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Two specific genes in Salmonella biofilm trigger colonial metamorphosis in the table eggs contents

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***Abstract*---**From hen-laying farms in Iraq, 100 collected white and brown egg contents were extracted. Salmonella spp. is present in 11 of the numbers isolates in white table egg content and six numbers brown table eggs. That represents a 17% proportion. The total count range of bacteria in egg contents was between 9.2 to 27 x106 log CFU/ ml. The 17 different isolates of Salmonella species. each isolate's biofilm formation is measured. At least one strain had a high capacity to generate biofilm (>0.400), two isolates had a moderate capacity (0.2–0.4), nine isolates had a low capacity (0.2–0.12), while 5 isolates did not produce biofilm (<0.11).The study is the first of its kind in Iraq and examines the gene expression targets csgD and bcsA that Salmonella spp. uses to produce biofilms.

***Keywords*---**Salmonella spp, biofilm, csgD, bcsA gene, white egg, brown egg.

Introduction

Table eggs are the best and easy source of food, containing quality protein, essential amino acids, essential vitamins, and minerals needed for good health (MAFF, 2009). Food poisoning associated with egg-borne pathogens may cause severe morbidity or mortality with diarrhea, vomiting, nausea, and abdominal cramps (Mitchell, 2005). Bacterial contamination can happen in three main parts of the egg (egg yolk, albumen, and shell membrane/eggshell) (Bahrouz, 2005). Eggs have a natural defense system against the contaminating microbes, such as cuticle, calcium hard shell, and shell membrane (Jerzy and Dagmara, 2009). The albumen contains several egg white proteins that have antimicrobial properties, especially the lysozyme. Ovomucoid is another proteinase that inhibits the ability

of bacteria to use the protein in albumen. Furthermore, the pH in albumen is about 9–10, and the viscosities of the egg white are not suitable for microbial growth (Froning, 1998).

Salmonella has been found in the ovaries of infected laying hens, including Salmonella Typhimurium in commercial flocks. Therefore, laying hens, if infected with Salmonella, may produce eggs with blood spots that could contain this pathogen. In an indirect survey, table eggs with blood spots procured from several commercial flocks were nearly twice as likely to contain Salmonella Enteritidis as eggs without blood spots (Sokołowicz, et al., 2018). Eggs can be infected by Salmonella via two major routes, vertical and horizontal. Vertical transmission (transovarial infection) occurs when the egg contents are contaminated with Salmonella during the formation of the egg before this is covered with the shell (Messens, 2005). The horizontal transmission includes trans shell infection of the contents of the egg during transit through the cloaca or after oviposition and fecal contamination of the external surface of the shell (EFSA, 2005). (Svobodová, et al,.2021) were able to demonstrate that Salmonella spp. has the ability to pass from the alimentary canal, via the blood, to the ovaries. Also, it has been suggested that in some instances, disease-causing bacteria may be excreted from the ovarian blood supply or may infect the ovary tissue.

Biofilms are structured aggregates of bacterial cells that are embedded in self-produced extracellular polymeric substances (EPS) (Ono, et al,.2014; Yawata, et al,.2014). Biofilm-forming cells usually differ from their planktonic counterparts and exhibit differences in gene expression (Doulgeraki, et al,.2017). Biofilm formation is one of the most important mechanisms utilized by Salmonella to survive in host cells(Steenackers H, et al., 2012) It also contributes to bacterial resistance to adverse environments and helps the bacteria to evade host immune responses(Jensen PØ et al., 2010; Bridier A, et al., 2011). Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product that enables it to produce end products, protein or non-coding RNA, and ultimately affect a phenotype, as the final effect (Wang, et al,.2017). These gene products are often proteins, but in non-protein-coding genes such as transfer RNA (tRNA) and small nuclear RNA (snRNA), the product is a functional non-coding RNA (De Silva,et al,.2016).In genetics, gene expression is the most fundamental level at which the genotype gives rise to the phenotype, i.e. observable trait (Nitzan, et al,.2019). The genetic information stored in DNA represents the genotype, whereas the phenotype results from the "interpretation" of that information. Such phenotypes are often expressed by the synthesis of proteins that control the organism's structure and development, or that act as enzymes catalyzing specific metabolic pathways.

All steps in the gene expression process may be modulated (regulated), including the transcription, RNA splicing, translation, and post-translational modification of a protein. Regulation of gene expression gives control over the timing, location, and amount of a given gene product (protein or ncRNA) present in a cell and can have a profound effect on the cellular structure and function (Roundtree, et al,.2017). In Salmonella the gene csgD is a central controlling regulator that can activate the transcription of csg BAC operons and encode the synthesis of curli fimbriae(Simm R. et al., 2014) This gene also promotes adrA gene transcription,

whose product interacts with bcsABZC-bcsEFG operons to synthesize cellulose (Liu Z. et al., 2014). Crl, a RpoS-binding factor, binding to alternative sigma factor RpoS, facilitates RNA polymerase holoenzyme formation (EσS), further enhancing CsgD expression (Robbe-Saule V. et al., 2006). Other regulators such as MlrA or OmpR could promote CsgD expression or transcription (Brown PK. Et al., 2001; Gerstel U. et al., 2006).

Materials and Methods

Specimen egg preparation

In the months of October through December (2021), 100 samples of table egg content (yolk and albumen) were collected. The egg was gathered from the field projects Al Hilla and Basmaia.Each egg was shattered in the laboratory after being sterilized on the smaller diameter end using 70% alcohol. The eggshell was removed, the albumen and yolk were separated, and each was put into a sterile container separately. Individual samples were homogenized, and 25 mL of albumen and 25 mL of yolk were then added to 225 mL of a 1 percent peptone solution in an Erlenmeyer flask (Moraes DMCI Duarte SCII Bastos TSAI Rezende CLGI Leandro NSMI Café MBI Stringhini JHI Andrade MAI, 2016).

Isolation of Bacteria

swabs were cultured on XLD agar. The plates were incubated aerobically at (37C° for) for18-24hrs. Then visual examination for growth detection, pigmentation, and colonial morphology. Isolated bacteria were purified by repeated subculture on XLD agar plates and incubated at (37C°) for 24 hrs. The pure bacteria were stored at (4C°). Salmonella spp. Identification using conventional PCR. Brain heart infusion broth with 15% glycerol was added to the samples and placed in an Eppendorf tube for long-term survival (for several months). The isolates were maintained frozen at -20 °C (deep freeze). After that, a genetic test is sent to a lab.

DNA extraction

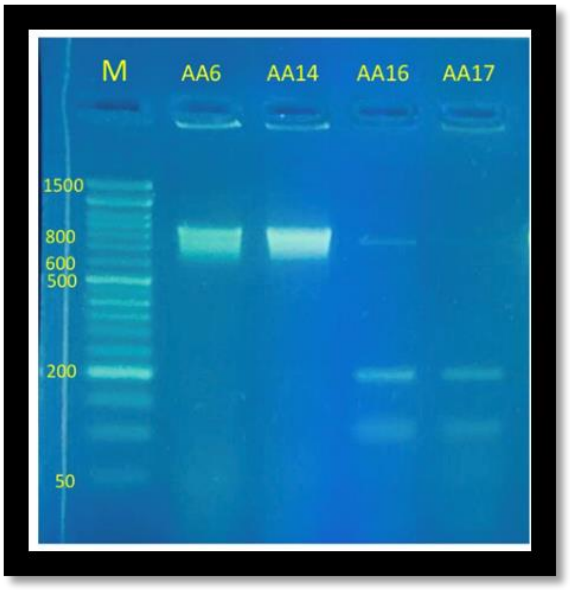
Genomic DNA of studied Salmonella isolates was extracted by using The G-spin Genomic DNA Extraction Kit supplied by Geneaid company as per manufacturer's instructions.

Biofilm assay using Microtiter plate method

The biofilm production test was conducted with few modifications according to a method performed by Piechota et al., 2018. Briefly, fresh pure colonies from each isolate were inoculated on Luria Bertani broth supplemented with 0.5% dextrose and incubated at 37°C for 24 hours without shaking. following incubation, about 108CFU/ml of the bacterial cell, the suspension was prepared (by diluting the culture to match the 0.5 McFarland standard). Then 200 µl of the suspension of each isolate was transferred into wells of 96-well sterile flat-bottom polystyrene plate and incubated without shaking at 37°C for 24 hours.

Results and Discussions

Salmonella spp. bacteria were isolated from the yolk and albumin of table eggs at rates of 3% in brown egg yolks and 3% with no growth. *Salmonella spp.* was found in 3 isolates of brown egg albumen, while 6 isolates did not grow. *Salmonella spp.* was found in white egg albumen in 5 isolates, with 2 isolates showing no growth, while 6 isolates of egg yolk had no growth. Since *Salmonella enteritidis* infection brought on by consuming contaminated eggs or egg products is still a serious health concern, a lot of study on contaminated eggshells and egg contents is focused on Salmonella. Depending on whether the research was based on eggs from naturally infected chickens or eggs from randomly picked table eggs, different levels of Salmonella prevalence have been seen on the eggshell and in the egg interior (De Reu,2008). *Salmonella spp.* enteric infection is a significant global source of diarrheal sickness, yet different studies report different rates of infection. Therefore, the findings of this study are more or less consistent with those of earlier researchers that looked into Salmonella from table eggs. On XLD agar *Salmonella spp.* small, spherical, red, and smooth, and with or without a black center. The present manuscript reports the development of a qPCR TaqMan assay that allows rapid and accurate detection of Salmonella cells in produce and eggs. The performance of the qPCR assay was comparable to that of the traditional BAM and USDA Salmonella culture methods.



Pic 1. PCR products of the amplification of the ITR region of Salmonella spp. The size of the PCR product is 855 bp. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 90, Time: 45 minutes. M: DNA ladder

The laboratorial evaluation of the Salmonella isolates described here demonstrated the effective differentiation of serotypes by the analysis of both rrn operons with the same discrimination power. Amplification and DNA sequencing resulted in samples with diverse DNA size fragments and different nucleotide sequences. Kappa agreement value obtained from the comparison between the

KWL scheme and both rrn operons was the same (0.792), indicating a moderate to strong correlalation 12; Pulido-Land´ınez et al., 2013, 2014).

Biofilm assay for Salmonella spp.

Biofilm formation is one of the most important mechanisms utilized by Salmonella to survive in host cells (Steenackers H, *et. al,*2012). Biofilm formation is an alive approach for microorganisms to conform to their dwelling environment, especially inside an adverse environment. Under biofilm protection, microbial cells in biofilms grow to be tolerant proof against antibiotic impregnable responses, which will increase the problems of the medical remedy of biofilm contamination. Clinical laboratory examinations confirmed a clean link between biofilm contamination and clinical of foreign bodies (Adrizain *et al.*, 2018). Crystal violet assay is considered suitable for evaluating biofilm formation because it yields reproducible results, which allows large numbers of strains and conditions to be studied at the same time. Furthermore, the method provides quantitative results by measuring the optical density of wells (Peeters et al., 2008; Pui et al., 2011). A correlation between microtitre plate assay and biofilm formation by Salmonella has been demonstrated (Patel and Sharma, 2010).

Pic 2. show biofilm assay for Salmonella spp

All 17 Salmonella isolates were subjected to a biofilm formation test using the Microtiter plate method of Piechota et al. (2018). The results showed that at least one isolate with a strong ability to produce biofilm (>0.400), 2 isolates with a moderate ability (0.2-0.4), and 9 isolates showed weak ability (<0.2 ≥0.12), while 5 isolates did not produce biofilm (<0.11). The table below shows the differences between Salmonella spp. ability to form biofilm layer:

Table 1-1
show differences between Salmonella spp. ability to form biofilm

Property	Non-biofilm producer (<0.12)	Weak producer (<0.2 ≥0.12)	Moderate producer (0.2-0.4)	Strong producer (>0.400)
Number of strains	5	9	2	1

The study of (Miryam Diez-Garcia *et.al,* 2012) showed that All *S. enterica* strains tested produced biofilms on polystyrene micro-well plates. In poultry products, Salmonella spp. was the main cause of early warnings in the European Union between January 2017 and May 2018 (RASFF, 2018). It is possible that these bacteria remain in the farm environment in a biofilm form. It is worth noting that microorganisms are highly resistant to disinfectants when structured as biofilms (Ziech et al., 2016). In general, disinfectants are not capable of eliminating the entire biofilm matrix, requiring further association with a mechanical process to disturb the matrix structure and expose the microorganisms to disinfectants (Maukonen et al., 2003; Srey et al., 2013). Biofilms increase microbial resistance to chemical, physical and biological agents and the ability to form biofilms has been identified as an important factor for the persistence of bacteria in food-processing facilities (Vestby et al., 2009).

Table 1-2
Show the strain of Salmonella spp. that produces biofilm

Features	Strains of Salmonella
High Biofilm production	AA14
Intermediate Biofilm production	AA6
Low Biofilm production	AA16
No biofilm production	AA17

Table (1-2) explained the strain of salmonella that produce biofilm were AA14 high biofilm production, AA6 intermediate biofilm production, AA16 low biofilm production, AA17 no biofilm production.

Gene expression for biofilm production strain of Salmonella spp.

Genes: csgD and bcsA genes are used as target genes that are responsible for producing biofilm, and gyrB gene is used as a housekeeping gene.

Table 1-3
Show CT values of the genes (calculated automatically by the Mx3005P Stratagene system)

Gene-Sample	CT	Gene-Sample	CT
csgD-AA14	28.07	bcs-AA14	29.96
csgD-AA14	28.45	bcs-AA14	30.15
csgD-AA6	27.99	bcs-AA6	30.68
csgD-AA6	27.87	bcs-AA6	29.82
csgD-AA16	28.58	bcs-AA16	29.02
csgD-AA16	28.27	bcs-AA16	29.53
csgD-AA17	37.65	bcs-AA17	31.27

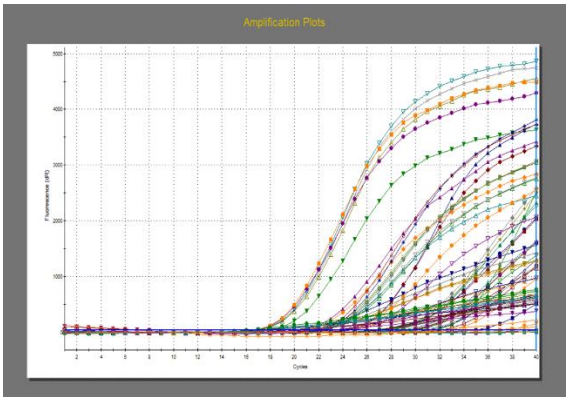
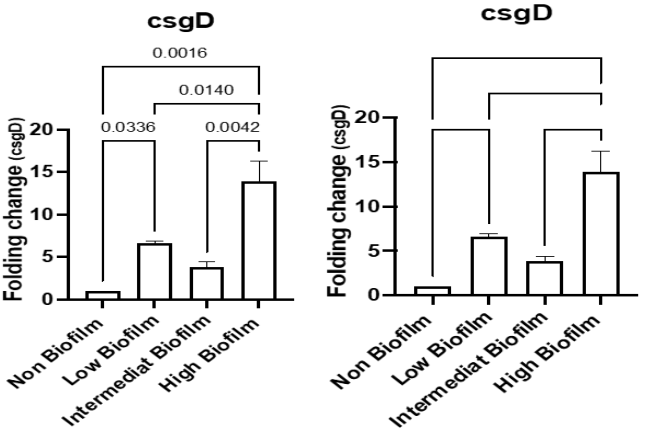


Figure 1. Amplification plot of three genes, SIRT-1, eNOS and GAPDH by the Mx3005P Stratagene system

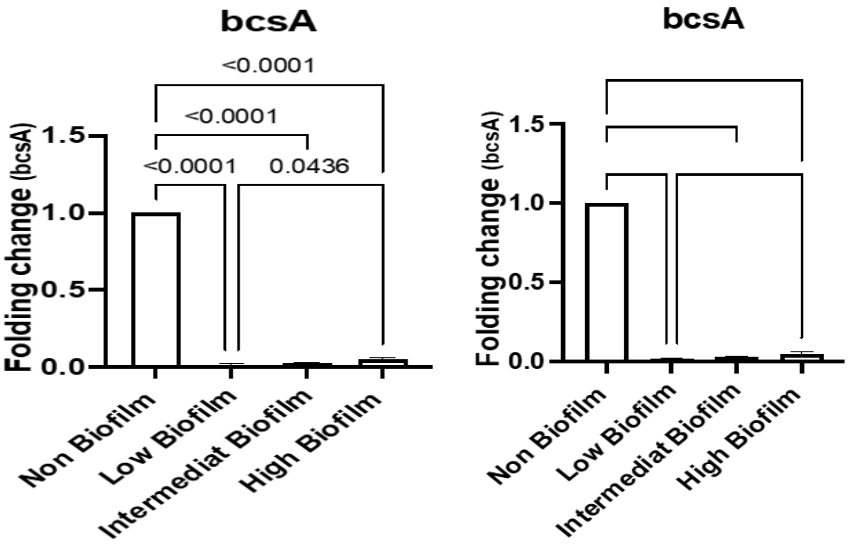
Gene used in our study
csgD gene

This gene responsible for biofilm produce in strains that produce biofilm.



bcsA gene

This gene is responsible for biofilm produced in strains that produce biofilm.



Our results were similar to results obtained (Chen S, *et al.,* 2021) they show that identification of Generated *S. Enteritidis* Mutants. The successful generation of *S. Enteritidis* mutant strains C50041□csgD, C50041□csgA, C50041□adrA, and C50041□bcsA was confirmed by using PCR. After each target gene had been replaced by the chloramphenicol resistance gene, the size of the altered chromosome was estimated to be about 1.2 kb. After the resistance gene had been knocked out, the size of the gene was observed to be about 200–400 bp, which is consistent with the expected value. The transcriptional levels of csgD, csgA, adrA, and bcsA genes were determined by relative qRT-PCR. The results showed that the four genes' transcriptional levels were significantly reduced in the mutants compared with the WT strain.

The growth rates of the *S. Enteritidis* mutant strains were similar to that of the wild type (WT) strain. Biofilm Formation of the *S. Enteritidis* Mutants: To examine the effects of the deleted genes on wild type strain biofilm formation, crystal violet staining tests were conducted. The WT strain C50041 and its mutant strains C50041□adrA and C50041□bcsA had similar amounts of circular staining in the plate well walls, whereas C50041□csgD and C50041□csgA had almost no circular staining. Quantifying the crystal violet staining results revealed that the OD550 values of C50041□csgD, C50041□csgA, and C50041□bcsA, but not that of C50041□adrA, were significantly lower than the OD550 value of the wild type strain (all $p < 0.01$), suggesting that biofilm formation was blocked after single mutations of csgD, csgA, or bcsA. in this study, the *S. Enteritidis* genes bcsA and csgA, which encode cellulose and curli fimbria, respectively (Simm R, *et al.,* 2014) as well as the genes csgD and adrA, which regulate the biofilm-related genes (Liu Z, *et al.,* 2014),were selected for investigation.

We report here that mutants of *S. Enteritidis* with deletion in csgD, csgA, and bcsA, but not of adrA, display defects in the level of biofilm formation by *S. Enteritidis*. Our results also suggest that genes related to biofilm formation (csgD, csgA, bcsA, and adrA) can alter the virulence of *S. Enteritidis* differently. The mortality rates of chickens infected with these strains indicate that deletions of csgD or bcsA attenuated the virulence of WT strain whereas deletion of csgA yielded the opposite result. Although the deletion of adrA had limited effects on the biofilm formation of *S. Enteritidis*, the LD50 value of the C50041□adrA mutant was much higher than that of the WT strain.

These data reveal that biofilm formation is related to bacterial virulence, and in *S. Enteritidis*, the synthesis of curli and cellulose could enhance its virulence. *S. Enteritidis* can be transmitted vertically through laying hens (Anastasiadou M, Michailidis G., 2016), and may cause persistent infection. Although previous studies have shown that the biofilms may be related to persistent Salmonella infections (Vestby LK, *et al.,* 2009), the relationship between biofilms and the vertical transmission of *S. Enteritidis* is still unclear. Here, we deleted four genes known to be related to biofilm formation and studied their roles in the vertical transmission of *S. Enteritidis* among laying hens. The results of our vertical transmission assay indicate that the genes csgD and bcsA significantly enhance the level of *S. Enteritidis* vertical transmission, whereas the genes csgA and adrA have limited effects. Within groups infected with the same *S. Enteritidis* strain, the percentages of produced eggs were higher in the intraperitoneal injection subgroup than in the crop gavage subgroup, which is consistent with the trends reported previously (Okamura M, *et al.,* 2007). Considering the biofilm makes the bacteria stuck somewhere, we speculated that biofilm could help Salmonella better and longer survival in the reproductive tract or on the egg or associated environment. In adverse, deletion of cellulose encoded by bcsA prevented biofilm formation and further decreased the adaption of *S. Enteritidis* in produced eggs, resulting in a decrease in bacterial penetration through the eggshell or by direct contamination of the egg contents before oviposition.

As the central regulator of biofilm formation, csgD regulates the expression of CsgA and AdrA, AdrA further controls the BcsA expression (Römling U, *et al.,* 1998). Therefore, the deletion of csgD could affect biofilm formation. However,

bcsA, in addition to being controlled by adrA, also can be regulated by other regulators, including the second messenger c-di-GMP, and sigma factor RpoS (Simm R, *et al.,* 2014). Therefore, deletion of adrA had limited effects on biofilm formation. Interestingly, cellulose, is encoded by bcsA, might be more important for the vertical transmission of *S. Enteritidis* compared with csgA-encoded curli fimbria, another biofilm component. These data are in line with our above virulence results and imply a different biological function between these two components. Overall, of the two genes studied here, csgD and bcsA had the strongest impacts on Salmonella spp. vertical transmission, the potential mechanisms will be studied in the future. Let's thus draw the conclusion that Pathogenic Salmonella bacteria are found in both white and brown egg content. Identification of the active and inactive genes responsible for the biofilm in each isolate and comparing them with the type of biofilm production. also suggest researching the virulence factors and bacteria's capacity for vertical transmission through eggs.

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