Determination of the Activity of the *fimF* Gene and Its N-Terminal Domain Disrupted Mutant on Biofilm Formation and Its Contribution to the Oxidative Stress Response in S. Typhimurium

Tuba Nur SÜRKAÇ[1,a], Nefise AKÇELİK[2,b], Mustafa AKÇELİK[1,c,✉]

[1]Ankara University, Faculty of Science, Department of Biology, Ankara, Türkiye; [2]Ankara University, Biotechnology Institute, Ankara, Türkiye

[a]ORCID: 0000-0001-6828-2663; [b]ORCID: 0000-0001-5541-1681; [c]ORCID: 0000-0002-1227-2324

ARTICLE INFO

Article History
Received : 13.11.2023
Accepted : 01.06.2024
DOI: 10.33988/auvfd.1390023

Keywords
Biofilm formation
fimF gene
N-terminal domain
Oxidative stress
Salmonella Typhimurium
Type 1 fimbriae

✉**Corresponding author**
akcelik@science.ankara.edu.tr

How to cite this article: Sürkaç TN, Akçelik N, Akçelik M (XXXX): Determination of the Activity of the *fimF* Gene and Its N-Terminal Domain Disrupted Mutant on Biofilm Formation and Its Contribution to the Oxidative Stress Response in *S.* Typhimurium. Ankara Univ Vet Fak Derg, XX (X), 000-000. DOI: 10.33988/auvfd.1390023.

ABSTRACT

Fimbriae is an important virulence factor which plays a key role in cell attachment and colonization of the intestinal mucosa during an infection of *Salmonella*, a pathogen that causes gastroenteritis and systemic infection in humans. In *S.* Typhimurium, type 1 fimbriae production strengthens the oxidative stress response. This study aimed to determine the effectiveness of the *fimF* gene and its N-terminal domain on biofilm formation in *S.* Typhimurium and their contribution to the oxidative stress response. Before the experiments to prove whether the N-terminal domain of the FimF protein is the region that determines the mechanism and function of the *fimF* gene; only the N-terminal domain of the *fimF* gene was cloned behind the pBAD promoter. As a result of biofilm experiments on polystyrene surfaces, it was determined that the biofilm production capacity was reduced significantly in mutant strains in terms of *fimF* and *dam* genes (P<0.01). In the oxidative stress response experiment conducted in the presence of hydrogen peroxide (H_2O_2), it was determined that the mutant strains were more resistant to hydrogen peroxide than the wild-type strain, therefore *Salmonella* cells perceived the absence of Dam methylase enzyme and FimF protein as a critical internal stress condition and produced strong responses to these stress conditions. As a result of comparative analysis of the N-terminal domain cloned mutant strain with the wild-type, it was proven that the N-terminal domain of the protein in question acts as an adapter protein, due to its close similarities with the wild-type.

Introduction

Due to their use of both human and animal systems as hosts and their ability to survive for long periods on the surfaces and tissues of plants, members of the *Salmonella* genus are important pathogens that seriously threaten public health all around the world. It is vital to restructure the approach to combating this pathogen based on biofilm forms, because at least 80% of infections and industrial pollution caused by *Salmonella* are due to these bacteria in biofilm forms, not planktonic. To change this strategy, it is necessary to first thoroughly define the genetic, physiological and environmental factors that contribute to biofilm formation (48).

The majority of bacterial infectious diseases in both humans and animals are caused by bacteria capable of adhering to mucosal surfaces (4, 35). Characterization of the *fim*, *lpf*, and *pef* fimbrial operons in *S.* Typhimurium has established that adhesins serve a variety of purposes during the early stages of an infection. Adhesins mediate initial contact with epithelial cells and thus play an important role in the pathogenicity of *S.* Typhimurium (3). The main function of fimbrial adhesins is to bind to a specific receptor on the host cell, such as a specific membrane protein, sugar residues, or lipid structures. All adhesins identified in *Salmonella* to date have been found to contain lectin-like functions. For instance, mannose

residues have been identified as specific targets of the fimbrial adhesin FimH, expressed by the *fim* operon. Fim fimbriae, the most common fimbrial adhesin in *Salmonella*, are the only fimbrial adhesins produced in laboratory static cultures (47). Fim fimbriae are a member of the type 1 fimbriae family that assembled the Chaperone-Usher pathway. There is strong evidence that type 1 fimbriae adhesins specifically bind to α-D-mannopyranosyl receptors on the cell surface (29). It has also been determined that free mannose molecules, which act as receptors, block host-specific adhesin binding in this mechanism (27, 35).

Type 1 fimbrial proteins in *S.* Typhimurium are encoded by the *fim* gene cluster (*fimAICDHFZYW*). *fimAICDHF* is expressed as a single transcriptional unit. An operon containing six structural genes (*fimA*, *fimI*, *fimC*, *fimD*, *fimH*, and *fimF*), three regulatory genes (*fimZ*, *fimY*, and *fimW*), and a tRNA specific for rare arginine codons (AGA and AGG) constitute the *fim* gene cluster (Figure 1). The structural genes belonging to the *fim* gene cluster are all expressed as a single transcript from the P*fimA* promoter, while the regulatory genes are all expressed from independent promoters. Regulatory genes carrying rare arginine codons are efficiently translated by tRNA encoded by *fimU* located at one end of the *fim* gene cluster (40). FimA (main subunit), FimI and FimH (adhesin) and FimF (adaptor) constitute the structural components of fimbriae. Fimbrial extensions consist of multiple copies of FimA. Chaperone-Usher proteins known as FimC and FimD, located on the cell surface, serve as assembly fimbriae. Fimbrial protein FimH, located at the tip of fimbriae, helps the adhesion of fimbriae (50).

The major structural subunit that polymerizes to form the fimbrial shaft is represented by FimA, a member of the type 1 fimbrial family linked to the *fim* gene cluster (5). FimH polypeptide is an adhesin molecule that recognizes mannose-containing host glycoprotein receptors and provides binding specificity to fimbriae, due to its binding region that will mediate binding to them, and is therefore responsible for binding to host cells. Mutants unable to produce this polypeptide have been proven to lack adhesion ability (1, 5, 47).

It has been determined that FimH found in *Salmonella* prefers enterocytes, unlike FimH found in *Escherichia coli*, which prefers to bind to epithelial cells (45). FimF found in *S.* Typhimurium and low-adhesion FimF variants found in *S.* Enteritidis were discovered to share the same properties (24).

FimF protein (Fimbrial-like protein), which acts as an adapter fimbrial protein in *S.* Typhimurium, is one of the structural components of type 1 fimbriae. The *fimF* gene, which encodes the FimF protein and is part of the operon encoding type 1 fimbrial proteins, plays an important role in the adhesion of proteins to a surface, environmental persistence, colonization for biofilm formation, and bacteria-host interactions for invasion (9).

The *Salmonella fim* gene cluster (*fimAICDHFZYW*) expresses the *fimF* gene, located within a single operon and regulated by the *fimA* promoter region (P*fimA*). Unlike the helical FimA core subunit, FimF is linear in structure (17). It has been discovered that the FimF protein, which plays a critical role in type 1 fimbriae biogenesis, is necessary for the initiation of fimbrial assembly. In studies conducted with the deleted *fimF* gene, which proves this phenomenon, it was determined that specific deletions of the genes encoding FimF and other adhesin proteins, which act as adapter proteins in both *E. coli* and *S.* Typhimurium, did not prevent fimbriae production, but these bacteria lost their adhesive properties (27).

Microorganisms form biofilm structures in order to survive and maintain their existence in the host and environment under certain stress conditions. Biofilm structures are defined as communities formed by microorganisms irreversibly adhering to interfaces such as abiotic or biotic or liquid-air interface phase. In other words, biofilm formation can also be expressed as a strategy of microorganisms to develop resistance against host defense mechanisms. These microbial cells have a self-produced extracellular polymeric matrix structure (EPS) on their outer surface, and thanks to this matrix, microorganisms increase their resistance to environmental stress conditions and their ability to adhere to surfaces (14, 15, 21, 33, 34). The first step in biofilm formation is adhesion to biotic and abiotic surfaces. It is known that type 1 fimbriae play a role as adhesive structures in HEp-2 cells, in the intestinal epithelium of some rodent species, in chicken intestinal epithelium, and in biofilm formations on plastic surfaces. Studies with *S.* Typhimurium have shown that strains containing type 1 fimbriae have a significantly higher adhesion and invasion rate on HeLa cells than strains without fimbriae (12, 16, 27).

Although type 1 fimbriae are the most studied fimbriae among members of the *Salmonella* genus, it is unknown whether the FimF protein has a function in virulence, biofilm formation, and host cell invasion (6, 38). In studies conducted to better understand the role of type 1 fimbriae in biofilm formation, there are very limited and conflicting data not only on the *S.* Typhimurium serovar but also on other *Enterobacteriaceae* members. Bacteria form biofilm structures to tolerate environmental stress conditions. In recent studies conducted with *S.* Typhimurium, it has been determined that type 1 fimbriae production strengthens the oxidative stress response (30). Considering the fact that the most common stress condition encountered by pathogens in host systems is oxidative stress, this study aimed to analyze the role of type 1 fimbriae on biofilm in comparison with the oxidative stress response.

DOI: 10.33988/auvfd.1390023

Table 1. Gene specific primer sequences.

fimF-N-ter-clon forward primer	5'- GATGTCGACCAACGTCGATTGCCACT -3'
fimF-N-ter-clon reverse primer	5'- TGACTGCAGTCAGGAAGGTCGCATCC -3'

Materials and Methods

Bacterial Strains and Culture Conditions: *S.* Typhimurium ATCC 14028 wild-type strain and its *fimF* and *dam* gene mutants were obtained from the Prokaryotic Genetic culture collection (Ankara University, Faculty of Science, Department of Biology). *S.* Typhimurium ATCC 14028 wild-strain and *fimF* gene mutant were grown in Luria Bertani (LB) medium by incubating at 37 ºC at 200 rpm for 18 hours. *S.* Typhimurium ATCC 14028 mutant strain, whose *fimF* and *dam* gene were deleted using the homologous region recombination technique (13), was grown on medium containing chloramphenicol (20 µg/mL^{-1}).

Obtaining the recombinant plasmid containing the N-terminal domain of the fimF gene: The *fimF* gene consists of 519 nucleotide pairs and this open reading frame (ORF) codes for 172 amino acids. *Sal*I and *Pst*I (Table 1) cutting sequences were added to primers specific to the N-terminal domain of this gene, which contains 151 nucleotide pairs and is not repeated elsewhere in the genome, to be used in cloning studies. Using these primers, the N-terminal region of the *fimF* gene was amplified from the chromosomal DNA template of the *S.* Typhimurium ATCC 14028 wild-type strain. To create cloning-specific sticky ends in the circular pBAD24 vector plasmid and *fimF* gene PCR products, *Sal*I and *Pst*I enzyme cuts were performed on these DNA molecules. The *fimF* PCR product was recombined by ligating the linearized pBAD24. T4 DNA ligase enzyme was used in ligation. Promega Biomath Calculators (Promega Corporation, USA) program was used to calculate the amount of insert to be added to the ligation reaction solution. In the ligation reaction, the insert/vector ratio was adjusted to 3/1. The recombinant plasmid was first transferred to the intermediate host, *E. coli* BL21 strain, and then to the *S.* Typhimurium 14028 *fimF* mutant strain by electroporation method. For proof of the accuracy of the transformants, plasmid isolation and colony PCR (#B100BG0021, Solis BioDyne, Estonia) reaction were performed (41).

Determination of bacterial cell aggregation: Active bacterial cultures, which were incubated at 37 ºC for 18 hours, were centrifuged at 6000 rpm for 10 minutes at 4 ºC at the end of the incubation. At the end of the centrifugation process, the supernatant was removed from the medium and the pellet was subsequently washed with 1 mL phosphate-buffered salt solution (PBS, pH 7.0 ± 2.0). This process was repeated twice. Then, the pellet was suspended in 10 mL of PBS and distributed in equal volume into three parallel tubes for each strain. The optical density of this suspension was adjusted to OD_{600}=0.2. Finally, the bacterial cultures in the tube were incubated at 20 ºC static conditions to make optical density (OD_{600}) measurements at different time intervals (0, 4, 20, and 24 hours).

The percentage of autoaggregation was determined using the formula: $[(1 - OD_{time} / OD_{T0}) \times 100]$ (44).

Determination of biofilm formation amounts and biofilm morphotypes on polystyrene surfaces: *S.* Typhimurium 14028 wild-type and mutant strains were incubated in salt-free LB Broth for 18 hours at 37 ºC. Active bacterial culture was added to 96-well polystyrene microplate wells at a concentration of 1×10^9 CFU/mL. Microplates were incubated under static conditions at 20 ºC for 72 hours. At the end of the incubation period, the wells were washed three times with PBS. After the washing process, the biofilm structures adhering to the wells were fixed with 95% methanol and kept at room temperature for 20 minutes. Biofilm structures were stained using 1% crystal violet for 15 minutes. The wells in the plates were washed with sterile distilled water to remove the dye that did not adhere to the biofilm structures. Glacial acetic acid (33%) was used to dissolve the dye adhering to the produced biofilm. The dye attached to the biofilm was measured using an ELISA reader (Biorad, USA) at OD_{595} nm. Final calculation was carried out by subtracting the arithmetic means of the controls (wells transferred to LB^{-NaCl} Broth only) from the average of the OD values determined for the tested strains. These experiments were conducted in three repeated measures and two replicates (43, 46).

To determine biofilm morphotypes, salt-free LB agar containing congo red (40 µg/mL) was used and inoculated culture was incubated at 20 ºC for a minimum of 8 days. At the end of the incubation, the colonies formed on the petri plate surface were visualized using a stereo microscope (Leica DMS1000, Germany). The entire study was carried out in six repeated measures and three replicates (36).

Determination of pellicle formation and cellulose production properties: *Salmonella* strains were incubated in salt-free LB agar and broth containing calcofluor (50 µg/mL) to determine the amount of cellulose production, and in salt-free LB broth to determine pellicle formation properties, at 20 ºC for a minimum of 8 days. At the end of the incubation, the calcofluorinated samples were

photographed under 366 nm UV light (Biometra TI 2, USA), and the pellicle test results were evaluated by photographing under white light. The entire study design was created as two repeated measures and two replicates (37, 42, 46).

To determine the cellulose production of the strains in glass coupons, first, salt-free LB medium with calcofluor (40 µg/mL) was added to six-well plates to which sterile glass coupons were added. Active cultures with optical density OD_{595}=0.2 were transferred to the wells at a ratio of 1/20 culture/medium. Microplates with glass coupon inserts were incubated at 20 ºC for 72 hours. At the end of the incubation, the glass coupons were washed three times with sterile 1x PBS and transferred to a sterile slide surface. The prepared preparation was visualized by fluorescence microscopy (Zeiss Axio Imager M1, Germany) (10).

Determination of resistance of bacteria to oxidative stress conditions: To determine the resistance of bacteria against oxidative stress conditions, active cultures were diluted 1:1000 in 25 mL LB medium. It was incubated at 37 ºC and 250 rpm until its optical density reached OD_{595}=0.4. At the end of the incubation, H_2O_2 was added to the cultures to a final concentration of 4 mM. The strains, whose initial concentration was adjusted as $1x10^6$ cells, were inoculated on LB agar medium at different time intervals (0, 1, 2, 3 and 4 hours) and incubated under static conditions at 37 ºC for 24 hours. Colony counts were made at the end of 24 hours and the results were expressed as CFU/mL (20, 31).

Statistical Analysis: The data of optical density and enumeration were presented as Mean ± Standard Deviation (SD). To test for any statistically significant difference between the genetically different groups, repeated measures one-way ANOVA was used, which was observed to be statistically significant at 1% confidence level ($p<\alpha=0.01$). For these analyses the native modules of the statistical programming language R were used.

Results

Obtaining S. Typhimurium 14028 fimF mutant strain carrying recombinant plasmid: The N-terminal domain of the *fimF* gene, which we aimed to determine its effect on biofilm production and its contribution to the oxidative stress response, was cloned into the pBAD24 vector plasmid with the arabinose-induced BAD promoter. As previously described, recombinant plasmids were transferred into target *Salmonella* cells by electroporation and electrotransformants were selected on ampicillin (100 µg/mL) containing LB agar (Figure 2).

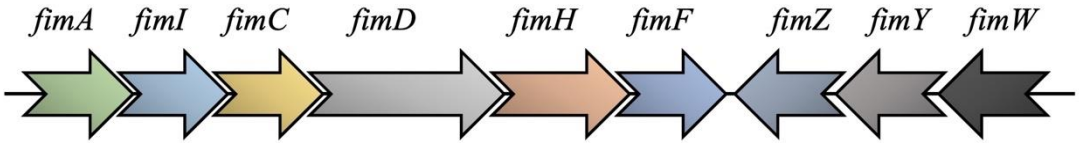

Figure 1. Diagram of the *fim* gene cluster of *S. enterica* serovar Typhimurium (8).

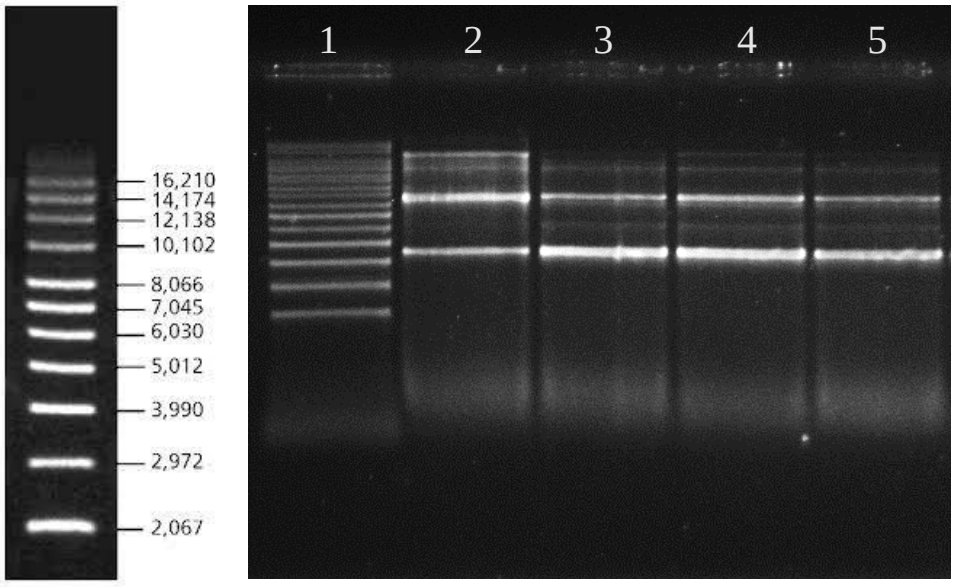

Figure 2. Recombination products.
(1) Supercoiled DNA Ladder (#D5292, Sigma, Germany), (2) pBAD24 plasmid vector, (3) Control Ligation Assay, (4-5) pBAD+$fimF^N$ transformants.

DOI: 10.33988/auvfd.1390023

Bacterial cell aggregation: Autoaggregation experiments were carried out by determining the optical densities (OD_{600}) of the aggregates formed at different time intervals. Autoaggregation amounts in *S.* Typhimurium ATCC 14028 wild-type strain increased by 30.3%, 33.6% and 39.3% at the 4th, 20th and 24th hours, respectively, compared to the starting time. These increase rates were determined as follows; 1.55%, 18.6% and 24.8%, at the 4th, 20th and 24th hours, respectively, in the *S.* Typhimurium 14028 strain in which empty pBAD vector was transferred; 5.50%, 17% and 18.5% in the Δ*dam* strain; 3.92%, 22.5% and 27.4% in the Δ*dam* (pBAD+*dam*) strain; 4.50%, 20.0% and 24.0% in the Δ*fimF* strain and 9.65%, 22.0% and 28.9% in the Δ*fimF* (pBAD+$fimF^N$) strain (Figure 3, Table 2).

Biofilm formation capacities of strains on polystyrene surfaces and their biofilm morphotypes: When the biofilm formation abilities of *S.* Typhimurium ATCC 14028 wild-type and mutant strains were examined on polystyrene surfaces; it was determined that, in case of transfer of the empty pBAD vector, the biofilm production ability of this strain was lower than that of the wild-type strain, with 64.2%, 4.56% and 28.7% lower biofilm formation at the 24th, 48th and 72nd hours, respectively. In biofilm experiments conducted with the same time periods, decrease rates were determined as; 80.1%, 50.9% and 69.7% in the Δ*dam* strain; 89.4%, 56.1% and 56.9% in the Δ*dam* (pBAD+*dam*) strain; 83.6%, 48.3% and 70.4% in the Δ*fimF* strain and 82.1%, 69.4% and 30.7% in the Δ*fimF* (pBAD+$fimF^N$) strain (Figure 4, Table 3).

The biofilm morphotypes produced by *S.* Typhimurium wild-type and mutant strains on solid medium containing Congo red were analyzed using qualitative observations. *S.* Typhimurium strains, including wild-type, wild-type (pBAD), and Δ*dam* (pBAD+*dam*), exhibited the 'rdar' biofilm morphotype, which contains curli fimbriae and cellulose in the biofilm EPS structure. In contrast, the Δ*fimF* and Δ*fimF* (pBAD+$fimF^N$) strains displayed the 'bdar' morphotype, which only contains curli fimbriae in the biofilm EPS structure. The *dam* mutant strain was similar to the rdar morphotype but formed an 'intermediate morphotype' due to reduced production of curli fimbriae (Figure 5).

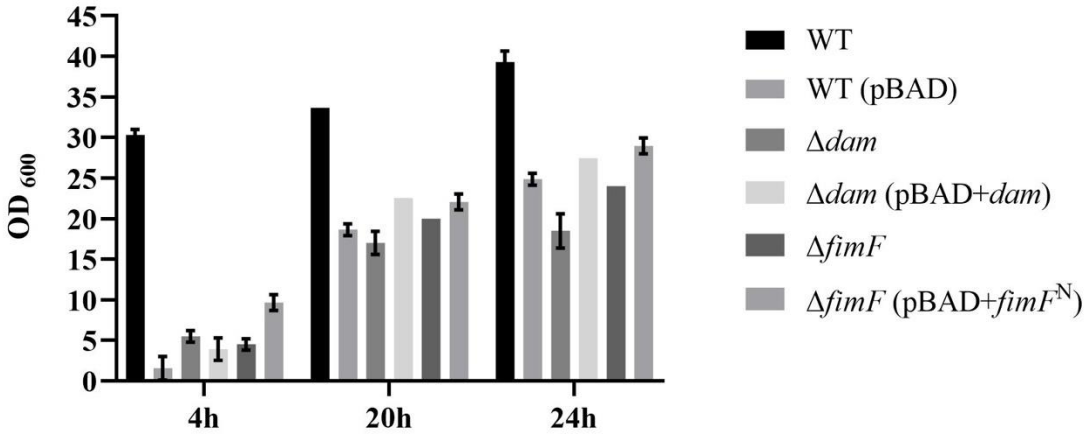

Figure 3. Autoaggregation characteristics of *S.* Typhimurium ATCC 14028 wild-type and mutant strains.

Table 2. Descriptive statistics of autoaggregation data of *S.* Typhimurium wild-type and mutant strains. The results were obtained using a formula to calculate the percentage of autoaggregation.

	MEAN			STD		
STRAIN	**4h**	**20h**	**24h**	**4h**	**20h**	**24h**
WT	28.93891	32.47588	37.94212	0.001155	0	0.002309
WT (pBAD)	4.761905	20.40816	26.53061	0.00611	0.002	0.002
Δ*dam*	4.347826	15.05017	20.73579	0.003055	0.00611	0.009165
Δ*dam* (pBAD+*dam*)	6.148867	21.68285	26.21359	0.005033	0.005774	0.006928
Δ*fimF*	4.682274	20.40134	23.74582	0.002	0.002309	0
Δ*fimF* (pBAD+$fimF^N$)	9.090909	21.36364	28.18182	0.004163	0.004163	0.004163

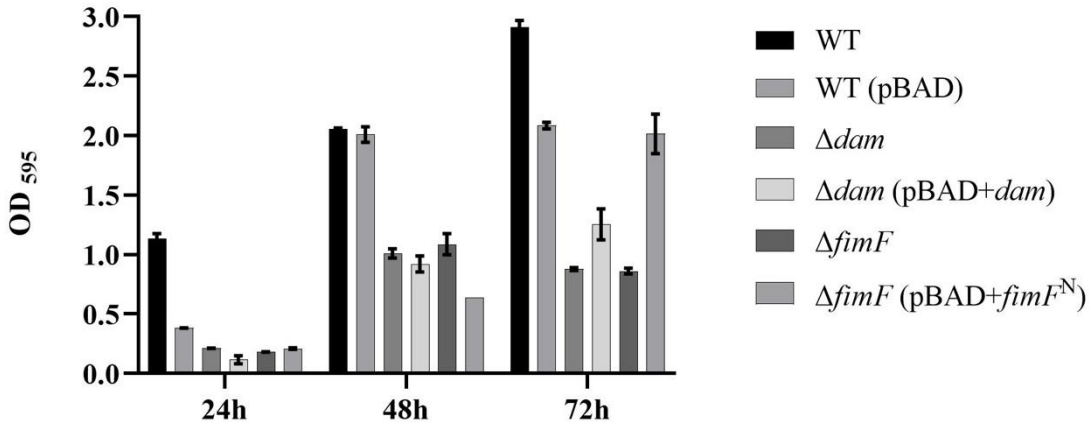

Figure 4. Biofilm production characteristics of *S*. Typhimurium ATCC 14028 wild-type and mutant strains on polystyrene surfaces.

Table 3. Descriptive statistics of biofilm production of *S*. Typhimurium wild-type and mutant strains.

	MEAN			STD		
STRAIN	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
WT	1.085	2.104	2.910	0.09266607	0.08828552	0.05939697
WT (pBAD)	0.388	2.008	2.073	0.0087178	0.065	0.02787472
Δ*dam*	0.215	1.032	0.879	0.0043589	0.0462313	0.01272792
Δ*dam* (pBAD+*dam*)	0.115	0.922	1.253	0.03252691	0.06788225	0.13010765
Δ*fimF*	0.177	1.087	0.861	0.00568624	0.08909545	0.02545584
Δ*fimF* (pBAD+*fimF*^{N})	0.194	0.643	2.016	0.02665208	0.01327906	0.16617009

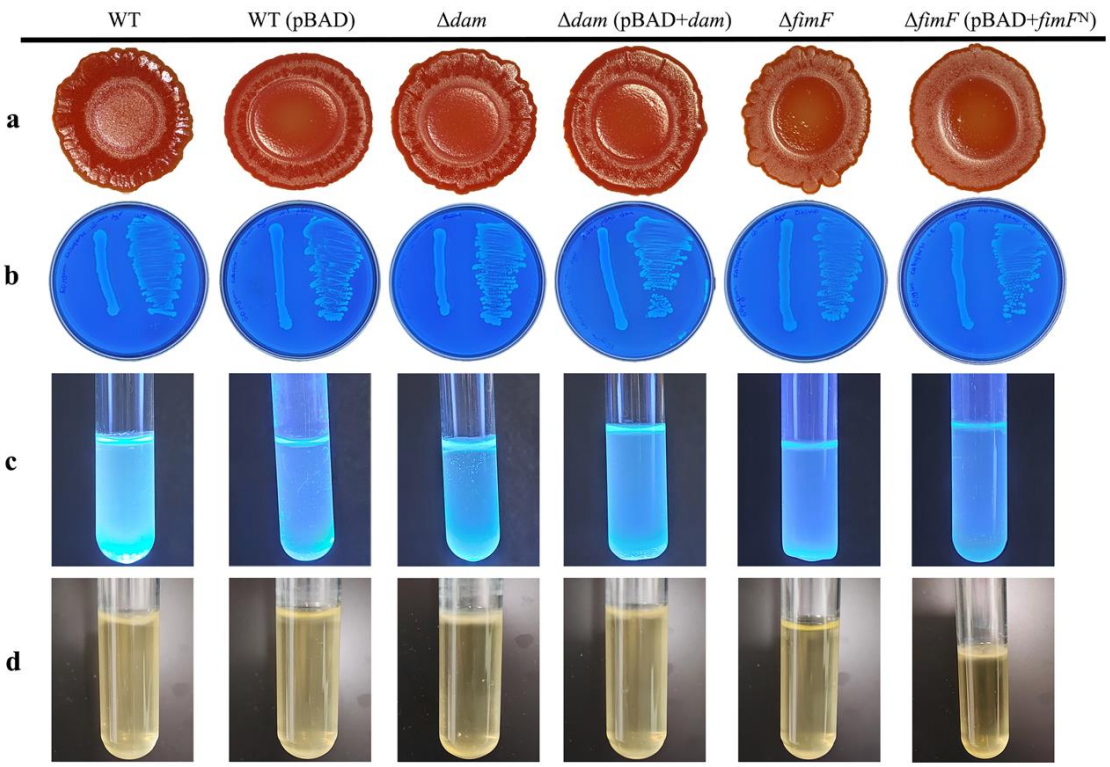

Figure 5. (a) Biofilm morphotypes of *S*. Typhimurium wild-type and mutant strains, (b) cellulose activity on salt-free LB agar containing chalcofluor (50 µg/mL) and (c) cellulose activity on salt-free LB broth containing the same amount of chalcofluor and (d) pellicle characteristics.

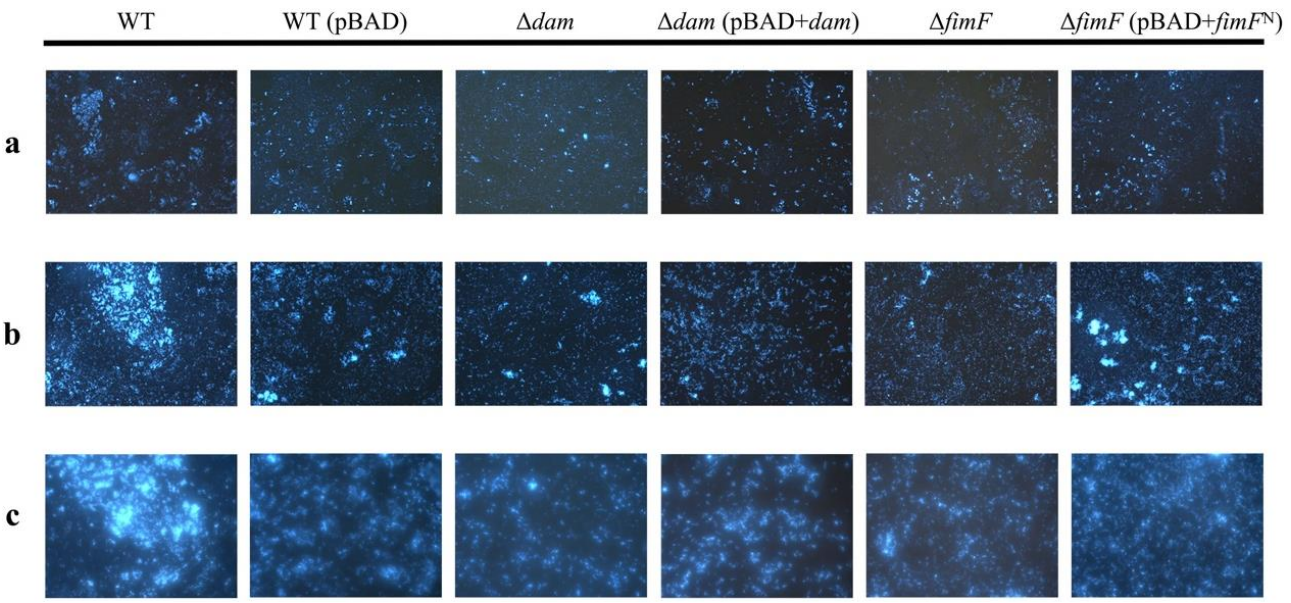

Figure 6. Fluorescence microscope image of the calcofluor binding ability of the pellicle structure formed by *S*. Typhimurium ATCC 14028 wild-type strain and mutant strains on glass coupons under (a) 5x, (b) 20x and (c) 40x objective.

Ability of bacteria to form pellicles and produce cellulose: In the experiment carried out to determine cellulose production, *Salmonella* strains were incubated in salt-free LB agar and liquid medium containing calcofluor, which is a cellulose indicator. *S*. Typhimurium wild-type and *dam* mutant strains gave strong luminescence due to cellulose production under 366 nm UV light, both in petri plates containing agar and in test tubes containing liquid medium. When all strains were compared among themselves according to their luminescence power, the wild-type strain gave the highest luminescence, while the *fimF* mutant gave the lowest luminescence.

Cellulose stabilizes the biofilm structures (pellicles) formed in the liquid-air interface against physical dispersive forces. As cellulose production increases, the pellicle structure gains firm physical properties. As a result of the experiment, it was determined that the pellicle structure of the wild-type strain with the rdar morphotype had a rigid pellicle structure, while the pellicle structure of the *fimF* mutant strain with the bdar morphotype and its completed construct created by cloning its N-terminal domain was fragile against physical factors such as shaking and mixing (Figure 5).

Identification of cellulose content of pellicle structures formed on glass slides: Calcofluor irradiation was used as a basis in the experiment conducted to determine the cellulose amounts of biofilm structures formed on glass slides. It was determined that the highest cellulose production was in the wild-type strain. It was observed that cellulose was produced in wild-type (pBAD) and Δ*dam* (pBAD+*dam*) and Δ*fimF* (pBAD+*fimF*^{N}) strains reexpressed with vector addition, with a close but very slight difference from the wild-type strain. Also, it was detected that all strains in this study produced cellulose, but less cellulose was produced in the *fimF* mutant compared to other strains (Figure 6).

Resistance to oxidative stress conditions: Bacterial growth rate of *Salmonella* strains in the study were evaluated in the presence of hydrogen peroxide (H_2O_2), which creates an oxidative stress environment. The number of viable wild-type strain in this environment increased from $1x10^6$ cells at the beginning to $5.3x10^8$ cells at the 60th minute. The number of bacteria growth remained constant at $5.3x10^8$ cells until the measurement time at the 120th minute. The amount of bacterial growth at the 180th minute increased by $2.6x10^8$ cells and the amount of bacterial growth at the 240th minute increased by $0.6x10^8$ cells. Using the wild-type strain as a reference, the percentage values of bacterial growth amounts of the complementation. Logarithmic transformation at base 10 (to rescale the magnitude) was applied to the resulting data to make a comparative analysis of the mutant and complementation strains with the wild-type strain. The amount of bacterial growth of the wild-type (pBAD) strain in the presence of hydrogen peroxide decreased by 12.1%, 15.6%, 6.20% and 5.82% at the 60th, 120th, 180th and 240th minutes, respectively. While the amount of bacterial growth of the Δ*dam* strain in the presence of hydrogen peroxide decreased by 14.9% and 9.44% in the 60th and 120th minutes, the amount of bacterial growth increased by 7.56% and 12.1% in the 180th and 240th minutes,

respectively. The amount of bacterial growth of the Δ*dam* (pBAD+*dam*) complementation strain in the presence of hydrogen peroxide at the 60th and 120th minutes decreased by 5.78% and 1.43%, while the amount of bacterial growth at the 180th and 240th minutes increased by 4.17% and 17.4%, respectively. While the amount of bacterial growth of the Δ*fimF* strain in the presence of hydrogen peroxide at the 60th minute decreased by 1.03%, the amount of bacterial growth in the presence of hydrogen peroxide at the 120th, 180th and 240th minutes increased by 2.01%, 10.2% and 20.8%, respectively. While a decrease of 0.32% and 4.11% was detected in the amount of bacterial growth of the Δ*fimF* (pBAD+*fimF*^{N}) strain in the presence of hydrogen peroxide at the 60th and 120th minutes, respectively, it was determined that there was an increase of 13.1% and 6.95% at the 180th and 240th minutes (Figure 7, Table 4).

Discussion and Conclusion

S. Typhimurium type 1 fimbriae characterized by mannose-sensitive haemagglutination, play an important role in adhesion to eukaryotic cells (32). The *S.* Typhimurium type 1 fimbriae *fim* gene cluster (*fimAICDHF*) consists of six structural genes organized as operons in the bacterial genome. Regulatory genes (*fimW*, *fimY* and *fimZ*) found in the *S.* Typhimurium genome and expressed by their own independent promoters control the regulation of the operon in question (40, 49). Chaperone-Usher proteins FimC and FimD undertake the task of bringing together the copies of FimA, the main structural subunit of type 1 fimbriae, while the FimH adhesin protein located at the tip of fimbriae gives fimbriae their adhesive properties (25). Although there is little similarity at the genetic and amino acid level between the FimF protein found in *S.* Typhimurium and the FimF protein found in *E. coli*, it is anticipated that the *S.* Typhimurium FimF protein plays a role in fimbriae biogenesis as an adapter protein, like FimF and FimG protein found in *E. coli*. The most important basis for this prediction is that the conserved amino acid sequences of the FimF protein found in *S.* Typhimurium are located in the same region as the *E. coli* adapter proteins (28, 32). The FimF protein is not essential for fimbriae production. However, if the protein in question is not expressed, fimbrial adhesion is either eliminated or reduced. Based on this fact, in studies conducted with many pathogens, including *Salmonella* and *E. coli*, it was determined that the FimF protein acts as an adapter in the addition of tip adhesins to the fimbrial

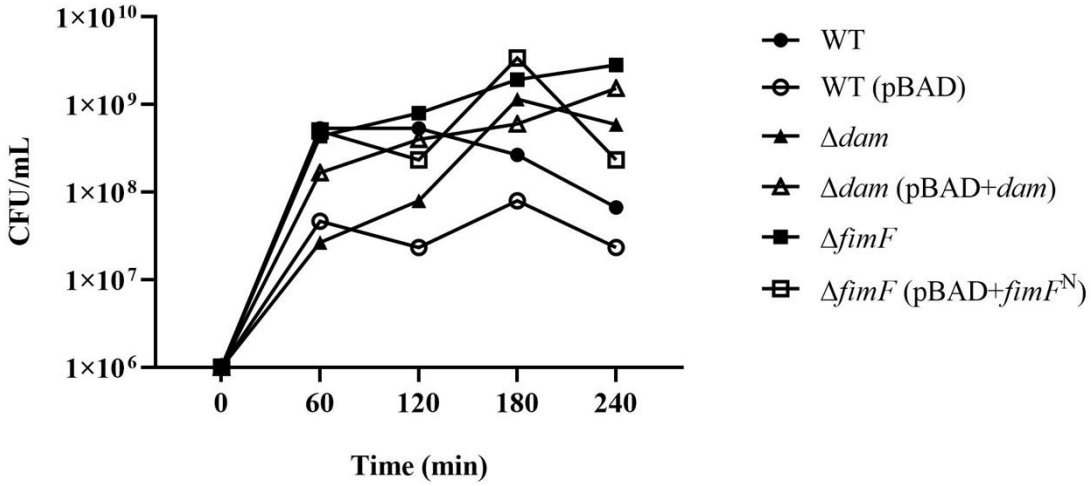

Figure 7. Responses of *S.* Typhimurium ATCC 14028 wild-type and mutant strains to stress condition in the environment containing 4mM hydrogen peroxide (H_2O_2).

Table 4. Growth characteristics of *S.* Typhimurium ATCC 14028 wild-type and mutant strains under oxidative stress conditions.

STRAIN	Initial Conc.	1h	2h	3h	4h
WT	1x10^{06} CFU/ml	5.3x10^{08} CFU/ml	5.3x10^{08} CFU/ml	2.7x10^{08} CFU/ml	6.7x10^{07} CFU/ml
WT (pBAD)	1x10^{06} CFU/ml	4.7x10^{07} CFU/ml	2.3x10^{07} CFU/ml	8.0x10^{07} CFU/ml	2.3x10^{07} CFU/ml
Δ*dam*	1x10^{06} CFU/ml	2.7x10^{07} CFU/ml	8.0x10^{07} CFU/ml	1.2x10^{09} CFU/ml	5.9x10^{08} CFU/ml
Δ*dam* (pBAD+*dam*)	1x10^{06} CFU/ml	1.7x10^{08} CFU/ml	4.0x10^{08} CFU/ml	6.0x10^{08} CFU/ml	1.5x10^{09} CFU/ml
Δ*fimF*	1x10^{06} CFU/ml	4.3x10^{08} CFU/ml	8.0x10^{08} CFU/ml	1.9x10^{09} CFU/ml	2.8x10^{09} CFU/ml
Δ*fimF* (pBAD+*fimF*^{N})	1x10^{06} CFU/ml	5.0x10^{08} CFU/ml	2.3x10^{08} CFU/ml	3.4x10^{09} CFU/ml	2.3x10^{08} CFU/ml

DOI: 10.33988/auvfd.1390023

structure and that adhesin binding activation cannot be achieved in the absence of this protein (39). However, in the study conducted with type 1 fimbriae adapter proteins of a number of pathogens, including *E. coli* (26), it was suggested that only the N-terminal ends of the adapter subunits act as adapters in the addition of fimbrial subunits. However, there is no experimental data regarding these predictions in *Salmonella*. The N-terminal domain of a protein regulates the function of that protein and ensures that the function is carried out correctly, and also determines the role of that protein in biological processes. In this study, in addition to the mutant of the *fimF* gene in the *S.* Typhimurium 14028 genome containing the chloramphenicol gene cassette, the use of a construct of the 151 bp long region located only at the 5' end of the gene in question cloned in front of the arabinose promoter of the pBAD24 vector was used, as in the literature data. It was aimed to determine whether it originates from the terminal domain or not. Previous studies conducted by our working group found that the expression rate of the *fimF* gene was significantly reduced in the *dam* mutant (22). Based on this finding, *S.* Typhimurium 14028 strain *dam* mutant was also included in the study to obtain information about the regulation characteristics as well as the function of the *fimF* gene.

As a result of our experimental studies, we found that biofilm formation in *dam* and *fimF* gene mutants decreased at statistically significant levels ($P<0.01$), and that both genes and therefore the proteins they encode have critical roles in the transition from the planktonic form to the biofilm form promoted under stress conditions in *Salmonella*. The failure to restore the biofilm defect caused by the *dam* mutation in the *dam* gene complementary strain is probably due to the much higher rate of *dam* expression from the inducible arabinose promoter of the pBAD vector compared to the wild-type. This is because over-expression of the *dam* gene is known to give the same phenotypic result as *dam* gene mutant strains (22). It has been determined that under conditions where *dam* expression is very high, the binding affinity of this protein to the 5'GATC3' series decreases due to competitive inhibition caused by the substrate concentration, and the *dam* mutant phenotype occurs in overexpressed constructs (2). On the other hand, the restoration of the biofilm phenotype at a statistically significant level ($P<0.01$) in the complementary strain in which the N-terminal domain of the *fimF* gene was transferred is strong evidence that this domain acts as an adaptor in the assembly of *fimF*.

The increased resistance to oxidative stress conditions in all mutants and complemented constructs compared to the wild-type strain contradicts literature data (11, 19, 23, 31). Both Dam methylase enzyme and type 1 fimbriae have critical roles in the pathogenicity and virulence of *Salmonella* (7, 11, 18, 27, 30). On the other hand, as can be clearly seen from the results of our study, the absence of dam methylase and *fimF* is perceived by the cells as an internal stress factor and probably for this reason, the cells create strong responses against oxidative stress.

Although the basic biofilm morphotype does not change in solid and liquid environments in the *dam* mutant, the formation of intermediate biofilm morphotypes that do not have a full rdar morphotype in solid environments and pellicle structures with reduced resistance to physical conditions in liquid environments has been evaluated as a decrease in cellulose production, one of the main components of EPS, in this mutant. As clearly seen in biofilm morphotyping tests, it was determined that the main elements that disrupted the stability of the biofilm and pellicle structure produced by the *fimF* mutant at a high level were due to the significant decreases in curli fimbriae and cellulose production in the *fimF* gene-deleted mutant. The failure to restore the biofilm morphotype in the complementation mutant created by cloning the N-terminal domain of the *fimF* gene into the pBAD vector is probably due to the fact that the mutant contains only a part of the gene in question instead of the full *fimF* gene.

In summary, as a result of the detailed genetic and functional analyzes carried out in this study, it has been proven that the N-terminal domain of the FimF protein functions as an adapter in type 1 fimbriae assembly and biofilm adhesion.

Acknowledgements

This study was derived from the MSc thesis of the first author.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

TNS, NA and MA conceived and planned the experiments. TNS carried out the experiments. TNS contributed to sample preparation. TNS, NA and MA contributed to the interpretation of the results. MA took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

References

1. **Amano A** (2010): *Bacterial adhesins to host components in periodontitis.* Periodontol, **52**, 12–37.
2. **Aya Castañeda M, Sarnacki SH, Noto Llana M, et al** (2015): *Dam methylation is required for efficient biofilm production in Salmonella enterica serovar Enteritidis.* Int J Food Microbiol, **193**, 15–22.
3. **Bäumler AJ, Tsolis RM, Heffron F** (1997): *Fimbrial adhesins of Salmonella typhimurium. Role in bacterial interactions with epithelial cells.* Adv Exp Med Biol, **412**, 149–158.
4. **Beachey EH** (1981): *Bacterial adherence: adhesin receptor interaction mediating the attachment of bacteria to mucosal surface.* J Infect Dis, **143**, 325-345.
5. **Boddicker JD, Ledeboer NA, Jagnow J, et al** (2002): *Differential binding to and biofilm formation on, HEp-2 cells by Salmonella enterica serovar Typhimurium is dependent upon allelic variation in the fimH gene of the fim gene cluster.* Mol Microbiol, **45**, 1255–1265.
6. **Bouckaert J, Mackenzie J, de Paz JL et al** (2006): *The affinity of the FimH fimbrial adhesin is receptor-driven and quasi-independent of Escherichia coli pathotypes.* Mol Microbiol, **61**, 1556–1568.
7. **Bourgeois JS, Anderson CE, Wang L, et al** (2022): *Integration of the Salmonella Typhimurium methylome and transcriptome reveals that DNA methylation and transcriptional regulation are largely decoupled under virulence-related conditions.* mBio, **13**, e0346421.
8. **Boyd EF, Hartl DL** (1999): *Analysis of the type 1 pilin gene cluster fim in Salmonella: its distinct evolutionary histories in the 5' and 3' regions.* J Bacteriol, **181**, 1301–1308.
9. **Campos-Galvão ME, Ribon AO, Araújo EF, et al** (2016): *Changes in the Salmonella enterica Enteritidis phenotypes in presence of acyl homoserine lactone quorum sensing signals.* J Basic Microbiol, **56**, 493–501.
10. **Castelijn GA, Van der Veen S, Zwietering MH, et al** (2012): *Diversity in biofilm formation and production of curli fimbriae and cellulose of Salmonella Typhimurium strains of different origin in high and low nutrient medium.* Biofouling, **28**, 51–63.
11. **Chatti A, Messaoudi N, Mihoub M, et al** (2012): *Effects of hydrogen peroxide on the motility, catalase and superoxide dismutase of dam and/or seqA mutant of Salmonella typhimurium.* World J Microbiol Biotechnol, **28**, 129–133.
12. **Crawford RW, Reeve KE, Gunn JS** (2010): *Flagellated but not hyperfimbriated Salmonella enterica serovar Typhimurium attaches to and forms biofilms on cholesterol-coated surfaces.* J Bacteriol, **192**, 2981–2990.
13. **Datsenko KA, Wanner BL** (2000): *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* PNAS, **97**, 6640–6645.
14. **Donlan RM** (2002): *Biofilms: microbial life on surfaces.* Emerg Infect Dis, **8**, 881-890.
15. **Flemming HC, Wingender J, Szewzyk U, et al** (2016): *Biofilms: an emergent form of bacterial life.* Nat Rev Microbiol, **14**, 563–575.
16. **Gonzalez-Escobedo G, Gunn JS** (2013): *Identification of Salmonella enterica serovar Typhimurium genes regulated during biofilm formation on cholesterol gallstone surfaces.* Infect Immun, **81**, 3770–3780.
17. **Gossert AD, Bettendorff P, Puorger C, et al** (2008): *NMR structure of the Escherichia coli type 1 pilus subunit FimF and its interactions with other pilus subunits.* J Mol Biol, **375**, 752–763.
18. **Guo Y, Gu D, Huang T, et al** (2020): *Essential role of Salmonella Enteritidis DNA adenine methylase in modulating inflammasome activation.* BMC Microbiol, **20**, 226.
19. **Hahn MM, González JF, Gunn JS** (2021): *Salmonella biofilms tolerate hydrogen peroxide by a combination of extracellular polymeric substance barrier function and catalase enzymes.* Front Cell Infect Microbiol, **11**, 683081.
20. **Hsu CY, Wu YL, Lin HC, et al** (2021): *A novel dibenzoxazepine attenuates intracellular Salmonella Typhimurium oxidative stress resistance.* Microbiol Spectr, **9**, e0151921.
21. **Jamal M, Ahmad W, Andleeb S, et al** (2018): *Bacterial biofilm and associated infections.* J Chin Med Assoc, **81**, 7–11.
22. **Keçeli Oğuz S, Has EG, Akçelik N, et al** (2023): *Phenotypic impacts and genetic regulation characteristics of the DNA adenine methylase gene (dam) in S. Typhimurium biofilm forms.* Res Microbiol, **174**, 103991.
23. **Kim JS, Liu L, Vázquez-Torres A** (2021): *The DnaK/DnaJ chaperone system enables RNA polymerase-DksA complex formation in Salmonella experiencing oxidative stress.* mBio, **12**, e03443-20.
24. **Kisiela D, Laskowska A, Sapeta A, et al** (2006): *Functional characterization of the FimH adhesin from Salmonella enterica serovar Enteritidis.* Microbiology (Reading, England), **152**, 1337–1346.
25. **Kisiela DI, Kramer JJ, Tchesnokova V, et al** (2011): *Allosteric catch bond properties of the FimH adhesin from Salmonella enterica serovar Typhimurium.* J Biol Chem, **286**, 38136–38147.
26. **Kloppsteck P, Hall M, Hasegawa Y, et al** (2016): *Structure of the fimbrial protein Mfa4 from Porphyromonas gingivalis in its precursor form: implications for a donor-strand complementation mechanism.* Sci Rep, **6**, 22945.
27. **Kolenda R, Ugorski M, Grzymajlo K** (2019): *Everything you always wanted to know about salmonella type 1 fimbriae, but were afraid to ask.* Front Microbiol, **10**, 1017.
28. **Lane MC, Mobley HL** (2007): *Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic Escherichia coli (UPEC) in the mammalian kidney.* Kidney Int, **72**, 19 –25.
29. **Leusch HG, Drzeniek Z, Markos-Pusztai Z, et al** (1991): *Binding of Escherichia coli and Salmonella strains to members of the carcinoembryonic antigen family: differential binding inhibition by aromatic alpha-glycosides of mannose.* Infect Immun, **59**, 2051–2057.

30. **Mannan T, Rafique MW, Bhatti MH, et al** (2020): *Type 1 fimbriae and motility play a pivotal role during interactions of Salmonella typhimurium with Acanthamoeba castellanii (T4 Genotype).* Curr Microbiol, **77**, 836–845.
31. **Morales EH, Calderón IL, Collao B, et al** (2012): *Hypochlorous acid and hydrogen peroxide-induced negative regulation of Salmonella enterica serovar Typhimurium ompW by the response regulator ArcA.* BMC Microbiology, **12**, 63.
32. **Nuccio SP, Baumler AJ** (2007): *Evolution of the chaperone/usher assembly pathway: fimbrial classification goes Greek.* Microbiol Mol Biol Rev, **71**, 551–575.
33. **O'Toole G, Kaplan HB, Kolter R** (2000): *Biofilm formation as microbial development.* Annu Rev Microbiol, **54**, 49–79.
34. **Percival SL, Malic S, Cruz H, et** al (2011): *Introduction to Biofilms.* Biofilms Vet Med, 41–68.
35. **Rehman T, Yin L, Latif MB, et al** (2019): *Adhesive mechanism of different Salmonella fimbrial adhesins.* Microb Pathog, **137**, 103748.
36. **Römling U, Rohde M** (1999): *Flagella modulate the multicellular behavior of Salmonella typhimurium on the community level.* FEMS Microbiol Lett, **180**, 91–102.
37. **Römling U, Rohde M, Olsén A, et al** (2000): *AgfD, the checkpoint of multicellular and aggregative behaviour in Salmonella typhimurium regulates at least two independent pathways.* Mol Microbiol, **36**, 10–23.
38. **Rosen DA, Pinkner JS, Walker JN, et al** (2008): *Molecular variations in Klebsiella pneumoniae and Escherichia coli FimH affect function and pathogenesis in the urinary tract.* Infect Immun, **76**, 3346–3356.
39. **Russell PW, Orndorff PE** (1992): *Lesions in two Escherichia coli type 1 pilus genes alter pilus number and length without affecting receptor binding.* J Bacteriol, **174**, 5923–5935.
40. **Saini S, Pearl JA, Rao CV** (2009): *Role of FimW, FimY, and FimZ in regulating the expression of type 1 fimbriae in Salmonella enterica serovar Typhimurium.* J Bacteriol, **191**, 3003–3010.
41. **Sambrook J, Russell DW** (2001): Mole Molecular Cloning, A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, New York.
42. **Solano C, García B, Valle J, et al** (2002): *Genetic analysis of Salmonella enteritidis biofilm formation: critical role of cellulose.* Mol Microbiol, **43**, 793–808.
43. **Stepanović S, Cirković I, Ranin L, et al** (2004): *Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface.* Lett Appl Microbiol, **38**, 428–432.
44. **Tareb R, Bernardeau M, Gueguen M, et al** (2013): *In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic Lactobacillus strains and interaction with foodborne zoonotic bacteria, especially Campylobacter jejuni.* J Med Microbiol, **62**, 637–649.
45. **Thankavel K, Shah AH, Cohen MS, et al** (1999): *Molecular basis for the enterocyte tropism exhibited by Salmonella Typhimurium type 1 fimbriae.* J Biol Chem, **274**, 5797–5809.
46. **Vestby LK, Møretrø T, Langsrud S, et al** (2009): *Biofilm forming abilities of Salmonella are correlated with persistence in fish meal- and feed factories.* BMC Vet Res, **5**, 20.
47. **Wagner C, Hensel M** (2011): *Adhesive mechanisms of Salmonella enterica.* Adv Exp Med Biol, **715**, 17–34.
48. **Worthington RJ, Richards JJ, Melander C** (2012): *Small molecule control of bacterial biofilms.* OBC, **10**, 7457–7474.
49. **Yeh KS, Tinker JK, Clegg S** (2002): *FimZ binds the Salmonella Typhimurium fimA promoter region and may regulate its own expression with FimY.* Microbiol Immun, **46**, 1–10.
50. **Zeiner SA, Dwyer BE, Clegg S** (2012): *FimA, FimF, and FimH are necessary for assembly of type 1 fimbriae on Salmonella enterica serovar Typhimurium.* Infect Immun, **80**, 3289–3296.