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The effect of *marT* gene on biofilm production of *Salmonella* Typhimurium

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Abstract: This study investigates the effect of MarT regulator protein on the production of *Salmonella* Typhimurium biofilm under in vitro culture conditions. The biofilm formation showed a statistically significant decrease compared to the wild-type strain at the two consecutive concentrations of arabinose (0.02% and 0.01%) in which the production of MarT protein was promoted to the highest level. When comparing the biofilm formation capacity to the wild type, the mutants containing a mutation in the genes investigated in terms of *marT* regulation showed statistically significant reductions, which were identified as *fliA* (59%), *fliC* (40%), *fimA* (46%), *fidL* (25%), and *misL* (25%) mutants. The biofilm structures of all strains used in this study were detected as the rdar morphotype in agar plates. Among the genes examined, *fimA*, *slsA*, *fliA*, *dps*, *cheA*, and *wzzB* genes were found to have a direct effect on the pellicle formation of *S.* Typhimurium. Finally, the biofilm formation features of the mutant strain, in which *marT* expression is induced, on glass, stainless steel, and polystyrene were also examined, and it was determined that the ability to produce biofilm decreased on all surfaces.

Key words: Biofilm, *Salmonella enterica* serovar Typhimurium, *marT*, Red/ET recombination system

1. Introduction

Salmonella species can form multicellular structures, commonly called biofilms, on various surfaces such as polymer, stainless steel, and glass, as well as biotic surfaces such as gallstones and epithelial cells (Costerton et al., 1999; Donlan and Costerton, 2002; Hall-Stoodley et al., 2006; Møretrø et al., 2009). Biofilms are the collection of microbial communities that adhere to a solid or living surface and are held together by a protective self-secreted matrix of extracellular polymeric substance (Costerton et al., 1995). It is known that bacteria within biofilms are well protected against environmental stress and are more resistant to antibiotics, disinfectant chemicals, and phagocytosis than the corresponding free-swimming or planktonic cells in suspension (Donlan, 2002; Høiby et al., 2010). As a result, they are extremely difficult to eradicate.

Biofilm-associated microorganisms have been linked to numerous human diseases, especially chronic diseases, and have been shown to colonize a wide variety of medical devices. Biofilm-detached cells are another problematic factor in infections caused by biofilms through the release of a group of bacteria from the biofilm community, which can then create another biofilm structure in different regions of the body (Stoodley et al., 2001). There is a great deal of research aiming to find effective strategies to prevent or control the formation of biofilms or to remove them once they form. Current combating strategies are designed to prevent initial colonization or minimize microbial cell attachment to the surface. Inhibition of genes involved in cell attachment and biofilm formation can become a new antibiofilm technology in the future. *Salmonella* is the most important agent causing acute foodborne disease through, for example, meat and eggs, making *Salmonella* a particular threat to human health. A significant fraction of foodborne salmonellosis is caused by *S.* Enteritidis and *S.* Typhimurium. The first stage in the disease is the attachment to and colonization of the intestinal epithelium of the host. Studies have shown that adhesins mediate *Salmonella* adherence to epithelial cells and its growth on the intestinal mucosa (Darwin and Miller, 1999; Ledeboer et al., 2006). The *S.* Typhimurium genome involves a number of determinants responsible for attachment, including 13 fimbrial operons and three adhesins from autotransporter families *shdA*, *misL*, and *sadA* (Wagner and Hensel, 2011).

As a consequence of DNA sequence analysis of serotype Typhimurium, the gene encoding the MisL protein was found on *Salmonella* pathogenicity island 3 (SPI3) and it is adjacent to the *marT*, *fidL*, *slsA*, and *rmbA* genes (Blanc-Potard et al., 1999). Studies have shown that MisL is required for intestinal colonization in chicks and mice (Morgan et al., 2004; Dorsey et al., 2005). While

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MisL expression is not detected by western blotting in *S.* Typhimurium when grown in vitro, the observation of the anti-MisL antibody in the serum of mice infected with serotype Typhimurium showed that the expression of MisL protein takes place under in vivo conditions (Dorsey et al., 2005). Moreover, the production of MisL is induced during macrophage infection by serotype Typhimurium (Eriksson et al., 2003). Expression of the MisL protein of serotype Typhimurium strains under in vitro conditions can be provided by cloning the *misL* gene to the vector system with an inducible arabinose promoter (Dorsey et al., 2005). Tükel et al. (2007) showed that the expression of MisL was activated in vivo by *marT*, a positive regulator of *misL*. Recently, Kroupitski et al. (2013) predicted that the expression of MisL autotransporter protein negatively affected biofilm formation in *S.* Typhimurium under in vivo conditions and highlighted the possible regulatory role of *marT* in biofilm formation. Since the biofilm-producing ability in serotypes of *Salmonella* is in close relationship with the global regulators, the determination of the efficiency of *marT* for biofilm production is the primary aim of this study.

Based on data obtained by Akkoç (2011), 11 genes were selected (Table 1) and tested for their effects on biofilm formation. Comparative microarray analysis revealed that 6 genes (*dps*, *yliH*, *cheA*, *cheM*, *fliA*, and *fliC*) are downregulated and 5 (*wzzB*, *fidL*, *misL*, *rmbA*, and *slsA*) are upregulated by the expression of the *marT* gene (Akkoç, 2011). In this study, we aimed to investigate the effects of the selected 11 genes on biofilm formation by performing Red recombinase system-based mutations. In brief, with the data obtained from this study, we aimed to identify new genes that have not previously been considered in relation to the effects on the formation of biofilm. Furthermore, in light of this information, this study intends to trace the development of new strategies for biofilm eradication.

2. Materials and methods

2.1. Bacterial strains, plasmid, and growth conditions

S. Typhimurium 14028 (wild-type strain), upregulated Δ*marT* mutant (MZ1627), and the pBAD24 plasmid used in this study were provided by Prof Dr Mustafa Akçelik, Department of Biology, Ankara University. *Salmonella* strains were cultured in Luria-Bertani broth (LB) at 37 °C for 18 h under shaking conditions.

2.2. In vitro production of MisL autotransporter protein

The pBAD24 vector was used to produce MisL autotransporter protein in which the *marT* gene, a positive transcriptional regulator, was cloned to downstream of the arabinose inducible BAD promoter. After the cloning of the *marT* gene, the pBAD24 vector was transformed to *marT* gene-blocked (Δ*marT*) *S.* Typhimurium strain 14028 (MZ1627). Overnight bacterial culture was inoculated into fresh LB medium and incubated until OD_{595} reached 0.2 for induction with arabinose. The induction of protein expression was achieved with the addition of L-arabinose

Table 1. Target genes containing mutations.

Gene name	Function
dps	DNA-binding protein from starved and stressed cells
yliH	Putative cytoplasmic protein
wzzB	Regulator of lipopolysaccharide O-chain length
misL	Autotransporter protein
slsA	Putative inner membrane protein
fidL	Putative inner membrane protein
rmbA	Putative cytoplasmic protein
cheM	Methyl accepting chemotaxis protein II (MCPII), aspartate chemoreceptor protein
cheA	Chemotaxis protein
fimA	Major type 1 subunit fimbrin
fliA	Transcription factor sigma 28 for class III flagellar operons
fliC	Flagellar biosynthesis; flagellin, filament structural protein
marT	Positive transcriptional regulator of MisL autotransporter protein

in an appropriate concentration (0.02%) to the growth medium of bacteria, which was then incubated for 30 min (Akçelik and Akçelik, 2011).

2.3. Microtiter plate assay

Biofilm formation assay was performed in 96-well polystyrene microtiter plates according to the method described by Woodward et al. (2000), with some slight modifications as suggested by Stepanović et al. (2000) and Vestby et al. (2009). Briefly, overnight cultures were diluted in LB without NaCl (LB^{wo}/NaCl) to OD_{595} = 0.2, and 30 µL of this suspension was transferred to each well of the polystyrene plates containing 100 µL of LB^{wo}/NaCl (3 parallels of each strain). The plates were incubated statically at 20.0 ± 1.0 °C for 48 h. After incubation, the plates were washed twice with sterile distilled water (SDW). The plates were dried at room temperature before being fixed with 130 µL of 98% methanol for 10 min. After incubation, methanol was removed and the wells were stained with 0.1% crystal violet. Following incubation at room temperature for 10 min, the plates were washed 3 times with SDW. Finally, optical densities (OD_{595}) were measured after the bound dye was dissolved by the addition of 33% glacial acetic acid. The results were calculated by subtracting the median OD_{595} of the triplicates of the control (test broth only) from the median OD_{595} of the triplicates of the sample.

2.4. Determination of the effects of *marT* gene on biofilm production

In order to determine the effect of the *marT* gene on the production of biofilm in *S.* Typhimurium, induction of the gene was carried out with the addition of arabinose. For this purpose, different concentrations of L-arabinose (0.02%, 0.01%, 0.005%, 0.0025%, 0.00125%, and 0.000625%) were added to the growth media of both wild-type *S.* Typhimurium 14028 and MZ1627 mutant strains, and these were incubated at 37 °C for 30 min with shaking at 200 rpm. Following incubation, wild-type *S.* Typhimurium 14028 and MZ1627 mutant strains were evaluated in terms of biofilm formation abilities, as described above.

2.5. Construction of mutant strains

Mutations in the *dps*, *yliH*, *wzzB*, *misL*, *slsA*, *fidL*, *rmbA*, *cheM*, *cheA*, *fliA*, *fliC*, and *fimA* genes were constructed using a method based on the Red/ET recombination system, according to the protocol recommended by the technical manual of the Quick and Easy *E. coli* Deletion Kit (Gene Bridges). Forward and reverse primers for the target genes were designed with the Primer3 program (http://frodo.wi.mit.edu) to provide 50 bp of upstream and downstream sequence homology. The 3′ ends of each primer included a sequence that was complementary to the DNA template provided with the kit, which contained a PGK-gb2-neo cassette flanked by FRT recombination sites. The sequences of the designed primers are shown in Table 2. After the generation of PCR products that contained the target genes and flanking sequences, the second step in the procedure was the transformation of expression plasmid pRedET into the *S.* Typhimurium 14028 strain. The expression of genes mediating Red/ET was induced with the addition of L-arabinose. After induction, the cells were prepared for electroporation, and the PCR product carrying the homology arms was electroporated into *S.* Typhimurium 14028 strain containing induced pRedET. Subsequently, clones of interest were selected on LB agar plates containing 15 µg/mL kanamycin. The obtained colonies were analyzed by colony PCR. This protocol is designed to quickly screen for plasmid insets directly from colonies. PCR amplifications were performed in a thermocycler (Techne TC-512) in 0.2-mL reaction tubes, each with 50 µL of reaction mixtures composed of 0.4 µM primer, 0.5 mM dNTP mix (Fermentas, Finland), 1X reaction buffer, 1.5 mM $MgCl_2$, 0.05 U of Taq Polymerase (Promega, USA), and one colony (picked with sterile toothpick) containing genomic DNA used as a template for PCR. PCR amplifications were performed as follows: 98 °C for 5 min (denaturation procedure, also lyses the bacterial cells), 60 °C (changes according to the primers used) for 30 s, 72 °C for 90 s, and a final elongation procedure at 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel electrophoresis, stained in ethidium bromide solution, and visualized under UV light.

2.6. Biofilm in liquid–air interface

All strains were cultured in LB at 37 °C for 18 h at 200 rpm. In order to study biofilm formation in liquid, i.e. pellicle formation at the liquid–air interface, 4.5 mL of LB^{wo}/NaCl was inoculated with 0.5 mL of an overnight culture, and incubated statically at 20 °C for 8 days. At the end of the incubation period, the strains were visually examined and categorized according to the presence of pellicles. Pellicles were also evaluated according to their appearance, as to whether they showed elastic, brittle, and rigid features.

2.7. Adhesion assays

The adhesion characteristics of wild-type and mutant strains were investigated using stainless steel coupons (type 304, 25 × 8 × 1 mm), glass slides (18 mm; Deckglaser cover glasses), and 24-well polystyrene microtiter plates. Surfaces of stainless steel and glass were chosen since these materials are widely used in the food industry. Stainless steel coupons were soaked in acetone for 30 min to remove grease and were rinsed in water before being soaked in 1 N NaOH for at least 1 h. The stainless steel coupons were subsequently rinsed in distilled water. All materials, glass slides, and stainless steel coupons were sterilized by autoclaving at 121 °C for 15 min (Chia et al., 2009). Stainless steel coupons were placed into 3.5 mL of LB^{wo}/NaCl broth, and glass slides were placed into 4 mL of LB^{wo}/NaCl broth media. Overnight cultures of wild-type 14028 strain were

Table 2. Primer sequences containing the homology sites of target genes and kanamycin cassette.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
fimA-f	GACGCTGGGTCAATACCGTACCGCCAGCTTTACGGCGA TTGGTAATACGAAATTAACCCTCACTAAAGGGCGG	fimA-r	CACTTTCGGATCGCAGTCATTCAGGACGATGGAGAAA GGCACCTGCGCAGTACGACTCACTATAGGGCTCG
rmbA-f	TTGCAATGGGCTGACAGTCTGGCAGACAGTGGCATGCA TATTGTCCTGATAAT TAACCCTCACTAAAGGGCGG	rmbA-r	TGCTTTTCAAAATCCAGTAATTGGCCAGCGGAGTTAAA CTTCTGTCAGAA TACGACTCACTATAGGGCTCG
fidL-f	CGCACTGGGGTGGTTTCCATCAGCGGAACATATTACG TCGATAATAAAATGAA TTAACCCTCACTAAAGGGCGG	fidL-r	TCTTTATTTTCACTCCATACATAGGAAACATCACGGCGG ATCACGCCGCTTAC GACTCACTATAGGGCTCG
slsA-f	GCTGATTCCGGAAATCCATGCGAATGCGCCCCACGCG CAATATGTCGCGC AATTAACCCTCACTAAAGGGCGG	slsA-r	TACCGCTTGTACAAAATCGGCATTATCCCAGGCATTAA TTTCCCCTTTACTACG ACTCACTATAGGGCTCG
MisL-f	CGTTGATTCGTCTGGGTGACGATGCAACTATCGAAAC ATCCGGCGCATCCAATTAACCCTCACTAAAGGGCGG	MisL-r	TTGTTGCCAAGCTCGGTTCTGGAGGAGGATGCTGCGT AAATCCCCGAAGA TACGACTCACTATAGGGCTCG
fliA-f	GCGGTCGACCGATATGACGCTTTGCAAGGAACGGC ATTTACCACTTACGC AATTAACCCTCACTAAAGGGCGG	fliA-r	AATCGCGGCTGCGTAATTCATCCAGCATCGCCCCACG AATACGCTGCACTTACGACTCACTATAGGGCTCG
fliC-f	ATCGACCGTGTATCCGGCCAGACTCAGTTCAACGGC GTGAAAGTCCTGGC AATTAACCCTCACTAAAGGGCGG	fliC-r	TAGTTTCACCGTCGTTGGCACCAACCTGGATGGTCA GGGTGTTGTCCTGCTAC GACTCACT
dps-f	ATGCGCGGTGCTAACTTTATTGCCGTTCATGAGATGCT GGATGGCTTCCG AATTAACCCTCACTAAAGGGCGG	dps-r	GCTGTACGGCGCGCTCGGCCATAGTATCCAGATGAT CGGTCAGTGCGGTATAC GACTCACTATAGGGCTCG
YliH-f	TGTCAGTCAGCGAAAGTGACACTCTGCGTGACAA TTTTTTTGAACTGAAT AATTAACCCTCACTAAAGGGCGG	YliH-r	TCCTGATCGAGATGCAGTCCCTGCCGTAAAGCGTGAT CGTGGAGCTCGCGTAC GACTCACTATAGGGCTCG
CheA-f	GCAATCTGGCTACTCTGACGGATGTCGTTAAGGGGGCGG ATTCACTGTCG AATTAACCCTCACTAAAGGGCGG	CheA-r	CAGAGCACCGCGACAATGTCATCTTCCGCGACGCT GCCATCCAGAGTTGCTAC GACTCACTATAGGGCTCG
CheM-f	TGTACCGCCAGACATTTGATCAAAGTGCTCATGACTACCG TTTTGCGCAA AATTAACCCTCACTAAAGGGCGG	CheM-r	ACCACCATCAAAATCAGCACCAGCACGACCGCAAG AACCCCCAGTTGCCATA CGACTCACTATAGGGCTCG
WzzB-f	CCGCTTTAGCTCTGCGTTTTCCGCATTATCGGAAGTGCT GGATAATCAGAAATTAA CCCTCACTAAAGGGCGG	WzzB-r	CAGCGCCTGCCCTTTTACCGACTGTTCAATGGTAAG CTTTTCCCGCTCTTTAATACGACTCACTATAGGGCTCG
*Kan-f	GATCGGCCATTGAACAAGAT	*Kan-r	TCGTCCTGCAGTTCATTCAG

*Kanamycin cassette-specific primer pairs designed to investigate the existence of kanamycin gene cassette for checking the accuracy of mutations.

diluted in LB^{wo}/NaCl to OD_{595} = 0.4, and the *marT* mutant strain (MZ1627) was diluted in LB^{wo}/NaCl to OD_{595} = 0.2. The promoter region of the mutant strain was induced with L-arabinose for 30 min, and OD_{595} was adjusted to 0.4. This suspension was transferred to three different media: 1) 1 mL was transferred to the test tubes containing stainless steel coupons, 2) 1.2 mL to the media with glass slides, and 3) 240 µL to each well of the polystyrene plate containing 800 µL of LB^{wo}/NaCl (3 parallels of each strain). Then all media were incubated statically at 20.0 ± 1.0 °C for 48 h. After 2 days of incubation, the stainless steel coupons and glass slides were removed aseptically and rinsed with gentle dipping into phosphate-buffered saline (PBS; Sigma) in order to eliminate the unattached cells. Sessile cells were detached from surfaces in a sterile test tube containing 3 mL of sterile PBS with a 20-kHz Vibra cell sonicator (VCX130, USA) for 3 min (Speranza et al., 2011). Viable and cultivable cells were enumerated by serial dilutions in PBS solution, and 10 µL of these suspensions was plated on LB agar and incubated at 37 °C for 24 h (Herigstad et al., 2001). The results of all experiments were evaluated as surface area (cm^2) per colony forming units (CFU) and presented as mean log CFU/cm^2.

2.8. Statistical analysis

The data analysis was performed using SPSS 13.0 (SPSS Inc., USA). Differences in the mean values among the groups were analyzed by one-way ANOVA, independent t-test, Mann–Whitney U-test, and Tukey test. All results were deemed significant if the P-value was below 0.05, the adopted level of significance.

3. Results

3.1. The effect of *marT* gene on biofilm production of *S. Typhimurium*

Our main goal in the determination of the incubation period for biofilm production was to detect the optimum time of biological activity of MarT protein induced by L-arabinose. After 72 h of incubation at pH 7.0 and 20 °C, the most appropriate time for maximum biofilm production was identified as 48 h. The highest differentiation in the means of biofilm production between the wild type 14028 strain and mutant MZ1627 strain arose after 48 h of incubation. By elongating incubation time to 72 h, the difference in biofilm production level was eliminated (Figure 1).

The induction of *marT* gene expression with 0.02% arabinose caused a decrease in the level of biofilm production at the end of hours 24 and 48. At these times, the determination of a statistically significant decrease in the biofilm production of the mutant strain compared to the wild type proved the negative regulation effect of the *marT* gene on biofilm production of *S.* Typhimurium. In order to reveal the direct effect of MarT protein on biofilm production, different concentrations of L-arabinose (starting with 0.02% arabinose and diluted by 1/2, 1/4, 1/8, 1/16, and 1/32, respectively) were added to the growth medium of both wild-type and mutant strains.

In these trials, due to the increased expression of *marT* depending on increased arabinose concentration, biofilm production was lowered in the mutant strain (MZ1627). Compared to the wild-type strain, a statistically significant reduction was found with the 0.01% and 0.02% concentrations of arabinose at a rate of 25% and 18.3%, respectively. On the other hand, no significant difference was found in the biofilm production of wild-type *S.* Typhimurium 14028 strain at any of the tested arabinose concentrations (Figure 2). All these data proved that the negative regulatory effect of the *marT* gene on biofilm production in *S.* Typhimurium is directly correlated with the level of expression of the gene in question.

3.2. Identification of genes responsible for biofilm production

To identify genes associated with biofilm formation, 12 genes whose expressions were regulated by the *marT* expression were chosen according to the microarray data. For this purpose, the phage lambda-based homologous recombination system site directed mutagenesis was created in 12 selected genes (*wzzB*, *fimA*, *rmbA*, *fidL*, *slsA*, *misL*, *fliA*, *fliC*, *dps*, *yliH*, *cheA*, and *cheM*) by introducing a neo cassette into these genes of *S.* Typhimurium (14028). Recombinations were confirmed by colony PCR. In this study, wild-type *S.* Typhimurium 14028 and the target gene mutants, disrupted by insertion of the kanamycin cassette, were compared in terms of biofilm formation properties on the polystyrene surface in order to identify the genes that affect the biofilm production level (Figure 3).

Based on the findings of this study, which were subjected to Tukey's multiple comparison test, the decrease in biofilm production of *fimA*, *fidL*, *misL*, *fliA*, and *fliC* gene mutants ($P < 0.05$) was found to be statistically significant.

Compared to the wild-type 14028 strain, the reductions in biofilm production that occurred as a result of mutations were calculated according to the formula given below (Figure 4).

$$(OD_{Wt} - OD_{Mutant}) / (OD_{Wt}) \times 100\%$$

The most powerful effect on the potential biofilm formation of mutations was seen on *fliA* (59%) and *fimA* (46%) mutant strains, while *fliC* (40%), *fidL* (25%),

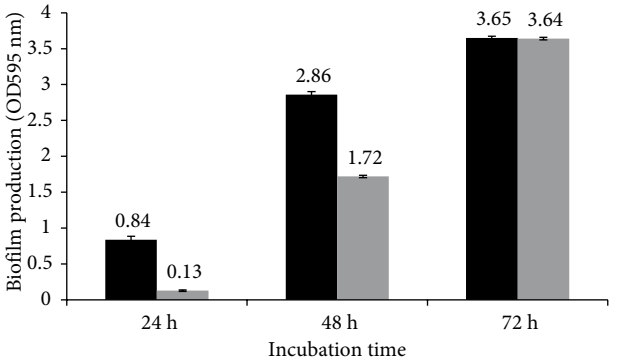

Figure 1. Biofilm production in an incubation-dependent manner in wild-type *S.* Typhimurium (14028) and mutant (MZ1627) strains (pH 7.0, incubation temperature 20 °C). Bars indicate standard error of the average. ■ 14028, ■ MZ1627.

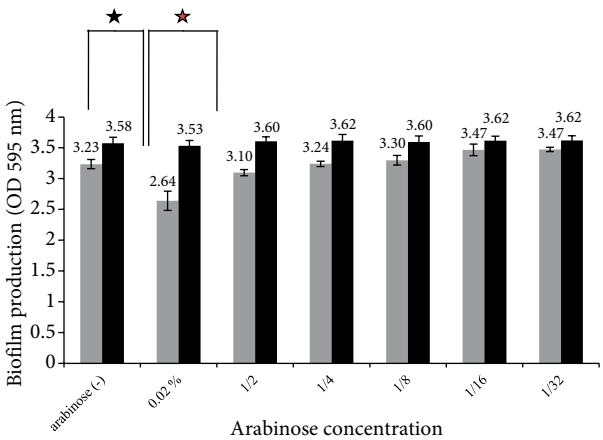

Figure 2. Biofilm production levels of *S.* Typhimurium 14028 and MZ1627 strains at different levels of MarT expression depending on the different arabinose induction. Stars indicate the statistically significant results of arabinose concentrations. Bars indicate standard error of the averages. ■ 14028, ■ MZ1627.

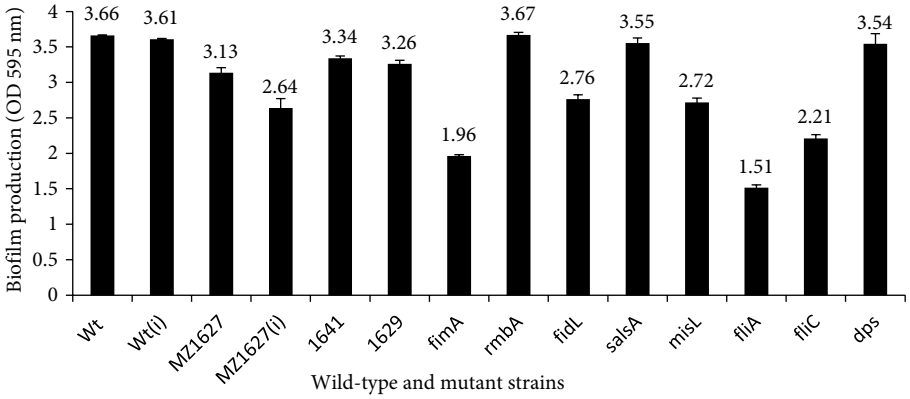

Figure 3. Effect of gene mutations on biofilm production. "i" indicates the *marT* expression by arabinose induction, "Wt" indicates wild-type (14028) strain, and bars indicate standard error of the average.

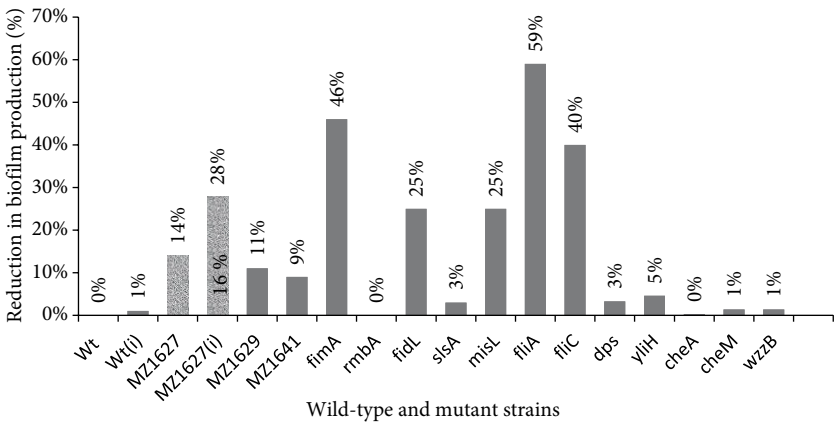

Figure 4. Reduction % in the biofilm production of mutant strains compared to wild-type 14028 strain.

and *misL* (25%) mutant strains followed. These findings indicate that *fliC*, *fimA*, *fliA*, *fidL*, and *misL* genes have an effect on biofilm production in *S.* Typhimurium that is independent of the induction of the *marT* gene expression.

3.3. Pellicle formation

All strains were evaluated according to their pellicle-forming abilities. Media turbidity, pellet at the bottom of the test tube, formation of ring structure between liquid–air interface, and physical features of pellicle (rigid, fragile, and elastic) were also visually evaluated after 8 days of incubation. Because all wild-type and mutant strains showed the rdar morphotype, they were expected to create an extremely tight pellicle structure in the liquid–air interphase. Contrary to this expectation, pellicle structure was not formed in some mutants [Δ1627(*i*), Δ*fimA*, Δ*slsA*, Δ*fliA*, Δ*dps,* Δ*cheA*, Δ*wzzB*)], and in others the tight pellicle structure was determined as a characteristic of the rdar morphotype (Wt, Δ*rmbA*, Δ*fidL*, Δ*misL*, ΔfliC, Δ*yliH*, Δ*cheM*) (Table 3).

3.4. Effect of *marT* gene induction on biofilm formation ability on different abiotic surfaces

Before and after the arabinose induction of wild-type *S.* Typhimurium 14028 and *marT* mutant MZ1627 strains, biofilm production abilities on stainless steel, glass, and polystyrene surfaces were investigated. Biofilm production was calculated as CFU/cm^2 on abiotic surfaces. This method assumes that each colony grows from a living bacterial cell. Biofilm production graphics were evaluated using the logarithmic values of CFUs and by comparison to each other (Figure 5).

The findings from this study have shown that the highest biofilm formation was carried out on polystyrene surface, followed by glass and stainless steel. Mutant MZ1627 strain showed lower biofilm production than the wild type.

Table 3. Pellicle formation characteristics of wild-type and mutant strains at liquid–air interphase.

Strains	Ring formation at liquid–air interphase	Pellicle formation at liquid–air interphase	Turbidity	Pellet at the bottom
Wt	(++)	Rigid	Clear	None
Δ*1627 (i)*	(+)	None	Cloudy	(++)
Δ*fimA*	(++)	None	Cloudy	(++)
Δ*rmbA*	(++)	Rigid	Clear	None
Δ*fidL*	(++)	Rigid	Clear	None
Δ*slsA*	(+)	None	Cloudy	(+)
Δm*isL*	(++)	Rigid	Clear	None
Δ*fliA*	(++)	None	Cloudy	(++)
Δ*fliC*	(++)	Rigid	Clear	(+)
Δ*dps*	(+)	None	Cloudy	(++)
Δ*yliH*	(+)	Rigid	Clear	None
Δ*cheA*	(+)	None	Cloudy	(++)
Δ*cheM*	(++)	Rigid	Clear	None
Δw*zzB*	(+)	None	Cloudy	(++)

(+): Thin ring formation/pellet formation.
(++): Thick ring formation/pellet formation.

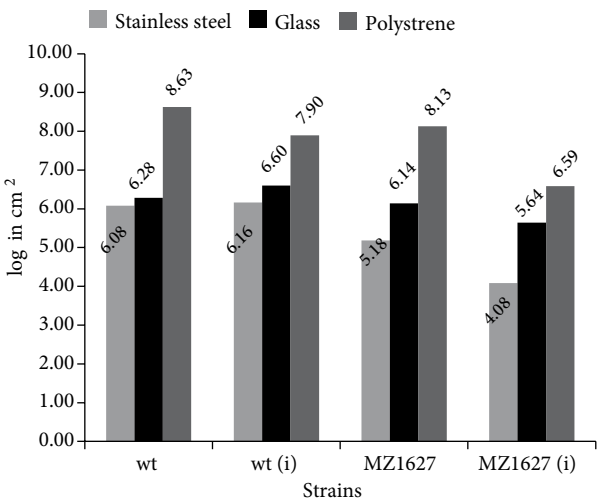

Figure 5. Comparison of biofilm adhesion to stainless steel, glass, and polystyrene surfaces. Biofilm formation properties of wild-type strain and *marT* expression-induced strain on glass, steel, and polystyrene were investigated, and a decrease in the production of biofilms on all surfaces of the *marT* expression-induced strain was determined. Stainless steel, glass, polystyrene.

4. Discussion

In this study, the effect of MarT regulator protein (and hence of the *marT* gene) on biofilm production in *S.* Typhimurium was determined. MarT is known as a regulatory protein in *S.* Typhimurium, which is not produced (or because of its low production, cannot be identified in western blot systems) under in vitro culture conditions; however, after the entry of the bacteria into the host system (in vivo conditions), its production is induced. It is also known to be involved in the positive transcriptional regulation of MisL autotransporter protein (Tükel et al., 2007). Because biofilm studies were carried out under in vitro conditions, the effect of MarT regulator protein was investigated in a mutant strain (*S.* Typhimurium MZ1627), in which an arabinose inducible promoter was recombined to the downstream of a pBAD plasmid. Under in vitro culture conditions, a statistically significant decrease in biofilm production compared to the wild type was determined with the two consecutive arabinose concentrations (0.02% and 0.01%) at which the production of MarT regulator protein was encouraged at the highest level (Tükel et al., 2007). Furthermore, the biofilm production capacity of the mutant strain (MZ1627) increased directly and proportionally to the reduction

in the concentration of arabinose (this also refers to the decline in the production of MarT protein) in the growth medium. In addition, the level of biofilm production of the mutant strain at the lowest concentration of arabinose was found to be very close to that of the wild-type strain. These data prove that biofilm production is negatively regulated by the *marT* gene in *S.* Typhimurium.

A total of 80% of all bacterial infections are caused by biofilm structures (de la Fuente-Núñez et al., 2012). Today, biofilms that are accepted as the predominant form of bacterial growth constitute the most complex examples of extracellular and intercellular organization. These properties of biofilms make them the focus of studies on the eradication of bacterial infections and the regulation of complex system networks. In studies conducted on the regulation of biofilm production in *Salmonella*, many of the functional genes, depending on internal or external circumstances, are found effective on this property. Evidence of participation of the *marT* gene in the negative regulation of the biofilm production in *S.* Typhimurium contributes to the field of science by being the first data in this area.

With the purpose of determining the effects of the genes investigated in terms of *marT* regulation under in vitro conditions on biofilm production, deteriorating mutants were obtained by inserting a kanamycin cassette into each gene, and the biofilm reproduction capabilities of these mutants were then compared to the wild-type strain. As a result of this experiment, statistically significant reductions compared to the wild type in biofilm production capacity were determined in *fliA* (59%), *fliC* (40%), *fimA* (46%), *fidL* (25%), and *misL* (25%) gene mutant strains, and also in the MZ1627 (16%) mutant strain, in which MarT expression was provided in the presence of arabinose. The decrease in the biofilm production capacity of *misL* and *fidL* gene mutants under in vitro conditions was a very interesting finding. So far, the presence of *marT* and *fidL* gene transcripts has been determined under in vitro conditions only in one study, and no study has performed the translation of the transcripts (Vandenbroucke et al., 2011). On the other hand, by using the recombinase-based in vivo expression technology, MisL autotransporter protein expression was shown to have a negative impact on biofilm production in *S.* Typhimurium (Kroupitski et al., 2013). These findings are in full compliance with our data, suggesting that biofilm production was prevented as a result of high expression level of the *marT* gene under in vitro conditions.

MarT is the primary transcriptional activator of the MisL autotransporter protein. However, the results obtained from mutational studies and the findings of Vandenbroucke et al. (2011) indicate that MisL and FidL proteins, albeit at low rates, are produced under in vitro conditions. Unlike under in vivo conditions, they contribute to the formation of biofilm even at this level (Vandenbroucke et al., 2011). Any variable effects of MisL and FidL proteins resulting from in vivo/in vitro phase differences on the biofilm production in *S.* Typhimurium will be understood by simply performing a detailed functional analysis of these genes.

Observation of high reduction in *fliA*, *fliC*, and *fimA* mutant strains (59%, 40%, and 46%, respectively) in terms of biofilm production on plastic surfaces compared to the wild-type strain proves the significant promotion of biofilm formation by these genes under in vitro conditions. To this day, the most detailed study on the effects of flagellin on the biofilm formation in *S.* Typhimurium showed that the major flagella filament protein (FliC), produced under in vitro and in vivo conditions, plays a role in the adhesion of *S.* Typhimurium cells to cholesterol and in the early-phase production of biofilm as an inducer; its inhibitory effect during biofilm development was also determined. This was explained by the *fliC* gene expression being stimulated during the initial stages of biofilm formation (biofilm adhesion) and suppressed at the stage of biofilm development (Crawford et al., 2010). In our study, a reduction in the biofilm production ability of the impaired *fliC* gene mutants confirmed this hypothesis. As a result of *fliC* (directly) or *fliA* gene degradation (indirectly), biofilm adhesion of these mutants occurs at low levels. *fliA* gene degradation affects biofilm formation indirectly by inhibiting the transcription of the *fliC* gene, because *fliA* encodes for sigma factor (sigma S), which recognizes the *fliC* promoter.

Determining the decrease in the capacity of biofilm production to 16% in the MZ1627 strain, which expresses MarT at a high level with the encouragement of arabinose, suggested that this reduction may be mediated by the cumulative effect of the negatively regulated *fliA*, *fliC*, and *fimA* genes. Thus, a decrease in the biofilm production capacity of these three mutants was also observed in our study.

In a previous study, the effect of chemotactic movement of *E. coli* besides flagellar movement on in vitro biofilm production was investigated. It was found that there was no difference between the *CheA-Z* mutant (motile but nonchemotactic) and wild-type strain in terms of biofilm production ability (Pratt and Kolter, 1998). The fact that *CheA* and *CheM* mutants had no impact on the biofilm production ability in our study supports the data.

The *fimA* gene encodes for the main subassembly of *Salmonella* type-1 fimbria. Type-1 fimbria plays an active role, along with other adhesins, in the adhesion of *Salmonella* cells, especially to biotic surfaces (Muller et al., 1991; Steenackers et al., 2012). Very few studies on the effect of type-1 fimbria on the biofilm production of *Salmonella* are available. Two studies, carried out on the

structural gene mutants of type-1 fimbriae, found that in vivo biofilm production was significantly inhibited, although the same condition has not been observed with in vitro production (Steenackers et al., 2012; Gonzalez-Escobedo and Gunn, 2013). Contrary to the findings in the literature, in our study the *fimA* gene, and therefore the *Salmonella* type-1 fimbriae, were found to promote the formation of biofilm on plastic (polystyrene) surfaces. The only research data that support these findings were found in the study of Teplitski et al. (2006). They reported that type-1 fimbriae promoted biofilm formation on plastic surfaces, and the biofilm formation capacity of the mutant strains in terms of type-1 gene dropped significantly.

In *Salmonella*, the examination of the pellicle structures is another test used for the macroscopic evaluation of biofilm structures. Composite pellicle structures resistant to physical-solving procedures (such as mixing) are created by biofilm-producing strains with rdar morphotype at the liquid–air interface in a biotic and abiotic environment. This enables the growth of anaerobic and microaerophilic bacteria in biofilm and thus provides advantageous conditions for the development of biofilms. It was determined that the occurrence of rigid pellicle structure requires a regulation of the functionality of the genes that are effective on the biofilm formation (Stepanović et al., 2004; Latasa et al., 2005; Møretrø et al., 2009; Vestby et al., 2009). In the trials investigating the effect of created mutations on the pellicle structure, no change in the properties of the pellicle structure was determined in *rmbA*, *fidL*, *misL*, *yliH*, *fliC*, *yliH*, and *cheM* gene mutants when compared to the wild type. However, it has been determined that the pellicle was not formed in the *fimA*, *slsA*, *fliA*, *dps*, *cheA*, and *wzzB* gene mutants. This is the first study indicating the direct effect of *fimA*, *slsA*, *fliA*, *dps*, *cheA*, and *wzzB* genes on pellicle formation in *S.* Typhimurium. These findings are significant in terms of their potential, which will allow for the development of new strategies in the eradication of generated biofilms at the liquid–air interface.

Finally, biofilm formation properties of the wild-type strain and *marT* expression-induced strain on glass, steel, and polystyrene have been investigated, and a decrease in the production of biofilms on all the surfaces of the *marT* expression-induced strain has been determined.

In conclusion, the present study has revealed that the *marT* gene decreases biofilm production in *S.* Typhimurium when optimal expression conditions are provided. In other words, MarT protein is one of the regulators that affect biofilm production negatively. In addition to the *marT* gene, *fliA*, *fliC*, *fimA*, *fidL*, and *misL* genes also contribute to the production of biofilm under in vitro conditions.

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