BACTERIAL PATHOGENICITY

Surface protein p104 is involved in adhesion of *Listeria monocytogenes* to human intestinal cell line, Caco-2

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Adhesion of *Listeria monocytogenes* to intestinal endothelial cells is an important initial event in the pathogenesis of infection which is not well understood. The suggestion has been made that some proteins, including internalin and actin polymerisation protein (ActA), and carbohydrate molecules mediate, at least in part, the adhesion of listeria to certain cultured mammalian cells. This study investigated the role of a *L. monocytogenes* cell-surface protein of 104 kDa (p104) in adhesion to human intestinal enterocyte-like Caco-2 cell lines by transposon (Tn916) mutagenesis and a p104-specific monoclonal antibody (MAb-H7). Genotypic and phenotypic characteristics of Tn916-transformed *L. monocytogenes* strains, AAMU530 and AAMU572, revealed that these strains did not express p104, and the transposon had been inserted at a single locus in the structural gene. Strains AAMU530 and AAMU572 yielded only 10 and 6.3% adhesion to Caco-2 cells. Coating of *L. monocytogenes* and *L. innocua* wild-type strains with MAb-H7 reduced adhesion to Caco-2 cells from 100% to 50 and 45%, respectively, whereas on isotype control MAb EM-7G1 had no effect. Western blot analysis with MAb-H7 indicated that p104 is present in all *Listeria* spp. except in *L. grayi*. Furthermore, p104 is also present in internalin (BUG8) and ActA (LUT12) deficient strains, suggesting that p104 is indeed different from internalin or ActA proteins. Cytotoxicity analysis of strains AAMU530 and AAMU572 demonstrated that these strains, although haemolytic and phospholipase-positive, were avirulent when tested with a hybridoma B-lymphocyte cell line. Loss of virulence could be attributed to the interruption of adhesion of mutant strains to the hybridoma cell line. These results strongly suggest that p104 is an adhesion factor in *L. monocytogenes* and possibly in other *Listeria* species and is involved in adhesion to intestinal cells.

Introduction

Listeria monocytogenes, a food-borne human pathogen, is responsible for infections such as septicaemia and meningitis in immunocompromised individuals and is one cause of abortion in pregnant women [1]. A recent report indicated that *L. monocytogenes* also causes gastro-enteritis in healthy individuals [2]. Transmission in man is frequently linked to food contaminated by listeria and entry into the host normally occurs in the gut. In animal studies (following intragastric inoculation) bacteria pass the gastrointestinal barrier and possibly penetrate the intestinal epithelial cells over-

laying Peyer's patches [3, 4]. The organism then disseminates to the brain and to the spleen, liver and other lymphatic systems.

A key aspect of the pathogenicity of *L. monocytogenes* is its ability to invade and multiply in phagocytic and non-phagocytic cells. Adhesion of *L. monocytogenes* to host cells is an essential event for invasion leading to infection. Adhesion is presumed to be mediated by *L. monocytogenes* cell-surface molecules with complementary receptors present on the eukaryotic cells [5]. Internalin (80 kDa) [6, 7], actin polymerisation protein (ActA, 90 kDa) [8], and other surface molecules [5, 9, 10] of *L. monocytogenes* have been shown to be partially responsible for adhesion to mammalian cells. Internalin A (InlA) is essential for entry into human enterocyte-like Caco-2 cells, whereas Internalin B (InlB) is required for entry into cultured

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hepatocytes such as TIB73 and HepG-2 and some epithelial cell lines including HeLa, HEp-2 and Vero cells [11]. The cellular receptor for InlA has been shown to be the adhesion molecule E-cadherin [12], but the interaction between InlA and E-cadherin leading to bacterial uptake by the host cells is not yet understood. A third member of the internalin family, named InlC, has recently been identified [13] and has been reported to encode a secreted protein of 297 amino acids that is homologous to InlA and InlB. InlC possibly plays a role in the late stage of infection rather than in the uptake of *L. monocytogenes* by non-phagocytic cells [7]. Another surface protein of 60 kDa (p60) present in all *Listeria* spp. has been reported to help *L. monocytogenes* to adhere and enter into mouse fibroblast (3T6) cell lines [14]. This protein apparently does not play any role in adherence or entry into Caco-2 cells [14, 15].

Several studies indicate that *L. monocytogenes* induces its own entry by initiating an 'internalisation signal' to the host cells to re-arrange cytoskeletal structure, thus allowing *L. monocytogenes* entry [16, 17]. Attachment of *L. monocytogenes* also causes tyrosine phosphorylation of host cell mitogen-activated protein kinase (MAP) which facilitates the internalisation process. Addition of protein kinase inhibitors, such as genistein, prevents *L. monocytogenes* internalisation; however, the MAP inhibitor does not inhibit bacterial adhesion to epithelial cells [16, 17].

In spite of extensive research on the pathogenesis of *L. monocytogenes* infection, adhesion of this organism to mammalian cells and tissues is poorly understood. The internalin and ActA molecules mentioned above are present only in *L. monocytogenes* and may be partly involved in adhesion to mammalian cells. However, reports by Meyer *et al.* [18] and Bunduki *et al.* [19] indicated that not only *L. monocytogenes*, but also *L. innocua*, *L. seeligeri*, *L. ivanovii* and *L. welshimeri* adhere to the primary intestinal endothelial cells. These studies suggested that there might be additional factors involved in adhesion of *Listeria* spp. to intestinal cells. In a preliminary report it was observed that *L. monocytogenes*, *L. innocua* and *L. welshimeri* also bind to Caco-2 and murine B-lymphocyte (Ped-2E9) cell lines [20]. Addition of a monoclonal antibody (MAb) raised against a surface protein of 104 kDa (p104) partially blocked the adhesion of *Listeria* to Caco-2 and Ped-2E9 cells [20].

In this study, the role of p104 in *L. monocytogenes* was examined by the employment of transposon mutagenesis (Tn916). Transconjugants deficient in p104 were studied for their ability to adhere to Caco-2 cells. In addition, MAbs were used to study the adhesion of *Listeria* to Caco-2 cells and to characterise p104 protein from various *Listeria* spp. Furthermore, the cytotoxicity of mutant strains ($\Delta p104$:Tn916) was evaluated in a tissue culture model [21].

Materials and methods

Mammalian cells

Human enterocyte-like Caco-2 (TIB37, ATCC) and murine hybridoma Ped-2E9 [21] cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (Atlanta Biologicals, Norcross, GA) 10% and glutamine 1% and incubated at 37°C in air with CO₂ 7% under humidified conditions.

MAbs

MAB-H7, specific for the p104 surface protein of *Listeria* spp., was developed by immunising mice with heat-killed *L. monocytogenes* cells [20]. MAB EM-7G1, specific for a 66-kDa cell surface protein of *L. monocytogenes*, was used in this study as a reference antibody [22]. Both MAbs (isotype IgG1) were purified by ammonium sulphate precipitation, followed by gel filtration chromatography (Sephacryl S-200). Protein concentrations were determined spectrophotometrically (absorption at 280 nm/1.4 × dilution factor) and concentrations for both antibodies were adjusted to 34 mg/ml.

Bacterial strains and transposon mutagenesis

L. monocytogenes F4244 (WT), BUG8 (Δ inlAB), LUT12 (Δ actA), *L. innocua* F4248, *L. ivanovii* ATCC 19119, *L. welshimeri* ATCC 35897, *L. seeligeri* SE31 and *L. grayi* ATCC 19120 strains were grown in brain heart infusion (BHI) broth with or without antibiotics. *Escherichia coli* CG120 carrying plasmid pAM120, which contains the transposon Tn916 [23], was used in this study. Conjugation of recipient strain *L. monocytogenes* F4244, erythromycin resistant (em^r) and donor strain *Enterococcus faecalis* CG110, tetracycline resistant (tet^r) carrying Tn916 was accomplished by the procedure described by Kathariou *et al.* [24]. Transconjugant colonies from agar plates were transferred to nitrocellulose membranes and immunoprobed with MAB-H7 [20]. Mutants that showed weak or no reaction were selected and confirmed biochemically with API *Listeria* strips (bioMérieux, Hazelwood, MO, USA). Cultures were also tested for phospholipase C (PLC) activity on agarose 2% medium containing egg-yolk 7%, 50 mM CaCl₂ and 50 mM dithiothreitol [25]; and for listeriolysin (LLO) activity on sheep blood 5% agar plates. Two mutant strains of *L. monocytogenes*, AAMU530 and AAMU572, were positive for PLC and LLO and were selected for further studies.

Genetic analysis of transconjugants

Plasmid (pAM120) carrying the Tn916 sequence was isolated from *E. coli* CG120 by the modified alkaline lysis or Quantum Prep Plasmid Miniprep (BioRad, Hercules, CA, USA) methods. Chromosomal DNA

was prepared with GES reagent (5 M guanidine isothiocyanate, 0.1 M EDTA, Sarkosyl 0.5% v/v) followed by purification with the Wizard Genomic DNA kit (Promega, Madison, WI, USA). Chromosomal and plasmid DNA were digested with restriction endonucleases according to the manufacturer's recommendations (Promega). DNA was separated by electrophoresis on agarose 0.6% gels in Tris-borate-EDTA buffer (0.04 M Tris-borate, 0.001 M EDTA, pH 8.0) [26].

PCR analysis. Insertion of Tn916 into the mutant strains was analysed by the use of transposon-specific *tetM* gene primers [27] in a PCR assay. A 10- μ l (1 μ g) portion of template DNA from *L. monocytogenes* WT, AAMU530, AAMU572 and *E. faecalis* CG110 (*tet*^r) was used for amplification in a total volume of 100 μ l in a Deltacycler I system (Ericomp, San Diego, CA, USA). The optimal PCR reaction mixture contained 100 pmol of each primer (left: 5' TTGATGCCCTT-TTGAATC 3'; right: 5' ACTGCATTCCACTTCC-CAAC 3'), 40 mM dNTPs, 10 μ l of 10 $\times$ reaction buffer, 5 units of Taq polymerase (Sigma) and 10 μ l of mineral oil. Initial denaturation at 94°C for 5 min was followed by 30 cycles of 94°C, 2 min; 60°C, 2 min and 72°C, 2 min. After final extension at 72°C for 10 min, the samples were stored at 4°C until analysis. The PCR products were separated by electrophoresis in agarose 1.2% gel.

Southern hybridisation. Southern hybridisation was performed according to the procedure of Southern [28] with the Genius System (Boehringer Mannheim, Indianapolis, IN, USA). *Hind*III-digested chromosomal DNA was electrophoresed and transferred to a nylon membrane (ICN, Costa Mesa, CA, USA). Pre-hybridisation of the membrane was carried out at 68°C. The transposon probe was made from the *Eco*RI restriction fragment (18 kb) of pAM120 which contains the Tn916 complete sequence [27]. The 18-kb fragment was purified from the agarose gel with a Prep-A-Gene DNA Purification System (BioRad), followed by non-radioactive random primed labelling with the Genius SystemTM (Boehringer Mannheim). Hybridisation products were detected by the enzyme immuno-assay method.

SDS-PAGE and Western blotting

L. monocytogenes WT, AAMU530, AAMU572, BUG8 and LUT12 strains along with other *Listeria* spp. were grown in BHI broth with or without tetracycline 10 μ g/ml for 18–24 h at 37°C. Cell-surface proteins were extracted with SDS-PAGE sample solvent [29] according to the method described by Bhunia *et al.* [30]. Protein samples were analysed in polyacrylamide 8% gels [29]. After electrophoresis (25 mA, 2 h), proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) by means of an electroblotter (BioRad). The

membranes were blocked with non-fat dry milk 5% for 2–12 h, probed with MAb-H7 (1 in 1000) and the colour was developed [30]. Simultaneously, duplicate gels were also stained with Coomassie Blue R250 or silver stain (BioRad).

Adhesion assay

L. monocytogenes WT and the mutants AAMU530 and AAMU572 were tested for their ability to adhere to Caco-2 monolayers [9]. Caco-2 cell monolayers in 24-well tissue culture plates were washed three times with 20 mM phosphate-buffered saline (PBS), pH 7.0, and resuspended with 0.4 ml of tissue culture medium per well. The monolayers were infected with 0.1 ml of test organisms (*c.* 1×10^8 cfu/well) and incubated at 37°C for 30 min. Non-adherent bacteria were eliminated by washing five times with PBS. Washed Caco-2 monolayers were treated with 0.5 ml of Triton X-100 0.5% for 10 min and the counts of adherent bacteria were determined by plating on BHI agar.

Adhesion of *Listeria* spp. to Caco-2 cells was also examined by Giemsa's stain. Plastic coverslips containing Caco-2 cell monolayers were transferred to 12-well tissue culture plates, inoculated with test organisms as described above and incubated at 37°C for 30 min. After being washed five times, the monolayers were appropriately stained with Giemsa's stain as described previously [14].

Effect of MAbs of adhesion

A 0.1-ml portion of each *L. monocytogenes* and *L. innocua* culture was allowed to react with 0.035 ml (34 mg/ml) of MAb EM-7G1 or H7 for 1 h at 37°C. The antibody-coated bacterial suspensions were then added to Caco-2 cells and incubated at 37°C for 30 min [31]. Caco-2 cells were washed three times, treated with Triton X-100 0.5% and bacterial counts were determined.

Cytotoxicity assay

Cytotoxic effects of mutants AAMU530 and AAMU572 and WT strains were determined on a hybridoma Ped-2E9 cell line by trypan blue (0.4%) staining and alkaline phosphatase (AP) release assay as reported previously [21, 32].

Results

Characterisation of transconjugants

Six broth mating experiments resulted in a total of 82 mutants, of which only two (AAMU530 and AAMU572) showed weak or no reaction with MAb-H7, suggesting that these strains possibly lacked the 104-kDa (p104) surface protein. Both strains were confirmed to be *L. monocytogenes* by API-Listeria

assay and were haemolytic (hly⁺) and phospholipase C positive (plc⁺).

PCR analysis of mutants with Tn916-specific primers revealed that AAMU530, AAMU572 and *E. faecalis* CG110 (positive control) gave a PCR product of 294 bp (Fig. 1a). No PCR product was observed from *L. monocytogenes* WT strain (negative control). This result demonstrated the insertion of Tn916 into the chromosome of the AAMU530 and AAMU572 strains (Fig. 1a).

In Southern hybridisation, the Tn916 probe hybridised with two complementary fragments (>12 and 6 kb) from the *Hind*III-digested chromosomal DNA of strains AAMU530 and AAMU572, thus confirming the single Tn916 insertion into the chromosome of the mutants (Fig. 1b) because transposon insertion in multiple sites would have generated more than two Tn916-probe-reactive bands. The extra, high mol. wt, faint bands in the AAMU530 and AAMU572 lanes were the products of incomplete digestion of the genomic DNA. Plasmid pAM120 (23.3 kb), harbouring Tn916, had a single *Hind*III restriction site and therefore, showed a single high mol. wt band. The additional light bands present in the lane were perhaps due to degradation of the plasmid during isolation or by prolonged enzymatic digestion. A genomic DNA preparation from strain WT did not show any hybridisation with the probe (Fig. 1b).

Western blotting

Western blotting with MAb-H7 indicated that the p104 protein was absent from strains AAMU530 and AAMU572, but was present in *L. monocytogenes* WT, BUG8 (Δ inlAB), LUT12 (Δ actA) and revertant strains of AAMU530 and AAMU572 (Fig. 2c). Furthermore, the MAb also reacted with protein extracts from *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri*, but had no reaction with *L. grayi* (Fig. 2b). Silver staining of SDS-PAGE gel showed that strain AAMU530 had a very faint band at 104 kDa, whereas no band was observed for strain AAMU572 at that position (Fig. 2a).

Adhesion of listeria to Caco-2 cells

Adhesion results (Table 1) demonstrated that strains AAMU530 and AAMU572 had only 10 and 6.3% adhesion to Caco-2 cells, respectively, values significantly lower than the 100% of adhesion strain WT ($p \leq 0.05$). *L. innocua* also showed adhesion to Caco-2 cells, but less efficiently than *L. monocytogenes* WT strain. Giemsa's staining results exhibited similar patterns of adhesion to the Caco-2 cells (data not shown). *L. monocytogenes* WT, showed 50% adhesion when coated with MAb-H7, which was significantly lower than that of *L. monocytogenes* cells treated with the control MAb EM-7G1 (Table 1). On the other

Fig. 1. (a) PCR confirmation of Tn916 insertion in *L. monocytogenes* mutant strains with *tetM* gene-specific primers. Lane 1, DNA standard; 2, *L. monocytogenes* WT; 3, AAMU530; 4, AAMU572; 5, *E. faecalis* CG110 carrying Tn916. (b) Southern hybridisation of *Hind*III-digested genomic DNA from *L. monocytogenes* mutant strains and plasmid pAM120 from *E. coli* CG120 harbouring Tn916 with an 18-kb Tn916 probe. Lanes 1, *L. monocytogenes* WT; 2, AAMU530; 3, AAMU572; 4, plasmid pAM120. Tn916 probe hybridised with two fragments of >12 (●) and 6 kb (◁) from AAMU530 (lane 2) and AAMU572 (lane 3) and one major band (●) from pAM120 linearised (23.3 kb) with *Hind*III (lane 4). The other bands in lane 4 are possibly the non-specific breakdown products of enzymic digestion.

hand, *L. innocua* cells treated with MAb-H7 had 45% and cells treated with EM-7G1 had 77% adhesion to Caco-2 cells; however, these differences in adhesion were not statistically significant at $p \leq 0.05$ (Table 1).

Fig. 2. (a) Silver-stained SDS-PAGE gel of cell-surface proteins from *L. monocytogenes* strains. Lane 1, LUT12- Δ actA; 2, BUG8- Δ inlAB; 3, revertant AAMU572; 4, revertant AAMU530; 5, AAMU572- Δ p104; 6, AAMU530- Δ p104; 7, WT; 8, mol. wt standard. Arrow-head in lane 6 indicates the presence of weak p104 protein band. (b) Western blot analysis of proteins from various *Listeria* species with MAb-H7. Lane 1, mol. wt standard; 2, *L. monocytogenes* WT; 3, *L. innocua*; 4, *L. ivanovii*; 5, *L. seeligeri*; 6, *L. welshimeri*; 7, *L. grayi*. (c) Western blot analysis of cell-surface proteins from *L. monocytogenes* strains with MAb-H7. Lane 1, WT; 2, AAMU530; 3, AAMU572; 4, BUG8; 5, LUT12; 6, revertant AAMU530; 7, revertant AAMU572. MAb showed no reaction with proteins from the mutant AAMU530 and AAMU572 strains.

Cytotoxicity profile of mutant strains

In the hybridoma cell viability assay, the percentage cell death for *L. monocytogenes* strains WT, AAMU530, AAMU572 and *L. innocua* was calculated to be 84, 70, 54 and 6%, respectively (Fig. 3). Similarly, in the AP assay, strains AAMU530 and AAMU572 induced AP releases of 16 and 15% respectively, which was significantly lower than the

Table 1. Effect of mutation (Tn916) and MAbs on adhesion of *Listeria* species to Caco-2 cells

Organisms	Adhesion	
	Average cfu/well* log ₁₀ mean cfu (SD)	Adhesion (%)†
<i>L. monocytogenes</i>		
F4244 (WT)	1.04×10^6 6.00 (0.12)	100
AAMU530 (Δ p104; Tn916)	1.04×10^5 5.00 (0.09)	10
AAMU572 (Δ p104; Tn916)	6.6×10^4 4.81 (0.18)	6.3
F4244 + EM-7G1‡	1.19×10^6 6.07 (0.02)	100
F4244 + MAb-H7‡	5.19×10^5 5.07 (0.09)	50
<i>L. innocua</i>		
F4248	4.11×10^4 4.61 (0.36)	100
F4248 + EM7G1‡	3.18×10^4 4.50 (0.36)	77
F4244 + MAb-H7‡	1.86×10^4 4.26 (0.47)	45

*Cfu/well is an average of six replicates from three experiments.

†Percentage adhesion calculations were based on the total adhesion by *L. monocytogenes* or *L. innocua* wild-type strains without any treatments using average cfu/well values.

‡MAb EM-7G1 and H7 are specific for 66-kDa and 104-kDa proteins, respectively, and belong to the IgG1 subclass.

Fig. 3. Cytotoxicity of *L. monocytogenes* mutant strains on a hybridoma B-lymphocyte line (Ped-2E9). Ped-2E9 cells were treated with PBS (control), *L. innocua* (Linn) and *L. monocytogenes* strains WT (Lm.WT), AAMU530 (A530) and AAMU572 (A572) for 5 h. Cytotoxic effects were determined by counting viable cells (■) after trypan blue staining or by calculating percentage alkaline phosphatase (■) release from Ped-2E9 cells [32]. Percentage cytotoxicity was determined based on the total alkaline phosphatase (AP) release from Triton-X-treated Ped-2E9 cells.

68% released by *L. monocytogenes* WT ($p \leq 0.05$). In contrast, *L. innocua* induced only 2% AP release (Fig. 3).

Discussion

Adhesion and invasion of professional and non-professional phagocytic cells by listeria are crucial steps in the pathogenesis of infection. *L. monocytogenes* penetrates certain mammalian cells with the help of internalin proteins (InlA and InlB) or may induce its own internalisation by activating host cell signal transduction pathways [33, 34]. Internalin-mediated entry is believed to be initiated through binding of the leucine-rich repeat region of internalin with a E-cadherin receptor on mammalian cells through a zipper mechanism [12, 35].

While many studies have explored the mechanisms of internalisation, only limited information is available as to the mechanisms of listeria adhesion to mammalian cells. Adhesion is an important key step in listeria pathogenesis, which is believed to be a complex process involving many cell-surface molecules [36], whose specific function(s) are not well understood. In general, adhesion properties in listeria are believed to be not only associated with pathogenic *L. monocytogenes* or *L. ivanovii*, but also with non-pathogenic *L. innocua*, *L. welshimeri* or *L. seeligeri*. To date, several cell surface molecules have been implicated in the adhesion of listeria to host cells. As discussed above, internalin [6] may be involved in adhesion; however, this protein is present only in *L. monocytogenes* and not in other *Listeria* spp. ActA protein, which is primarily responsible for bacterial motility inside the host cell cytoplasm, was shown to help *L. monocytogenes* attachment to a heparan sulphate proteoglycan receptor in macrophage and Chinese hamster ovary (CHO) cells [8].

Another surface protein p60, present in all *Listeria* spp., was reported to be responsible for adhesion to and invasion of fibroblast (3T6) cells, but not to the epithelial (Caco-2) cells [14]. A study by Hess *et al.* [15] suggested that p60 and internalin possibly acted in concert during *L. monocytogenes* invasion. Their *in-vivo* study with an internalin-deficient, p60-secreting *L. monocytogenes* strain showed no variation in adhesion or invasion of mouse intestinal cells and consequently they concluded that there might be additional factor(s) responsible for adhesion and invasion by *L. monocytogenes*. Cowart *et al.* [9] indicated that *L. monocytogenes* surface carbohydrate molecules, such as α -D-galactose, initiate adhesion to hepatocarcinoma (HepG-2) cells *via* a galactose receptor. Recently, Maganti *et al.* [10] showed that N-acetylneuraminic acid was also involved in adhesion to murine peritoneal macrophages. Meyer *et al.* [18] and Bunduki *et al.* [19] showed that *L. monocyto-*

genes, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri* show varying degrees of adhesion to intestinal epithelial cells. However, there is no report on identification or characterisation of the bacterial factors responsible for such adhesion. Summarising the above information leads to the conclusion that several factor(s) may possibly be involved in listeria adhesion to mammalian cells.

This study reports the identification of a surface protein, p104, that is involved in the adhesion of *Listeria* spp. to Caco-2 cells. Two mutant strains, AAMU530 and AAMU572, were developed with the aid of a conjugative transposon (Tn916). These strains were haemolytic (*hly*⁺) and phospholipase C-positive (*plc*⁺) and showed no reaction with a p104-specific MAb-H7 in Western immunoblot, suggesting that these strains lacked p104 expression. The complete absence of p104 expression in strain AAMU572 was also confirmed by silver staining of SDS-PAGE; however, a faint 104-kDa band was visible upon silver staining for AAMU530 proteins. Lack of expression of p104 is due to the insertion of Tn916 into the structural gene, which was confirmed by PCR and Southern hybridisation (Fig. 1). Furthermore, Southern hybridisation results indicated that Tn916 had been inserted in only a single locus in the chromosome of each of the mutants. Thus, the mutant strains obtained in this study acquired a site-specific mutation for p104 without interference to the other structural or physiological genes. Strains AAMU530 and AAMU572 lost Tn916 when cultured in antibiotic-free media and resulting revertant strains expressed p104 proteins similarly to strain WT (Fig. 2). This study further confirmed the insertion of Tn916 in the chromosome of mutant strains.

Western blotting of protein extracts from internalin (BUG8)- and ActA (LUT12)-deficient strains indicated that these two strains reacted with MAb-H7, suggesting that p104 is distinct from internalin and ActA (Fig. 2c). The present study also examined the expression of p104 in other *Listeria* spp. by Western blotting and showed that this protein is present in *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri*, but not in *L. grayi* (Fig. 2b). This study further suggested that unlike the internalin and ActA proteins, p104 is present in both pathogenic and non-pathogenic *Listeria* spp.

The adhesion assay data illustrated that the mutants AAMU530 and AAMU572 had significantly reduced adherence to Caco-2 cells compared with the parent strain (Table 1). However, as the mutant strains ($\Delta p104$) still showed some adhesion to Caco-2 cells, it is conceivable that other factors are possibly involved in the adhesion process besides p104. Among the mutants, strain AAMU530 showed relatively higher adhesion (10%) than strain AAMU572 (6.3%) ($p \leq 0.05$). This discrepancy may be caused by the

partial expression of p104 in AAMU530 as evidenced in the silver-stained gel (Fig. 2a). Studies with MABs revealed that MAB-H7 (specific for p104) also partially inhibited the adhesion of wild-type *L. monocytogenes* and *L. innocua* to Caco-2 cells. On the other hand, an isotype control, MAB EM-7G1 (specific for the 66-kDa surface protein), did not interfere with adhesion. Adhesion of *L. innocua* to Caco-2 cells without antibody treatment was also studied and it was less than that of *L. monocytogenes* WT. Bunduki *et al.* [19] also observed the lesser adherence of *L. innocua* to primary intestinal endothelial cells. These findings strongly suggest that p104, a previously unreported protein, plays an important role in the adhesion of *L. monocytogenes* to Caco-2 cells. Furthermore, p104 is also possibly responsible for adhesion of *L. innocua* to Caco-2 cells. Although not tested, p104 may act as an adhesion factor in other *Listeria* spp. including *L. ivanovii*, *L. welshimeri* and *L. seeligeri*, as they also express p104 (Fig. 2b).

Although the $\Delta p104$ mutant strains were hly⁺ and plc⁺, cytotoxicity data indicated that these mutants exhibited a significantly reduced virulence, suggesting that the absence of p104 in these mutants may have resulted in reduced adherence, and thus a lowered cytotoxic effect. In a separate experiment, the p104-specific MAB also inhibited the cytotoxic effect of *L. monocytogenes* on Caco-2 and Ped-2E9 cells (data not shown). Again, the reduced cytotoxic effect may be due to the blocking of p104 by MAB-H7, leading to a decrease in adhesion of *L. monocytogenes* and thus interfering with the cytotoxic effect on mammalian cells. Collectively, these results suggest that p104 is an important adhesion factor in *L. monocytogenes* and possibly in other *Listeria* spp., and is responsible for adhesion to intestinal Caco-2 cells. Presumably, p104 acts in concert with other molecules to ensure attachment to mammalian cells.

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