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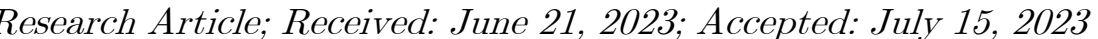

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DETECTION TARGET GENES IN COMBATING BIOFILM FORMS IN *SALMONELLA* TYPHIMURIUM 14028

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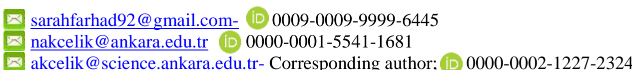
ABSTRACT. In this study, the relationship of *hilA*, *invA*, *adrA*, *spiC*, *otsB* and *csgD* genes, which are known to play critical roles in the pathogenicity and virulence of *Salmonella* strains, with biofilm formation was investigated by examining the changes in the expression levels of these genes during the transition from planktonic form to biofilm form. When the virulence gene expressions between the *S*. Typhimurium 14028 mutant, which lost its ability to form biofilms due to *csgD* gene deletion, and the wild type strain were compared, it was determined that the expression levels of *hilA*, *invA* and *adrA* genes increased, whereas the expression levels of *spiC*, *otsB* and *csgD* genes decreased. These data indicate that all examined genes play critical activation or inhibition roles in biofilm regulation as well as pathogenicity and virulence. On the other hand, in the mutant strain; The increase in the expression levels of *hilA*, *invA* and *adrA* genes shows that inhibitors of the proteins encoded by these genes have the potential to be of practical use in the prevention and control of infections caused by both biofilm-forming and non-biofilm-forming *Salmonella* strains.

1. INTRODUCTION

Salmonella is an important genus of pathogenic bacteria that has many hosts and causes different diseases in these hosts, including different subspecies and serovariates. In addition to the serovariates of species and subspecies of this genus adapted to certain host organisms, serovariates with wide host ranges can also be found. These serovariates can cause local or systemic diseases with very different courses from limited gastroenteritis caused by non-typhoid *Salmonella* (NTS) serovariates to typhoid fever, which can result in fatal intestinal perforation. Although it has been determined that plant material is a serious source of contamination today; animal products, especially poultry products, are identified as the main sources of *Salmonella* sp. [1].

The clinical course and outcome of salmonellosis is characterized by a complex host-bacteria interactions. Efficiency of host response to infection by NTS *Salmonella* serovariates, it varies depending on many factors such as nutritional status, age, gastric pH, genetic predisposition, and both innate and adaptive immunity [2]. Bacterial features that contribute to the severity of the disease can be defined as serotype, infectious dose, physiological state of bacterial cells,

Keywords. Salmonella, biofilm, gene expression, qRT-PCR



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antimicrobial resistance (AMR), gene inactivation and virulence factors. It is possible to summarize virulence factors of *Salmonella* as toxins, capsule, flagella, fimbrial structures, effector proteins and their secretion systems. These factors are encoded by *Salmonella* Pathogenicity Islands (SPIs), virulence plasmids or prophages (temperate phages). To date, 24 SPIs have been identified whose functionality has been described at different stages of *Salmonella* infection. Among these SPIs, SPI-1 and SPI-2 are the ones whose genetic and phenotypic features have been studied the most. SPI-1 is common in both *S. enterica* and *S. bongori* and encodes a type three secretion system (T3SS) that allows the displacement of effector molecules involved in the invasion of host cells. SPI-2, found in many subspecies of *S. enterica* but not in *S. bongori*, contains an additional T3SS genes involved in the translocation of effector molecules important for the intracellular survival of these bacteria. Other SPIs are variably present in *S. enterica* subspecies, and some encode other secretion systems such as T1SS and T6SS, other effector molecules and fimbriae [3].

The much higher resistance of biofilm forms of these bacteria to antimicrobial agents compared to planktonic strains increases the likelihood of failures in the prevention and treatment of the spread of *Salmonella* infections and limits therapeutic options in industrial and clinical applications when treatment is required. In addition to their high resistance to adverse environmental conditions, biofilms that develop on equipment and tools used in food industry and medical treatment also constitute reservoirs of pathogenic and food spoilage microorganisms that increase the risk of microbial contamination [4,5,6]. For these reasons, *Salmonella* biofilm infections originating from the food industry and hospitals cause serious health problems and economic losses all over the world [6].

Biofilm structures, which are a multicellular organization-like life form, are the result of genetic and therefore physiological reprogramming of bacteria attached to a surface in solid-air or liquid-air intermediate phases, surrounded by an exopolymeric matrix that functions as a protection against stress conditions and a nutrient store. The formation of functional differentiation in cells of the same species in the biofilm structure is the main reason why these structures are defined as multicellular bacterial life forms [7]. The fact that the resistance of the biofilm structures of bacteria to all known bacterial control agents is much higher than the planktonic forms of the same bacteria has brought about the necessity of determining new control agents against these structures. In this regard, the detection or development of natural or synthetic antibiofilm agents is one of the most commonly used strategies. Here, the fact that the biofilm matrix generally does not allow the penetration of the agents in question or significantly prevents their entry into the biofilm structure is the main reason limiting the chance of combating new agents [8,9,10]. The safest way to overcome this handicap is to determine the genetic expression differences that occur in the transition from the planktonic form to the biofilm form in a bacterium and to develop specific control agents for the gene products to be selected in this direction. In this way,

both the prevention of the formation of biofilm structures and the eradication of the formed biofilms will be possible in a safer way [11].

In this study, the expression levels of *otsB, spiC, adrA, csgD, hilA* and *invA* genes that play a role in virulence and pathogenicity in *S.* Typhimurium 14028 wild-type strain and its mutant with impaired biofilm forming ability (14028*ΔcsgD*) were examined, and the efficacy of these genes in the transition to biofilm form was investigated. Therefore, it is aimed to lay the foundations of an effective antibiofilm strategy that will target both biofilm structures and pathogen-associated molecular patterns (PAMPs).

2. MATERIALS AND METHODS

2.1 Biomaterial

S. Typhimurium 14028 strain was obtained from American Type Culture Collection (ATCC, 10801 University Blvd, Manassas, VA, USA), and the *csgD* gene mutant of this strain, which cannot form biofilm, was obtained from Ankara University Biotechnology Institute. Luria-Bertani (LB) broth and agar (Merck, Rahway, NJ 07065 USA) media were used for the activation and routine production of bacteria from stock cultures. Stock cultures were stored in LB broth media containing 40% glycerol at -80 ^{0}C.

2.2. Formation and measurement of biofilm structures

After single colonies of the bacteria to be tested were suspended in TSB medium, the density of the suspension was standardized by comparison with the 1.0 McFarland standard (ie. 3.0 X 10^8 cfu/mL). Cultures were diluted 1:30 in freshly prepared growth medium to obtain a bacterial concentration of approximately 1.0 X 10^7 cfu/mL in sterile polypropylene or glass tubes. Thereafter, 150 µL volumes of 1/30 dilutions were added to each well of a 96-well microtiter plate (Corning® Thermowell PCR 96 well plates, Merck, Rahway, NJ 07065 USA). After the autoclaved PEGs (Merck, Rahway, NJ 07065 USA) were placed in the growth medium, the microtiter plates were covered with aluminum foil and incubated at 20 ºC for 24, 48 and 72 hours. After the supernatants were removed at the end of the incubation period, the wells were washed three times with phosphate-buffered saline (PBS, pH 7.0±2.0). After washing, 140 µL of 95 % methanol was added to fix the biofilm structures attached to the PEGs and kept at room temperature for 20 minutes. Biofilm structures were stained for 15 minutes using 1% crystal violet. The plates were washed with sterile distilled water, and the microplates were dried at room temperature after removing the dye that did not adhere to the biofilm structures. 140 µL (33%) glacial acetic acid was added to the wells to dissolve the dye bound to the produced biofilm, and the plates were incubated at room temperature for 30 minutes. At the end of the incubation, the amount of dye attached to the biofilm was determined at OD_{595} nm in the ELISA reader (Biorad, USA). The final calculation was performed by subtracting the average of the OD values of the control (wells containing LB-

NaCl broth only) group from the mean of the OD values determined for the strains tested. These trials were carried out in 3 parallel and 2 repetitions [12].

2.3. Quantitative real time PCR (QRT-PCR)

S. Typhimurium 14028 and its mutant obtained by deletion of *csgD* regulator gene were inoculated at 1% in LB broth medium and incubated at 37 ºC under shaking conditions until the optical density (OD_{600}) value reached approximately 0.6 in the growth medium. High Pure RNA Isolation (Promega) kit was used for total RNA detection from bacterial cultures. Purity and quantification of the obtained RNAs were determined using the ND-1000 spectrophotometer (Thermo Scientific / USA) device, and electrophoresis was performed in 2% agarose gel at 100 V constant electric current for 1 hour. RNA gels containing 0.2 µg/mL EtBr were visualized under UV light. Molecular size determination was performed using the GeneRuler 1 kb (kilobase) DNA Ladder (Thermo Scientific / USA).

cDNA synthesis with RNA samples obtained from *S.* Typhimurium 14028 and *csgD g*ene mutant was performed using cDNA synthesis Kit (Roche, Germany). The reaction mixtures used in cDNA synthesis and the applied temperature cycle were given Tables 1, 2 and 3. The resulting cDNAs were stored at -20 °C until qRT-PCR experiments.

TABLE 1. Denaturation solution

Contents	Concentration	Final Concentration	Volume (µL)
RNA	-	1000 ng/µL	-
Random primer	600 pmol/µL	60 µM	2
Water (PCR grade)	-	-	Volume is made up 13 µL

TABLE 2. Reverse transcription solution

Contents	Concentration	Final Concentration	Volume (µL)
Reverse transcription buffer	5X	1X (8 mM $MgCl_2$)	4
RNase inhibitor	40 U/µL	20 U	0.5
dNTP mix	10 mM for each nucleotide	1 mM for every nucleotide	2
Reverse transcriptase	20 U/mL	10 U	0.5
Final volume	-	-	**20**

TABLE 3. Temperature cycle for reverse transcription reactions

Steps	Temperature (°C)	Time (min)	Number of cycle
Elongation 1	25	10	1
Elongation 2	50	60	1
Inactivation	85	5	1

2.4 Determination of gene expression levels

The genes whose expression levels were investigated in QRT-PCR experiments and the primers designed specifically for these genes are given in Table 4. LightCycler 480 (Roche, Germany) device was used for all QRT-PCR experiments performed in this study. 5 X HOT FIREPol EvaGreen QRT-PCR Supermix (Solis BioDyne, Estonia) kit was used as the amplification mix for all the genes given in Table 5. The amplification mix for QRT-PCR is given in Table 6. Amplification reactions were performed in 96-well plates in a total volume of 10 µL. The reaction mixture containing the same volume of ddH2O without the cDNA template was used as a negative control (NK). The program used for amplification in Light Cycler 480 device performed as given in Table 6 [13].

TABLE 4. Genes and primers

Target Genes and Their Functions	Primer	Reference
adrA, Regulator for cellulose production	F: GGCTGGGTCAGCTACCAG R: CGTCGGTTATACACGCCCG	[14]
csgD, Regulator for curli fimbria synthesis	F: ACGCTACTGAAG ACC AGG AAC R: GCATTCGCCACGCAGAATA	[15]
hilA, Virulence regulator	F: CATGGCTGGTCAGTTGGAG R: CGTAATTCATCGCCTAAACG	[16]
spiC, Encodes an efector protein. For host cell invasion	F: CTGTGGCTTTCAGTGGTCAG R: TGCGTTGTCCGGTAGTATTTC	[16]
invA, Host cell invasion	F: CACGCTCTTTCGTCTGGCA R: TACGGTTCCTTTGACGGTGCGA	[17]
otsB, stress response, trehalose production	F: TTAACCGTATCCCCCGAACTC R: CCGCGAGACGGTCTAACAAC	[18]

TABLE 5. Amplification mixture for QRT-PCR

Contents	Final Concentration	Volume (µL)
EvaGreen qPCR supermix (5X)	1X	2
Forward primer	10 pmol / µL	0.5
Reverse primer	10 pmol / µL	0.5
cDNA (1:10)	10 ng /µL	1
ddH_2O	-	6
Final volume	-	10

TABLE 6. qRT-PCR programme for LightCycler 480

Steps	Process	Temperature (^{0}C)	Time
First denaturation	First denaturation	95	15 dk
Amplification (40 cycles)	Denaturation	95	15 sn
	Annealing	58*	20 sn
	Elongation	72	20 sn
Melting curve	Denaturation	95	30 sn
	Annealing	60	30 sn
	Elongation	99	30 sn
Cooling	Cooling	40	30 sn

*Primer binding Temperature™ was determined as 58 ºC for all tested genes and 55 ºC for the reference gene 16S rDNA.

2.5 Statistical analyses

Statistical analyzes of the data obtained from the study were performed using Graph Pad Prism Version 5.10 (Graph Pad Software Inc., San Diego, CA, USA). In this program, the data were first subjected to Dunnett post hoc and then one-way ANOVA analysis. The normalization of the data obtained from the live cell counts was performed on the basis of log10 [19]. In the interpretation of all statistical studies, the threshold P value was taken as <0.05.

3. RESULTS AND DISCUSSION

3.1. Biofilm formation characteristics of wild type strain and its *csgD* gene deleted mutant

In the study conducted to determine the biofilm production capacities of *S.* Typhimurium 14028 wild type and its *csgD* mutant strain, the highest amount of biofilm production was determined at 72 hours in the incubation periods tried. The amount of biofilm production of the *csgD* gene mutant strain was reduced by approximately 90-95% ($p<0.05$) compared to the wild type at all incubation times tried (Figure 1). These measurement values in the mutant are within the definition of the strain that does not have the ability to produce biofilms [20,21,22].

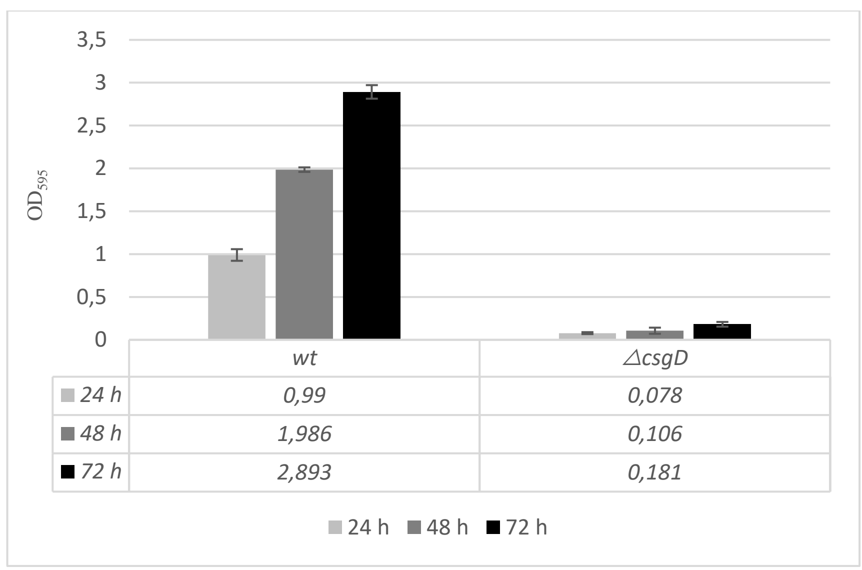

FIGURE 1. Biofilm production capacities of *S.* Typhimurium 14028 wild type strain and its *csgD* mutant.

3.2. Changes in expression levels of virulence and pathogenicity-related genes in *S.* Typhimurium 14028 wild type strain and its non-biofilm forming mutant (14028*ΔcsgD*)

In order to determine the changes in virulence and pathogenicity between *S.* Typhimurium 14028 wild type strain and its biofilm-forming mutant (14028*ΔcsgD*), expression levels of *otsB, spiC, adrA, csgD, hilA* and *invA* genes were investigated. For the qRT-PCR experiments carried out in this direction, total RNA isolations from all strains were performed in the first step and the purity of the isolated RNA samples were checked on agarose gels (Figure 2).

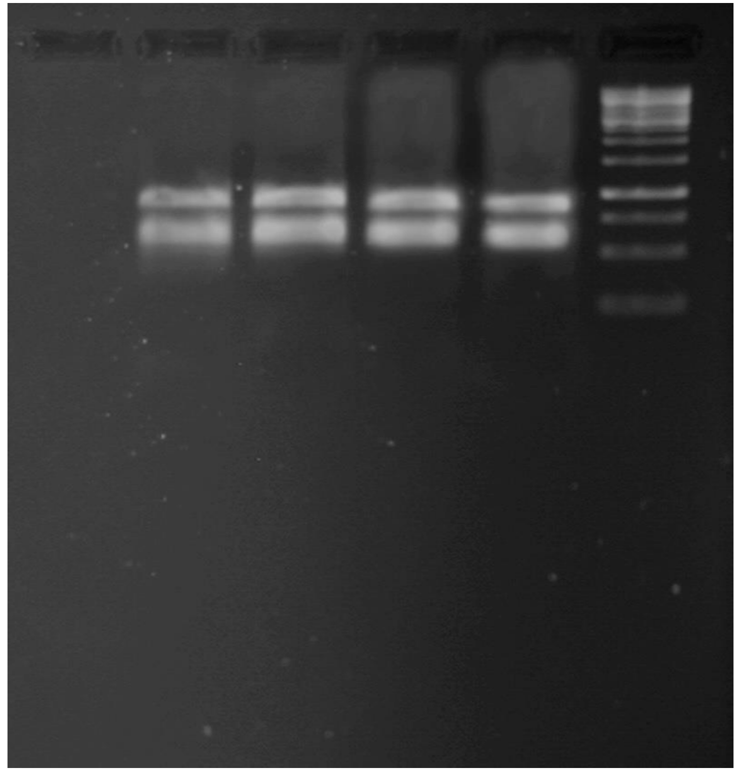

FIGURE 2. Agarose gel image of total RNAs isolated from *S.* Typhimurium 14028 and *ΔcsgD* mutant.

In the next step, cDNA synthesis was performed from the RNA samples determined to be of sufficient purity and concentration, and the obtained cDNA samples were used in qRT-PCR experiments. In order to analyze the qRT-PCR data obtained from the experiments, the amplification efficiencies of the primers were added to the normalization calculations as a variable. In summary, Ct values obtained for each primer pair using serial dilutions prepared with cDNA samples obtained from *S.* Typhimurium 14028 wild type strain were used as calibrators. Expression level of 16S rRNA gene, which is a housekeeing gene, was used as qRT-PCR control (Table 7 and Figure 3).

TABLE 7. Ct values of genes whose expression levels were investigated in wild type and mutant strains

CT Values	*otsB*	*spiC*	*adrA*	*csgD*	*hilA*	*invA*	*16S RNA*
14028 WT	24,56	24,47	24,49	25,22	24,54	24,47	30,22
	24,51	24,44	24,44	25,16	24,56	24,51	30,57
	24,54	24,36	24,33	25,24	24,64	24,48	32,14
14028*ΔcsgD*	28,31	28,57	21,01	28,18	20,71	21,34	31,145
	28,13	28,06	20,99	27,02	20,61	21,48	30,87
	28,14	28,3	20,99	27,74	20,65	21,49	31,81

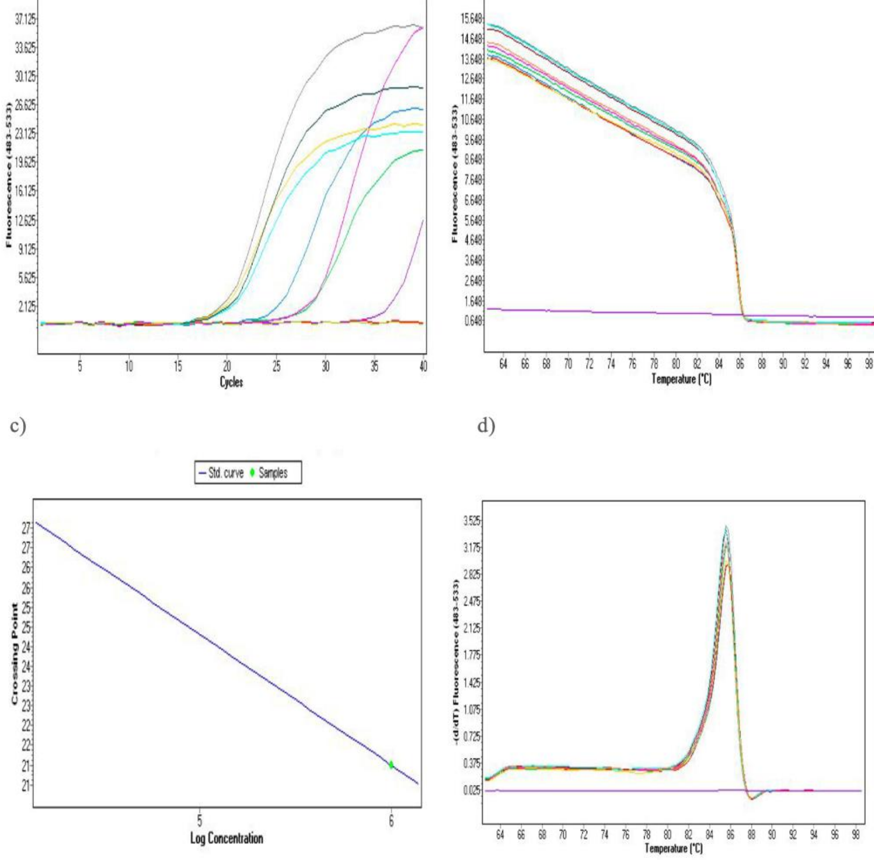

FIGURE 3. Results of the standardization process performed in qRT-PCR experiments. a) amplification curves, b) melting curves, c) standard curve, d) melting peaks.

After these standardization and normalization measurements, fold changes in the expression levels of the genes investigated using the Livak method were determined. As a result of these studies, the expression levels of the investigated genes in *S.* Typhimurium 14028 wild-type strain and its biofilm deficient mutant (14028*ΔcsgD*) were defined comparatively. According to these data, the expression levels of *spiC, otsB* and *csgD* genes were decreased by 12.25, 10.26 and 4.41 folds (*p≤0.05), and the expression levels of *adrA, hilA* and *invA* genes increased by 13.19, 18.66 and 10.18 folds, respectively, in mutant strain (Figure 4).

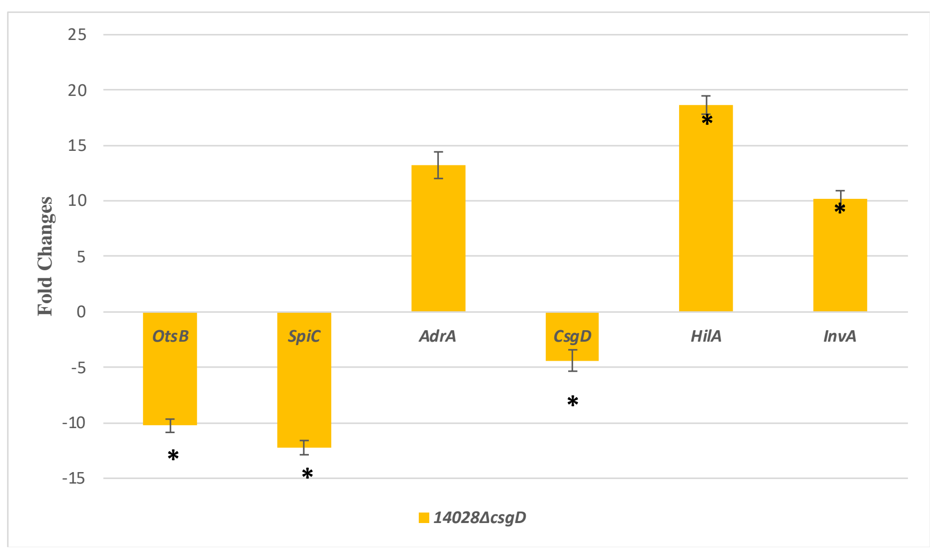

FIGURE 4. Fold changes of normalized gene expression levels in *S.* Typhimurim 14028 *csgD* mutant compared to wild-type strain.

When the data obtained from these trials were examined; The *hilA* gene, which is a member of the Ompr/ToxR family and acts as a transcriptional activator of genes related to host cell invasion in *S.* Typhimurium [23], the *invA* gene, which is the positive regulator of the Type 3 Secretory System (T3SS) [24] and the *adrA* gene, which encodes an enzyme that participates in cyclic di ganosyl monophosphate (c-di-GMP) metabolism in *Salmonella*, and which in this way is active in the biosynthesis of cellulose[25], expression levels were found to increase. This indicates that environmental adaptation is achieved by increasing host cell invasion functions in mutants whose environmental persistence has decreased due to loss of biofilm formation ability. These findings may shed light on the evolution of *Salmonella* pathogenicity, with expression levels of genes involved in whole cell invasion generalized by comparative analysis. These findings also show that inhibitors of the proteins encoded by these genes have the potential to be of practical use in the prevention and control of infections caused by both biofilm-forming and non-biofilm-forming *Salmonella* strains.

On the other hand, the decrease in the expression level of the *spiC* gene [26], which encodes a translocator protein in *Salmonella* and whose role in pathogenicity is controversial, but which is claimed to be involved in traffic blocking in cases where cell trafficking is disrupted, indicates that the gene in question has functions different from those suggested in the literature. In addition, the decrease in the expression of the *otsB* gene [27], which encodes the acid stress and low water activity resistance protein in the mutant that has lost the ability to form biofilm, is a new evidence supporting the relationship of these functions with biofilm structures. Finally, the detection of decreased expression levels of the *csgD* gene [28], which is the transcriptional regulator of the coiled fimbria, which is an important component of the biofilm matrix in *Salmonella* serovariates, is evidence that the target function of the mutant used is impaired.

4. CONCLUSIONS

In our study, differences in the expression levels of certain virulence genes were investigated between the *S.* Typhimrium 14028 wild strain and its biofilm-deficient mutant (14028*ΔcsgD*) to identify pathogen-associated molecular patterns (PAMP) to be targeted to combat *Salmonella* biofilm forms. These expression differences clearly showed that the expression levels of three important genes known to play a role in *Salmonella* pathogenicity and virulence (*invA, hilA* and *adrA*) were increased in the biofilm-producing mutant of *S.* Typhimurium 14028 strain. This indicates that these genes play important roles not only in pathogenicity and virulence, but also in the transition from the planktonic form to the biofilm form. In the light of these data, it is possible to say that choosing and testing natural or synthetic compounds with strong inhibitory activity against the products of *invA, hilA* and *adrA* genes as antibiofilm agents has a strong potential to combat biofilm structures.

Author Contribution Statements SFN- data collection and analysis, interpretation of results and draft manuscript preparation. NA-study conception and design, data analysis, interpretation of results, manuscript editing. MA-study conception and design, interpretation of results, manuscript editing. All authors reviewed the results and approved the final version of the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

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