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Inhibition of ROCK activity allows InlF-mediated invasion and increased virulence of *Listeria monocytogenes*

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Summary

Listeria monocytogenes **is an intracellular bacterial pathogen that causes life-threatening disease. The mechanisms used by *L. monocytogenes* to invade non-professional phagocytic cells are not fully understood. In addition to the requirement of bacterial determinants, host cell conditions profoundly influence infection. Here, we have shown that inhibition of the RhoA/ROCK pathway by pharmacological inhibitors or RNA interference results in increased *L. monocytogenes* invasion of murine fibroblasts and hepatocytes. InlF, a member of the internalin multigene family with no known function, was identified as a *L. monocytogenes*-specific factor mediating increased host cell binding and entry. Conversely, activation of RhoA/ROCK activity resulted in decreased *L. monocytogenes* adhesion and invasion. Furthermore, virulence of wild-type bacteria during infection of mice was significantly increased upon inhibition of ROCK activity, whereas colonization and virulence of an *inlF* deletion mutant was not affected, thus supporting a role for InlF as a functional virulence determinant *in vivo* under specific conditions. In addition, inhibition of ROCK activity in human-derived cells enhanced either bacterial adhesion or adhesion and entry in an InlF-independent manner, further suggesting a host species or cell type-specific role for InlF and that additional bacterial determinants are involved in mediating ROCK-regulated invasion of human cells.**

Introduction

Listeria monocytogenes is a food-borne, intracellular bacterial pathogen that causes listeriosis, a disease characterized by gastroenteritis, meningitis, encephalitis and maternofetal infections. The frequent contamination of food by *L. monocytogenes* makes listeriosis a serious public health concern because of the high mortality rate (20–30%) (Lorber, 1997). *L. monocytogenes* has a broad host range and is able to infect humans and animals. During infection, bacteria disseminate from the intestine to the blood, allowing spread to internal organs and eventually the brain. *L. monocytogenes* can infect a variety of tissues and cell types, including phagocytic and non-professional phagocytic cells, such as epithelial, endothelial, fibroblasts, hepatocytes and neurons (Vazquez-Boland *et al.*, 2001). Following entry into host cells, bacteria escape the phagocytic vacuole, replicate within the host cell cytosol and spread via actin-based motility to neighbouring cells.

To date, several bacterial factors have been shown to be involved in infection. Entry of bacteria into non-professional phagocytic host cells is facilitated by several bacterial surface proteins such as InlA and InlB, both members of the internalin multigene family. InlA interacts with E-cadherin on the surface of host cells and has been shown to be required for invasion of intestinal tissue *in vivo* using a transgenic mouse model (Lecuit *et al.*, 2001). Furthermore, InlA was shown to play a key role for crossing the human maternofetal barrier, although it was shown not to be essential for this role in the pregnant guinea pig infection model (Bakardjiev *et al.*, 2004; Lecuit *et al.*, 2004). InlB, important for liver and splenic colonization *in vivo* (Shen *et al.*, 2000; Lecuit *et al.*, 2004), triggers bacterial entry by interacting with the hepatocyte growth factor receptor (Met) and two other cellular components: gC1q-R and proteoglycans (Braun *et al.*, 2000; Shen *et al.*, 2000; Jonquieres *et al.*, 2001). Less is known about the precise function of the remaining 23 identified members of the internalin multigene family. However, deletion mutants of *inlC* or the *inlGHE* gene cluster display reduced virulence in the mouse infection model (Engelbrecht *et al.*, 1996; Raffelsbauer *et al.*, 1998). During tissue culture infections, *inlC* and the *inlGHE* gene cluster were shown to have a supportive role in InlA-mediated invasion (Bergmann *et al.*, 2002). Additional studies have determined that InlH and InlJ play a role in *L. monocytogenes* virulence, as mutant strains are attenuated in colonization of mice (Schubert *et al.*, 2001; Sabet *et al.*, 2005). No roles in infection have been determined

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thus far for the products of the *inlC2*, *inlD* and *inlF* genes as *in vitro* and *in vivo* infection studies with the respective null mutants have not revealed a phenotype (Dramsi *et al*., 1997). In addition to internalins, several other *L. monocytogenes* surface proteins have been shown to be involved in infection. Several adhesins and invasion-mediating factors, including FbpA, the autolysin Ami, ActA, Lap and Vip (Milohanic *et al*., 2001; Dramsi *et al*., 2004; Bierne and Cossart, 2007), have been characterized.

Whereas numerous bacterial determinants that facilitate *L. monocytogenes* infection have been characterized, less is known about the host cell factors that are required for infection. Our laboratory has recently performed a genome-wide RNA interference screen in *Drosophila* cells to identify host factors required for the pathogenesis of *L. monocytogenes* (Agaisse *et al*., 2005). The identified factors spanned a wide range of associated cellular functions. Interestingly, knockdown of several gene products caused an increase in infection by *L. monocytogenes*, including the small GTPase Rho1, the *Drosophila* homologue of mammalian RhoA. Small GTPases of the Rho family act as molecular switches in signal transduction and thereby regulate a variety of cellular processes. The major downstream targets of RhoA are Rho kinases (ROCKs), serine-threonine protein kinases that are involved in many fundamental cellular processes, such as cell adhesion, motility, contractility, gene expression and cytokinesis (Noma *et al*., 2006). ROCKs are important regulators of cell growth, migration and apoptosis via control of actin cytoskeletal assembly (Riento and Ridley, 2003). By affecting tight and adherent junctions through actin cytoskeletal contractions, ROCKs can also regulate macrophage phagocytic activity and endothelial cell permeability (Wojciak-Stothard *et al*., 2001; Wojciak-Stothard and Ridley, 2002). In mammalian cells, two ROCK isoforms have been identified: ROCK-I (also known as ROKβ or p160ROCK) and ROCK-II (also known as ROKα or Rho kinase) (Ishizaki *et al*., 1996; Matsui *et al*., 1996). ROCKs are comprised of an N-terminal catalytic kinase domain, a central coiled-coil domain that includes the Rho-binding domain, and a C-terminal pleckstrin homology domain that is split by a cysteine-rich region (Nakagawa *et al*., 1996). ROCK-I and ROCK-II share 65% homology in overall amino acid sequence and 92% homology in their kinase domains. ROCK-I mRNA is preferentially expressed in lung, liver, spleen, kidney and testes, whereas ROCK-II mRNA is highly expressed in the heart and brain (Nakagawa *et al*., 1996; Di Cunto *et al*., 2000).

Several effectors for ROCKs have been proposed. Myosin light chain (MLC) is one of the major downstream targets of ROCKs and phosphorylation of MLC controls the assembly of actin-myosin structures (Kureishi *et al*., 1997; Yoneda *et al*., 2005; Li *et al*., 2006; Samarin *et al*., 2007). The myosin binding subunit on MLC phosphatase is another important downstream target, resulting in the subsequent phosphorylation of MLC by inhibiting the phosphatase activity (Somlyo and Somlyo, 2000). In addition, ROCKs can also phosphorylate LIM kinase, ERM proteins, adducin and the Na-H exchanger NHE1 (Maekawa *et al*., 1999; Riento and Ridley, 2003). The overall physiological effects of ROCKs are to enhance actin–myosin association through increasing MLC phosphorylation and preventing actin depolymerization. In addition, ROCKs are involved in the regulation of other cellular functions independent of their effects on the actin cytoskeleton, such as inhibition of insulin signaling and regulation of cell size (Farah *et al*., 1998; Sordella *et al*., 2002). Although extensively studied, limited information exists about the specific role of each ROCK isoform in the various cellular processes. While previously assumed to be functionally redundant, recent studies suggest isoform-specific functions (Coleman *et al*., 2001; Sebbagh *et al*., 2001; Riento *et al*., 2003; 2005; Yoneda *et al*., 2005; 2007).

ROCKs have also become an important therapeutic target as increased ROCK activity has been shown to be involved in various diseases, including hypertension, cancer and neurological disorders (Mueller *et al*., 2005; Noma *et al*., 2006). Recently, the ROCK inhibitor Fasudil (HA-1077) has been approved and marketed in Japan for treatment in humans of systemic and pulmonary arterial hypertension, angina pectoris, acute ischemic stroke and cerebral vasospasm (Masumoto *et al*., 2001; 2002; Shimokawa *et al*., 2002; Fukumoto *et al*., 2005; Shibuya *et al*., 2005).

In this study, we investigated the impact of the RhoA/ROCK pathway on *L. monocytogenes* infection of mammalian cells. We have shown that depending on the host cell type, inhibition of RhoA and ROCK activity increases bacterial adhesion or both adhesion and entry into host cells. Conversely, increases in RhoA and ROCK activity results in decreased *L. monocytogenes* adhesion and invasion. Furthermore, we identified InlF as a bacterial factor involved in mediating adhesion and invasion of murine fibroblasts and hepatocytes under ROCK inhibition conditions. Interestingly, inhibition of ROCK activity in human-derived host cells enhanced either bacterial adhesion or adhesion and entry in an InlF-independent manner, suggesting a possible species-specific activity for InlF. Last, we have also demonstrated that inhibition of ROCK activity during *in vivo* infection of mice increased the virulence of *L. monocytogenes* in an InlF-dependent manner, implying potential effects of clinically used ROCK inhibitors, such as Fasudil, on susceptibility to *L. monocytogenes* infection.

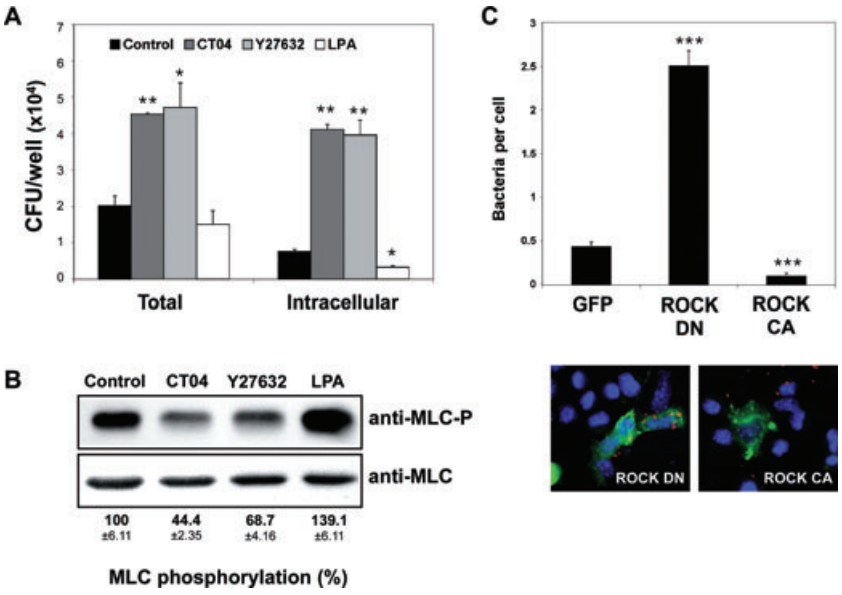


Fig. 1. RhoA and ROCK activity affects *L. monocytogenes* invasion.
A. L2 cells were treated for 5 h with 2 μg ml^{-1} CT04 or for 30 min with 10 μM Y27632, 10 μM LPA or with DMSO (control) prior to infection with wild-type *L. monocytogenes* (10403S). At 1 h post infection, cell-associated (total) bacteria were quantified as described in *Experimental procedures*. For determination of intracellular bacteria, samples were washed and subsequently incubated with medium containing 50 μg ml^{-1} gentamicin for an additional 1 h to kill extracellular bacteria prior to quantifcation of intracellular bacteria. Data represent the mean cfu and standard deviations (SD) of one of three experiments performed in triplicate with similar results. $*P < 0.05$, $**P < 0.01$ compared with control.
B. Western blot analysis of L2 cell extracts treated as in (A) with 2 μg ml^{-1} CT04, 10 μM Y27632, 10 μM LPA or DMSO (control). Phospho-MLC antibody (MLC-P, upper panel) or MLC 2 antibody (MLC, lower panel) was used. Shown is a representative autoradiograph. MLC phosphorylation was quantified by densitometry as described in *Experimental procedures* and normalized against total MLC protein. Numerical values indicate the means ± SD of the percentage of MLC phosphorylation compared with control samples of three independent experiments.
C. L2 cells seeded on glass coverslips were transfected with *myc*-tagged ROCK constructs or the respective control vector expressing GFP. ROCK DN denotes dominant negative ROCK, whereas ROCK CA denotes constitutively active ROCK. Twenty-four hours post transfection, cells were infected with wild-type *L. monocytogenes* for 1 h. Coverslips were washed several times to remove unbound bacteria and then analysed by immunofluorescence microscopy. Transfected cells were identified by detection of Myc-tag expression (green). Cell-associated bacteria (red) were quantified by counting 100 transfected cells for each sample. Cell nuclei are shown in blue. The upper graph indicates the means ± SD of bacteria associated per transfected L2 cell ($n = 3$). $***P < 0.001$ compared with GFP.

Results

RhoA and ROCK activity affect L. monocytogenes *invasion*

Results of a genome-wide RNAi screen in *Drosophila* cells indicated that depletion of the small GTPase Rho1 (homologue of human RhoA) increases *L. monocytogenes* infection efficiency (Agaisse *et al.*, 2005). Using a chemical inhibitor approach, we evaluated the impact of RhoA and its major downstream effector target ROCK on *L. monocytogenes* infection in mammalian host cells. Treatment of host cells with CT04 (Exoenzyme C3 transferase from *Clostridium botulinum*) is commonly used to selectively inactivate RhoA, while Y27632 is a specific inhibitor of ROCK activity. Furthermore, lysophosphatidic acid (LPA) is known to activate RhoA and consequently ROCK (Mills and Moolenaar, 2003; Riento and Ridley, 2003; Moolenaar *et al.*, 2004; Bian *et al.*, 2006). To determine a role for RhoA and ROCK activity in the early stages of *L. monocytogenes* infection, L2 murine fibroblast cell monolayers were incubated with CT04, Y27632 or LPA prior to infection, and bacterial host cell association (adhesion and entry) as well as host cell entry (intracellular) were determined by gentamicin protection assay (Fig. 1A). Inhibition of RhoA as well as ROCK activity led to a twofold increase in cell-associated bacteria and a over fivefold increase in intracellular bacteria compared with untreated L2 cells. In contrast, treatment with the RhoA/ROCK activator LPA reduced the number of cell-associated bacteria by ~1.5-fold and the number of intracellular *L. monocytogenes* by over twofold. Cell culture medium is known to contain LPA, present in supplemented serum at concentrations ranging from 2 to 10 μM, which can function to stimulate RhoA activity (Moolenaar, 1995). Interestingly, incubation of L2 cells in serum-free medium prior to and during infection increased adhesion and entry of *L. monocytogenes* to a similar level as CT04 or Y27632 treatment (data not shown). To confirm the effect of drug treatment on RhoA/ROCK pathway activity, the phosphorylation level of MLC, a major downstream

target of ROCK, was examined. Western blot analysis of L2 cell lysates showed, respectively, a decrease of 55.6% and 31.3% in MLC phosphorylation upon CT04 and Y27632 treatment, whereas LPA treatment increased phosphorylation of MLC by 39.1% (Fig. 1B).

Although Y27632 is selective for ROCK, when applied at higher concentrations, Y27632 has been shown to affect other kinases, such as protein kinase C-related kinase 2, citron kinase, MSK1 and protein kinase N (Ishizaki *et al*., 2000). To verify the association of the observed effects on *L. monocytogenes* infection with inhibition of ROCK activity, we analysed the effect of two additional ROCK inhibitors, HA-1077 (Fasudil) and HA-1100 (Shimokawa *et al*., 1999; Davies *et al*., 2000; Sward *et al*., 2000). Treatment with all three inhibitors resulted in a similar phenotype (Fig. S1). The number of cell-associated (total) as well as intracellular bacteria increased in a dose-dependent manner upon inhibitor treatment. To further confirm the impact of ROCK activity on *L. monocytogenes* infection, L2 cells were transfected with dominant negative (ROCK DN) or constitutive active ROCK (ROCK CA) and then infected with *L. monocytogenes* (Fig. 1C). ROCK DN expressing cells contained 4.6-fold more bacteria associated per L2 cell, whereas ROCK CA expression decreased the number of associated bacteria per L2 cell by 5.2-fold compared with transfected control cells (GFP-expressing vector). Taken together, these data indicated that infection of murine L2 fibroblasts by *L. monocytogenes* is regulated by RhoA and ROCK activity. Inhibition of RhoA or ROCK activity increases bacterial host cell association and entry. Conversely, activation of RhoA and ROCK results in decreased *L. monocytogenes* host cell association and entry.

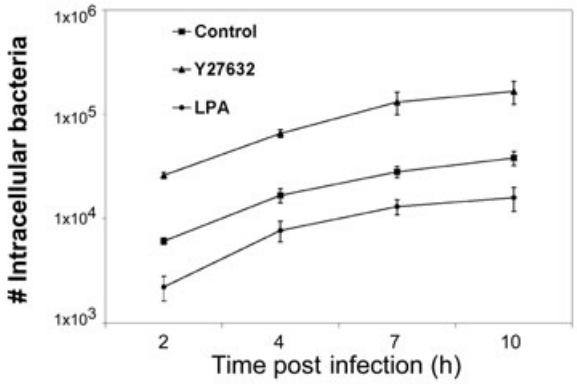
ROCK activity does not affect intracellular growth or cell-to-cell spread of L. monocytogenes

Following entry into host cells, *L. monocytogenes* replicates within the cytosol and spreads cell to cell via actin-based motility. To determine whether ROCK activity affects intracellular replication, L2 cells treated with Y27632 or LPA were infected with bacteria and intracellular growth was examined (Fig. 2A). To ensure that Y27632 and LPA are efficiently active over the course of the infection, drug-containing medium was replaced at 3-h intervals. Compared with the untreated control, the initial number of intracellular bacteria at 2 h post infection was increased by Y27632 treatment and decreased upon LPA treatment, which is consistent with the RhoA/ROCK-regulated effect on bacterial entry observed in Fig. 1A. However, the rates of intracellular growth over the 10-h infection period were similar under all conditions examined (Fig. 2A). In addition, cell-to-cell spread was assayed by analysing plaque formation in L2 cell monolayers treated with Y27632 or LPA. Although the number of plaques per monolayer was increased over sevenfold following Y27632 treatment, and decreased over threefold following LPA treatment, plaque sizes were not significantly altered compared with control samples (Fig. 2B, upper panel). Because Y27632 and LPA activity may be decreased over the infection period for the plaquing assay and the experimental design does not allow for replacement of drug-containing medium, spreading efficiency of bacteria within drug-treated L2 cells was further analysed by determining the size of foci of infection. As shown in Fig. 2B (lower panel) and Fig. S2, the foci size at 12 h and 24 h post infection was not significantly altered by Y27632 or LPA treatment compared with control samples. These data indicated that neither intracellular replication nor cell-to-cell spread of *L. monocytogenes* was affected by alterations in RhoA/ROCK activity.

Depletion of ROCK-I or ROCK-II isoforms increases L. monocytogenes *invasion*

There are two isoforms of ROCK, ROCK-I and ROCK-II, which are encoded by separate genes. However, ROCK inhibitors, such as Y27632, HA-1100 and HA-1077, target both isoforms equally. To determine whether one specific isoform of ROCK is involved in regulating *L. monocytogenes* infection, expression of ROCK-I and ROCK-II was individually downregulated in L2 cells using RNAi. ROCK-I- or ROCK-II-specific shRNA constructs were used for transfection of L2 cells. An shRNA construct targeting GFP was included as a control. Western blot analysis using antibodies for each ROCK isoform was used to confirm ROCK-specific dowregulation (Fig. 3, bottom panel). Seventy-two hours post transfection, L2 cells were infected with wild-type *L. monocytogenes*, and host cell-associated (total) as well as intracellular bacteria were quantified using gentamicin protection assays. As shown in Fig. 3 (upper graph), downregulation of either ROCK-I or ROCK-II resulted in increased L2 cell-associated and intracellular bacteria. Compared with cells transfected with shRNA targeting GFP, host cell-associated bacteria were increased by twofold while intracellular bacteria were increased 5.7-fold in cells transfected with the shRNA targeting ROCK-I. Downregulation of ROCK-II expression increased the number of cell-associated bacteria by 2.7-fold and intracellular bacteria by 7.2-fold. Furthermore, concurrent downregulation of both ROCK isoforms enhanced total cell-associated bacteria by 3.1-fold and intracellular bacteria by 10.1-fold compared with the control. However, co-transfection of shRNAs targeting ROCK-I and ROCK-II resulted in less efficient downregulation of each isoform (Fig. 3, lower panel). These data demonstrate that decreased expres-

A



B

	Plaque size (%)	Number of plaques
Control	100.00 ±7.07	22.67 ±4.26
Y27632	99.79 ±6.09	179.00 ±14.45
LPA	98.10 ±6.61	7.50 ±1.50

	Foci size (%)	
	12h	24h
Control	100.00 ±13.60	100.00 ±14.13
Y27632	98.25 ±14.55	101.50 ±12.87
LPA	102.80 ±11.62	99.83 ±11.30

Fig. 2. ROCK activity does not affect intracellular growth or cell-to-cell spread of *L. monocytogenes*.
A. Intracellular growth of *L. monocytogenes* in L2 cells. L2 cell monolayers seeded onto glass coverslips were treated with 10 μM Y27632, 10 μM LPA or DMSO (control) and infected with wild-type *L. monocytogenes* (10403S). At 1 h post infection, coverslips were washed and medium containing 10 μM Y27632, 10 μM LPA, or DMSO and gentamicin was added and replaced at 4 and 7 h post infection. At the indicated times post infection, coverslips were removed and the number of intracellular bacteria determined as described in *Experimental procedures*. Data shown represents the means ± SD of one of three independent experiments performed in triplicate with similar results.
B. Cell-to-cell spread in L2 fibroblasts. For plaque formation assays, L2 cell monolayers seeded in six-well dishes were treated with 10 μM Y27632, 10 μM LPA or DMSO (control). Wild-type *L. monocytogenes* (10403S) were added to monolayers for 1 h. The infected monolayers were washed with PBS, and a medium-agarose overlay containing 10 μM Y27632, 10 μM LPA, or DMSO and gentamicin was added to kill extracellular bacteria. Intracellular growth and cell-to-cell spread of bacteria were visualized after 72 h by the formation of clearing zones (plaques) within the L2 monolayers. The number of plaques/well was determined and the diameters of 10 plaques/sample were measured. Data represent the mean percent diameter of plaques ± SD compared with the control and the mean number of plaques formed/well ±SD. Data are from three independent experiments performed in duplicate. For microscopy analysis of foci of infection, L2 cell monolayers seeded onto glass coverslips were treated with 10 μM Y27632, 10 μM LPA or DMSO (control) and infected with GFP-expressing wild-type *L. monocytogenes* (10403S). At 1 h post infection, coverslips were washed and medium containing 10 μM Y27632, 10 μM LPA, or DMSO and gentamicin was added and replaced at 4, 8 and 12 h post infection. At 12 h and 24 h post infection, coverslips were fixed, nuclei stained and analysed by fluorescence microscopy. The diameters of 15 foci/sample were measured. Data represent the mean percent diameter of foci ±SD compared with the control. Data are from three independent experiments.

sion of both ROCK isoforms resulted in similar increases in *L. monocytogenes* invasion, suggesting that a common downstream mechanism is responsible.

Impact of myosin II activity on L. monocytogenes *infection*

ROCKs are important regulators of diverse cellular functions, including cell contraction, actin cytoskeleton organization, cell adhesion and motility. A principle substrate of ROCKs is MLC, the regulatory subunit of myosin II. Phosphorylation of MLC results in stimulation of actin–myosin interactions. We used the myosin II-specific inhibitor blebbistatin (Straight *et al.*, 2003) to elucidate a role of actin–myosin interactions for the observed increase in *L. monocytogenes* host cell association and entry. Pretreatment of L2 cells with blebbistatin increased bacterial host cell association and entry to similar levels observed when L2 cells were treated with Y27632 (Fig. 4). Simultaneous incubation of L2 cells with both Y27632 and blebbistatin did not result in an additive effect, suggesting that the same myosin II-dependent pathway is involved in mediating the increase in cell-associated and intracellular bacteria observed with each inhibitor. These data suggest that actin–myosin structures are involved in regulating *L. monocytogenes* interaction with host cells, and that disruption of actin–myosin interactions leads to increased host cell association and entry.

The ROCK-dependent effects on L. monocytogenes *infection are host cell type-specific*

To determine whether the impact of ROCK activity on *L. monocytogenes* invasion is restricted to murine fibroblasts, various murine and human cell lines, including both professional and non-professional phagocytic cells, were treated with the ROCK inhibitor Y27632 and the effect on *L. monocytogenes* infection assessed. Y27632-induced changes in cell-associated and intracellular bacteria were quantified by gentamicin protection assay (Table 1). For murine fibroblast cell lines (L2, L929 and NIH 3T3), as well as for the murine hepatocyte cell line TIB 75 and the human fibroblast cell line WI38, Y27632 treatment increased host cell association between 1.88- and 2.61-fold and host cell entry between 3.45- and 5.44-fold. Interestingly, Y27632 treatment of HeLa and Hep2 human epithelial cell lines resulted in increased cell association (~2.5-fold), but did not significantly affect host cell

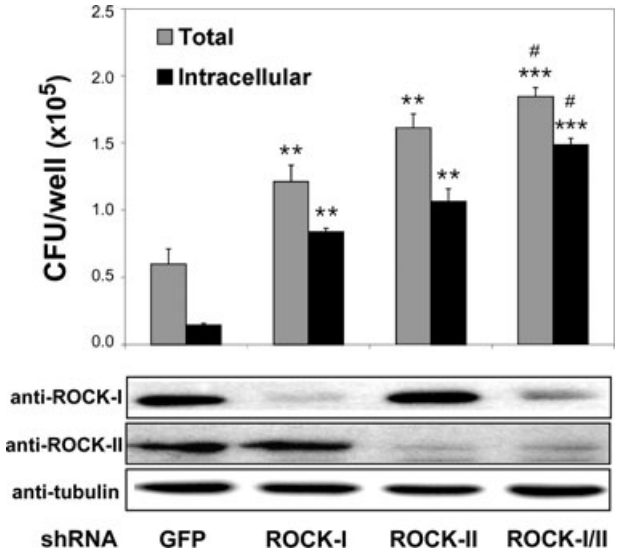


Fig. 3. Depletion of either ROCK isoform increases *L. monocytogenes* invasion. Expression of ROCK-I and ROCK-II was downregulated in L2 cells by RNAi using shRNA constructs targeting GFP, ROCK-I, ROCK-II or both ROCK-I and ROCK-II. Seventy-two hours post transfection, L2 cells were infected with wild-type *L. monocytogenes* (10403S) for 1 h and cell-associated (total) and intracellular bacteria were quantified by gentamicin protection assay. The upper graph indicates the means ±SD cfu per well for one of the three representative experiments performed in triplicate with similar results. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with GFP control. # $P < 0.05$ compared with ROCK-I and ROCK-II samples. Bottom panels: shRNA-transfected L2 cell lysates were analysed by Western blot using antibodies specific for ROCK-I, ROCK-II or tubulin.

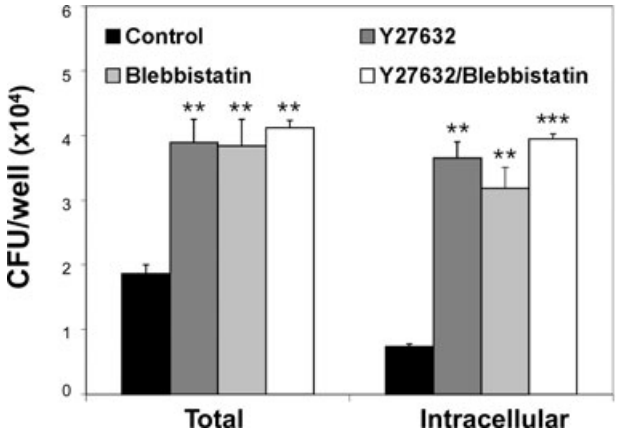


Fig. 4. Inhibition of myosin II activity increases *L. monocytogenes* invasion. L2 cells were treated for 30 min with DMSO (control), 10 μM Y27632, 10 μM blebbistatin or both Y27632 and blebbistatin prior to infection with wild-type *L. monocytogenes* (10403S) for 1 h. Cell-associated (total) and intracellular bacteria were quantified by gentamicin protection assay. Data represents the means ± SD cfu per well for one of the three experiments performed in triplicate with similar results. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control samples.

entry (Table 1). No significant changes in *L. monocytogenes* host cell association or entry were observed for cell lines 1308.1 (murine epithelial), HEK293 (human epithelial), HepG2 (human hepatocyte) or for any of the macrophage cell lines examined (murine RAW 264.7, RAW 309 Cr.1; human U937). In addition, treatment of cell lines with higher concentrations of Y27632 (30 μM) gave similar results (data not shown). These data reveal a cell type-specific effect of ROCK activity inhibition on *L. monocytogenes* infection, resulting in two distinct phenotypes. One phenotype, which was observed for murine fibroblasts and hepatocytes, as well as for human fibroblast cells, resulted in increased bacterial host cell association and entry. The second phenotype, observed with HeLa and Hep2 human epithelial cells, was restricted to increased host cell association (adhesion) with no affect on bacterial entry into host cells. Furthermore, host cell

Table 1. Cell type-specific effects of ROCK activity inhibition on *L. monocytogenes* infection.

Classification	Species	Cell line	Cell type	Total	Intracellular
Non-professional phagocytic	Murine	L2	Fibroblast	**2.61** ± 0.04	**5.44** ± 0.43
		L929	Fibroblast	1.88 ± 0.09*	**3.88** ± 0.17
		NIH 3T3	Fibroblast	**2.35** ± 0.08	**3.45** ± 0.52
		1308.1	Epithelial	0.97 ± 0.08	1.11 ± 0.21
		TIB 75	Hepatocyte	**2.40** ± 0.06	**4.93** ± 0.10
	Human	WI38	Fibroblast	**2.16** ± 0.06	**4.88** ± 1.17
		HeLa	Epithelial	**2.54** ± 0.26	1.26 ± 0.04
		Hep2	Epithelial	**2.48** ± 0.13	1.02 ± 0.03
		HEK-293	Epithelial	1.04 ± 0.05	0.65 ± 0.17
		HepG2	Hepatocyte	1.12 ± 0.04	0.97 ± 0.01
Phagocytic	Murine	RAW 264.7	Macrophage	0.99 ± 0.05	1.14 ± 0.09
		RAW 309 Cr.1	Macrophage	0.89 ± 0.01	0.90 ± 0.08
	Human	U937	Macrophage	0.86 ± 0.05	0.87 ± 0.03

*$P < 0.01$; bold type indicates changes in cfu greater than twofold with $P < 0.05$. Monolayers of cell lines were treated with 10 μM Y27632 or DMSO (control) for 30 min prior to washing of monolayers and infection with wild-type *L. monocytogenes* 10403S. Cell-associated (total) and intracellular bacteria were quantified by gentamicin protection assay as described in *Experimental procedures*. Data presented represent the means ± SD fold change in cfu upon Y27632 treatment compared with control samples for one of the three experiments performed in triplicate with similar results.

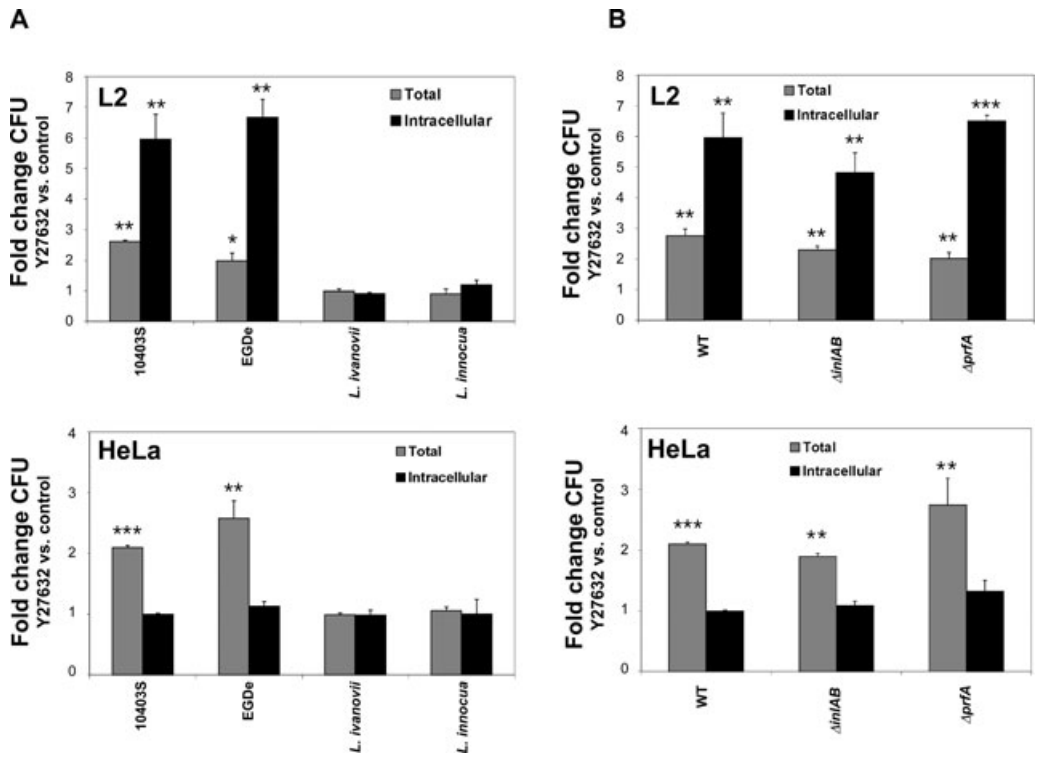


Fig. 5. Increased *L. monocytogenes* infection upon inhibition of ROCK activity is independent of InlA, InlB and PrfA. L2 or HeLa cell monolayers were treated with 10 μM Y27632 or DMSO (control) and subsequently incubated with *Listeria* strains for 1 h. Cell-associated (total) and intracellular bacteria were quantified by gentamicin protection assay. Data represent the means ±SD fold change in cfu upon Y27632 treatment compared with control samples for one of the three experiments performed in triplicate with similar results.
A. *L. monocytogenes* strains 10403S or EGDe, *L. ivanovii* or *L. innocua* were used in infections.
B. *L. monocytogenes* 10403S deletion mutants in *inlAB* or *prfA* were used in infections. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control (control fold change = 1).

association and uptake by the phagocytic cell lines tested were not increased upon Y27632 treatment.

The ROCK-dependent effects on host cell infection may be L. monocytogenes*-specific*

To determine if the increase in host cell association and bacterial uptake following inhibition of ROCK activity was specific to *L. monocytogenes*, various bacterial species, including non-invasive (*Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*) and invasive (*E. coli inv*; *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*) were analysed for alterations in host cell association and entry into Y27632-treated host cells. Infections of L2 and HeLa cell lines, representative of the two distinct *L. monocytogenes* invasion phenotypes observed following Y27632 treatment (Table 1), were performed. As seen in Fig. S3, none of the additional bacterial strains examined were altered for host cell association or entry in Y27632-treated L2 or HeLa cells. Again, *L. monocytogenes* was the only bacterial species examined found to be affected in host cell interactions by inhibition of ROCK activity, suggesting that alterations in interactions with host cells following inhibition of ROCK activity may be *L. monocytogenes*-specific.

To investigate the potential species specificity of the ROCK-dependent effects on bacteria/host cell interactions, various *Listeria* strains were analysed for infection of Y27632-treated L2 and HeLa cells. Bacterial strains examined included another pathogenic *Listeria* species, *L. ivanovii*, a non-pathogenic member of the *Listeria* genus, *L. innocua*, and an additional *L. monocytogenes* strain, EGDe (Fig. 5A). As observed for *L. monocytogenes* 10403S, infection of host cells with EGDe was similarly affected by Y27632 treatment. Host cell association and bacterial uptake were increased in L2 cells, whereas only the number of cell-associated (adhered) bacteria was observed to increase during infection of HeLa cells. *L. ivanovii* and *L. innocua* did not show alternations in cell association or uptake upon Y27632 treatment of host cells. Based upon these data, we speculate that interactions between a *L. monocytogenes*-specific factor and a host cell receptor(s) mediate the ROCK-dependent effects on bacterial adhesion and uptake.

To gain insight into the identity of the putative *L. monocytogenes*-specific invasion determinant, *L. mono-*

cytogenes deletion mutants were analysed in gentamicin protection assays. Two principal *L. monocytogenes* invasion factors are InlA and InlB. To determine whether these determinants were involved in mediating the Y27632-dependent increase in infection, an *inlAB* deletion mutant (10403S Δ*inlAB*) was analysed. Although the Δ*inlAB* strain showed decreased infection of both L2 and HeLa cells (50% of 10403S; data not shown), a similar increase in host cell association (HeLa) or cell association and entry (L2) was observed upon Y27632 treatment (Fig. 5B). Furthermore, the PrfA transcriptional activator positively regulates expression of many *L. monocytogenes* virulence determinants (Dussurget *et al*., 2002; Milohanic *et al*., 2003). To determine whether PrfA regulates expression of the putative invasion factor, a *prfA* deletion mutant (10403S Δ*prfA*) was analysed. The increase in Y27632-dependent host cell association and uptake was not abolished during infection by the Δ*prfA* strain (Fig. 5B). Taken together, these data suggest that the increase in *L. monocytogenes* infection upon inhibition of ROCK activity is not mediated by InlA or InlB, nor is it dependent upon a PrfA-regulated factor.

InlF mediates L. monocytogenes *invasion of Y27632-treated host cells*

We next used a genetic screening approach to identify the *L. monocytogenes* factor(s) mediating the ROCK-dependent increase in infection. *L. innocua* does not show significant invasion of untreated or Y27632-treated L2 cells (Fig. 5A). Therefore, a *L. monocytogenes* genomic expression library was transformed into *L. innocua*, and bacteria were used to infect Y27632-treated L2 cells. *L. innocua* transformants demonstrating at least a threefold increase in invasion of Y27632-treated L2 cells were isolated and further analysed by DNA sequencing as described in *Experimental procedures*. A transforming clone (YInv-4A) harbouring a 2.2-kb *L. monocytogenes* DNA insert was found to encode InlF (Lmo0409), a member of the internalin multigene family with unknown function. Compared with mock-treated host cells, *L. innocua* YInv-4A showed a 2 ± 0.22-fold increase in L2 cell-associated bacteria and a 3.75 ± 0.28-fold increase in bacterial entry into Y27632-treated L2 cells.

Role of InlF in L. monocytogenes *infection*

To further investigate the role of InlF in host cell infection, an *inlF* in-frame deletion mutant in 10403S (Δ*inlF*) was constructed. Infection of L2, TIB 75, WI38 and HeLa cells by Δ*inlF* bacteria was analysed by gentamicin protection assay (Fig. 6). During infection of L2 and TIB 75 cells under mock-treated conditions (control), Δ*inlF* displayed similar host cell association and entry efficiencies as wild-type bacteria (Fig. 6). However, following infection of Y27632-treated cells, Δ*inlF* did not show a significant increase in host cell association or uptake (Fig. 6). Furthermore, during infection of WI38 and HeLa cells, no differences were observed between wild-type and Δ*inlF* bacteria under untreated or Y27632-treated conditions. These data indicated that InlF mediates the Y27632-dependent increase in infection of L2 murine fibroblasts and TIB 75 murine hepatocytes, but that an additional *L. monocytogenes* factor(s) is required to facilitate Y27632-dependent increased adherence and/or entry of human-derived WI38 fibroblasts and HeLa epithelial cells.

Based on phylogenetic and serological studies, *L. monocytogenes* strains have been divided into three evolutionary groups, Lineages I, II and III. Attempts have been made to correlate lineage affiliation with pathogenic potential, including host specificity and virulence. Interestingly, Lineage I strains have been described as lacking *inlF* (Tsai *et al*., 2006; Jia *et al*., 2007). We analysed infection of L2 cells by various *L. monocytogenes* Lineage I strains, FSL J1-194 (Tsai *et al*., 2006; Jia *et al*., 2007), FSL R2-503 (Borucki *et al*., 2004; Ducey *et al*., 2007) and FSL N1-017 (Gray *et al*., 2004) (Fig. 7A). Compared with 10403S (Lineage II), none of the Lineage I strains examined showed a significant increase in L2 cell association or entry upon Y27632 treatment, further implicating a role of InlF for increased infection following inhibition of ROCK activity. Moreover, a Y27632-dependent increase in adherence to HeLa cells was still observed following infection with Lineage I strains (data not shown), further indicating that a *L. monocytogenes* factor(s) besides InlF can mediate Y27632-dependent increased adherence to HeLa cells.

As additional confirmation for InlF mediating increased invasion following inhibition of ROCK activity, we analysed infection of L2 cells with *L. monocytogenes* strains complemented for expression of InlF. *L. monocytogenes* 10403S-derived Δ*inlF*, Lineage I strain FSL J1-194 and *L. innocua* harbouring a plasmid encoding InlF demonstrated an increase in host cell association and entry upon Y27632 treatment (Fig. 7B). Infection of L2 cells with *Listeria* strains lacking InlF and harbouring the cloning vector alone was not affected by Y27632 treatment. Taken together, the data shown in Figs 6 and 7 indicate a role of InlF for increased infection of L2 cells following inhibition of ROCK activity.

Inhibition of ROCK activity in vivo *increases* L. monocytogenes *virulence and is dependent on InlF*

Infection of BALB/c mice was used to determine the impact of inhibiting ROCK activity and a role of InlF for *L. monocytogenes* virulence during *in vivo* infection. As HA-1077 (Fasudil) is an approved drug for clinical use,

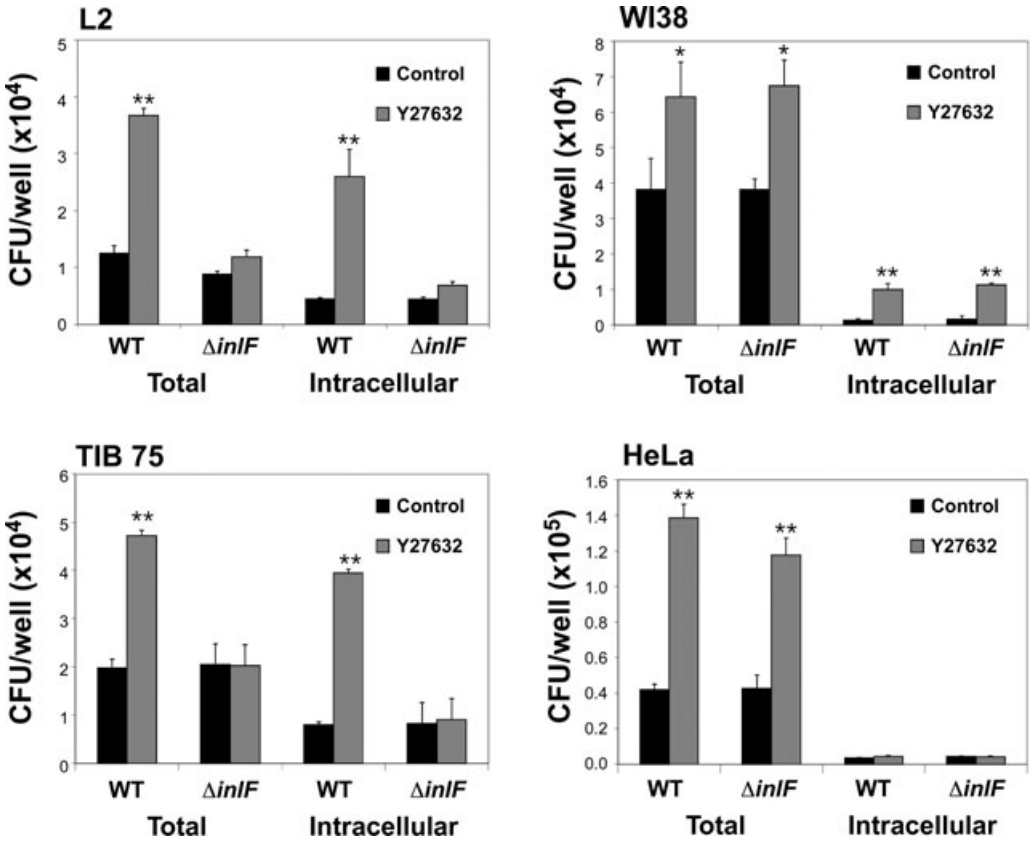


Fig. 6. Increased infection of L2 and TIB 75 cells following inhibition of ROCK activity is InlF-dependent. L2, TIB 75, WI38 or HeLa cells were treated with DMSO (control), or 10 μM Y27632 prior to infection with wild-type (WT) *L. monocytogenes* 10403S or an isogenic *inlF* in-frame deletion mutant (Δ*inlF*) for 1 h. Cell-associated (total) and intracellular bacteria were quantified by gentamicin protection assay. Data represents the means ±SD cfu per well for one of the three experiments performed in triplicate with similar results. *$P < 0.05$, **$P < 0.01$ compared with control samples.

HA-1077 was used for ROCK activity inhibition studies in mice. Animals were treated with HA-1077 followed by intravenous injection of wild-type or Δ*inlF* bacteria. The number of bacteria present in the liver and spleen was determined 72 h post infection (Fig. 8). The wild-type bacterial burden in organs was significantly increased when mice were treated with HA-1077 prior to infection, yielding a 30-fold increase in bacterial numbers in both the livers and spleens of infected mice. In addition, a fourfold increase in colonization of liver and spleen was observed in HA-1077-treated mice at 24 h post infection (data not shown), suggesting that early events in infection were involved in mediating the increase in bacterial burden. In the absence of inhibitor treatment, Δ*inlF* colonized organs similar to wild-type bacteria. In contrast to infection with wild type, the increase in bacterial burden observed in liver or spleen following treatment with HA-1077 was completely abolished during infection with Δ*inlF* bacteria. These results suggest that inhibition of ROCK activity in mice can increase *L. monocytogenes* infection *in vivo* and is dependent on expression of InlF.

Discussion

We recently determined that RNAi knockdown of the small GTPase Rho1 in *Drosophila* cells resulted in increased infection by *L. monocytogenes* (Agaisse *et al.*, 2005). In this report, we investigated the impact of the homologous RhoA/ROCK pathway for *L. monocytogenes* infection of mammalian host cells. Inhibition of RhoA/ROCK activity increased host cell association and entry into murine fibroblast and hepatocyte cell lines. Conversely, activation of RhoA/ROCK activity resulted in decreased *L. monocytogenes* invasion. Interestingly, downregulation of expression of either ROCK isoform resulted in increased *L. monocytogenes* infection (Fig. 3). The mechanism by which inhibition of ROCK activity leads to increased infection is currently unknown. However, ROCKs affect a wide range of cellular functions and recent studies report isoform-specific activities (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001; Riento *et al.*, 2003; Yoneda *et al.*, 2005; 2007) and distinct isoform distribution within cells (Riento *et al.*, 2003; Yoneda *et al.*, 2005). Therefore, it is unclear

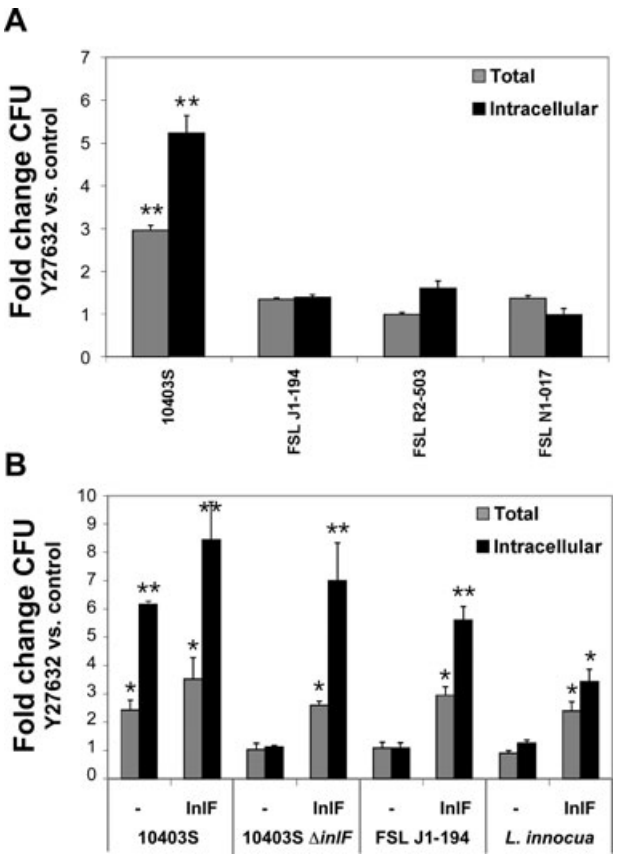


Fig. 7. Expression of InlF in *Listeria* mediates increased infection of Y27632-treated L2 cells.
A. L2 cells were treated with 10 μM Y27632 or DMSO (control) and infected with *L. monocytogenes* (10403S) or Lineage I strains FSL J1-194, FSL R2-503 or FSL N1-017 for 1 h. Cell-associated (total) and intracellular bacteria were quantified by gentamicin protection assay.
B. *L. monocytogenes* 10403S, Δ*inlF*, FSL J1-194 or *L. innocua* were transformed with plasmid pAM-*inlF* (InlF) or pAM401spacOid-BamHI (–) and analysed for infection of L2 cells treated with 10 μM Y27632 or DMSO (control) as in A. Data represent the means ± SD fold change in cfu upon Y27632 treatment compared with control samples for one of the three experiments performed in triplicate with similar results. $*P < 0.05$, $**P < 0.01$ compared with the control (control fold change = 1).

whether a common pathway or distinct isoform-specific functions are involved in the regulation of *L. monocytogenes* infection.

Studies with the myosin II-specific inhibitor blebbistatin strongly suggest that the assembly stage of myosin–actin structures has an impact on *L. monocytogenes* infection. Similar to inhibition of ROCK activity, disassembly of myosin–actin filaments resulted in increased host cell association and bacterial uptake (Fig. 4). Myosin–actin assembly is regulated by phosphorylation of MLC, a major substrate for ROCKs (Kureishi *et al.*, 1997). Both ROCK-I and ROCK-II isoforms were shown to phosphorylate MLC *in vitro* and *in vivo*. Furthermore, siRNA-mediated ROCK depletion resulted in a substantial reduction of MLC phosphorylation (Yoneda *et al.*, 2005; Li *et al.*, 2006; Samarin *et al.*, 2007). Therefore, it is possible that a common function of ROCK-I and ROCK-II, such as regulation of myosin–actin assembly, accounts for the increase in *L. monocytogenes* infection. The mechanism by which myosin–actin structures may contribute to the regulation of *L. monocytogenes* infection is being further investigated.

Several bacterial species were examined for ROCK-dependent interactions with host cells, including invasive and non-invasive bacteria. Within the spectrum of bacteria tested in this study, an increase in host cell association or entry following inhibition of ROCK activity was only observed with *L. monocytogenes* (Fig. S3). In addition, interaction of *L. monocytogenes* with phagocytic host cells was not affected by inhibition of ROCK activity (Table 1). Therefore, our studies suggest a specific receptor–ligand interaction leading to invasion of host cells. Given that increased infection was also observed following blebbistatin treatment, we hypothesize that alterations in myosin–actin structures may lead to surface exposure of a putative host cell receptor as has been described for integrins (Carragher *et al.*, 2006). Another possibility is that receptor conformation and/or membrane localization is affected by alteration in myosin–actin structures (Wojciak-Stothard *et al.*, 1999; Rodriguez-Fernandez *et al.*, 2001; Shewan *et al.*, 2005; Charrasse *et al.*, 2006; Lawler *et al.*, 2006). As a consequence, a putative receptor could be made more accessible resulting in bacterial adhesion and uptake. An impact of RhoA and ROCK activity in infection has been shown for some pathogenic organisms. Binding of the fungal pathogen *Cryptococcus neoformans* to endothelial cells was increased upon Y27632 treatment (Chen *et al.*, 2003) and expression of a dominant negative RhoA-enhanced host cell invasion by *Vibrio parahaemolyticus* (Akeda *et al.*, 2002). Nonetheless, the mechanisms governing these interactions are not known. In addition, vaccinia virus has been shown to directly inhibit RhoA/ROCK signalling to facilitate virus morphogenesis and motility (Valderrama *et al.*, 2006). Our ongoing studies are aimed at determining if *L. monocytogenes* can actively interfere with RhoA or ROCK activity during infection.

We have also identified InlF as a *L. monocytogenes*-specific factor that mediates increased invasion of murine-derived cell lines, such as fibroblasts and hepatocytes, following inhibition of ROCK activity (Fig. 6). Although a role for fibroblasts in naturally occurring *L. monocytogenes* infection has not been described thus far, hepatocytes represent a cell type crucial for host colonization *in vivo* (Vazquez-Boland *et al.*, 2001). InlF is one of the 25 members of the internalin multigene family present in *Listeria* species (Hamon *et al.*, 2006). However, a role for only a few internalin family members has been

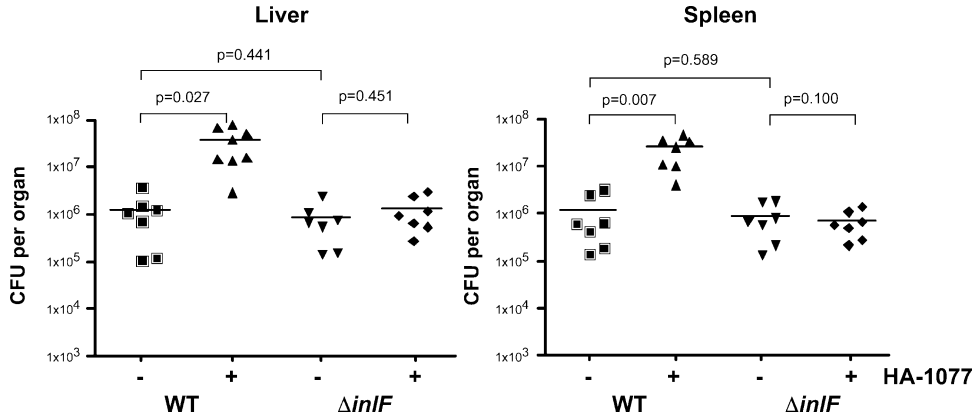


Fig. 8. Inhibition of ROCK activity increases virulence in mice and is dependent on expression of InlF. Groups of seven BALB/c mice were injected intravenously with 10 mg kg^{-1} HA-1077 (+) or PBS (–), and 5 h later infected via intravenous injection with wild-type 10403S (WT) or Δ*inlF* bacteria (1×10^4 bacteria/animal). At 72 h post infection, the bacterial burden in the liver and spleen was determined. Each symbol represents the cfu per organ of one mouse. Horizontal bar indicates the mean value per group. p represents the statistical significance between bracketed groups.

described during infection. InlA has been shown to promote invasion of human non-phagocytic cells expressing E-cadherin (Gaillard *et al*., 1991; Mengaud *et al*., 1996), while InlB mediates entry into several host cell types by interacting with multiple cellular receptors, including hepatocyte growth factor receptor (Met) and gC1q-R (Braun *et al*., 2000; Shen *et al*., 2000). Furthermore, interactions between host cell receptors and InlA or InlB are species-specific (Lecuit *et al*., 1999; Khelef *et al*., 2006). Transcription of *inlF* has been shown to be PrfA- and σ^B-independent and upregulated by high salt (McGann *et al*., 2007). While *inlF* is present in *L. monocytogenes*, the gene is absent in *L. innocua* and *L. ivanovii* strains. This is consistent with our results indicating that increased infection following inhibition of ROCK activity was not observed with *L. innocua* and *L. ivanovii* or abolished upon deletion of *prfA* in *L. monocytogenes* (Fig. 5). Prior studies have shown that deletion of *inlF* did not affect invasion of Caco-2 and HepG2 cells or virulence in mice (Dramsi *et al*., 1997). Nonetheless, we have identified a condition where host cells are susceptible to InlF-mediated infection by *L. monocytogenes* both *in vitro* and *in vivo*. Consistent with previous reports (Dramsi *et al*., 1997), an *inlF* mutant did not show any defect in host cell association or entry under standard cell culture conditions (Fig. 6).

Based on genotypic and epidemiological characteristics, *L. monocytogenes* isolates can be grouped into three evolutionary divisions (Lineages I, II and III). Only Lineages I and II strains have been isolated from humans, although all three lineages are represented in animal isolates. Interestingly, *inlF* is present in all Lineage II isolates, including 10403S and EGDe, but was not found in Lineage I strains (Dramsi *et al*., 1997; Tsai *et al*., 2006). Consistent with a role of InlF in *L. monocytogenes* infection, none of the Lineage I strains examined in this study demonstrated increased infection following inhibition of ROCK activity (Fig. 7A). Previous attempts to correlate lineages with pathogenic potential suggest that in animals, Lineage I strains represent a narrower spectrum for pathogenesis than Lineage II strains (Pohl *et al*., 2006). The absence of *inlF* in Lineage I strains may contribute to this outcome. However, no such correlation was found in humans as epidemiological studies do not indicate a higher or specific pathogenic potential of Lineage II strains in humans (Jeffers *et al*., 2001). The fact that *inlF* is highly conserved within Lineage II strains may support its potential importance and suggests that the presence of *inlF* in Lineage II strains may represent an advantage for infection of hosts other than humans.

In vitro infection of various host cell lines demonstrated that inhibition of ROCK activity resulted in two host cell type-specific phenotypes. For murine fibroblasts and hepatocytes, as well as for human fibroblasts, increased host cell association and entry was observed, whereas for the human epithelial cell lines HeLa and Hep2, an increase in adhesion but not in entry was observed (Table 1). It is possible that a different or altered host cell receptor–bacterial ligand interaction is responsible for increased adhesion, but not entry into epithelial cells. This is consistent with our results indicating that InlF is not involved in mediating increased adhesion to HeLa cells (Fig. 6). In addition, InlF was not involved in increased infection of WI38 human fibroblasts (Fig. 6), suggesting a possible species-specific interaction of InlF with murine cells. Future studies will be aimed at identifying *L. monocytogenes* factors mediating increased infection of human-derived cells upon inhibition of ROCK activity.

In addition to *in vitro* studies, we determined that inhibition of ROCK activity *in vivo* using HA-1077 (Fasudil)

treatment of mice enhanced *L. monocytogenes* infection leading to increased colonization of the liver and spleen (Fig. 8). Importantly, with Δ*inlF* bacteria, the increase in bacterial burden in organs upon infection of HA-1077-treated mice was completely abolished, supporting a functional role of InlF during infection. Although consistent with *in vitro* infection data, we can only speculate on the mechanism supporting an InlF-mediated increase in colonization following inhibition of ROCK activity *in vivo*. In tissue culture infection models, host cell association and entry of *L. monocytogenes* into murine fibroblasts and hepatocytes were affected by ROCK activity dependent on expression of InlF. Whether ROCK activity has the same impact on host cell invasion in the *in vivo* mouse model needs to be investigated. In addition, colonization of both liver and spleen was similarly affected by inhibitor treatment, suggesting a systemic effect. Although less pronounced, an increase in colonization of organs was detectable at 24 h post infection in inhibitor-treated mice. Therefore, the increase in bacterial burden observed at 72 h post infection (Fig. 8) may be due to alterations in initial colonization events. Currently, it is unclear at which stage of infection or which host cell types are involved in mediating enhanced colonization following inhibition of ROCK activity in mice. Our *in vitro* infection studies with murine TIB 75 hepatocytes (Fig. 6) suggest that enhanced bacterial entry of host tissue may account for increased liver colonization in inhibitor-treated mice. Consequently, other organs or host cell types may be similarly affected, as inhibition of ROCK activity appears to systemically affect *L. monocytogenes* infection in mice. Previous studies have shown that inhibition of ROCK activity can attenuate innate immune responses *in vivo*, such as leukocyte infiltration, neutrophil migration and the production of pro-inflammatory cytokines and chemokines (Bao *et al*., 2004; Tasaka *et al*., 2005; Thorlacius *et al*., 2006). However, in this study, a repression of general innate immune responses is unlikely to be the cause of increased colonization in mice as infection with Δ*inlF* bacteria was not altered by drug treatment. Detailed investigation of colonization events during the course of *in vivo* infection is currently being performed to elucidate the mechanism of enhanced *L. monocytogenes* pathogenesis following inhibition of ROCK activity.

In conclusion, we have shown that inactivation of the host cell kinase ROCK leads to increased *L. monocytogenes* infection *in vitro* and *in vivo*. Furthermore, although a role in infection has not been defined for the majority of internalin family members, we have identified InlF as a *L. monocytogenes*-specific factor involved in mediating enhanced infection in a host cell type/species-specific manner following inhibition of ROCK activity. Besides the expression of specific bacterial invasive determinants, the susceptibility of host cells to invasion will determine which tissues *L. monocytogenes* can infect. Inhibition of ROCK activity in cultured murine host cells provides an *in vitro* model where the function of InlF as an invasin becomes apparent. Current studies are aimed at identifying the host receptor(s) for InlF. Taken together, this study represents a valuable approach to reveal the function of a bacterial virulence factor that may be relevant under certain host cell conditions or during infection of specific host species. Several host conditions can influence the success of infection, primarily through alterations in the function of the immune system. Individuals who are immunocompromised are more susceptible to *L. monocytogenes* infection (Vazquez-Boland *et al*., 2001). Nonetheless, there are also adult patients for whom no obvious predisposing condition has been identified. The potential relevance of ROCK activity in *L. monocytogenes* infection is not readily apparent as information regarding *in vivo* ROCK activity is limited. However, animal studies have shown that under certain physiological conditions, ROCK activity can be downregulated, as demonstrated for aorta and myometrium tissues in pregnant rats (Cario-Toumaniantz *et al*., 2003; Katoue *et al*., 2006). More importantly, the ROCK inhibitor Fasudil is an approved drug already therapeutically used in patients with cardiovascular diseases, and the range of applications is likely to expand, as ROCKs have become an attractive drug target for treatment of cancer and neurological disorders (Mueller *et al*., 2005; Noma *et al*., 2006). As adequate epidemiological data are not yet available, whether treatment of humans with ROCK inhibitors enhances susceptibility to *L. monocytogenes* infection is unknown, but may now potentially be considered as a risk factor.

Experimental procedures

Bacteria strains and media

Bacterial strains and plasmids used in this study are listed in Table 2. *L. monocytogenes*, *L. innocua* and *L. ivanovii* strains were grown in Brain Heart Infusion (BHI) medium (Difco, Detroit, MI). *S. enterica* serovar Typhimurium IR715 (ATCC 14028), *P. aeruginosa* PAK1 and *B. subtilis* were grown in Luria–Bertani (LB) broth or on LB agar. *S. flexneri* 2457T, containing the large *Shigella* virulence plasmid, was grown in Tryptic Soy Broth. *E. coli* strains were grown in LB broth at 37°C with shaking. Antibiotics were used at the following concentrations: chloramphenicol at 20 μg ml^{-1} for selection of pAM401 and 100 μg ml^{-1} carbenicillin for pCON1 derivatives in *E. coli*; chloramphenicol 7.5 μg ml^{-1} for selection of pAM401 and pCON1 derivatives in *L. monocytogenes* and *L. innocua*; kanamycin at 30 μg ml^{-1} for maintaining pJP2 in *E. coli*.

Cell culture

The human-derived epithelial cell lines HeLa (ATCC CCL-2) and HEp-2 (ATCC CCL-23), the murine-derived epithelial cell

Table 2. Bacterial strains used in this study.

Strain	Species	Description	Source or reference
10403S	*L. monocytogenes*	Wild-type strain	Bishop and Hinrichs (1987)
DH-L1252	*L. monocytogenes*	10403S expressing GFP	This study
DH-L368	*L. monocytogenes*	10403S Δ*inlAB*	H. Shen
DH-L371	*L. monocytogenes*	10403S Δ*prfA*	H. Shen
DH-L478	*L. monocytogenes*	Wild-type strain EGDe	M. Loessner
DH-L1681	*L. monocytogenes*	10403S with pAM401spacOid-BamHI	This study
DH-L1676	*L. monocytogenes*	10403S with pAM-*inlF*	This study
DH-L1674	*L. monocytogenes*	10403S Δ*inlF*	This study
DH-L1682	*L. monocytogenes*	10403SΔ*inlF* with pAM401spacOid-BamHI	This study
DH-L1677	*L. monocytogenes*	10403SΔ*inlF* with pAM-*inlF*	This study
DH-L1668	*L. monocytogenes*	Lineage I strain FSL J1-194 (DUP-1042B)	Tsai *et al.* (2006)
DH-L1669	*L. monocytogenes*	Lineage I strain FSL R2-503 (G6054)	Ducey *et al.* (2007)
DH-L1671	*L. monocytogenes*	Lineage I strain FSL N1-017	Gray *et al.* (2004)
DH-L1683	*L. monocytogenes*	DH-L1668 with pAM401spacOid-BamHI	This study
DH-L1678	*L. monocytogenes*	DH-L1668 with pAM-*inlF*	This study
DH-L1353	*L. ivanovii*	Wild-type strain FSL C2-010	ATCC C2-010
DH-L657	*L. innocua*	Wild-type *L. innocua*	N. Freitag
DH-L1684	*L. innocua*	DH-L657 with pAM401spacOid-BamHI	This study
DH-L1679	*L. innocua*	DH-L657 with pAM-*inlF*	This study
DH-1664	*Salmonella typhimurium*	Wild-type strain IR715	ATCC 14028
DH-P1665	*Pseudomonas aeruginosa*	Wild-type strain PAK1	Takeya and Amako (1966)
DH-B1666	*Bacillus subtilis*	Wild-type strain PY79	Youngman *et al.* (1983)
DH-1667	*Shigella flexneri*	Wild-type strain 2457T, serotype 2a	Labrec *et al.* (1964)
DH-E112	*E. coli*	JM109(DE3)	Promega
DH-E182	*E. coli*	XL1-Blue	Stratagene
DH-E123	*E. coli*	pCON1 in JM109	Freitag (2000)
DH-E652	*E. coli*	pJP2 (pACYC184 with *Y. pseudotuberculosis* invasin) in XL1-Blue	J. Pratt [Isberg *et al.* (1987)]
DH-E659	*E. coli*	pAM401spacOid in XL1-Blue	Gründling *et al.* (2003)
DH-E969	*E. coli*	pAM401spacOid-BamHI in XL1-Blue	This study
DH-E1673	*E. coli*	pCON1/ Δ*inlF* in XL1-Blue	This study
DH-E1675	*E. coli*	pAM-*inlF* in XL1-Blue	This study

line 1308.1, the murine-derived fibroblast cell lines L2 and L929, the murine-derived macrophage cell lines RAW 264.7 and RAW 309 Cr.1, and the human-derived macrophage cell line U937 (ATCC CRL-1593.2) were cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% FBS (HyClone, Logan, UT), 55 μM 2-β-mercaptoethanol, 1 mM sodium pyruvate and 2 mM glutamine. The human kidney epithelial cell line HEK293, the murine fibroblast cell line NIH 3T3 and the murine hepatocyte cell line TIB 75 (ATCC TIB 75) were propagated in DMEM medium (Mediatech, Herndon, VA) supplemented with 10% FBS, 55 μM 2-β-mercaptoethanol, 1 mM sodium pyruvate and 2 mM glutamine. The human hepatocyte cell line HepG2 was cultured in DMEM medium (Mediatech, Herndon, VA) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine and 1× non-essential amino acids. The human fibroblast cell line WI38 was grown in MEM medium (Mediatech, Herndon, VA) supplemented with 10% FBS, 2 mM glutamine and Earle's BSS adjusted to contain 1.5 g l^{-1} sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. All cell lines were maintained at 37°C in a 5% CO_2-air atmosphere.

DNA constructs and transfection

pCAG-*myc*-based plasmids expressing dominant negative (ROCK-I DN) and constitutive active (ROCK-I CA) ROCKs were a gift from Dr L. Luo (Stanford University, Stanford, CA). The control vector pCAG-GFP (Matsuda and Cepko, 2004) was purchased from Addgene (Plasmid 11150, Addgene, Cambridge, MA). Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Cells were used for experiments 24 h post transfection. pLKO.1-based shRNA constructs targeting GFP, murine ROCK-I and ROCK-II were kindly provided by Dr William Hahn (Dana-Farber Cancer Institute, Boston, MA). The gene-specific hairpin sequences are as follows: GFP: 5′-CGCAAGCT GACCCTGAGTTC-3′; ROCK-I: 5′-GACATTTGAAGTTAGC AGA-3′; ROCK-II: 5′-CTCGTCAACCTTATGAGTA-3′. Transfection of shRNA constructs was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. L2 cells were transfected twice at approximately 24-h intervals with 4 μg of DNA per 1×10^6 cells. Transfected cells were used for experiments 72 h after the first transfection.

Antibodies and reagents

The antibodies used were: rabbit polyclonal anti-phospho-MLC (Ser19) (#3671, Cell Signaling, Danvers, MA); rabbit polyclonal anti-MLC 2 (FL-172), goat polyclonal anti-ROCK-I (C-19), goat polyclonal anti-ROCK-II (C-20) and rabbit polyclonal anti-tubulin (H-235) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-Myc (M4439, Sigma, St. Louis, MO); and rabbit polyclonal anti-*L. monocy-*

togenes (Difco, Detroit, MI). For microscopy, all secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Bio-Rad, Hercules, CA) or anti-goat (Santa Cruz Biotechnology) secondary antibodies were used for Western blot analyses. Hoechst stain was purchased from Sigma. Reagents used were: Rho inhibitor CT04 (Cytoskeleton, Denver, CO); Y27632 (Biomol, Plymouth Meeting, PA); HA-1077 (Fasudil; LC-Laboratories, Woburn, MA); HA-1100 (Hydroxyfasudil; Calbiochem, San Diego, CA); Blebbistatin and Lysophosphatidic acid (LPA, Sigma, St. Louis, MO).

Western blot analyses

Total L2 cell lysates were resolved by 7% (ROCK detection) or 12% (MLC detection) sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with Tris-buffered saline containing 0.1% Tween 20 and 4% bovine serum albumin, membranes were probed with specific antibodies. Proteins were visualized with peroxidase-coupled secondary antibody using the ECL system (Amersham, Piscataway, NJ). Autoradiographs were imaged using the Typhoon 9400 scanner (Amersham). Quantitative analysis was performed using the ImageQuant TL software program (GE Healthcare Bio-Sciences, Piscataway, NJ). Mean values and standard deviation were calculated from three independent experiments. The control value was set as 100% and all other values were calculated with respect to the control.

Microscopy

L2 cell monolayers seeded on glass coverslips were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 50:1. At 1 h post infection, monolayers were washed three times with PBS and fixed with 3.2% paraformaldehyde for 15 min at room temperature. Subsequently, the monolayers were permeabilized with 0.1% Triton X-100 in PBS for 15 min and then blocked with 1% bovine serum albumin in PBS for 30 min at room temperature. For immunodetection, coverslips were co-incubated with rabbit polyclonal antibody specific for *L. monocytogenes* and mouse monoclonal antibody against Myc, washed with PBS, followed by treatment with Rhodamine Red X-conjugated donkey anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG and Hoechst stain. Coverslips were mounted and analysed by fluorescence microscopy. Images were acquired using MetaMorph imaging software (Molecular Devices, Downingtown, PA). For each acquisition, a Z-series was obtained and the collected Z-stack was merged into one plane using the 'Stack Arithmetic: Maximum' command of MetaMorph. Appropriate colours were assigned to each fluorescent image (blue for Hoechst, green for FITC and red for Rhodamine Red X). The colour images were scaled and ultimately overlaid using the 'Overlay' command of MetaMorph.

Gentamicin protection assay

Host cells were grown in 24-well cell culture dishes to 70–80% confluency. On the day of infection, monolayers were pre-incubated with or without inhibitors for the indicated time periods and then incubated with bacteria from 14- to 16-h cultures that had been pelleted, washed in PBS and resuspended in cell culture medium. Infected monolayers were incubated for 1 h or 30 min (macrophage cell lines) in a 5% CO_2-air atmosphere at 37°C. Cultures were washed three times with medium and processed further to determine cell-associated and intracellular colony-forming units (cfu). To quantify total cell-associated bacteria, washed monolayers were immediately lysed with 1% Triton X-100 in PBS. Bacteria were suspended by vigorous pipetting and cfu in lysates were determined by plating dilutions on agar plates. To determine bacterial internalization, extracellular bacteria were selectively killed by incubating washed monolayers for 1 h in culture medium containing gentamicin followed by Triton X-100 lysis and plating for viable intracellular bacteria. For macrophage cell lines, a concentration of 10 μg ml^{-1} gentamicin was used. For all other cell lines, 50 μg ml^{-1} gentamicin was applied. The following MOIs were used for infections with *Listeria* strains: MOI 50:1 for L2, L929, NIH3T3, WI38, 1308.1, HeLa, Hep2 and HepG2; MOI 5:1 for HEK293; MOI 1:1 for RAW 264.7, RAW 309 Cr.1 and U937. L2 and HeLa cell infections with *B. subtilis*, *E. coli*, *E. coli inv*, *S. enterica* serovar Typhimurium, *P. aeruginosa* and *S. flexneri* were performed using a MOI of 50:1.

Intracellular growth assay

A total of 2.0×10^6 host cells were seeded 1 day prior to infection in 60-mm-diameter culture dishes containing 12-mm-diameter round glass coverslips. 10 μM Y27632, 10 μM LPA or DMSO was added to host cells 30 min prior to infection. Wild-type *L. monocytogenes* (10403S) was grown 14–16 h in 3 ml of BHI medium at 30°C without shaking. The bacterial culture was washed once with PBS and used for infection of host cells at a MOI of 50:1. At 1 h post infection, monolayers were washed three times with RPMI medium, and RPMI-10% FBS medium containing DMSO, 10 μM Y27632 or 10 μM LPA and 50 μg ml^{-1} gentamicin was added and replaced at 4 and 7 h post infection. The number of cfu per coverslip was determined at the indicated time points by separately placing coverslips, in triplicate, into 15-ml conical tubes containing 5 ml of sterile water. Following vortexing of conical tubes, dilutions of lysates were plated on LB agar plates.

Plaque assay in L2 cells

Twenty-four hours prior to infection, 2×10^6 L2 cells were seeded in each well of a six-well dish. 10 μM Y27632, 10 μM LPA or DMSO (control) was added to L2 cells 30 min prior to infection. Fourteen- to sixteen-hour *L. monocytogenes* cultures were washed with PBS and 2×10^5 bacteria in culture medium were added to each well. At 1 h post infection, monolayers were washed twice with PBS and a 0.7% agarose medium overlay in DMEM, 5% FBS, 30 μg ml^{-1} gentamicin, and either 10 μM Y27632, 10 μM LPA or DMSO was applied. At 48 h post infection, a second agarose medium overlay was applied that contained 187 μg ml^{-1} neutral red and 30 μg ml^{-1} gentamicin. At 72 h post infection, plates were scanned to

Table 3. Primer sequences used in this study.

Number	Sequence	Restriction site[a]
256	5′-ACGCTCGCGACTAACAGCACAAGAGCGGAAAGATG-3′	NruI
257	5′-CGGGATCCGTCGACCAGATAAAATATTTCTAGAACACCTCC-3′	BamHI
660	5′-GCTCTAGATCGAGCCGGTCAACGGAAAT-3′	XbaI
661	5′-CTCCAAATATAAAACGCGGATGAACGGTCATTATGGTGGTGATTT-3′	–
662	5′-AAATCACCACCATAATGACCGTTCATCCGCGTTTTATATTTGGAG-3′	–
663	5′-GGGGATCCTTGCTACTTTGGATGGTGGTG-3′	BamHI
664	5′-CGCGGATCCAGGAGGAAAAATATGAAATCTAAAAATAATTATTTCAAAC-3′	BamHI
665	5′-AAGTCGACTTATGCTTTTTTTCTCCAAATATAAAA-3′	–

a. The indicated restriction endonuclease site is underlined within each oligonucleotide sequence.

digital images, and the diameters of 10 plaques per well were determined using Adobe Photoshop 6.0 software.

Analysis of foci of infection

L2 cell monolayers seeded onto glass coverslips were treated with 10 μM Y27632, 10 μM LPA or DMSO (control) and infected with strain DH-L1252, a variant of GFP-expressing wild-type 10403S *L. monocytogenes* DH-L1039 (Agaisse *et al*., 2005), at a MOI of 1:1. After 1 h of infection, coverslips were washed three times with PBS and medium containing 10 μM Y27632, 10 μM LPA, or DMSO and 50 μg ml^{-1} gentamicin was added. At 4, 8 and 12 h post infection, the medium was replaced with fresh medium containing 10 μM Y27632, 10 μM LPA, or DMSO and 10 μg ml^{-1} gentamicin. At 12 and 24 h post infection, coverslips were removed, fixed and stained with Hoechst. Samples were analysed by fluorescence microscopy using a 20× objective. The diameters of 15 foci per sample were determined using Adobe Photoshop 6.0 software.

In vivo *virulence studies*

Six- to eight-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were injected intravenously with HA-1077 in PBS (10 mg kg^{-1}; approximately 250 μg/animal) or PBS only (Rikitake *et al*., 2005; Shin *et al*., 2007). After 5 h, mice were infected intravenously with 1×10^4 *L. monocytogenes*. At 24 h or 72 h post infection, mice were humanely euthanized and livers and spleens were sterilely dissected and homogenized in 5 ml of PBS. The number of cfu/organ was determined by plating dilutions of the organ homogenates on LB agar.

Expression library construction and selection

Using primer pair 256 and 257 (Table 3), the SPAC/lacOid promoter/operator region was polymerase chain reaction (PCR)-amplified from plasmid pAMspacOid (Gründling *et al*., 2003), digested with NruI and BamHI and cloned into the multicopy plasmid vector pAM401 (Wirth *et al*., 1986), which had been digested with the same enzymes, resulting in the plasmid pAMspacOid-BamHI (DH-E969). A *L. monocytogenes* 10403S expression library was constructed by digesting chromosomal DNA with Sau3AI. The 1- to 4-kb fragments were ligated into pAMspacOid-BamHI that had been digested with BamHI. After transformation into *E. coli* XL1-Blue, plasmid DNA from approximately 2×10^4 pooled single colonies was isolated and transformed into *L. innocua*. A total of 1.1×10^4 independent colonies were generated by selective growth on BHI agar plates containing 7.5 μg ml^{-1} chloramphenicol. Colonies were pooled and amplified by growth in BHI broth with 7.5 μg ml^{-1} chloramphenicol for 4 h at 37°C. The library was enriched for invasion-mediating clones by infecting Y27632-treated L2 cells at an MOI of 100:1 for 1 h. After an additional hour of infection in the presence of 50 μg ml^{-1} gentamicin, intracellular clones were isolated by lysing infected L2 cells with 1% Triton X-100 in PBS. Clones were amplified by 14- to 16-h growth at 37°C and used for additional infection of L2 cells. This enrichment was repeated twice. After the third round of infection, isolated intracellular clones were plated directly on BHI plates containing 7.5 μg ml^{-1} chloramphenicol. Single colonies were tested individually for invasion (see *Gentamicin protection assay*) and putative clones were analysed by plasmid isolation and sequencing of DNA inserts.

Mutagenesis and cloning of inlF

Primer pair 660 and 661 was used with wild-type 10403S genomic DNA to amplify ~1.0 kB of the region upstream of *inlF* including DNA sequence encoding the first 10 amino acids of InlF. Primers 662 and 663 were used to amplify ~1 kB of DNA sequence downstream of *inlF* and encoding the last 10 amino acids of InlF. The 5′ and 3′ PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and used as templates for a splicing by overlap extension (SOE) PCR reaction (Horton *et al*., 1989). The flanking primers, 660 and 663, were used to amplify a ~2.0-kB PCR product containing an in-frame deletion of sequences encoding amino acids 11–810 of InlF. The SOE PCR product was gel-purified, digested with XbaI and BamHI, ligated to plasmid pCON1 digested with the same restriction enzymes, and transformed into XL1-Blue to create strain DH-E1673. The resulting plasmid, pCON1/Δ*inlF* was introduced into wild-type 10403S by electroporation, and allelic exchange was performed (Camilli *et al*., 1993) to generate strain DH-L1674. To create a complementing InlF construct, the *inlF* gene was cloned into pAMspacOid-BamHI (DH-E969). *inlF* carrying an optimized *ermC* RBS was amplified from 10403S genomic DNA using primers 664 and 665.

The resulting PCR product was gel-purified, digested with BamHI, ligated into pAMspacOid-BamHI digested with BamHI and EcoRV, and transformed into XL1-Blue, creating strain DH-E1675. The resulting plasmid pAM-*inlF* was sequenced and transformed into 10403S, 10403S Δ*inlF*, *L. innocua* and FSL J1-194 by electroporation, creating stains DH-L1676, DH-L1677, DH-L1678 and DH-L1679. As a control, the empty pAMspacOid-BamHI vector was also transformed in the same strains, creating strains DH-L1681, DH-L1682, DH-L1683 and DH-L1684 respectively.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test (two-tailed, unpaired). Differences were considered significant at $P < 0.05$.

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