

# Evidence for subpopulations of *Listeria monocytogenes* with enhanced invasion of cardiac cells

Francis Alonzo, III,<sup>1</sup> Linda D. Bobo,<sup>2</sup> Daniel J. Skiest<sup>3</sup>  
and Nancy E. Freitag<sup>1</sup>

Correspondence  
Nancy E. Freitag  
nfreitag@uic.edu

<sup>1</sup>Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL, USA

<sup>2</sup>Adult Infectious Diseases, Washington University School of Medicine, St Louis, MO, USA

<sup>3</sup>Division of Infectious Diseases, Baystate Medical Center – Tufts University School of Medicine, Springfield, MA, USA

Cardiac infections caused by the foodborne bacterium *Listeria monocytogenes* represent a significant but poorly studied facet of disease. It is not known whether *L. monocytogenes* cardiac infections stem solely from host susceptibility, or whether bacterial isolates exist that exhibit a tropism for cardiac tissue. Here we examine the cardio-invasive capacity of a recent *L. monocytogenes* cardiac case strain (07PF0776) as well as nine additional outbreak and clinical isolates. Mice infected with the cardiac isolate 07PF0776 had 10-fold more bacteria recovered from heart tissue than those infected with *L. monocytogenes* strain 10403S, a well-characterized clinical isolate originally obtained from a human skin lesion. Additional *L. monocytogenes* isolates exhibited varied capacities to colonize the hearts of mice; however, those with the highest efficiency of mouse cardiac invasion also demonstrated the highest levels of bacterial invasion in cultured myoblast cells. Our findings strongly suggest that subpopulations of *L. monocytogenes* strains have acquired an enhanced ability to target and invade the myocardium.

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## INTRODUCTION

*Listeria monocytogenes* is a foodborne, facultative, intracellular bacterial pathogen that infects humans and causes disease following translocation across the gut epithelial barrier (Swaminathan & Gerner-Smith, 2007). While healthy individuals usually exhibit mild forms of disease such as gastroenteritis, immunocompromised individuals and elderly patients often suffer more severe forms of illness, which include meningoencephalitis and septicæmia, with 20–30% of these patients succumbing to infection despite antibiotic treatment (Mead *et al.*, 1999; Vázquez-Boland *et al.*, 2001). A significant but much less documented sequela of *L. monocytogenes* infections involves the heart. This type of infection is estimated to occur in at least 7–10% of those with systemic disease; however, it is exceedingly rare in HIV-AIDS (acquired immunodeficiency syndrome) patients (0.5%) (Berenguer *et al.*, 1991; Brouqui & Raoult, 2001; Bruschi, 2001; Kales & Holzman, 1990). Cardiac infection with *L. monocytogenes* most often manifests as endocarditis or invasive

myocarditis (Antolín *et al.*, 2008; Haddad *et al.*, 2007; Hill *et al.*, 2006; Lindholm, 2008; Llanwarne *et al.*, 2007; Pocar *et al.*, 2009; Rua Galisteo *et al.*, 2007). Approximately 60% of endocarditis cases are associated with individuals who have prior valvular abnormalities (Antolín *et al.*, 2008), and 50% of those infected require valve replacement due to severe damage and failure (Brouqui & Raoult, 2001).

The death rate from cardiac illness is estimated to be up to 35% despite treatment, and yet very little is known regarding *L. monocytogenes* colonization of cardiac tissue and its resultant pathologies (Antolín *et al.*, 2008). Although less common in comparison to *Staphylococcus aureus* or viridans streptococci-associated heart infections, bacterial endocarditis due to *L. monocytogenes* infection still requires careful consideration for diagnosis and treatment in susceptible individuals due to its high mortality (Brouqui & Raoult, 2001; Hill *et al.*, 2006). Additionally, cases of bacterial myocarditis and abscess formation due to *L. monocytogenes* infection have been described (Haddad *et al.*, 2007; Hood & Baxter, 1999; Makaryus *et al.*, 2004; McCue & Moore, 1979). Although myocarditis and abscess formation represent a smaller proportion of *L. monocytogenes* cardiac cases, these cases are noteworthy in that the organism colonizes what had previously been assumed to be a non-permissive site for

Abbreviations: LLS, listeriolysin S; RBC, red blood cell.

The GenBank/EMBL/DDBJ accession numbers for the DNA sequence data for the previously unpublished *hly*, *inIA* and *inIB* genes are HQ703556–HQ703578.

bacterial replication (Luo *et al.*, 2003). While the majority of patients with endocarditis or invasive myocarditis due to *L. monocytogenes* can be considered immunocompromised (Brusch, 2001), at least two documented cases of *Listeria*-associated cardiac illness have occurred in patients who were apparently completely healthy prior to infection (Adler *et al.*, 2009; Haddad *et al.*, 2007).

It is not known whether *L. monocytogenes* cardiac manifestations occur as a result of patient predisposition or whether they result from strains that possess enhanced colonization capacities. A study by Luo *et al.* (2003) suggested that the heart is not normally a tissue that is permissive for *L. monocytogenes* colonization. Here we describe the examination of a series of clinical and food outbreak *L. monocytogenes* isolates for cardiac infection, including a recent isolate (07PF0776) cultured from an HIV-infected patient who had a non-resuscitatable asystolic arrest due to invasive *L. monocytogenes* infection of the heart. Our studies indicate that a subpopulation of *L. monocytogenes* strains (that includes 07PF0776) exhibit an enhanced capacity to colonize cardiac tissue.

## METHODS

**Bacterial strains and culture conditions.** All bacterial strains used are described in Table 1 along with serotype and source. J4403, J5043 and J4901 were obtained courtesy of Dr Lewis Graves at the CDC. Clinical isolate 07PF0776 was obtained from both the Massachusetts Public Health Laboratory and from the CDC (where it is classified as J4533). Isolate NWI4b was obtained courtesy of the Children's Memorial Hospital and Northwestern Memorial Hospital Chicago, IL, USA, from Dr Stanley Schulman (Adler *et al.*, 2009). All other strains were derived from laboratory stocks, and the original sources from which they were isolated are indicated. Prior to experiments, all strains were grown statically overnight from a single colony in 2 ml Brain Heart Infusion (BHI) broth (Difco Laboratories) at 37 °C.

**Case report and isolation of *L. monocytogenes* 07PF0776.** A 51-year-old HIV-infected man with dyslipidaemia and hypertension presented with chest pain, dyspnoea, transient fevers and chills for 3

weeks, and evidence of inferior ischaemia by electrocardiogram on admission. Cardiac markers indicated that his myoglobin B fraction was elevated. The patient's CD4<sup>+</sup> T-cell count was 207 cells mm<sup>-3</sup> with an HIV RNA level of 310 765 copies ml<sup>-1</sup>, and he had not been on any antiretroviral or preventative treatment for opportunistic infection for over 1 year. Intermittent chest pain continued, and on the second hospital day his temperature rose to 39.2 °C transiently, but he was not started on antibiotics. On day three, his vital signs were normal that morning, but 1 h later he became asystolic and could not be resuscitated. Five of six blood cultures later grew *L. monocytogenes*.

The organism (from here on referred to as 07PF0776) was isolated from blood cultures from the patient by the Baystate Health Microbiology Laboratory. The isolate was biochemically characterized as *L. monocytogenes* using standard procedures by the Massachusetts Public Health Department and the CDC Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, and confirmed by AccuProbe (GenProbe) (Weaver, 1989). Serological typing was by the method of Seeliger & Höhne (1979). PFGE was performed using *Ascl* and *Apal* restriction endonuclease digests of bacterial DNA in accordance with the CDC PulseNet standardized protocol (Graves & Swaminathan, 2001). Computer-assisted analysis of PFGE patterns and comparison with patterns in the PulseNet national database was performed using Molecular Analyst Fingerprinting Plus with Data Sharing Tools Version 1.6 (Bio-Rad Laboratories) (Graves & Swaminathan, 2001; Graves *et al.*, 2005). The CDC pattern identifiers for the cardiac isolate are GX6A.1019 for the *Ascl* pattern and GX6A.12.0088 for the *Apal* pattern.

Gross and microscopic autopsy analysis was performed by the Baystate Health Center Department of Pathology (Springfield, MA, USA) on the cardiovascular, respiratory, gastrointestinal, hepatobiliary systems and pancreas, urogenital, endocrine, haematopoietic, musculoskeletal and central nervous systems of the deceased. Haematoxylin, Brown and Brenn, acid-fast bacilli and Gomori methenamine-silver stains were used for analysis. Gross examination revealed an enlarged heart with left ventricular hypertrophy, and an interventricular septal abscess of 3.5 × 3.5 × 3.0 cm (Fig. 1a, b). There were no valvular or other lesions in the heart or great vessels, and the coronary arteries were free of obstruction. Clusters of *L. monocytogenes* were seen by Brown and Brenn stain along with a neutrophilic infiltrate (Fig. 1c). It was difficult to discern from the specimen whether the bacteria were intracellular, extracellular, or both. There were also clusters of *L. monocytogenes* within the hepatic

**Table 1.** Clinical and outbreak isolates used in this study

Strain no.	Designation	Isolate type	Serotype
NF-L100	I0403S	Virulent laboratory strain (skin lesion)	1/2a
NF-L101	EGDe	Virulent laboratory strain (guinea pig)	1/2a
NF-L725	ScottA	Virulent laboratory strain (foodborne)	4b
NF-L828	F2379	Foodborne – Los Angeles (Jalisco cheese)	4b
NF-L831	OIS4b	Foodborne – Sweden (Brie cheese)	4b
NF-L832	OIJ4b	Outbreak – Japan	4b
NF-L1403	07PF0776	Clinical isolate – cardiac interventricular septal abscess	4b
NF-L1590	J4403	Clinical isolate – pericardial fluid	1/2b
NF-L1591	J4901	Clinical isolate – heart tissue	NT
NF-L1592	J5043	Clinical isolate – patient blood	4b
NF-L1776	NWI4b	Clinical isolate – cardiac pseudotumour	ND

NT, Not typable; ND, not determined.

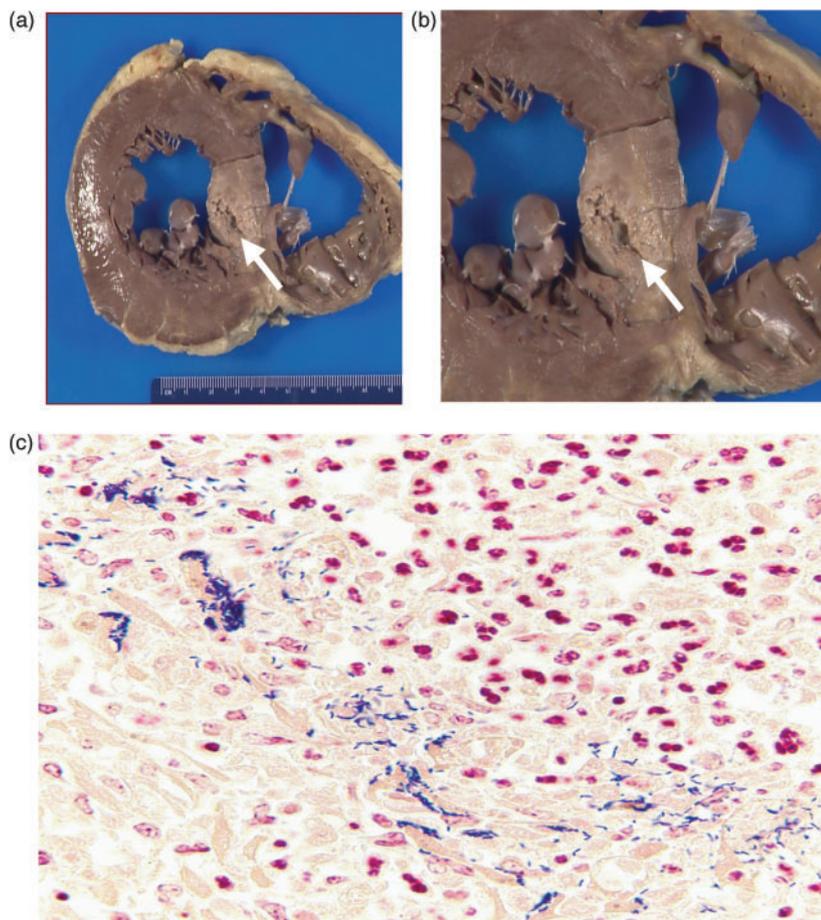
sinusoids (data not shown). There was no sign of infection in the other organs. The cause of death was likely to be a fatal arrhythmia due to death of myocardial cells in the interventricular septum and disruption of the main impulse conduction pathway of the heart from the invasive infection.

**Mouse intravenous infections.** All animal procedures were IACUC approved and performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Intravenous inoculation of mice via tail vein was performed as previously described (Alonzo *et al.*, 2009) with some modifications. Six to eight week old female ND4 Swiss Webster mice (Harlan) were injected with 0.2 ml PBS [0.144 g  $\text{KH}_2\text{PO}_4 \text{ ml}^{-1}$ , 9.00 g  $\text{NaCl ml}^{-1}$ , 0.795 g  $\text{Na}_2\text{HPO}_4$  (anhydrous)  $\text{ml}^{-1}$ ] containing  $2 \times 10^4$  c.f.u. of each strain of *L. monocytogenes*. For time-course experiments, 15 mice were inoculated and at 24, 48 and 72 h post-inoculation, five mice were sacrificed and livers, spleens and hearts were dissected. Organs were homogenized and dilutions of the tissue homogenate were spread onto BHI agar plates to enumerate bacterial burdens to each organ. For the multistrain comparisons of bacterial burden to the heart, mice were inoculated as described, but were sacrificed at only the 24 h time point. All animal experiments were repeated a minimum of three times. Statistics were calculated using a one-way ANOVA with Tukey's multiple comparison test, and  $P < 0.05$  was considered significant.

**Intracellular growth in tissue culture cells.** The H9C2 cell line (ATCC CRL-1446) was kindly provided by Dr David Engman

(Northwestern University, Chicago, IL, USA) and serves as a rat myoblast cell line routinely used for cardiac-related pathogenesis studies (Hyland *et al.*, 2008; Lee *et al.*, 2006; Tyler *et al.*, 2005). H9C2 and J774 mouse macrophage-like cells were maintained in DMEM supplemented with penicillin ( $100 \mu\text{g ml}^{-1}$ ), streptomycin ( $100 \mu\text{g ml}^{-1}$ ), L-glutamine ( $292 \mu\text{g ml}^{-1}$ ) and 10% fetal bovine serum (Hyclone) and grown at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . Monolayers of cells were grown on acid-washed coverslips in DMEM without antibiotics. Cells were infected with *L. monocytogenes* at an m.o.i. of 100 bacteria to 1 myoblast (H9C2) or 0.1 bacteria to 1 macrophage (J774). After 1 h, cells were washed three times with  $37^\circ\text{C}$  PBS followed by the addition of  $37^\circ\text{C}$  DMEM containing gentamicin ( $15 \mu\text{g ml}^{-1}$ ). At the time points indicated, coverslips were removed and lysed in 5 ml  $\text{H}_2\text{O}$  with vigorous vortexing, or were processed for fluorescence microscopy. Dilutions of lysates were spread onto LB agar for enumeration of bacterial c.f.u. Fluorescence staining was performed as previously described (Mueller & Freitag, 2005) and images were obtained on a DeltaVision microscope (Applied Precision). Images are representative of at least ten independently viewed fields and were captured using SoftWorx image software (Applied Precision).

**Invasion assays.** H9C2 cells were infected at an m.o.i. of 100:1. After 1 h, cells were washed three times with  $37^\circ\text{C}$  PBS followed by removal of coverslips for enumeration of total bacterial c.f.u.  $37^\circ\text{C}$  DMEM containing gentamicin ( $15 \mu\text{g ml}^{-1}$ ) was then added to the remaining cells and allowed to incubate for an hour at  $37^\circ\text{C}$ . Additional coverslips were then removed and intracellular bacterial



**Fig. 1.** Gross and microscopic examination reveals the presence of an abscess within the interventricular septum (IVS) of the heart as well as bacillary micro-organisms in and around cardiac tissue. (a) Cross-section of patient's heart. The white arrow denotes the location of an abscess within the IVS. (b) Enlarged view of the abscess shown in (a). (c) Brown and Brenn stain of the cardiac septal abscess showing both the presence of many bacillary organisms and a pronounced neutrophilic infiltrate.

c.f.u. were enumerated. The ratio of intracellular bacteria to that of the total inoculum was calculated and indicated as per cent invasion. Assays were conducted three times in triplicate.

**L2 plaque assays.** Plaque assays were conducted as previously described (Sun *et al.*, 1990). A monolayer of L2 fibroblast cells was infected with *L. monocytogenes* at an m.o.i. of 30:1 for 1 h followed by the addition of gentamicin ( $10 \mu\text{g ml}^{-1}$ ) in a DMEM/0.7% agarose overlay. After 72 h, plaques were visualized by staining with Neutral Red and measured with a micrometer. A minimum of three plaque assays were performed with approximately 25 plaques measured per experiment.

**Haemolytic activity.** Haemolytic activity and phospholipase activity were measured as described by Alonzo *et al.* (2009). Briefly, overnight cultures of *L. monocytogenes* in LB were diluted 1:10 into 10 ml fresh LB and grown for 5 h at 37 °C with shaking. After 5 h, OD<sub>600</sub> readings were measured and ~1.2 ml bacterial culture was centrifuged at maximum speed in a microcentrifuge. After centrifugation, supernatants were removed and bacterial pellets were discarded. The supernatants from cultures with higher optical densities after 5 h of growth were normalized by diluting into fresh LB, thus accounting for differences in bacterial abundance between strains. Serial dilutions of culture supernatants were made into PBS + 1 mM DTT (pH 5.0) followed by the addition of sheep red blood cells (RBCs). After incubation at 37 °C for 30 min, supernatant/RBC mixtures were centrifuged at maximum speed for 1 min and the dilution resulting in 50% lysis of RBCs based on visual examination of RBC pellets was recorded. Haemolytic units are defined as the reciprocal of the culture supernatant dilution required for 50% RBC lysis. Experiments were conducted a minimum of three times in triplicate.

**Phospholipase activity.** Phospholipase activity was assessed by streaking single colonies of each *L. monocytogenes* strain onto egg yolk agar plates containing 25 mM glucose 6-phosphate, 2.5% egg yolk and 0.2% activated charcoal (to enhance PrfA-dependent *plcB* expression) (Alonzo *et al.*, 2009). After 72 h, the white precipitates surrounding the bacterial streaks were examined. Strain 10403S was considered the standard to which all other strains were compared. Thus, a strain with increased phospholipase activity had a larger zone of opacity than 10403S, while a strain with decreased phospholipase activity exhibited a smaller zone.

**Bacterial swimming motility.** Motility of isolates was measured in a soft agar assay as described by Shetron-Rama *et al.* (2003). One microlitre of an overnight culture of each isolate was injected into a BHI soft agar plate (0.3% agar) and incubated at 37 °C for 48 h. Motile distance was determined by measuring the diameter of the zones of bacterial growth for each strain tested. Measurements are from a minimum of three independent experiments.

**DNA sequencing and construction of cladogram relationship trees.** Bacterial genomic DNA was isolated using a DNeasy Blood/Tissue kit (Qiagen). Oligonucleotides used for sequencing *hly* were as follows: 1SEQllo, GAGTAATAAACTAATGTGCG; 2SEQllo, AACATTTCGTTACCTTCAGG; 3SEQllo, GAGAAAAGAAGAAATCCA-TC; 4SEQllo, AAAAGCTATTACCATGATGAT; 5SEQllo, AATTCA-ATTCATCCATGGCA; 6SEQllo, ATTTCTATTTTTCACAAGTGG. Oligonucleotides for sequencing *inlA* and *inlB* were designed as previously described (Van Stelten *et al.*, 2010). Sequencing was carried out by the DNA Services Facility at the University of Illinois at Chicago and analysed using the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). DNA sequence data for previously unpublished *hly*, *inlA* and *inlB* genes have been submitted to GenBank and assigned accession numbers HQ703556–HQ703578.

## RESULTS

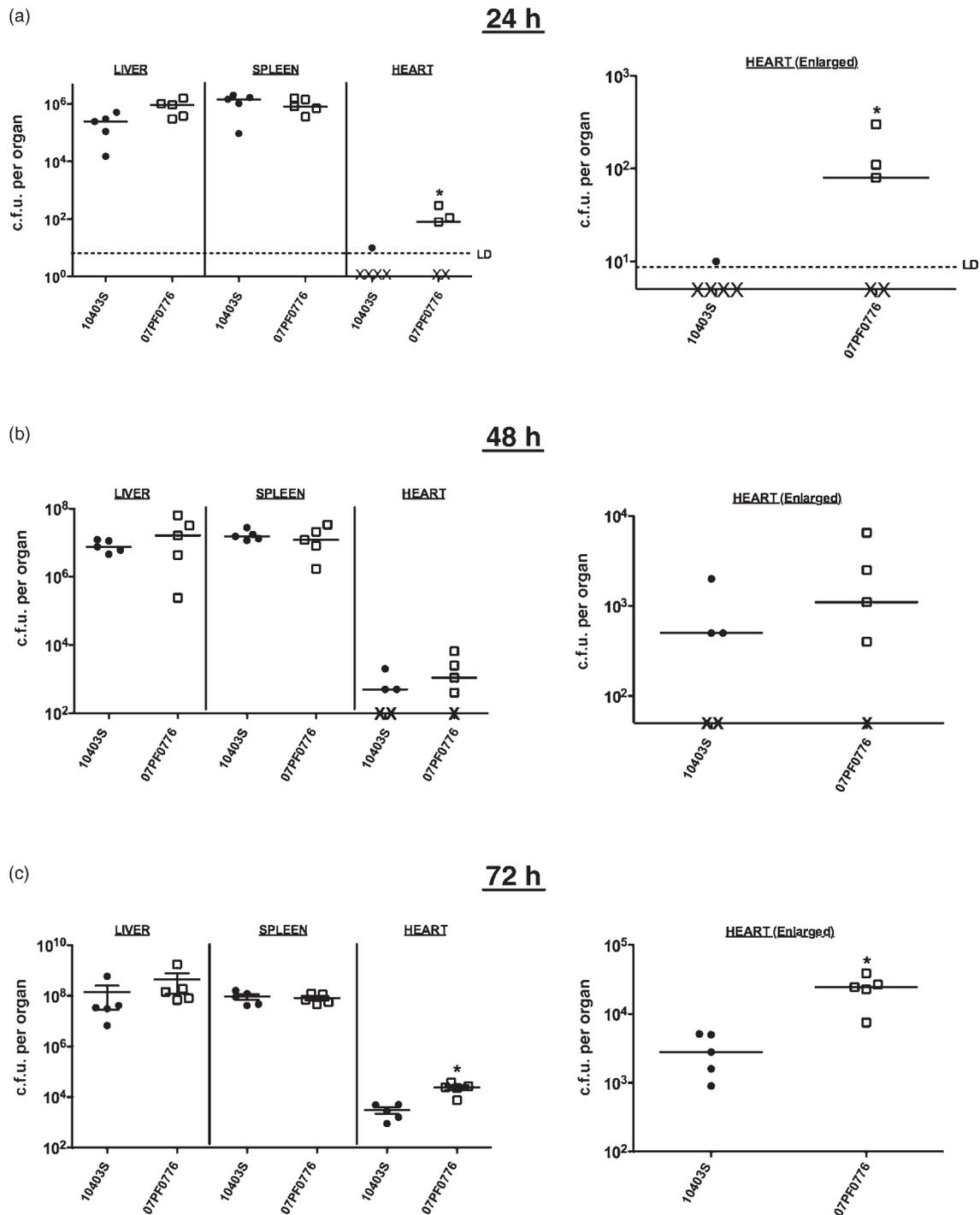
### *L. monocytogenes* human cardiac isolate 07PF0776 exhibits an enhanced capacity for colonization of heart tissue in mice

Although host physiology and immunity are critical components of infectious disease progression, pathogens have also been observed to acquire traits that increase their pathogenic capacity. Given that *L. monocytogenes* cardiac infections are rare in HIV-AIDS individuals (Berenguer *et al.*, 1991; Brouqui & Raoult, 2001; Bruschi, 2001; Kales & Holzman, 1990), we investigated whether a recent HIV-AIDS patient cardiac infection isolate, 07PF0776, exhibited an enhanced ability to target cardiac tissue. Mice were intravenously infected with 07PF0776 and at various time points post-infection the bacterial burdens in hearts, livers and spleens were compared to those of animals infected with an independent and well-characterized *L. monocytogenes* human skin lesion isolate, strain 10403S (Bishop & Hinrichs, 1987; Edman *et al.*, 1968).

At 24 h post-infection, strains 10403S and 07PF0776 produced strikingly similar bacterial burdens in the livers and spleens of infected mice, and both strains established infection in these tissues with similar efficiencies (Fig. 2). In contrast to the recovery of bacteria from liver and spleen, mice infected with 07PF0776 had increased bacterial burdens in the heart as well as an increased efficiency of heart colonization when compared to animals infected with 10403S (Fig. 2a). Whereas only one of five mice infected with 10403S contained detectable numbers of bacteria within the heart, three of five mice infected with 07PF0776 had bacteria recovered from cardiac tissue. Animals infected with 07PF0776 were observed to have 10–15-fold more bacteria in the heart than the animals for which 10403S could be recovered. At 48 h post-infection, the bacterial burdens in the livers and spleens of animals infected with either 07PF0776 or 10403S were nearly identical, and differences in bacterial burdens in the hearts were less pronounced and not of statistical significance (Fig. 2b). The 07PF0776 case isolate still exhibited a greater efficiency of heart infection than 10403S with four out of five 07PF0776-infected mice having detectable bacterial c.f.u. recovered from the hearts in comparison with three of five mice infected with 10403S (Fig. 2b). At 72 h post-infection, however, the difference in bacterial burdens in the hearts of animals infected with 07PF0776 was 10-fold greater than observed for those infected with 10403S, while the numbers of bacteria recovered from the livers and spleens remained nearly identical (Fig. 2c). These data indicate an enhanced proclivity of strain 07PF0776 for cardiac infection in comparison to 10403S.

### Additional *L. monocytogenes* isolates can be grouped based on their capacity for cardiac colonization

Based on the enhanced ability of 07PF0776 to colonize the hearts of infected mice, it seemed of interest to determine



**Fig. 2.** Strain 07PF0776 exhibits an enhanced ability to colonize cardiac tissue of infected mice. Bacterial burdens present within livers, spleens and hearts at 24 h (a), 48 h (b) and 72 h (c) following intravenous inoculation of  $2 \times 10^4$  c.f.u. of *L. monocytogenes*. X below the dashed limit of detection (LD) line represents mice that either contained no bacteria within the heart or whose bacterial burdens were below the limit of detection. Statistics were calculated using a one-way ANOVA with Dunnett's post-test. \*,  $P < 0.05$ . ●, 10403S; □, 07PF0776.

whether cardiac infection by *L. monocytogenes* represents a trait shared by many strains or alternatively one that is limited to a few strains or even unique to 07PF0776. Nine

additional clinical and outbreak strains of *L. monocytogenes* were thus compared for their capacity to establish cardiac infections (Table 1). Included in this comparison were

strains isolated from patients with cardiac-associated *Listeria* infections (J4403, J4901 and NWI4b) and a blood isolate (J5043) as well as the first genome sequenced strain (EDGe) and a variety of strains isolated from independent foodborne outbreaks (ScottA, OIS4b and OIJ4b). The comparison included strains of serotypes 1/2a, 1/2b and 4b, and one strain designated non-typable (Table 1). This sampling of isolates thus represented the two serotypes most commonly associated with systemic disease (1/2a and 4b), as well as isolates directly associated with cardiac disease and isolates obtained from food as well as blood samples. Each isolate was examined for its ability to colonize the hearts of intravenously infected mice. Consistent with the results obtained for strains 07PF0776 and 10403S, similar numbers of bacteria were recovered from the livers and spleens of infected animals for each isolate at 24 h post-infection (Fig. 3a, b). No statistically significant differences in bacterial c.f.u. were detected for isolates recovered from either organ.

Interestingly, striking differences were observed when strains were examined for colonization of mouse hearts (Fig. 3c). Isolate 07PF0776 had a high efficiency of heart infection (11 out of 14 mice with detectable c.f.u. within the heart) as well as the largest numbers of bacterial c.f.u. recovered. An additional cardiac isolate, J4403, was also recovered with high efficiency from the hearts of infected mice (nine out of ten animals infected) and in numbers that resembled those of 07PF0776 (Fig. 3c). The remaining strains were found to vary in their ability to colonize heart tissue. Indeed, there were no detectable bacteria in the hearts of the majority of infected animals for several strains, including the cardiac-associated patient isolates NWI4b and J4901 (Fig. 3c). It is of interest to note that isolates recovered most abundantly from the hearts of infected animals were not recovered from the livers or the spleens in increased numbers in comparison to other strains. In contrast, the isolates recovered in the largest numbers from the livers and/or spleens of mice had the lowest numbers of bacteria recovered from the heart (F2379, EGDe, J5043 and 10403S) (Fig. 3a–c). Taken together, these data indicate that cardiac isolates J4403 and 07PF0776 appeared better able to target, infect and/or replicate within cardiac tissue than the other examined isolates, including isolates associated with other patient cardiac infections.

### **A majority of *L. monocytogenes* isolates replicate within the cytosol of infected cardiac tissue culture cells**

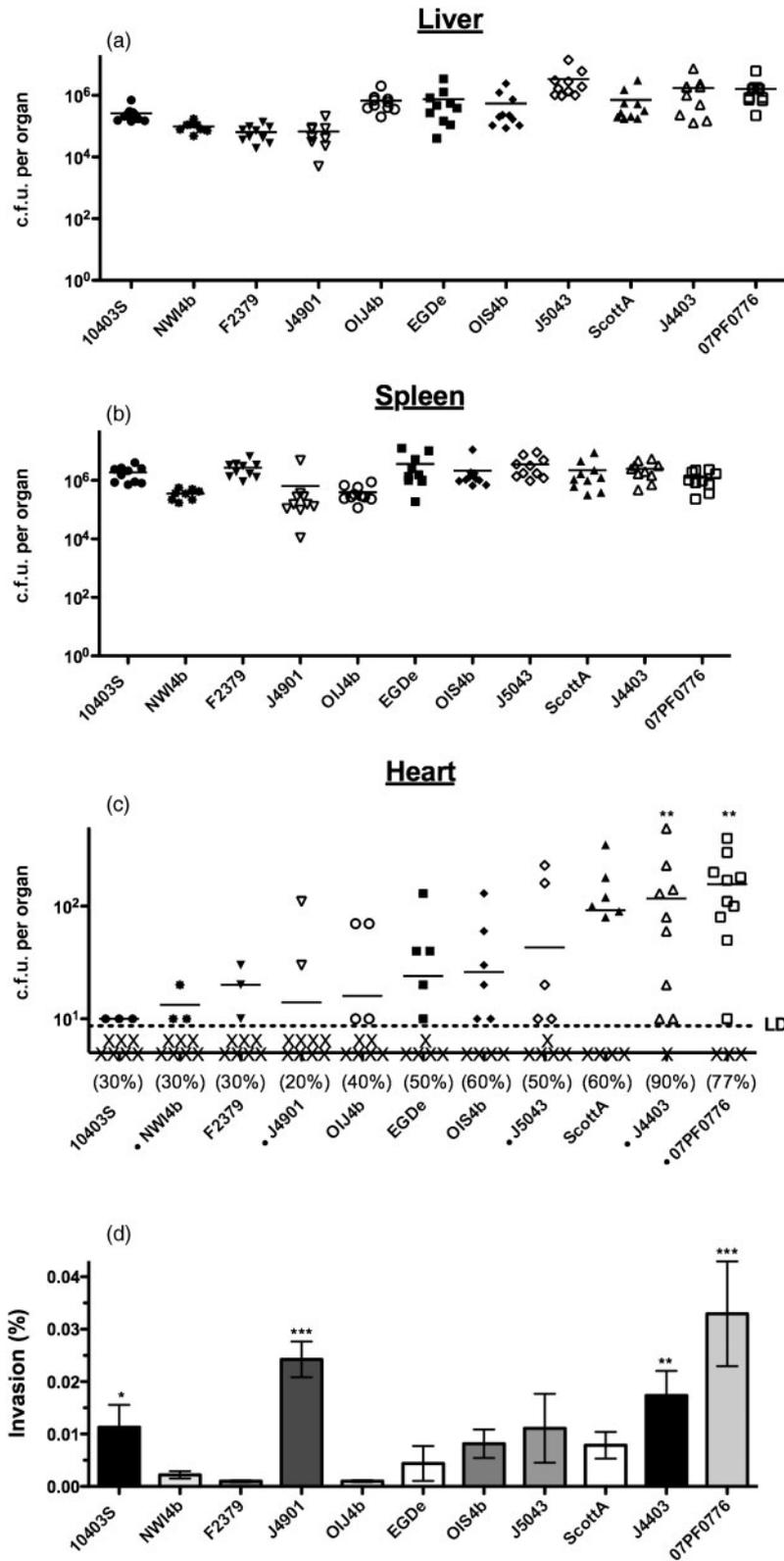
To compare the ability of selected *L. monocytogenes* isolates to invade host cells, escape the phagosome and replicate within the cytosol, bacterial replication was examined in murine J774 macrophage-like tissue culture cells as well as in H9C2 rat myoblast tissue culture cells. Five strains were selected as representative of bacteria with differing efficiencies of heart colonization (Fig. 3). Strains 10403S and F2379

were selected as strains displaying low-level infection of cardiac tissue *in vivo*, and J4403, ScottA and 07PF0776 were selected as three strains exhibiting a higher efficiency of cardiac infection. All five isolates had similar rates of replication in J774 cells although modest differences in the efficiency of bacterial uptake into macrophages were observed (Fig. 4a).

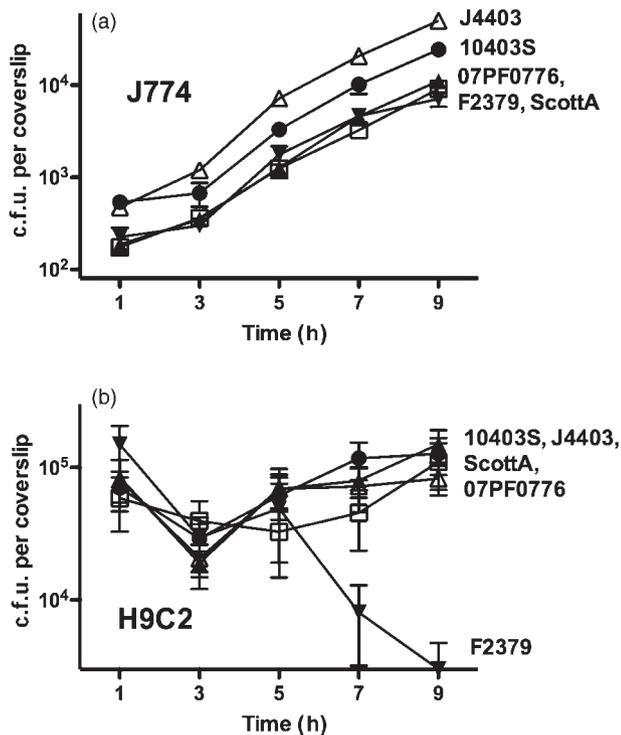
In contrast to the results observed for the infection of macrophage-like cells, differences in bacterial intracellular replication were observed for the selected isolates in rat myoblast H9C2 cells (Fig. 4b). All strains were capable of cell entry (albeit with varied efficiencies) with reduced rates of intracellular bacterial replication in comparison with the rates of growth observed in J774 cells (Fig. 4b). In J774 macrophages, the doubling times for all strains were between approximately 60 and 75 min. In the rat myoblast cells, the doubling times were increased approximately two- to threefold with ScottA having the shortest doubling time (120 min) and 07PF0776 the longest (195 min). One strain, F2379, exhibited a dramatic decrease in bacterial numbers beginning at 5 h post-infection, potentially reflective of bacterial cytotoxicity (Fig. 4b). All strains of *L. monocytogenes* were capable of phagosome lysis and entry into the myoblast cytosol based on bacterial association with host cell actin (Fig. 5a–e).

### ***L. monocytogenes* strains that target the heart *in vivo* exhibit enhanced invasion of cardiac cells *in vitro***

The *L. monocytogenes* isolates were examined for several activities associated with virulence, including secreted haemolytic and phospholipase activities, efficiency of cell-to-cell spread and the presence of listeriolysin S (LLS) as well as flagella-mediated swimming motility (an activity that has been associated with enhanced colonization of the intestinal epithelium) (O'Neil & Marquis, 2006) (Table 2). Strains were additionally examined for their ability to invade a H9C2 rat myoblast cell line in tissue culture (Table 2 and Fig. 3d). While variability in plaque size, haemolytic activity, phospholipase activity, swimming motility and the presence/absence of LLS was observed for all strains tested, no phenotype appeared to directly correlate with the ability of a strain to establish cardiac colonization in animals (Table 2). In contrast, the efficiency of bacterial invasion of H9C2 cells was found to be significantly increased for both cardiac colonizers 07PF0776 and J4403 (0.033% and 0.017%, respectively) compared to that of the other isolates (ranging from 0.001% to 0.008%, with the exception of J4901, and to a lesser degree, 10403S) (Fig. 3d). In fact, a striking correlation appeared to exist between the efficiency of H9C2 invasion for nearly all strains tested and the overall bacterial burdens in the hearts of infected mice. These findings suggest that the *L. monocytogenes* isolates that efficiently target the heart for replication may do so as a result of an increased capacity for cardiac cell invasion.



**Fig. 3.** Multistrain comparisons reveal a subset of strains with enhanced invasion characteristics. Mice were intravenously inoculated with  $2 \times 10^4$  c.f.u. of 1 of 11 isolates of *L. monocytogenes*. Bacterial burdens to the liver (a), spleen (b) and heart (c) were quantified 24 h post-inoculation. The percentage of mice containing quantifiable bacteria within the heart is indicated beneath the scatter plot for each individual strain. X below the dashed limit of detection (LD) line represents mice that either contained no bacteria within the heart or whose bacterial burdens were below the limit of detection. (d) Measurement of bacterial invasion efficiency by gentamicin protection of H9C2 cardiomyoblasts. Per cent invasion is the ratio of total intracellular bacteria to the bacterial inoculum. Statistics were calculated using a one-way ANOVA with Tukey's multiple comparison test. Statistically significant increases in burden compared to the five least invasive strains are indicated: \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.0001$ . ●, 10403s; \*, NW14b; ▼, F2379; ▽, J4901; ○, OIJ4b; ■, EGDe; ◆, OIS4b; ◇, J5043; ▲, ScottA; △, J4403; □, 07PF0776. Strains marked with a small black circle are of cardiac or patient blood origin.



**Fig. 4.** *L. monocytogenes* strains invade and replicate within cardiomyoblasts. (a) Intracellular growth in J774 macrophage-like cells (m.o.i. 0.1:1) of representative cardio-invasive/non-invasive strains of varied origin and serotype. (b) Intracellular growth in H9C2 rat myoblasts infected at an m.o.i. of 100:1. ●, 10403S; △, J4403; ▲, ScottA; □, 07PF0776; ▼, F2379.

#### Amino acid sequence alignments of key invasion proteins reveal extensive conservation for cardio-invasive and non-invasive strains, and suggest potential polymorphisms that might contribute to altered tissue tropism

InlA and InlB are the two most well-characterized bacterial invasion proteins associated with *L. monocytogenes* and they directly facilitate bacterial uptake into specific host cell types (Bierne *et al.*, 2007). As few as two amino acid substitutions within InlA have been shown to dramatically alter species-specific invasive capacity by enhancing bacterial binding to host cell receptors (Wollert *et al.*, 2007). To determine whether there were detectable amino acid polymorphisms in either InlA or InlB that could potentially correlate with cardiac cell invasion, each gene was amplified from the *L. monocytogenes* isolates and amino acid sequences were compared along with those of the conserved virulence protein listeriolysin O. The construction of cladogram relationship trees suggested a potential phylogenetic link between strains with increased cardio-invasive capacity, particularly for InlA, where J4403 and 07PF0776 were most closely linked (Fig. 6a). Additionally, phylogenetic linkage based on InlA sequence

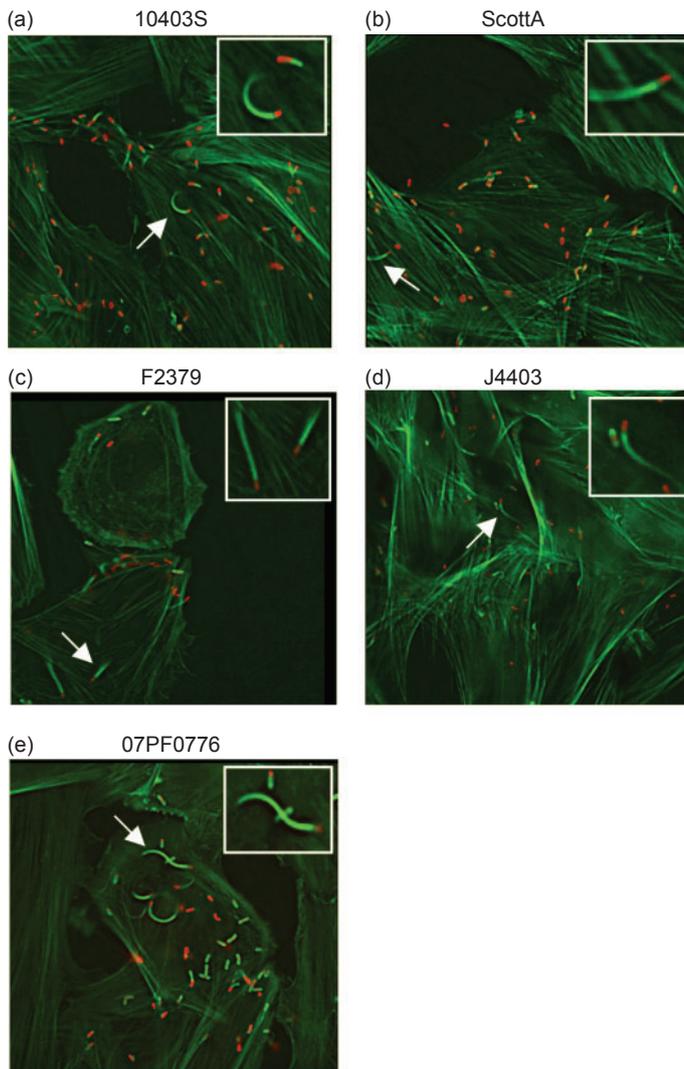
suggested relationships between strains with the highest and lowest levels of cardiac invasion (compare Fig. 6a to Fig. 3c and d). It should be noted that isolate OIJ4b contained both a stop codon in *inlA* and significant rearrangements of *inlB* sequence that complicated alignment and comparison (Fig. 6b). Amino acid sequence comparisons based on cladogram relationships revealed five unique amino acid polymorphisms associated with cardio-invasive isolates for either InlA (L589I and D744Y) or InlB (L138I, M/S251T and N375D) (Fig. 6d). Overall, the isolates of *Listeria* that efficiently target cardiac tissue *in vivo* appear more closely related than those that do not, and these isolates share amino acid polymorphisms that may indicate a link of InlA and/or InlB to enhanced cardiac cell invasion.

## DISCUSSION

Although systemic *L. monocytogenes* infections in humans have primarily been associated with the central nervous system, the placenta and the developing fetus, a significant percentage of invasive disease cases involve cardiac tissue (Brouqui & Raoult, 2001; Bruschi, 2001). Despite this association, cardiac disease resulting from *L. monocytogenes* infections remains poorly defined. While host physiology and immune status influence the course and extent of *L. monocytogenes* infection, it is possible that some bacterial isolates possess an enhanced ability to colonize alternative tissue sites. Here we have presented evidence to suggest that subpopulations of *L. monocytogenes* exhibit an enhanced capacity for establishing cardiac disease *in vivo* resulting from increased bacterial invasion of cardiac cells, a trait potentially linked to amino acid polymorphisms in InlA and/or InlB.

While isolates 07PF0776 and J4403 appeared to efficiently target the hearts of infected mice, it remains possible that these isolates are capable of invading other host sites in addition to the heart, liver and spleen. Work by Hardy *et al.* (2004, 2006) has shown that *L. monocytogenes* 10403S colonizes the mouse gall bladder, a location with the potential to serve as a reservoir for reactivation of illness. *Listeria* has also been reported in the bone marrow of infected mice and humans (de Bruijn *et al.*, 1998; Hardy *et al.*, 2009; Khan *et al.*, 2001). It would thus appear that the number of body sites that have the potential to serve as foci for *Listeria* replication may be more diverse than originally appreciated. Efforts to determine the progression and distribution of 07PF0776 and J4403 in animal infection models are currently under way.

The apparent correlation between the ability of *L. monocytogenes* to invade cardiac cells in tissue culture and its capacity to colonize the hearts in infected mice suggests that *L. monocytogenes* might establish infections at novel body sites by acquiring cell type-specific invasive capacities. As *L. monocytogenes* has the capacity to spread intracellularly from one cell type to another, the colonization of a tissue



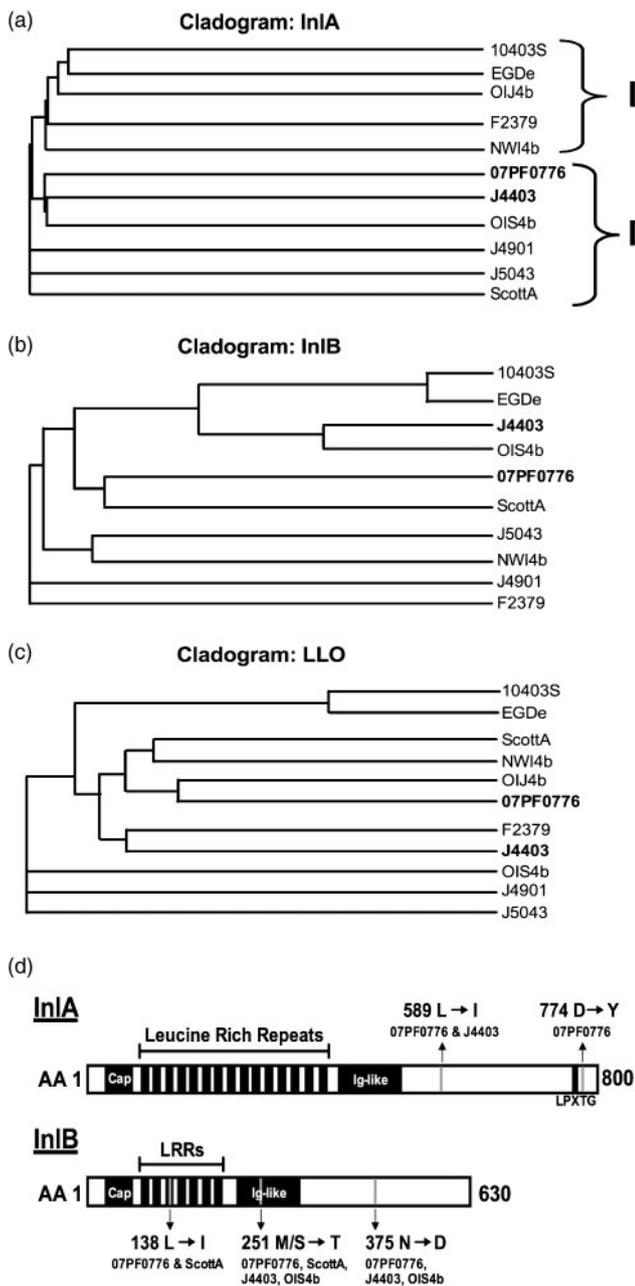
**Fig. 5.** *L. monocytogenes* mediates phagosome escape and entry into the cytosol of infected cardiomyoblasts. (a–e) *L. monocytogenes* 10403S, J4403, ScottA, 07PF0776 and F2379 were used to infect H9C2 cells at an m.o.i. of 100 : 1 for 6.5 h. Cells were then dually stained with 7-nitrobenz-2-oxa-1,3-diazole-phalloidin (actin – green) and a secondary antibody conjugated to rhodamine (for all *Listeria* strains – red). Bacterial association with host cell actin is indicative of cytosolic bacteria.

would not be expected to be limited by whether a strain is capable of direct bacterial invasion at that location. We speculate that some organs such as the heart, which moves

blood rapidly through its chambers, might have limited contact with *L. monocytogenes*-infected cells present within the circulation. Bacteria free in the blood, however, may

**Table 2.** Virulence characteristics of the selected clinical and outbreak isolates used in this study

Strain	Haemolytic activity (HU)	Phospholipase activity	Motility (cm)	Plaque diameter (%)	LLS	Invasivity (H9C2)	Heart colonization
10403S	+ (84.4)	+	+ (16.2)	+ (100)	–	+	–
EGDe	+ (100.0)	+	+/- (9.75)	+ (89%)	–	+	+/-
ScottA	++ (120.0)	–	+ (17.5)	+ (102%)	–	++	++
F2379	+ (70.0)	+	+ (13.0)	+ (90%)	+	+	–
OIS4b	++ (120.0)	+	+/- (10.75)	+ (94%)	+	++	+/-
OIJ4b	+++ (320.0)	+++	+ (15.75)	++ (120%)	+	+	+/-
07PF0776	++ (160.0)	+	+ (12.2)	++ (117%)	+	+++	+++
J4403	++ (120.0)	+	+ (14.75)	++ (121%)	–	+++	+++
J4901	++ (140.0)	+	+ (16.25)	– (65%)	+	+++	–
J5043	– (37.5)	+	+ (14.75)	+ (102%)	+	++	+/-
NWI4b	+ (70.0)	–	+ (13.25)	– (70%)	+	+	–



**Fig. 6.** Amino acid sequence alignment and cladogram grouping suggest that amino acid polymorphisms within InIA and InIB may contribute to the cardio-invasive phenotypes of highly invasive strains. Cladograms of the amino acid sequence alignments from (a) InIA, (b) InIB and (c) listeriolysin O (LLO). Alignments and cladograms were constructed using the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). For InIA comparisons, brackets indicate strains with low-level *in vivo* cardiac colonization (I) or mid- to high-level colonization (II). For InIB, strain OIJ4b was omitted due to substantial rearrangement of the internalin sequence resulting in insufficient sequence coverage and misalignment. (d) Schematic illustrating the unique amino acid polymorphisms present in both InIA and InIB that may influence cardiac cell invasive capacity.

more efficiently target the heart if they are capable of direct cardiac cell attachment and invasion. One notable exception to the apparent link between myoblast invasive capacity and cardiac infection was isolate J4901, which exhibited high efficiency invasion of H9C2 cells but was unable to establish cardiac infections in mice. This strain was, however, isolated from a human cardiac infection, indicating that it is capable of targeting cardiac tissue in other circumstances.

Amino acid comparisons of the bacterial invasion proteins InIA and InIB suggest that the cardio-invasive strains form phylogenetically similar subsets, particularly when grouped based upon InIA sequence. A closer comparison of amino acid polymorphisms between strains revealed differences that may individually or in combination influence cardiac cell invasion. As an example, the InIA L589I polymorphism was present in both 07PF0776 and J4403; while this represents a conservative amino acid substitution, it is interesting that this InIA polymorphism has not been previously identified as a natural variant. Additionally, the InIB polymorphisms could potentially influence invasion independently or in concert with one another and/or with InIA to increase cardiac cell invasive capacity. It is worth noting that the most cardio-invasive isolate (07PF0776) contains all five amino acid polymorphisms, while less invasive isolates contain a subset. We are currently investigating whether these amino acid polymorphisms serve to alter the invasive capacity of *L. monocytogenes*; however, it remains possible that other yet to be defined bacterial factors contribute to cardiac disease. An enhanced understanding of bacterial factors that contribute to tissue tropism could reduce cardiac morbidity and mortality through rapid diagnostic and therapeutic intervention.

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