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 non-multidrug-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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant information</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028s</td>
<td></td>
<td>ATCC</td>
</tr>
<tr>
<td>14028s <em>phoP</em></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>14028s <em>invA</em></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>14028s <em>invA</em></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>VT4Tx</td>
<td>Vermont DT104 bovine isolate</td>
<td>USDA, Athens, Ga.</td>
</tr>
<tr>
<td>3016</td>
<td>Georgia DT104 poultry isolate</td>
<td>USDA, Athens, Ga.</td>
</tr>
<tr>
<td>2745</td>
<td>Washington DT104 human isolate</td>
<td>J. Gay, Washington State University</td>
</tr>
<tr>
<td>4157</td>
<td>Washington DT104 bovine isolate</td>
<td>J. Gay, Washington State University</td>
</tr>
</tbody>
</table>
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 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.

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 *invF*, 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 ~10⁸ 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 10⁸ 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 10⁸ CFU (Table 2). PCR analysis of this strain using primers for four loci known to be required for *Salmonella* virulence (*spvC, hilA, sodC1*, 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 ~10⁸ CFU containing a 1:1 mixture of *S. enterica* serovar Typhimurium 14028s and each of the four *S. enterica* serovar
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 \( \mu \text{g/ml} \), to distinguish \textit{S. enterica} serovar Typhimurium 14028s (chloramphenicol susceptible) from the multidrug-resistant DT104 isolates (chloramphenicol resistant). The number of CFU of \textit{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 \textit{S. enterica} serovar Typhimurium DT104 isolate to the CFU of \textit{S. enterica} serovar Typhimurium 14028s recovered from the spleen and liver. None of the four \textit{S. enterica} serovar Typhimurium DT104 isolates tested were able to colonize the spleen or liver of infected mice as well as \textit{S. enterica} serovar Typhi-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**FIG. 2.** Susceptibility to reactive oxygen and nitrogen species. (A and B) \textit{Salmonella} cultures were grown overnight in LB medium, diluted in saline, and plated (100 \( \mu \text{l} \) containing \( 10^6 \) CFU) on M9 minimal plates containing 0.2% glucose. Then 15 \( \mu \text{l} \) 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 \( \mu \text{l} \) 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 \textit{Salmonella} strains. HEp-2 cells were grown to confluence and infected at a multiplicity of infection of 10 to 50 with \textit{S. enterica} serovar Typhimurium 14028s, \textit{S. enterica} serovar Typhimurium DT104 isolates, and \textit{hilA} and \textit{invA} mutant strains of \textit{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 \( \mu \text{g/ml} \) 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.
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.

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