculosis* (Deb *et al.*, 2002), *Leptospira interrogans* (Artiushin *et al.*, 2004), *Helicobacter pylori* (Lazowska *et al.*, 2000), *Babesia microti* (Lodes *et al.*, 2000), *Vibrio cholerae* (Hang *et al.*, 2003) and *Escherichia coli* O157 : H7 (John *et al.*, 2005).

METHODS

Bacterial culture. A single colony of *L. monocytogenes* LI0521 (serotype 4b), maintained on trypticase soy blood agar plates at 4 °C, was cultured in 1 ml Luria–Bertani (LB) broth containing 50 mM MOPS (pH 7.5) (LB-MOPS) at 37 °C for 22 h. The bacteria were subcultured at a dilution of 1 : 100 overnight for infection experiments. The cell number was determined from the OD reading, with an OD₆₂₀ of 0.61 equivalent to 1 × 10⁹ bacteria ml⁻¹ (Schlech, 1993), and confirmed by plating serial dilutions of cells onto LB-MOPS agar plates. *E. coli* strains NovaBlue (DE3) (Novagen), DH5α (Invitrogen) and InVzF' (Invitrogen), used for cloning experiments,

were cultured at 37 °C on LB plates or in LB broth supplemented with carbenicillin (50 µg ml⁻¹) or kanamycin (50 µg ml⁻¹) as required.

Antisera from infected animals. On day 1 after the collection of pre-immune sera, two groups of New Zealand female rabbits (two animals per group) were inoculated intravenously with 1 × 10⁶ viable listerial cells in 0.25 ml saline and with 1 × 10⁹ heat-killed (75 °C for 30 min) bacteria in 0.25 ml saline, respectively. At 30 days post-infection (p.i.), each group of animals was infected or immunized as on day 1. Each rabbit was bled weekly up to and including day 44 p.i. ELISA analysis (see later) of the serially collected antisera indicated a high titre of IgG antibodies in all of the animals at day 44 p.i. The rabbits were sacrificed at 45 days p.i. and a large volume of blood (serum) was collected through cardiac puncture. Sera were stored at -20 °C until use.

Assessment of the rabbit antibody response by ELISA. ELISA was performed to analyse the IgG antibody responses in rabbits experimentally exposed to *L. monocytogenes*. Each serum sample was tested in duplicate. Nunc Immuno Maxisorp microtitre plates (VWR) were sensitized overnight at 4 °C with soluble listerial antigens prepared as described previously (Yu, 2004) and diluted 1 : 100 in PBS. Plates were then washed with PBS containing 0.05 % Tween 20 (PBST). Serum samples were serially diluted in PBS containing 1 % BSA, added to the wells (100 µl per well) and incubated for 1.5 h at room temperature. After washing with PBST, 100 µl horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory) diluted 1 : 1000 in PBS was added to the wells and incubated for 1 h at room temperature. After a final wash with PBST, 100 µl of a substrate solution containing 1 mM 2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid) and 0.015 % H₂O₂ in 50 mM sodium citrate buffer (pH 4.5) was added to each well. Following incubation with moderate shaking for 10 min at room temperature, the A₄₁₄ was determined using a Labsystems Multiskan Bichromatic plate photometer.

Construction of an expression library. All DNA manipulations were performed essentially according to established procedures (Sambrook & Russel, 2000). A protein expression library for *L. monocytogenes* was constructed in pSCREEN1b⁺ΔEcoRV (Yu, 2004), a plasmid vector derived from the pSCREEN T-Vector (Novagen). Expression of inserted DNA fragments under the control of the T7 promoter from the vector yields a polypeptide with an N-terminal fusion including a six-histidine (His₆) tag. The genomic DNA was isolated from 40 ml of an overnight culture using a GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Biosciences) with some modifications. Briefly, cell pellets were resuspended in 1 ml PBS and treated with lysozyme (Sigma-Aldrich) at 2.5 mg ml⁻¹ at 37 °C for 1 h, followed by incubation with proteinase K (Sigma-Aldrich) at 2 mg ml⁻¹ for 1 h. Subsequently, DNA was extracted from 600 µl of the treated cell suspension according to the supplier's instructions, dissolved in 100 µl DNA hydration solution (from the kit) and quantified using a PicoGreen dsDNA Quantification Reagent kit (Molecular Probes) with a λ DNA standard following the manufacturer's instructions. Fragments (1.5–2.5 kb) resulting from partial digestion of the genomic DNA (24 µg) with *Sau3AI* were separated by gel electrophoresis in 1 % low-melting-point agarose, purified using a QIAquick gel extraction kit (Qiagen) and partially filled in with dGTP and dATP using Klenow large fragment (New England Biolabs). The DNA fragments were then ligated, at an insert : vector ratio of 1 : 1, into the *XhoI* site of the pSCREEN1b⁺ΔEcoRV vector, which had been pre-cut with *XhoI* and partially filled in with dCTP and dTTP. Transformation of *E. coli* NovaBlue (DE3) competent cells with 1 µl aliquots of the ligation mixture yielded a library of recombinant clones for expression screening. The presence of insert in any randomly selected clone was determined by colony PCR using the SP6 promoter primer (5'-GATTTAGGTGACACTATAG-3') and T7

terminator primer (5'-GCTAGTTATTGCTCAGCGG-3') in a *Taq* DNA polymerase reaction mixture (Lin, 1999). The mixture (50 µl) was incubated in a Techne Progene thermocycler at 95 °C for 5 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min.

Library screening. The *L. monocytogenes* expression library was screened with R α L and R α K essentially as described in the manual of the ColonyFinder Immunoscreening kit (Invitrogen). Colonies on LB plates were overlaid with an OPTITRAN nitrocellulose membrane (Schleicher & Schuell) for 1 min and probed with R α L. A second membrane was overlaid on the same plate for 4 min and probed with R α K. Both antisera were a 1 : 1 mixture from two infected or immunized rabbits and used at a pre-determined dilution (1 : 400) in TBST [20 mM Tris/HCl (pH 7.5), 500 mM NaCl and 0.05 % Tween 20]. Bound antibodies were detected using alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and a substrate solution [10 ml AP buffer: 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂) containing nitro blue tetrazolium (33 µg ml⁻¹) and 5-bromo-4-chloro-3-indolyl phosphate (17 µg ml⁻¹). Clones exhibiting reactivity only with R α L were identified as true positives after three rounds of screening.

DNA sequence determination and analysis. Recombinant plasmids were prepared from 5 ml of *E. coli* cultures (positive clones) using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions and used as templates for the determination of insert sequences at the University of Guelph automatic DNA sequencing facility (Guelph, Ontario, Canada). Both strands of the insert were first sequenced with the SP6 primer and the T7 terminator primer and completed with additional primers derived from the initially determined sequences. Oligonucleotide primers were designed using the GENERUNNER software package (Hastings Software) and were custom synthesized (Sigma Genosys). All possible ORFs in the sequenced DNA were identified using the software packages available online at the ExPASy Molecular Biology Server (<http://ca.expasy.org/>). Putative promoters upstream of an ORF were identified using a neural network promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Analysis of the deduced amino acid sequence from an ORF was carried out with INTERPROSCAN, SIGNALP 3.0 and PFAM HMM at the ExPASy website (<http://ca.expasy.org/tools/>). Protein sequence alignment and comparison were performed using the MEGALIGN module of LASERGENE (DNASTAR).

PCR cloning. For the identified immunoreactive clones containing partial ORFs, PCR was used to amplify the unknown flanking sequences in order to obtain the complete ORFs. To accomplish this, one of the PCR primers was synthesized based on the sequence flanking similar ORFs in the published genome sequences of *L. monocytogenes* and *Listeria innocua* (Glaser *et al.*, 2001). The other primer was synthesized based on the determined sequences of the inserts. PCR was performed using the Expand High Fidelity PCR system (Roche Diagnostics) according to the manufacturer's instructions. Briefly, a 100 µl reaction mixture containing 0.2 mM dNTP, 1 × PCR buffer 1, 0.25 µM forward primer, 0.25 µM reverse primer, 1 µl *L. monocytogenes* genomic DNA (0.1 µg µl⁻¹) and 1 µl of a *Taq* and *Pwo* DNA polymerase mixture was incubated in a Techne Progene cyler at 94 °C for 2 min, followed by 35 cycles of amplification at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 2 min, with a final extension at 72 °C for 10 min. The PCR products were gel purified using a QIAquick Gel Extraction kit and inserted into the TA cloning vector pCR2.1 (Invitrogen). Both strands of the insert in the recombinant plasmids were first sequenced with the M13 forward primer (5'-GTAAACGACGACGAGT-3') and M13 reverse primer (5'-CAGGAACAGCTATGAC-3') and completed with additional primers derived from the initially determined sequences.

SDS-PAGE and Western blotting. SDS-PAGE was performed using the method described by Laemmli (1970), using a 4 % stacking gel and a 12 % resolving gel in a Bio-Rad minigel apparatus (Bio-Rad). Total *E. coli* proteins for SDS-PAGE were prepared by lysing bacterial cells in SDS/β-mercaptoethanol sample buffer at 100 °C for 10 min. For Western blot analysis, the separated proteins were electroblotted onto a nitrocellulose membrane using a Bio-Rad Trans-Blot SD semi-dry transfer cell according to the manufacturer's manual. The membranes were blocked with 3 % skimmed milk powder in PBST for at least 1 h, washed and then incubated at room temperature for 1 h with rabbit antiserum (R α L or R α K) at a dilution of 1 : 400 in PBST containing 3 % BSA. The membranes were then washed five times with TBST and incubated for 1 h with AP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1 : 5000 in PBST containing 3 % BSA. The membranes were washed as above and immunostained with 10 ml AP buffer containing nitro blue tetrazolium (25 µg ml⁻¹) and 5-bromo-4-chloro-3-indolyl phosphate (25 µg ml⁻¹) for 10 min.

RESULTS AND DISCUSSION

Identification of *E. coli* clones expressing R α L-reactive proteins

Evaluation by colony PCR of the *L. monocytogenes* protein expression library constructed prior to immunoscreening revealed that 20 out of 23 randomly selected clones (87 %) contained DNA inserts of about 1.5–2.0 kb. R α L and R α K, used for differential screening of the library, exhibited a comparable antibody titre (1 : 3200 to 1 : 6400) at 44 days p.i., as evaluated by ELISA. The initial screening of 35 000 clones with both sera identified 165 clones that reacted exclusively with R α L. Each of these positive clones was subjected to a second and third round of screening, resulting in the identification of 31 stable clones that reacted with R α L but not with R α K. The inserted DNA fragments from all of the positive clones were sequenced, leading to the classification of these clones into eight groups, each containing either identical or overlapping inserts (Fig. 1). Western blot analysis of total cellular proteins from one or two representative clones (pSCRN34, pSCRN114, pSCRN54, pSCRN159, pSCRN79, pSCRN88, pSCRN93, pSCRN92, pSCRN119, pSCRN130 and pSCRN100) in each group demonstrated the reactivity of encoded proteins with R α L (Fig. 2a) but not with R α K (Fig. 2b). These results were consistent with the findings of the library immunoscreening with R α L and R α K. Further Western blot analysis of total proteins from all of the representative clones with an anti-His mAb (Qiagen) showed that the reactive proteins migrated to positions corresponding to those of the major bands recognized by R α L with the exception that the mAb failed to detect any protein bands from clone pSCRN100 (data not shown).

Identity and sequence characteristics of R α L-reactive proteins expressed from positive clones

To elucidate the identity of R α L-reactive proteins expressed from the positive clones, protein sequence similarity

Fig. 1. Schematic illustration of the genes encoding RxL-reactive proteins in the cloned genomic DNA of *L. monocytogenes* serotype 4b. The GenBank accession numbers for the nucleotide sequences reported in (a)–(h) are EF409978 (a), EF409981 (b), EF409979 (c, e), EF409982 (d), EF409983 (f), EF409980 (g) and EF409984 (h).

searching was performed using BLAST with amino acid sequences deduced from the ORFs (within the inserts) that satisfied the following criteria: (i) a truncated ORF was inserted in frame with the start codon of the N-terminal fusion (including a His₆ tag) to express a fusion protein under the control of the vector's T7 promoter; (ii) the fusion protein was recognized by both anti-His mAb and RxL on Western blots; (iii) if the above criteria were not met, a complete or partial ORF with the upstream sequence containing a putative promoter predicted by a neural network promoter predation program (http://www.fruitfly.org/seq_tools/promoter.html) was considered to specify an RxL-reactive protein. The BLAST search results revealed the identity of RxL-reactive proteins encoded by 31 positive clones as InlA, two putative internalins (InlC2 and InlD) and five novel proteins of unknown function designated here as immunogenic surface proteins (Isp) IspA, IspB, IspC, IspD and IspE (Fig. 1).

A truncated InlA was defined by ten positive clones with identical DNA inserts of ~1.5 kb (nt 1692–3195) (Fig. 1a). A DNA fragment upstream of the incomplete *inlA* ORF was amplified by PCR and cloned into pCR2.1 to create pCR227-228 for sequencing. With the additional sequence from this recombinant plasmid, the complete ORF of *inlA* (nt 504–2906) with a 503 bp 5'-flanking sequence was obtained (GenBank accession no. EF409978). Eighty-five nucleotides downstream from the *inlA* stop codon was another incomplete ORF encoding the InlB protein (Dramsai *et al.*, 1995). The *L. monocytogenes* serotype 4b InlA was similar to the InlAs from other *L. monocytogenes* strains, including a length of 800 aa, a calculated molecular mass of 86.598 kDa and a predicted pI of 4.673, with an N-terminal domain containing 14 leucine-rich repeats and a C-terminal sorting signal comprising a conserved LPXTG motif (aa 767–771), followed by a hydrophobic domain (~20 aa) and a tail of positively charged amino acids.

Fig. 2. Western blot analysis of the recombinant proteins expressed from selected immunoreactive clones with rabbit antisera. Proteins transferred onto nitrocellulose membranes were probed with R α L (a) or R α K (b) at a dilution of 1 : 400. Each lane contained total proteins from cells equivalent to 1 ml culture with an OD₅₉₀ of 0.02. All clones except for pSCRN100 expressed a truncated *L. monocytogenes* protein with an N-terminal fusion. *E. coli* harbouring the cloning vector pSCREEN1b⁺ΔEcoRV was used as a control.

InlC2 was expressed in a truncated form from three immunoreactive clones with identical inserts of ~900 bp (nt 533–1472) (Fig. 1c). To obtain the full-length ORF of *inlC2*, two PCR products were generated and inserted into pCR2.1 (pCR293–263 and pCR262–291) for sequencing. A sequence of 1832 bp was assembled from these clones, which contained the 1647 bp ORF of *inlC2* and a 184 bp 3'-flanking sequence. The serotype 4b InlC2 protein had a length of 548 aa, a calculated molecular mass of 58.684 kDa and a predicted pI of 4.665. Its sequence showed 94.9, 94.0 and 94.2 % identity to those of EGD strain InlC2 (Drams et al., 1997), EGD-e InlH (Glaser et al., 2001) and EGD (serotype 1/2a) InlD (NCBI protein accession no. CAC20635), respectively.

A truncated form of InlD was encoded by six R α L-reactive clones with identical inserts of ~1.7 kb (nt 500–2288) (Fig. 1e), which were overlapped by identical inserts of ~2.2 kb (nt 1–2288) from two other positive clones. A 2288 bp sequence derived from the inserts of these overlapping clones contained a 1707 bp *inlD* ORF with 5'- and 3'-flanking sequences of 388 and 193 bp. There was a partial ORF 229 bp upstream of *inlD*, with its deduced amino acid sequence giving 100 % identity over 59 C-terminal residues to InlC2 (this study). Thus there was an InlC2-coding gene upstream of *inlD*. The sequences from Fig. 1(c and e) were combined and deposited in GenBank (GenBank accession no. EF409979). The serotype 4b InlD comprised 568 aa with a calculated molecular mass of 61.441 kDa and a predicted pI of 4.391. It shared strong homology (99.5 % identity) with the EGD strain InlD (NCBI protein accession no. AAB67970; Drams et al., 1997) and 61.4 and 64.1 % identity to *L. monocytogenes* EGD-e InlH (Glaser et al., 2001) and EGD (serotype 1/2a) InlD (NCBI protein accession no. CAC20635), respectively.

A truncated IspA was expressed from four immunoreactive clones with identical inserts of ~1.9 kb (nt 1140–3073) and one positive clone with a smaller overlapping insert of ~1.1 kb (nt 1140–2256) (Fig. 1g). To resolve the complete ORF of *ispA*, a PCR cloning strategy was used to generate pCR253–254 for sequencing. This resulted in a sequence of 3073 bp, which contained a 1770 bp *ispA* ORF with 5'- and 3'-flanking sequences of 155 and 1148 bp (GenBank accession no. EF409980). IspA comprised 589 aa with a calculated molecular mass of 63.578 kDa and a predicted pI of 4.092, and showed 93.5 % identity to Lin0372, a probable cell-surface protein of *L. innocua* Clip11262 (Glaser et al., 2001). This protein had sequence characteristics similar to an internalin protein, including a signal peptide (aa 1–31, predicted by SIGNALP 3.0), nine leucine-rich repeats with 26 aa per repeat (aa 94–291), a region of two inter-repeats (B repeat, aa 384–502) and an LPXTG sorting motif (aa 559–563) at the C-terminus. There was another incomplete ORF (1018 bp; in the reverse orientation), 133 bp downstream from *ispA*, whose translated product was similar to *L. innocua* Lin0373 or *L. monocytogenes* Lmo0354 (Glaser et al., 2001) based on a BLAST similarity search.

A truncated form of IspB was encoded by a partial ORF within the insert (~1.4 kb) of an immunoreactive clone (Fig. 1b; nt 820–2179). The complete ORF of *ispB* was obtained by sequencing the inserts from two recombinant plasmids, pCR268–279 and pCR257–317, which were created using a PCR cloning strategy. This resulted in a 2371 bp sequence composed of a 1962 bp ORF of *ispB* and 5'- and 3'-flanking sequences of 270 and 139 bp (GenBank accession no. EF409981). The deduced sequence of IspB comprised 653 aa with a calculated molecular mass of 74.693 kDa and a predicted pI of 6.224. This protein had 99.8 and 97.7 % identity to Lmo0927 and Lin0927, hypothetical transmembrane proteins of *L. monocytogenes* EGD-e and *L. innocua* Clip11262 (Glaser et al., 2001),

respectively. IspB exhibited significant homology (62 and 23 % identity) to the sulfatases of *Bacillus anthracis* A20121 and *E. coli* K1 (Hoffman *et al.*, 2000; Read *et al.*, 2003), respectively. This protein contained a C-terminal domain (aa 253–574) similar to a sulfatase domain (predicted by INTERPROSCAN). Disruption or deletion of the sulfatase gene *aslA* from a cerebrospinal isolate of *E. coli* K1 (the causal agent of neonatal *E. coli* meningitis) reduced the ability of this pathogen to invade the brain microvasculature (Hoffman *et al.*, 2000). *L. monocytogenes* is capable of invading the blood–brain barrier and causing meningitis, implying that, although its sulfatase activity is as yet unproven, IspB may have a similar role in invasion of the brain microvasculature.

IspC was expressed in a truncated form from two immunoreactive clones with identical inserts of ~1.3 kb (Fig. 1d; nt 1–1325). The C-terminal sequence (325 aa) of IspC deduced from its incomplete ORF showed 32.3 and 32.6 % identity to the corresponding regions of *L. monocytogenes* and *L. innocua* amidases Ami (Glaser *et al.*, 2001), respectively. In addition, IspC had 68.9 and 35.4 % identity to the corresponding regions of the putative amidase-like proteins Lin1064 and Lmo1076 of *L. innocua* and *L. monocytogenes*, respectively (Glaser *et al.*, 2001). Using additional unpublished sequence data (H. Dan and M. Lin), the complete ORF of *ispC* was obtained (GenBank accession no. EF409982). There was an incomplete ORF (nt 1041–1325), 65 bp downstream from the *ispC* ORF, in the reverse orientation, encoding a protein similar to Lin1065, a putative protein of *L. innocua* (Glaser *et al.*, 2001).

A truncated form of IspD was encoded by a partial ORF within the insert (~1.8 kb) from an R_zL-reactive clone (Fig. 1f; nt 1–1812). The deduced C-terminal amino acid sequence (570 aa) of IspD showed 32.9, 33.9 and 30.0 % identity, respectively, to the corresponding regions of ORFA, a putative protein of *Listeria seeligeri* (GenBank accession no. CAA65738), Lmo0333, a putative peptidoglycan protein of *L. monocytogenes* EGD-e (Glaser *et al.*, 2001), and Lin0665, a putative protein of *L. innocua* (Glaser *et al.*, 2001), which is highly similar to the *L. seeligeri* ORFA. The 570 aa C-terminal region of IspD contained two leucine-rich repeat units (aa 16–70) with 14 residues per repeat and four polycystic kidney disease repeats (Hughes *et al.*, 1995). Given that leucine-rich and polycystic kidney disease repeat domains have been shown or inferred to have specific molecular binding functions (Hughes *et al.*, 1995; Kajava, 1998; Cabanes *et al.*, 2002), this suggests that IspD has a similar role in bacterium–host interactions and/or signalling. A complete *ispD* ORF was obtained using additional unpublished sequence data (L. Wang, T. Abujamel and M. Lin) (GenBank accession no. EF409983).

A truncated form of IspE was expressed from an immunoreactive clone with an insert of ~1.5 kb (Fig. 1h; nt 1–1473; GenBank accession no. EF409984) using a partial ORF (in the opposite direction to the vector's T7

promoter, nt 1–883), presumably directed by a putative promoter in the 590 bp sequence upstream of the ORF. The deduced N-terminal amino acid sequence (294 aa) of IspE showed 92.2 % identity to the corresponding region of Lmo0835, a putative peptidoglycan-bound protein of *L. monocytogenes* EGD-e (Glaser *et al.*, 2001), and contained a signal peptide (aa 1–33, predicted by SIGNALP 3.0) and two repeats (aa 58–107 and aa 85–237) of unknown function. Another partial ORF (nt 876–1473) oriented in the same direction as the *ispE* ORF and overlapping it by 8 bp was found in the insert, encoding a protein similar to the putative protein Lmo0834 of *L. monocytogenes* (Glaser *et al.*, 2001).

The present study has provided the first example of the identification of protein targets of the humoral immune response to *L. monocytogenes* infection by differential immunoscreening of a protein-expression library. The five novel proteins reported here of unknown function exhibited immunogenicity during rabbit infection, suggesting that they are probably expressed on the cell surface and involved in the interaction with host cells during the infection process, and are thus referred to as immunogenic surface proteins (Isp). Some of these proteins have a cell-wall-anchoring domain or motif for surface localization. Like InlA and the putative internalins InlC2 and InlD, both IspA and IspD contained a cell-wall-anchoring motif, LPXTG (Cabanes *et al.*, 2002), at their C-terminal end. IspC contained a C-terminal cell-wall-anchoring domain made up of several Gly–Trp modules as found in the autolysins Ami and Auto of *L. monocytogenes* (Cabanes *et al.*, 2004; Braun *et al.*, 1997; McLaughlan & Foster, 1998) and in the internalin InlB (Braun *et al.*, 1997). IspB and IspE are likely to be a transmembrane sulfatase and a peptidoglycan-bound protein, respectively. With the exception of InlA (Boerlin *et al.*, 2003), seven other proteins (InlC2, InlD, IspA, IspB, IspC, IspD and IspE) were, for the first time, shown to be expressed as novel targets of the humoral immune response to *L. monocytogenes* infection. Given that rabbits are recognized as a good animal model of human infection with *L. monocytogenes*, the occurrence of a similar antibody response to these proteins in listeriosis patients may be expected. These proteins may be gene products induced only in infection or may be significantly upregulated *in vivo* during infection. Co-expression of these novel proteins during infection with a known virulence factor, InlA, appears to suggest that they are involved in *L. monocytogenes* virulence and pathogenesis. These proteins could be useful candidates for investigating humoral immunity against listerial infection and may aid in the development of vaccines and serodiagnostic assays.

ACKNOWLEDGEMENTS

We acknowledge J. Devenish for his assistance in *L. monocytogenes* culture. We are also grateful to L. Kealey, B. Davis and B. Tripp for their assistance in animal infection and immunization experiments, and M. Mallory and E. Tangorra for their technical support.

REFERENCES

- Artiushin, S., Timoney, J. F., Nally, J. & Verma, A. (2004). Host-inducible immunogenic sphingomyelinase-like protein, Lk73.5, of *Leptospira interrogans*. *Infect Immun* **72**, 742–749.
- Bannantine, J. P. & Stabel, J. R. (2001). Identification of two *Mycobacterium avium* subspecies *paratuberculosis* gene products differentially recognised by sera from rabbits immunised with live mycobacteria but not heat-killed mycobacteria. *J Med Microbiol* **50**, 795–804.
- Berche, P., Reich, K. A., Bonnichon, M., Beretti, J. L., Geoffroy, C., Raveneau, J., Cossart, P., Gaillard, J. L., Geslin, P. & other authors (1990). Detection of anti-listeriolysin O for serodiagnosis of human listeriosis. *Lancet* **335**, 624–627.
- Bhunia, A. K. (1997). Antibodies to *Listeria monocytogenes*. *Crit Rev Microbiol* **23**, 77–107.
- Boerlin, P., Boerlin-Petzold, F. & Jemmi, T. (2003). Use of listeriolysin O and internalin A in a seroepidemiological study of listeriosis in Swiss dairy cows. *J Clin Microbiol* **41**, 1055–1061.
- Braun, L., Dramsi, S., Dehoux, P., Bierne, H., Lindahl, G. & Cossart, P. (1997). InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. *Mol Microbiol* **25**, 285–294.
- Braun, L., Ghebrehwet, B. & Cossart, P. (2000). gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*. *EMBO J* **19**, 1458–1466.
- Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L. & Cossart, P. (2002). Surface proteins and the pathogenic potential of *Listeria monocytogenes*. *Trends Microbiol* **10**, 238–245.
- Cabanes, D., Dussurget, O., Dehoux, P. & Cossart, P. (2004). Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. *Mol Microbiol* **51**, 1601–1614.
- Deb, D. K., Dahiya, P., Srivastava, K. K., Srivastava, R. & Srivastava, B. S. (2002). Selective identification of new therapeutic targets of *Mycobacterium tuberculosis* by IVIAT approach. *Tuberculosis (Edinb)* **82**, 175–182.
- Dramsi, S., Biswas, I., Maguin, E., Braun, L., Mastroeni, P. & Cossart, P. (1995). Entry of *Listeria monocytogenes* into hepatocytes requires expression of inlB, a surface protein of the internalin multigene family. *Mol Microbiol* **16**, 251–261.
- Dramsi, S., Dehoux, P., Lebrun, M., Goossens, P. L. & Cossart, P. (1997). Identification of four new members of the internalin multigene family of *Listeria monocytogenes* EGD. *Infect Immun* **65**, 1615–1625.
- Edelson, B. T. & Unanue, E. R. (2000). Immunity to *Listeria* infection. *Curr Opin Immunol* **12**, 425–431.
- Edelson, B. T., Cossart, P. & Unanue, E. R. (1999). Cutting edge: paradigm revisited: antibody provides resistance to *Listeria* infection. *J Immunol* **163**, 4087–4090.
- Gentschev, I., Sokolovic, Z., Kohler, S., Krohne, G. F., Hof, H., Wagner, J. & Goebel, W. (1992). Identification of p60 antibodies in human sera and presentation of this listerial antigen on the surface of attenuated salmonellae by the HlyB-HlyD secretion system. *Infect Immun* **60**, 5091–5098.
- Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloeker, H., Brandt, P. & other authors (2001). Comparative genomics of *Listeria* species. *Science* **294**, 849–852.
- Grenningloh, R., Darji, A., Wehland, J., Chakraborty, T. & Weiss, S. (1997). Listeriolysin and IrpA are major protein targets of the human humoral response against *Listeria monocytogenes*. *Infect Immun* **65**, 3976–3980.
- Handfield, M., Brady, L. J., Progulske-Fox, A. & Hillman, J. D. (2000). IVIAT: a novel method to identify microbial genes expressed specifically during human infections. *Trends Microbiol* **8**, 336–339.
- Hang, L., John, M., Asaduzzaman, M., Bridges, E. A., Vanderspurt, C., Kirn, T. J., Taylor, R. K., Hillman, J. D., Progulske-Fox, A. & other authors (2003). Use of in vivo-induced antigen technology (IVIAT) to identify genes uniquely expressed during human infection with *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **100**, 8508–8513.
- Hoffman, J. A., Badger, J. L., Zhang, Y., Huang, S. H. & Kim, K. S. (2000). *Escherichia coli* K1 *aslA* contributes to invasion of brain microvascular endothelial cells in vitro and in vivo. *Infect Immun* **68**, 5062–5067.
- Hughes, J., Ward, C. J., Peral, B., Aspinwall, R., Clark, K., San Millan, J. L., Gamble, V. & Harris, P. C. (1995). The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. *Nat Genet* **10**, 151–160.
- John, M., Kudva, I. T., Griffin, R. W., Dodson, A. W., McManus, B., Krastins, B., Sarracino, D., Progulske-Fox, A., Hillman, J. D. & other authors (2005). Use of in vivo-induced antigen technology for identification of *Escherichia coli* O157 : H7 proteins expressed during human infection. *Infect Immun* **73**, 2665–2679.
- Kajava, A. V. (1998). Structural diversity of leucine-rich repeat proteins. *J Mol Biol* **277**, 519–527.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lazowska, I., Trzeciak, L., Godlewska, R., Hennig, E., Jagusztyn-Krynicka, K., Popowski, J., Regula, J. & Ostrowski, J. (2000). In search of immunogenic *Helicobacter pylori* proteins by screening of expression library. *Digestion* **61**, 14–21.
- Lin, M. (1999). Molecular analysis of flaB, a periplasmic flagellar core protein gene in pathogenic leptospires. *J Biochem Mol Biol Biophys* **2**, 181–187.
- Lodes, M. J., Houghton, R. L., Bruinsma, E. S., Mohamath, R., Reynolds, L. D., Benson, D. R., Krause, P. J., Reed, S. G. & Persing, D. H. (2000). Serological expression cloning of novel immunoreactive antigens of *Babesia microti*. *Infect Immun* **68**, 2783–2790.
- Lorber, B. (1997). Listeriosis. *Clin Infect Dis* **24**, 1–9.
- Mackaness, G. B. (1962). Cellular resistance to infection. *J Exp Med* **116**, 381–406.
- McLaughlan, A. M. & Foster, S. J. (1998). Molecular characterization of an autolytic amidase of *Listeria monocytogenes* EGD. *Microbiology* **144**, 1359–1367.
- Meier, J. & Lopez, L. (2001). Listeriosis: an emerging food-borne disease. *Clin Lab Sci* **14**, 187–192.
- Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M. & Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* **84**, 923–932.
- Ochsenbein, A. F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H. & Zinkernagel, R. M. (1999). Control of early viral and bacterial distribution and disease by natural antibodies. *Science* **286**, 2156–2159.
- Read, T. D., Peterson, S. N., Tourasse, N., Baillie, L. W., Paulsen, I. T., Nelson, K. E., Tettelin, H., Fouts, D. E., Eisen, J. A. & other authors (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423**, 81–86.
- Rocourt, J. & Bille, J. (1997). Foodborne listeriosis. *World Health Stat Q* **50**, 67–73.
- Rollins, S. M., Peppercorn, A., Hang, L., Hillman, J. D., Calderwood, S. B., Handfield, M. & Ryan, E. T. (2005). In vivo induced antigen technology (IVIAT). *Cell Microbiol* **7**, 1–9.

Sambrook, J. & Russel, D. W. (2000). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schlech, W. F., III (1993). An animal model of foodborne *Listeria monocytogenes* virulence: effect of alterations in local and systemic immunity on invasive infection. *Clin Invest Med* **16**, 219–225.

Shen, Y., Naujokas, M., Park, M. & Ireton, K. (2000). InIB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell* **103**, 501–510.

Suk, K., Das, S., Sun, W., Jwang, B., Barthold, S. W., Flavell, R. A. & Fikrig, E. (1995). *Borrelia burgdorferi* genes selectively expressed in the infected host. *Proc Natl Acad Sci U S A* **92**, 4269–4273.

Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J. & Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* **14**, 584–640.

Yu, W. (2004). *The gene products of Listeria monocytogenes induced specifically during rabbit infection*. MSc Thesis, University of Ottawa, Ottawa, Canada.