Identification of new loci involved in adhesion of Listeria monocytogenes to eukaryotic cells

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Insertional mutagenesis was performed with Tn1545 in the genetic background of an inlAB deletion mutant to identify new adhesion determinants in Listeria monocytogenes. Four insertion mutants defective in adhesion to eukaryotic cells were identified. Insertion sites were cloned by inverse-PCR and sequenced. The genetic organization of insertion regions was further analysed by screening and sequencing DNA fragments from a HindIII library and by searching databases. Three adhesion-defective mutants each had one copy of Tn1545 inserted into their chromosome. The insertion sites were different in the three mutants: (i) upstream from two ORFs in tandem, similar to dfp and priA of Bacillus subtilis, respectively; (ii) within an ORF encoding a putative 126 amino-acid-polypeptide with no significant similarity to any known protein; (iii) within an ORF similar to a B. subtilis ORF with no known function, just upstream from an operon similar to an ABC (ATP-binding cassette) transporter operon from B. subtilis. The excisants obtained from these mutants using the excision reporter plasmid pTCR9 recovered full adhesion capacity. A fourth mutant was the most severely defective in adhesion. It had five Tn1545 insertions, one of which was upstream from dfp and priA, and another of which was upstream from ami, a gene encoding a surface-exposed autolysin with a C terminus similar to that of InlB. Ami was clearly involved because an ami null mutant constructed in an EGDΔinlA–F background was adhesion-defective. Thus new regions involved in the adhesion of L. monocytogenes to eukaryotic cells were identified. Further study is required to define more accurately the roles of these regions in the adhesion process itself.

Keywords: Listeria monocytogenes, adhesion loci, insertional mutagenesis, microbial pathogenesis

INTRODUCTION

Listeria monocytogenes is a ubiquitous Gram-positive, food-borne pathogen responsible for serious infections in humans (Gray & Killinger, 1966; Farber & Peterkin, 1991). Infection of pregnant women may result in abortion, stillbirth and neonatal meningitis or sepsis (Gellin et al., 1991). Meningitis, meningoencephalitis and bacteraemia are the most common presentations in non-pregnant adults (Nieman & Lorber, 1980; Gellin et al., 1991). In a murine model of infection, L. monocytogenes is a facultative intracellular parasite and immunity is cell-mediated (Kaufmann, 1993). The molecular and cellular basis of the intracellular life of this parasite has been largely elucidated (Portnoy et al., 1992; Sheehan et al., 1994). After entering the cell via a membrane-bound vacuole, L. monocytogenes rapidly gains access to the cytosol and propels itself by mediating...
actin assembly. Actin-based motility enables the bacteria to infect adjacent cells, where a new cycle of cell infection is initiated. A number of genes involved in the escape of bacteria from the vacuole and in intra- and intercellular spread have been identified. They are clustered in a single chromosomal region and are all coordinately regulated by the PrfA regulator protein.

*L. monocytogenes* infects macrophages (Mackness, 1962) and a wide range of non-professional phagocytes, including epithelial cells (Gaillard et al., 1987), endothelial cells (Drevets et al., 1995), hepatocytes (Rosen et al., 1989; Wood et al., 1993; Dramsi et al., 1995; Gaillard et al., 1996) and fibroblasts (Havell, 1986; Kuhn & Goebel, 1989). The uptake of *L. monocytogenes* by macrophages involves the binding of the complement components C1q and C3b, deposited at the bacterial surface, to the complement receptors expressed on the macrophage surface (Alvarez-Dominguez et al., 1992; Drevets & Campbell, 1991; Drevets et al., 1992). Type I macrophage scavenger receptors may also be involved in *Listeria* binding in the absence of complement, probably via the recognition of bacterial lipoteichoic acid (Dunne et al., 1994).

*L. monocytogenes* enters non-professional phagocytes via a zipper-like mechanism (Finlay & Cossart, 1997). The entry process involves the expression of an operon composed of two genes, *inlA* and *inlB*, under the control of PrfA (Gaillard et al., 1991; Dramsi et al., 1993b). The *inlA* gene encodes an 800-amino-acid protein, internalin, which is required for entry into human enteroocyte-like Caco-2 cells (Gaillard et al., 1991; Dramsi et al., 1993a). The cellular receptor for internalin has been identified as the adhesion molecule E-cadherin (Mengaud et al., 1996). The *inlB* gene is very similar to *inlA* and encodes a 630-amino-acid protein (Gaillard et al., 1991; Dramsi et al., 1995). InlB is required for entry into the hepatocytic cell lines TIB73 and HepG-2, and the epithelial cell lines HeLa, HEP-2 and Vero (Dramsi et al., 1995). The cellular receptor recognized by InlB is unknown.

In recent years, most studies have focused on listerial ligands directly involved in cell invasion. Little attention has been paid to listerial ligands mediating adhesion to cell surfaces (adhesins). Cowart et al. (1990) reported that virulent but not avirulent strains of *L. monocytogenes* adhered to Hep-G2 hepatocytes via lectin–substrate interaction. Evidence has also been provided that *L. monocytogenes* interacts with the cell surface via proteoglycans, with the at least partial involvement of the *Listeria* surface protein ActA (Alvarez-Dominguez et al., 1997). More recently, Pandiripilly et al. (1999) have shown the role of a cell-surface protein of 104 kDa (p104) in adhesion of *L. monocytogenes* to the human intestinal cell line Caco-2. *Listeria* adhesins may be essential at various stages of the infection process: they may be involved in the colonization of the gastrointestinal tract; they may restrict *Listeria* to appropriate target cells or tissues, such as the central nervous system and placenta; and they may be involved in cell invasion, activating host cell signal transduction pathways or triggering the synthesis of a target cell receptor required for invasion.

In most cell models, *L. monocytogenes inlAB* mutants adhere 10–20 times more efficiently than wild-type *Listeria innocua* (Gaillard et al., 1996). This suggests the existence of species-specific adhesion determinants other than *inlAB*. To identify these genetic determinants, we used a strategy based on insertional mutagenesis in the background of an *inlAB* deletion mutant, followed by screening for the loss of ability to adhere to eukaryotic cells. We report herein the isolation and characterization of these mutants.

**METHODS**

*L. monocytogenes* strains and growth conditions. The wild-type, serotype 1/2a, EGD strain BUG 600 and its isogenic *inlAB* deletion mutant, BUG 949 (Dramsi et al., 1995), were kindly provided by P. Cossart (Institut Pasteur, Paris, France). The *ami* null mutant, BUG 1505 (Braun et al., 1997), constructed from the EGDΔinlA–F strain BUG 1080 (Dramsi et al., 1997), was also provided by P. Cossart. BUG 949-SmR is a spontaneous streptomycin-resistant mutant (MIC > 1000 mg l\(^{-1}\)) obtained from BUG 949 by one-step selection on agar containing streptomycin (1000 mg l\(^{-1}\); this study). *L. monocytogenes* BM4140, serotype 7, was used as the donor of Tn1545 for transposon mutagenesis (Gaillard et al., 1986). *L. innocua* CLIP 11262 (Collection des Listeria de l’Institut Pasteur, Paris, France) was used as a negative control in adhesion assays. Strains were grown in brain-heart infusion (BHI) broth (Difco Laboratories) with shaking or on BHI agar, at 37 °C.

**Culture of cell lines.** The human melanoma cell line SK-MEL 28 (ATCC HTB 72), obtained from R. Gabathuler and W. A. Jefferies (University of British Columbia, Vancouver, Canada), was used between passages 20 and 40. This cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM; 25 mM glucose; Gibco), containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). The human colon carcinoma cell line Caco-2 (ATCC HTB 37), used between passages 25 and 35, was propagated as previously described (Gaillard & Finlay, 1996). The human hepatocellular carcinoma cell line HepG-2 (ATCC HB 8065), obtained from S. Dramsi and P. Cossart (Institut Pasteur, Paris, France), was propagated as described by Dramsi et al. (1995). All incubations were carried out in a 10% CO\(_2\) atmosphere at 37 °C. Cells were seeded at 8 × 10\(^5\) cells cm\(^{-2}\) onto 12 mm diameter glass coverslips in 24-well plates for adhesion assays, and in 24-well tissue culture plates (Falcon) for invasion assays. Monolayers were used 24 h after seeding.

**Insertional mutagenesis and screening.** Insertional mutagenesis was achieved by transferring Tn1545 from *L. monocytogenes* BM4140 to *L. monocytogenes* BUG 949-SmR. Eighteen-hour cultures of the donor and recipient strains in BHI broth were mixed in a 1:1 ratio. Drops (25 μl) of this mixture were deposited on a membrane filter (HA, 0.4 µm; Millipore) and incubated for 8 h at 37 °C in BHI broth containing streptomycin and erythromycin (10 mg l\(^{-1}\)). Ten clones were picked randomly from each selection plate and subcultured on BHI agar containing streptomycin and erythromycin (10 mg l\(^{-1}\)) and tetracycline (10 mg l\(^{-1}\)). One colony was picked and cultured for 18 h at 37 °C in BHI broth containing streptomycin and erythromycin in 96-well microtitre plates. The plates were kept at −80 °C until required. For screening, mutants were...
grown in BHI broth containing erythromycin for 18 h at 37 °C. Bacterial culture (10 µl) was added to cell monolayers in 24-well tissue culture plates, previously washed once with DMEM and overlaid with 500 µl DMEM. The cells were incubated for 1 h at 37 °C, washed three times with PBS pH 7.2 (BioMérieux), fixed with methanol and stained with methyl blue. Adherent bacteria were counted by examining 100 cells in randomly selected microscope fields.

**Adhesion assay.** Bacteria from 18-h-cultures in BHI broth were pelleted by centrifugation, washed once and diluted appropriately in DMEM. Cells were inoculated with bacteria at a m.o.i. of approximately 100 bacteria per cell. They were incubated for 1 h, washed three times with PBS, fixed with 3% (w/v) parafomaldehyde in PBS for 30 min and washed three times with PBS. Cells were then processed for fluorescence labelling. Different conditions were used in some experiments: (i) bacterial inocula prepared from 4- and 8-h cultures; (ii) infection ratios of 10 and 500 bacteria per cell; (iii) incubation times of 15 min, 30 min and 3 h. For immunolabelling of *Listeria*, cells were incubated sequentially with a rabbit antiserum against listerial O antigen 1/2 (provided by J. Rocourt, Institut Pasteur, Paris, France) diluted 1/1000, and a CY3-labelled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1/1000. Dilutions were made in 1% bovine serum albumin in PBS. Incubations were carried out for 30 min at room temperature and were followed by three washings in PBS. Coverslips were mounted on slides and examined by fluorescence microscopy with a Leica DMRB microscope. Each assay was carried out in triplicate and repeated three times. Adherent bacteria were counted by examining 500 cells in randomly selected microscope fields.

**Quantitative invasion assay and F-actin staining of bacteria.** Cells were inoculated with bacteria at a m.o.i. of approximately 100 bacteria per cell, as described above. They were incubated for 1 h to allow the bacteria to enter, and were then washed twice and overlaid with fresh DMEM containing gentamicin (10 mg l⁻¹) to kill extracellular bacteria. At intervals, cells were washed twice and processed for either counting of bacteria or F-actin staining. For counting, cells were lysed by adding cold distilled water. The titre of viable bacteria released from the cells was determined on agar plates. Each experiment was carried out in triplicate and repeated three times. For F-actin staining, cells were fixed with 3% paraformaldehyde, washed three times and permeabilized for 5 min in 0.1% (v/v) Triton X-100 (Sigma) in PBS. Rhodamine-phallloidin (Molecular Probes) was used as previously described (Gaillard & Finlay, 1996).

**Phenotypic characterization of mutants.** Agglutination with anti-listerial O 1/2 antiserum was carried out on slides. Phage-typing was performed at the Centre National de Référence des *Listeria* (Institut Pasteur, Paris, France), as described by Audurier et al. (1979). Carbohydrate fermentation was assessed using API 50CH strips (API-System). Haemolysis and lecinthinase activity were scored on blood agar and egg-yolk agar plates, respectively. Phosphatidylinositol phospholipase C phenotype was also scored on plates, as described previously (et al. 1985). Bacterial cultures (1 ml; OD₆₀₀ 0.6) were centrifuged and the pellet suspended in 1 X SDS-PAGE sample buffer [130 mM Tris/HCl pH 6.8, 1% (w/v) SDS, 7% (v/v) 2-mercaptoethanol, 7% (w/v) sucrose, 0.01% (w/v) bromophen blue]. SDS-PAGE was carried out as described elsewhere (Laemmli, 1970) in 10% (w/v) polyacrylamide minigels (Mini Protein II; Bio-Rad). Protein concentrations were measured by the method of Bradford (1976). Proteins were stained with Coomassie brilliant blue. Western blotting was carried out as previously described (Gholizadeh et al., 1996). Western blots were probed with rabbit affinity-purified anti-AMI antibody (Braun et al., 1997) diluted 1/1000, and anti-rabbit horseradish-peroxidase-conjugated secondary antibody. Antibody binding was detected by adding 0.05% (w/v) diaminobenzidine tetrahydrochloride (Sigma) and 0.03% (v/v) hydrogen peroxide.

**General genetic manipulations.** Total DNA from *Listeria* cells was prepared as previously described (Poyart-Salmeron et al., 1992). pUC18 (Yanisch-Perron et al., 1985) was used to clone *Listeria* DNA fragments in *Escherichia coli* strain DH5α (Woodcock et al., 1989). E. coli strains containing pUC vector derivatives were grown in Luria–Bertani media (Difco Laboratories) containing ampicillin (100 mg ml⁻¹). Plasmid DNA from *E. coli* was prepared by rapid alkaline lysis (Birnboim & Doly, 1979). Isolation of DNA fragments, DNA cloning and restriction analysis were performed by standard techniques (Sambrook et al., 1989). Restriction enzymes and ligase were purchased from New England Biolabs and were used as recommended by the manufacturer. DNA was amplified with Taq DNA polymerase (Promega) for 35 cycles of 60 s at 95 °C, 60 s at 55 °C and 90 s at 72 °C in a Gene Amp System 9600 thermal cycler (Perkin-Elmer). Southern blots were performed under high-stringency conditions as previously described (Gaillard et al., 1991). Tn1545-specific sequences were detected by probing with a 0.83 kb fragment internal to the int gene (Poyart-Salmeron et al., 1992).

**Isolation of excisants.** The excision reporter plasmid pTCR9 (Celli et al., 1997) was used to select excisants from insertional mutants. This plasmid confers resistance to erythromycin. It contains the chloramphenicol resistance gene cat; insertionally inactivated with pAT112, an integrative vector containing the attachment site of Tn1545. pTCR9 can be used as a reporter plasmid because the transposon-encoded proteins required for the excision of Tn1545 diffuse intracellularly and promote the excision of pAT112 from pTCR9. pTCR9 was transferred from *Enterococcus faecalis* JH2-2(pTCR9) to *L. monocytogenes* mutants by conjugation using published methods (Celli et al., 1997). Excisants were directly selected on BHI agar containing chloramphenicol (10 mg l⁻¹) and streptomycin (1000 mg l⁻¹). The excision of Tn1545 from the chromosome of chloramphenicol-resistant clones was assessed by Southern blotting. Excisants were cured of pTCR9 by subculturing bacteria in BHI broth at 42 °C.

**Cloning of chromosomal sequences bordering insertion sites.** This was done by inverse-PCR. Chromosomal DNA from *Listeria* cells was digested with *SalI*. Digestion products were ligated, generating circular molecules containing the right end (about 1.9 kb) of Tn1545. DNA was amplified by PCR using the primers attR-Tn1545 (5’-CGGATCCCGTT-AAGTATCTTCCATAGT-3’) and 5’-CGTTGCAAGGCAATCC-3’). The corresponding amplifications were digested with *SalI* and inserted into the BamHI site of pUC18.

**Nucleotide sequencing and sequence analyses.** DNA was sequenced using Taq DiDeoxy terminators and by the DyePrimer Cycling Sequence protocol developed by Applied Biosystems (Perkin Elmer), with fluorescent dideoxynucleotides and primers. Fluorescent primers were purchased from Life Technologies. Labelled extension products were analysed on an ABI Prism system (Applied Biosystems-Perkin Elmer). Sequences were analysed with DNA Strider 1.2. Protein and nucleotide databases were searched using the programs BLASTN and BLASTX (National Center for Biotechnology
Information, Los Alamos, NM), available via the Internet. Protein sequences were aligned with CLUSTAL v.

RESULTS

Adhesion of the inlAB mutant BUG 949 to eukaryotic cells

The adhesion capacity of BUG 949 was studied in various cell assay systems. Table 1 shows the results of adhesion assays after a 1 h incubation at an initial ratio of 100 bacteria per cell. BUG 949 adhered only slightly less well to Hep-G2 and SK-MEL 28 cells than did the wild-type strain, EGD. BUG 949 and EGD differed far more in the Caco-2 cell system. However, in all cell systems tested, BUG 949 was 10–20 times more adherent than L. innocua CLIP 11262. Similar results were obtained with various bacteria/cell ratios (10 to 500 bacteria per cell) and incubation times (15 min to 3 h).

BUG 949 was 50–100 times less invasive than EGD in the cell models tested (approx. 1–2% versus 0.05% intracellular bacteria at 2 h). However, the intracellular growth rates of the two strains were similar, with an apparent doubling time of approximately 1 h between 2 and 8 h (data not shown). F-actin staining of cells at 4 and 8 h also showed that BUG 949 retained the ability to induce actin assembly (data not shown). Thus, as previously reported with the murine embryonic hepatocyte line TIB73 (Gaillard et al., 1996), BUG 949 remained able to enter eukaryotic cells and behaved normally once inside them.

Isolation of mutants defective in adhesion to eukaryotic cells

A bank of 2000 insertion mutants was constructed from BUG 949-SmR and screened for adhesion to SK-MEL 28 cells. Preliminary experiments showed that BUG 949-SmR was about five times less adherent than BUG 949. This was the case for all streptomycin-resistant mutants obtained from BUG 949, probably caused by misreading at the translation step of protein synthesis due to mutations involved in streptomycin resistance. The bank was screened with SK-MEL 28 cells for a number of practical reasons. SK-MEL 28 cells grow as individuals, making it particularly easy to evaluate the adhesion of Listeria to them. They also grow fast, making it possible to prepare large quantities of cells in a short time.

On screening the bank, we identified 26 mutants that appeared to adhere poorly to cells based on methyl blue staining. We reassessed adhesion by immunofluorescence in controlled conditions and found a significant reduction in adhesion for only four mutants (G48, I59, B147 and J810), obtained from independent mating experiments. These mutants were indistinguishable from their parents with respect to the following characters: morphology and colony appearance; hemolytic, lecithinase and phosphatidylinositol phospholipase C activities; growth rate in BHI broth; serovar and phage type. Excisants from mutants G48, B147 and J810 (strains G48ex, B147ex and J810ex, respectively) were easily selected with the pTCR9 reporter system (Celli et al., 1997). Despite several attempts, this approach was unsuccessful with I59, consistent with there being five copies of Tn1545 inserted into the chromosome of this mutant (see below).

Mutant I59 was the most severely impaired in adhesion to SK-MEL 28 cells (Fig. 1; one tenth the adhesion of the parent BUG 949-SmR). The other mutants were three to four times less adherent than BUG 949-SmR and the corresponding excisants. Similar results were obtained with various quantities of inoculum (10 to 500 c.f.u. cell⁻¹) and incubation times (15 min to 3 h), and with bacteria grown to exponential, late exponential and stationary phases (data not shown). The ability of the

Table 1. Adhesion of the inlAB mutant BUG 949 to eukaryotic cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>L. monocytogenes</th>
<th>L. innocua</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGD</td>
<td>BUG 949</td>
</tr>
<tr>
<td>Hep-G2</td>
<td>44 (16)</td>
<td>32 (18)</td>
</tr>
<tr>
<td>Caco-2</td>
<td>150 (46)</td>
<td>45 (18)</td>
</tr>
<tr>
<td>SK-MEL 28</td>
<td>17 (11)</td>
<td>14 (6)</td>
</tr>
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was incubated for 1 h at 37 °C with approximately 100 bacteria per cell, washed and processed for fluorescence immunolabelling of bacteria. Results are expressed as mean ± SD numbers of adherent bacteria per cell (three determinations).

Fig. 1. Adhesion of insertion mutants to SK-MEL 28 cells. Cells were incubated for 1 h at 37 °C with approximately 100 bacteria per cell, washed and processed for fluorescence immunolabelling of bacteria. Results are expressed as mean ± SD numbers of adherent bacteria per cell (three determinations).
Adhesion loci in *L. monocytogenes*

![Fig. 2. Invasiveness of BUG 949-SmR (●) and insertion mutants I59 (○), B147 (■), J810 (□) and G48 (▲) in the SK-MEL 28 cell model. SK-MEL 28 monolayers were incubated for 1 h at 37 °C with approximately 100 bacteria per cell. After washing, the cells were reincubated for 8 h in fresh culture medium containing gentamicin (10 mg l⁻¹). At intervals, the cells were washed again, lysed and viable bacteria were counted on agar plates. Results are expressed as mean ± SD numbers of viable bacteria per well (three determinations).](image)

The G48 mutant did not appear to be defective in cell invasion. Although it interacted less efficiently with the cell surface, this mutant fully retained the ability to trigger uptake by cells. Consistent with its adhesion phenotype, mutant I59 was much less invasive than its parent, the parent being about 20 times more invasive. Mutants B147 and J810 showed intermediate invasion levels. All mutants induced actin polymerization (data not shown). The mutants also interacted less efficiently with cells in the Caco-2 and Hep-G2 cell systems (data not shown). As in the SK-MEL 28 cell model, mutant I59 was the most severely impaired in both adhesion and invasion.

**Cloning and sequencing of insertion sites (Fig. 3)**

The mutants were analysed by Southern blotting, using a 0.83 kb fragment internal to the *int* gene of Tn1545 as a probe (data not shown). The probe hybridized to a single band in each of three different enzyme digests of DNA prepared from mutants G48, B147 and J810. This indicates that a single copy of Tn1545 had inserted into the chromosome of each of these mutants. Southern blot experiments carried out with mutant I59 showed five bands of various molecular sizes for each of the enzymes tested, suggesting the insertion of at least five copies of Tn1545 in this mutant.

For each mutant, the *Sau3AI* fragment containing the right part of Tn1545 and the flanking listerial region was ligated to form circular molecules, amplified by PCR, and sequenced both directly and after cloning into pUC vectors. The genetic organization of potentially important insertion regions was studied further. Libraries of chromosomal *HindIII* DNA fragments were constructed from BUG 949 and screened by colony
hybridization, using appropriate PCR-amplified Listeria-specific DNA fragments as probes. Plasmids containing HindIII fragments from the insertion regions were obtained and used for sequence determination. Sequence data were completed by screening databases if necessary.

**Mutant G48.** Transposon Tn1545 had inserted 19 bp upstream from a 1200 bp ORF similar to the dfp (DNA and flavoprotein) gene from Bacillus subtilis (60% identity at the amino acid level). Listeria dfp is preceded by a potential RBS (TGAGG) located 15 bp upstream from the predicted start codon. Four bp downstream from dfp, and in the same orientation, lies an ORF. The first 1776 bp of this ORF, from the predicted start codon, were sequenced and found to encode a polypeptide with significant similarity (64% identity over a 592-amino-acid overlap) to the protein encoded by the priA gene of B. subtilis, which is involved in primosome formation. No potential RBS was detected between dfp and priA, indicating that these genes may be translated together. The sequence of Listeria DNA flanking the left end of Tn1545 was available from the European Listeria Genome Consortium database. The Tn1545 insertion was 130 bp downstream from a 207 bp ORF similar to the rpoZ gene of B. subtilis (encoded proteins 54% identical).

**Mutant I59.** The sequences of five insertion sites were determined. A first copy of Tn1545 had inserted at the same site as in mutant G48, 19 bp upstream from dfp. A second copy had inserted 34 bp upstream from ami, a gene recently characterized in L. monocytogenes that encodes an autolytic amidase (Braun et al., 1997; McLaughlan & Foster, 1998). Downstream from ami, and apparently transcribed convergently to it, lies an ORF similar to pyrG, a CTP synthase gene from B. subtilis. The third insertion was inside an ORF of which 348 bp was sequenced. The deduced amino acid sequence was 54% identical to β-glucoside permease II ABC (ATP-binding cassette) component encoded by the bgIP gene of B. subtilis. The fourth insertion was within an ORF of which 258 bp was sequenced. The deduced amino acid sequence was 64% identical to the product of the spoIIIJ gene of B. subtilis. The last insertion had occurred within a putative ORF, ORF1, of which 80 bp was sequenced. No significant similarity was found to sequences available in the public databases.

On Western blots of protein extracts from I59 probed with anti-Ami antibody, a major band at approximately 100 kDa was absent (Fig. 4). Thus, not unexpectedly, the insertion upstream from ami in I59 appears to prevent the expression of this gene.

**Mutant B147.** Tn1545 had inserted in a region containing two ORFs similar to the nrgB and nrgA genes of B. subtilis (encoded proteins 53% and 66% identical, respectively), and an ORF, partially sequenced, similar to the aspRS gene of B. subtilis (identity score of 70% over an 117-amino-acid overlap). nrgB and nrgA were arrayed in tandem and were convergent with aspRS. Sequence analysis of the region between nrgB and aspRS indicated the presence of a putative 381 bp ORF in which Tn1545 appeared to have inserted. This ORF, ORF2, showed no significant similarity with any known gene. ORF2 is preceded by a potential RBS (GGAGG). A putative −10 box (TATAAT) is also present. ORF2 encodes a 126-amino-acid polypeptide (predicted Mr 14171) with no prominent features. The ORF2 protein sequence contains an N-terminal stretch with all the characteristics ascribed to bacterial signal peptides, with the most likely cleavage site between positions 24 and 25 (YQH-AS).

**Mutant J810.** Tn1545 had inserted within an ORF, ORF4, the complete sequence of which was obtained from the European Listeria Genome Consortium database. ORF4 encodes a 187-amino-acid polypeptide 31% identical to the product of a gene of B. subtilis with no known function, yuaB. Downstream from ORF4 and transcribed in the same direction, lies an operon similar to an ABC transporter operon of B. subtilis involved in the processing of glycine betaine, carnitine and choline. ORF3, of which 366 bp was sequenced, lies 248 bp upstream from ORF4 and is transcribed in the same direction. The deduced amino sequence was 34% identical to the predicted product of the gene in the nfnB–entD intergenic region of E. coli.

**Loss of adhesion of an ami null mutant constructed in EGDΔinlA–F**

We tried to determine which insertions made I59 adhesion-defective. The insertion in the dfp region was probably involved because the same event in the G48 mutant was also clearly associated with an adhesion-defective phenotype. However, I59 tended to be more...
severely defective in adhesion to cells than G48, suggesting that other insertion events were also involved. We focused our attention on ami as it was the only gene encoding a surface molecule to be identified in the insertion regions in IS9. We investigated the role of ami in adhesion by assessing an ami null mutant constructed in EGDΔAmI–F (Braun et al., 1997). This mutant adhered five to six times less efficiently than its parent [mean (±SD) number of adherent bacteria: Hep-G2, 5 (3) versus 25 (12) bacteria per cell islet; Caco-2, 7 (4) versus 38 (14) bacteria per cell islet; SK-MEL 28, 2 (2) versus 13 (8) bacteria per cell]. However, the ami mutant invaded cells and grew intracellularly with the same efficiency as its parent (data not shown). Thus, the disruption of ami affected the adhesion of the bacteria but not their potential for invasion.

DISCUSSION

Insertional mutagenesis was performed to identify new genes involved in the adhesion of L. monocytogenes to eukaryotic cells. We used an inlAB mutant as genetic background to prevent functional complementation by internalin or InlB. Indeed, in most cell models, these proteins also act as adhesins and may mask other adhesion systems. Only 4 of the 2000 insertion mutants screened were adhesion-defective. This is unlikely to be due to the use of Tn1545. As Tn1545 insertions are not totally random, we performed both Tn1545 and Tn917 mutagenesis at the beginning of our work. Tn917 mutagenesis was discontinued after about 800 insertion mutants were screened without the isolation of an adhesion-defective mutant (data not shown). The small proportion of adhesion-defective mutants in our bank suggests that few genomic regions are required for adhesion to cells in L. monocytogenes. Alternatively, some adhesion determinants essential to the viability of bacterial cells, such as lipoteichoic acids, may not have been identified by insertional mutagenesis.

Three adhesion-defective mutants isolated from our bank had one copy of Tn1545 inserted in their chromosome. Their phenotype was clearly related to transposon insertion as excisants selected with the excision reporter plasmid pTCR9 (Celli et al., 1997) recovered full adhesion capacity. In one mutant, Tn1545 had integrated just upstream from two genes in tandem, one homologous to dfp and the other to priA of B. subtilis. The role of dfp and/or priA in Listeria adhesion, if any, is likely to be indirect. The dfp gene product of E. coli is a 45 kDa flavoprotein that may be involved in oxidation/reduction reactions. Previous studies have shown that dfp mutations affect DNA synthesis and pantothenate metabolism in E. coli (Spitzer & Weiss, 1985; Spitzer et al., 1988). priA is involved in primosome formation. In the second mutant, the insertion had occurred within a putative ORF encoding a 126-amino-acid polypeptide with a typical signal peptide. This polypeptide has no other notable characteristics and has no significant similarity to any known protein. We are currently trying to determine how it contributes to Listeria adhesion. In the third mutant harbouring one transposon copy, Tn1545 had inserted within a gene similar to a B. subtilis gene with no known function, just upstream of an ABC transporter operon. The role of this region in Listeria adhesion is also under investigation by our group.

The fourth adhesion-defective mutant had five copies of Tn1545 in its chromosome. One insertion was upstream from dfp, at the same location as that found in one of the multicopy mutants. However, the multicopy mutant was much more severely defective in adhesion than the monocopy mutant, suggesting a role for at least one of the other insertions. We thought it likely that the insertion upstream from ami, which was associated with a lack of Ami in bacterial cells, was involved. This was confirmed by showing that the inactivation of ami in an EGDΔAmI–F background was associated with a marked reduction in the adhesion capacity of bacterial cells. A loss of adhesion in ami null mutants has not previously been reported (Braun et al., 1997; McLaughlan & Foster, 1998). A slight reduction in motility was the only reported phenotypic change (McLaughlan & Foster, 1998). However, the adhesion of mutants to eukaryotic cells was not evaluated in these previous studies. Our results are consistent with a recent study showing the role of a Listeria cell-surface protein of 104 kDa in adhesion to Caco-2 cells (Pandiripally et al., 1999). It is tempting to speculate that this adhesion factor may be Ami.

The ami gene was recently cloned and sequenced by using two different approaches (Braun et al., 1997; McLaughlan & Foster, 1998; Braun et al., 1997) were searching for proteins with a cell-surface anchoring region similar to that of InlB. They identified ami by Southern blotting of L. monocytogenes EGD chromosomal DNA using the 3' end of the inlB gene as a probe. McLaughlan & Foster (1998) were aiming to characterize the major autolysins of L. monocytogenes to elucidate their physiological functions. They cloned ami from an expression library screened for lytic-enzyme-producing clones. The ami gene product is a 102 kDa autolysin with three characteristic domains: (i) a 30-amino-acid putative sequence signal, (ii) a 179-amino-acid N-terminal domain similar to the alanine amidase domain of the Atl autolysin of Staphylococcus aureus, (iii) a C-terminal domain (between amino acids 262 and 917) homologous to the C-terminal anchoring domain of InlB, with eight repeats of approximately 80 amino acids starting with the dipeptide GW (GW repeats).

The role of Ami in Listeria adhesion is unclear. We cannot exclude the possibility that ami mutation affects bacterial adhesion by altering cell-surface composition due to a dysfunction in autolytic activity. However, a direct role is possible because Ami is exposed at the bacterial surface (Braun et al., 1997). This is consistent with recent data showing adhesive properties in two staphylococcal autolysins, the autolysin AtlE of Staphylococcus epidermidis (Heilmann et al., 1997) and the
autolysin/adhesin Aas of Staphylococcus saprophyticus (Hell et al., 1998). Both ArIE and Aas consist of an amidase and a glucosaminidase domain separated by a region containing GW repeats. Subcloning experiments have mapped the adhesive activities of Aas to the region connecting the two enzymic domains (Hell et al., 1998).

Like Aas, and probably ArIE, Ami may promote the attachment of L. monocytogenes to eukaryotic cells via its GW-repeat-containing domain.

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REFERENCES


Adhesion loci in L. monocytogenes


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