

# 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*

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## 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  $\alpha$ -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 *PfimA* 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 (*PfimA*). 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.

**Table 1.** Gene specific primer sequences.

<i>fimF</i> -N-ter-clon forward primer	5'- GATGTCGACCAACGTCGATTGCCACT -3'
<i>fimF</i> -N-ter-clon reverse primer	5'- TGA CTGCAGTCAGGAAGGTCGCATCC -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 °C 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<sup>-1</sup>).

**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. *SalI* and *PstI* (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, *SalI* and *PstI* 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 °C for 18 hours, were centrifuged at 6000 rpm for 10 minutes at 4 °C 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<sub>600</sub>=0.2. Finally, the bacterial cultures in the tube were incubated at 20 °C static conditions to make optical density (OD<sub>600</sub>) measurements at different time intervals (0, 4, 20, and 24 hours).

The percentage of autoaggregation was determined using the formula:  $[(1 - OD_{\text{time}} / OD_{T0}) \times 100]$  (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 °C. Active bacterial culture was added to 96-well polystyrene microplate wells at a concentration of 1×10<sup>9</sup> CFU/mL. Microplates were incubated under static conditions at 20 °C 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<sub>595</sub> nm. Final calculation was carried out by subtracting the arithmetic means of the controls (wells transferred to LB<sup>-NaCl</sup> 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 °C 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 °C 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 °C 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).

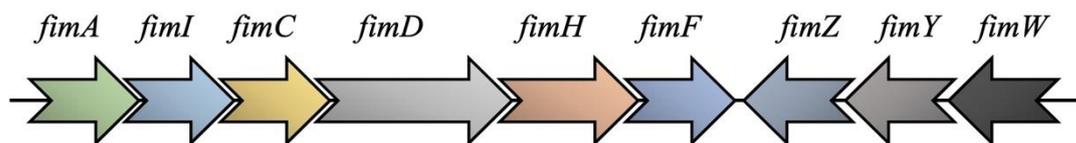
**Determination of resistance of bacteria to oxidative stress conditions:** To determine the resistance of bacteria against oxidative stress conditions, active cultures were diluted 1:1000 in 25 mL LB medium. It was incubated at 37 °C and 250 rpm until its optical density reached  $OD_{595}=0.4$ . At 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  $1 \times 10^6$  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 °C for 24 hours. Colony counts were made at the end of 24 hours and the results were expressed as CFU/mL (20, 31).

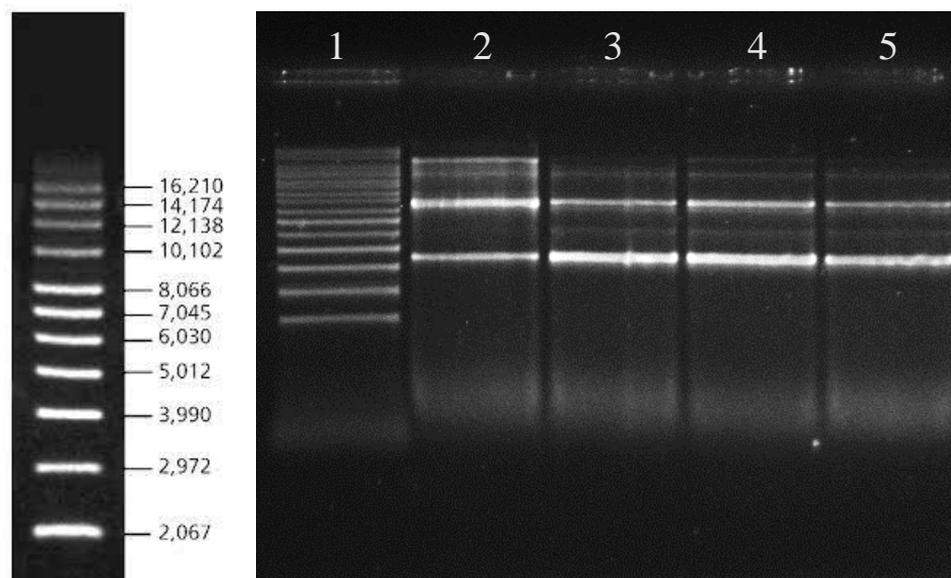
**Statistical Analysis:** The data of optical density and enumeration were presented as Mean ± Standard Deviation (SD). To test for any statistically significant difference between the genetically different groups, repeated measures one-way ANOVA was used, which was observed to be statistically significant 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*<sup>N</sup> transformants.

**Bacterial cell aggregation:** Autoaggregation experiments were carried out by determining the optical densities (OD<sub>600</sub>) 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 4<sup>th</sup>, 20<sup>th</sup> and 24<sup>th</sup> hours, respectively, compared to the starting time. These increase rates were determined as follows; 1.55%, 18.6% and 24.8%, at the 4<sup>th</sup>, 20<sup>th</sup> and 24<sup>th</sup> hours, respectively, in the *S. Typhimurium* 14028 strain in which empty pBAD vector was transferred; 5.50%, 17% and 18.5% in the  $\Delta dam$  strain; 3.92%, 22.5% and 27.4% in the  $\Delta dam$  (pBAD+*dam*) strain; 4.50%, 20.0% and 24.0% in the  $\Delta fimF$  strain and 9.65%, 22.0% and 28.9% in the  $\Delta fimF$  (pBAD+*fimF*<sup>N</sup>) 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 24<sup>th</sup>, 48<sup>th</sup> and 72<sup>nd</sup> 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  $\Delta dam$  strain; 89.4%, 56.1% and 56.9% in the  $\Delta dam$  (pBAD+*dam*) strain; 83.6%, 48.3% and 70.4% in the  $\Delta fimF$  strain and 82.1%, 69.4% and 30.7% in the  $\Delta fimF$  (pBAD+*fimF*<sup>N</sup>) 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  $\Delta dam$  (pBAD+*dam*), exhibited the 'rdar' biofilm morphotype, which contains curli fimbriae and cellulose in the biofilm EPS structure. In contrast, the  $\Delta fimF$  and  $\Delta fimF$  (pBAD+*fimF*<sup>N</sup>) 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).

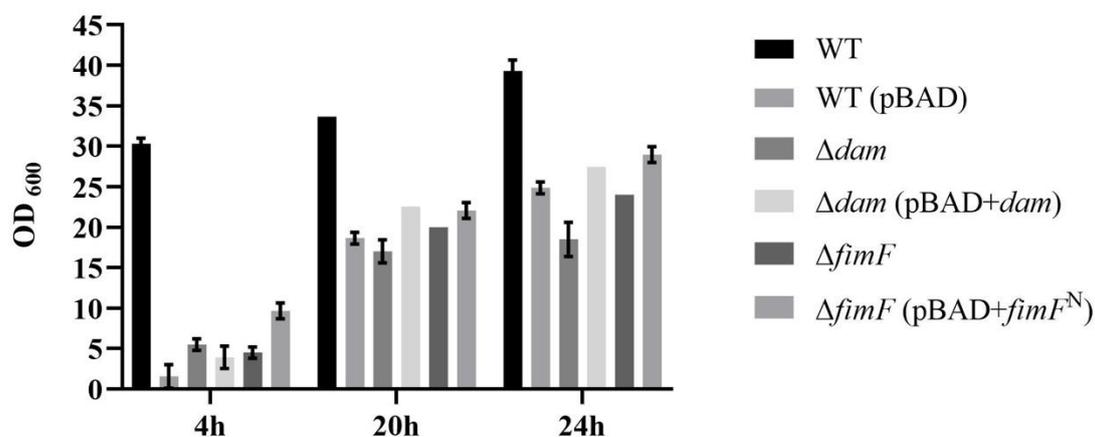
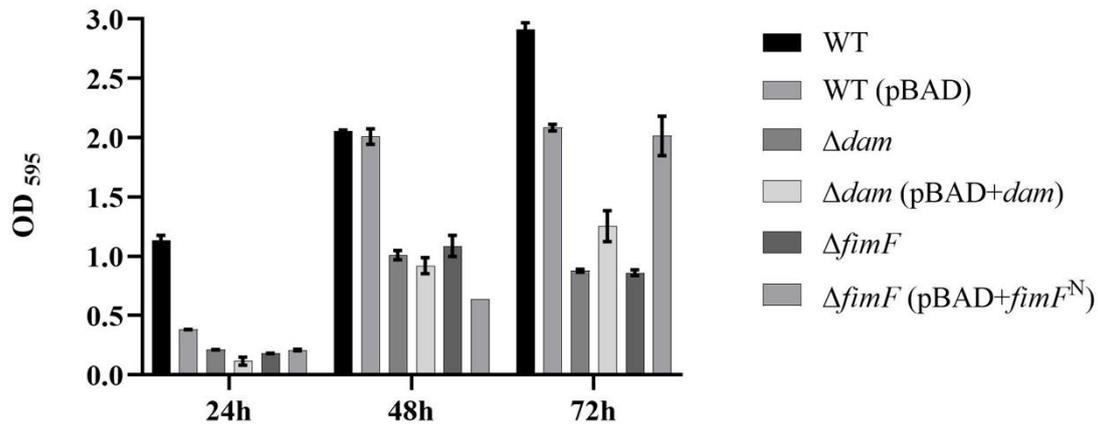


Figure 3. Autoaggregation characteristics of *S. Typhimurium* ATCC 14028 wild-type and mutant strains.

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.

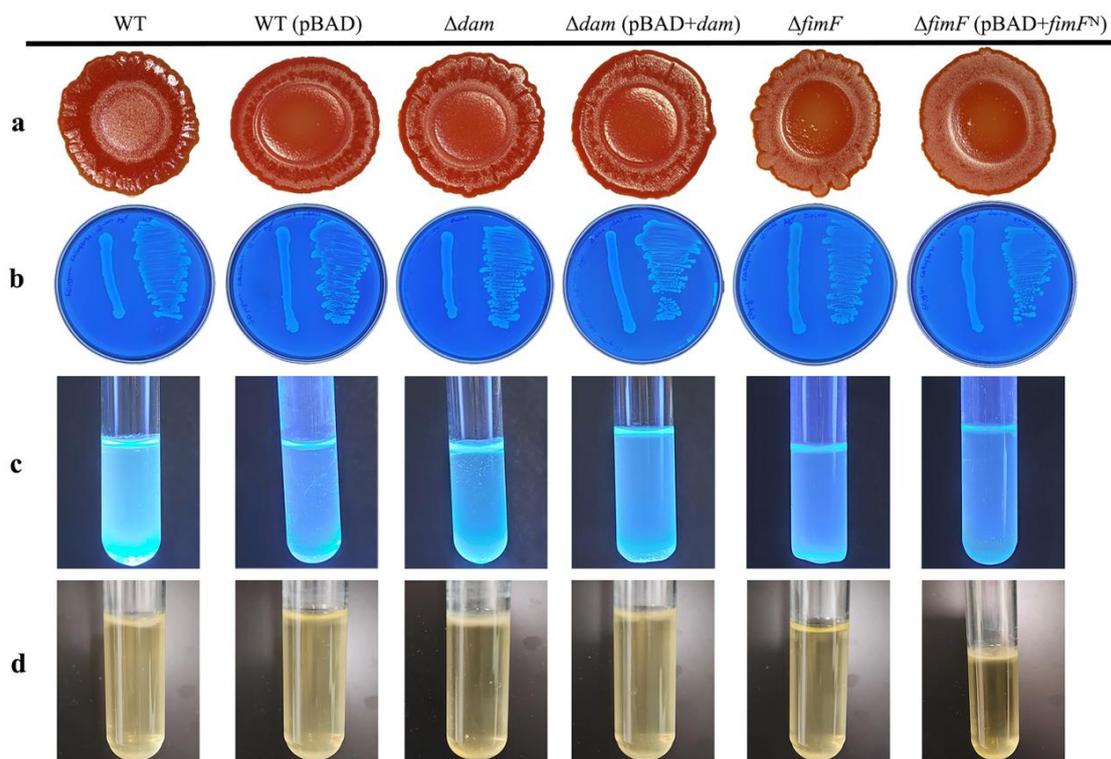
STRAIN	MEAN			STD		
	4h	20h	24h	4h	20h	24h
WT	28.93891	32.47588	37.94212	0.001155	0	0.002309
WT (pBAD)	4.761905	20.40816	26.53061	0.00611	0.002	0.002
$\Delta dam$	4.347826	15.05017	20.73579	0.003055	0.00611	0.009165
$\Delta dam$ (pBAD+ <i>dam</i> )	6.148867	21.68285	26.21359	0.005033	0.005774	0.006928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



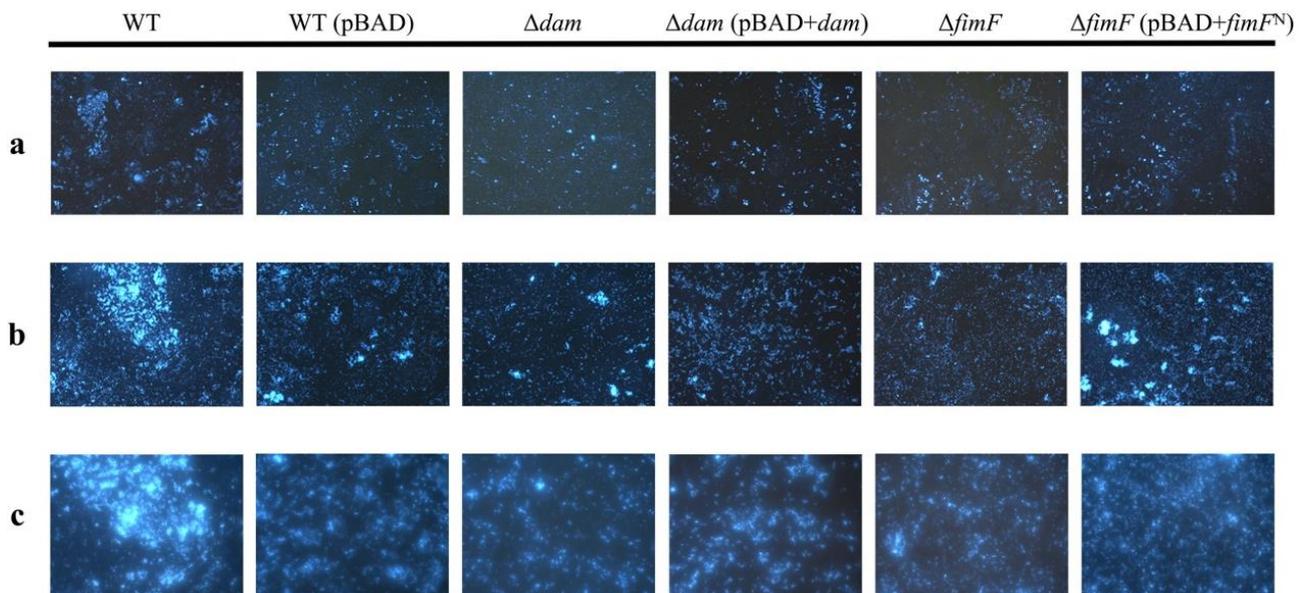
**Figure 4.** Biofilm production characteristics of *S. Typhimurium* ATCC 14028 wild-type and mutant strains on polystyrene surfaces.

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

STRAIN	MEAN			STD		
	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
$\Delta dam$	0.215	1.032	0.879	0.0043589	0.0462313	0.01272792
$\Delta dam$ (pBAD+ <i>dam</i> )	0.115	0.922	1.253	0.03252691	0.06788225	0.13010765
$\Delta fimF$	0.177	1.087	0.861	0.00568624	0.08909545	0.02545584
$\Delta fimF$ (pBAD+ <i>fimF</i> <sup>N</sup> )	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

$\Delta dam$  (pBAD+*dam*) and  $\Delta fimF$  (pBAD+*fimF*<sup>N</sup>) 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).

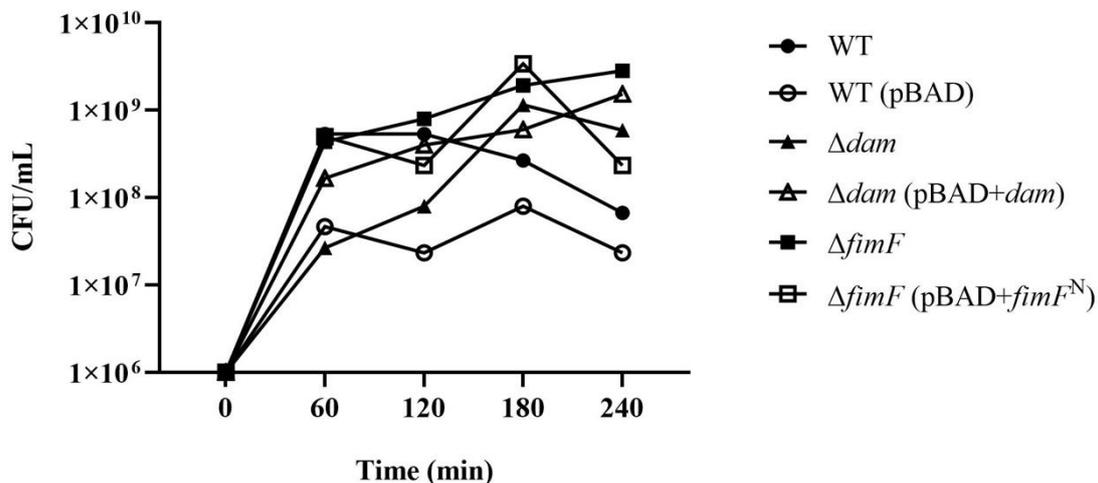
**Resistance to oxidative stress conditions:** Bacterial growth rate of *Salmonella* strains in the study were evaluated in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which creates an oxidative stress environment. The number of viable wild-type strain in this environment increased from 1x10<sup>6</sup> cells at the beginning to 5.3x10<sup>8</sup> cells at the 60<sup>th</sup> minute. The number of bacteria growth remained constant at 5.3x10<sup>8</sup> cells until the measurement time at the 120<sup>th</sup> minute. The amount of bacterial growth at the 180<sup>th</sup> minute increased by 2.6x10<sup>8</sup> cells and the amount of bacterial growth at the 240<sup>th</sup> minute increased by 0.6x10<sup>8</sup> 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 60<sup>th</sup>, 120<sup>th</sup>, 180<sup>th</sup> and 240<sup>th</sup> minutes, respectively. While the amount of bacterial growth of the  $\Delta dam$  strain in the presence of hydrogen peroxide decreased by 14.9% and 9.44% in the 60<sup>th</sup> and 120<sup>th</sup> minutes, the amount of bacterial growth increased by 7.56% and 12.1% in the 180<sup>th</sup> and 240<sup>th</sup> minutes,

respectively. The amount of bacterial growth of the  $\Delta dam$  (pBAD+*dam*) complementation strain in the presence of hydrogen peroxide at the 60<sup>th</sup> and 120<sup>th</sup> minutes decreased by 5.78% and 1.43%, while the amount of bacterial growth at the 180<sup>th</sup> and 240<sup>th</sup> minutes increased by 4.17% and 17.4%, respectively. While the amount of bacterial growth of the  $\Delta fimF$  strain in the presence of hydrogen peroxide at the 60<sup>th</sup> minute decreased by 1.03%, the amount of bacterial growth in the presence of hydrogen peroxide at the 120<sup>th</sup>, 180<sup>th</sup> and 240<sup>th</sup> 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  $\Delta fimF$  (pBAD+*fimF<sup>N</sup>*) strain in the presence of hydrogen peroxide at the 60<sup>th</sup> and 120<sup>th</sup> minutes, respectively, it was determined that there was an increase of 13.1% and 6.95% at the 180<sup>th</sup> and 240<sup>th</sup> 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 <sup>6</sup> CFU/ml	5.3x10 <sup>8</sup> CFU/ml	5.3x10 <sup>8</sup> CFU/ml	2.7x10 <sup>8</sup> CFU/ml	6.7x10 <sup>7</sup> CFU/ml
WT (pBAD)	1x10 <sup>6</sup> CFU/ml	4.7x10 <sup>7</sup> CFU/ml	2.3x10 <sup>7</sup> CFU/ml	8.0x10 <sup>7</sup> CFU/ml	2.3x10 <sup>7</sup> CFU/ml
$\Delta dam$	1x10 <sup>6</sup> CFU/ml	2.7x10 <sup>7</sup> CFU/ml	8.0x10 <sup>7</sup> CFU/ml	1.2x10 <sup>9</sup> CFU/ml	5.9x10 <sup>8</sup> CFU/ml
$\Delta dam$ (pBAD+ <i>dam</i> )	1x10 <sup>6</sup> CFU/ml	1.7x10 <sup>8</sup> CFU/ml	4.0x10 <sup>8</sup> CFU/ml	6.0x10 <sup>8</sup> CFU/ml	1.5x10 <sup>9</sup> CFU/ml
$\Delta fimF$	1x10 <sup>6</sup> CFU/ml	4.3x10 <sup>8</sup> CFU/ml	8.0x10 <sup>8</sup> CFU/ml	1.9x10 <sup>9</sup> CFU/ml	2.8x10 <sup>9</sup> CFU/ml
$\Delta fimF$ (pBAD+ <i>fimF<sup>N</sup></i> )	1x10 <sup>6</sup> CFU/ml	5.0x10 <sup>8</sup> CFU/ml	2.3x10 <sup>8</sup> CFU/ml	3.4x10 <sup>9</sup> CFU/ml	2.3x10 <sup>8</sup> CFU/ml

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.

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### Ethical Statement

This study does not present any ethical concerns.

### 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.

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