Assessment of the pathogenic potential of two Listeria monocytogenes human faecal carriage isolates

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Two human faeces carriage isolates of Listeria monocytogenes (H1 and H2) were compared to reference strains (ScottA and LO28) with regard to their lethality in 14-day-old chick embryos, their haemolytic and phospholipase (phosphatidylcholine-phospholipase C and phosphatidylinositol-phospholipase C) activities and their invasiveness towards Caco-2 cells. Experimental infection of chick embryos allowed discrimination of the strains into those exhibiting high virulence (ScottA and H2), those exhibiting slightly attenuated virulence (LO28) and those exhibiting low virulence (H1). A similar percentage mortality and time to death for embryos was observed when they were infected with H2 as was seen with infection by the reference strain ScottA. Therefore, human carriage strain H2 was considered potentially pathogenic. In contrast to H2 and ScottA, H1 exhibited low virulence. Using the tissue-culture cell-line model, it was found that carriage strain H1 was unable to enter Caco-2 cells efficiently, even though it was similar to the virulent strains in terms of the enzymic activities involved in pathogenicity. Detection of the internalins InlA and InlB, involved in the internalization of L. monocytogenes in the host cells, by immunoblot indicated that a truncated form of InlA was produced by H1. Taken together, these data provide a starting point for the study of the behaviour of two types of human faeces carriage strains and their characterization.

Keywords: virulence, internalin, chick-embryo assay, Caco-2 cell-culture assay

INTRODUCTION

Listeria monocytogenes is a ubiquitous Gram-positive rod that causes serious infections in humans and animals. The most common route of infection by L. monocytogenes is via the gastrointestinal tract (Schlech, 1984), as evidenced by several outbreaks of listeriosis caused by the ingestion of contaminated food materials (Schlech et al., 1983). Numerous reports have been published dealing with the prevalence of L. monocytogenes in food products (Ryser & Marth, 1999). Despite frequent exposure of the population to this bacterium (Notermans et al., 1998), the probability of contracting listeriosis is low, since the incidence of the disease ranges from 1–6 to 6 cases per million population (Rocourt et al., 2000).

One explanation for the relatively low incidence of the disease may be the limitation of listeriosis cases to high-risk groups, which include pregnant women, neonates and immunocompromised adults. However, a few cases of listeriosis have been identified in immunocompetent patients (Schlech et al., 1983; Goulet et al., 1993; Kelly et al., 1999), indicating that (i) L. monocytogenes has the potential to infect immunocompetent individuals, and (ii) the immunocompetent status of an individual alone can not explain the low risk of infection.

Differences in the human-pathogenic potentials of L. monocytogenes isolates may also be involved in their ability to infect individuals. Most of our knowledge

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Abbreviations: ACA, acriflavine–ceftazidime agar; Pc-PLC, phosphatidylcholine-specific phospholipase C; Pi-PLC, phosphatidylinositol-specific phospholipase C.

The GenBank accession number for the sequence reported in this paper is AF468816.
about the pathogenicity of *L. monocytogenes* comes from the many studies that have been carried out using mouse models. The molecular and cellular mechanisms involved in the intracellular life of *L. monocytogenes* have been identified using complementary *in vitro* tests such as tissue-culture assays (Gaillard et al., 1987) and mutagenesis strategies. Virulence of *L. monocytogenes* is due to the expression of several genes (Braun & Cossart, 2000) responsible for the ability of the pathogen to penetrate cells, to proliferate in them and to spread in them. The entry process involves the expression of two proteins, InlA (internalinin) and InlB, encoded by two genes, *inlA* and *inlB*, respectively, which are organized as an operon. These surface proteins are both necessary and sufficient for entry of the bacterium into various culture cell lines (Gaillard et al., 1991; Dramsi et al., 1995). A second genetic locus is involved in the functions essential for intracellular survival of the bacterium. This locus encodes listeriolysin O, phospholipases (phosphatidylinositol-specific phospholipase C (Pi-PLC) and phosphatidylcholine-specific phospholipase C (Pc-PLC)) and ActA. All of these cell-surface and secreted proteins are co-ordinately regulated by the pleiotropic transcriptional activator PrfA.

Several studies have shown heterogeneity within the virulence of *L. monocytogenes* isolates (Del Corral et al., 1990; Brosch et al., 1993; Wiedmann et al., 1997; Van Langendonck et al., 1998; Norrung & Andersen, 2000; Barbour et al., 2001), without any systematic correlation between the level of virulence and the type characteristics (i.e. serovars, phagovars, ribovars and DNA macrorestriction patterns) or the origin (i.e. human, animal, category of food or environment) of the strains. These studies dealt generally with the comparison of strains isolated from clinical and food environments. However, little attention has been paid to the potential virulence of human carriage strains of *L. monocytogenes*.

The detection of *L. monocytogenes* carriage strains has been demonstrated, particularly in human faeces. The majority of epidemiological investigations have focused only on the rate of faecal carriage of *L. monocytogenes* strains, and it has been estimated that between 1 and 6% of the general population carry this bacterium (Durst & Zimanyi, 1976; Kampelmacher & van Noorle Jansen, 1972; Schuchat et al., 1993). To better understand their occurrence, the characterization of human faecal carriage strains of *L. monocytogenes* is needed. The well-characterized LO28 strain was recovered from the faeces of healthy pregnant women. Analysis of this strain has shown that a mutation in *inlA* results in a truncated protein being released into the culture medium (Jonquieres et al., 1998). Surprisingly, this human carriage strain has shown a high level of virulence in the mouse model.

In this work, the virulence of two human faecal carriage strains of *L. monocytogenes*, H1 and H2, was compared to that of the well-known reference strains ScottA and LO28, to try to explain the lack of listeriosis symptoms in immunocompetent individuals.

Three different approaches were considered to try to explain the differences observed in the degrees of virulence between the human faecal carriage strains and the reference strains. First, the pathogenicity of the strains was characterized by inoculating 14-day-old chick embryos via the chorioallantoic route, as this test appears to be a reliable alternative to the mouse bioassay for the assessment of the pathogenicity of *Listeria* spp. (Terplan & Steinmayer, 1989; Notermans et al., 1998). Second, the virulence-associated determinants, including haemolytic and phospholipase (Pc-PLC and Pi-PLC) activities, were analysed and compared among the isolates. Third, the efficiency of the isolates for adhesion to, invasion of, and growth within human Caco-2 intestinal cells was investigated and evaluated. At the same time, we analysed the production of InlA and InlB proteins, to further characterize the internalization process of these human faecal carriage strains.

**METHODS**

**Listerial strains.** All *L. monocytogenes* strains, except ScottA, were isolated from human faeces. *L. monocytogenes* strains Scott A (CIP 103575, serotype 4b), a strain isolated from a human outbreak in an epidemic in Massachusetts (USA) in 1983, and LO28 (serotype 1/2c), a carriage strain recovered from the faeces of a healthy pregnant woman, were chosen as reference strains. In our laboratory, two faecal carriage *L. monocytogenes* strains, H1 (serogroup 1) and H2 (serogroup 4), were originally isolated from two human patients without listeriosis. H1 was isolated from a healthy pregnant carrier, whose twins were born healthy; H2 was isolated from a three-year-old infant carrier (Rousset et al., 1994).

A strain of *Listeria welshimeri* was used as a negative control in the virulence assays – it was also isolated in our laboratory from the faeces of a healthy human (Rousset et al., 1994).

**Culture medium and growth conditions.** Overnight static cultures of *Listeria* cells grown at 37 °C in brain heart infusion (BHI) broth (Biomérieux) were used for the chicken-embryo bioassays and cell-culture assays. For Western-blot analysis, protein samples were derived from cultures grown to stationary phase at 37 °C in MCDB 202 (Cryo Bio Systems) supplemented with 50 mg glucose l−1, 10% yeast nitrogen base without amino acids (YNB; Difco) and 1% trace elements (Cryo Bio Systems).

Viable counts were performed on acriflavine–ceftazidime agar (ACA; 10 mg acriflavine l−1, Sigma; 50 mg ceftazidime l−1, Glaxo Wellcome) plates incubated for 48 h at 37 °C.

**Chicken embryo methodology**

**Chicken embryos.** Fertile eggs were purchased from IFFA-CREDO. Eggs were incubated for 14 days, as described by Buncic & Avery (1996), at 37–5 °C in a rotary egg incubator (Grumbach S84 model).

**Chorioallantoic inoculation of embryos.** This procedure was carried out using the technique of Buncic & Avery (1996). Strains of *L. monocytogenes* were grown in BHI at 37 °C to an OD600 of 1.0 and were harvested by centrifugation at 6000 g for 10 min at room temperature. The cell pellets were gently suspended in PBS (Dulbecco’s phosphate buffer saline with 1 mg glucose ml−1 and 36 mg sodium pyruvate l−1; Life Technologies) at pH 7.2, to obtain an initial cell density of 3 × 105 to 3 × 108 c.f.u. ml−1. Serial dilutions of the cell suspensions were prepared in PBS, and embryos were inocu-
lated with 0·1 ml of a 10⁻³ dilution via the chorioallantoic membrane, after boring an artificial hole through the shell. The inoculum dose was confirmed by enumeration of the viable count on ACA plates. At least five embryos were used per strain tested. For each strain tested, controls were performed in which eggs were inoculated with sterile PBS. This was to test the viability of the chick embryos.

Inoculated eggs were incubated in a horizontal position at 37·5 °C, and the vitality of the embryos was monitored daily for 6 days by using transillumination (CL TH model); death was recognized by a loss of all visible blood vessel structure, a gelling of the suspending liquids and an absence of any reflexive movement of the embryo. The calculation of the mean time to death was made on the basis of the mean date of death of the five eggs (Norrung & Andersen, 2000). Calculation of the mortality rate completed the appreciation of the level of virulence for each strain tested.

**Strain characterization.** The test strains of *L. monocytogenes* were spot-inoculated (10⁹ bacteria spot⁻¹) onto appropriate agar plates for the observation of three distinct enzymic activities.

Haemolytic activity was tested on Bacto Colombia blood agar base EH medium (mBAP; Difco) supplemented with 5% (v/v) saline-washed horse red blood cells. Plates were incubated for 48 h at 37 °C after inoculation and were observed for the development of zones of haemolysis around the colonies (Fujisawa & Morì, 1994).

Pe-PLC activity was tested by inoculating bacteria onto BHI agar base EH medium (mBAP; Difco) supplemented with 5% (v/v) fresh egg yolk (Coffey et al., 1996). Plates were incubated for 48 h at 37 °C and were observed for the development of zones of opacity, due to degraded egg-yolk lecithin, around the colonies.

Pi-PLC activity was determined as described by Notermans et al. (1991). After growth at 37 °C on TY agar (1% tryptone; 0·5% yeast extract; 1% NaCl; 1% agar), plates were covered with the substrate 1% 1-α-phosphatidylinositol (Sigma) in 0·7% agarose. Plates were re-incubated at 37 °C and were observed for 6 days for the development of turbid haloes (the insoluble diacylglycerol) around the colonies.

**Cells.** The human colon carcinoma cell-line Caco-2 (ECACC no. 86010202) was used between passages 36 and 42. The cells were routinely grown in 25 cm² plastic tissue-culture flasks (Greiner) at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ air. The culture medium used for growth of the cell line was Dulbecco’s modified Eagle minimum essential medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, 1% (v/v) non-essential amino acids and antibiotics (100 U penicillin ml⁻¹; 100 µg streptomycin ml⁻¹). All reagents were purchased from Invitrogen (Life Technologies).

**Infection of Caco-2 cells.** Confluent cell monolayers were trypsinized and adjusted in 24-well tissue-culture plates (Greiner), to obtain almost semi-confluent monolayers of Caco-2 cells after 3 days of incubation at 37 °C (10⁶ cells well⁻¹). Cells were incubated in medium without antibiotics for 12 h and were washed with fresh medium just before use. After overnight growth in BHI, the lissiral cells were centrifuged (6000 g for 10 min at room temperature) and resuspended in DMEM before infection of the Caco-2 monolayers. The Caco-2 monolayers were infected with 300 µl of the bacterial suspension (m.o.i. of 100 bacteria cell⁻¹), according to the method of Van Langendonck et al. (1998).

Adhesion of the *L. monocytogenes* cells to the Caco-2 cells was allowed to occur for 30 min at 37 °C (Santiago et al., 1999). The cell monolayers were then washed five times to remove non-adherent bacteria. Adherent bacteria were harvested, after lysis of the cell monolayers with 0·5 ml Triton X-100 (0·1% in cold PBS) for 10 min. The c.f.u. values for viable bacteria were determined by plating suitable dilutions of the lysates onto ACA plates. The plates were subsequently incubated for 48 h at 37 °C.

For entry and intracellular growth assays, monolayers were infected with bacteria for 2 h at 37 °C. After incubation, non-adherent bacteria were removed from the monolayers by washing them three times with PBS. Cell monolayers were then covered with fresh DMEM containing gentamicin at a bactericidal concentration (100 µg ml⁻¹), to kill extracellular adherent bacteria. After a contact time of 1·5 h at 37 °C, the rate of bacterial entry into the Caco-2 cells was determined by plate counts, following washing of the Caco-2 cells with 0·5 ml Triton X-100 (0·1% in cold PBS) to lyse the cells. It was considered that counts obtained 3·5 h after the onset of infection represented the number of bacteria that had been internalized. To determine the rate of intracellular growth, DMEM supplemented with 5 µg gentamicin ml⁻¹ was used as the growth medium, and the incubation time was increased to 19·5 h (Van Langendonck et al., 1998). The rate of replication was calculated as the ratio of the number of bacteria internalized after 21·5 h of infection (i.e. the intracellular growth rate) to the number of bacteria internalized after 3·5 h of infection (i.e. the rate of entry).

**Preparation of culture supernatants and extraction of bacterial surface proteins.** The technique of Kocks et al. (1992) was used for these procedures. Following the growth of cultures in MCD medium 202 supplemented with 50 mg glucose 1⁻¹, 10% YNB and 1% trace elements, bacteria were removed by two centrifugations (each at 6000 g for 10 min at room temperature), to obtain the culture supernatant proteins. Supernatant proteins were precipitated overnight by the addition of 16% (v/v) trichloroacetic acid (TCA) at 4 °C.

For SDS extracts, the cell pellets were washed in 10 mM Tris/HCl (pH 8), centrifuged and immediately resuspended in 1% (v/v) SDS in 10 mM Tris/HCl (pH 8). They were then incubated for 15 min with gentle shaking (1000 r.p.m.). The suspensions were then centrifuged, and the bacterial surface proteins in the supernatants were precipitated with 16% (v/v) TCA and allowed to stand at 4 °C overnight.

All precipitated protein fractions were washed twice in 90% acetone, suspended in Laemmli sample buffer (Laemmli, 1970) and heated at 95 °C for 7 min, prior to SDS-PAGE and Western blotting analyses.

**SDS-PAGE and immunoblotting.** SDS-PAGE was done by the method of Laemmli (1970) with 12·5% polyacrylamide gels. Equal amounts (10 µg) of the samples were loaded into each lane of the gel. After electrophoresis (90 min at 20 mA), the proteins were transferred onto a nitrocellulose membrane (PROTEAN; Schleicher and Schuell) using the semi-dry electroblotting method. The membrane was stained with 0·2% Ponceau red, to confirm the transfer efficiency. Membrane hybridizations were performed with diluted (1:1000) mouse mAbs directed against InlA (mAb L7.7) or InlB (mAb 2H5) (Braun et al., 1997, 1999; Mengaud et al., 1996a). For immunodetection of the hybridized proteins, bound primary antibodies were revealed with an anti-mouse-immunoglobulin–horseradish-peroxidase conjugate (diluted 1:1000; Sigma). Peroxidase activity was detected using the ECL kit (Amersham Pharmacia).

**DNA sequencing.** For inlA from *L. monocytogenes* strain H1, sequencing was performed on DNA fragments generated by
PCR. The primers used for PCR were seq01 (5'-AATCTAG-CACACGGTTGCGG-3') and seq02 (5'-TGTGACCTTCTTTACGGGC-3'), and they generated a 733 bp gene fragment. The PCR product was sequenced in both orientations by Genome Express. The nucleotide sequence was deposited in the GenBank database and has been assigned accession number AF468816. This sequence was compared to the internalin (inlA and inlB) gene sequence of Gaillard et al. (1991) (accession no. M67471).

RESULTS

Virulence of L. monocytogenes strains towards chick embryos

The chick embryo model was chosen to study the virulence of Listeria strains from various origins. The inoculum dose, determined by plate count, ranged from 1·5 to 2·5 log₁₀ c.f.u. egg⁻¹. Inoculation of 14-day-old chick embryos via the chorioallantoic fluids distinguished highly virulent strains (100% mortality rate with a mean time to death ranging from 2 to 3 days) from strains producing a lower mortality rate [higher mean time to death (>3-5 days)] (Fig. 1).

As expected, the epidemic strain ScottA was fully pathogenic in 14-day-old chick embryos (mortality 100%), whereas the asymptomatic carriage reference strain LO28 was less pathogenic (mortality 80%). L. welshimeri, a species avirulent in humans, was avirulent in chick embryos (mortality 0%) and was used as a control strain.

When compared to the reference strains, H2, a carriage strain, was found to be highly virulent, showing the same level of virulence as ScottA. In contrast, the virulence of strain H1 was strongly attenuated, since four out of the five chick embryos inoculated with this strain were still alive after 6 days.

Enzymic potential for virulence of carriage strains

Table 1 summarizes the characteristics of the five cultures studied, with respect to their serogroups, their haemolytic activities on horse-blood agar and their Pc-PLC and Pi-PLC activities.

As expected, L. welshimeri tested negative in the three enzymic assays, since this non-pathogenic Listeria species does not possess the genes necessary for these activities. All of the L. monocytogenes strains tested showed clear haemolysis on Colombia blood agar base supplemented with horse blood. Carriage strains H1 and H2 supported similar high levels of haemolytic activity, whereas ScottA demonstrated the lowest haemolytic activity.

In the same way, L. monocytogenes carriage strains H1 and H2 exhibited lecithin degradation (Pc-PLC activity) and were also positive for Pi-PLC activity. Surprisingly, ScottA gave the lowest activity for these two phospholipase activities, when compared to the other L. monocytogenes strains.

Efficiency of human L. monocytogenes carriage strains in infecting Caco-2 cells

The ability of L. monocytogenes strains to adhere to, enter into and multiply in intestinal epithelial cells was evaluated using an in vitro model with human enterocyte-like Caco-2 cells. The results of kinetic studies for ScottA, LO28, H1, H2 and L. welshimeri are summarized in Fig. 2. Results are expressed as the percentage of listerial adhesion to Caco-2 cells, the percentage of listerial entry into Caco-2 cells or the percentage of intracellular growth of Listeria isolates within Caco-2 cells, to take into account the differences in the initial cell numbers between strains (between 3 x 10⁶ and 5 x 10⁷ bacteria well⁻¹). The rate of replication was calculated from the percentages of entry and intracellular growth.

Under the test conditions, the kinetics of Caco-2 cell infection by ScottA and H2 (i.e. the most virulent strains) were similar. These two strains were the most efficient in entering Caco-2 cells, but had the lowest rate of replication. After 3-5 h of contact with Caco-2 cells, the rate of entry was 3·7 and 15·2% for ScottA and H2, respectively; 21·5 h post-infection, the rate of replication was 2·8 and 2·0 for ScottA and H2, respectively.

In contrast, L. welshimeri showed low adhesion values (1·3% of deposited bacteria) and extremely low entry values (0·06% of deposited bacteria), even though its
Table 1. Strains of *Listeria* used in this study, and their characteristics

<table>
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<tr>
<th>Species/category of isolate</th>
<th>Serogroup</th>
<th>Activity</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Haemolytic</td>
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<tr>
<td><em>L. monocytogenes</em> reference epidemic strain</td>
<td>4b</td>
<td>+</td>
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<tr>
<td>ScottA</td>
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<td></td>
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<tr>
<td><em>L. monocytogenes</em> carriage strains</td>
<td>1/2c</td>
<td>+</td>
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<tr>
<td>LO28</td>
<td>1</td>
<td>+</td>
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<tr>
<td>H1</td>
<td>4</td>
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<tr>
<td>H2</td>
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<tr>
<td><em>L. welshimeri</em></td>
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* + + + , Strong activity; + + , moderate activity; + , weak activity; − , no activity.

However, bacteria that reached the Caco-2 cytoplasm had a high rate of replication (87.5 for LO28 and 8.2 for H1 21.5 h post-infection). The relatively high intracellular replication levels observed for LO28, H1 and *L. welshimeri* within Caco-2 cells might be related to the relatively low entry level. However, cell infection performed with a higher m.o.i. showed that these strains exhibited the same intracellular rate of replication (data not shown). Therefore, the high rate of replication for LO28 in Caco-2 cells (rate of multiplication > 80) might be the reason why the mortality rate for chick embryos reached 80%, compared to 20% for H1, whose rate of replication was ten times lower than that of LO28.

The lower levels of invasion and dissemination of strains LO28 and H1 compared to those of ScottA and H2 could be due, at least partly, to an impaired ability to adhere to Caco-2 cells. This led us to examine the synthesis of two surface proteins, InlA and InlB, in LO28 and H1, which have been implicated in adhesion mechanisms.

**Analyses of *L. monocytogenes* human carriage strains surface proteins (internalin InlA and InlB) required for entry into epithelial cells**

The presence of the surface proteins InlA and InlB in H1, H2, ScottA and LO28 was analysed in culture supernatants and SDS-extracts by using two distinct specific mAbs, L7.7 for the production of InlA (Mengaud *et al*., 1996a) and B4-6 for the production of InlB (Braun *et al*., 1997) (Fig. 3).

As expected, InlB was produced by the carriage strains H1 and H2 and the reference strains ScottA and LO28, and had an apparent estimated molecular mass of 60 kDa in the SDS-extract fractions.

In culture supernatants and SDS-extract fractions from H2 and ScottA, InlA was produced with a molecular mass of 80 kDa. In contrast, InlA from strains H1 and LO28 had an apparent molecular mass of approximately 47 and 60 kDa, respectively; these two proteins were therefore considered truncated. A higher amount of InlA was detected in the LO28 culture supernatant than in its SDS-extract fraction. Surprisingly, compared to the

in intracellular growth ability inside the Caco-2 cells was higher than that of ScottA and H2 (rate of replication of 18.45, 21.5 h post-infection).

Among the carriage strains with attenuated virulence towards chick embryos, the weakly attenuated strain LO28 and the strongly attenuated strain H1 showed results that were closer to the behaviour of *L. welshimeri* than to the behaviour of *L. monocytogenes* ScottA. These strains were characterized by extremely low adhesion values (<1% of deposited bacteria) and, consequently, low entry levels, which were about 0.8% for LO28 and 0.11% for H1 3.5 h post-infection.
The present study focused on the characterization of the entry mechanisms of two *L. monocytogenes* human faeces carriage strains, to determine if their lack of virulence in humans could be linked to one or more components implicated in their internalization into host cells, the first step of infection.

First, we assessed the ability of the chick-embryo test to classify our isolates according to their level of virulence. Our results confirmed the occurrence of *L. monocytogenes* strains with attenuated virulence. To the best of our knowledge the present investigation demonstrated, for the first time, attenuated virulence of *L. monocytogenes* LO28. This highlights the importance of the choice of the in *vivo* model used for the study of pathogenicity. In fact, the chick-embryo assay could be a better model than the mouse or rat models, which are not considered appropriate for evaluating the virulence of *L. monocytogenes* strains such as LO28. Indeed, as noted by Lecuit et al. (1999), cells expressing mouse or rat E-cadherin (mEcad) do not allow InlA-dependent entry, whereas cells expressing human E-cadherin (hEcad), guinea pig or chicken E-cadherin (LCAM) are totally permissive to invasion by InlA-producing bacteria.

Furthermore, although H1 and H2 seem to be avirulent in humans, H2 was fully virulent in chick embryos, with its level of virulence similar to that of the epidemic strain ScottA. For H2, the differences in virulence against human cells and chick-embryo cells may be due to a low dose of ingested bacteria for the human and/or to the performance of the human immune system. In comparison, H1 seems to have a reduced capacity to invade both human and chick-embryo cells. This led us to examine more precisely the virulence-associated enzymic properties of the strains, to determine if the differences in pathogenicity between H1 and H2 could be correlated with differences in enzymic activities (i.e. haemolytic and phospholipases activities).

Our results demonstrate that, on the basis of three enzymic assays (for haemolytic, Pi-PLC and Pc-PLC activities), human carriage strains H1 and H2 have the enzymic potential necessary that contributes towards pathogenicity, since levels of the three enzymic activities observed for the two strains were higher than those of epidemic reference strain ScottA. These results suggested that either the production or the activity of virulence factors in the host is different from that observed in *vivo*, or that other putative virulence factors are responsible for the differences observed in the degrees of virulence produced by the different strains.

Consequently, we decided to use an *in vitro* approach to follow the steps of infection of host epithelial cells, to discover what impaired function could be responsible for the differences in pathogenicity observed between the strains. Strains virulent in the chick embryos, i.e. ScottA and H2, entered Caco-2 cells with great penetration efficiency, whereas strains with attenuated virulence towards the chick embryos, i.e. LO28 and H1, showed low entry levels.

On the one hand, our results demonstrated significant differences in the behaviour of the two carriage strains (H1 and H2) with regard to their kinetics within Caco-2 cells. On the other hand, when compared to *in vivo* tests, human enterocyte-like cell-line Caco-2 seems to be a reliable alternative model to the rat and mouse models.

Therefore, the human carriage strain H2 seems to be potentially as pathogenic as the known pathogenic strain ScottA, producing InlA with a molecular mass of 80 kDa, whereas the other carriage strain, H1, produced a truncated form of InlA (molecular mass of approximately 47 kDa) and was less virulent. The truncated InlA of H1 (and also of LO28) could explain its reduced ability for adhesion to Caco-2 cells and, consequently, probably reduces its ability to be fully pathogenic towards chick embryos.

**DNA sequencing**

DNA sequencing of *inlA* from strain H1 showed 12 silent mutations. One supplementary single point mutation was detected, consisting of the substitution of a cytosine for a thymidine at position 1414 (this position is based on the first translated codon). This point mutation created a nonsense codon (TAG) in the coding sequence, which could lead to the production of a protein with a theoretical molecular mass of 47 kDa.

**DISCUSSION**

The present study focused on the characterization of the entry mechanisms of two *L. monocytogenes* human faeces carriage strains, to determine if their lack of virulence in humans could be linked to one or more components implicated in their internalization into host cells, the first step of infection.
allowing the elucidation of altered function(s) in the progress of bacteria through the host cell. Using the human model it was shown that H1 entered Caco-2 cells poorly, as did LO28, with levels of entry as low as those observed for L. welsbmeri. The low level of entry of L. monocytogenes LO28 into mouse cells has been reported previously (Mengaud et al., 1996b), and this low level of cell entry has already been attributed to a truncated form of InlA – and to the resulting soluble form of InlA (Jonquieres et al., 1998) – but this truncated protein did not affect the virulence of LO28 in the mouse model.

Western-blot analysis showed that H1 had a truncated InlA protein (as was the case for LO28), with an apparent molecular mass of 47 kDa, whereas the InlA protein of H2 was entire, with a molecular mass of 80 kDa. An mAb (B4-6) raised against InlB allowed us to confirm that there were no problems concerning the production of InlB from the locus inlAB.

It was found that a nonsense mutation was responsible for the truncated form of the H1 InlA. Taking into account that mAb L7.7 is specific for the N-terminal part of InlA, the absence of its C-terminal part, and consequently the absence of the C-terminal part of the inter-repeat region, could lead to impaired folding of the leucine-rich repeat (LRR) region. Both the inter-repeat region and the LRR region are necessary for internalization-mediated entry into host cells (Lecuit et al., 1997). The two strains with apparently attenuated virulence towards chick embryos (H1 and LO28) produced truncated forms of InlA, which could be responsible for the low level of entry into Caco-2 cells. Therefore, our preliminary results support the hypothesis that InlA has a more critical role in clinical forms of listeriosis than previously thought.

Our finding of single point mutations within the InlA sequence clearly confirms the hypothesis of Chakraborty et al. (1994), who worked with environmental strains of L. monocytogenes. These authors showed that naturally occurring virulence-attenuated isolates were detected in hosts for only short periods of time, as a result of mutations occurring within virulence genes. They suggested that carriage strains, similar to strain H1 described in this study, could induce protection to subsequent infection by more pathogenic variants, such as H2. Manohar et al. (2001) reinforced this point of view by showing that gut colonization of mice with actA-negative mutants of L. monocytogenes could stimulate a humoral mucosal immune response. These findings could be a partial explanation for the widespread resistance to listeriosis seen in humans exposed to L. monocytogenes. Another reason for the absence of listeriosis in carrier H2 could be due either to the presence of additional physiological factors or to conditions that are required for the proliferation and implantation of L. monocytogenes in the mucosa of the human intestine, such as the presence of compounds that are not involved in its internalization or its growth within cells but which are still linked to its ability to colonize the host, or which affect its survival upon penetration of the intestinal mucosa (Barbour et al., 2001; Cotter et al., 2001). Moreover, the microbial interactions between L. monocytogenes and enteric commensal bacteria could explain the absence of listeriosis, as suggested by Zachar & Savage (1979) and Cebra (1999).

Finally, this study allowed us to characterize the two opposite behaviours of two human faecal carriage strains, H1 and H2. H1 has attenuated virulence towards chick embryos and can not be translocated through the intestinal wall. This phenotype could be due, in part, to a mutation in inlA. H2 should be considered potentially pathogenic with regard to its virulence in chick embryos, its abilities to express virulence-associated determinants and its invasion efficiency within Caco-2 cells. Indeed, this strain may have encountered particular circumstances within the host’s intestinal tract that prevented the development of listeriosis. Whether the truncation of internalin within human carriage L. monocytogenes strains is a rare event remains to be investigated.

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