Salmonella enterica subsp. enterica Serovar Heidelberg Food Isolates Associated with a Salmonellosis Outbreak Have Enhanced Stress Tolerance Capabilities

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ABSTRACT Salmonella enterica serovar Heidelberg is currently the 12th most common serovar of Salmonella enterica causing salmonellosis in the United States and results in twice the average incidence of blood infections caused by nontyphoidal salmonellae. Multiple outbreaks of salmonellosis caused by Salmonella Heidelberg resulted from the same poultry processor, which infected 634 people during 2013 and 2014. The hospitalization and invasive illness rates were 38% and 15%, respectively. We hypothesized that the outbreak strains of Salmonella Heidelberg had enhanced stress tolerance and virulence capabilities. We sourced nine food isolates collected during the outbreak investigation and three reference isolates to assess their tolerance to heat and sanitizers, ability to attach to abiotic surfaces, and invasiveness in vitro. We performed RNA sequencing on three isolates (two outbreak-associated isolates and a reference Salmonella Heidelberg strain) with various levels of heat tolerance to gain insight into the mechanism behind the isolates’ enhanced heat tolerance. We also performed genomic analyses to determine the genetic relationships among the outbreak isolates. Ultimately, we determined that (i) six Salmonella Heidelberg isolates associated with the foodborne outbreak had enhanced heat tolerance, (ii) one outbreak isolate with enhanced heat tolerance also had an enhanced biofilm-forming ability under stressful conditions, (iii) exposure to heat stress increased the expression of Salmonella Heidelberg multidrug efflux and virulence genes, and (iv) outbreak-associated isolates were likely transcriptionally primed to better survive processing stresses and, potentially, to cause illness.

IMPORTANCE This study provides a deep analysis of the intrinsic stress tolerance and virulence capabilities of Salmonella Heidelberg that may have contributed to the length and severity of a recent salmonellosis outbreak. Additionally, this study provides a comprehensive analysis of the transcriptomic response of S. enterica strains to heat stress conditions and compares baseline stationary-phase gene expression among outbreak- and non-outbreak-associated Salmonella Heidelberg isolates. These data can be used in assay development to screen isolates for stress tolerance and subsequent survival. This study adds to our understanding of the strains associated with the outbreak and informs ongoing regulatory discussions on Salmonella in poultry.

KEYWORDS RNA-seq, Salmonella enterica serovar Heidelberg, genomics, heat tolerance, outbreak, poultry, stress tolerance

Salmonellae are frequently associated with poultry and eggs (1–3), though contact with live beef calves (4) and ingestion of contaminated pork (5), fruits and vegetables (6, 7), spices (8), and peanut butter (9) have all been implicated in salmonellosis.
outbreaks. However, *Salmonella enterica* serovars Heidelberg, Typhimurium, and Enteritidis are all commonly associated with poultry and eggs, especially chicken and turkey (10, 11). No serovar of *S. enterica* is currently considered an adulterant in raw poultry. However, to meet USDA performance standards, no more than 9.8% of young broilers (5/51 sampled), 25% of ground chicken samples, and 15.4% of chicken parts sampled can be positive for *S. enterica* (12). To achieve this benchmark, poultry processors employ a number of pathogen reduction steps during slaughter and processing, including the use of high temperatures and the use of antimicrobials in scald, chill, and wash water (13, 14). Nonetheless, these measures do not entirely eliminate *S. enterica* from the finished product.

Some isolates may be more likely to survive processing control strategies due to intrinsic characteristics. For instance, *Salmonella Senftenberg* 775W is extremely heat resistant (15), which would result in better survival of improper cooking or processing measures. Additionally, acid tolerance varies considerably among *S. enterica* strains, which could affect the efficacy of some antimicrobials (13, 15). Finally, *S. enterica* strains have widely different abilities to form biofilms, which are far more resistant to stress than planktonic cells, providing protection to the bacteria from mechanical stress, heat, sanitizers, and desiccation (16–24). Proper handling kills *Salmonella*. However, research indicates that few U.S. consumers use meat thermometers when cooking poultry, and many wash carcasses in the kitchen sink and share cutting boards between poultry and other foods (25–27). Consequently, bacteria which survive pathogen reduction measures during processing can cross-contaminate food, non-food-contact surfaces, and ready-to-eat foods, resulting in potential illness. In view of this, understanding the intrinsic factors that may support the survival of the pathogen from processing control strategies is crucial to improving their efficacy, especially for serovars causing invasive illnesses.

Recently, an outbreak of salmonellosis resulting from *Salmonella* Heidelberg in chicken underscored the importance of studying differences in stress tolerance among salmonellae. This outbreak was traced back to a single processor in the western United States and lasted for more than a year, despite the processor meeting and eventually surpassing performance standards (2, 28, 29). In fact, as the company increased efforts to reduce the *S. enterica* prevalence in its products, the percentage of *S. enterica* isolates detected overall dropped, but the percentage of *S. enterica* isolates belonging to the outbreak strains increased over almost 5 months before declining (29). Seven *Salmonella* Heidelberg strains were implicated in the outbreak, and several of these were multidrug resistant (MDR) (2). The outbreak had an above-average hospitalization rate and invasive illness rate for *Salmonella* Heidelberg (a 38% versus 26% average hospitalization rate and a 15% versus 13% average invasive illness rate), suggesting that the strains might have had above-average virulence (2, 30). Given the length and scope of the outbreak, we hypothesized that the outbreak strains of *Salmonella* Heidelberg may have had enhanced stress tolerance capacities.

The purpose of this study was to identify intrinsic *Salmonella* Heidelberg characteristics that may have contributed to the length, scope, and severity of the 2013-2014 *Salmonella* Heidelberg outbreak from chicken (2, 28, 29). To accomplish this, we assessed the heat tolerance, sanitizer tolerance, and attachment ability of nine food isolates associated with the outbreak and compared these characteristics to those of reference strains. We also evaluated the invasion capacity of four outbreak-associated food isolates and defined the transcriptomic profile of three *Salmonella* Heidelberg isolates (two outbreak-associated food isolates and strain SL476) during stationary phase and after heat shock to identify potential mechanisms of enhanced heat and stress tolerance. Finally, we compared the phenotypic and transcriptomic characteristics with epidemiological and genomic data to draw broader inferences about the isolates.

**RESULTS**

*Genomic analysis of Salmonella* Heidelberg outbreak-associated isolates. (i) Isolates contain multiple plasmids and resistance genes. All six isolates (R1-0001,
R1-0002, R1-0003, R1-0006, R1-0007, and SL476) had evidence of at least one plasmid (Table 1). Reads mapping to ColpVC, a plasmid found in *Salmonella* Enteritidis (GenBank accession no. JX133088.1), were the most common, followed by reads mapping to an IncHI2/IncHI2A resistance plasmid from *Serratia marcescens* (GenBank accession no. BX664015.1). Four isolates had reads mapping to an IncI1 plasmid from *Salmonella* Typhimurium (GenBank accession no. AP005147.1), while two had reads mapping to an IncX1 plasmid from *Escherichia coli* (GenBank accession no. EU370913.1). Finally, R1-0002 had reads mapping to an IncA/C2 bla<sub>CYM-2</sub> resistance plasmid from *Klebsiella pneumoniae* (GenBank accession no. JN157804.1). PlasmidFinder failed to recognize the second recognized SL476 plasmid; a BLAST analysis of the nucleotide sequences in the National Center for Biotechnology Information (NCBI) database identified it as matching *Escherichia coli* strain Sanji plasmid pSJ_3 (GenBank accession no. CP011066.1). All *Salmonella* Heidelberg isolates possessed the fosfomycin resistance gene (*fosA7*). Aminoglycoside resistance gene *aac(6\(^{-}\)=)-Iaa* was also present across the isolates (Table 1). However, its nucleotide sequence had only 97.3% similarity with the nucleotide sequence of the reference gene. Alignment of the isolate sequences with the reference nucleotide sequence revealed that their sequences all contained seven point mutations leading to the following amino acid substitutions: R8K, H10N, D12E, H42Y, V76A, T105A, and S117T. All of these are within the predicted acetyltransferase domain. Furthermore, all isolates contained a missense mutation in *parC* (T57S) associated with resistance to nalidixic acid and ciprofloxacin.

(ii) Genomic analyses reveal isolate relationships. With a seven-gene multilocus sequence typing (MLST) scheme, all *Salmonella* Heidelberg isolates typed as MLST sequence type (ST) 15 (Table 1). However, using core genome MLST (cgMLST), the isolates all had different STs. A phylogenetic analysis of the seven outbreak isolates and SL476 demonstrated that isolate pairs from the same pulsed-field gel electrophoresis (PFGE) type generally clustered together (Fig. 1). When the strains were compared, isolates from PFGE type JF6X01.0041 were most closely related to JF6X01.0258 isolate R1-0006, while JF6X01.0045 isolates clustered most closely with JF6X01.0326 isolate R1-0007. SL476 (PFGE type unknown) was not closely related to any of the isolates. Analyzing the nucleotide difference (ND) matrix, there were substantial differences between isolates of the same PFGE strains (see Table S1 posted at [https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507](https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507)). There were 134 total NDs among the isolates, with 58 NDs appearing between isolates of JF6X01.0045 (R1-0002, R1-0004), while isolates from JF6X01.0041 (R1-0001, R1-0005) had 24 NDs and R1-0003 and R1-0006 (JF6X01.0258) had 22 NDs. The isolates differed from SL476 by 109 to 134 NDs. A larger analysis including food isolates from two other PFGE types involved in the outbreak (see Fig. S1 posted at [https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507](https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507)) showed that the isolates with PFGE patterns matching those of isolates involved in the 2013-2014 outbreak clustered more closely with each other than with SL476, regardless of their collection year.

The majority of *Salmonella* Heidelberg outbreak-associated isolates have enhanced heat tolerance in stationary phase. We assessed the heat tolerance of nine *Salmonella* Heidelberg food isolates associated with the outbreak as well as SL476 and *Salmonella* Typhimurium 14028 at 56°C, the temperature of a hard (i.e., high-heat) scald in poultry processing (Table 2) (31). There were no significant differences in heat tolerance among isolates in logarithmic phase (data not shown). However, six of the nine outbreak-associated *Salmonella* Heidelberg isolates showed significantly increased heat tolerance (as determined by a repeated-measures analysis of variance [ANOVA] for the survivor curves) compared to that of *Salmonella* Typhimurium 14028 (\( P < 0.05 \)). R1-0006 and R1-0007 showed significantly higher heat tolerance than *Salmonella* Heidelberg SL476 and *Salmonella* Typhimurium 14028 (\( P < 0.05 \)) in stationary phase (Fig. 2).

There was no association between antibiotic resistance and enhanced heat tolerance (see Table S2 posted at [https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507](https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507)). R1-0007 is a pansusceptible isolate, while R1-
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result for the following isolates:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SL476</td>
</tr>
<tr>
<td>Genome SRA no.</td>
<td>ERR351248</td>
</tr>
<tr>
<td>Salmonella serovar</td>
<td>Heidelberg</td>
</tr>
<tr>
<td>MLST type&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>cgMLST type&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5466</td>
</tr>
<tr>
<td>Plasmid(s) detected&lt;sup&gt;c&lt;/sup&gt;</td>
<td>IncI1</td>
</tr>
<tr>
<td>Antibiotic resistance gene(s)</td>
<td>detected&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>aac(6')-Iaa, aph(6)-Ia, aph(3')-Ia</td>
</tr>
<tr>
<td>Beta-lactam</td>
<td>bla&lt;sub&gt;TEM-1b&lt;/sub&gt;</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>fosA7</td>
</tr>
<tr>
<td>Phenicol</td>
<td>—</td>
</tr>
<tr>
<td>Sulfonamide</td>
<td>sul1</td>
</tr>
<tr>
<td>Tetraacycline</td>
<td>tet(B)</td>
</tr>
<tr>
<td>(point mutation)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>As determined by the Center for Genomic Epidemiology’s MLST (v.2.0) (32).
<sup>b</sup>As determined by the Center for Genomic Epidemiology’s PlasmidFinder (v.2.0) (76).
<sup>c</sup>As determined by the Center for Genomic Epidemiology’s cgMLSTFinder (v.1.0) (36).
<sup>d</sup>As determined by the Center for Genomic Epidemiology’s ResFinder (v3.1) (77).
<sup>e</sup>—, no resistance element found.
<sup>f</sup>NCBI lists two plasmids for the isolate (pSL476_3 and pSL47691); however, pSL476_3 is not recognized by PlasmidFinder.
0006 is resistant to kanamycin, streptomycin, and tetracycline (Table 2). Antibiotic-resistant isolates R1-0001, R1-0002, and R1-0005 did not have enhanced heat tolerance, while antibiotic-resistant isolates R1-0003, R1-0006, and R1-0009 and pansusceptible isolates R1-0004, R1-0007 and R1-0008 had significantly higher heat tolerance than *Salmonella* Typhimurium ATCC 14028 (\( P < 0.05 \)), and R1-0006 and R1-0007 had significantly higher heat tolerance than SL476 (\( P < 0.05 \)).

**Salmonella Heidelberg outbreak-associated isolates attach to an abiotic surface better under stress conditions.** We assessed the attachment capability of 10 *Salmonella* Heidelberg isolates, 4 *Salmonella* Typhimurium isolates, 4 *Salmonella* Newport food isolates, *Salmonella* Typhimurium ATCC 14028, and *Salmonella* Enteritidis at 21°C (in 1\( \times \) tryptic soy broth [TSB]; see Fig. S2 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507] and 1/20\( \times \) TSB) and at 4°C. At 21°C in 1/20\( \times \) TSB (Fig. 3A), serovar and time were significant (\( P < 0.05 \)). *Salmonella* Heidelberg attached significantly less than *Salmonella* Typhimurium, Enteritidis, and Newport (adjusted \( P \) value \( P_{\text{adj}} < 0.05 \). Attachment efficacy increased significantly as time increased from 24 h to 120 h \( P_{\text{adj}} < 0.05 \). At the isolate level, *Salmonella* Enteritidis isolate R1-0010 had the highest attachment, which was significantly higher than that of R1-0001, R1-0002, R1-0003, R1-0005, and SL476 \( P_{\text{adj}} < 0.05 \). *Salmonella* Heidelberg R1-0002 had the lowest attachment, which was significantly lower than that of all the isolates except SL476 and R1-0005 \( P_{\text{adj}} < 0.05 \). However,
heat-tolerant *Salmonella* Heidelberg isolates R1-0006 and R1-0007 did not attach significantly less than *Salmonella* Enteritidis R1-0010. When this experiment was carried out to 9 days, we found similar results (see Fig. S3 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507).

**FIG 2** Survival of *Salmonella* Heidelberg outbreak-related isolates and *S. enterica* reference strains at 56°C during stationary phase. Error bars represent the standard deviation from at least three biological replicates.

**TABLE 2** Characteristics of isolates studied

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Serotype</th>
<th>PulseNet PFGE pattern</th>
<th>Antimicrobial resistance profilea</th>
<th>Isolate typeb</th>
<th>Isolate source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUL R1-0001</td>
<td>Heidelberg</td>
<td>JF6X01.0041</td>
<td>Gen, Kan, Str, Fis, Tet</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0002</td>
<td>Heidelberg</td>
<td>JF6X01.0045</td>
<td>Amp, Chl, Gen, Kan, Str, Fis, Tet</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0003</td>
<td>Heidelberg</td>
<td>JF6X01.0258</td>
<td>Gen, Kan, Str, Fis, Tet</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0004</td>
<td>Heidelberg</td>
<td>JF6X01.0045</td>
<td>Pansusceptible</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0005</td>
<td>Heidelberg</td>
<td>JF6X01.0041</td>
<td>Gen, Kan, Str, Fis, Tet</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0006</td>
<td>Heidelberg</td>
<td>JF6X01.0258</td>
<td>Kan, Str, Tet</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0007</td>
<td>Heidelberg</td>
<td>JF6X01.0326</td>
<td>Pansusceptible</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>PUL R1-0008</td>
<td>Heidelberg</td>
<td>JF6X01.0122</td>
<td>Pansusceptible</td>
<td>Outbreak associated</td>
<td>USDA-ARS stock collection, IL</td>
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<tr>
<td>PUL R1-0009</td>
<td>Heidelberg</td>
<td>JF6X01.0022</td>
<td>Amp, Chl, Gen, Kan, Str, Fis, Tet</td>
<td>Outbreak associated</td>
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<tr>
<td>PUL R1-0010</td>
<td>Enteritidis</td>
<td>Unknown</td>
<td>Unknown</td>
<td>No information</td>
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<tr>
<td>SL476</td>
<td>Heidelberg</td>
<td>Unknown</td>
<td>Gen, Amp, Kan, Str, Tet, Aug, Cep, Smx</td>
<td>Reference strain</td>
<td>Salmonella Genetic Stock Centre, Calgary</td>
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<tr>
<td>ATCC 14028</td>
<td>Typhimurium</td>
<td>Unknown</td>
<td>Pansusceptible</td>
<td>Reference strain</td>
<td>M. Singh lab, Purdue University</td>
</tr>
<tr>
<td>B-65297</td>
<td>Newport</td>
<td>JJPX01.0422</td>
<td>None listed</td>
<td>Food isolate</td>
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<tr>
<td>B-65298</td>
<td>Newport</td>
<td>JJPX01.0422</td>
<td>Aug, Amp, Fox, Tio, Axo, Chl, Str, Fis, Tet, Cot</td>
<td>Food isolate</td>
<td>USDA-ARS stock collection, IL</td>
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<tr>
<td>B-65299</td>
<td>Newport</td>
<td>JJPX01.0422</td>
<td>None listed</td>
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<td>B-65300</td>
<td>Newport</td>
<td>JJPX01.0422</td>
<td>Aug, Amp, Fox, Tio, Axo, Chl, Str, Fis, Tet, Cot</td>
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<tr>
<td>B-65301</td>
<td>Typhimurium</td>
<td>JPPX01.0552</td>
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<tr>
<td>B-65302</td>
<td>Typhimurium</td>
<td>JPPX01.0552</td>
<td>None listed</td>
<td>Food isolate</td>
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</tr>
<tr>
<td>B-65303</td>
<td>Typhimurium</td>
<td>JPPX01.0552</td>
<td>None listed</td>
<td>Food isolate</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
<tr>
<td>B-65304</td>
<td>Typhimurium, O:5-negative variant (Copenhagen)</td>
<td>JPPX01.0552</td>
<td>None listed</td>
<td>Food isolate</td>
<td>USDA-ARS stock collection, IL</td>
</tr>
</tbody>
</table>

aAntimicrobial abbreviations: Gen, gentamicin; Amp, ampicillin; Chl, chloramphenicol; Kan, kanamycin; Str, streptomycin; Fis, sulfisoxazole; Fox, cefoxitin; Tio, cefotilur; Axo, ceftriaxone; Tet, tetracycline; Aug, amoxicillin-clavulanic acid; Cep, cephalothin; Smx, sulfamethoxazole.

bOutbreak-associated food isolate collected during an outbreak investigation from products of the company under investigation with a PFGE pattern matching that of the outbreak strain.
At 4°C in 1/20× TSB (Fig. 3B), serovar, time, and serovar × time were significant ($P < 0.05$). Salmonella Typhimurium had the highest attachment among the serovars, while Newport had the lowest ($P_{\text{adj}} < 0.05$). Salmonella Heidelberg had significantly lower attachment than Salmonella Typhimurium, but its attachment was not significantly different from that of Salmonella Enteritidis or Newport ($P_{\text{adj}} < 0.05$). Salmonella Enteritidis attached significantly better than Salmonella Newport ($P_{\text{adj}} < 0.05$). Attachment at 120 h was significantly lower than at both 24 h and 72 h ($P_{\text{adj}} < 0.05$). At the isolate level at 4°C, heat-tolerant isolate Salmonella Heidelberg R1-0006 had the highest attachment among all the isolates, while SL476 had the lowest attachment, and Salmonella Typhimurium 14028 had attachment comparable to that of most Salmonella Heidelberg and Newport isolates ($P_{\text{adj}} < 0.05$).
Salmonella Heidelberg food isolates do not have enhanced tolerance to a PAA-based sanitizer or increased in vitro invasiveness. Isolates rarely survived or grew in the presence of peroxycetic acid (PAA) at concentrations greater than 50 ppm, indicating that they were sensitive to the sanitizer at label use concentrations (see Table S3 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507).

We intended to investigate the in vitro virulence of all the outbreak-associated isolates, in addition to the reference isolates (12 total isolates), in a Caco-2 cell model; however, 5 Salmonella Heidelberg outbreak-associated strains grew in up to 250 μg/ml gentamicin when incubated in Dulbeco modified Eagle medium with 10% fetal bovine serum. Consequently, we tested resistance to amikacin disulfate, an aminoglycoside to which the isolates were anticipated to be sensitive. Seven of the 12 isolates were resistant to 250 μg/ml amikacin disulfate (data not shown). Consequently, we proceeded with standard gentamicin protection assays for the six gentamicin-susceptible isolates and omitted the other six from our in vitro studies. We were able to assess the invasiveness of four Salmonella Heidelberg outbreak-associated isolates; two isolates were resistant to both gentamicin and amikacin and therefore were not assessed. We also assessed reference strains SL476 and 14028. While strain was a significant factor in the model (P = 0.049), there were no significant differences in mean invasiveness among the six strains after adjusting for multiple comparisons. Invasiveness ranged from approximately 3% for R1-0009 to just over 1.2% for R1-0006 (see Fig. S4 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507).

Heat stress increases expression of some HSPs, virulence, and stress responses genes in Salmonella Heidelberg R1-0006, R1-0007, and SL476. (i) Increased expression of HSPs. The heat shock protein (HSP) genes hscBA were upregulated in all three isolates, but more so in SL476 than in R1-0006 and R1-0007 (Fig. 4). Conversely, stress response regulator rpoH was expressed at a higher level in R1-0006 and R1-0007 than in SL476. Only R1-0006 showed significantly increased expression of the heat shock protein gene dnaJ, while R1-0007 was the only isolate with significant increases.
in the expression of *ibpB*. SL476 showed lower differential expression of the heat shock protein gene *ibpA*; R1-0006 and R1-0007 showed less differential expression of the genes encoding heat shock proteins GroS, HslR, and SigE. All three isolates showed significantly increased expression of the negative regulators of SigE, RseBC and stress-associated chaperone ClpP. R1-0006 and R1-0007 also had significantly decreased expression of *sigE* and increased expression of negative regulator *rseA*.

Among the genes encoding stress tolerance systems, most genes were significantly less expressed during heat stress (Fig. 4), including genes for osmotic stress, cold shock, oxidative stress, and acid stress. Expression of cold shock protein genes *cspAE* was decreased among all three isolates, and *cspC* expression was decreased in SL476 and R1-0006. However, R1-0006 had increased expression of phage shock protein genes *pspABDE*, fusaric acid resistance gene *SeHa_C1611*, and stress-associated gene *deaD*. R1-0006 and R1-0007 also had significantly decreased expression of *rseA*.

**(ii) Heat stress induces increased expression of virulence genes.** During heat shock, multiple genes associated with *S. enterica* virulence showed significantly increased expression among the three isolates (Fig. 5). Salmonella pathogenicity island 1 (SPI-1) genes *invI*, *epaR*, and *yopJ* (avrA) had significantly increased expression across all three isolates. Additionally, SPI-1 genes *invE*, *epaOQR*, *spaS*, and *orgB* had significantly increased expression in SL476. In R1-0006, SPI-1 genes *invG*, *epaR*, and *orgB* showed significantly increased expression; however, *invH*, *sicA*, *prgHIU*, *sopB*, and *sopE2* all had significantly decreased expression compared to that during stationary phase. In R1-0007, SPI-1 genes *epaQR* and *inv* also showed significantly increased expression, but *invFH*, *sicA*, *sipAB*, *iapB*, *prgHIU*, *sopB*, *sopE*, and *sopE2* all had significantly decreased expression under heat stress. Multiple SPI-2 genes had increased expression among the three isolates (Fig. 5), but the patterns of gene expression varied substantially. SL476 showed the greatest number of SPI-2 genes with significantly increased expression; expression of *sseDEFGJ*, and *ssaCDEJKLOV* significantly increased. In R1-0006, the genes
siB, ssaT, and sseDFG showed significantly increased expression. R1-0007 had the fewest SPI-2 genes with significantly increased expression; sseF had significantly higher expression under heat stress, while ssaG and pipB2 had significantly lower expression. Other secretion systems were also affected (Fig. 5). SPI-5 gene pipA had increased expression across all three isolates. SL476 also showed significantly increased expression of SPI-3 gene mgtC and SPI-5 genes pipAB. Additionally, four type VI secretion system (T6SS) genes had significantly increased expression across all isolates.

Three other putative virulence genes—sinH, nleB, and the gene for a putative attachment/invasion locus (SeHa_C0439)—had significantly increased expression during heat stress. Conversely, invasion response regulator uvrY and a virK homologue (SeHa_C1038) showed significantly decreased expression across all three isolates. SL476 and R1-0006 also showed significantly reduced expression of cold shock protein genes cspCE, which have been shown to affect virulence (33), and in SL476 and R1-0007 the expression of another copy of virK (SeHa_C2956) was also significantly decreased. R1-0007 showed significantly reduced expression of virK but significantly increased expression of putative virulence gene mviM.

(iii) **Heat stress upregulates multidrug efflux pumps.** During heat stress, the genes for multiple antibiotic resistance or multidrug efflux pumps were differentially expressed in all three isolates (Fig. 6). The patterns of expression for antibiotic resistance genes varied across the isolates. Streptomycin resistance gene strB was expressed at a significantly higher level in SL476, while mig-14, which may be involved in polymyxin resistance (34), was expressed at significantly lower levels. Conversely, the expression of streptomycin resistance gene strA was significantly decreased in R1-0006; however, SeHa_C2520, potentially encoding antibiotic resistance gene norA, had significantly increased expression. Multidrug efflux pump genes macAB and mdfA had significantly increased expression across all isolates. The genes mdtB, sbmA, acrD, dinF, and envR had significantly increased expression in SL476, while the expression of mdtHIJK and acrR was significantly decreased under heat shock conditions. In R1-0006, multidrug transporter genes mdtG, dinF, emrB, and envR and acriflavine/quaternary ammonium efflux pump gene smvA showed significantly increased expression under heat stress, but...
expression of the master efflux pump gene tolC, as well as mdtHJ, acrR, and marR, was significantly decreased. MDR efflux pump genes mdtG, mdtM, mdtQ, dinF, emrB, envR, SeHa_C0564, SeHa_C2503, and smvA also had significantly increased expression in R1-0007, but marAR, acrR, mdtJK, mprA, and ramA had significantly lower expression than they did under control conditions.

Heat-tolerant isolates R1-0006 and R1-0007 have higher expression of stress response systems, virulence genes, and multidrug efflux pumps in stationary phase than SL476. (i) Increased expression of stress response genes. HSP gene hslR expression was increased in both R1-0006 and R1-0007 during stationary phase (Fig. 7) compared to that in SL476 during stationary phase. In R1-0006, HSP genes hspQ, hslR, dnaJ, clpB, and clpX were also expressed at significantly higher levels. Acid stress chaperone gene hdeB was also expressed at significantly higher levels. R1-0007 showed significantly higher expression of HSP genes hslR and hslU, while the sigE negative regulator gene rseA and HSP gene cpxP were expressed at significantly lower levels than in SL476 during stationary phase. Putative disulfide bond chaperone gene dsbI and heat-induced spheroplast protein Y gene spy were also expressed at significantly lower levels in R1-0007.

A number of stress-related genes were expressed at significantly higher levels in R1-0006 during stationary phase (Fig. 7), including general stress response protein gene ycfR (bhsA), phosphate starvation protein gene psiF, two copies of phage shock protein gene psdD, and oxidative stress protein genes soxR and dps. Acid stress response genes aaeAB were also expressed at higher levels. Multiple genes involved in osmotic stress (e.g., osmBCYW) were expressed at significantly higher levels. Other genes expressed at significantly higher levels included the gene encoding parquat-inducible protein B (pqiB_1), comA (ydeJ, SeHa_C1685), and the general stress protein 39 gene (SeHa_C3405). Cold shock protein gene cspD, universal stress protein gene uspF, phosphate starvation protein gene psiB, and copper resistance gene copD were all expressed at significantly lower levels in R1-0006 than in SL576.
Fewer stress-related genes were differentially expressed in R1-0007 in stationary phase compared to SL476 gene expression levels (Fig. 7). oxyR regulon gene dps, phosphate starvation stress protein gene psiF, phage shock protein gene pspD, a putative secondary copy of the pspD gene, and comA (ydeJ, SeHa1685) were expressed at significantly higher levels in R1-0007. Conversely, cold shock protein gene cspD, telluride resistance gene tehB, and phosphate starvation protein gene psiB were downregulated.

(ii) Outbreak-associated isolates have higher expression of virulence genes than SL476 in stationary phase. Multiple SPI-1 and SPI-5 genes were expressed at significantly higher levels in R1-0006 and R1-0007 than in SL476 in stationary phase (Fig. 8). Several fimbrial genes were also expressed at significantly higher levels. In contrast, most identified type IV secretion system (T4SS) genes were expressed at significantly lower levels. SPI-1 virulence genes expressed at significantly higher levels included spaP (epaP), sitAB, sicA, invAGHIJR, spaS, and effector genes sipAB and sopAB; SPI-1 effector genes sopE and invR (SeHa_C3091) were expressed at significantly lower levels (Fig. 8). SPI-2 gene ssaEJ, SeHa_C1543, and effector genes pipB and pipB2 were expressed at significantly higher levels in R1-0006, while ssaT, trtB, and pipA were expressed at significantly lower levels. Four putative T6SS (SPI-6) genes were differentially expressed. ompA, sciS (icmF, SeHa_C0323), and a gene matching virG (SeHa_C0348) were expressed at significantly higher levels, while an hcp gene (SeHa_C3375) was expressed at significantly lower levels. Additionally, virulence-associated genes zinc transcriptional regulator gene zur, surface-exposed virulence protein gene bigA, virulence genes nleB, phoP, and virK, and virulence-associated flagellar genes srfABC were expressed at significantly lower levels, while psiF was expressed at significantly higher levels.

Fewer SPI-1 virulence genes were expressed at significantly higher levels in R1-0007 than in R1-0006 compared to their levels of expression in SL476 (Fig. 8). SPI-1 genes expressed at significantly higher levels included spaP (epaP), sitAB, sicA, invAGHIJR, spaS, and effector genes sipAB and sopAB. SPI-2 genes ssaCEKQ and effector genes pipB and pipB2 were all expressed at significantly higher levels. ompA and sciS (icmF, SeHa_C0323), involved in expression of T6SS genes, and mgtAC and slsA of SPI-3 were also expressed at significantly higher levels in R1-0007. Finally, R1-0007 had significantly higher expression of virulence-associated genes nleB and psiF and significantly lower expression of virulence regulator gene phoP and virulence genes srfABC and mviM.
R1-0006 has higher stationary-phase expression of multidrug efflux pumps than SL476. *pmrD*, involved in resistance to polymyxin (35), was expressed at significantly lower levels during stationary phase in R1-0006 than in SL476, as was aminoglycoside resistance gene *aac(6\(^{-}\))Iaa* (Fig. 9). R1-0006 had significantly higher levels of expression of multidrug efflux pump genes *mdtAB*, *mdtG*, *mdtK*, *baeS*, and *acrB* than SL476, while *acrR* was expressed at significantly lower levels. Only the efflux pump gene *emrD* was expressed at significantly lower levels in R1-0007 than in SL476. *marAR* was expressed at significantly higher levels in both isolates R1-0006 and R1-0007 than in SL476, and multidrug-efflux pump gene *mdtIJ* and the putative multidrug efflux gene SeHa_C1561 were expressed at significantly lower levels in the two isolates than in SL476.

**DISCUSSION**

Genotypic analyses. Determining genetic differences among the isolates was tricky. A nucleotide difference (ND)-based phylogenetic analysis showed that isolate pairs from the same PFGE type typically clustered together, while cgMLST (36) divided all the isolates into unique STs. A search of the EnteroBase database (36) for the STs in this study found that SL476, R1-0001, R1-0002, R1-0004, R1-0005, R1-0006, and R1-0007 were the only isolates in their STs, while there was just one other isolate in R1-0003’s ST. This suggests that the cgMLST classification system employed by EnteroBase may be overly stringent.

Phylogenetic analyses better illuminated the differences between the isolates. The ND matrix showed the largest differences (58 NDs) between isolates with PFGE type JF6X01.0045; the data indicate that R1-0002 is an MDR isolate, while R1-0004 is pansusceptible. The isolates with PFGE pattern JF6X01.0041 (R1-0001, R1-0005) had identical antibiotic resistances but differed by 24 single nucleotide polymorphisms (SNPs), while R1-0003 and R1-0006 (JF6X01.0258) differed by 22 NDs, with R1-0006 being resistant to only three of the five antimicrobials that R1-0003 had resistance to. Overall, the data indicated that isolate pairs from the same strain were not necessarily highly related. This is not surprising, as the outbreak involved multiple processing establishments, which were in turn sourced from multiple farms (28, 29). Hoffmann et al. found as few as 17 SNPs between isolates from the 2011 outbreak associated with ground turkey (37, 38), while Keefer et al. found that retail and clinical *Salmonella* Heidelberg isolates with the same PFGE profile had 35 SNPs different among them (39). This makes JF6X01.0045 isolates unusually dissimilar for isolates from the same strain.
within an outbreak, while isolate pairs from strains JF6X01.0258 and JF6X01.0041 differ within normal parameters. While SL476 was quite dissimilar from the outbreak-associated isolates, the NDTree phylogenetic analysis pipeline determined it to be the most appropriate reference genome for the isolates, affirming it as a good reference for this study.

All isolates, regardless of their phenotypic antibiotic resistance profile, contained aminoglycoside resistance gene \textit{aac(6')-Iaa}, which codes for a deactivator of kanamycin and amikacin (40). However, this gene does not provide clinical-level resistance to kanamycin or amikacin (40); in fact, this class of aminoglycoside resistance genes is often not expressed in \textit{S. enterica} (41, 42). Consequently, its presence alone does not adequately explain the high levels of amikacin resistance that we uncovered when attempting to use amikacin for cell invasion assays. However, alignment of the isolate sequences with the reference sequence revealed that the isolates shared seven point mutations leading to amino acid substitutions. These mutations may have increased amikacin inactivation, especially as several mutations changed the charge type or size of the amino acid R group. Additionally, this gene was expressed in SL476 and R1-0007 and at low levels (mean, 25 copies) in R1-0006 as well. This may explain the resistance to amikacin that we uncovered when attempting to use it for invasion assays.

All the isolates also had a T57S mutation in topoisomerase, encoded by \textit{parC}. This mutation is associated with resistance to ciprofloxacin and nalidixic acid, which none of the isolates were phenotypically resistant to. However, the literature indicates that \textit{gyrA} mutations are primarily responsible for resistance to these antimicrobials (43–45). The T57S mutation in the protein encoded by \textit{parC} does not seem to yield clinical levels of fluoroquinolone resistance, and it is rarely found in strains without \textit{gyrA} mutations (42, 45, 46). One study remarked that certain \textit{parC} mutations were associated with specific serovars of \textit{S. enterica} (45). However, Keefer et al. did not find it among the 12 \textit{Salmonella} Heidelberg isolates that they studied (39), suggesting that it is not specifically associated with \textit{Salmonella} Heidelberg as a serovar. Consequently, the significance of this mutation in our isolate set is unclear. However, this underscores that antibiotic resistance data from genomic analyses must be interpreted with caution in the absence of phenotypic data.

\textbf{Enhanced heat tolerance may contribute to processing intervention survival and therefore to outbreak scope and duration.} The heat tolerance of SL476 was similar to that found in published data for \textit{Salmonella} Enteritidis, but with more extreme tailing of the survival curve (15, 47, 48), while \textit{Salmonella} Typhimurium ATCC 14028 had a somewhat lower heat tolerance than SL476 (not statistically significant). R1-0006 and R1-0003 were from strain JF6X01.0258, a strain that caused 186/634 outbreak illnesses, yet represented less than 1% of the \textit{Salmonella} Heidelberg isolates in PulseNet prior to the start of the outbreak (28). This strain was also associated with illnesses from rotisserie chicken (28). Consequently, R1-0006’s enhanced heat tolerance may be epidemiologically important.

Several recent studies have investigated the transcriptional response of \textit{S. enterica} to stress. Pin et al. exposed \textit{Salmonella} Typhimurium strain 4/74 stationary-phase cultures to 45°C or pH 5 for 30 min and compared the microarray data from RNA collected (i) during exposure to these conditions, (ii) immediately after reversal to normal conditions, and (iii) at 30 min postreversal with those for control cultures exposed to 25°C and pH 7 (49). They found that acidification changed the transcriptional levels of only a few genes, while heat stress caused widespread transcriptional changes (49). They also found that 85% of the genes upregulated by heat shock remained upregulated 30 min after the reversal to 25°C and that this was associated with increased tolerance to further heat stress or acidification (49). This agrees with our results, which also found global transcriptional changes after heat shock. While R1-0006 and R1-0007 had varied upregulation of HSPs and stress response systems during heat shock, they also had significantly higher (≥2-fold increase, \textit{P} < 0.05) expression of HSPs and stress proteins in stationary phase at 37°C, potentially priming for adaptation to and survival during heat stress. Sirsat et al. investigated the transcriptome of mid-log-phase \textit{Salmonella}
Typhimurium ATCC 14028 after 10 min at 48°C using a partial microarray (1,152 genes assessed) and also found that several heat shock proteins and stress proteins were upregulated; however, due to the different growth phase and temperature, these results are not directly comparable to our data (50).

Milillo et al. studied the transcriptional response of stationary-phase Salmonella Typhimurium LT2 to heated sodium propionate or sodium acetate (2.5% antimicrobial, pH 4, 55°C) for 1 min (51). They found that multiple genes for heat shock proteins or molecular chaperones were downregulated (51). The authors attributed this to the presence of membrane damage, citing a study which found inhibition of heat shock proteins following membrane damage (51). These results are somewhat similar to the heat shock results that we found for our isolates and suggest that we may have been observing some membrane damage in our isolates. This is not unexpected, given the temperature (56°C). While we did not find data from previous work to which to compare our stationary-phase transcriptomic data, stationary phase is known to induce stress tolerance within S. enterica (52, 53). Therefore, it may be that our more stress-tolerant strains activate this general stress response to stationary phase at higher levels and that this provides cross protection to other stresses.

Salmonella Heidelberg is not considered exceptionally heat tolerant among the salmonellae (15), which is further supported by the fact that SL476 had heat tolerance similar to that of Salmonella Enteritidis (47). Lianou and Koutsoumanis found no significant differences in heat tolerance among S. enterica serovars in a study that included Salmonella Heidelberg, Enteritidis, Typhimurium, and Senftenberg (15). Humpheson et al. noted that within heat-stressed monocultures of Salmonella Enteritidis, a subpopulation of bacteria possessed enhanced heat tolerance, resulting in a survivor tail (47). This suggests that the heat tolerance is often a phenotypic phenomenon and that the isolates that we studied simply had a higher subpopulation of heat-tolerant survivors. In contrast, Nguyen et al. recently identified a heat resistance island in Salmonella Senftenberg, indicating a genotypic basis for heat tolerance in some salmonellae (54). We did not find this island in our heat-tolerant isolates, which is unsurprising, as to date it has been identified only in Salmonella Senftenberg (54). We also did not investigate promoter sequences for mutations which might have led to enhanced gene expression, but we recognize that this could contribute to differential expression. Further, some genes in S. enterica are known to have multiple transcriptional start sites.

**Heat stress does not consistently enhance virulence gene expression.** Heat stress has been observed to increase virulence gene expression under some circumstances in S. enterica. When Sirsat et al. investigated the transcriptome of mid-log-phase Salmonella Typhimurium ATCC 14028 after 10 min at 48°C using a partial microarray, they found that a number of genes involved in virulence were also upregulated (50). Conversely, when Milillo et al. studied the transcriptional response of stationary-phase Salmonella Typhimurium to heat and acid stress by exposing Salmonella Typhimurium LT2 to heated 2.5% sodium propionate or sodium acetate, pH 4, at 55°C for 1 min (51), they found that transcription of virulence genes was not increased, a result that they attributed to membrane damage (51). Our results had some similarities with those of both studies, with upregulation of some virulence genes (including genes from SPI-1 and SPI-2) under heat stress but with a pattern which varied across isolates. Consequently, the effect of heat stress on virulence gene expression may be isolate dependent.

**Stress tolerance may provide an adaptive advantage for attachment under adverse conditions but is not associated with sanitizer tolerance.** Attachment ability is known to be highly variable among and within S. enterica serovars. Previous investigators detected serovar-specific differences in attachment; however, they also found high variation within isolates of the same serovars (16, 24). Keelara et al. compared the attachment of Salmonella Typhimurium, Infantis, and Derby strains in a standard crystal violet microtiter assay and found that fewer Salmonella Typhimurium
strains than strains of the other two serovars exhibited moderate or strong attachment (55). Conversely, our *Salmonella* Typhimurium isolates had the highest overall attachment capacity in this study. Studies of *Salmonella* Heidelberg have generally found it to be an average biofilm former (56, 57). In contrast, our data indicated that all *Salmonella* Heidelberg isolates except R1-0001 and R1-0005 were weak or nonattaching biofilm formers, using the categories and conditions defined by Stepanović et al. (24 h of attachment at 35°C; we tested the isolates at 37°C; data not shown) (16). However, the fact that R1-0006 demonstrated greater attachment than the other isolates under stressful conditions (e.g., cold, nutrient-poor conditions) may indicate that at least some of the outbreak isolates are better able to form biofilms as a response to certain stressful conditions, such as a chiller in a processing plant or a grocery store cold case.

As enhanced sanitizer tolerance could increase *S. enterica* survival in the poultry processing environment, we tested *Salmonella* Heidelberg, Typhimurium, and Newport food isolates, as well as two reference strains, for increased tolerance to a PAA-based sanitizer (see Table 2 for the complete isolate list). None of the isolates that we studied showed tolerance to PAA-based sanitizers (see Table S3 posted at https://www.researchgate.net/publication/33369668_AJE2_Supplemental_data_20190507). Some *Salmonella* Typhimurium isolates can develop tolerance to peracetic acid compounds (58), and studies in other pathogens indicate that multidrug-resistant pathogens may be more likely to be sanitizer tolerant (59, 60). However, it is crucial to note that the conditions under which we studied sanitizer tolerance did not contain organic soils common in the poultry processing environment, which can reduce sanitizer efficacy (61). Furthermore, studying the sanitizer tolerance of biofilms from these isolates would also have been useful; however, it was beyond the scope of this study.

**Isolates with enhanced tolerance to one stress are more likely to tolerate other stresses.** In our study, heat-tolerant isolate R1-0006 showed the highest attachment levels of any isolate in vitro under conditions of cold and nutrient stress. R1-0006 also demonstrated higher expression of heat shock proteins, general stress systems, osmotic stress systems, and some MDR efflux systems. Ultimately, these data suggest that the phenotypic characteristics of this isolate contributed to its fitness and higher overall prevalence than the other *S. enterica* strains and serovars during the first three phases of the Food Safety and Inspection Service (FSIS) monitoring period (29). Multiple studies have shown that enhanced tolerance to one stress may cross protect the organism against other stresses. For instance, Humphrey et al. found that *Salmonella* Enteritidis isolates that were more tolerant of heat, acid, or hydrogen peroxide also better survived desiccation (62). Others have demonstrated that the preexposure of *Salmonella* Enteritidis (63) and Typhimurium (49) to sublethal temperatures increased their tolerance to both heat and acid in subsequent exposures, indicating an overlap in stress protection systems. Hamilton et al. studied the transcriptome of *Salmonella* Typhimurium in a nonstatic biofilm system and found that, in addition to upregulation of attachment genes, *Salmonella* Typhimurium upregulated genes involved in the heat and oxidative stress response (64).

**Heat stress may provide cross protection against antimicrobial stress.** In addition to showing significantly increased expression of heat shock proteins and stress genes, all *Salmonella* Heidelberg isolates had higher expression of efflux pumps under heat stress. This may indicate that these isolates are more likely to develop antimicrobial tolerance under high-stress conditions, especially since overexpression of *mdfA* can produce resistance to tetracycline, chloramphenicol, doxorubicin, and norfloxacin, *macAB* overexpression provides resistance to erythromycin, and the *emrAB* system provides resistance to nalidixic acid (65). In agreement with this, the AcrAB-TolC antibiotic efflux system has been shown to be upregulated in response to bile and oxidative stress (66, 67). Additionally, *macAB* have been shown to play a role in virulence in *Salmonella* Typhimurium and are induced by conditions inside macrophages (65). Consequently, upregulation of these pumps during exposure to stress could increase virulence and antibiotic tolerance or even resistance.
Epidemiological and in vitro data do not support enhanced virulence among outbreak strains. Given the length and scope of the outbreak (28, 29), we hypothesized that the outbreak-associated Salmonella Heidelberg isolates might also have increased virulence; however, we found no evidence of increased invasiveness among the isolates tested. This is similar to the findings in other studies of increased invasiveness in Salmonella Typhimurium DT104, a multidrug-resistant strain associated with increased hospitalization risks (68, 69). Studies with other model systems may be warranted to more effectively evaluate virulence potential.

Whether antibiotic-resistant isolates are more virulent than pansusceptible isolates is disputed (68, 70). In this outbreak, the strains that caused the most hospitalizations and highest blood infection percentages displayed MDR phenotypes (28, 29). Conversely, apart from strain JF6X01.0672, which caused two cases of disease (neither of which resulted in blood infections), the lowest percentage of blood infections was found for pansusceptible strains (or strains for which most isolates were pansusceptible) (28, 29). Due to the high gentamicin resistance of our MDR isolates, we were unable to obtain in vitro results for comparison; however, epidemiological data from this outbreak (28) indicate that MDR isolates of Salmonella Heidelberg may be more invasive.

Data from PulseNet listing the numbers of isolates of each strain (i.e., PFGE PulseNet pattern) derived from blood, stool, and other sites in patients during the outbreak showed that there were substantial differences in blood infection percentages among the seven strains, with the percentage of blood infections varying from 0% to 17% (72). Of the six strains represented in our study, four had blood infection percentages of between 14 and 17% (see Table S2 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507) (72).

The strain responsible for the highest number of outbreak illnesses was JF6X01.0258 (R1-0003, R1-0006) (28); this strain was also associated with increased heat tolerance in our in vitro assays. However, it did not have a high hospitalization rate (31% versus an average for this outbreak of 38%) and had an expected invasive illness rate (14% versus an average of 15% for this outbreak) (72). Heat-tolerant strain JF6X01.0326 (R1-0007) had a 36% hospitalization rate but only a 6% invasive illness rate (28, 72). Finally, heat-susceptible strain JF6X01.0041 (R1-0001, R1-0005) caused 103 illnesses and had a 49% hospitalization rate and a 16% invasive illness rate (28). Invasive illnesses rates (for which the percentage of blood isolates is a proxy measurement [30]) are likely a more accurate epidemiological measure of isolate virulence than hospitalization rates, as hospitalization may be affected by factors unrelated to isolate virulence, such as patient age, climate, or poor hydration management. However, regardless of the measure used, there does not appear to be a strong relationship between heat tolerance and virulence potential.

Summary. Combined, these data suggest that some outbreak-associated Salmonella Heidelberg isolates may be uniquely primed to survive processing-type stresses, but considerable variation existed among the isolates. Data from this work will inform future risk assessments and industry best practices for Salmonella Heidelberg and other S. enterica serovars and may contribute to the development of gene expression-based assays to identify isolates more likely to survive processing stresses.

MATERIALS AND METHODS

Strains used in this study. The strains sourced and used in this study are described in Table 2. All strains were stored in 20% glycerol at −80°C. Strains were recovered in tryptic soy broth (TSB) or tryptic soy agar (TSA) at 37°C (Becton, Dickinson, Franklin Lake, NJ). We obtained nine food-derived isolates representing six of the seven outbreak strains from the USDA Food Safety and Inspection Service through the USDA Agricultural Research Service (28). These isolates were compared to Salmonella Heidelberg reference strain SL476, Salmonella Typhimurium reference strain ATCC 14028, and a Salmonella Enteritidis strain.

Genomic analyses. The U.S. Food and Drug Administration sequenced seven of the nine outbreak isolates that we studied and placed their raw read genomic sequences in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) collection (Table 1). Reads were downloaded and transferred to Purdue’s Research Computing clusters. Reads were trimmed using the Trimmmomatic trimmer tool to a quality score (Q) of 20 (73) and assembled using the SPAdes (v.3.10.1)
algorithm (74), and the assemblies were assessed using the Quast (v.3.2) program (75). Assemblies were submitted to Center for Genomic Epidemiology (CGE) servers to search for plasmids (PlasmidFinder [v.2.0] [76]) and antibiotic resistance genes and mutations (ResFinder [v.3.1] [77]) and to determine the MLST of the isolates (MLST [v.2.0] [32]). All CGE programs were run on the default settings. As an additional typing mechanism, raw reads were submitted to analysis with the CGE cgMLSTFinder tool (v.1) (36). To further assess isolate relationships, the (i) original 8 isolates and (ii) a fuller set of 12 isolates (the same isolates plus 4 isolates from 2013 with PFGE profiles matching those of the two other 2013-2014 outbreak strains [JF6X01.0022, JF6X01.0122]) were downloaded from the NCBI SRA and submitted to NDtree (v.1.2), a raw reads-based phylogenetic tree tool (78–80). NDtree was allowed to choose the best reference genome and chose that with GenBank accession no. NC_011083 (SL476) as the template. The phylogenetic tree was visualized using the FigTree (v.1.4.4) program (available from http://tree.bio.ed.ac.uk/software/figtree/).

**Heat shock assays. **S. enterica cultures were isolated on TSA and grown overnight for 18 to 24 h. A single colony was inoculated into 5 ml of TSB and grown for 16 h at 37°C at 200 rpm; overnight cultures were serially diluted to 10^6 CFU/ml. Cells were grown to log phase (6 h) or stationary phase (12 h), as determined by the use of standard growth curves. Logarithmic- and stationary-phase cultures were aliquoted into 2 ml microcentrifuge tubes (WVR, Radnor, PA) and heat shocked in a 56°C water bath (WVR, Radnor, PA). Aliquots were removed, serially diluted, and plated on tryptic soy agar (Becton, Dickinson, Franklin Lake, NJ) for 0, 2.5, 5, and 7.5 min (log phase) and for 0, 15, 30, 45, and 60 min (stationary phase) of total exposure to 56°C. The time to temperature was determined to be 2.4 min using a thermocouple (Omega, Norwalk, CT) and was accounted for in the total exposure time. The plates were incubated at 37°C for 36 ± 1 h. Experiments were conducted in biological triplicate. Statistically significant differences between isolates and reference strains were determined by repeated-measures analysis of variance (ANOVA) followed by Dunnett’s test, with α equal to 0.05. Analysis was performed in RStudio (v.0.98.1091) software.

**Salmonella attachment assays.** Attachment to abiotic surfaces as an indicator of biofilm-forming potential was measured using a standard crystal violet assay as previously described (81, 82). Isolated colonies of 10 Salmonella Heidelberg isolates, 4 Salmonella Typhimurium isolates, 4 Salmonella Newport food isolates, Salmonella Typhimurium ATCC 14028, and Salmonella Enteritidis were transferred to TSB and incubated at 37°C for 16 to 24 h to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Polystyrene 96-well plates (Corning, Corning, NY) were prepared with 1× (nutrient-rich conditions) or 1/20× (nutrient-limited conditions) TSB, inoculated, and incubated statically at 4°C or 21°C for 1, 2, or 5 days. Attachment in 1/20× TSB at 21°C at 5, 7, and 9 days was also assessed (see Fig. S2 and S3 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507). Attached cells were stained with 0.4% crystal violet and indirectly quantified by determination of the OD_{600}. Experiments were conducted in biological triplicate. Statistical analyses were performed in SAS (v.9.4) software (SAS Institute, Cary, NC). The OD_{600} was log_{10} transformed to fit a generalized linear mixed model with the Proc Glimmix procedure. Analysis of attachment capacity was conducted at 4°C (n = 538), and 21°C (n = 536), respectively, at both the serovar and isolate levels (α = 0.05). The main effects “serovar,” “isolate,” “time,” “serovar × time,” and “isolate × time” were tested to determine a significant correlation with attachment capacity. Tukey pairwise comparisons and least-squares means with the Tukey adjustment were used to identify significant correlations.

**PAA tolerance assay.** Tolerance to a peracetic acid-based sanitizer (BioSide HS, 15%; Madison Chemical Co., Madison, IN) was determined for 18 isolates (Table 2) using a microtiter-based assay as described by Wang et al. (83). Briefly, isolated colonies were grown overnight on TSA at 37°C and then suspended in TSB to an OD_{600} of 0.6 to 0.7. A 1:100 dilution of the suspension was transferred to 2× TSB for a final concentration of 1× TSB; 50 μl of the diluted suspension was transferred to assay plates containing 50 μl of each sanitizer solution for final concentrations of 25, 50, 100, and 250 ppm sanitizer. The plates were incubated statically at 37°C. The plates were scanned at OD_{600} at 0 and 24 h to determine the MIC. Experiments were conducted in biological triplicate. Isolates that consistently grew in ≥50 ppm PAA after 24 h (OD_{600} > 0.1) were considered tolerant (see Table S3 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507). Analysis is limited to descriptive statistics.

**Caco-2 cell invasion assays.** Caco-2 cell (gentamicin protection) invasions assays were conducted as previously described (84–86). Briefly, stationary-phase cultures were isolated on TSA and grown overnight for 18 to 24 h. A 0.1 ml of the diluted suspension was transferred to assay plates containing 1 ml of the diluted suspension was transferred to assay plates containing 0.1 ml of the diluted suspension was transferred to assay plates containing 1× TSB; 50 μl of the diluted suspension was transferred to each plate of the diluent for 1, 2, or 5 days. Attached cells were stained with 0.4% crystal violet and indirectly quantified by determination of the OD_{600}. Experiments were conducted in biological triplicate. Statistical analyses were performed in SAS (v.9.4) software; significance was defined as a P value < 0.05. Analysis was limited to descriptive statistics.

**RNA sequencing and data processing.** RNA was extracted from stationary-phase (37°C; control) and heat-shocked (5.1 min of total exposure at 56°C) R1-0006, R1-0007, and SL476 cells using the TRIzol reagent (Ambion, Foster City, CA) in triplicate for each isolate and condition. A Ribo-Zero kit (Illumina, San Diego, CA) was used to remove rRNA; TURBO DNase (Thermo Fisher, Waltham, MA) was used to remove DNA. DNA removal was assessed using quantitative reverse transcription-PCR for rpoD and gyrB (71, 84–86). Primers, probes, and 2X master mix were purchased from Integrated DNA Technologies (IDT), Coralville, IA (see Table S4 posted at https://www.researchgate.net/publication/333609668_AJE2_Supplemental_data_20190507). RNA samples with no reverse transcriptase were used to assess residual DNA contamination. cDNA libraries were constructed using a ScriptSeq (v.2) library kit for bacteria using the low-input protocol with the RiboZero kit (Illumina, San Diego, CA), and samples were sequenced on an Illumina HiSeq 2500 platform (San Diego, CA) by the Purdue University Genomics Core (West Lafayette, IN).
Transcriptomic analyses were performed on Purdue University Research Computing clusters. Reads were trimmed to Q20, and adaptors were removed using the Trimomatic (v.0.36) trimmer tool (73). The trimmed reads were aligned with the SL476 index sequence (created from NCBI RefSeq genome accession no. NC_011083.1 using the Bowtie2 [v.2.1.0] program) using TopHat (v.2.1.1) software (87); read counts were obtained using the HTSeq (v.0.6.1) package (88). Differential expression between control and heat shock samples was determined using the DESeq2 (v.1.12.4) program (89). For all comparisons, genes with a base mean read count of <30 were removed due to low read counts. Genes with a log, fold change in expression of >1 and a P<0.05 were considered significantly differentially expressed (DE). Transcriptomes were annotated using the KEGG online database for SL476 as well as a publicly available general feature format (GFF) of SL476. Unannotated gene calls were made using the National Center for Biotechnology Information (NCBI) online protein BLAST (blastp suite) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to search the nonredundant protein sequences database. For proteins with ambiguous results, protein sequences (per KEGG SL476 data) of top gene hits were aligned with sequences in the UniProt database (90) using the EMBOS Needle gene-wise aligner (protein setting, http://www.ebi.ac.uk/Tools/psa/emboss_needle/) (91). Where genes had multiple names, both were included with the current or most common name first.

Data availability. RNA sequencing data (raw read files) for this project are available for download from NCBI GEO under accession number GSE103418 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103418).

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