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In Vitro and In Vivo Assessment of *Salmonella enterica* Serovar Typhimurium DT104 Virulence

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Multidrug-resistant *Salmonella enterica* serovar Typhimurium phage type DT104 has become a widespread cause of human and other animal infection worldwide. The severity of clinical illness in *S. enterica* serovar Typhimurium DT104 outbreaks has led to the suggestion that this strain possesses enhanced virulence. In the present study, in vitro and in vivo virulence-associated phenotypes of several clinical isolates of *S. enterica* serovar Typhimurium DT104 were examined and compared to *S. enterica* serovar Typhimurium ATCC 14028s. The ability of these DT104 isolates to survive within murine peritoneal macrophages, invade cultured epithelial cells, resist antimicrobial actions of reactive oxygen and nitrogen compounds, and cause lethal infection in mice were assessed. Our results failed to demonstrate that *S. enterica* serovar Typhimurium DT104 isolates are more virulent than *S. enterica* serovar Typhimurium ATCC 14028s.

Food-borne bacterial infections due to *Salmonella* remain a serious threat to human health in both developing and industrialized countries. Between 1997 and 1998, 37,842 cases of human salmonellosis were reported to the Centers for Disease Control and Prevention. The estimated number of human *Salmonella* infections in the United States exceeds 1.4 million annually (21). In 1995, 24% of all reported *Salmonella* infections in the United States were caused by *Salmonella enterica* serovar Typhimurium, second only to serovar Enteritidis phage group 4. Of the *S. enterica* serovar Typhimurium isolates, 32% were found to be multidrug-resistant *S. enterica* serovar Typhimurium DT104 (17), characterized as resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (1). *S. enterica* serovar Typhimurium DT104 was first isolated in England in 1984 but is now routinely isolated worldwide (22, 24). A British study conducted in 1994 reported a 3% mortality rate due to infection with *S. enterica* serovar Typhimurium DT104 (1), a rate substantially higher than in historical controls. However, in a separate study examining *Salmonella* bacteremia in England and Wales from 1994 to 1996, the percentage of fatal salmonellosis cases due to *S. enterica* serovar Typhimurium DT104 was no greater than those due to nonmultidrug-resistant *S. enterica* serovar Typhimurium (23). Attention became focused on *S. enterica* serovar Typhimurium DT104 in the United States when members of a Vermont family became gravely ill after consuming contaminated unpasteurized milk from their own dairy herd (C. R. Friedman, R. C. Brady, M. J. Celotti, S. E. Schoenfeld, R. H. Johnson, P. D. Galbraith, J. K. Carney, K. Robbins, and L. Slutsker, presented at Int. Conf. Emerg. Infect. Dis., Atlanta, Ga., 8 to 11 March 1998).

Because of the reported severity of disease caused by this organism and the increased frequency of isolation, *S. enterica* serovar Typhimurium DT104 has been proposed to have enhanced virulence in domestic animals and humans (24). Previous work by Carlson et al. (7, 8) indicated that most multidrug-resistant *S. enterica* serovar Typhimurium isolates do not have enhanced ability to invade or adhere to human epithelial tissue culture cells. In this study, we have examined additional in vitro and in vivo phenotypes associated with *Salmonella* virulence.

The ability of *Salmonella* to survive and replicate in host phagocytes is an essential component of *Salmonella* virulence.

TABLE 1. Strains used in this study

Strain	Relevant information	Reference or Source
14028s		ATCC
14028s *phoP*		14
14028s *hilA*		2
14028s *invA*		15
VT4Tx	Vermont DT104 bovine isolate	USDA, Athens, Ga.
3016	Georgia DT104 poultry isolate	USDA, Athens, Ga.
2745	Washington DT104 human isolate	J. Gay, Washington State University
4157	Washington DT104 bovine isolate	J. Gay, Washington State University

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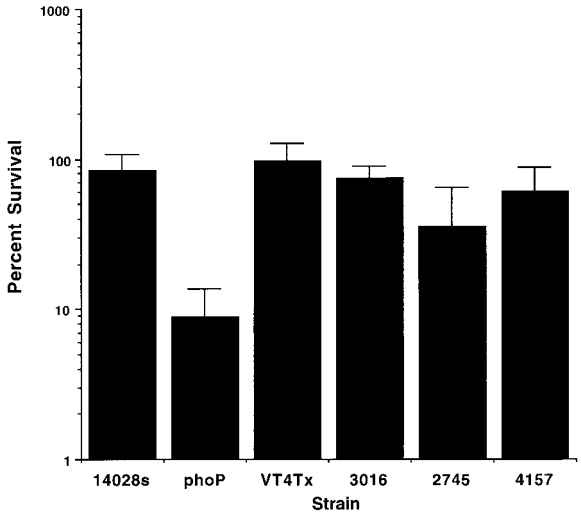


FIG. 1. Macrophage survival. Survival of *Salmonella* strains was determined in BALB/c (*ity*^{s}) peritoneal macrophages. Peritoneal macrophages were harvested from mice 4 days after injection with 5 mM sodium periodate and plated at a density of 3×10^5 to 5×10^5/well. They were infected 24 h later at a multiplicity of infection of 5:1 with opsonized *S. enterica* serovar Typhimurium 14028s, *S. enterica* serovar Typhimurium DT104 isolates, or macrophage-sensitive mutant *phoP S. enterica* serovar Typhimurium. Extracellular bacteria were killed using amikacin (100 μg/ml) since the DT104 isolates are resistant to gentamicin. Results are expressed as percent survival and represent the average of two independent assays. Error bars indicate standard deviation.

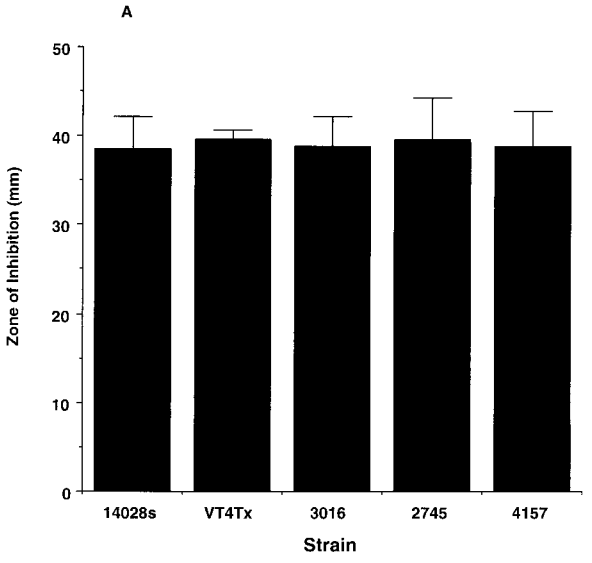
Mutants that are unable to survive in macrophages are attenuated for virulence in vivo (14). We compared several *S. enterica* serovar Typhimurium DT104 isolates from different geographic locales for their ability to survive within murine macrophages. The strain numbers and source of these strains are shown in Table 1. The intracellular survival of *S. enterica* serovar Typhimurium DT104 isolates and control strains in peritoneal macrophages from BALB/c mice was determined as previously described (5). Macrophages were infected with opsonized *S. enterica* serovar Typhimurium, and survival was measured 18 h postinfection. A *phoP S. enterica* serovar Typhimurium mutant was used as a macrophage-sensitive control (13). *S. enterica* serovar Typhimurium DT104 isolates were able to survive and replicate in activated murine macrophages at levels similar to those of *S. enterica* serovar Typhimurium 14028s (Fig. 1).

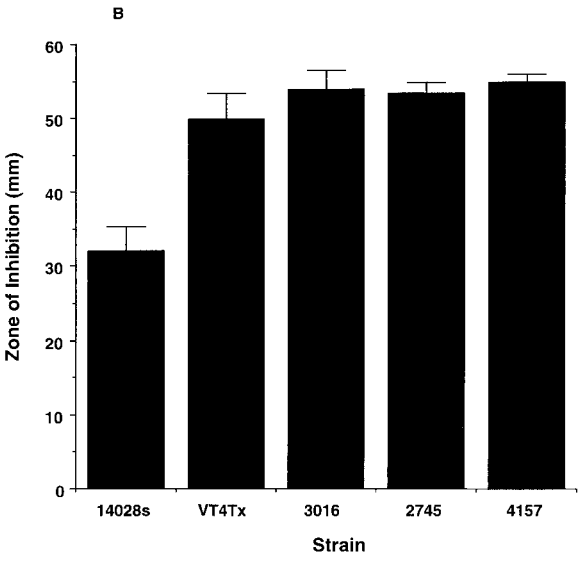
Another important characteristic of *Salmonella* is its ability to resist reactive oxygen and nitrogen species produced by host phagocytes (4, 6, 11, 18). The resistance of *S. enterica* serovar Typhimurium DT104 isolates to reactive oxygen species and reactive nitrogen species was assayed as previously described by DeGroote et al. (10–12) and Lu et al. (20). *S. enterica* serovar Typhimurium DT104 isolates did not demonstrate enhanced susceptibility to hydrogen peroxide compared with *S. enterica* serovar Typhimurium 14028s (Fig. 2A). Similar results were observed for paraquat susceptibility (Fig. 2B). *S. enterica* serovar Typhimurium DT104 isolates showed similar resistance to acidified nitrite after 3 h of incubation (Fig. 2C) (20). Furthermore, all DT104 isolates were able to grow to the same extent in acidified (pH5) Luria-Bertani (LB) broth lacking sodium nitrite (data not shown).

The ability of *Salmonella* to invade the intestinal epithelium is a necessary step for the initial phase of *Salmonella* infection. To determine the relative invasive ability of *S. enterica* serovar Typhimurium DT104 isolates, standard epithelial cell invasion assays were performed with cultured HEp-2 cells, a human epithelial carcinoma cell line. As shown in Fig. 3, *S. enterica* serovar Typhimurium 14028s and DT104 isolates invaded HEp-2 cells to a similar extent, while the noninvasive *S. enterica* serovar Typhimurium mutants, *hilA* and *invA*, showed significantly reduced invasion, as previously described (16, 19). Thus, the invasive properties of the *S. enterica* serovar Typhimurium DT104 isolates tested are not significantly different from those of *S. enterica* serovar Typhimurium 14028s.

The standard in vitro assays described above indicate that *S. enterica* serovar Typhimurium DT104 isolates do not demonstrate an increased ability to invade tissue culture cells, survive within murine macrophages, or withstand reactive oxygen or nitrogen species. However, in vivo virulence cannot always be predicted from in vitro phenotypic assays (22). Therefore, we tested whether DT104 isolates exhibit increased in vivo virulence relative to the well-characterized strain *S. enterica* serovar Typhimurium 14028s in the murine model of *Salmonella* infection, including a competitive infection assay. The virulence of *S. enterica* serovar Typhimurium DT104 isolates in susceptible mice was compared to that of *S. enterica* serovar Typhimurium 14028s. *Salmonella*-susceptible BALB/c mice were infected orally with different inocula of one of four geographically diverse *S. enterica* serovar Typhimurium DT104 isolates or *S. enterica* serovar Typhimurium 14028s. Following oral administration of $\sim 10^8$ CFU, the ability of *S. enterica* serovar Typhimurium VT4Tx (a bovine strain isolated from the Vermont outbreak) to cause lethal infection in mice was essentially identical to that of *S. enterica* serovar Typhimurium 14028s. Similar observations were made following oral administration of clinical DT104 isolates 3016 (a chicken isolate from Georgia) and 4157 (a bovine isolate from Washington State) (Table 1). However, strain 2745, a human clinical DT104 isolate (from Washington State), did not cause lethal infection in BALB/c mice following inoculation of 10^8 CFU. *Salmonella*-resistant C3H/HeN mice that were infected with *S. enterica* serovar Typhimurium DT104 isolates did not succumb to infection or show clinical signs of salmonellosis when the bacterium was given at doses ranging up to 10^8 CFU (Table 2). PCR analysis of this strain using primers for four loci known to be required for *Salmonella* virulence (*spvC, hilA, sodCI*, and *invF*) (9, 11, 15, 19) indicated that strain 2745 carried these genes and suggested that its decreased virulence is not attributable to the absence of these loci. *S. enterica* serovar Typhimurium DT104 isolates had similar in vitro growth characteristics in LB broth, formed smooth colonies on LB agar plates, and grew well on M9 minimal medium supplemented with 0.2% glucose (data not shown).

A competitive-infection assay was performed to further compare the virulence of the *S. enterica* serovar Typhimurium DT104 isolates with that of *S. enterica* serovar Typhimurium 14028s. Groups of four BALB/c mice were orally infected with $\sim 10^8$ CFU containing a 1:1 mixture of *S. enterica* serovar Typhimurium 14028s and each of the four *S. enterica* serovar





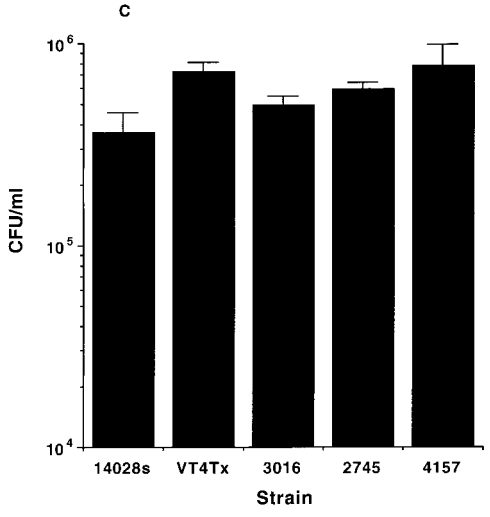


FIG. 2. Susceptibility to reactive oxygen and nitrogen species. (A and B) *Salmonella* cultures were grown overnight in LB medium, diluted in saline, and plated (100 μl containing 10^6 CFU) on M9 minimal plates containing 0.2% glucose. Then 15 μl of 3% hydrogen peroxide (A) or paraquat (1 mM) (B) was spotted onto 6-mm-diameter paper disks, the disks were placed onto the bacterial lawn, and the plates were incubated overnight at 37°C. The zone of inhibition was measured on two axes, averaged, and plotted. (C) Susceptibility to acidified nitrite was determined by adding 20 μl of an overnight culture to 2 ml of acidified LB (pH 5) with or without 20 mM sodium nitrite. Bacteria were incubated at 37°C with aeration for 3 and 6 h, and viable cells were enumerated by plating dilutions onto LB agar.

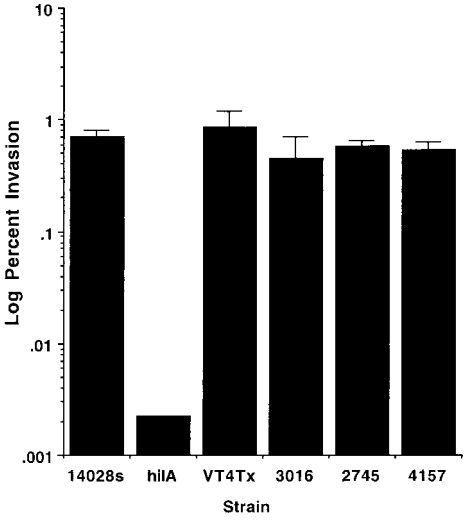


FIG. 3. Invasion of HEp-2 cells by *Salmonella* strains. HEp-2 cells were grown to confluence and infected at a multiplicity of infection of 10 to 50 with *S. enterica* serovar Typhimurium 14028s, *S. enterica* serovar Typhimurium DT104 isolates, and *hilA* and *invA* mutant strains of *S. enterica* serovar Typhimurium grown overnight in LB without aeration at 37°C. Nonadherent bacteria were removed by washing three times with phosphate-buffered saline; then, RPMI plus 10% fetal calf serum containing amikacin at 100 μg/ml was added and the plates were incubated for an additional 1 h. The cells were washed three times with phosphate-buffered saline and lysed with 1% Triton X-100. Bacterial invasion was determined by plating serial dilutions onto LB agar. The results, expressed as percent invasion, are representative of three independent assays. Error bars indicate standard deviation.

Typhimurium DT104 isolates: VT4Tc, 3016, 2745, and 4157. On days 4 and 6 postinfection, mice were euthanized and tissues were collected for bacterial enumeration. The tissues were homogenized in 10 ml of sterile water, and 10-fold serial dilutions were plated on XLD (Difco) and XLD containing chloramphenicol at 20 μg/ml, to distinguish *S. enterica* serovar Typhimurium 14028s (chloramphenicol susceptible) from the multidrug-resistant DT104 isolates (chloramphenicol resistant). The number of CFU of *S. enterica* serovar Typhimurium 14028s per organ was calculated by subtracting the number of colonies on the XLD-chloramphenicol plates from the number of colonies on the corresponding XLD plates. The competitive index (CI) was calculated as the ratio of the CFU of each *S. enterica* serovar Typhimurium DT104 isolate to the CFU of *S. enterica* serovar Typhimurium 14028s recovered from the spleen and liver. None of the four *S. enterica* serovar Typhimurium DT104 isolates tested were able to colonize the spleen or liver of infected mice as well as *S. enterica* serovar Typhi-

TABLE 2. Lethality and competitive infection of *S. enterica* serovar Typhimurium 14028s and DT104 isolates for BALB/c mice

Strain	Dose (CFU)[a]	Mortality (no. dead/ total no.)[a]	Dose (CFU)[b]	Spleen CI[b]	Liver CI[b]
14028s	3.6×10^6	0/3			
	3.6×10^7	1/3			
	3.6×10^8	3/3			
VT4Tx	2.8×10^6	0/3	1.4×10^8	0.3620 ± 0.701[c]	0.3627 ± 0.698[c]
	2.8×10^7	2/3			
	2.8×10^8	3/3			
3016	3.1×10^6	0/3	1.3×10^8	0.0108 ± 0.022	0.0046 ± 0.008
	3.1×10^7	1/3			
	3.1×10^8	3/3			
2745	2.8×10^6	0/3	1.3×10^8	0.0039 ± 0.005	0.0506 ± 0.096
	2.8×10^7	0/3			
	2.8×10^8	0/3			
4157	3.0×10^6	0/3	1.3×10^8	0.0306 ± 0.051	0.0124 ± 0.015
	3.0×10^7	3/3			
	3.0×10^8	2/3			

[a] Groups of three 6-week-old BALB/c female mice (Charles River Laboratories) were infected orally with *S. enterica* serovar Typhimurium ATCC 14028s or DT104 isolates grown overnight at 37°C in LB broth. Prior to oral infection, food and water were withheld from the mice for 4 h. Bacteria were diluted in phosphate-buffered saline and administered to the mice by allowing them to drink 20 μl from the end of a pipette tip. Infected mice were observed daily for up to 3 weeks, and moribund mice were euthanized.

[b] For the competitive infection assay, each *S. enterica* serovar Typhimurium DT104 isolate was mixed 1:1 with *S. enterica* serovar Typhimurium ATCC 14028s for coinfection of four BALB/c female mice per group. The mice were challenged using the same procedure as described in footnote *a*. Results are listed as the median CI for day 6 postinfection ± standard deviation.

[c] Variation due to one mouse having a CI greater than 1.

murium 14028s did during mixed infections, as demonstrated by a CI ratio of less than 1 (Table 2). Nevertheless, three of the four DT104 strains were able to cause lethal infections in mice when administered singly.

In conclusion, we have utilized in vitro and in vivo virulence assays to compare four geographically diverse *S. enterica* serovar Typhimurium DT104 clinical isolates with a well-characterized virulent *S. enterica* serovar Typhimurium strain. *S. enterica* serovar Typhimurium DT104 isolates from Washington, Vermont, and Georgia did not demonstrate enhanced resistance to reactive oxygen or nitrogen species, nor were these isolates able to survive and replicate in activated murine macrophages or invade cultured epithelial cells to a greater extent than *S. enterica* serovar Typhimurium ATCC 14028s. When tested for virulence in susceptible mice, most DT104 isolates showed similar lethality, although one DT104 isolate (2745) was unable to cause lethal infection. In a mixed-infection assay, none of the *S. enterica* serotype Typhimurium DT104 isolates demonstrated an enhanced ability to compete with *S. enterica* serovar Typhimurium ATCC 14028s. The increasing frequency of *S. enterica* serovar Typhimurium DT104 isolation from both humans and domestic animals cannot be attributed to enhanced virulence-associated phenotypes detectable by conventional assays. Of course, the conditions that permit *S. enterica* serovar Typhimurium DT104 to disseminate efficiently under field conditions cannot be completely replicated in the laboratory.

At present it is not clear whether the increased prevalence of *S. enterica* serovar Typhimurium DT104 in many parts of the world is more likely to be a result of its resistance to multiple antimicrobial agents per se (3) or to greater competitive fitness related to other, unknown factors. Nevertheless, the continued widespread use of antimicrobial agents in the production of food animals is likely to provide a potent selection pressure for the emergence and persistence of multidrug-resistant strains such as *S. enterica* serovar Typhimurium DT104.

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REFERENCES

1. **Akkina, J. E., A. T. Hougue, R. J. Angulo, R. Johnson, K. E. Petersen, P. K. Saini, P. J. Fedorka-Cray, and W. E. Schlosser.** 1999. Epidemiologic aspects, control, and importance of multiple-drug resistant *Salmonella typhimurium* DT104 in the United States. J. Am. Vet. Med. Assoc. **214:**790–798.
2. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Coordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
3. **Bjorkman, J., D. Huge, and D. I. Andersson.** 1998. Virulence of antibiotic-resistant *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **95:**3949–3953.
4. **Buchmeier, N. A., S. Bossie, C.-Y. Chen, F. C. Fang, D. G. Guiney, and S. J. Libby.** 1997. SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. Infect. Immun. **65:**3725–3730.
5. **Buchmeier, N. A., and F. Heffron.** 1989. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. Infect. Immun. **57:**1–7.
6. **Buchmeier, N. A., S. J. Libby, Y. Xu, P. Lowewen, J. Switala, D. G. Guiney, and F. C. Fang.** 1995. DNA repair is more important than catalase for *Salmonella* virulence in mice. J. Clin. Investig. **95:**1047–1053.
7. **Carlson, S. A., M. Browning, K. E. Ferris, and B. D. Jones.** 2000. Identification of diminished tissue culture invasiveness among multiple antibiotic resistant *Salmonella typhimurium* DT104. Microb. Pathog. **28:**37–44.
8. **Carlson, S. A., R. M. Willson, A. J. Crane, and K. E. Ferris.** 2000. Evaluation of invasion-conferring genotypes and antibiotic-induced hyperinvasive phenotypes in multiple antibiotic resistant *Salmonella typhimurium* DT104. Microb. Pathog. **28:**373–378.
9. **Chikami, G. K., J. Fierer, and D. G. Guiney.** 1985. Plasmid-mediated virulence in *Salmonella dublin* demonstrated by use of a Tn5-*oriT* construct. Infect. Immun. **50:**420–424.
10. **De Groote, M. A., and F. C. Fang.** 1995. NO inhibitions: antimicrobial properties of nitric oxide. Clin. Infect. Dis. **21**(Suppl. 2)**:**S162–S165.
11. **De Groote, M. A., U. A. Ochsner, M. U. Shiloh, C. Nathan, J. M. McCord, M. C. Dinauer, S. J. Libby, A. Vazques-Torres, Y. Xu, and F. C. Fang.** 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide sythanse. Proc. Natl. Acad. Sci. USA **94:**13997–14001.
12. **De Groote, M. A., T. Testerman, Y. Xu, G. Stauffer, and F. C. Fang.** 1996. Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. Science **272:**414–417.
13. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
14. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
15. **Galán, J. E., and R. I. Curtiss.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
16. **Galan, J. E., C. Ginocchio, and P. Costeas.** 1992. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. J. Bacteriol. **174:**4338–4349.
17. **Hosek, G., D. Leschinsky, S. Irons, and T. J. Safrank.** 1997. Multidrug-resistant *Salmonella* serotype Typhimurium—U.S., 1996. Morb. Mortal. Wkly. Rep. **46:**308–310.
18. **Humphreys, S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts.** 1999. The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella typhimurium*. Infect. Immun. **67:**1560–1568.

19. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Indentification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89:**1847–1851.
20. **Lu, S., A. R. Manges, Y. Xu, F. C. Fang, and L. W. Riley.** 1999. Analysis of virulence of clinical isolates of *Salmonella enteritidis* in vivo and in vitro. Infect. Immun. **67:**5651–5657.
21. **Mead, P. S., L. Slutsker, V. Dietz, L. R. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
22. **Threlfall, E. J., J. A. Frost, L. R. Ward, and B. Rowe.** 1996. Increasing spectrum of resistance in multiresistant *Salmonella typhimurium*. Lancet **347:**1053–1054.
23. **Threlfall, E. J., L. R. Ward, and B. Rowe.** 1998. Multiresistant *Salmonella typhimurium* DT104 and salmonella bacteraemia. Lancet **352:**287–288.
24. **Villar, R. G., M. D. Macek, S. Simons, P. S. Hayes, M. J. Goldoft, J. H. Lewis, L. L. Rowan, D. Hursh, M. Patnode, and P. S. Mead.** 1999. Investigation of multidrug-resistant *Salmonella* serotype DT104 infections linked to raw-milk cheese in Washington state. JAMA **281:**1811–1816.

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