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Research Paper

Transcriptome Analysis of *Salmonella* Heidelberg after Exposure to Cetylpyridinium Chloride, Acidified Calcium Hypochlorite, and Peroxyacetic Acid

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ABSTRACT

The application of RNA sequencing in commercial poultry could facilitate a novel approach toward food safety with respect to identifying conditions in food production that mitigate transcription of genes associated with virulence and survivability. In this study, we evaluated the effects of disinfectant exposure on the transcriptomes of two field isolates of *Salmonella* Heidelberg (SH) isolated from a commercial broiler processing plant in 1992 and 2014. The isolates were each exposed separately to the following disinfectants commonly used in poultry processing: cetylpyridinium chloride (CPC), acidified calcium hypochlorite (aCH), and peroxyacetic acid (PAA). Exposure times were 8 s with CPC to simulate a poultry processing dipping station or 90 min with aCH and PAA to simulate the chiller tank in a poultry processing plant at 4°C. Based on comparison with a publicly available annotated SH reference genome with 5,088 genes, 90 genes were identified as associated with virulence, pathogenicity, and resistance (VPR). Of these 90 VPR genes, 9 (10.0%), 28 (31.1%), and 1 (1.1%) gene were upregulated in SH 2014 and 21 (23.3%), 26 (28.9%), and 2 (2.2%) genes were upregulated in SH 2014 challenged with CPC, aCH, and PAA, respectively. This information and previously reported MICs for the three disinfectants with both SH isolates allow researchers to make more accurate recommendations regarding control methods of SH and public health considerations related to SH in food production facilities where SH has been isolated. For example, the MICs revealed that aCH is ineffective for SH inhibition at regulatory levels allowed for poultry processing and that aCH was ineffective for inhibiting SH growth and caused an upregulation of VPR genes.

Key words: Differential gene expression; Disinfectant; Poultry processing; RNA sequencing; *Salmonella*

Salmonella is a major foodborne pathogen worldwide and is highly associated with contaminated poultry products. The Centers for Disease Control and Prevention *(3)* estimates that in the United States *Salmonella* causes approximately 1.2 million foodborne illnesses, 23,000 hospitalizations, and 450 deaths per year. *Salmonella enterica* subsp. *enterica* serovar Heidelberg (SH) is one example of a *Salmonella* serovar that has been linked to poultry-associated outbreaks in humans and continues to be isolated in poultry processing plants *(34, 43)*.

Currently, the common methodology for evaluating the efficacy of disinfectants against *Salmonella* requires identifying the inhibitory concentration and or log reduction of the bacteria due to the action of the disinfectant *(28)*. However, *Salmonella* isolates from poultry products and processing plants can be both tolerant to disinfectants and resistant to antibiotics despite not being challenged with antibiotics during poultry production and/or processing *(22, 23, 25, 42)*. Positive correlations have been found between tolerance to disinfectants and resistance to antibiotics in poultry products *(22, 23)*. In *Salmonella,* the development of increased tolerance to disinfectants and the simultaneous increased resistance to antibiotics after stepwise exposure to disinfectants is well established *(2, 21, 24)*. Therefore, although the mechanisms conferring antimicrobial resistance after exposure to disinfectants are complex, an understanding of the effects of disinfectants on *Salmonella* is becoming more important as antimicrobial resistant and disinfectant tolerant bacteria are becoming more prevalent in the food chain *(27, 38)*.

High-throughput RNA sequencing (RNA-seq) is one tool that can be used to monitor changes in gene expression due to stressors such as disinfectants. From a food safety perspective, RNA-seq could be used to determine critical food safety parameters in a food system environment, with

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TABLE 1. *MICs for SH, regulatory ranges, and experimental concentrations for each disinfectant*

	MIC for SH 2014 (ppm)		MIC for SH 1992 (ppm)		Concn (ppm)	
Disinfectant	Bacteriostatic	Bactericidal	Bacteriostatic	Bactericidal	Regulatory	Experimental
CPC	300	600	300	300	500–8,000	62.5
aCH	>3,200	>3,200	>3,200	>3,200	20–50	20
PAA	900	4,600	900	3,700	200–2,000	130

the ultimate goal of identifying conditions in food production that mitigate transcription of genes associated with antimicrobial resistance and virulence. Until recently, high-throughput sequencing technologies such as RNA-seq were very expensive and time-consuming, making them impractical for food safety applications. However, with rapid advances in sequencing technologies, high-throughput sequencing is becoming more cost effective and practical for use in food safety systems *(37, 39)*.

In this study, two field strains of SH isolated from a commercial poultry processing plant were challenged with three disinfectants commonly used in the chiller tank and dipping stations in poultry processing plants. The aim of this study is to analyze virulence, pathogenicity, and resistance (VPR) gene expression in SH challenged with subinhibitory concentrations of cetylpyridinium chloride (CPC), acidified calcium hypochlorite (aCH), and peroxyacetic acid (PAA) to evaluate the risk of these disinfectants increasing VPR expression in SH.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Two SH strains (California Animal Health Food Safety Laboratory, Davis) that were originally isolated from a commercial processing plant in 2014 and 1992 were used for this study. Frozen stock cultures for both strains were made using 50% glycerol and then stored at –80°C. Overnight cultures were prepared by inoculating 10 mL of Trypticase soy broth (TSB; BD, Sparks, MD) with the SH stock cultures and incubating them overnight at 37°C. Colony counts were performed by plating and incubating on Trypticase soy agar (TSA; BD) plates at 37°C.

Determining the disinfectant concentration for the SH challenge in preparation for RNA extraction. SH was challenged with CPC, aCH, and PAA concentrations of 62.5, 20, and 130 ppm, respectively (disinfectants were provided by a commercial poultry company and diluted with TSB in the laboratory), prior to RNA extraction. These concentrations were previously determined to be below the MIC *(6)*. Based on the MICs, CPC had a bacteriostatic concentration of 300 ppm for both SH strains and bactericidal concentrations of 600 and 300 ppm for SH 2014 and SH 1992, respectively (Table 1). PAA had a bacteriostatic concentration of 900 ppm for both strains and bactericidal concentrations of 4,600 and 3,700 ppm for SH 2014 and SH 1992, respectively (Table 1). The MIC assay could not be used to determine the bacteriostatic and the bactericidal concentrations for aCH because growth occurred even at the highest concentration tested, which was 3,200 ppm (Table 1). The results of the MIC assay indicated that CPC and PAA were effective for the control of SH, whereas aCH was completely ineffective. Challenged SH cultures were spread plated on TSA until 5×10^8 CFU/mL was achieved. After the disinfectant challenge but before the RNA extraction, SH was plated on TSA to determine the CFU. All groups had the same log CFU except for the CPC group from SH 2014, which had a 2-log decrease. The disinfectants used in this study were chosen because of their use in the chiller tank after slaughter and at the dipping stations during second processing. The temperature of 4°C and contact times of 8 s for CPC and 90 min for both aCH and PAA were also based on how these disinfectants are typically used in a commercial poultry processing plant.

Planktonic SH challenge with CPC in preparation for RNA extraction. An aliquot (1,000 μL) of each of the overnight SH cultures was used to inoculate 99 mL of TSB. The resultant culture was then incubated at 37°C and 100 rpm for 3.5 h. The mid-log culture was then split into two groups, CPC treatment and a no-treatment control. Technical triplicates were made for each group. The CPC group was challenged with 62.5 ppm of CPC for 8 s (plus 12 min for washing steps), whereas the control group had no disinfectant or additional diluent added. For this study, the 62.5-ppm CPC group will be referred to as the 8-s group even though the contact time with the disinfectant was technically closer to 12 min. Both groups were kept at 4°C during the 8-s challenge. Immediately after the challenge, all samples were centrifuged for 5 min at 10,000 rpm (Sorvall Legend X1R, Thermo Fisher Scientific, Waltham, MA) and then reconstituted with TSB. Two additional washing steps were added to increase the RNA integrity scores. All samples were vortexed until the pellets were completely dissolved in preparation for RNA extraction and plating.

Planktonic SH challenge with aCH and PAA in preparation for RNA extraction. Similar to the CPC challenge steps, a 1,000-μL aliquot of each overnight SH culture was used to inoculate 99 mL of TSB. The resultant culture was then incubated at 37°C and 100 rpm for 3.5 h. The mid-log culture was then divided into three groups: aCH, PAA, and no treatment. The aCH and PAA groups were challenged with 20 ppm of aCH and 130 ppm of PAA, respectively, for 90 min at 4°C. The no-treatment control group was kept at 4°C for 90 min and received no disinfectant or additional diluent. The samples were then washed and prepared for RNA extraction as described for the CPC group.

RNA extraction. The RNeasy Protect Bacteria Mini Kit (Qiagen, Valencia, CA) was used for the extraction of total RNA. After reconstituting the culture in the appropriate volume of TSB (determined for a cell pellet size of 5×10^8 as recommended in the protocol), 500 μL of the culture was transferred to 2-mL tubes each containing 1 mL of RNA Protect Bacteria reagent (Qiagen). The samples were then vortexed for 5 s and incubated at room temperature. After 5 min, samples were centrifuged for 10 min at 5,000 × *g*. This modification was made to improve the RNA integrity number. The supernatant was removed, and the pellets were immediately placed in the Qiacube (Qiagen) for RNA extraction.

RNA quality assurance. After RNA extraction, samples were washed again with RNAClean XP beads (Agencourt, Beckman Coulter, Palo Alto, CA) following the single tube format protocol to obtain 260/280 ratios above a minimum of 1.8. Nucleic acid quantification was performed using a Take3 plate (Biotek, Winooski, VT). The next day after validating for growth and counting colonies, RNA integrity was assessed using the a bioanalyzer (Agilent, Santa Clara, CA). When samples had concentrations and RNA integrity numbers below 20 ng/μL, they were concentrated using the RNA Clean and Concentrator Kit 5 (Zymo Research, Irvine, CA). For this experiment, which involved RNA extractions from SH exposed to disinfectants, RNA integrity scores above 5 were deemed acceptable for sequencing. Although this cutoff is relatively low, the combination of extracting RNA from bacteria exposed to disinfectants and previous findings that SH has intervening sequences that make evaluating RNA integrity challenging were taken into account *(31)*.

RNA-seq. When RNA concentrations and integrity scores were acceptable, 30 samples were sent to the DNA Technologies and Expression Analysis Core facility (University of California, Davis) for sequencing. One microgram of the total RNA from each sample was ribodepleted using Gram-Negative Bacteria Ribo Zero kit (Illumina, San Diego, CA). Strand-specific and barcode-indexed RNA-seq libraries were generated from the ribodepleted RNA using a stranded RNA-seq library preparation kit (Kapa, Cape Town, South Africa) and barcoded adapters (NEXTflex, Bioo Scientific, Austin, TX) following the instructions of the manufacturer. The RNA-seq libraries were PCR amplified with HiFi polymerase (Kapa), cleaned up with a 1× volume of AMPure XP beads (Beckman Coulter), and then quantified by fluorometry (Qubit, LifeTechnologies, Thermo Fisher). Quality assurance of each library was determined using a Bioanalyzer 2100 instrument (Agilent), and the library was pooled in equimolar ratios according to the fluorometric measurements. The pooled library was quantified by quantitative PCR with a Library Quant kit (Kapa) and sequenced on one lane of single-end 50 on a HiSeq 3000 apparatus (Illumina) generating on average 13.5 million single-end reads passing the chastity filter per sample.

RNA-seq accession numbers. RNA-seq data were submitted to the National Center for Biotechnology Information SRA database and can be found under study number SRP091888 with the following accession numbers: SRX2255480, SRX2255481, SRX2255482, SRX2255483, SRX2255484, SRX2255485, SRX2255486, SRX2255487, SRX2255488, SRX2255489, SRX2255490, SRX2255491, SRX2255492, SRX2255493, SRX2255494, SRX2255495, SRX2255496, SRX2255497, SRX2255498, SRX2255499, SRX2255500, SRX2255501, SRX2255502, SRX2255503, SRX2255504, SRX2255505, SRX2255506, SRX2255507, SRX2255508, and SRX2255509.

Data analysis. The demultiplexed Illumina reads were aligned to the *Salmonella enterica* subsp. *enterica* serovar Heidelberg strain 41578 gene set (consisting of 5,088 rRNA, tRNA, and protein-coding genes) obtained from the Pathosystems Resource Integration Center *(40)* using the Burrows-Wheeler short read aligner v. 0.6.2 with default parameters *(18)*. Overall, 97 to 98% of the reads aligned to the genome. With the exception of one sample, 78 to 80% of the reads aligned to known genes. Reads that uniquely aligned to a gene with a minimum mapping quality of 10 were counted using Samtools idxstats v. 1.2 *(19)*. The 5,088 genes were filtered to 4,623 genes by removing genes expressed at <0.2 reads per kilobase of transcript per million mapped reads.

Differential expression analyses were conducted using the limma-voom *(17, 33)* bioconductor pipeline, using a model with variables for strain, treatment, chilling, and all two- and three-way interactions of these variables. Differentially expressed genes were detected with a cutoff value of $\log_2$ (fold change) >1 or less than -1 and an adjusted $P < 0.001$.

The *Kyoto Encyclopedia of Genes and Genomes* (KEGG) enrichment analyses were conducted using the KEGGREST Bioconductor package (https://bioconductor.org/packages/release/bioc/html/KEGGREST.html). Pathway enrichment was tested by comparing P values of genes in a given pathway with P values of genes not in the pathway using a one-sided Wilcoxon rank-sum test (i.e., testing whether genes in the pathway have lower P values in a differential expression analysis than genes not in the pathway). KEGG pathways with $P < 0.05$ were considered significantly enriched.

Selection of VPR genes. Of 4,623 genes, 90 genes were selected based on their KEGG orthology (KO) category *(15, 16)*. For this study, virulence and pathogenicity genes ($n = 27$) included genes in the KO categories bacterial invasion of epithelial cells ($n = 9$) and *Salmonella* infection ($n = 18$). Resistance genes ($n = 63$) included genes in the KO categories β-lactam resistance ($n = 21$), cationic antimicrobial peptide (CAMP) resistance ($n = 34$), and vancomycin resistance ($n = 8$). These 90 genes are referred to here as VPR genes.

RESULTS

Because of the batch effects from completing the CPC (plus control) RNA experiments on different days from the aCH and PAA (plus control) RNA experiments, genes were compared only when those extractions were done during the same experiment. Only those samples that were processed on the same day were compared because variations in incubation, challenge, and RNA extraction conditions between days could be completely confounded with the experimental effects of interest *(30)*. Therefore, only SH samples with and without aCH and PAA within the same SH field strain were compared directly, and only SH samples with or without CPC within the same SH field strain were compared directly. The structure of the "Results" section reflects the appropriate comparisons due to batch effects. When comparing gene expression within a batch, genes with false discovery rate adjusted P values of <0.001 were considered significant.

Effects of CPC on the 2014 SH field strain. After a contact time of about 12 min (8 s at 4°C plus 12 min for washing steps), there was a 10.0% (9 of 90 genes) increase in upregulation of VPR genes in SH 2014 exposed to CPC relative to the unexposed control isolate (Table 2). Although 12.7% (8 of 63) of resistance genes were upregulated, only 3.7% (1 of 27) of virulence and pathogenicity genes were upregulated (Table 2). In contrast, no down-regulation of VPR genes was found in SH exposed to CPC relative to the control (Table 2).

Effects of CPC on the 1992 SH field strain. After a contact time of about 12 min (8 s at 4°C plus 12 min for washing steps), there was a 23.3% (21 of 90 genes) increase in the upregulation of VPR genes in SH 1992 exposed to

TABLE 2. *Number of VPR and non-VPR genes that were upregulated and down-regulated for each disinfectant for SH 2014*[a]

		No. (%) of genes after treatment with:					
		CPC		aCH		PAA	
Gene type	No. of genes	Upregulated	Down-regulated	Upregulated	Down-regulated	Upregulated	Down-regulated
Virulence and pathogenicity	27	1 (3.7)	0	2 (7.4)	0	1 (3.7)	0
Resistance	63	8 (12.7)	0	26 (41.3)	0	0	0
Total VPR genes	90	9 (10.0)	0	28 (31.1)	0	1 (1.1)	0
Total non-VPR genes	4,533	331 (7.3)	124 (2.7)	1,145 (25.3)	54 (1.2)	96 (2.1)	125 (2.8)

[a] Onefold cutoff, $P < 0.001$.

CPC relative to culture not exposed to CPC (Table 3). Although 40.7% (11 of 27) of virulence and pathogenicity genes were upregulated, only 15.9% (10 of 63) of resistance genes were upregulated. However, 3.3% (3 of 90) of virulence and pathogenicity genes and 0% of resistance genes were down-regulated in SH exposed to CPC relative to SH not exposed to CPC (Table 3).

Effects of aCH on the 2014 SH field strain. After a contact time of about 90 min at 4°C, there was a 31.1% (28 of 90 genes) increase in the upregulation of VPR genes in SH 2014 exposed to aCH relative to SH not exposed to aCH (Table 2). Although 41.3% (26 of 63) of resistance genes were upregulated, only 7.4% (2 of 27) of virulence and pathogenicity genes were upregulated. In contrast, no down-regulation of VPR genes was found in SH exposed to aCH relative to SH not exposed to aCH (Table 2).

Effects of aCH on the 1992 SH field strain. After a contact time of about 90 min at 4°C, there was a 28.9% (26 of 90 genes) increase in the upregulation of VPR genes in SH 1992 exposed to aCH relative to SH not exposed to aCH (Table 3). Although 39.7% (25 of 63) of resistance genes were upregulated, only 3.7% (1 of 27) of virulence and pathogenicity genes were upregulated. Contrastingly, there was no down-regulation of VPR genes in SH exposed to aCH relative to SH not exposed to aCH (Table 3).

Effects of PAA on the 2014 SH field strains. After a contact time of 90 min at 4°C, the majority of VPR genes (98.9%, 89 of 90 genes) in SH 2014 had no significant upregulation or down-regulation (Table 2). Only one gene (3.7%) from the virulence and pathogenicity category was upregulated, and none of the VPR genes were down-regulated.

Effects of PAA on the 1992 SH field strains. After a contact time of 90 min at 4°C, the majority of VPR genes (97.8%, 88 of 90 genes) had no significant upregulation or down-regulation (Table 3). Of the remaining 2.2% of the VPR genes that had significant differential gene expression, 2.2% (2 of 90) of the VPR genes were upregulated and 0.0% were down-regulated when comparing the SH 1992 strain that was exposed to PAA relative to the SH strain not exposed to PAA (Table 3). Upregulation was observed in 3.2% (2 of 63) of the resistance genes (Table 3).

Effects of aCH and PAA on the 2014 SH field strain. The aCH-challenged SH 2014 was more likely to upregulate VPR genes than was the PAA-challenged SH 2014 (Table 4). SH exposed to aCH had more VPR genes (31.1%, 28 of 90 genes) that were upregulated than did SH exposed to PAA (1.1%, 1 of 90 genes) (Table 2). The biggest differences were in the expression of genes associated with resistance. The aCH-exposed resistance genes (41.3%, 26 of 63 genes) and virulence and pathogenicity genes (7.4%, 2 of 27 genes) were most likely to be upregulated, whereas the PAA-exposed resistance genes (0.0%) did not have significant upregulation, and only (3.7%, 1 of 27) of the virulence and pathogenicity genes had upregulation (Table 2).

Effects of aCH and PAA on the 1992 SH field strain. The aCH-challenged SH 1992 was more likely to upregulate VPR genes than was the PAA-challenged SH 1992 (Table 4). SH exposed to aCH had more VPR genes (28.9%, 26 of

TABLE 3. *Number of VPR and non-VPR genes that were upregulated and down-regulated for each disinfectant for SH 1992*[a]

		No. (%) of genes after treatment with:					
		CPC		aCH		PAA	
Gene type	No. of genes	Upregulated	Down-regulated	Upregulated	Down-regulated	Upregulated	Down-regulated
Virulence and pathogenicity	27	11 (40.7)	3 (11.1)	1 (3.7)	0	0	0
Resistance	63	10 (15.9)	0	25 (39.7)	0	2 (3.2)	0
Total VPR genes	90	21 (23.3)	3 (3.3)	26 (28.9)	0	2 (2.2)	0
Total non-VPR genes	4,533	631 (13.9)	348 (7.5)	981 (21.6)	66 (1.5)	100 (2.2)	13 (0.3)

[a] Onefold cutoff, $P < 0.001$.

90 genes) that were upregulated than did SH exposed to PAA (2.2%, 2 of 90 genes) (Table 3). The biggest differences were in the expression of genes associated with resistance. The aCH-exposed resistance genes (39.7%, 25 of 63 genes) and virulence and pathogenicity genes (3.7%, 1 of 27 genes) were most likely to be upregulated, whereas the PAA-exposed resistance genes (3.2%, 2 of 63 genes) did not have significant upregulation, and 0.0% of the virulence and pathogenicity genes had upregulation (Table 3).

Effects on non-VPR genes. Although the above results focused on the 90 VPR genes, the RNA-seq results provided data regarding 4,533 annotated non-VPR genes (Tables 2 and 3). In summary, the non-VPR genes had levels of significant upregulation and down-regulation similar to those of the VPR genes for each disinfectant. Like the VPR genes, the non-VPR genes had the highest amount of upregulation when SH was exposed to aCH followed by CPC and PAA in both SH strains (Tables 2 and 3).

KEGG pathway analysis. In this study, the bacterial invasion of epithelial cells and *Salmonella* infection pathways from KEGG were analyzed to further support the differential gene expression results. Information on the remaining pathways that were significantly enriched ($P < 0.05$) in SH 2014 (Supplemental Table 1S) and SH 1992 (Table 2S) is available as supplemental material. Only two significantly enriched pathways ($P < 0.05$) were identified, and both were in CPC-challenged SH 1992 (Table 5).

DISCUSSION

Differential gene expression in the two disinfectant-challenged field strains of SH relative to the nonchallenged strains was observed for both VPR and non-VPR genes (Tables 2 and 3). Differential gene expression of both the VPR and non-VPR genes also was noted when comparing how each SH strain responded to aCH and PAA (Tables 2 and 3).

Although SH was not challenged with antibiotics, genes related to antibiotic resistance were upregulated especially when the strains were challenged with aCH; 41.3% (26 of 62 genes) and 39.7% (25 of 63 genes) of the resistance genes were upregulated in the 2014 and 1992 SH strains, respectively (Table 2 and 3). Of the β-lactam, CAMP, and vancomycin resistance genes, 28.6% (6 of 21 genes), 55.9% (19 of 34 genes), and 12.5% (1 of 8 genes), respectively, were upregulated in aCH-challenged SH 2014 (Table 4). Similarly, 23.8% (5 of 21 genes), 52.9% (18 of 34 genes), and 25% (2 of 8 genes), respectively, were upregulated in aCH-challenged SH 1992 (Table 4).

This upregulation of resistance genes is not surprising. Previous studies revealed that SH has key genetic sequences related to antibiotic and heavy metal resistance *(8),* and this phenomenon of nonantibiotic substances, such as disinfectants or heavy metals, selecting for antibiotic-resistant bacteria can occur and may be a component of multidrug resistance in bacteria *(12, 32)*. One explanation for this phenomenon is that some resistant determinants can cluster together and thus coselect for antibiotic and heavy metal resistance *(38)*. This type of coresistance or coselection is an important factor to consider because both copper and zinc are used as growth promoters instead of in-feed antibiotics in some poultry facilities *(41)* and may become more common in the United States now that the U.S. Food and Drug Administration Veterinary Feed Directive has been fully implemented *(36)*.

Cross-resistance, or resistance to a variety of substances via a physiological adaptation (as opposed to genetic linkage, which occurs with coresistance), is another important mechanism of bacterial resistance to antimicrobials *(27)*. Examples of cross-resistance mechanisms are reduced cell permeability, production of neutralizing enzymes, target alteration, and overactive efflux pumps, which can pump a broad spectrum of substances such as antibiotics, biocides, and other inhibitors out of the cell and create multidrug-resistant bacteria *(35, 38)*.

Although CPC-challenged SH 2014 and 1992 also had upregulated resistance genes (12.7%, 8 of 63 genes, and 15.9%, 10 of 63 genes, respectively), these strains were less upregulated than were aCH-challenged SH 2014 and 1992 (41.3%, 26 of 63, and 39.7%, 25 of 63 genes, respectively) (Tables 2 and 3). Nevertheless, genes with gene products such as multiple antibiotic resistance proteins, multidrug resistance proteins, and heavy metal resistance proteins were primarily upregulated in both aCH- and CPC-challenged SH (data not shown). Genes associated with the efflux pump and multidrug resistance such as acriflavine resistance protein E (*acrE*), *acrF* (data not shown), and multiple antibiotic resistance protein A (*marA*) (Table 4) were upregulated, especially in aCH-treated SH. Similarly, genes such as *acrA, marA,* and *marR* (Table 4) were upregulated especially in CPC-treated SH. Chen et al. *(4)* found that among other genes, *acrA, acrB, acrE, acrF,* and multidrug efflux protein D gene (*emrD*) were significantly upregulated in fluoroquinolone-resistant *Salmonella* Typhimurium. In general, active efflux pumps play an important role in resistance to a variety of substances, including antibiotics, detergents, and other inhibitors, particularly in gram-negative bacteria such as *Salmonella (29)*. Overactive efflux pumps and changes to the outer membrane have been proposed as broad-spectrum mechanisms conferring tolerance and/or resistance to antimicrobials in *Salmonella* after exposure to CPC *(22, 26)*. In particular, overexpression of the acrAB efflux system in *Salmonella* Typhimurium plays a role in resistance to the fluoroquinolone ciprofloxacin, among others *(11)*. Although other mechanisms of resistance should be considered, such as mutations in quinolone resistance determining regions of gyrase and topoisomerase IV that are closely correlated with high resistance to fluoroquinolone in *Escherichia coli (13),* fluoroquinolone resistance observed in *Salmonella* Typhimurium could not be explained by these mutations alone *(10)*. Giraud et al. *(11)* found that *Salmonella* Typhimurium was highly resistant to ciprofloxacin despite lacking mutations known to cause fluoroquinolone resistance in *E. coli* and that active efflux was closely correlated with ciprofloxacin resistance. Therefore, upregulation of genes associated with active efflux pumps and antimicrobial resistance alone suggests that antimicrobial resistance can be a potential concern in

TABLE 4. *Effects of disinfectant on selected VPR genes in Salmonella Heidelberg (SH) field strains from 2014 and 1992*[a]

KO category	Gene ID	Gene product	CPC		aCH		PAA	
			2014	1992	2014	1992	2014	1992
Bacterial invasion of epithelial cells	SEEH1578_00730	Secreted protein	−0.57	−1.11[b]	0.36	−0.25	−0.61	0.03
	SEEH1578_14510	G-nucleotide exchange factor SopE	−0.17	1.00[c]	−0.33	−0.18	0.18	−0.13
	SEEH1578_14685	Inositol phosphate phosphatase SopB (EC 3.1.3.−)	−0.27	0.97	0.09	−0.11	−0.29	−0.29
	SEEH1578_18540	G-nucleotide exchange factor SopE	−0.45	1.32[c]	0.23	0.02	0.08	−0.10
	SEEH1578_21900	Adherence and invasion outer membrane protein (Inv, enhances Peyer's patches colonization)	0.61	−0.66	0.33	0.22	0.07	0.22
	SEEH1578_23220	Type III secretion injected virulence protein (YopE)	0.59	1.68[c]	−0.35	−0.34	−0.10	−0.37
	SEEH1578_23225	Type III secretion host injection protein (YopB); cell invasion protein SipD (*Salmonella* invasion protein D)	0.60	2.13[c]	−0.34	−0.27	−0.12	−0.29
	SEEH1578_23230	Type III secretion negative modulator of injection (YopK, YopQ, controls size of translocator pore); cell invasion protein SipC (effector protein SipC)	0.21	1.65[c]	−0.25	−0.09	−0.13	−0.32
	SEEH1578_23235	Cell invasion protein SipB	0.07	1.47[c]	−0.25	−0.21	−0.10	−0.24
Salmonella infection	SEEH1578_07550	Cytochrome c552 precursor (EC 1.7.2.2)	−0.02	0.79	3.10[c]	0.91	2.21[c]	0.20
	SEEH1578_08005	Nitrite-sensitive transcriptional repressor NsrR	0.25	−1.49[b]	0.40	0.11	−0.10	0.28
	SEEH1578_15355	SifA protein	−0.03	−1.10[b]	0.39	−0.39	−0.15	0.30
	SEEH1578_16225	Type III secretion effector SseB	−0.03	0.32	0.65	0.13	0.20	−0.09
	SEEH1578_16235	Secretion system effector SseC	0.33	0.08	0.95	0.28	−0.02	0.31
	SEEH1578_16240	Secretion system effector SseD	0.52	0.61	0.76	0.01	−0.33	−0.31
	SEEH1578_16255	Type III secretion effector SseF	0.98	0.34	1.28	0.51	0.62	0.77
	SEEH1578_16260	Type III secretion effector SseG	0.64	0.14	0.81	0.04	−0.06	0.20
	SEEH1578_17400	Secreted effector J SseJ	0.82	−0.49	1.36	0.29	0.51	0.67
	SEEH1578_19070	Flagellin (FliC)	0.19	1.01[c]	−0.40	−0.34	0.20	−0.06
	SEEH1578_20475	Leucine-rich repeat protein	0.56	−0.07	0.54	0.40	0.08	0.33
	SEEH1578_22105	Flavohemoglobin/nitric oxide dioxygenase (EC 1.14.12.17)	0.55	0.33	0.49	0.46	0.20	−0.04
	SEEH1578_22670	Flagellin (FliC)	0.20	1.10[c]	−0.44	−0.11	0.21	−0.10
	SEEH1578_22705	Secreted effector protein	0.54	1.29[c]	1.77[c]	1.37[c]	−0.07	0.40
	SEEH1578_22995	Anaerobic nitric oxide reductase transcription regulator NorR	1.02[c]	1.22[c]	0.99	0.96	0.24	0.34
	SEEH1578_23000	Anaerobic nitric oxide reductase flavorubredoxin	0.19	0.28	1.17	0.86	0.21	−0.10
	SEEH1578_23005	Nitric oxide reductase FlRd-NAD(+) reductase (EC 1.18.1.−)	0.71	1.55[c]	0.11	0.35	0.06	0.84
	SEEH1578_23125	Type III secretion injected virulence protein (YopP, YopJ, induces apoptosis, prevents cytokine induction, inhibits NFkb activation)	0.19	0.95	0.00	0.26	0.03	−0.12
β-Lactam resistance	SEEH1578_02075	Type I secretion outer membrane protein, TolC precursor	0.19	0.77	0.51	0.83	−0.12	−0.03
	SEEH1578_03545	Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.−.−)	0.59	−0.44	−0.21	−0.53	−0.15	0.17
	SEEH1578_04770	Putative β-lactamase (EC 3.5.2.6)	0.10	−0.03	0.71	0.35	−0.14	−0.02
	SEEH1578_09640	Cell division protein FtsI (peptidoglycan synthetase) (EC 2.4.1.129)	0.34	0.59	0.79	0.91	−0.16	0.39
	SEEH1578_10675	Protein with similarity to RtcB	1.06[c]	−0.67	0.06	−0.32	−0.32	0.45
	SEEH1578_11165	RND efflux system, outer membrane lipoprotein	0.35	−0.52	0.98	1.04	0.02	0.38

TABLE 4. *Continued*

KO category	Gene ID	Gene product	CPC		aCH		PAA	
			2014	1992	2014	1992	2014	1992
	SEEH1578_11650	AmpG permease	0.86	0.07	−0.16	−0.36	−0.23	0.10
	SEEH1578_11800	Acriflavine resistance protein B (AcrB)	0.41	0.50	0.80	0.91	0.06	0.19
	SEEH1578_11805	Acriflavine resistance protein A (AcrA)	0.89	1.06[c]	0.86	0.99	0.09	0.15
	SEEH1578_12630	Penicillin-binding protein 2 (PBP-2)	1.25[c]	−0.25	0.43	0.14	−0.02	0.54
	SEEH1578_14195	Outer membrane porin OmpF	−0.31	−0.51	−0.26	−0.79	−0.12	−0.32
	SEEH1578_15280	β *N*-acetyl-glucosaminidase (EC 3.2.1.52)	0.39	0.64	0.24	0.44	0.40	0.20
	SEEH1578_17660	Periplasmic murein peptide-binding protein MppA	0.40	0.92	0.69	0.72	0.63	0.05
	SEEH1578_17980	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1)	0.31	0.26	1.90[c]	1.00	0.51	0.93
	SEEH1578_17985	Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)	0.75	0.71	1.84[c]	1.14[c]	0.46	0.98
	SEEH1578_17990	Oligopeptide transport system permease protein OppC (TC 3.A.1.5.1)	1.38[c]	0.83	1.78[c]	1.54[c]	−0.11	1.03[c]
	SEEH1578_17995	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	1.84[c]	1.09[c]	1.94[c]	1.90[c]	−0.53	1.02[c]
	SEEH1578_18000	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	0.89	0.69	2.25[c]	2.36[c]	−0.31	0.94
	SEEH1578_18435	Cell division protein FtsI (Peptidoglycan synthetase) (EC 2.4.1.129)	0.92	−0.69	1.68[c]	1.08[c]	−0.12	0.24
	SEEH1578_18855	Penicillin-binding protein 2 (PBP-2)	0.81	0.04	0.58	0.12	−0.10	0.40
	SEEH1578_20610	Outer membrane porin OmpC	0.67	1.36[c]	−0.50	−0.59	0.35	0.13
CAMP resistance	SEEH1578_01005	*N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	1.24[c]	−0.64	0.39	0.31	−0.61	0.38
	SEEH1578_02110	Periplasmic thiol:disulfide interchange protein, DsbA-like	0.67	0.44	1.06[c]	0.39	0.23	0.34
	SEEH1578_03445	Peptidyl-prolyl *cis-trans* isomerase PpiA precursor (EC 5.2.1.8)	1.00[c]	−0.01	1.39[c]	1.85[c]	−0.65	0.07
	SEEH1578_04260	Phosphoethanolamine transferase specific for the outer Kdo residue of lipopolysaccharide	0.04	−0.99	2.00[c]	1.71[c]	−0.32	0.38
	SEEH1578_06040	Periplasmic thiol:disulfide interchange protein DsbA	0.12	0.75	1.40[c]	1.66[c]	−0.09	0.20
	SEEH1578_06335	Copper sensory histidine kinase CpxA	0.92	−0.41	1.91[c]	1.94[c]	−0.04	0.51
	SEEH1578_06340	Copper-sensing two-component system response regulator CpxR	0.39	−0.29	1.96[c]	2.09[c]	0.10	0.51
	SEEH1578_07635	Sensor protein BasS (activates BasR)	0.68	0.05	3.33[c]	2.47[c]	0.63	0.56
	SEEH1578_07640	Two-component transcriptional regulatory protein BasR (activated by BasS)	0.43	0.89	3.52[c]	2.49[c]	0.85	0.51
	SEEH1578_07645	Phosphoethanolamine transferase EptA	1.27[c]	1.25[c]	3.52[c]	2.43[c]	0.49	0.44
	SEEH1578_07960	*N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	0.67	0.16	0.49	0.69	−0.15	0.33
	SEEH1578_10165	HtrA protease/chaperone protein	0.43	−0.24	3.29[c]	3.11[c]	−0.27	0.83
	SEEH1578_10260	Acyl-[acyl-carrier-protein]—UDP-*N*-acetylglucosamine *O*-acyltransferase (EC 2.3.1.129)	−0.26	−0.71	−0.08	−0.36	−0.18	0.12
	SEEH1578_10325	Copper homeostasis protein CutF precursor/lipoprotein NlpE involved in surface adhesion	0.39	0.73	0.92	0.95	−0.35	0.17
	SEEH1578_12570	Lipid A acylation protein PagP, palmitoyltransferase	0.89	2.47[c]	1.35[c]	1.04[c]	0.40	−0.41
	SEEH1578_15310	L,D-transpeptidase YcfS	1.52	0.17	3.53[c]	4.27[c]	−0.39	0.07
	SEEH1578_15385	Sensor histidine kinase PhoQ (EC 2.7.13.3)	0.56	1.27[c]	0.91	0.95	−0.07	−0.14
	SEEH1578_15390	Transcriptional regulatory protein PhoP	−0.35	1.36[c]	0.79	0.98	0.05	−0.26
	SEEH1578_16815	Multiple antibiotic resistance protein MarA	1.02[c]	1.40[c]	1.34[c]	0.76	0.02	0.35

TABLE 4. *Continued*

KO category	Gene ID	Gene product	CPC		aCH		PAA	
			2014	1992	2014	1992	2014	1992
	SEEH1578_17725	Peptide transport periplasmic protein SapA	0.58	0.86	0.25	0.48	−0.07	−0.11
	SEEH1578_17730	Peptide transport system permease protein SapB	0.69	1.06[c]	−0.04	0.17	0.10	−0.36
	SEEH1578_17735	Peptide transport system permease protein SapC	0.29	0.71	−0.02	0.26	0.01	−0.05
	SEEH1578_17740	Peptide transport system ATP-binding protein SapD	0.16	0.53	−0.09	0.30	−0.06	−0.08
	SEEH1578_17745	Peptide transport system ATP-binding protein SapF	0.22	−0.24	0.11	0.18	−0.02	−0.06
	SEEH1578_20760	UDP-4-amino-4-deoxy-L-arabinose—oxoglutarate aminotransferase (EC 2.6.1.87)	0.04	0.30	3.07[c]	2.82[c]	0.65	0.47
	SEEH1578_20765	Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase (EC 2.4.2.53)	0.18	0.20	2.94[c]	2.91[c]	0.63	0.47
	SEEH1578_20770	UDP-4-amino-4-deoxy-L-arabinose formyltransferase (EC 2.1.2.13)/UDP-glucuronic acid oxidase (UDP-4-keto-hexauronic acid decarboxylating) (EC 1.1.1.305)	0.50	0.33	2.48[c]	3.01[c]	0.43	0.68
	SEEH1578_20775	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD (EC 3.5.1.n3)	0.65	0.32	2.34[c]	2.97[c]	0.14	0.78
	SEEH1578_20780	Undecaprenyl phosphate-α-4-amino-4-deoxy-L-arabinose arabinosyl transferase (EC 2.4.2.43) @ melittin resistance protein PqaB @ polymyxin resistance protein PmrK	0.40	0.15	2.19[c]	2.63[c]	0.01	0.78
	SEEH1578_20785	Undecaprenyl phosphate-aminoarabinose flippase subunit ArnE	0.11	0.20	1.87[c]	2.18[c]	0.19	0.55
	SEEH1578_20790	Undecaprenyl phosphate-aminoarabinose flippase subunit ArnF	−0.29	0.93	0.83	1.00[c]	0.29	−0.06
	SEEH1578_20795	Signal transduction protein PmrD	−0.37	1.04[c]	0.69	0.60	0.29	−0.25
	SEEH1578_21300	Outer membrane protease E	0.27	0.95	1.16	0.89	0.03	0.27
	SEEH1578_21575	*N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	0.32	0.34	0.44	0.34	0.03	0.16
Vancomycin resistance	SEEH1578_07405	Alanine racemase (EC 5.1.1.1)	0.41	0.05	0.35	−0.09	0.00	0.05
	SEEH1578_09650	UDP-*N*-acetylmuramoyl-tripeptide—D-alanyl-D-alanine ligase (EC 6.3.2.10)	0.70	0.72	0.76	0.54	0.57	0.52
	SEEH1578_09655	Phospho-*N*-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13)	0.86	0.43	0.44	0.42	0.23	0.47
	SEEH1578_09670	UDP-*N*-acetylglucosamine—*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase (EC 2.4.1.227)	0.40	−0.40	0.57	0.47	−0.36	0.49
	SEEH1578_09680	D-alanine-D-alanine ligase (EC 6.3.2.4)	0.05	−0.47	0.53	0.28	−0.02	0.28
	SEEH1578_11320	D-alanine-D-alanine ligase (EC 6.3.2.4)	0.48	0.88	−0.06	−0.32	−0.05	0.03
	SEEH1578_17245	D-alanyl-D-alanine dipeptidase	0.32	0.75	1.30[c]	1.23[c]	0.08	−0.19
	SEEH1578_18270	Alanine racemase (EC 5.1.1.1)	−0.22	−0.83	0.85	1.13[c]	0.05	0.63

[a] Shown are $\log_2$-fold change values that were used to determine whether the genes were upregulated or down-regulated in the challenged field strains (onefold cutoff). Most values are not significantly different. Acidified calcium hypochlorite (aCH) and peroxyacetic acid (PAA) results within the same strain can be compared directly, but cetylpyridinium chloride (CPC) samples cannot be compared directly.

[b] Down-regulation ($P < 0.001$, $\log_2$-fold change less than −1).

[c] Upregulation ($P < 0.001$, $\log_2$-fold change >1).

TABLE 5. *Number of upregulated and down-regulated genes in selected significant enriched KEGG pathways from SH 1992 treated with CPC*[a]

Pathway	*P* value	No. (%) of upregulated genes	No. (%) of down-regulated genes	Total genes
Bacterial invasion of epithelial cells	2.95E–04	6 (66.7)	1 (11.1)	9
Salmonella infection	0.01	11 (44.0)	2 (8.0)	25

[a] Onefold cutoff, $P < 0.001$.

aCH- and CPC-treated SH, although the concern is greater for aCH-treated SH.

Although previous findings and our data suggest that upregulation of selected resistance genes is a bigger issue in SH challenged with aCH, upregulation of virulence and pathogenicity genes is also likely to occur when SH is exposed to CPC (Tables 2 and 3). The bacterial invasion of epithelial cells KEGG pathway was enriched only in SH 1992 treated with CPC; 66.7% (6 of 9) of the genes were upregulated, and 11.1% (1 of 9 genes) were down-regulated (Table 5). Because virulence is closely correlated with increased type III secretion synthesis *(20),* the three type III secretory virulence genes included in the bacterial invasion of epithelial cells should be an area of focus. For those three genes, CPC caused more significant upregulation in the SH 1992 strain than did aCH and PAA (Table 4). All the type III secretory genes from the bacterial invasion of epithelial cells KO category were upregulated in the CPC-treated SH 1992, and none were significantly upregulated in any of the other treatments (Table 4). The *Salmonella* infection pathway also was enriched in SH 1992 treated with CPC; 44.0% (11 of 25) of the genes were upregulated, and 8.0% (2 of 25 genes) were down-regulated (Table 5). In contrast to the bacterial invasion of epithelial cells pathway, the *Salmonella* infection pathway had no type III secretion genes that were upregulated (Table 4). However, two *fliC* (flagellin) genes, which are advantageous for gut colonization by modifying the swimming pattern of *Salmonella* to increase contact with the gut *(14),* were upregulated (Table 4).

In previous studies, CPC has been an effective disinfectant for the control of *Salmonella* even at short contact times of 10, 17, 23, and 90 s *(5, 34)*. In our laboratory, minimum bacteriostatic disinfectant concentrations for CPC and PAA were within the regulatory ranges stipulated by the U.S. Department of Agriculture, Food Safety and Inspection Service (Table 1) *(6)*. Although these results suggest that CPC and PAA should be considered equally effective for the control of SH, the addition of RNA-seq data related to VPR differential gene expression should also be considered. Consequently, based on VPR regulation and survivability, PAA may be a better disinfectant because it is less likely to upregulate VPR genes and thereby less likely to induce antibiotic resistance. In both SH 2014 and SH 1992, PAA caused less upregulation of VPR genes than did CPC (Tables 2 and 3). In SH 2014, 1.1% (1 of 90) of VPR genes were upregulated after PAA treatment, whereas 10% (9 of 90) of VPR genes were upregulated after CPC treatment. Similarly, in SH 1992, 2.2% (2 of 90) of VPR genes were upregulated after PAA treatment, whereas 23.3% (21 of 90) of VPR genes were upregulated after CPC treatment. This observation that PAA is less likely to upregulate VPR genes is supported by results of a study by Biswal et al. *(1),* who found that the prevalence of antimicrobial resistance genes in *Escherichia coli* decreased after treatment with PAA. When Colla et al. *(7)* challenged a total of 20 SH isolates from 2005 and 2009 with three disinfectants commonly used in processing plants, these authors found that isolates from 2009 had increasing resistance to chlorhexidine and quaternary ammonium but the same field strains were not resistant to PAA. One potential explanation for this result is the relative lack of upregulation of VPR genes for SH exposed to PAA. Therefore, SH remained relatively susceptible to PAA, which is consistent with previous reports regarding reduced *Salmonella* growth *(5, 34)*.

Although preharvest approaches for the control of *Salmonella* are considered the most efficacious *(9),* in regard to a disinfectant at the minimum it is essential to do no harm at the processing plant with respect to foodborne pathogen control. However, the results from this study indicate that the SH that survives exposure to the disinfectant aCH can cause over 28% of the genes associated with VPR to be upregulated (Tables 2 and 3) and that aCH is not efficacious for inhibiting or killing these strains (Table 1). Therefore, increased resistance may occur when exposing SH to aCH as opposed to PAA and CPC. Consequently, from a food safety perspective the ability to couple MIC testing with RNA-seq analysis could be helpful for assessing both disinfectant efficacy and risk with respect to upregulation of VPR genes in bacteria that are challenged with subinhibitory concentrations of disinfectants. Evaluation of disinfectants solely based on their lethal effects ignores what is happening with gene expression and phenotypically with surviving bacteria that can affect human health. Therefore, the goal of disinfectant use should not be focused only on killing or mitigating foodborne pathogens but should also focus on use of disinfectants that are less likely to increase VPR gene expression.

RNA-seq could be an additional tool in food processing for making risk-based decisions when selecting disinfectants. Recent advances in sequencing technologies have made RNA-seq less expensive and less time-consuming. Resources such as extension specialists and genome core facilities at universities could help facilitate RNA-seq experiments for processing plants that may not have the capacity to run these analyses. From a hazard analysis and critical control points (HACCP) perspective, RNA-seq could be used to determine critical food safety parameters in a way never previously investigated in a food system environment with the ultimate goal of identifying conditions in food production that mitigate transcription of genes

associated with virulence and survivability. Based on our results for SH control, it may be useful to explore the idea of using CPC to kill SH in the dipping stations followed by a rinse and a subsequent PAA dip. How this combination of disinfectants would affect VPR expression is unknown. Future studies should evaluate VPR gene expression after exposure to a combination of disinfectants commonly used during poultry processing. Different time points on the processing line also should be evaluated for temporal effects and to determine how often RNA-seq analyses should be done; the present study included only one time point on the processing line immediately after disinfectant challenge. Another management practice to consider is rotation between disinfectants based on MIC and RNA-seq results. This study represents a novel approach toward postharvest control of pathogenic bacteria with the goal of facilitating the transition from lab-based RNA-seq studies to implementation within the HACCP program of a particular food processor.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/0362-028X.JFP-18-235.s1.

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