Investigations of *Salmonella enterica* Serovar Newport Infections of Oysters by Using Immunohistochemistry and Knockout Mutagenesis

Christopher M. Morrison, Sharon M. Dial, William A. Day, Jr., and Lynn A. Joens

Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, Arizona, USA

The consumption of raw oysters is an important risk factor in the acquisition of food-borne disease, with *Salmonella* being one of a number of pathogens that have been found in market oysters. Previous work by our lab found that *Salmonella* was capable of surviving in oysters for over 2 months under laboratory conditions, and this study sought to further investigate *Salmonella*’s tissue affinity and mechanism of persistence within the oysters. Immunohistochemistry was used to show that *Salmonella* was capable of breaching the epithelial barriers, infecting the deeper connective tissues of the oysters, and evading destruction by the oysters’ phagocytic hemocytes. To further investigate the mechanism of these infections, genes vital to the function of *Salmonella*’s two main type III secretion systems were disrupted and the survivability of these knockout mutants within oysters was assayed. When the *Salmonella* pathogenicity island 1 and 2 mutant strains were exposed to oysters, there were no detectable deficiencies in their abilities to survive, suggesting that *Salmonella*’s long-term infection of oysters does not rely upon these two important pathogenicity islands and must be due to some other, currently unknown, mechanism.

*Salmonella enterica* is a prime example of an organism that has evolved versatile traits which allow it to live within a diverse range of hosts. Mammals, birds, and reptiles are all well-known reservoirs of *Salmonella*, but the list of possible hosts includes invertebrate animals like arthropods (29), nematodes (1, 12, 45), and certain protozoa (6, 39, 52). In conjunction with this diversity of possible hosts, *Salmonella* is capable of a range of host-microbe interactions. The same serovar can act as a harmless commensal in one host, cause a self-limiting gastroenteritis in another, and trigger a possibly life-threatening systemic infection in yet another host (49).

For humans, *Salmonella enterica* is one of the leading causes of food-borne disease in the United States (13, 41), with over 1 million cases each year (48). In particular, the Newport serovar is the third most commonly isolated serovar, comprising 10% of all *Salmonella* cases in 2007 (13), and the incidence of *Salmonella* serovar Newport infections rose dramatically between 1997 and 2002 (14), with most of the public health attention focused on the cattle industry as a likely contributor to this increase (2, 22, 43).

While much is understood about the interactions between *Salmonella* and vertebrates, much less is known about its relationships with invertebrates, which could be an overlooked and important aspect of *Salmonella*’s ecology (50). Of direct concern to human health is the relationship between *Salmonella* and mollusks and, in particular, oysters, which are typically consumed raw. The consumption of oysters is a major risk factor for the acquisition of food-borne diseases (11), and previous work in our lab found *Salmonella* in roughly 7% of market oysters from around the United States tested at two different times of the year (8), with one particular pulsed-field gel electrophoresis (PFGE) genotype of the *Salmonella* Newport serovar predominating (7). A more recent survey conducted by our lab found a 1.2% prevalence of *Salmonella* within oysters served in restaurants in Tucson, AZ (9). This survey, conducted 5 years after the original study by Brands et al. (8), also isolated the same PFGE genotype of *Salmonella* serovar Newport from oysters. Further work by our group established that *Salmonella* serovar Newport was capable of surviving within oysters for at least 2 months within a laboratory setting, but there did not appear to be any fitness advantage within oysters for this predominating genotype of *Salmonella* serovar Newport compared to other strains of *Salmonella* (33). The same work also showed that *Salmonella* survived within oysters significantly better than a nonpathogenic strain of *Escherichia coli* in two different tank environments (33).

Extensive studies of the *Salmonella* genome and those of its relatives have led to the discovery of several genomic islands important for pathogenesis. The two most important and well studied of these genomic islands are aptly named *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). Each of these genomic clusters contains numerous genes required for infection that are absent from *Salmonella*’s avirulent relatives and are believed to have originated from horizontal gene transfer (26). The first, SPI-1, contains genes required to construct and operate a type III secretion system (T3SS) used in the initial attachment and invasion of epithelial cells (26, 28, 32). One critical gene product, InvA, has been shown to be a key component of the inner membrane portion of the T3SS and may function to form a channel through which effector molecules can be delivered (16, 20). *invA* mutants fail to translocate SPI-1 effector molecules (16) and show significant attenuation in mouse models of disease due to decreased invasion of the intestines (20). The second pathogenicity island, SPI-2, contains a suite of genes that encodes a separate and mostly independent T3SS that allows the organism to survive and replicate within phagocytes (28, 30, 51). Similar to InvA, SsaV is thought to play a role in the inner membrane channel of the SPI-2 T3SS (30). *ssaV* mutants are unable to cause systemic disease in animal models of disease (51) and have been shown to be defective.
in translocating SPI-2 effector molecules, thereby reducing their fitness in a variety of assays (4, 10, 19, 24, 35, 42, 47).

This study sought to further examine the mechanisms by which *Salmonella* interacts with oysters. Through the use of immunohistochemistry (IHC), we were able to determine that *Salmonella* was taking up residence within the connective tissues of the oyster, a finding that suggested an infective process. Based on those results and the findings of others that showed that SPI-1 and SPI-2 were potentially important in colonizing other invertebrate hosts (6, 45), we hypothesized that *Salmonella*’s SPI-1 and SPI-2 would also play an important role in establishing an infection within oysters. To test this hypothesis, we disrupted the two T3SSs and exposed oysters to the deficient mutants using our previously established methods (33).

**MATERIALS AND METHODS**

**Bacterial strains and cultivation.** Strains used in these experiments are summarized in Table 1. Strains were grown in LB broth and on LB agar plates, supplemented with 30 μg/ml chloramphenicol (ICN Biomedicals, Aurora, OH), 50 μg/ml kanamycin (Shelton Scientific, Shelton, CT), and/or 100 μg/ml dianaminopimelic acid (DAP; Sigma, St. Louis, MO) when applicable. Xylose-lysine-deoxycholate (XLD) agar was used for oyster experiments. LB and XLD media were obtained from Difco Laboratories (Becton, Dickson and Company, Sparks, MD). Broth cultures were grown in a shaking incubator at 37°C unless otherwise stated.

**Immunohistochemistry.** Oysters were acquired, acclimated, maintained, and exposed to 8.15 × 10^7 CFU/ml of *S. enterica* serovar Newport LAI160311 as described previously (33). Every 5 days for a total of 30 days postexposure, three randomly selected oysters were aseptically shocked and dissected, and their tissues were fixed for approximately 48 h in Davidson’s fixative modified by replacing the deionized water with 25 ppt artificial seawater (27). The tissues were then routinely processed by following the standard procedures of the Arizona Veterinary Diagnostic Lab. Endogenous peroxidase-like activity was quenched by placing the slides in a 3% hydrogen peroxide solution for 30 min. After rinsing in deionized water, the slides were blocked for either 4 h at room temperature or overnight at 4°C in a solution of 5% skim milk in phosphate-buffered saline (PBS). Following blocking, the slides were exposed to a 1:500 dilution of primary antibodies for 90 min. The primary antibodies (Difco Laboratories) were a polyclonal rabbit anti-*Salmonella* antisera specific for the C2 lipopolysaccharide (LPS) antigen of the *Salmonella* Newport serovar or a polyclonal rabbit anti-*E. coli* antisera specific for the 157 LPS antigen of enterohemorrhagic *E. coli*, which was used as a negative control. Next, an anti-rabbit peroxidase-conjugated polymer (Envision; Dako-Cytomation, Carpinteria, CA) was applied for 30 min, followed by application of 3,3′-diaminobenzidine (DAB; Dako) for 5 min to produce a brown precipitate at the location of primary antibody accumulation. The slides were then counterstained with hematoxylin (Dako) for 5 min, before dehydration and placement of a coverslip. Incubation steps were done using a Dako Autostainer apparatus to automate and standardize incubation times across samples. The contrast and white balance of all photomicrographs were adjusted equally, and the images were cropped using Photoshop CS4 software. No other alterations to the images were made.

**Generation of mutants.** Knockout mutants were constructed via allelic exchange using the pDS132 suicide vector (R6K ori oriT sacB) as described elsewhere (36). PCR, using the primers listed in Table 2, was used to amplify roughly 1-kb fragments of *S. enterica* serovar Newport SL317 chromosomal DNA upstream and downstream of the genes targeted for disruption as well as the antibiotic resistance genes used to mark the gene deletions. These fragments were digested with the appropriate restriction enzymes and sequentially cloned into the pBC KS+ cloning vector. For the invA knockout, the cat gene from pBC, which confers resistance to chloramphenicol, was spliced between the homologous fragments upstream and downstream of the invA gene, while the ssaV knockout similarly used a kanamycin resistance gene from the EZ-Tn5 transposon. A derivative of the pDS132 plasmid, dubbed pDS132-Kan, was made by removing the cat gene on the backbone pDS132 and replacing it with the same kanamycin resistance gene used for ssaV through a series of partial digestions and ligations. The roughly 3-kb constructs were then subcloned from the pBC vector into pDS132 or pDS132-Kan using PCR primers with new flanking cut sites compatible with the suicide vector. pDS132-ΔssaV was introduced into wild-type *Salmonella* Newport

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**TABLE 1 Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmonella</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typhimurium LT2</td>
<td>Used as positive control in cell culture assays</td>
<td>33</td>
</tr>
<tr>
<td>Newport LA160311</td>
<td>Multidrug-resistant oyster isolate</td>
<td>8, 33</td>
</tr>
<tr>
<td>Newport SL317</td>
<td>Pan-susceptible wild-type strain</td>
<td>33</td>
</tr>
<tr>
<td>Newport ΔinvA</td>
<td>SL317 invA::Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>Newport ΔssaV</td>
<td>SL317 ssaV::Cm'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α MCR</td>
<td>General cloning strain</td>
<td>Lab collection</td>
</tr>
<tr>
<td>SM10 Δpir</td>
<td>Propagation of π-dependent plasmids</td>
<td>Lab collection</td>
</tr>
<tr>
<td>χ7213</td>
<td>DAP-dependent strain used for conjugating π-dependent suicide vectors (denoted strain MGN617 in the reference)</td>
<td>40</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBC KS+</td>
<td>Cm' ori fI, general cloning plasmid; source of cat gene</td>
<td>Lab collection</td>
</tr>
<tr>
<td>pUC18-EZ-Tn5</td>
<td>Source of Kan' gene</td>
<td>Lab collection</td>
</tr>
<tr>
<td>pDS132</td>
<td>Cm' ori R6K sacB, π-dependent counterselectable suicide vector</td>
<td>36</td>
</tr>
<tr>
<td>pDS132-Kan</td>
<td>Replacement of cat gene from pDS132 with Kan' gene</td>
<td>This study</td>
</tr>
<tr>
<td>pBCΔinvA</td>
<td>pBC carrying invA deletion allele, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>pBCΔssaV</td>
<td>pBC carrying ssaV deletion allele, Cm' Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pDS132-Kan-ΔinvA</td>
<td>pDS132-Kan carrying invA deletion allele, Cm' Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pDS132-ΔssaV</td>
<td>pDS132 carrying ssaV deletion allele, Cm' Kan'</td>
<td>This study</td>
</tr>
</tbody>
</table>

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SL317 via electroporation using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA) set to 2.5 kV, 200 µF, and 25 µF. Potential double-crossover mutants were then screened with a variety of PCR primer pairs to confirm the desired mutant allele.

pDS132-Kan-ΔinvA was introduced into a DAP-dependent pir-positive χ7213 strain of E. coli, and the transformed χ7213 strain was then used to introduce the suicide vector into wild-type Salmonella Newport SL317 via conjugation, which proved far more efficient than electroporation, as described elsewhere (40). Putative double-crossover mutants obtained after sacB counterselection were then examined with a panel of PCR tests to confirm that the desired mutations had been obtained.

Invasion assays. Invasion assays were performed by following the work of others with limited modifications (20, 21). Briefly, the nonpolarized human intestinal epithelial cell line Henle-407 (25) was grown in basal medium Eagle (BME; Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (DMEM-FBS) in a 37°C humidified CO2 incubator. Overnight bacterial cultures were then diluted in PBS and incubated for 24 h. Overnight bacterial cultures were then diluted 1:1 in DMEM-FBS, resulting in an MOI of between 10 and 20. After a 30-min incubation, the cells were washed three times with PBS and then incubated for 90 min in DMEM-FBS supplemented with 100 µg/ml of gentamicin. After 2 h, the cells were washed three times with PBS and 0.1% deoxycholate in PBS was added to half the wells for each strain to lyse the cells. The lysate was collected, and 10-fold serial dilutions in PBS were plated on LB agar to determine the concentration of bacteria surviving within the cells. Each strain of bacteria was tested in quadruplicate, and the entire experiment was repeated at least three independent times.

Macrophage survival assays. Macrophage survival assays were conducted as described elsewhere, with a few modifications (5, 17, 18, 46). Briefly, the mouse macrophage-like cell line J774.A1 (38) was grown in Dulbecco’s modification of Eagle’s medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (DMEM-FBS) in a 37°C humidified CO2 incubator supplemented with 5% CO2. The J774.A1 cells were seeded into 24-well tissue culture dishes with approximately 5 × 105 cells per well and incubated for 24 h. Overnight bacterial cultures were then diluted in PBS to an OD600 of 0.1 and opsonized for 15 min with the addition of heat-inactivated normal mouse serum (Sigma, St. Louis, MO). This solution was further diluted 1:10 in DMEM-FBS, resulting in an MOI of between 10 and 20. After a 30-min incubation, the cells were washed three times with PBS and then incubated for 90 min in DMEM-FBS supplemented with 100 µg/ml of gentamicin. After 2 h, the cells were washed three times with PBS and 0.1% deoxycholate in PBS was added to half the wells for each strain to lyse the cells. The lysate was collected, and 10-fold serial dilutions in PBS were plated on LB agar to determine the concentration of bacteria surviving in the macrophages. The other half of the wells had their media replaced with DMEM-FBS supplemented with 100 µg/ml of gentamicin and were incubated for another 20 h, at which point the lysis and quantification steps were repeated for the remaining wells. The fold increase was calculated by dividing the number of bacteria remaining at 20 h by the number initially counted at 2 h. Each strain of bacteria was tested in quadruplicate, and the entire experiment was repeated at least three independent times.

Survival of mutants in oysters. Pacific oysters (Crassostrea gigas) were acquired, maintained, exposed to bacteria, and analyzed as described previously (33). The oysters were exposed to approximately 106 CFU/ml of...
each strain of bacteria and left for 24 h, before being returned to their tanks. On postexposure sample days, one oyster per tank (3 tanks per group) was aseptically shucked, weighed, and homogenized into a 1:5 dilution of sterile PBS. The resulting homogenate was then serially diluted and plated on XLD agar, and the plate was incubated at 37°C to determine the number of CFU per gram of oyster meat. On test days 20 and 30, homogenates were simultaneously plated on LB agar with chloramphenicol and kanamycin to confirm that the mutant salmonellae detected on the XLD agar plates were the proper strains.

**Statistics.** As described previously (33), CFU data were transformed to a normal distribution by taking the base-10 logarithm to account for the skew inherent in the quantification of exponential growth prior to performing any statistical analysis (31). To determine if any observed differences between strains of bacteria were statistically significant in either the cell culture or the oyster assays, one-way analysis of variance (ANOVA) tests for independent samples were conducted followed by a Tukey honestly significant difference test. All tests were performed using the open-source statistical software R.

**RESULTS**

Localization of *Salmonella* within oyster tissues. Immunohistochemical analysis using *Salmonella*-infected oysters and anti-*Salmonella* antiserum was performed in order to determine where the salmonellae reside during their long-term interactions with oyst-

ers. At 5 days postexposure (D.P.E.) to the salmonellae, staining of oyster tissues with anti-*Salmonella* antibody showed antibody-antigen complexes localized to three places within the oysters. The first area of staining, shown in Fig. 1A, identifies the bacteria as being closely associated with or inside the pseudostratified epithelial cells lining the gut and in darker-staining foci within the gut lumen. The second area of prominent staining, seen in Fig. 1B, occurs in the digestive glands, which are blind-ended tubules where most of the oyster’s nutrient absorption occurs. The staining is specific to the columnar digestive cells of the gland and appears to be absent from the pyramidal basophil cells. The third site of staining occurs within the vesicular connective tissue throughout the body of the oyster, shown in Fig. 1C. In stained areas of connective tissue, there is often a corresponding infiltration of hemocytes, identified by their small dark-blue-staining nuclei. At 10 and 15 D.P.E., the distribution of staining remained the same but the extent of the staining around the gut mucosa and in the digestive glands declined. Beyond 15 D.P.E., the staining is almost exclusively within the connective tissue of the oyster, including within the oysters’ hemocytes (Fig. 2). Two controls were run alongside the anti-*Salmonella* antibody tests of *Salmonella*-exposed tissues. The first control used a different antibody, an anti-*E. coli* antibody, on the same *Salmonella*-exposed tissues to confirm that the antibody was specific for *Salmonella*. These experiments occasionally showed faint staining in the same areas identified by the anti-*Salmonella* antibodies. This is most likely due to the polyclonal antiserum recognizing similar epitopes found in both *Salmonella*’s O:2 and *E. coli*’s O:157 LPS molecules. The second control was to process tissues from unexposed oysters in an identical fashion to that for the *Salmonella*-exposed tissues using the anti-*Salmonella* antibodies to make sure that the antibodies were not binding to antigens found within the oyster tissue itself, and these controls resulted in no observable staining.

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**FIG 1** Photomicrographs of IHC stained oyster tissues. (A) Gut epithelium; (B) digestive gland tissue; (C) vesicular connective tissue. Left column, oysters exposed to *Salmonella* and stained with an anti-*Salmonella* antibody (Ab); right column, negative controls consisting of *Salmonella*-exposed oysters stained with an anti-*E. coli* negative-control antibody (top) and unexposed oysters stained with anti-*Salmonella* antibodies (bottom). Magnifications, ×200 (A and C) and ×400 (B).

**FIG 2** Photomicrographs of IHC stained oyster tissues. Each time point, 5 days postexposure (A), 15 days postexposure (B), and 30 days postexposure (C), shows staining with anti-*Salmonella* antibodies. Magnifications, ×1,000.
In contrast, the concentration dropped to 4.72 CFU/ml by the second time point. The wild-type strain started with a concentration of 4.77 CFU/ml, but the increase during the course of the assay. The wild-type Salmonella from an average log number of CFU/ml of 4.75 to 5.34, a 3.88-fold increase during the course of the assay. The wild-type Salmonella showed significantly reduced compared to the other strains (P < 0.01).

Confirmation of mutant phenotypes. To investigate whether the same major virulence factors used in mammalian infections, namely, SPI-1 and SPI-2, were important in establishing an infection in oysters, ΔinvA and ΔssaV mutants were constructed in Salmonella Newport. Cell culture assays were done to confirm the expected mutant phenotypes prior to exposing these strains to oysters. Predictably, the ΔinvA mutant constructed in this experiment was deficient in its ability to invade the Henle-407 intestinal epithelial cell line (Table 3). Specifically, the ΔinvA strain had an average of 2.72 log CFU/ml remaining within the cells, compared to 5.83 log CFU/ml for the Salmonella Typhimurium LT2 strain, which was used as a positive control, 6.09 log CFU/ml for wild-type Salmonella Newport, and 6.11 for the ΔssaV mutant strain. The log-transformed CFU/ml values for each well of each strain were used in the ANOVA test described above to determine that the amount of invasion seen with the ΔinvA mutant was significantly reduced compared to the other strains (P < 0.01).

Also as expected, the ΔssaV mutant was able to invade intestinal cells with similar efficiency as wild type but had reduced macrophage survival abilities (Table 3). In particular, the ΔssaV mutant started with a concentration of 4.77 CFU/ml, but the concentration dropped to 4.72 CFU/ml by the second time point. In contrast, the Salmonella Typhimurium control strain grew from an average log number of CFU/ml of 4.75 to 5.34, a 3.88-fold increase during the course of the assay. The wild-type Salmonella Newport strain had a 3.02-fold increase, from 5.76 to 6.24 log CFU/ml, and the ΔinvA mutant also had a 3.00 fold-increase, from 5.03 to 5.51 CFU/ml, during its time within the macrophages. The fold increase values for each well of each strain were used in the ANOVA test previously described to determine that the survival ability of the ΔssaV mutant was statistically significantly reduced compared to the other strains (P < 0.01).

Survival of T3SS mutants in oysters. Once the phenotypic deficiencies were confirmed in vitro, oysters were exposed to the mutant strains to identify any survival deficiencies in vivo. Figure 3 summarizes the concentration of Salmonella found within the oysters over the course of the 30-day experiment. The oysters were exposed to an average of either 3.94 × 10⁶ CFU/ml of the wild-type strain, 4.39 × 10⁷ CFU/ml of the ΔinvA mutant, or 4.91 × 10⁸ CFU/ml of the ΔssaV mutant. Five days into the experiment, the oysters exposed to wild-type Salmonella Newport contained an average of 1.06 × 10⁵ CFU/g of oyster meat, the oysters exposed to the ΔinvA mutant contained an average of 7.26 × 10⁴ CFU/g, and the oysters exposed to the ΔssaV mutant contained an average of 1.69 × 10⁴ CFU/g. From those average values, the amount of bacteria within the oysters declined slowly, consistent with the results of our earlier studies (33), until the levels of bacteria after 30 days were 4.34 × 10² CFU/ml for wild type, 4.36 × 10² CFU/ml for the ΔinvA strain, and 6.91 × 10² CFU/ml for the ΔssaV strain. Using the ANOVA techniques described above, there were no statistically significant differences between any of the strains at any time point.

**TABLE 3 Cell culture assays**

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of Salmonella Typhimurium-positive control</th>
<th>Invasion</th>
<th>Macrophage survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type SL317</td>
<td>104.37</td>
<td>77.82</td>
<td></td>
</tr>
<tr>
<td>SL317 ΔinvA</td>
<td>46.66⁺</td>
<td>77.18</td>
<td></td>
</tr>
<tr>
<td>SL317 ΔssaV</td>
<td>104.75</td>
<td>25.03⁺</td>
<td></td>
</tr>
</tbody>
</table>

⁺ Statistically significant difference compared against the other two strains (P < 0.01).

**FIG 3 Survival of mutant Salmonella in oysters.** Pacific oysters (Crassostrea gigas) were exposed to two mutant strains of Salmonella Newport, along with a wild-type control. Every 5 days for 30 days, the numbers of viable bacteria remaining within the oysters were enumerated. The means of three independent replicates, done in triplicate, ±95% confidence intervals are displayed.
invasive infection, we used IHC on tissues from oysters exposed to *Salmonella* to determine where the bacteria resided. The results of the IHC clearly showed that *Salmonella* was not just within the lumen of the oyster’s gut but was also within cells of the digestive gland and eventually beyond the epithelial barrier and into the connective tissue of the oyster. The consistent presence of *Salmonella* within hemocytes throughout the study also suggests that *Salmonella* is potentially capable of surviving within the oysters’ hemocytes for long periods of time. The timeline and distribution of the *Salmonella* suggested a typhoid-like infection with initial invasion into the columnar epithelial cells of the digestive gland, followed by uptake into and survival within hemocytes, which then disperse the bacteria into other areas of the host. These results were not surprising because our previous work demonstrated significant differences between the survivability of *Salmonella Newport* in oysters and that of a nonpathogenic strain of *E. coli*, which suggested that virulence factors could play a role in the survival of *Salmonella* in oysters (33).

Since *Salmonella* was invading the oysters’ tissues, we wanted to determine if the infection utilized the same well-known virulence factors encoded within SPI-1 and SPI-2 that *Salmonella* uses to infect mammalian hosts. It was plausible that the genes encoded in these pathogenicity islands could have evolved in such a way that the functionality of the T3SS could confer a selective advantage in a broad range of hosts, especially considering evidence that *Salmonella*’s T3SS provides fitness within other more primitive hosts like the amoeba *Acanthamoeba polyphaga* (6) and the nematode *Caenorhabditis elegans* (45).

In order to test this hypothesis, knockout alleles were constructed in *invA* of SPI-1 and *ssaV* of SPI-2. While most research on SPI-1 and SPI-2 has been conducted using *Salmonella* Typhimurium strains, we conducted our mutagenesis in the same *Salmonella* serovar, Newport, that was used in the IHC work. Due to complications that we encountered trying to introduce marked mutations into the multidrug-resistant strain isolated from oysters, *Salmonella* Newport LAJ160311, we instead mutated a pan-susceptible strain, *Salmonella* Newport SL317, previously shown to survive within oysters just as well as the LAJ160311 strain (33).

After the wild-type and mutant strains of *Salmonella* were introduced into the oysters, there was never a significant difference in survivability between the wild-type and the mutants throughout the 30-day study, indicating that neither *invA* nor *ssaV* and, by extension, neither SPI-1 nor SPI-2 are necessary for *Salmonella*’s survival within oysters. Given the apparent nature of the oysters’ infection, these results were surprising, but there are many possible explanations for why this might be the case. While it is clear that *Salmonella* gains entry into the epithelial cells of the digestive glands, this may be an intentional part of the oyster’s digestive process and not due to invasion by the bacteria, since the digestive glands of the oyster are known to endocytose and digest food particles found within the lumen of the digestive glands (34). Once the bacteria have reached the connective tissue, finding *Salmonella* within circulating hemocytes is also not unexpected, given their immunological role within oysters. It has been established that an oyster’s phagocytic hemocytes respond to bacterial infections (3, 15), including infections by *Salmonella*, as shown by the hemocyte infiltration in areas of oyster tissue that stained positive for *Salmonella* in this study (Fig. 1C). However, it is known that members of the *Vibrio* genus can evade the actions of hemoocytes and other immune effectors and establish persistent infections within oysters (23, 37, 44).

At no time during any of the previous oyster studies (33) or in this one did the average number of CFU per gram of oyster ever increase during the course of the experiment, implying that the bacteria may merely be resisting digestion and destruction through mechanisms independent of either SPI-1 or SPI-2. Shea et al. describe some salmonellae as entering a viable but nonreplicating state upon phagocytosis by macrophages (42), a description that would certainly fit the consistent patterns of viability seen when *Salmonella* was introduced into oysters in our lab.

The oyster represents a unique niche for *Salmonella*, where environmental conditions like salinity, pH, temperature, nutrient composition and concentrations, and host defenses result in selective pressures that are distinctly different from those in the niches of reptiles, birds, and mammals that are more traditionally associated with *Salmonella*. This study has further clarified the nature of the interaction between *Salmonella* and oysters and eliminated the most obvious virulence factors for establishing the infection observed, but further study is necessary to more fully understand the mechanism of this atypical *Salmonella* infection.

**ACKNOWLEDGMENTS**

This research was supported by a grant from the USDA-CSREES Epidemiological Approaches for Food Safety Initiative (award no. 2006-35212-17040).

We acknowledge Arlette Schneider for outstanding technical support during the oyster studies. We also thank the following people for their kind gifts of strains and plasmids: *E. coli* DH5α MCR and pBC KS+ were gifts from Stephen Billington, and *E. coli* χ7213 was a gift from Kenneth Roland. We also thank Margarette Cooper and Kerry Cooper for their critical evaluations of the manuscript.

**REFERENCES**


