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Genes of *Salmonella enterica* Serovar Enteritidis Involved in Biofilm Formation

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Abstract: Although biofilms contribute to bacterial tolerance to desiccation and survival in low-moisture foods, the molecular mechanisms underlying biofilm formation have not been fully understood. This study created a mutant library from *Salmonella* Enteritidis using mini-Tn*10* transposon mutagenesis. The biofilm-forming potential of acquired mutants was assessed before the genomic DNA of the mutants that formed significantly ($p \leq 0.05$) less biofilm mass than their wildtype parent strain was extracted for deep DNA sequencing. The gene of each mutant interrupted by mini-Tn*10* insertion was identified by aligning obtained sequencing data with the reference Genbank sequences using a BLAST search. Sixty-four mutant colonies were selected, and five mutants that formed the least amount of biofilm mass compared to the wildtype parent strain were selected for sequencing analysis. The results of the BLAST search revealed that the gene interrupted by mini-Tn*10* in each mutant is responsible for the biosynthesis of aldehyde dehydrogenase (EutE), cysteine desulfurase (SufS or SufE), a transporter protein, porin OmpL, and a ribbon–helix–helix protein from the CopG family, respectively. Knock-off mutant construction is a possible approach to verify the potential of the identified genes to serve as targets of antimicrobial intervention to control *Salmonella* colonization on low-moisture foods and in their production environment.

Keywords: *Salmonella*; biofilm; low moisture foods; transposon mutagenesis

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1. Introduction

Foodborne *Salmonella* infection is an important public health issue, accounting for over 1.2 million cases, 23,000 hospitalizations, and 450 deaths each year in the United States [1]. Globally, nontyphoidal *Salmonella* causes ca. 150 million illnesses and ca. 60,000 deaths each year [2]. The symptoms of *Salmonella* infections vary from self-limited gastroenteritis to life-threatening systemic complications [3]. Outbreaks of foodborne *Salmonella* infection have historically been associated with animal products such as poultry, meat, or dairy products [3]. Recently, however, the pathogen has been implicated in outbreaks associated with fresh produce, including low-moisture products such as cereals, chocolates, seeds, peanuts, and almonds [4,5]. These foods have been erroneously considered to be safe and unable to support the growth of pathogenic microorganisms [4,5]. It is now well documented that pathogens like *Salmonella* can survive for days or even years under desiccation and in low-moisture foods [6].

The control of *Salmonella* in/on low-moisture foods is challenging because the pathogen has developed stronger cross-tolerance to multiple environmental stressors such as heat and chemical sanitizers compared to the pathogen in high-moisture foods [7]. Moreover, low-moisture foods have a relatively longer shelf life, and most of them are ready-to-eat products that do not require an additional killing step to eliminate the pathogen before consumption [8]. For this reason, once *Salmonella* is present in/on low-moisture food or in the production environment, it has a high probability of leading to long-lasting and widespread outbreaks.

Several cell-defensive strategies, including the synthesis of certain extracellular polymeric substances and the formation of biofilms, can protect *Salmonella* cells from desiccation [5]. Biofilm formation is a food safety concern unlimited to low-moisture foods. However, when bacterial cells with extraordinary biofilm-forming abilities are introduced to low-moisture food, they could persist much longer than those lacking such capabilities [9]. In particular, *Salmonella* cells able to synthesize extracellular polysaccharides, one of the major components of the biofilm matrix, have been reported to play an important role in survival under desiccation [10,11].

Although it is well documented that mutations in genes involved in the biosynthesis of curli, as well as cellulose and other exopolysaccharides, could affect cell initial attachment and biofilm formation [12–14], genetic determinants encoding many other cellular components that may have critical roles in biofilm formation remain unknown. To further understand the underlying molecular mechanisms of biofilm formation by *Salmonella*, mini-Tn*10* transposon mutagenesis was used initially to create a library of *S. enteritidis* mutants with randomly interrupted genes, and subsequently, the biofilm-forming ability of the wildtype *Salmonella* strain and its mutants was assessed and compared. To identify the defective gene of the mutants with a reduced ability to form biofilms, the genomic DNA sequence of the mutants was compared, using a BLAST search with the reference sequences of *S. enteritidis* deposited in Genbank.

2. Materials and Methods

2.1. Materials and Culture Conditions

The wildtype *Salmonella* strains used, and their mutants created, in this study are listed in Table 1. *Salmonella enterica* subsp. *enterica* serotype Enteritidis PT 30 isolated from a raw almond-related outbreak [15] and *Escherichia coli* strain BW20767 [16] were used in this study. The *S. enteritidis* strain was the causative agent of the 168 laboratory-confirmed cases of *Salmonella* infection (157 in Canada, 11 in the United States) between October 2000 and July 2001. It is a known biofilm producer, but its biofilm-forming ability relative to *Salmonella* reference strains is unknown. The *E. coli* BW 20767 was obtained from Dr. Nikki Freed at Massey University. Cultures of *S. enteritidis* and *E. coli* preserved at −80 °C were inoculated on tryptic soy agar (TSA; BBL/Difco, Sparks, MD, USA) and incubated at 37 °C for 18 h. The resulting culture was transferred to fresh TSA plates two consecutive times and incubated under the conditions described above. When necessary, the growth media were supplemented with antibiotics, kanamycin at 50 µg/mL, ampicillin at 100 µg/mL, and/or nalidixic acid at 30 µg/mL (MP Biomedicals, Santa Ana, CA, USA).

Table 1. Bacterial strains used in this study.

Strains	Description	Reference
S. enteritidis PT 30	The raw almond-associated outbreak strain; amp^s, kan^s, nal^r	[15]
E. coli BW20767	*pir+*; kan^r on mini-Tn*10*; amp^r on pJA1; nal^s on the chromosome	[16]
SE-L3	With a mini-Tn*10* insertion	This study
SE-L19	With a mini-Tn*10* insertion	This study
SE-L29	With a mini-Tn*10* insertion	This study
SE-S15	With a mini-Tn*10* insertion	This study
SE-S26	With a mini-Tn*10* insertion	This study

2.2. Construction of Salmonella enteritidis Mutants via Mini-Tn10 Transposon Mutagenesis

Mini-Tn*10* transposon mutagenesis was performed using a previously published protocol with some modifications [16]. The *E. coli* BW20767 strain harboring a kanamycin-resistant marker (kan^r) on the mini-Tn*10* transposon, an ampicillin-resistant marker (amp^r) on the suicidal plasmid pJA1, and a nalidixic acid-sensitive marker (nal^s) on the chromosome was used as the donor strain and the *S. enteritidis* almond outbreak strain (amp^s, kan^s, nal^r) was used as the recipient strain. The donor and recipient were grown individually in tryptic soy broth (TSB) at 37 °C for 18 h, and the resulting cultures were mixed in a 1:1 (*v*/*v*) ratio. The mixture (100 µL) was spotted on TSA and incubated at 37 °C for 18 h.

To activate the transposon, the resulting culture mixture was grown in TSB containing 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG; Fisher Scientific, Waltham, MA, USA) at 37 °C for 6 h. Following the incubation, the culture was streaked on TSA supplemented with kanamycin and nalidixic acid and incubated at 37 °C overnight. Cells that were resistant to kanamycin and nalidixic acid on TSA were randomly selected. To select *S. enteritidis* mutants generated by transposon mutagenesis, the susceptibility of cells to ampicillin on TSA was examined. Colonies that were resistant to kanamycin and nalidixic acid but sensitive to ampicillin were streaked on XLT4 agar and MacConkey agar. Black colonies on XLT4 agar with supplements that were colorless on MacConkey agar were randomly selected, and each colony was transferred to TSB amended with two antibiotics, kanamycin and nalidixic acid, and incubated at 37 °C overnight before being used in the biofilm study. The microbiological media used in this study were purchased from Becton Dickinson (Sparks, MD, USA).

2.3. Assessment of Biofilm-Forming Capability of S. enteritidis Mutants

The biofilm-forming ability of the mutants was assessed using the crystal violet binding assay [17,18]. In brief, mutants and their wildtype parent were grown for 18 h in Luria–Bertani no salt (LBNS) broth with or without kanamycin and nalidixic acid, respectively. Each cell suspension was diluted (1:40; v/v), and the resulting cell suspension (2 mL) was inoculated into a 24-well polystyrene tissue culture plate for biofilm development. The plates were incubated for 7 days at 25 °C. Following the incubation, the cultures in LBNS were withdrawn, and loosely attached cells were removed by rinsing with sterilized water three times. The biofilm mass was fixed with 95% ethanol (Koptec, King of Prussia, PA, USA) for 10 min and air-dried for 10 min at ambient temperature. The fixed biofilms were stained with 2% crystal violet (Fisher Scientific, Waltham, MA, USA) for 15 min, and the excess stain was then rinsed off with running tap water, followed by drying the plates for 1 h at room temperature. The crystal violet stain in stained biofilms was extracted with an ethanol and acetone mixture (80:20; VWR International, LLC, Randor, PA, USA). The absorbance of the ethanol–acetone solution was measured using a spectrophotometer (Thermo Fisher Scientific, Freemont, CA, USA) at a wavelength of 550 nm. The assay was replicated three times with duplicates each time. Significant differences in the absorbance values of crystal violet in the ethanol and acetone solution were analyzed using a one-way analysis of variance (ANOVA) and Fisher's least significant difference test ($p \leq 0.05$). Mutant colonies with a significantly lower amount of biofilm mass in comparison to their wildtype parent were selected for sequencing analysis.

2.4. Mini-Tn10 Transposon Insertion Sites in S. enteritidis Mutants

The genomic DNA of mutants that formed significantly less ($p \leq 0.05$) biofilm mass compared to their wildtype parent was extracted. In brief, each selected mutant was cultivated in 1 mL of TSB containing kanamycin and nalidixic acid for 18 h. The resulting culture was rinsed twice with sterile distilled water after centrifuging for 2 min at 14,000× g. After final washing and discarding the supernatant, the cell pellet was resuspended in sterilized distilled water (0.5 mL). Lysis buffer (20 μL) containing 20% (w/v) SDS, 0.5 M EDTA, and 1 M Tris-Cl was added to the cell suspension, followed by agitation at ambient temperature for 2 h on a Clay Adams brand @ Nutator (BD Biosciences, Bedford, MA, USA). A mixture of 0.5 mL of phenol and chloroform at a 1:1 ratio (v/v) was added to the sample for DNA purification, and the sample was agitated on the Nutator for 30 min. The sample was then centrifuged for 2 min at 14,000× g, and the supernatant containing DNA was collected. The DNA was precipitated with 1 mL of 100% ethanol overnight at −20 °C and precipitated DNA was collected after centrifugation under the conditions described above and dried subsequently at room temperature. The DNA dissolved in sterilized distilled water (30 μL) was submitted for purification and whole-genome sequencing at CD Genomics (Shirley, NY, USA, https://www.cd-genomics.com, accessed on 20 April 2024). The chemicals used for DNA extraction were from Fisher Scientific unless specified.

PilotEdit software (version 9.3.0; http://Pilotedit.com, accessed on 20 April 2024) was used to analyze the acquired sequencing data. DNA sequences containing the sequence of IS10, TTTTTACCAAAATCATTAGGGGATT, were compared to the *E. coli* mini-Tn*10*-based transposon cassette (accession no. AJ601386.1) and *S. enteritidis* (accession no. CP050716.1) using a BLAST search (https://blast.ncbi.nlm.nih.gov, accessed on 20 April 2024).

3. Results

3.1. Libraries of S. enteritidis Mutants

The kanamycin resistance gene (*kan^r*) on the mini-Tn*10* transposon carried by the suicidal plasmid pJA1 was successfully inserted into the chromosome of the *S. enteritidis* recipient (*nal^r*, *amp^s*). Sixty-four mutant colonies were selected from TSA supplemented with kanamycin, as well as nalidixic acid, the antibiotic resistance marker on the recipient chromosome. Cells of the mutant colonies were later confirmed to be sensitive to ampicillin, the gene of which was located on the suicidal plasmid that delivered the mini-Tn*10* to the recipient cells (Figure 1). The mutant colonies had the morphological characteristics of *Salmonella* on XLT4 and MacConkey agar plates (Figure 1).

Figure 1. Cultures of a selected *Salmonella* mutant on tryptic soy agar supplemented with 100 µg/mL ampicillin (**left**) and XLT-4 agar (**middle**), as well as the cultures of the *E. coli* donor BW20767 [pink] and the *Salmonella* mutant [colorless] (**right**) on MacConkey agar.

3.2. Selection of Mutants with Reduced Ability to Form Biofilms

Figure 2 shows the A_{550} values of the solutions of crystal violet extracted from the biofilms formed by the wildtype and mutant *Salmonella* cultures. Among tested mutants, SE-L3, SE-L19, SE-L29, SE-S15, and SE-S26 formed significantly ($p \leq 0.05$) less biofilm mass compared to their wildtype parent. However, the biofilm masses from the five mutants were not significantly ($p > 0.05$) different from one another.

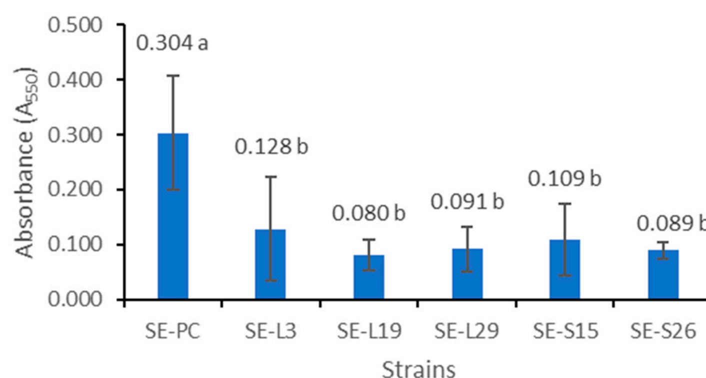

Figure 2. Biofilm mass developed by wildtype and mutant *S. enteritidis*. The data represent the A_{550} values of the solutions of crystal violet extracted from biofilm mass. The error bars represent the standard deviations of the means. Means followed by different letters are significantly different ($p \leq 0.05$). SE-PC: positive control from the wildtype parent strain.

3.3. Identification of the Mini-Tn10 Insertion Sites on the S. enteritidis Chromosome

The identified mini-Tn*10* insertion sites in examined mutants are reported in Table 2. The genetic locations of mini-Tn*10* insertion are shown in a circular chromosome map (Figure 3). In the SE-L3 mutant, the mini-Tn*10* interrupted the *eutE*, a gene responsible for the biosynthesis of aldehyde dehydrogenase, while in SE-L19, SE-L29, SE-S15, and

SE-S26, the transposon was found in the genes that encode for a transporter protein, outer membrane protein L, a ribbon–helix–helix protein from the CopG family, and cysteine desulfurase, respectively (Table 2).

Table 2. Alignment of acquired DNA sequences, interrupted by mini-Tn*10* in *Salmonella* mutants, with the reference Genbank sequences using a BLAST search *.

Mutant Name	Length of Analyzed Sequence (Base)	Position on Analyzed Sequence	Name of Matching Gene Products in the GenBank	Genbank Accession Number	Position of the Corresponding Sequence on the Reference Genome	Match (%)	Gap (%)
SE-L3	263	76–159	*E. coli* kanamycin-resistant Tn*10*-based transposon cassette	AJ601386.1	2516–2599	98	0
		153–263	Aldehyde dehydrogenase (EutE)	CP050716.1	1347001–1347111	100	0
SE-L19	262	74–150	*E. coli* kanamycin-resistant Tn*10*-based transposon cassette	AJ601386.1	2523–2599	100	0
		151–262	Transporter	CP050716.1	229143–229254	100	0
SE-L29	264	77–154	*E. coli* kanamycin-resistant Tn*10*-based transposon cassette	AJ601386.1	2522–2599	100	0
		153–264	Porin OmpL	CP050716.1	4505153–4505264	100	0
SE-S15	265	80–163	*E. coli* kanamycin-resistant *Tn*10-based transposon cassette	AJ601386.1	2515–2599	99	1
		157–265	Ribbon–helix–helix protein from the CopG family	CP050716.1	4031581–4031689	99	0
SE-S26	268	78–156	*E. coli* kanamycin-resistant *Tn*10-based transposon cassette	AJ601386.1	2521–2599	100	0
		155–268	Cysteine desulfurase (SufS) Cysteine desulfuration protein (SufE)	CP050716.1	2147129–2147242	100	0

* The identities of the genes interrupted by mini-Tn*10* were confirmed by Refseq NZ_WIBZ01000004.1 for SE-L3 (positions 21265–21155) and SE-L29 (positions 10837–10948); NZ_WIBO01000007.1 for SE-L29 (positions 24526–24425); NZ_WIBO0100003.1 for SE-S5 (positions 3411–3519); and NZ_WIBO01000022.1 for SE-S26 (positions 46285–46398).

In detail, bases 153 to 263 of the acquired DNA sequence from SE-L3 corresponded to bases 1347001 to 1347111 on an *S. enteritidis* genome (accession no. CP050716.1) and shared 100% identity and 0% gap with the genes encoding for the aldehyde dehydrogenase (EutE). In SE-L19, bases 151–262 of the sequencing data matched the sequence of a gene encoding for a transporter protein that corresponds to bases 229143 to 229254 on *S. enteritidis* genome CP050716.1 with 100% identity and 0% gap. The gene encoding for porin OmpL was interrupted by mini-Tn*10* in the SE-L29 mutant. Bases 153 to 264 of the sequence obtained from SE-L29 shared 100% identity and 0% gap with the DNA sequence corresponding

to bases 4505153 to 4505264 on *S. enteritidis* genome CP050716.1. In the SE-S15 mutant, bases 157–265 of the obtained sequence shared 99% identity and 0% gap with the gene encoding for a ribbon–helix–helix protein from the CopG family. In the SE-S26 mutant, bases 155–268 of the collected sequencing data corresponded to bases 2147129 to 2147242 of the reference *S. enteritidis* genome and matched sufS and sufE, genes that are involved in cysteine desulfuration.

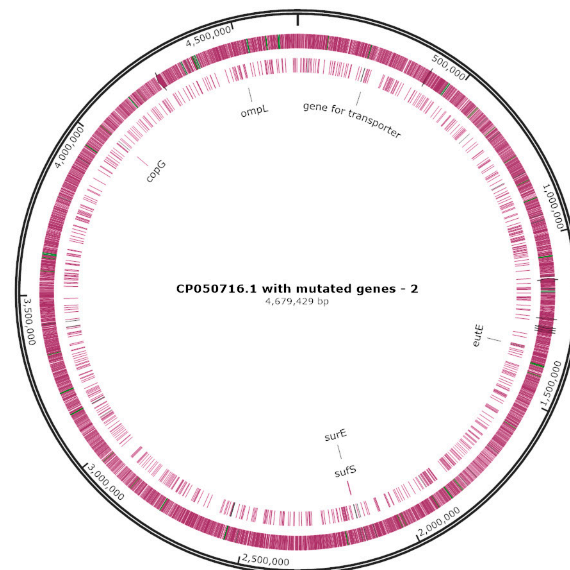

Figure 3. Mini-Tn*10* insertion locations in the SE-L3, SE-L19, SE-L29, SE-S15, and SE-S26 mutants on a circular chromosome map of *S. enteritidis* (accession no. CP050716.1).

Furthermore, bases 76–159, 74–150, 77–154, 80–163, and 79–156 of the acquired sequences from mutants SE-L3, L19, L29, S15, and S26, respectively, matched the *E. coli* kanamycin-resistant Tn*10*-based transposon cassette (accession no. AJ601386.1).

4. Discussion

The mini-Tn*10* in the SE-L3 mutant interrupted a gene that encodes for aldehyde dehydrogenase, EutE, which is involved in ethanolamine degradation (Table 2). Ethanolamine is a prevalent compound on the cell membrane of the host intestine [19]. Intestinal-associated bacteria including *Escherichia*, *Enterococcus*, and *Salmonella* can metabolize ethanolamine as a carbon and nitrogen source, and the ability of cells to metabolize ethanolamine contributes to pathogenesis by outcompeting bacteria that cannot utilize it [20,21].

The genes required for ethanolamine utilization are clustered on the ethanolamine utilization operon (*eut*). EutBC is an enzyme of ethanolamine ammonia lyase that degrades ethanolamine into acetaldehyde (carbon source) and ammonia (nitrogen source). EutE, an aldehyde dehydrogenase, converts acetaldehyde into acetyl-CoA, which can be used in a myriad of metabolic reactions including the tricarboxylic acid (TCA) cycle. In addition, acetyl-CoA can be phosphorylated into acetyl phosphate by phosphate acetyltransferase, Pta, subsequently forming acetate by acetate kinase (AcaK) to generate ATP [20,22]. Thus, mutations in *eutE* can result in changes in the cellular concentration of acetaldehyde, acetyl-CoA, and downstream metabolites of acetyl-CoA such as acetyl phosphate in bacterial cells.

Acetyl phosphate has been shown to play important roles in the biosynthesis of flagella and curli, extracellular polymeric substances required for biofilm formation [22,23]. Acetyl phosphate transfers the phosphoryl group into the two-component signaling system, OmpR [24,25] and RcsB [26]. OmpR, a response regulator, is required for the regulation of the *csgD* promoter. CsgD is a biofilm regulator that is responsible for the biosynthesis of organelles involved in bacteria motility such as flagella and for the production of cellulose and curli [27]. RcsB is nevertheless an activator of colanic acid production, which is one of the major extracellular polymeric substances facilitating the maturation of biofilms [28]. It

has been reported that the phosphorylation of RcsB acts as a key signal to switch planktonic cells to a sessile state in *S. typhimurium* [29].

Previous studies have shown that changes in the cellular level of acetyl-CoA through manipulation of the genes regulating acetyl-CoA metabolism affect cells' ability to form biofilms [30,31]. For example, mutations in the acetyl-CoA positive regulatory genes, *ackA* (encoding for acetate kinase), *pta* (encoding for phosphotransacetylase), and *ldhA* (encoding for lactate dehydrogenase), caused the cells of *E. coli* to form more biofilms, while a mutation in the acetyl-CoA negative regulatory gene, *pflA*, decreased the cells' ability to form biofilms [30]. In *E. coli*, the acetyl-CoA level was negatively regulated by PpiB, which belongs to the superfamily of peptidyl-prolyl cis/trans isomerase. Mutants with a defective *ppiB* produced a higher level of acetyl-CoA and produced more biofilm than the wildtype strain [32]. In the current study, the SE-L3 mutant with a defective gene encoding EutE might have been unable to convert acetaldehyde into acetyl-CoA, and consequentially, the cells of SE-L3 had a poorer ability to form biofilms.

In the SE-L19 and SE-L29 mutants, mini-Tn*10* insertions disrupted the coding region of the genes encoding a transporter and porin OmpL, respectively. Bacteria have both passive and active transport systems. Passive transport allows passive diffusion of small molecules using channels such as porins in outer membranes, while active transport moves molecules against a concentration gradient, which requires ATP as an energy source [33,34]. Bacterial transporters are responsible for the uptake of essential nutrients including polysaccharides, amino acids, minerals, and vitamins, as well as the secretion of various molecules including toxins and antimicrobial agents out of the cell [35]. Thus, transporter proteins play critical roles in bacterial growth and antimicrobial resistance [36]. In addition to supporting survival against toxic compounds, the role of ABC transporters in biofilm development has been proposed in many microorganisms. For example, mutations in *pstS*, *pstC*, and *pstA* in the *pst* operon, encoding for the high-affinity phosphate-specific transporter in the ABC transporter family, decreased the amount of biofilm formed by bacteria such as *Pseudomonas aureofaciens* [36] and *Proteus mirabilis* [37]. The Pst system is required to repress the expression of phosphate (Pho) regulon; disruption of the Pst systems leads to constitutive expression of the Pho regulon [36,38]. Pho regulon expression has been demonstrated to inhibit biofilm formation by expression of RapA phosphodiesterase that lowers the level of c-di-GMP [30–40], a second messenger in an intracellular signaling system that controls bacterial lifestyle switching between sessile biofilm and planktonic cells [41]. In *Streptococcus mutans*, the deletion of *pstS* caused a reduction in extracellular polysaccharide production, which is necessary for adhesion and biofilm formation, and a reduced ability to attach to certain surfaces [38]. Similarly, the *lapEBC*-encoded ABC transporter plays important roles in the export of adhesion, LapA, and biofilm maturation in *Pseudomonas fluorescens* as the mutations within the *lapEBC* cluster caused the loss of detectable LapA on the cell surface and defects in irreversible attachment, thus forming a less matured biofilm structure compared to wildtype cells [42]. Collectively, these observations indicate that the mutations in the phosphate transporter and adhesin LapA transporter negatively regulate biofilm formation, possibly by activating the Pho regulon, which in turn decreases the cellular level of c-di-GMP and disrupts cell adhesion and colonization on surfaces, the events critical for the establishment of a biofilm community.

The outer membrane porin L (OmpL) is homologous to YshA [43], which is highly conserved and widely distributed among different *Salmonella* serovars [44]. The potential role of YshA in biofilm formation has been discussed [45]. In a previous study, the *yshA*-mutant cells of *S. typhimurium* accumulated less biofilm mass on a polyvinyl chloride plate than the wildtype cells in LB no salt broth [45]. The *yshA* is part of the *yihU-yshA* operon, which is required for the assembly and translocation of the O-antigen, one of the main constituents of the extracellular matrix of *Salmonella* [46]. Previous studies have shown that the O-antigen capsule is crucial for *Salmonella* attachment and biofilm formation on various surfaces [47,48].

The mass of biofilm was significantly lower when the gene encoding for a ribbon–helix–helix (RHH) protein from the CopG family was disrupted in the SE-L15 mutant (Figure 1). The RHH motif is a common structural motif of prokaryotic antitoxin proteins [49]. The link between the toxin–antitoxin system and biofilm formation has been explored. MazEF, for example, is one of the antitoxin–toxin systems in *E. coli* and many other pathogenic bacteria including *S. enterica* [50]. MazE, the antitoxin, is a putative transcriptional repressor of the CopG family [51]. It neutralizes MazF, a stable toxin that cleaves RNA to inhibit protein translation and interfere with vital cellular processes [52]. A significant increase in biofilm formation was observed in a *Staphylococcus aureus* strain lacking *mazF* expression [53]. Loss of MazF also caused increased expression of intercellular adhesion gene cluster (*ica*) and production of polysaccharide intercellular adhesin. The decrease in biofilm mass formed by the SE-S15 mutant with the knock-off gene encoding for the RHH protein of the CopG family could be due to an impaired RHH motif-containing antitoxin that releases the toxin, resulting in the inhibition of adhesion gene expression and subsequently biofilm formation.

The expression of *sufS* and *sufE* was interrupted in the SE-S26 mutant. SufS and SufE are part of the sulfur formation (SUF) system, involved in iron–sulfur (FeS) cluster biogenesis under iron starvation and oxidative stress in many bacteria [53,54]. The SUF system is encoded by the *sufABCDSE* operon, which encodes two protein complexes, SufSE and SufBCD, and one SufA protein [54]. SufS is a cysteine desulfurase that forms a complex with SufE, which has an unknown function. The SufSE complex mobilizes sulfur atoms from cysteine and provides sulfur atoms to the SufBCD complex to assemble an FeS cluster [54], which is a cofactor of several enzymes such as aconitase, succinate dehydrogenase, and glutamate synthase [54–56].

Aconitase is an enzyme that catalyzes the isomerization of citrate to isocitrate in the TCA cycle [55,57]. Mutating the gene encoding for aconitase resulted in a decrease in biofilm formation, possibly due to the interference of citrate flow in the TCA cycle [57]. Glutamate synthase is an FeS cluster-containing enzyme that connects the TCA cycle to nitrogen metabolism by converting 2-ketoglutarate, a TCA cycle intermediate, into glutamate, which contributes to ammonium assimilation and the biosynthesis of nitrogen-containing compounds such as amino acids [58]. *B. subtilis* cells with a mutation in *gltA*, the gene encoding for glutamate synthase, formed thin and flat pellicles and defective biofilms compared to the wildtype cells [58]. The biofilm defect in mutant cells could have resulted from an accumulation of citrate as evidenced by the restored biofilms in a *gltA* mutant with limited citrate synthase activity [58]. The relationship between citrate concentration in growth media and biofilm formation has been studied previously [59]. A high citrate concentration (2%) was bactericidal, while a low concentration (0.2%) facilitated biofilm formation [59]. Additionally, the accumulation of citrate in *gltA* mutant cells could cause iron shortage by chelating [58]. Iron shortage can induce a planktonic mode of growth in the liquid phase, while a high iron concentration stimulates cell aggregation and biofilm formation [60]. Thus, the insertion of mini-Tn*10* into *sufSE* could likely fail to activate FeS assembly-dependent metabolic enzymes, aconitase, and glutamate synthase, subsequently interfering with the TCA cycle, resulting in an iron shortage due to the accumulation of TCA cycle intermediates and, consequently, defective biofilm formation.

Other than those identified in the current study, other *Salmonella* genes that play a role in biofilm formation have been identified in previous studies. Mutations in *flgK* and *rfbA*, encoding flagella and lipopolysaccharide production, respectively, in *S. typhimurium* DT104, resulted in decreased biofilm formation [45]. Solano et al. found that the synthesis of exopolysaccharide cellulose encoded by *bcsABZC* and *bcsEFG* operons was required for biofilm formation by *S. enteritidis* [13]. Barak et al. reported that mutations in the genes of *S. enteritidis* regulating curli, cellulose, and pili production reduced the ability of pathogenic cells to attach to plant tissues [12]. Chen and Wang found that the level of biofilm mass significantly decreased when *cdg*, *trx2*, and *rtx* were interrupted by mini-Tn*10* insertion, while the interruption of *fadI* gene significantly increased the level of biofilm formation [61].

This study identified some of the genes that are involved in biofilm formation by *S. enteritidis*, namely those that are involved in the biosynthesis of aldehyde dehydrogenase (EutE), cysteine desulfurase (SufS or SufE), a transporter protein, porin OmpL, and a ribbon–helix–helix protein from the CopG family. The research could serve as a starting point for a series of future studies. Knock-off mutants could be constructed to verify the function of the genes. If successful, the proteins encoded by the genes characterized in the current study could serve as potential targets for the development of antimicrobial interventions to prevent pathogen contamination in low-moisture foods and their production environment.

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Conflicts of Interest: The authors declare no conflicts of interest.

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