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Effects of *dam* and *seqA* genes on biofilm and pellicle formation in *Salmonella*

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ABSTRACT

In this study, the effects of *dam* and *seqA* genes on the formation of pellicle and biofilm was determined using five different *Salmonella* serovars *S.* Group C1 (DMC2 encoded), *S.* Typhimurium (DMC4 encoded), *S.* Virchow (DMC11 encoded), *S.* Enteritidis (DMC22 encoded), and *S.* Montevideo (DMC89 encoded). *dam* and *seqA* mutants in *Salmonella* serovars were performed by the single step lambda red recombination method. The mutants obtained were examined according to the properties of biofilm on the polystyrene surfaces and the pellicle formation on the liquid medium. As a result of these investigations, it was determined that the biofilm formation properties on polystyrene surfaces decreased significantly ($p < 0.05$) in all tested *dam* and *seqA* mutants, while the pellicle formation properties were lost in the liquid medium. When pBAD24 vector, containing the *dam* and *seqA* genes cloned behind the inducible arabinose promoter, transduced into *dam* and *seqA* mutant strains, it was determined that the biofilm formation properties on the polystyrene surfaces reached to the natural strains' level in all mutant strains. Also, the pellicle formation ability was regained in the liquid media. All these data demonstrate that *dam* and *seqA* genes play an important role in the formation of biofilm and pellicle structures in *Salmonella* serovars.

KEYWORDS

dam; *seqA*; biofilm; pellicle; *Salmonella*

Introduction

The *Salmonella* genus belongs in the Enterobacteriaceae family and contains two species: *Salmonella enterica* and *Salmonella bongori* [1]. Poultry meat, minced meat, sausage, egg products, aquaculture, ice cream, milk powder and cream are among the main sources of transmission of *Salmonella* serovars, which is one of the main causes of foodborne infections. Apart from that, sauces, cucumbers, puddings and traditional dairy products also have an important place among the foods that are at risk for transmission of *Salmonella* [2–4].

Salmonella is one of the most frequently isolated foodborne pathogens and is associated with three distinct human disease syndromes, bacteremia, typhoid fever, and enterocolitis [5]. According to World Health Organization reports, Foodborne diarrhoeal disease agents caused 230,000 (95% UI 160,000–320,000) deaths, particularly non-typhoidal *Salmonella enterica* (NTS) which causes diarrhoeal and invasive disease [6]. Currently, around 2,600 *Salmonella* serotypes have been described and listed in the White-Kauffmann-Le Minor (WKL) scheme and most of these serotypes can adopt within a variety of animal hosts, including humans [7–9].

Biofilms are multicellular-like life forms that are formed by the irreversible attachment of microorganisms embedded in an extracellular polymeric matrix to biotic or abiotic surfaces or interfaces [10,11]. The main cause of persistent *Salmonella* infections is the

ability of many members of these bacteria to form a biofilm on biotic and abiotic surfaces. Biofilm-based bacteria are often very difficult to remove from surfaces and equipment because they are highly resistant to environmental stress conditions such as disinfectants, antibiotics, drying, pH, temperature and surface concentration [12,13]. Therefore, the development of effective strategies for biofilm formation and prevention and the eradication of *Salmonella* infections through the identification of factors affecting the biofilm structure is of great importance regarding agricultural production, human and animal health and environment as well as many industry sectors, especially the food industry.

The structure of the subpopulations of bacteria is often controlled by epigenetic mechanisms. Many bacterial biofilm structures are also controlled by epigenetic mechanisms [14,15]. The most important enzyme group that plays a role in the formation of epigenetic mechanisms is DNA methylases. DNA adenine methyl transferase enzyme (Dam methylase) methylates at the adenine N-6 position in the 5'GATC3' sequences required for the expression of virulence genes of many bacteria, including *S. enterica* [16–18]. However, there is very limited information on the effect of *dam* methylation on biofilm formation in *Salmonella* serovars. Aya Castaneda et al. [19] found that the productivity of biofilm production in *dam* mutants of *S. Enteritidis* was significantly decreased.

As a result of quantitative real-time PCR experiments, researchers have found that the expression of the *csgA* and *csgD* genes, known to play a direct or indirect role in the production of curli fimbriae and cellulose, the key components of the biofilm matrix, was significantly reduced in these mutants [19].

Another protein that regulates the initiation of *dam* methylation after DNA replication and thus the start of DNA replication is the SeqA protein. Because the SeqA protein binds to the hemimethylated 5' 'GATC3' sequences with a higher affinity than the Dam methylase, it regulates the initiation of replication by preventing Dam methylase binding in the early stages of replication. It has also been determined that the SeqA protein also participates in the transcriptional regulation of different genes in bacteria. It was found that the SeqA protein promotes this activity by increasing the superhelical turns in the binding sites of transcription factors and by lowering the DNA binding activities of the transcription factors [17,20,21]. Chatti et al. [22] have determined that virulence reduced significantly in the *seqA* mutants of *S. typhimurium* regarding wild type strain. In another study, conducted by the same investigators using *S. typhimurium* with *dam* and *seqA* mutants, it was determined that the membrane phospholipids undergo structural changes and the ability to produce biofilm in *dam* mutants, but wild-type and *seqA* mutants did not show significant alteration of Fatty acids (FAs) profile after UV-C treatment [23]. Authors concluded that the alteration in FAs composition of membrane leads to changes in several characteristics of strains, such as changes in the levels of biofilm formation and the motility of *S. typhimurium* strains.

This study aimed to determine the efficacy of *dam* and *seqA* genes on biofilm production and pellicle formation of five different *Salmonella* serovars in light of the limited literature data summarized above.

Materials and methods

Bacterial strains and vectors

Salmonella strains and vectors used in this study are listed in Table 1. pKD3 and pKD46 vectors are kindly provided by Assistant Professor Evren Doruk ENGİN, Ankara University, Biotechnology Institute, Turkey. DMC (initials of researchers, isolated *Salmonella* strains) encoded *Salmonella* strains were previously

isolated by Avşaroğlu et al. [24]. For planktonic culture conditions, *Salmonella* strains were grown at 37°C for 12h with agitation of 200 rpm. For biofilm culture conditions, bacteria were grown in Luria Bertani (LB) broth without salt (LB/NaCl⁻), to maintain optimum conditions for biofilm production at 20°C under static conditions. When necessary, the medium was supplemented with ampicillin (Sigma) and chloramphenicol to reach a final concentration of 100 µg/ml and 25 µg/ml, respectively (Table 2).

Construction of *dam* and *seqA* mutants

dam and *seqA* gene deletions using a single step lambda Red recombination method were performed similarly to the previously described method [25]. The mutagenic primers, which are designed for PCR amplification of the insertion cassette on the template plasmid pKD3, contain homology arms (50 nucleotides in length) to the given target regions. The PCR products were amplified by PCR using the Forward primer (F): 5' TGCGACTCCTGGTTGGTACAGAGCCAGCAGTTC GTCCA CCTTTTACGTGGTGTAGGCTGGAGCTGCTTC'3' and Reverse primer (R): 5'CGCGCTTTTTTGAAGTGGGC AGGGGAAAATACCCTCTGCTTGATGATATGGGAATTA-GCCATGGTCC'5 for *dam* gene and for the *seqA* gene, Forward primer (F): 5'GACATCGCACTGGATTAAG ATGAAAACGATTGAAGTTGATGATG AACTCTGTGTAGGC TGGAGCTGCTTC'3 and Reverse primer (R): 5'GCAAAC CTTTCAATCAATTCCGCCGGAATTGCATTGACTGCATG-ATGTATGGGAATTAGCCATGGTCC'3. After generating PCR products that contained the target genes and antibiotic resistance cassette (Figure 1), the second step in the protocol that is the transformation of expression plasmid pKD46 into the *Salmonella* strains was applied [25]. The expression of genes mediating Red/ET recombination (via expression of phage-derived protein pairs from Redα/Redβ from λ phage) was induced by the addition of 50 µL 10% L-arabinose. Following the induction, the PCR product carrying the homology arms was electroporated into the competent wild-type *Salmonella* strains, containing induced pRed/ET, Red/ET expression plasmid (Bio-Rad Gene Pulser; 0.2 cm cuvette, 2.5 kV, 25 µF, 200 ohms). After that, cells were recovered in 1 mL LB medium for 3 h at 37°C. Recombinants were identified by plating on LB agar medium containing chloramphenicol (25 µg/mL) antibiotic. The mutations were confirmed by sequence analyses (data not shown). The high-frequency

Table 1. *Salmonella* strains and their serotype informations used in this study.

Strain Codes	Serovar Name	Source
DMC2	<i>Salmonella</i> Group CI	Avşaroğlu et al. 2007
DMC4	<i>Salmonella</i> Typhimurium	
DMC11	<i>Salmonella</i> Virchow	
DMC22	<i>Salmonella</i> Enteritidis	
DMC89	<i>Salmonella</i> Montevideo	

Table 2. Vectors; their features and antibiotic resistance characteristics used for the construction of *dam* and *seqA* mutants and for controlling the expression of *dam* and *seqA* genes.

Plasmids	Features of Plasmids	Resistance
pKD46	Red-expression vector	Amp ^R
pKD3	Chloromphenicol cassette vector	Amp ^R , Chl ^R
pBAD24	Expression vector	Amp ^R

Figure 1. Amplified Chloramphenicol cassettes with homology arms to the *dam* (Line 2) and *seqA* (Line 3) Line1: GeneRuler™ 1 kb Plus Ladder (Thermo Scientific™). Fragment sizes of DNA ladder: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 bp.

generalized transducing bacteriophage P22 was used for transduction of the *dam* and *seqA* genes back into mutant strains. In this manner, the mutant strains were converted back to wild type strains [26]. Stock cultures were maintained at -80°C .

Cloning the *dam* and *seqA* genes into the pBAD24 vector

The pBAD24 vector was used to control the expression of Dam and SeqA proteins by cloning the *dam* and *seqA* genes to downstream of the arabinose-inducible BAD promoter. Primer pairs to be used in the amplification of the *dam* and *seqA* genes from *S. enterica* serovar Typhimurium 14028 strain, were designed by adding *Sall* and *PstI* restriction endonuclease recognition sequences (Table 3). Genomic DNA was isolated from *Salmonella* cultures by using cetyltrimethylammonium bromide (CTAB) method that

was described by Wilson [27]. PCR experiments were performed by using the GeneAmp9700 Thermocycler (Applied BioSystems, USA). PCR products were electrophoresed in 1% agarose gels and then stained with EtBr. Amplified PCR products (*dam* and *seqA* genes) were purified with High Pure PCR Product Purification kit (Roche Chem., USA). The pure and circular pBAD24 vector, which is 4500 base pairs (bp), and the purified PCR products were cut by *Sall* and *PstI* enzymes to create sticky ends. Following purification of the linear pBAD24 vector and PCR products obtained after restriction enzyme digestion, phosphodiester bonds were allowed to form between the adhesive ends by using the DNA ligase enzyme. Binding reactions of the DNA fragments were performed in a total volume of 20 μL using T4 DNA ligase enzyme and buffer solutions (Fermentas, Thermo Fisher Scientific, USA) of this enzyme. The recombinant plasmids obtained after the addition of the *dam* or *seqA* genes were transferred to the competent *S. Typhimurium* 14028 strain by using electroporation as described above. Since the pBAD24 vector used in the study carried ampicillin (100 $\mu\text{g}/\text{mL}$) resistance, amp-containing LB agar was used as the selective medium. pBAD24 vectors containing the *dam* and *seqA* genes were transferred to *dam* and *seqA* mutants of wild-type strains by electroporation [28]. Plasmid DNA isolation from probable transformants was performed using Plasmid DNA Purification Kit (Qiagen), and isolated plasmids were analyzed on 1% agarose gel electrophoresis, stained in EtBr solution, and visualized under UV light. Supercoiled DNA ladder (Sigma) was used as a size marker.

Microplate assay for biofilm formation

The bacteria were inoculated 1% in 5 mL of LB/NaCl⁻ medium and activated for 18 hours at 37°C with shaking at 200 rpm. Next, 30 μL of these dilutions were transferred to 96-well microtiter plate wells containing 100 μL LB/NaCl⁻ by adjusting the bacterial cultures to $\text{OD}_{595} = 0.2$ (10^8 colony forming unit per milliliter) (Multiskan Go, Thermo Fisher Scientific, Finland). Plates were allowed to incubate at 20°C for 24, 48, 72 and 96 hours. At the end of the incubation period, wells were washed 3 times with phosphate buffered saline (PBS, Sigma) and planktonic cells were removed. Then 130 μL of 1% crystal violet was added to wells for staining of the

Table 3. Primers used for amplification of *dam* and *seqA* genes for cloning into the pBAD24 vector.

Primer	Primer Sequences 5'→3'	T _m (°C)	Amplicon Size
<i>dam</i> F (<i>Sall</i>)	5 - GGTCGACTT ATTTTCTTGCAGGCGTTG -3'	68.3	837 bp
<i>dam</i> R (<i>PstI</i>)	5 -GCTGCAGATGAAAAAATCGCGCTTT-3'	61.9	
<i>seqA</i> F (<i>Sall</i>)	5 - GGTCGACTT GTATTCAAGAAACATCG-3'	68.7	543 bp
<i>seqA</i> R (<i>PstI</i>)	5 -GCTGCAGTTAAATTGTTCCGCAACCTT-3'	63.7	

biofilm structure and subjected to incubation for 30 minutes. At the end of this period, the wells were washed 3 times with distilled water, and then 130 μL of 33% (v/v) glacial acetic acid was added to dissolve the dye bound to the biofilm structure [29]. Biofilm formation tests were performed in 2 biological and 3 technical replicates. For biological replications, experiments were performed on 2 different days, while 3 individual measurements were performed on the same day for technical replication. The dye density was measured at OD595, and the values obtained from the wells containing only LB/NaCl⁻ used as controls from the average of the obtained values were subtracted.

Determination of pellicle formation

In experiments in which the entire pellicle structure of wild-type and mutant strains was examined, 0.5 mL of the active cultures of *Salmonella* strains were inoculated into 4.5 mL of LB/NaCl⁻ broth and allowed to incubate at 20°C for 8 days (optimum time and temperature for the production of the pellicle). Experiments with mutant strains were followed by an induction step. Samples to be worked for this purpose were grown overnight in appropriate conditions, then were inoculated onto fresh medium and expected to grow to OD600 = 0.1. Subsequently, 0.01% arabinose was added to the growth medium for induction of the promoter region and incubation step was performed at 37°C for 30 min. Following the incubation, the same protocol for wild-type strains was also applied to mutant strains, i.e. incubation at 20°C for 8 days. The formation of the pellicle of the strains were visually examined every day on the basis of the formation of the ring structure in the liquid air intermediate phase, the resistance against the formed pelagic mixing process, the cloudiness which is the indicator of the presence of planktonic cells in the medium and the cell accumulation at the base of the tubes [30,31].

Statistical analysis

All statistical analysis was performed by Minitab statistics software, version 15 (USA). Experiments were performed in triplicate. Differences in the mean values among the groups were analyzed by one-way ANOVA and independent t-test. All results were determined significant if the p-value was below 0.05, the adopted level of significance.

Results

Controlled expression of *dam* and *seqA* genes

To control the expression levels of the *dam* and *seqA* genes, which we aimed to determine the effect

on biofilm production, the intended genes were cloned to downstream of the arabinose-inducible BAD promoter found in the pBAD24 vector. As defined before, the recombinant plasmids were transferred to the competent *Salmonella* cells by electroporation and electrotransformants were selected on ampicillin (100 $\mu\text{g}/\text{mL}$) containing LB agar. Once colonies harvested on selective medium, their plasmid contents and the presence of *dam* and *seqA* genes on recombinant plasmids were examined. Since obtained band patterns were at the expected sizes, 5043 bp for *seqA* gene and 5340 bp for *dam* gene, it was concluded that *seqA* and *dam* genes were successfully cloned into the multiple cloning site of pBAD24 vector (4500 bp) between *Sall* and *PstI* restriction sites (Figure 2). Clones determined to contain the desired vectors were used in further investigations.

The effect of *dam* and *seqA* genes on biofilm production of *Salmonella* serovars

The biofilm production capacities of wild-type strains and their *dam* and *seqA* mutants were determined by microtiter plate assays. After incubation at 20°C for 24, 48, 72, and 96 hours, it was determined that 72 h incubation is the most appropriate time for maximum biofilm production of DMC11, DMC22 and DMC89 encoded strains, while 96 h determined as the most appropriate time for maximum biofilm production of DMC2 encoded strain.

As a result of these experiments; biofilm production capacities of *dam* and *seqA* mutants decreased significantly in all tested incubation times compared to wild type strains regarding statistical significance ($p < 0.05$). The induction of *dam* and *seqA* gene expressions with 0.01% arabinose caused an increase in the biofilm production of mutant strains at the end of 24, 48, 72, and 96 hours.

It was found that the ability to form biofilm in all mutants were almost 100% restored similarly to wild-type strains, after transferring the *dam* and *seqA* genes via cloning pBAD24 (pBAD24+ *dam* and pBAD24+ *seqA*) vector to the *dam* and *seqA* mutants and inducing gene expression with addition of 0.01% arabinose (Figure 3, Table 4). All these results proved that the *dam* and *seqA* genes are necessary for biofilm production of tested *Salmonella* serovars.

Pellicle formation

All wild-type and mutant strains were evaluated for their pellicle forming abilities. Physical features of pellicle (rigid, fragile and elastic), media turbidity, pellet at the bottom of the test tube and formation of ring structure between liquid-air interface were also visually evaluated after 8 days of incubation.

Figure 2. The plasmid content of *S. Typhimurium* DMC4 transformant.

Line 1: Supercoiled DNA ladder (Sigma-Aldrich). Fragment sizes of DNA ladder: 16210, 14174, 12138, 10102, 8066, 7045, 6030, 5012, 3990, 2972, 2067 bp, Line 2,5 and 6: Empty pBAD24 vector, Line 3: *dam*⁺ pBAD24 vector, Line 4: *seqA*⁺ pBAD24 vector. Data not shown for other serovars since plasmid contents were the same for individual *dam*⁺ pBAD24 and *seqA*⁺ pBAD24 containing transformants.

When the properties of the pellicle formation in the liquid-air intermediate phase were examined, it was found that pellicle formation was observed in the all tested serovars of DMC2 (*S. Group C1*), DMC4 (*S. Typhimurium*), DMC11 (*S. Virchow*), DMC22 (*S. Enteritidis*) and DMC89 (*S. Montevideo*) ($p < 0.05$). The ability to form a pellicle in the *dam* and *seqA* mutants of the same serovar was lost. On the other hand, parallel to the results obtained from biofilm formation characteristics on polystyrene surface, after transferring the pBAD24 vector containing the *dam* and *seqA* genes to the *dam* and *seqA* mutants, it was determined that if these transformants were induced in the presence of 0.01% arabinose, the ability of pellicle formation was regained ($p < 0.05$) (Figure 4).

Discussion

Salmonella enterica is an important pathogen of various organisms such as reptiles, birds, and mammals and is also an important food-borne pathogen of humans [32]. *Salmonella enterica* are the leading cause of a wide variety of infections ranging from gastroenteritis to bacteremia and typhoid fever [33].

Bacterial biofilms are described as bacterial communities that can be attached to abiotic or biotic surfaces and enclosed with self-produced extracellular matrix substances [34]. Since bacteria in biofilms are well protected against environmental stress, more resistant to antimicrobial chemotherapies than free-living bacteria, consequently they are extremely difficult to eradicate [35].

The Dam adenine methyltransferase enzyme encoded by the *dam* gene recognizes the GATC

sequences on the double-stranded DNA and is methylated at position N6. This hemimethylated state of the newly synthesized DNA: allows time for the repair of incorrectly matched bases, the regulation of gene expression, the suppression of the onset of chromosomal replication, and the activation or suppression of cell cycle-related processes [36]. The SeqA protein requires at least two hemimethylated GATC sequences on the same side of the DNA to demonstrate high binding activity. Here it is determined that the SeqA protein function as a nucleoid organizing protein [36]. Proteins involved in the formation and maintenance of chromosome structure, such as SeqA protein, are known to function as global regulators [37].

In certain promoters, the DNA methylation state in the specific GATC sequences either activates or represses the transcription by affecting the binding of either RNA polymerases or transcription factors [38–40]. It was determined that the SeqA protein involved in chromosome segregation, chromosome replication and nucleoid segregation, regulates transcription of Dam methylase, and also acts as a transcriptional coactivator by facilitating binding of the *cII* transcription factor to the lambda *p₁* and *p_{AQ}* promoters [41–43]. This function is performed either as a GATC methylation or as a co-activator. For example, it was determined that the SeqA protein activates the transcription factor *cII* of PR and PaQ promoters in lambda phage by binding to CII as activator coactivator. It is also known that the competition of the OxyR repressor and the SeqA protein for the hemimethylated series regulates the phase variation in the *E. coli agn43* gene [43]. It was reported that Dam and SeqA activity participate in the regulation of different genes

Figure 3. Biofilm production capacities of wild type strains, their *dam* and *seqA* mutants and arabinose induced pBAD24+ *dam* and pBAD24+ *seqA* transformants following the incubation for 24 h (a), 48 h (b), 72 h (c), 96 h (d) ($p < 0.05$).

Table 4. % Biofilm productions of all tested serovars at different incubation time periods.

Strain	24 hours	48 hours	72 hours	96 hours
DMC2	100%	100%	100%	100%
DMC2 Δdam	0.25%	1,13%	0,1%	0,98%
DMC2 $\Delta seqA$	0%	0,64%	0,5%	0,55%
DMC2 Δdam + pBAD24(<i>dam</i>)	91.7%	69,3%	100%	84,5%
DMC2 $\Delta seqA$ + pBAD24(<i>seqA</i>)	96.3%	79,7%	100%	93,1%
DMC4	100%	100%	100%	100%
DMC4 Δdam	16%	19,7%	82,1%	73%
DMC4 $\Delta seqA$	27.8%	15,0%	57,7%	24,8%
DMC4 Δdam + pBAD24(<i>dam</i>)	78.2%	83%	100%	88,2%
DMC4 $\Delta seqA$ + pBAD24(<i>seqA</i>)	91.7%	93,8%	95,4%	90%
DMC11	100%	100%	100%	100%
DMC11 Δdam	1%	1,1%	1,12%	1,35%
DMC11 $\Delta seqA$	1.9%	4,24%	0,96%	3,2%
DMC11 Δdam + pBAD24(<i>dam</i>)	89.7%	60,5%	71%	58,8%
DMC11 $\Delta seqA$ + pBAD24(<i>seqA</i>)	98.9%	67,9%	88,5%	77,2%
DMC22	100%	100%	100%	100%
DMC22 Δdam	0%	0%	0,6%	1,07%
DMC22 $\Delta seqA$	8.7%	0,078%	0,34%	5,83%
DMC22 Δdam + pBAD24(<i>dam</i>)	88.4%	75,07%	98,5%	81,6%
DMC22 $\Delta seqA$ + pBAD24(<i>seqA</i>)	94.9%	87,6%	98,3%	88,2%
DMC89	100%	100%	100%	100%
DMC89 Δdam	1,71%	1,03%	4,5%	1,78%
DMC89 $\Delta seqA$	47,2%	0,64%	1,03%	2,19%
DMC89 Δdam + pBAD24(<i>dam</i>)	93,9%	87,9%	88,5%	89,97%
DMC89 $\Delta seqA$ + pBAD24(<i>seqA</i>)	98,5%	96,19%	96,4%	92,57%

in *Salmonella* and significantly reduce adhesion and invasion in mutant strains for *dam* and *seqA* genes [20,22,42,44]. It was shown in certain bacteria, such as *Salmonella typhimurium*, *Escherichia coli* and *Acinetobacter baumannii*, that biofilm formation is under epigenetic regulation [14,19,45]. However, there are a few articles describing the effect of *dam* methylation on biofilm formation in *Salmonella* [19,23]. Aya Castaneda et al. [19] have found that DNA methylation in *S. enteritidis* upregulates the expression of biofilm production factors such as cellulose and curli by modifying the *csgD* expression. But, it was determined that *seqA* genes not be effective in biofilm formation [23].

In this study, it was determined that the formation of biofilm on the polystyrene surfaces by *dam* mutants of different *Salmonella* serovars significantly reduced in relation to the relationship of DNA methylation with biofilm production in *Salmonella*. Restoring the biofilm formation ability to the same mutants when the cloned *dam* gene was induced by arabinose addition confirms the results obtained after the *dam* mutation. On the other hand, for the first time, this study showed that the SeqA protein also plays a role in the regulation of biofilm formation in *Salmonella* serovars. Badie et al. [32] performed comparative genomic analysis between *Salmonella* pathogenic and nonpathogenic isolates. They determined that altered levels of Dam differentially affect several virulence-associated phenotypes such as bacterial virulence gene expression, motility, flagellar synthesis in *Salmonella* pathogenic strain 14028 compared to the avirulent laboratory strain, LT2. Since biofilm formation can be described as a virulence factor, our results are in line with the finding of Badie et al. [32].

Verification tests carried out using pBAD24 vector, mentioned above, yielded the same results when carried out with the *seqA* mutants. Several researchers determined the effect of *dam* and/or *seqA* mutations on the membrane composition of *Salmonella Typhimurium* [23,46]. In both *dam* and *seqA* mutants, the Dam methylase and SeqA proteins encoded by the respective genes probably perform these activities by altering the activity of the RNA polymerase or transcription factors in the promoter regions. It was previously determined that SeqA and Dam proteins might regulate fatty acid biosynthesis of *Salmonella Typhimurium* and membrane fluidity might cause changes in flagella and exopolysaccharide which are involved in the biofilm formation [23]. In our work, the changes observed in the biofilm formation of *dam* and *seqA* mutants may also lead to changes in the composition of the cell membranes due to these mutations. Lobner-Olesen et al. [36] assessed the transcription patterns in *Escherichia coli*, via high-density oligonucleotide array, with various levels of Dam and SeqA proteins. They determined similar transcription profiles in SeqA-deficient and Dam-overproducing cells and concluded that other protein(s) can carry out the function of SeqA in *dam* mutants. The reason of similar results obtained at *dam* and *seqA* mutants may be due to abovementioned protein(s), carry out the function of SeqA. However, there is a need for further work to obtain certainty of this determination.

The results of the biofilm formation on the polystyrene surfaces have also been confirmed by the pellicle formation tests in liquid air interface carried out with these bacteria. Apart from a few strains, the ability to form pellets was lost in the *dam* and/or *seqA* mutants of the serovar. When *dam* and *seqA* genes cloned pBAD24

Figure 4. Pellicle formation abilities of wild type *S. Group CI* (DMC2, a) *dam* (b) *seqA* (c) mutants and arabinose induced pBAD24+ *dam* (d) and pBAD24+ *seqA* (e) transformants (the first arrow is pellicle structure and second is ring structure).

vector was transferred to the mutant strains, the pellicle-forming properties were restored. In summary, the same effect of the *dam* and *seqA* genes on biofilm formation on polystyrene surfaces was obtained for pellicle formation. These data are strong evidence that the *dam* and *seqA* genes participate in the regulation of biofilm formation in different *Salmonella* serovars.

The data we obtain from our research is the key to understanding the biology of *Salmonella* biofilm production and providing significant contributions to combat biofilm processes. To the best of our knowledge, this is the first report that determines the role of *dam* and *seqA* genes on the biofilm forming abilities of five different *Salmonella* serovars (*S. Group CI*, *S. Tyhimurium*, *S. Virchow*, *S. Enteritidis*, and *S. Montevideo*). Also, the role of these two genes have been identified in the pellicle

structure. These findings in practical terms indicate that SeqA protein and Dam adenine methylase inhibitors can be used to attenuate biofilm formation.

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Disclosure statement

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