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Cellular Response of *Campylobacter jejuni* to Trisodium Phosphate

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The highly alkaline compound trisodium phosphate (TSP) is used as an intervention to reduce the load of *Campylobacter* on poultry meat in U.S. poultry slaughter plants. The aim of the present study was to investigate the cellular responses of *Campylobacter jejuni* NCTC11168 when exposed to sublethal concentrations of TSP. Preexposure of *C. jejuni* to TSP resulted in a significant increase in heat sensitivity, suggesting that a combined heat and TSP treatment may increase reduction of *C. jejuni*. A microarray analysis identified a limited number of genes that were differently expressed after sublethal TSP exposure; however, the response was mainly associated with ion transport processes. *C. jejuni* NCTC11168 *nhaA1* (Cj1655c) and *nhaA2* (Cj1654c), which encode orthologues to the *Escherichia coli* NhaA cation/proton antiporter, were able to partially restore TSP, alkaline, and sodium resistance phenotypes to an *E. coli* cation/proton antiporter mutant. In addition, inhibition of *r*esistance-*n*odulation-cell *d*ivision (RND) multidrug efflux pumps by the inhibitor PaβN (Phe-Arg β-naphthylamide dihydrochloride) decreased tolerance to sublethal TSP. Therefore, we propose that NhaA1/NhaA2 cation/proton antiporters and RND multidrug efflux pumps function in tolerance to sublethal TSP exposure in *C. jejuni*.

Chemical decontamination has been used widely for decades in the U.S. poultry slaughter plants to reduce the presence of human pathogens on the meat (22). Currently, the highly alkaline compound trisodium phosphate (TSP) is applied as a dip or spray of prechilled or chilled poultry carcasses (6, 22) and is effective against *Campylobacter*, *Salmonella*, and *Listeria* (6). In the European Union (EU), an ongoing debate questions the safety of chemical decontamination, and more data, such as the development of resistance, cross protection, and virulence, are needed for the risk assessments required by the EU (8).

The alkalinity of TSP causes bacterial death through disruption of cell membranes (18, 30), and it acts as a detergent that removes fat from the surface of poultry carcasses that further increases the killing action of TSP (13). Sublethal concentrations of TSP may be encountered by the bacteria when the compound is inadequately distributed on the surface of the carcass or inactivated by excessive amounts of organic material or when leftover TSP residues are present on the carcass during storage (1, 2, 31). A major concern is that exposure to sublethal concentrations of TSP may increase bacterial tolerance to food processing interventions, preservation treatments, and antibacterial conditions within the human hosts.

Campylobacter jejuni is one of the most frequently reported causes of bacterial food-borne infections in developed countries (7, 9), leading to self-limiting acute gastroenteritis. The consumption and handling of poultry meat products is the major source of human campylobacteriosis, and the use of TSP during poultry meat processing is known to reduce levels of *C. jejuni* (4, 28, 29, 37). The aim of the present study was to show whether sublethal TSP exposure affects gene expression and the physiology of *C. jejuni*, including cross-protection to other environmental stresses.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. *C. jejuni* strains were routinely grown on blood agar base II (Oxoid) supplemented with 5% calf blood or in brucella broth (BB; Difco, Broendby, Denmark) at 37°C under microaerobic conditions (6% O_2, 6% CO_2, 4% H_2, and 84% N_2) or (5% O_2, 10% CO_2, and 85% N_2). *De novo* protein synthesis was inhibited by chloramphenicol (128 μg/ml). *Escherichia coli* strains were grown at 37°C in modified Luria-Bertani broth (LBK), where KCl substituted NaCl (10). When appropriate kanamycin (30 μg/ml), erythromycin (200 μg/ml), chloramphenicol (30 μg/ml), or ampicillin (50 or 100 μg/ml) was added. Trisodium phosphate (TSP; Riedel-de Häen 04278) was used at concentrations of weight/volume (%).

E. coli CTN40 carrying cation/proton antiporter mutations from strain KNabc was constructed by P1 transduction and selection for relevant resistance markers and confirmed by lack of growth in LBK 0.2 M NaCl (21). NCTC11168 chromosomal DNA was used as a template for amplification of a 2,346-bp *nhaA1* (Cj1655c)-*nhaA2* (Cj1654c) fragment using DreamTaq DNA polymerase (Fermentas, St. Leon-Rot, Germany) and the primers (Eurofins MWG GmbH, Ebersberg Germany) CTR1-up (5′-TATGGATCCTTAGGAGCAAGGATGCAAATG-3′; BamHI site underlined) and CTR9-down (5′-TATCTCGAGTATGCCTCATCAATCC CCTTA-3′; XhoI site underlined). The *nhaA1-nhaA2* fragment was cloned into pCR2.1 (Invitrogen, Naerum, Denmark) and moved to pET-21(+) (Novagen) using BamHI and XhoI (Fermentas) resulting in pCTN1.

Survival during heat stress after exposure to sublethal TSP. A 90 ml overnight culture of *C. jejuni* was adjusted to optical density at 600 nm (OD_{600}) of 0.8, split in two, centrifuged for 5 min at 8,000 rpm, inoculated into BB alone or BB plus 0.6% TSP, and incubated under aerobic conditions at 37°C for 30 min. Cultures were washed twice in phosphate-buffered saline (PBS; pH 7.4 [CM7733; Oxoid, Greve, Denmark]) and resuspended in BB. Cultures were diluted into BB preheated to 48°C, and samples were withdrawn and placed on ice. Samples were 10-fold serial diluted and CFU determined. When no colonies were detected, CFU was set to half the detection limit (1.2 $\log_{10}$ CFU/ml). The mean $\log_{10}$ CFU/ml

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
C. jejuni NCTC11168	Human clinical isolate	National Collection of Type Cultures (United Kingdom)
E. coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL* (Str^r) *endA1* λ$^-$	Invitrogen
KNabc	TG1 *nhaA*::Kan *nhaB*::Erm *chaA*::Cam	21
ER2566	F$^-$ *fhuA2* [*lon*] *ompT lacZ*::*T7gene1 gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10* R(*mcr-73*::miniTn*10*-Tet^s)2 R(*zgb-210*::Tn*10*)(Tet^s) *endA1* [*dcm*] λ$^-$	NEB
CTN40	*E. coli* ER2566 *nhaA*::Kan *nhaB*::Erm *chaA*::Cam	This study
CTN15	CTN40 containing pET-21(+)	This study
CTN58	CTN40 containing pCTN1	This study
CTN3	CTN40 containing pTLJ03/*nhaA1*	This study
CTN9	CTN40 containing pTLJ03	This study
Plasmids		
pCR2.1-TOPO	Cloning vector; Amp^r	Invitrogen
pCR2.1-TOPO/*nhaA1-nhaA2*	pCR2.1-TOPO containing *nhaA1* and *nhaA2* genes from *C. jejuni* NCTC11168	This study
pET-21(+)	Transcription vector, *T7* promoter, *lacI* coding sequence; Amp^r	Novagen
pCTN1	pET-21(+) containing *nhaA1 and nhaA2* genes from *C. jejuni* NCTC11168	This study
pTLJ03	Transcription vector, *T7* promoter, *lacI* coding sequence; Amp^r	Geneservice (27)
pTLJ03/*nhaA1*	*nhaA1* in pTLJ03, plasmid obtained from *C. jejuni* NCTC11168 ORF library	Geneservice (27)

[a] Resistance: Str^r (streptomycin), Amp^r (ampicillin), Kan^r (kanamycin), Erm^r (erythromycin), Cam^r (chloramphenicol). Sensitive: Tet^s (tetracycline).

± the standard deviation (SD) at each sampling time of three independent experiments is shown.

Collection of samples and RNA extraction. *C. jejuni* were inoculated into BB to an OD_{600} of 0.05 and incubated for 8 h until reaching exponential phase (an OD_{600} of 0.32 to 0.50). The culture was concentrated and split into two portions, one in BB and another in BB plus 0.6% TSP (pH 9.2). After 5 and 30 min, the samples were collected, and RNAprotect bacterial reagent (Qiagen, Copenhagen, Denmark) was added. Total RNA

TABLE 2 Microarray analysis of gene expression in *C. jejuni* NCTC11168 in brucella broth with 0.6% TSP compared to brucella broth (pH 7)

	Relative gene expression[a] at:					
	5 min		30 min			
Systematic	Fold change	*P*	Fold change	*P*	Product	Additional functional description
Cj0008	**2.2**	**0.000**	1.8	0.090	Hypothetical protein Cj0008	
Cj0039c	1.3	0.024	**1.8**	**0.000**	GTP-binding protein typA homolog	
Cj0044c	–1.3	0.026	**–2.0**	**0.000**	Hypothetical protein Cj0044c	
Cj0045c	1.0	0.859	**–1.7**	**0.000**	Putative iron-binding protein	
Cj0176c	2.5	0.001	**3.2**	**0.000**	Putative lipoprotein	In operon with Cj0173-Cj0175c, which is involved in scavenging iron from lactoferrin and transferrin[b]
Cj0403	1.3	0.045	**1.5**	**0.001**	Hypothetical protein Cj0403	
Cj0608	**2.4**	**0.000**	1.4	0.004	Putative outer membrane protein	
Cj0717	1.3	0.115	**1.7**	**0.000**	Hypothetical protein Cj0717	
Cj0770c	1.1	0.298	**2.0**	**0.000**	Putative periplasmic protein	Contains a conserved domain characteristic of the periplasmic component of an ABC transporter[c]
Cj0771c	1.3	0.089	**1.8**	**0.000**	Putative periplasmic protein	
Cj1000	1.8	0.018	**1.7**	**0.000**	Putative transcriptional regulator (LysR family)	
Cj1241	–1.5	0.001	**–1.6**	**0.000**	Putative transmembrane transport protein	
Cj1436c	–1.4	0.025	**–1.6**	**0.000**	Putative aminotransferase	
Cj1467	–1.3	0.098	**–2.0**	**0.001**	Hypothetical protein Cj1467	
Cj1471c	–1.7	0.006	**–2.3**	**0.001**	Putative type II protein secretion system E protein	Essential for DNA uptake and natural transformation[d]
Cj1483c	–1.4	0.326	**–1.8**	**0.000**	Putative lipoprotein	
Cj1533c	1.6	0.002	**1.8**	**0.000**	Putative helix-turn-helix containing protein	
Cj1646	–1.5	0.070	**–2.0**	**0.000**	Putative ABC transport system permease protein	

[a] Relative gene expression is presented as the mean ratio of fluorescence intensity of TSP exposed cells to that of pH 7-exposed cells. Genes with the fold change shown in boldface indicate significant differential expression (Student *t* test with a *P* value of <0.05 adjusted with the Benjamini-Hochberg false-discovery-rate multiple testing correction). All *P* values were calculated using the Student *t* test.

[b] Function described previously (19, 36).

[c] Information revealed from bioinformatic analysis.

[d] Function described previously (38).

was extracted using an RNeasy minikit (Qiagen) according to the manufacturer's instructions with an additional on-column DNase I digestion (RNase-Free DNase Set; Qiagen) and the addition of lysozyme. The purity and integrity of the RNA was confirmed with a 2100 Bioanalyzer (Agilent Technologies).

Microarray hybridizations and data analysis. *C. jejuni* NCTC11168 whole-genome microarrays (v2.1.0 with PCR reporters for each of 1,654 genes spotted in duplicate) were supplied by the Microarray Group at St. George's Hospital Medical School, London, United Kingdom (BμG@S Group [http://www.bugs.sghms.ac.uk]). Probe labeling and microarray hybridization followed the standard RNA protocols from the BμG@S Group (34). Total RNA (5 μg) derived from *C. jejuni* both exposed to TSP and unexposed was labeled with Cy3 and Cy5, respectively. Purified Cy3/Cy5-labeled cDNA was hybridized on the microarrays underneath a LifterSlip (Eirie Scientific). Slides were scanned with a GMS418 microarray scanner (Genetic Microsystems). ImaGene software v5.5 (BioDiscovery) was used for quantification of the fluorescence from each spot. The data were further processed using MAVI Pro v2.6.0 software (MWG Biotech) and analyzed using GeneSpring GX v7.3.1 software (Agilent Technologies). The data from triplicate biological experiments at each sampling time were combined. Significant differential gene regulation was identified by using a *P* value cutoff of 0.05 and application of the Benjamini-Hochberg false discovery rate.

Growth of *C. jejuni* in the presence of TSP and a multidrug efflux pump inhibitor. *C. jejuni* was inoculated to an OD_{600} of 0.05 into either BB, BB plus 0.4% TSP, BB plus 64 μg of PaβN/ml (Phe-Arg β-naphthylamide dihydrochloride Sigma, Broendby, Denmark), or BB plus 0.4% TSP and 64 μg of PaβN/ml. The mean OD_{600} ± the SD from three independent experiments is presented.

Functional complementation of the *E. coli* cation/proton antiporter mutant. The OD_{600} of *E. coli* CTN39, CTN40, CTN15, and CTN58 was adjusted to 0.05 in LKB plus 0.2% TSP, LKB plus 0.2 M NaCl, and LBK (pH 8.5)–(KOH). To CTN15 and CTN58, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce *nhaA1-nhaA2* expression or as a control. The mean OD_{600} is presented.

RESULTS AND DISCUSSION

Genome-wide gene expression during sublethal TSP exposure. The gene expression profile of *C. jejuni* in response to sublethal TSP exposure was investigated by microarray analysis and triplicate biological replicates identified 27 significantly differentially regulated genes (see Table S1 in the supplemental material), 18 of which showed ≥1.5-fold differential expression in at least 1 time point (Table 2). Noteworthy, most gene functions were associated with transport processes, such as iron uptake, DNA uptake, and efflux. The most highly upregulated gene (3.2-fold) was Cj0176c belonging to the operon Cj0173c-Cj0176c that plays a role in uptake of iron from transferrin proteins (19, 20). A gene, Cj1471c, characterized to function in DNA uptake and transformation (38) showed the largest downregulation (2.3-fold) in response to TSP. Thus, in *C. jejuni*, sublethal TSP exposure elicits a limited transcriptional response that mainly is associated with transport processes.

***nhaA1* and *nhaA2* function as cation/proton antiporters in tolerance to TSP.** In other bacteria, NhaA cation/proton antiporters play an essential role in alkaline pH homeostasis by exchanging cytoplasmic cations with external protons (23). *C. jejuni* NCTC11168 encodes two cation/proton antiporter orthologues, NhaA1 and NhaA2 that show 46 and 49% identity to *E. coli* NhaA, respectively, and 56% similarity to each other. Although an *E. coli* cation/proton antiporter mutant was unable to grow in 0.2% TSP (pH 8.2), the presence of the *C. jejuni* genes *nhaA1* and *nhaA2* partly restored its ability to grow at the wild-type level (Fig. 1A).

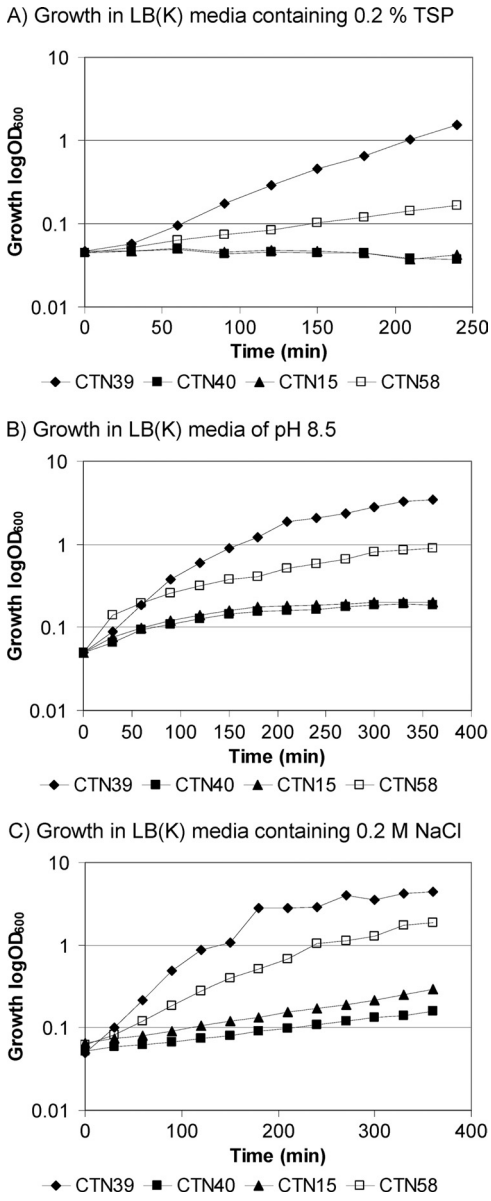

FIG 1 Complementation capacity of *C. jejuni* NCTC11168 *nhaA1* and *nhaA2* genes in a TSP, alkali, and Na^+-sensitive *E. coli* cation/proton antiporter mutant. The *E. coli* strains tested were wt (CTN39, ♦), cation/antiporter Δ*nhaA* Δ*nhaB* Δ*chaA* mutant (CTN40, ■), cation/antiporter Δ*nhaA* Δ*nhaB* Δ*chaA* mutant containing plasmid pCTN1 with the *C. jejuni* NCTC11168 *nhaA1* and *nhaA2* genes (CTN58, □), and cation/antiporter Δ*nhaA* Δ*nhaB* Δ*chaA* mutant containing negative control vector pET-21(+) (CTN15, ▲). The *E. coli* strains were grown in LB(K) medium containing 0.2% TSP (A), pH 8.5 adjusted with KOH (B), or 0.2 M NaCl (C) for up to 360 min at 37°C. One representative of four experiments under each condition is presented, and similar results were obtained in all four experiments.

Furthermore, alkaline conditions (pH 8.5) and NaCl (0.2 M) inhibited growth of the *E. coli* cation/proton antiporter mutant, but the presence of *C. jejuni nhaA1* and *nhaA2* partly restored its ability to grow as the wild type (Fig. 1B and C). We attempted to determine the contribution of each of the *C. jejuni nhaA* genes to TSP tolerance, and even though the plasmids were unstable, we found that *nhaA1* partly complemented the growth deficiency of the *E. coli* cation/proton antiporter mutant in the presence of TSP

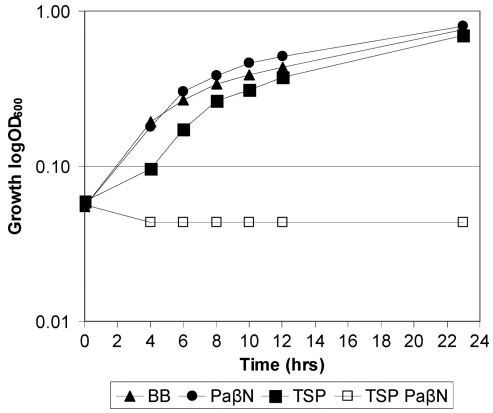

FIG 2 Growth of *C. jejuni* NCTC11168 in the presence of TSP and the efflux pump inhibitor PaβN. *C. jejuni* cultures were incubated under microaerobic conditions at 37°C for 24 h in either BB (▲), BB containing 0.4% TSP (■), BB containing 64 μg of PaβN/ml (●), or BB containing 0.4% TSP and 64 μg of PaβN/ml (□). Growth was measured as the OD_{600}, and data are presented as the means from three independent experiments where the SD was below an OD_{600} of 0.01 in all experiments.

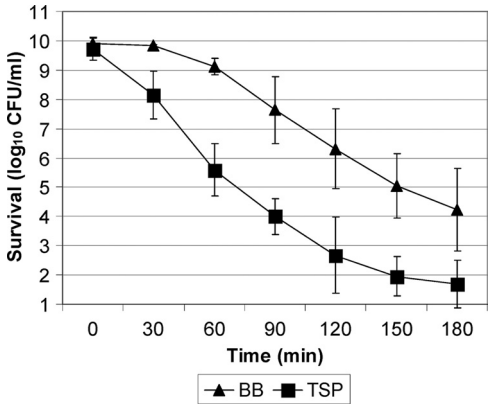

FIG 3 Effect of sublethal TSP treatment on the survival of *C. jejuni* NCTC11168 during heat stress. *C. jejuni* cells were incubated in BB (▲) or BB containing 0.6% TSP (■) for 30 min in aerobic conditions at 37°C. Subsequently, the cells were resuspended in fresh BB and incubated at 48°C for 3 h, where survival was determined as CFU/ml, and data are presented as the mean $\log_{10}$ CFU/ml ± the SD of three experiments.

(0.2%) and NaCl (0.2 M) (data not shown), suggesting that *nhaA1* itself is able to function as a cation/proton antiporter. Finally, the ability of *nhaA1* and *nhaA2* to provide TSP tolerance may be ascribed to their function in tolerance to alkaline as well sodium ions.

Our microarray data show that transcription of the *C. jejuni nhaA* genes are not induced when exposed to TSP. However, noteworthy, changes in the external pH affect the transport activity of *E. coli* NhaA (3, 24), thus regulating the activity posttranscriptionally, and a similar mechanism may ensure regulation of the activity of the *C. jejuni* cation/proton antiporters. Interestingly, bioinformatic analysis of sequenced *C. jejuni* genomes revealed that both NhaA1 and NhaA2 are present and conserved in all genomes, suggesting that both genes have an important role in this species.

Multidrug efflux pump inhibitor PaβN sensitizes *C. jejuni* to TSP. The upregulation of an efflux protein (Cj0608) during sublethal TSP exposure generated the hypothesis that efflux in general may be involved in tolerance to TSP. To further investigate this, we exposed *C. jejuni* to TSP in the presence of PaβN that inhibits multidrug efflux pumps of the *r*esistance-*n*odulation-cell *d*ivision (RND) superfamily (16, 17). We found that *C. jejuni* NCTC11168 was unable to grow in the presence of 0.4% TSP and 64 μg of PaβN/ml, whereas growth in 64 μg of PaβN/ml was similar to growth in the control media and 0.4% TSP only had a marginally effect on the initial growth rate (Fig. 2). Thus, inhibition of RND multidrug efflux pumps by PaβN decreases *C. jejuni* tolerance to TSP, suggesting that PaβN directly inhibit TSP transport mediated by RND multidrug efflux pumps. Alternatively, these results may merely reflect that *C. jejuni* cannot tolerate both the competitive inhibition of transport activity imposed by PaβN and the alkaline TSP that may act as indirect inhibitor of efflux pumps through deprivation of energy (16, 25, 33). In addition, the *C. jejuni* genome contains functionally uncharacterized putative efflux proteins such as Cj0035c containing the conserved domain of a multidrug efflux pump of the major facilitator superfamily that may have a dual function in a multidrug efflux protein and cation/proton antiporter similar to *E. coli* Mdfa (15).

Sublethal TSP exposure increases sensitivity of *C. jejuni* to heat stress. In the transmission from farm to fork, *C. jejuni* most likely encounters high-temperature stress, for instance, hot water or steam treatment in the slaughterhouse (5, 12, 26). During heat stress at 48°C, we observed that the viability of cells pretreated with TSP decreased immediately after onset of the heat stress, whereas cells with no prior TSP treatment were unaffected for the first 30 min of the heat stress (Fig. 3), but survival was not affected by the presence of chloramphenicol (data not shown). A number of additional strains were tested, and they also showed increased sensitivity to heat stress when exposed to TSP (data not shown). In contrast, TSP exposure did not affect survival significantly after 20 min of peroxide stress (data not shown). In conclusion, sublethal exposure to TSP increases the sensitivity of *C. jejuni* to heat stress under aerobic conditions independently of protein synthesis. In contrast, *de novo* protein synthesis during alkaline exposure participated in induction of thermotolerance in *Salmonella enterica* serovar Enteritidis (11, 31), and previous studies of food-borne pathogens (*S. enterica* serovar Enteritidis, *E. coli* O157:H7, *V. parahaemolyticus*, and *L. monocytogenes*) have demonstrated that cells surviving TSP or a comparable alkaline treatment acquired increased thermotolerance (14, 31, 32, 35). Collectively, the observed differences among bacterial species emphasizes the importance of carefully assessing the impact of sublethal TSP exposure on survival during subsequent stressful interventions for all relevant pathogens on poultry meat.

Concluding remarks. In light of our observations, new decontamination procedures may be designed to obtain increased reduction of *C. jejuni*, such as a combination of TSP and heat treatment. Finally, the importance of efflux pumps as well as cation/proton antiporters such as NhaA1 and Nha2 may be interesting to investigate further to identify inhibitors or combined treatments that will provide a high reduction of *C. jejuni* even when low concentrations of TSP are used.

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