Published in final edited form as:

Vet Microbiol. 2007 March 10; 120(3-4): 328–333.

Characterization of *Salmonella enterica* serovar Typhimurium DT104 invasion in an epithelial cell line (IPEC J2) from porcine small intestine

David R. Brown* and Lisa D. Price

Department of Veterinary and Biomedical Sciences, University of Minnesota, 1988 Fitch Avenue, St. Paul, Minnesota 55108-6010

Abstract

Salmonella Typhimurium DT104 is an emerging enteric pathogen in swine of increasing medical importance. In this study, the time course and the actin-dependent host signaling processes necessary for invasion of a *S. Typhimurium* DT104 field isolate were investigated in IPEC J2 epithelial cells derived from porcine small intestine. Internalized bacteria were quantified by a gentamicin resistance assay. DT104 internalization into epithelial monolayers increased steadily between 15 – 120 min after apical inoculation. Internalization was reduced by the *Rho* GTPase inhibitor mevastatin, the N-WASP inhibitor wiskostatin and the actin-disrupting agent cytochalasin D, but not the Rac1 GTPase inhibitor NSC-23766. Early DT104 invasion of porcine enterocytes appears to be mediated by Rac1 GTPase-independent changes in epithelial actin assembly.

Keywords

actin; intestinal epithelium; time course; neural Wiskott-Aldrich syndrome protein; *Rho GTPase*

1. Introduction

Non-typhoidal *Salmonella* infections are a leading cause of food-borne illness in the United States, ranging from enteritis to septicemia. The multidrug-resistant *S. Typhimurium* definitive type 104 (DT104) is the most common *Salmonella* phage-type isolated worldwide from both humans and animals, including swine and is of increasing public health concern (Helms et al., 2005). *Salmonellae* possesses several crucial virulence factors that have been mapped to *Salmonella* pathogenicity islands (SPI). Gene products encoded in SPI-1 form components of the type III secretion system that is required for *Salmonella* invasion of epithelial cells, the primary cellular barrier to intestinal infection (Schlumberger and Hardt, 2006). The process of *Salmonella* internalization into enterocytes and disruption of barrier function at mucosal surfaces involves the activation of epithelial *Rho* GTPases by SPI-1 effector proteins and induction of cytoskeletal actin reorganization (Tafazoli et al., 2003; Patel et al., 2005). The small GTPases Rac1 and cell division cycle (Cdc) 42 in particular have been implicated in *Salmonella* invasion of polarized epithelial cells through interactions with host Wiskott-Aldrich syndrome proteins (WASP)/WASP-family verprolin-homologous (WAVE) proteins

*Corresponding author: David R. Brown, Ph.D. Department of Veterinary and Biomedical Sciences, University of Minnesota, 1988 Fitch Avenue, St. Paul, Minnesota 55108-6010, Telephone: (612) 624-0713, FAX: (612) 625-0204, E-mail: brown013@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

which link GTPase activation to actin assembly (Criss et al., 2001; Unsworth et al., 2004; Shi et al., 2005).

In the present investigation, we characterized the internalization of a *S. Typhimurium* DT104 field isolate in monolayer cultures of IPEC J2 epithelial cells, which are derived from the swine small intestine and have been used in previous studies of epithelial interactions with enteric pathogens (McOrist et al., 1995; Sonntag et al., 2005; Schierack et al., 2006; Skjolaas et al., 2006). Specifically, we determined the time course for internalization of *S. Typhimurium* DT104 and its susceptibility to *Rho* GTPase, WASP and actin inhibitors. The results of these studies indicate that *S. Typhimurium* is rapidly internalized into high resistance epithelial cell monolayers through a Rac1-independent process.

2. Materials and methods

2.1. Bacteria

A field isolate of *Salmonella enterica* subsp. *enterica* serovar Typhimurium var. Copenhagen (a strain resistant to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline, i.e. R-type ACSSuT; Minnesota Dept. of Health isolate number #E02-000392) was kindly provided by Dr. Jeffrey Bender (Center for Animal Health and Food Safety, University of Minnesota, St. Paul, MN) and subsequently identified as definitive type 104 (DT104) by the Centers for Disease Control and Prevention (Atlanta, GA). Bacteria were stored in 4% (vol/vol) glycerol/phosphate-buffered saline (PBS) until time of culture and grown overnight in Luria-Bertani (LB) medium at 37°C in a humidified 5% CO₂ atmosphere.

Spectrophotometric evaluations indicated that overnight incubation was sufficient for all bacteria to reach the stationary growth phase. Overnight cultures (300 µl) were inoculated into fresh LB (30 ml) and incubated for 3–4 hours to obtain mid-log phase cultures. Inocula (100 µl) were added to the apical chamber.

2.2. Drugs

[(1S,7R,8S,8aR)-8-[2-[(2R,4R)-4-Hydroxy-6-oxo-oxan-2-yl]ethyl]-7-methyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl] (2S)-2-methylbutanoate (mevastatin; also known as Compactin) and N'-[2-(5-diethylaminopentan-2-ylamino)-6-methyl-pyrimidin-4-yl]-2-methyl-quinoline-4,6-diamine (NSC-23766) were purchased from Tocris Cookston (Ellisville, MO); 1-(3,6-dibromocarbazol-9-yl)-3-dimethylaminopropan-2-ol (wiskostatin) and cytochalasin D were purchased from EMD Biosciences-Calbiochem (San Diego, CA). Mevastatin and cytochalasin D were solubilized in ethanol, wiskostatin was dissolved in dimethylsulfoxide, and NSC-23766 was dissolved in distilled water.

2.3. Culturing of IPEC-J2 epithelial cells

IPEC J2 cells (passages 40–66) were derived from porcine jejunal epithelial cells and were a kind gift from Dr. Bruce Schultz (Dept. of Anatomy and Physiology, Kansas State University, Manhattan, KS). The cells were grown and maintained in 50% Dulbecco's Modified Eagle Medium and 50% Nutrient Mixture F12 (Ham) (1:1 DMEM/F12; Invitrogen Life Technologies, Carlsbad, CA), 5% fetal bovine serum, 5 µg/ml insulin (Sigma), 5 ng/ml epidermal growth factor (Sigma), 0.1% streptomycin and 0.1% penicillin (Invitrogen). IPEC J2 cells were seeded at a density of 5 x 10⁶ per well on Costar Transwell plates (surface area = 4.7 cm²) coated with rat tail collagen (Sigma). The cells were maintained in an atmosphere of 5% CO₂ at 37 °C. The culture medium was changed on alternate days after cells adhered to filters, and replaced with antibiotic-free medium 12 h prior to each experiment. Electrical resistance of monolayers was determined on a daily basis after seeding (approximately 5 – 6 days).

2.4. Determination of *Salmonella* internalization

Bacterial invasion in IPEC-J2 cells was determined in six or more cell monolayers from at least two different passages within 6–7 days after initial seeding after the method of Elsinghorst (1994). Prior to bacterial exposure, monolayers were rinsed three times for 5 min in sterile PBS and subsequently bathed in 1:1 DMEM/F12 prior to bacterial inoculation of the apical medium. The apical surface of the IPEC J2 monolayers was exposed to an inoculum of *S. Typhimurium* for periods ranging from 2 – 120 min at 37 °C under a humidified 5% CO₂ atmosphere. The mean ratio of salmonellae to epithelial cells (i.e. the multiplicity of infection) was approximately 0.1. The effects of drugs on bacterial internalization in IPEC J2 monolayers were measured after exposure of the apical surface to *S. Typhimurium* DT104 for 30 min. Mevastatin or NSC23766 or their respective solvents (in control experiments) were added to the apical medium 20 hr prior to apical addition of salmonellae, whereas cytochalasin D and wiskostatin were added to the apical medium 30 min prior to bacterial addition. Monolayers were rinsed three times for 5 min with sterile PBS and subsequently incubated in PBS containing 100 µg/ml gentamicin for 80 min to eliminate extracellular bacteria. After incubation in gentamicin solution, monolayers were rinsed three times for 5 min in sterile PBS. One milliliter of PBS containing 0.1% Triton-X was added to each Transwell filter, which was then placed on an orbital shaker for 5 min. The resulting cell suspension was vortexed, serially diluted and spread-plated on Difco XLD agar (Becton-Dickinson, Sparks, MD). The total number of bacteria associated with cell monolayers, representing adherent and internalized salmonellae, was determined in monolayers exposed to *Salmonella*, but untreated with gentamicin. Black bacterial colonies were counted using a Quebec darkfield colony counter (Leica, Inc., Buffalo, NY).

2.5. Data analysis

Data are expressed as means ± SE of electrical resistance (in ohms) or the log₁₀ of colony forming units (CFU) recovered per monolayer of cultured IPEC J2 cells. Statistical analyses of data were performed by using the PRISM computer software program (Version 4.0; GraphPad Software, San Diego, CA). Comparisons between a control mean and a single treatment mean were made by a two-tailed, paired *t* test. Comparisons of multiple means were made by analysis of variance (ANOVA). In all cases, the limit for statistical significance was set at *P* < 0.05.

3. Results and Discussion

3.1. Electrical characteristics of porcine IPEC J2 epithelial cells and time course of *S. Typhimurium* DT104 adherence and internalization

As reported previously (Scheirack et al., 2006), the electrical resistance of IPEC J2 cell monolayers increased steadily over the culture period. Upon reaching confluency, the monolayers attained a high transepithelial electrical resistance (Fig. 1). *S. Typhimurium* DT-104 in the mid log growth phase rapidly adhered to and invaded cell monolayers after its inoculation in the apical medium. Bacterial internalization into monolayers increased steadily between 15 – 120 min after inoculation. The total number of salmonellae recovered from cell monolayers increased by approximately 1.1 log units over this time period, whereas counts of intracellular *Salmonella* recovered from gentamicin-treated monolayers increased by 3.5 log units (Fig. 2).

Salmonellae are known to invade the follicle-associated epithelium (i.e. Peyer's patches) in the small intestine that is associated with the induction of mucosal immune responses, although they are capable of invading the non-follicular mucosa as well (Watson et al., 1995; Bolton et al., 1999; Schauser et al., 2004). After *S. Typhimurium* contact with the epithelial cell membrane, the translocation of invasion effector molecules into host cells occurs within

seconds (Schlumberger and Hardt, 2006). Internalization of a *S. Typhimurium* DT104 field isolate into high resistance epithelial cell monolayers was detectable within 2 – 5 min after luminal bacterial exposure, rapidly increased between 15 – 60 min and slowed after 60 min. To our knowledge, these represent the first data on the time course for *Salmonella* invasion in the IPEC J2 cell line. This time-related pattern in bacterial invasion is similar for other strains of *S. Typhimurium* examined in some lines of intestinal and non-intestinal cultured epithelial cells (Francis et al., 1992; Kusters et al., 1993; Mills and Finlay, 1994). Invasion of *S. Typhimurium* into enterocytes is detectable as early as 5–10 min after inoculation into porcine small intestinal loops (Meyerholz and Stabel, 2002; Schauser et al., 2004). In contrast to the number of organisms recovered from gentamicin-treated cells, the total number of salmonellae recovered from cell monolayers that were not treated with the antibiotic (representing both adherent and internalized organisms) remained relatively high and increased to a lesser extent over this time interval.

3.2. Effects of inhibitors of host signaling pathways on *S. Typhimurium* internalization

Internalization of *S. Typhimurium* DT104 was significantly reduced in monolayers pretreated with the *Rho* GTPase prenylation inhibitor mevastatin (Fig. 3). This drug decreases the activity of the cytosolic *Rho* GTPases Rac1 and Cdc42 by inhibiting the post-translational, C-terminal geranylgeranylation of these enzymes that is necessary for their proper intracellular localization and function (Greenwood et al., 2006). The ability of *S. Typhimurium* to disrupt barrier function in monolayer cultures of Manin-Darby canine kidney (MDCK) epithelial cells has previously been shown to be decreased by prenylation inhibitors of *Rho* family GTPases (Tafazoli et al., 2003).

Rac1 has been linked to SopE-induced *S. Typhimurium*-induced actin reassembly and invasion in non-intestinal COS, MDCK and HeLa cell lines (Hardt et al., 1998; Criss et al., 2001; Unsworth et al., 2004). The potential role of Rac-1 in *Salmonella* invasion of porcine enterocytes has not been investigated previously. The number of recoverable, internalized salmonellae remained unaltered in IPEC J2 monolayers pretreated with NSC23766, a specific Rac1 GTPase inhibitor (Fig. 3; Gao et al., 2004). This result suggests that Rac1 is not involved in the process of *S. Typhimurium* internalization in IPEC J2 cells and possibly intestinal epithelial cells in general. Cdc42 may alternatively serve a role in the internalization process. This hypothesis is buttressed by the observation that DT104 internalization in this cell line was significantly reduced by wiskostatin, a specific N-WASP inhibitor which maintains the molecule in an inactive conformation (Fig. 3; Peterson et al., 2004; Leung et al., 2006). N-WASP, which is activated preferentially by Cdc42, is coupled to the host actin-related protein (Arp) 2/3 complex mediating actin nucleation evoked by SopE and other SPI-1 effector molecules (Schlumberger and Hardt, 2006). In contrast, Rac1 activates the Arp 2/3 complex through interactions with WAVE proteins. The WAVE signalling pathway is critical for *S. Typhimurium* invasion in MDCK cells, but there is little or no information on its role in intestinal cell lines (Criss et al., 2001; Shi et al., 2005). The finding that wiskostatin was not as effective as mevastatin in reducing *S. Typhimurium* invasion may be related to its inability to stabilize all cellular N-WASP in an inactive conformation or its degradation by epithelial peptidases. It would be of interest in future studies to examine the effects of other *Rho* GTPase inhibitors, such as Clostridium difficile toxin B, on invasion of salmonellae in IPEC J2 and other intestinal epithelial cell lines. In delineating a specific role for Cdc42 in *Salmonella* internalization, it will be necessary to assess invasion of salmonellae in cells expressing constitutively active or dominant negative mutant forms of Cdc42, and in wild-type cells treated with specific Cdc42 inhibitors when these are developed.

Cytochalasin D decreased *S. Typhimurium* DT104 internalization in IPEC J2 cells (Fig. 3). This result is in general agreement with those obtained in other studies of *Salmonella* entry

into epithelial cells (Patel et al., 2005). However, *Salmonella* internalization into porcine jejunal Peyer's patches appears to be insensitive to cytochalasin D (Green and Brown, 2006; D.R. Brown and L.D. Price, unpublished data). This suggests that *Salmonella* internalization is dependent upon different cellular mechanisms in non-follicular and follicular mucosae of the small intestine.

The results of this study complement and extend those of previous investigations of *Salmonella* interactions with IPEC J2 cell monolayers (Schierack et al., 2006; Skjolaas et al., 2006). In addition to delineating the signal transduction pathway leading to *Salmonella* invasion in porcine intestinal epithelial cells, the results suggest that the intracellular signalling processes underlying *Salmonella* internalization may vary between polarized epithelial cells derived from different tissue sources (e.g. intestine vs. kidney). Additional investigations will be required to determine how the present findings in porcine intestinal cells relate to the intracellular mechanisms underlying the invasion of *S. Typhimurium* DT104 in the intact porcine intestine.

Acknowledgements

This work was supported in part by National Institutes of Health grant R01 DA-10200.

References

- Bolton AJ, Martin GD, Osborne MP, Wallis TS, Stephen J. Invasiveness of *Salmonella* serotypes Typhimurium, Choleraesuis and Dublin for rabbit terminal ileum in vitro. *J Med Microbiol* 1999;48:801–810. [PubMed: 10482290]
- Criss AK, Ahlgren DM, Jou TS, McCormick BA, Casanova JE. The GTPase Rac1 selectively regulates *Salmonella* invasion at the apical plasma membrane of polarized epithelial cells. *J Cell Sci* 2001;114:1331–1341. [PubMed: 11256999]
- Elsinghorst EA. Measurement of invasion by gentamicin resistance. *Methods Enzymol* 1994;236:405–420. [PubMed: 7968625]
- Francis CL, Starnbach MN, Falkow S. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. *Mol Microbiol* 1992;6:3077–3087. [PubMed: 1360615]
- Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc Natl Acad Sci U S A* 2004;101:7618–7623. [PubMed: 15128949]
- Green BT, Brown DR. Differential effects of clathrin and actin inhibitors on internalization of *Escherichia coli* and *Salmonella choleraesuis* in porcine jejunal Peyer's patches. *Vet Microbiol* 2006;113:117–122. [PubMed: 16326046]
- Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nature Rev Immunol* 2006;6:358–370. [PubMed: 16639429]
- Hardt WD, Chen LM, Schuebel KE, Bustelo XR, Galán JE. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* 1998;93:815–826. [PubMed: 9630225]
- Helms M, Ethelberg S, Mølbak K. the DT104 Study Group. International *Salmonella* Typhimurium DT104 infections, 1992–2001. *Emerg Infect Dis* 2005;11:859–867. [PubMed: 15963280]
- Kusters JG, Mulders-Kremers GAWM, van Doornik EM, van der Zeijst BAM. Effects of multiplicity of infection, bacterial protein synthesis, and growth phase on adhesion to and invasion of human cell lines by *Salmonella typhimurium*. *Infect Immun* 1993;61:5013–5020. [PubMed: 8225577]
- Leung DW, Morgan DM, Rosen MK. Biochemical properties and inhibitors of (N-)WASP. *Methods Enzymol* 2006;406:281–296. [PubMed: 16472665]
- McOrist S, Jasni S, Mackie RA, Berschneider HM, Rowland AC, Lawson GH. Entry of the bacterium ileal symbiont intracellularis into cultured enterocytes and its subsequent release. *Res Vet Sci* 1995;59:255–260. [PubMed: 8588102]

- Meyerholz DK, Stabel TJ, Ackermann MR, Carlson SA, Jones BD, Pohlenz J. Early epithelial invasion by *Salmonella enterica* serovar Typhimurium DT104 in the swine ileum. *Vet Pathol* 2002;39:712–720. [PubMed: 12450202]
- Mills SD, Finlay BB. Comparison of *Salmonella typhi* and *Salmonella typhimurium* invasion, intracellular growth and localization in cultured human epithelial cells. *Microb Pathog* 1994;17:409–423. [PubMed: 7752882]
- Patel JC, Rossanese OW, Galan JE. The functional interface between *Salmonella* and its host cell: opportunities for therapeutic intervention. *Trends Pharmacol Sci* 2005;26:564–570. [PubMed: 16182381]
- Peterson JR, Bickford LC, Morgan D, Kim AS, Ouerfelli O, Kirschner MW, Rosen MK. Chemical inhibition of N-WASP by stabilization of a native autoinhibited conformation. *Nature Struct Mol Biol* 2004;11:747–755. [PubMed: 15235593]
- Schauser K, Olsen JE, Larsson LI. Immunocytochemical studies of *Salmonella* Typhimurium invasion of porcine jejunal epithelial cells. *J Med Microbiol* 2004;53:691–695. [PubMed: 15184542]
- Schierack P, Nordhoff M.; Pollmann M, Weyrauch KD.; Amasheh S, Lodemann U.; Jores J, Tachu B.; Kleta S, Blikslager A.; Tedin K, Wieler LH. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. *Histochem Cell Biol* 2006;125:293–305. [PubMed: 16215741]
- Schlumberger MC, Hardt WD. *Salmonella* type III secretion effectors: pulling the host cell's strings. *Curr Opin Microbiol* 2006;9:46–54. [PubMed: 16406778]
- Shi J, Scita G, Casanova JE. WAVE2 signaling mediates invasion of polarized epithelial cells by *Salmonella* typhimurium. *J Biol Chem* 2005;280:29849–29855. [PubMed: 15929989]
- Skjolaas KA, Burkey TE.; Dritz SS, Minton JE. Effects of *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC) on chemokine and cytokine expression in swine ileum and jejunal epithelial cells. *Vet Immunol Immunopathol* 2006;111:199–209. [PubMed: 16473412]
- Sonntag AK, Bielaszewska M, Mellmann A, Dierksen N, Schierack P, Wieler LH, Schmidt MA, Karch H. Shiga toxin 2e-producing *Escherichia coli* isolates from humans and pigs differ in their virulence profiles and interactions with intestinal epithelial cells. *Appl Environ Microbiol* 2005;71:8855–8863. [PubMed: 16332882]
- Tafazoli F, Magnusson KE, Zheng L. Disruption of epithelial barrier integrity by *Salmonella enterica* serovar typhimurium requires geranylgeranylated proteins. *Infect Immun* 2003;71:872–881. [PubMed: 12540569]
- Unsworth KE, Way M, McNiven M, Machesky L, Holden DW. Analysis of the mechanisms of *Salmonella*-induced actin assembly during invasion of host cells and intracellular replication. *Cell Microbiol* 2004;6:1041–1055. [PubMed: 15469433]
- Watson PR, Paulin SM, Bland AP, Jones PW, Wallis TS. Characterization of intestinal invasion by *Salmonella typhimurium* and *Salmonella dublin* and effect of a mutation in the *invH* gene. *Infect Immun* 1995;63:2743–2754. [PubMed: 7790093]

Fig. 1.

Transepithelial resistance of IPEC-J2 cell monolayers over days in culture. Data represent the mean $\pm$ S.E.M. of electrical resistance measured across 4 randomly-chosen Transwell plates at passages 52–56 (filled diamonds) and 62–66 (unfilled diamonds). Resistances determined across six monolayers per plate were averaged each day after initial cell plating. Cells were seeded at a density of 5×10^6 cells and grown on Transwell polyester filters (pore size = $0.4 \mu\text{m}$; surface area = 4.7 cm^2) coated with rat tail collagen. Electrical resistance increased significantly over time ($F = 3.98$, total $df = 39$; $P = 0.011$, two-way ANOVA), but did not differ between passages ($P = 0.918$).

Fig. 2.

Time course for recovery of *S. Typhimurium* DT104 field isolate in IPEC-J2 cells treated (left, filled circles) or (right, unfilled circles) untreated with gentamicin representing internalized and total bacterial recovery respectively. Each circle in the aligned dot plot represents recovery of internalized or total numbers of salmonellae from a single monolayer. Bacteria in mid log growth phase were added to the apical medium at a mean ($\pm$ S.E.) inoculum of 6.74 ± 0.09 $\log_{10}$ CFU/monolayer in 6 – 9 pairs of cell monolayers for each time point. Numbers of both internalized and total bacteria associated with monolayers increased with time of exposure to the apical surface ($P < 0.0001$, one way ANOVA, $n = 9$ groups).

Fig. 3.

Effects of *Rho* GTPase, N-WASP and actin inhibitors on internalization of the *S. Typhimurium* DT104 field isolate into IPEC J2 cell monolayers. Inhibitors were added to the apical medium for 30 min (50 μ M wiskostatin or 10 μ M cytochalasin D) or 20 hr (50 μ M mevastatin or 100 μ M NSC-23766). Bacteria in mid log growth phase were added to the apical medium at a mean ($\pm$ S.E.) inoculum of 6.61 ± 0.12 log₁₀ CFU/monolayer in 6–8 pairs of cell monolayers. Filled shapes in the aligned dot plot represent recovery of internalized salmonellae from individual monolayers pretreated with an inhibitor and exposed to *S. Typhimurium* DT104 for 30 min. Unfilled shapes represent recovery of internalized salmonellae from individual monolayers pretreated with the relevant vehicle used to solubilize the inhibitor which served as paired controls for each inhibitor treatment condition. Wide horizontal bars indicate mean log₁₀ CFU/g and shorter horizontal bars indicate standard error of the mean for each condition. * $P < 0.05$ and ** $P < 0.01$ vs. solvent control, paired t test.