marT AS A GLOBAL REGULATORY GENE FOR BIOFILM FORMATION IN *SALMONELLA TYPHIMURIUM*

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ABSTRACT

marT AS A GLOBAL REGULATORY GENE FOR BIOFILM FORMATION IN *SALMONELLA TYPHIMURIUM*

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In this study 14 different genes (*fimA, fimD, fimF, fimH, stjB, stjC, csgA, csgD, ompC, sthB, sthE, rmbA, fliZ* ve *yaiC*) thought to be related to *marT* gene at *Salmonella enterica* serotype Typhimurium 14028 and each involved in the biofilm formation and besides this, MZ1627, a derivative of the same strain that is disrupted in terms of *marT* gene was examined according to its regulation characteristics with *marT*. For the comparative analysis of the target genes, each of the gene was mutated through antibiotic gene cassette insertion separately, as repeating the same process to test the autoregulation function of *marT* gene, 15 different mutant genes in total were obtained. Then, each of these strain's expression level was determined in the presence and absence of the *marT* induction by transforming recombinant plasmid (pBAD24+*marT*) into these strains where *marT* gene's expression is controlled by cloning it into the arabinose-induced BAD promoter. According to QRT-PCR results, *marT* gene has an important role in terms of regulation of the examined 14 genes. Again these results indicated that, the protein encoded by *marT* gene is at the same time an autoregulator which organize its own promoter positively, also in general, it suggested that, MarT protein not only regulates the expression of the misL gene encoding an autotransporter protein, but also acts as a global regulator in

Salmonella. In this study, strains carrying mutant genes subjected to MarT regulation, compared to parental strain 14028, have also been investigated for their biofilm-forming capacity on polystyrene surfaces. As a result of 24 and 48 hours of incubation, it was observed that there was a statistically significant ($p < 0.05$) decrease in the biofilm formation capacities of each mutant strain. It has been found that *fliZ, ompC, rmbA, stjB* and *stjC* genes are directly related to biofilm formation in *Salmonella* and that there is a serious decrease in biofilm formation in their absence. In the next step, the attachment properties of the mutant strains to the cell surface compared to the parental strain were examined and Caco-2 and HEp-2 cells were used for this. According to the results, single gene mutations are not sufficient for an effective attachment.

One of the most important findings of this study is that the global regulator MarT also controls *csgD* expression, which is the main regulatory gene for biofilm formation. In our "post antibiotics" era caused by the increasing antibiotic resistance crisis, it is thought that the development of chemical inhibitors specific to MarT will play a pioneering role in finding new solutions targeting biofilm formation in *Salmonella* Typhimurium infections.

Keywords: Adhesion, Biofilm formation, Regulation, *marT, Salmonella Typhimurium*

ÖZ

SALMONELLA TYPHIMURIUM'DA BİYOFİLM OLUŞUMU İÇİN YENİ BİR REGÜLATÖR GEN: *marT*

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Bu çalışmada, *Salmonella enterica* serotip Typhimurium 14028'de *marT* geni ile ilgili olabileceği düşünülen ve her biri biyofilm oluşumunda rol alan 14 farklı gen (*fimA, fimD, fimF, fimH, stjB, stjC, csgA, csgD, ompC, sthB, sthE, rmbA, fliZ* ve *yaiC*) ve bunların yanısıra aynı suşun *marT* geni bakımından bozulmuş olan bir türevi olan MZ1627 suşu *marT* ile regülasyon özelliklerine göre incelenmiştir. Adı geçen aday genlerin karşılaştırmalı analizleri için herbir gen antibiyotik kaset mutajenezi (insersiyonu) yoluyla ayrı ayrı mutasyona uğratılmış, aynı işlem, otoregülatör fonksiyonun test edilmesi amacıyla *marT* geni için de tekrarlanarak toplam 15 farklı mutant suş elde edilmiştir. Daha sonra bu suşların her biri *marT* geninin arabinoz ile indüklenen BAD promotoru altına klonlanarak ekspresyonunun kontrol altına alındığı rekombinant plasmid (pBAD24+*marT*) ile transforme edilerek her bir tekli genin ekspresyon seviyesinin *marT* indüksiyonu varlığında ve yokluğunda nasıl değiştiği belirlenmiştir.

QRT-PCR sonuçlarına göre, *marT* geni, incelenen 14 genin regülasyonu açısından önemli bir role sahiptir. Yine bu sonuçlar, *marT* geni tarafından kodlanan proteinin aynı zamanda kendi promotorunu da pozitif olarak düzenleyen bir oto-regülatör

olduğuna işaret etmiş, genel anlamda da MarT proteininin sadece ototransporter bir protein kodlayan *misL* gen ekspresyonunu düzenlemekle kalmayıp aynı zamanda *Salmonella*'da global bir regülatör görevi gördüğünü düşündürmüştür.Bu çalışmada, MarT regülasyonuna tabi tutulan mutant genleri taşıyan suşlar, parental suş 14028'e kıyasla, polistiren yüzeylerde biyofilm oluşturma kapasiteleri de incelenmiştir.

24 ve 48 saatlik inkübasyonlar sonucunda, her bir mutant suşa ait biyofilm oluşum kapasitelerinde istatistiksel açıdan ($p< 0.05$) dikkate değer bir düşüş olduğu gözlenmiştir. *fliZ, ompC, rmbA, stjB* ve *stjC* genlerinin *Salmonella*'da biyofilm oluşumuyla doğrudan ilişkili oldukları ve yokluklarında biyofilm oluşumunda ciddi bir düşüş olduğu anlaşılmıştır. Bir sonraki aşamada mutant suşların parental suşa kıyasla hücre yüzeylerine tutunma özellikleri incelenmiş ve bunun için Caco-2 ve HEp-2 hücreleri kullanılmıştır. Elde edilen sonuçlara gore, tutunmanın etkili bir şekilde gerçekleşmesi için tek gen mutasyonları yeterli olmamaktadır.

Global regülatör MarT'nin, biyofilm oluşumu için başlıca düzenleyici gen olan *csgD* ekspresyonunu da kontrol etmesi, bu çalışmanın en önemli bulgularından birisidir. Giderek artan antibiyotik dirençliliği krizinin neden olduğu "antibiyotikler sonrası" çağımızda, MarT'ye spesifik kimyasal inhibitörlerin geliştirilmesiyle *Salmonella* Typhimurium enfeksiyonları özelinde biyofilm oluşumunu hedef alan yeni çözümler bulunması yönünde öncü bir rol oynayacağı düşünülmektedir.

Anahtar Kelimeler: Adhezyon, Biyofilm oluşumu, Regülasyon, *marT, Salmonella Typhimurium*

To my family,

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CHAPTER 1

1 INTRODUCTION

Biofilms are the microorganismal communities that show multicellular-like behaviour by attaching to surfaces (Mah and O'Toole, 2001). The microorganisms which produce biofilm are embedded in extracellular polymeric matrix (EPS) that they produce. EPS generally consists of polysaccharides, proteins, nucleic acids and lipids. EPS also generates the mechanic stability of biofilm, the sticky form, attachment of the surface and the three dimensional polymer networks which provide the contact between the cells (Flemming and Winender, 2010). The function of the biofilms is to protect the microorganisms against the external factors and/or to collect the nutrients. Biofilm is of great importance in food industry due to the negative effects on both the industrial manufacturing processes and health. The damage on medical and industrial tools, the loss of energy and product during the manufacturing processes and the stubborn infections caused by microbial biofilms are among the most important problems all over the world (Fujishige, 2006).

The food sourced *Salmonella enterica* type *Enterobacteriaceae* family members have the ability to form biofilm on biotic and abiotic surfaces (Pourty and Gunn 2003, Ledeboer and Jones 2005). Targeting the bacterial source of biofilms is a must to minimize the industrial problems, to control the infections caused by the genus *Salmonella* which has up to 2500 serotypes determined until today in humans and animals. Bacterial biofilms have higher level of resistance characteristics than their planktonic forms against environmental stress conditions and the bacterial struggled agents (Barhart and Chapman, 2006). The main functional components of extracellular polymeric matrix (EPS) in *Salmonella* biofilms are protein structured curly fimbria and cellulose. These components

together or one by one play a key role in surface attachment and cell clustering in biofilm (Olsen *et al*., 1993, Romling *et al*., 1998, Vival *et al*., 1998, Pringent-Combaret *et al*., 2001, Cookson *et al*., 2002). In *Salmonella*, the expression of curly fimbria has mutual regulation systems with cellulose production (Zoaj *et al*., 2001, Romling *et al*., 2002).

1.2 The general characteristics of the genus *Salmonella*

The genus *Salmonella*, as a member of *Enterobacteriaceae* family, is Gram negative, rod-shaped, facultative aerobic, and has three species, namely *Salmonella enterica*, *Salmonella bongari* and *Salmonella subterranean* (Su *et a*l., 2007). *S. enterica* which has the most widespread disease-causing agent with six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp.*indica* (Branner *et al*., 2000). These bacteria have high adaptation affinity some stress conditions like acidity, high temperature and low water activity, and usually grow in a wide temperature range of 7-48 ^{0}C (Kaufman 1998, White *et al*., 2006). *Salmonella enterica* subsp. *enterica* serovar Typhimurium is responsible for the majority of salmonellosis cases worldwide (Hernandez *et al*., 2012).

The major source of infections caused by intracellular pathogen *Salmonella* are meat and meat products, raw egg, unpasteurized milk and salad sauces. The unprocessed herbal products are also reported as an important contamination source. The symptoms caused by *Salmonella* infections which are generally described as “salmonellosis” include diarrhea, cramps, nausea, vomiting and fever. These symptoms may be local (bowel) or penetrating to lymph and bloodstream, the *Salmonella* capable of protecting themselves from the host’s immune system it may cause more serious symptoms. For instance, while many of the serotypes of *S. enterica* cause local gastroenteritis, (*S. Typhimurium, S. Enteridis, S. Virchow*…), *S. Typhi* causes a systemic infection in humans which is called typhoid fever. These

bacteria have ability to pass the epithelial tissue and enter into the macrophages, they can stay in macrophages, and then proliferate and spread to mesenteric lymph nodes, spleen and liver (Zhang *et al*., 2000).

The invasion of *Salmonella* shows huge differences from other pathogenic bacteria. After *Salmonella*-contaminated food is consumed by a person, the bacterium invades the dendritic cells and the enterocytes through colonizing on the epithelial barrier of intestine. The serotypes of *Salmonella* pass the barrier successfully with the help of their complex membrane structures, encounter the macrophages and replace with the M cells as secreting *Salmonella* pathogenicity island 1 (SPI-1) proteins coded by bacterial adhesion type three secretion system 1 (T3SS-1) or as using the fimbria, they invade the macrophages (Hurley *et al*., 2014). *Salmonella* sinks into macrophages, forming SCV (*Salmonella* containing vacuole) (Hurley *et al*., 2014). It changes SCV membrane contents such as lipids and proteins. That's how *Salmonella* can live on, reproduce and escape from the host's antimicrobial defence mechanism (Kolodziejek and Miller, 2005).

Many studies have indicated that *Salmonella* has the capability to attach to some surfaces like plastics, metals, etc and produce biofilm on these surfaces (Sinde and Carballo, 2000; Stepanovic *et al*., 2004). The biofilm formation is accepted as one of the most important features of the pathogen as it directly affects the development and durability.

There are some main steps in biofilm production in *Salmonella*. These steps include the attachment to the surface, the colony development, the biofilm maturation including the process of three-dimensional mushroom like structures formed by microcolonial cells and the disintegration of the biofilm. After dispersion of the cells, the organism finds new hosts and continues its life cycle (Stepanovic *et al*, 2004). The members of *Salmonella* species can maintain the vividness at the end of various disinfection treatments thanks to their biofilm formation characteristic (Ronner *et al*.,

1993, Moreto *et al.,* 2003). Although in the factories where food production takes place make large investments to eradicate the *Salmonella* contaminations, it was determined that some of the clones can maintain their presence for years in the industrial plant.

1.3 The general features of biofilm

Water composes the biggest part (97%) of the content of the biofilm matrix. The other components in the matrix are; 1-2% of exopolysaccharides, 1-2% of globular glycoproteins and other proteins, 1-2% of nucleic acid, lipid and phospholipids. These percentages can differ according to the organism's type, physical characteristics, nature of the growth environment, and the variety of the fluid (Allison, 2003). Biofilms protect bacteria from the environmental effects such as temperature, moisture, pH changes and the effect of ultraviolet rays. Storing up the nutrients and facilitating removal of the wastes are among other advantages of biofilms (Post *et al.,* 2004).

Besides being dense surfaces, biofilms do also have a granular nature where special water channels above and below the microcolonies distribute water and nutrients. The cellular waste material is also transported to the biofilm base with these channel systems. Passive diffusion is another mechanism for transport within the biofilms. In addition, the water channels transfer oxygen to the areas in biofilm (Davey and O'toole, 2000). In genome of *S.* Typhimurium, there are 12 distinct operons which are shown to be important in curly fimbria (csg) and biofilm formation. For instance, by using the HEp-2 cells and the chicken intestinal tissue, it was determined that the type 1 fimbria is required for the biofilm production and the attachment to the epithelial cell layers (Boddicker *et al.,* 2002).

The curly fimbria is highly important for biofilm formation by *Salmonella*. It plays a role in the first attachment to a surface and encourages the cell to cell interaction

(Barnhart and Chapman, 2006). The *Salmonella* biofilms have a high amount of cellulose in their extracellular matrix which also contains O-antigenic capsule (O-Ag capsule), fimbria subunits and lipopolysaccharides (Crawford *et al.,* 2010).

For biofilm formation in *Salmonella* spp., some other crucial structures include Fim, Lpf, Pef, Bcf, Csg (fimbria type 1, long polar fimbria, plasmid encoded fimbria, bovine colonization factor, curly fimbria, respectively). Besides this, colonic acid is also necessary for *Salmonella* type bacteria while producing biofilm on epithelial cells (Ledeboer and Jones, 2005; Ledeboer *et al.,* 2006).

1.4 The mechanism of biofilm formation

In nature, even the simplest biofilm layer might have a pretty complicated dynamic (O'Toole *et al*., 2000). The improvement of biofilm continues in the areas where nutrient flow shows continuity. However, when the nutrients in the environment are depleted the surface connections get weakened and the microbial cells turn back into their planktonic forms. The starvation facilitates transition to the planktonic to look for new nutrient sources, to become adapted to the environment and then spread (Kolter *et al*., 1993).

Biofilm forming process comprises five steps. These are; initial attachment, irreversible attachment, colony development, biofilm maturation and biofilm dispersal (O'Toole *et al*., 2000).

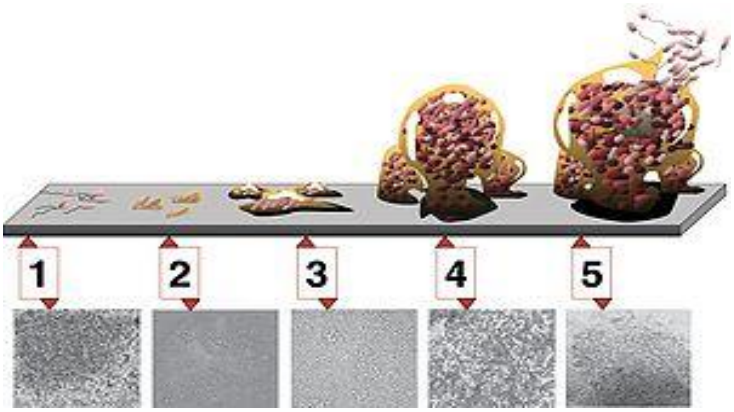


Figure1.1 Biofilm forming steps (Monroe, 2007). Initial attachment, 2. Irreversible attachment, 3. Colony development, 4. Biofilm maturation, 5. Biofilm dispersal

1.4.1 Initial attachment

In this phase, the bacterial cells do not contact with the surface. They are in close distance and a rapprochement takes place for which the structure of the surface (Donlan, 2002), the surface load (Abdallah *et al*., 2009), hydrophobicity (Donlan, 2002), pH, temperature (Nilsson *et al*., 2011) and the presence of nutrients (Grestel and Römlin, 2001, Donlan, 2002) play critical roles.

1.4.2 Irreversible attachment

In the irreversible attachment phase the dipole-dipole interactions which are short distance interactions with the surface, covalent bonds, hydrophobic and hydrogen interactions are formed.

Microorganisms can attach to surfaces irreversibly with their flagella, pili-like organelles and EPS. In this phase, the gathering of the planktonic bacteria on the surface happens as mediated by the signal molecules (quorum sensing) (Poulsen, 1999).

1.4.3 Colony development

The next phase of biofilm formation is the surface colonization. One attached bacterium starts to divide, thus forming a colony on a surface (the first colony), and other bacteria begin to form their own colonies on the same surface (secondary colonies). As the biofilm develops, an increase is seen in the mass of encapsulated microorganisms in their polymeric matrix (Poulsen, 1999).

1.4.4 Biofilm maturation

Microbial mass in biofilm can be transformed into mushroom or tower-like structures with the effect of nutrients (Chmielewski and Frank, 2003, Klausen *et al*., 2003). There are water channels which act like circulation system to deliver nutrients and remove the metabolic wastes between microcolonies (Kumar *et al*., 1998, Poulsen, 1999). For cells to form this structure, 10 days or more is needed (Stoodley *et al*., 2002).

1.4.5 Biofilm dispersal

In biofilm formation, the last phase is the detachment. Microorganisms in this phase return back to their planktonic state (Sauer *et al*.,2002).

The increased flow force (Stoodley *et al.*, 2002), internal enzymatic corruption, revelation of EPS or surface binding proteins-like processes are seen in the cell, playing a role in separation of biofilm cells (Kaplan *et al*., 2004). At the same time, the running out of the nutrients is also accepted as another reason for cell detachments (O'Toole *et al*., 2000). This detachment can either happen due to the external forces or rupturing of the cells (Poulsen, 1999).

1.5 The interaction of cells in the biofilm structures through signal molecules (Quorum Sensing; QS)

Biofilm forming is not a random event where bacteria only get together, attach to significant surface, then adhere there and maintain their lives together with the other species on that surface. Many organisms send signals to each other to coordinate their activities.

With the process called quorum sensing which is an important mechanism in biofilm formation, bacteria can measure the signal molecule density they produce, sense the amount of other microorganisms around them and enable to transfer these data to other bacteria (Camara, 2007). In another words, with QS, bacteria determine the bacterial population in their environment. As the amount of bacteria attaching to the surface increases, this signal's local concentration increases and with this increase, a number of processes point out the beginning of biofilm formation.

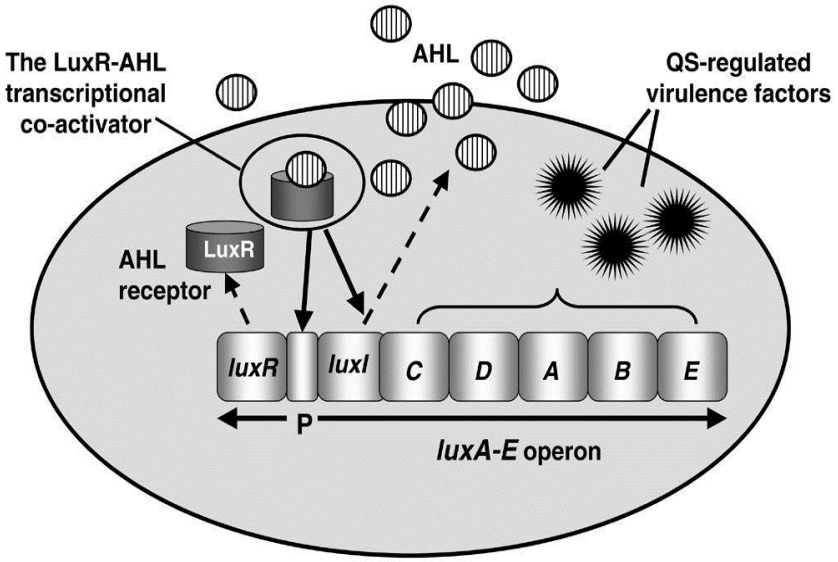


Figure 1.2. Bacterial connections with the signal molecules (Asad and Opal, 2008)

Quorum sensing (QS) also have some other important regulatory actions including antibiotic and pigment (secondary metabolites) biosynthesis, virulence factor

formation, sporulation, competence, cell separation and infections (March and Bentley, 2004). This mechanism providing cellular interaction is mediated by auto-inducer (AI) molecules (Asad and Opal, 2008).

QS take place in two ways as between species and inner species. Gram negative bacteria use N-acil homoserine lactose (AHL, AHLs, acyl-HSL or HSL), Gram positive bacteria mostly use oligopeptides as auto-inducers in QS mechanism (Donabedian, 2003; Wong, 2013). Some microorganisms use more than one type of QS molecules. Figure 1.2 illustrates the relation between AI formation and regulation of virulence factor biosynthesis in Gram-negative pathogens.

In addition, the ability of Gram-negative and Gram-positive bacteria to use auto-inducer-2 (AI-2's) signal molecules in QS systems is common. (Raffa, 2005). In studies conducted on QS systems, it has been determined that *Salmonella enterica* conducts intercellular communication with AI-2 signals. (Donabedian, 2003).

1.6 Regulation of biofilm formation

1.6.1 CsgD

Salmonella which is the gastrointestinal food origin pathogen has the ability to produce biofilm on different surfaces such as plants, tissues and organs of the animal host as well as industrial and medical equipments. CsgD regulates the transcription of *csgBAC* operon coding for the structural subunits of curly fimbria and indirectly, it contributes to the cellulose production with the activation of *adrA* operon transcription (Römling *et al*., 2000; Zakikhany *et al*., 2010). The AdrA protein is a diguanylate cyclase that binds to cellulose and synthesizes the secondary messenger circular diguanosine monophosphate (cyclic diguanosine monophosphate, c-di-GMP) that activates cellulose, and in the post-transcriptional stage it regulates the

bcsABZC operon where genes transcribed in cellulose biosynthesis mechanism as changing the concentration of c-di-GMP (Zogaj *et al*., 2001; Simm *et al.*, 2004).

CsgD is an integral part of the curly fimbria biosynthesis system, which is formed by differential transcription of the csgBAC and csgDEFG operons.

The high nucleotide and protein level of similarity in curly fimbria of *S*. Typhimurium and *E. coli* points to the evolution from a mutual ancestor (Römling *et al*, 1998). The comparative genetic analysis made in the zone between *csgD-csgB* genes showed that there is a high level of similarity in the members of *Salmonella* except for *S. bongori*. This situation is a sign that the changes in the zone between *CsgD-CsgB* had originated from the natural mutations after the genetic drift (Davidson, 2008).

There is a relation between the activation of *csgD* and STM2123 and STM3388 proteins (complex DEF/EAL domain). STM2123 is a component required for the activation of *csgD* at the beginning of biofilm formation. It was determined that STM3388 protein provides a positive contribution to biofilm production from the phase of maturation. In *S*. Typhimurium, proteins (STM1703, STM1827, STM3611 and STM4264) which include other four important EAL domains show activity in the expression of *csgD* similarly (Simm *et al.*, 2007). In some studies, conducted with the mutants of these proteins, a significant increase in the expression of *csgD* was found due to the increase in c-di-GMP at the cellular level. In this context, the view has arisen that cellular c-di-GMP levels can control different targets in the regulation of these proteins and biofilm formation. (Simm *et al.*, 2007).

This data emphasis that, ci-di-GMP has a very important role by taking part in curly fimbria and cellulose biosynthesis through *csgD* in the virulence and motility in biofilm production (Solano *et al.*, 2002).

1.6.2 BarA/SirA and Csr system

BarA/SirA system is a highly protected system in gama-proteobacteria (Teplitski *et al*., 2003, 2006). This system is a response regulator participated to SirA, a FixJ family protein in *Salmonella* which is phosphorylated by sensor kinase BarA (Altier *et al.*, 2000., Pernesti *et al.,* 2001) or cellular acetyl phosphate (Lawhon *et al*., 2002). SirA is responsible for the transcriptional activation of csrB and csrC sRNAs. Both sRNAs are the part of the Csr system in *Salmonella* and they are in an antagonistic interaction with the RNA binding protein CsrA.

Teplitski (2006) determined that the double mutants of *sirA, fimI, csrB* and *csrC* cannot perform biofilm production on plastic surfaces but flhDC mutants produce much more biofilm. This study revealed that SirA has a role on the expression of flagellar or type I fimbrial components which contribute to biofilm production in positive way in transcriptional level and Csr system has regulatory roles in posttranscriptional level. It was determined that SirA-P activates csrB and csrC, fim operon and hilA at the transcriptional level. Increasing level of *csrB/csrC* inhibits the activation of CsrA. Decreasing activity of CsrA encourages the production of biofilm by causing the reduction in expression of the proteins such as FlhDC and HilA which might then inhibit the biofilm production (Teplitski *et al.*, 2006). CsrA at the same time reduces the expression of *fim*. With the reduction of CsrA, the type I fimbria biosynthesis increases, thus biofilm production becomes enhanced.

1.6.3 PhoPQ and RstA

The system of PhoPQ of *Salmonella* is a double system formed from cytoplasmic response regulator PhoP and sensor kinase PhoQ localized in inner membrane (Kato and Groisman, 2008). As a result of activation of PhoP, the modification of LPS is controlled with more than 120 genes' direct or indirect expression which are related to many functions such as transporting magnesium, invasion of epithelium cells and remaining alive in macrophages (Kato and Roisman, 2008., Charles *et al*., 2009).

Another PhoPQ depended factor which contributes the regulation of biofilm is the regulation of RpoS by PhoPQ system indirectly. RpoS regulates the synthesis of the components related to movement and CsgD in transcriptional level as well as biofilm production. PhoP stabilizes RpoS as acting *ira*P's transcriptional activator. As coding a product which interacts with RssB, *ira*P provides the stability of RpoS. (Tu *et al*., 2006). PhoP at the same time activates the expression of RstA (Choi *et al*., 2009). This protein induces the disintegration of RpoS indirectly through ClpXP-SsrB proteolytic pathway. RstA is the response regulator of the RstA/rstB double system (Cabeza *et al*., 2007). The counter effects of IraP and RstA take an active role in the expression arrangement of RpoS depending on extracellular signals. The activation of RstA by PhoP represents other alternatives to PhoPQ dependent biofilm regulation. The expression of high level of RstA causes its negative regulation as binding to csgD promoter in *E. coli* (Ogasawara *et al*., 2007). The finding of the binding motif of RstA in csgD operon in *Salmonella* is a proof that RstA inhibits the expression of *csgD* directly (Cabeza *et al*., 2007).

1.7 The Present Study

One of the most important characteristics which play a role in *Salmonella* virulence is biofilm formation. That is why the determination of the presence of global regulators which have roles in biofilm formation has become the key point of the fight with biofilms. In this study, the role of *marT* gene with a global regulatory potential was investigated. As based on an earlier microarray study of Akkoç *et al*, 2009, we aimed to understand the role of *marT* gene on 14 (*fimA, fimF, fliZ, fimH, fimD, sthB, sthE, csgA, csgD, yaiC, stjC, stjB, rmbA* and *ompC*) biofilm production related genes' expression as well as the biofilm formation and adherence (attachment) characteristics of each of the respected mutant general.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Material

2.1.1 Bacterial strains and their maintenance

Wild type *Salmonella* Typhimurium 14028, *S.* Typhimurium MZ1627 (*ΔmarT* strain containing *marT* gene at the downstream of the arabinose inducible BAD promoter of $pBAD_{24}$ vector), *S.* Typhimurium MZ1629 (*ΔmarT* strain containing empty $pBAD_{24}$ vector) were obtained from the culture collection of Prokaryotic Genetics Laboratory, Ankara University. Wild type and mutant *Salmonella* were grown in Luria Bertani (LB- Sigma, USA) media. Bacterial stock cultures were stored in sterile microfuge tubes at -80 ° C with the addition of 60 % glycerol.

S. Typhimurium MZ1627, a derivative of *S.* Typhimurium 14028, was used to determine the transcription levels of the target genes depending on *marT* induction by arabinose. The former studies based on microarray analyses performed by Akçelik (2011) have documented the genes that have significant changes in expression levels related to the induction of *marT* gene (*csgA, csgD, ompC, fimA, fimD, fimF, fimH, fliZ, stjB, stjC, marT, rmbA, sthB, sthE, yaiC*), as listed with their functions in Table 2.1. The target genes' specific primers designed and used in Q-RT PCR assays are tabulated in Table 2.3.

Table 2.1 The target genes and their functions

Gene	Function
fimA	Fimbria's main structural subunit (Zeiner *et al*., 2012)
marT	The positive regulator of MisL autotransporter protein (Akçelik *et al*., 2007)
fimH	Fimbrial adhesion (Zeiner *et al*., 2012)
rmbA	Possible cytoplasmic protein (Kayser *et al*., 1999)
stjC	Fimbrial chaperone protein (Akkoç *et al.,* 2009; Yüksel *et al.,* 2012)
stjB	Fimbrial assembly protein (Akkoç *et al.,* 2009; Yüksel *et al*., 2012)
sthB	Fimbrial chaperone protein (Townsend *et al*., 2001; Latasa *et al*., 2005)
sthE	Major fimbrial protein SthE (Townsend *et al*., 2001; Latasa *et al*., 2005)
csgD	Transcriptional regulator CsgD (Liu *et al*., 2014)
csgA	Curli major subunit (Liu *et al*., 2014)
fimF	Adhesion (Zeiner *et al*., 2012)
fimD	Outer membrane usher protein (Zeiner *et al*., 2012)
ompC	Attachment to host cells (Negm and Pistole 1999)
fliZ	Flagellar regulatory protein FliZ (Chubiz *et al*., 2010)
yaiC	Diguanylate cyclase AdrA(diguanylate cyclase involved in the regulation of flagella rotation) (Cowles *et a*l., 2016)

Table 2.2 Plasmids used in this study

Strains	**Relevant Characteristic(s)**	**Source or reference**
pRedET	Red/ET expression plasmid, Amp^R	Gene Bridges, Quick & Easy *E. coli* Gene Deletion Kit
$pBAD_{24}$	Bacterial expression vector with BAD promoter from arabinose operon, which can be induced and repressed, Amp^R	Townsend et al., 2001
$pBAD_{24}$ + *marT*	pBAD24 vector contains *marT* gene under control of the arabinose inducible pBAD/araC promoter, Amp^R	Townsend et al., 2001
pET-6XHis-(-30)GFP	GFP expressing vector with IPTG induction. High copy number vector with pET28 backbone, Amp^R	Addgene plasmid # 62,936; https://n2t.net/addgene:62936; RRID:Addgene_62936
pET28aGFP	pET28a vector, expressing GFP copied from pET-6XHis-(-30)GFP plasmid	This study

Table 2.3 Gene specific primers used in Q-RT PCR assays

Gene	**Primers**	**PCR Product (bp)**
csgA	F: 5'-CCAGGGTGCGGATAACAGTA-3 R: 5'-CCAACCTGACGCACCATTAC -3	177
csgD	F: 5'-GCGATGAGTGAGTAATGCGG-3 R: 5'-GGCCTCATATTAACGGCGTG-3	176
fimA	F: 5'-TGTGCCGTCAGCACTAAATC-3' R: 5'-TGGTGTTATCTGCCTGACCA-3'	181
fimD	F:-5'-AGGTAATCATCAGCCTCCCG-3 R: 5'-CAGCCGTTTAATCCACTCCG-3'	160
fimF	F: 5'-GATCAACGTCGATTGCCACT-3' R:5'-TGATCAGGAAGGTCGCATCC-3	151
fimH	F: 5'-ATCGGCGCGTCGTTATTTAG-3' R: 5'-ATTATCGGACACCATCGCCT-3	168
fliZ	F: 5'-TCAGAACTGGCGGTAAAGGG - R: 5'-GCAATTACCTCAGCGAGCAA -	190
ompC	F: 5'-CGTAACCGTTGCTGATGTCC-3' R: 5'-GCAGCGCAGTATTCTCAGAC-3	193
sthB	F:-5'-TATCGCTAACCACATCGCCT-3' R: 5'-GAACTTCGCTGAACTGACGG-3	200
sthE	F:-5'-GCGACTACAGCCATCCATTG-3 R: 5'-TGGCGCTTATTCTGCTGTTC-3'	194

Table 2.3. (continued)

stjB	F:5'-AAGTCTAATTTCCCCGGGCA-3' R: 5'-GGCACAAGACTATCAAGCGG-3	166
stjC	F: 5'-TCACACCATTGTCGTCAGGT -3 R: 5'-TACTCAGCGTCCCCTTTGTT-3'	189
yaiC	F:-5'-GAGCATGAATCTGATGGGCG-3 R: 5'-ACTTACCCAGGCGAACAGAA-3	187
rmbA	F:-5'-TTGCAATGGGCTGACAGTCT-3 R: 5'-TGCTTTTCAAAATCCAGTAA-3'	100
16S	F:-5'-CCGTCAATTCCTTTGAGTTT-3' R: 5'-AGAGTTTGATCCTGGCTCAG-3	919

2.1.2 Restriction endonucleases and buffer solutions

Restriction endonucleases and buffer solutions were provided from Fermentas, EU.

2.2 Methods

2.2.1 Bacterial genomic DNA isolation

S. Typhimurium 14028 was inoculated (1%) to and grown in LB at 37ºC by shaking at 200 rpm for 18 hours. A 1.5 mL aliquot from this culture was transferred into a microcentrifuge tube. The bacterium was precipitated by centrifuging at 12000 rpm for 2 minutes. After supernatant was removed, bacterial pellet was dissolved in 567 µL of Tris-EDTA (TE) buffer and mixed with 30 µL of 10% of sodium dodecyl sulphate (SDS) and proteinase K enzyme solution. The mixture was incubated for

1 hour at 37ºC. Then 100 µL of 5M NaCl and 80 µL of CTAB (cetyltrimethyl ammonium bromide)/NaCl solution were added and mixed properly by using micro-pipette tips until white particles are formed. The mixture was incubated at 65ºC for 10 minutes and 750 µL of chloroform/isoamyl alcohol (24/1 volume/volume) was added and mixed. The mixture was then centrifuged at 12000 rpm for 5 minutes and the supernatant was transferred into a new microcentrifuge tube. Then 750 µL of phenol/chloroform/isoamyl alcohol (25/24/1 volume/volume) was added and the resulting mixture was centrifuged again at 12000 rpm for 5 minutes. After centrifugation, the supernatant was transferred into a new microcentrifuge tube and 350 µL of isopropanol was added. The mixture was mixed gently until having a white precipitate. The tubes were next centrifuged again at 12000 rpm for 5 minutes. The supernatant was removed, and the pellet was washed with 70 % of ethanol (350 µL) and centrifuged again at 12000 rpm for 5 minutes. The supernatant was removed and th chromosomal DNA dried at room temperature was dissolved in 100 µL of TE buffer (Asubel et al., 1994, Sambrook and Russel, 2001).

2.2.2 Plasmid isolation

In general, the cells were grown in LB medium containing appropriate antibiotics at 37 °C by shaking at 200 rpm for 18 hours. The isolation of the plasmids was performed by using GeneJet Plasmid Miniprep (Fermentas, #K0503) kit by following the manufacturer's protocol and the electrophoresis was run in a 0.7% of agarose gel at 100 Volts. After gel electrophoresis, the DNA bands were stained in a buffer containing 0.2 g/mL of ethidium bromide (Sigma Chem. Co., USA) for 30 minutes. After destaining, the gel was visualized under UV (366 nm) and Syngene Gene Genius Gel Imaging System was used to obtain gel images. For molecular size determination, supercoiled molecular standard mix (Sigma, D5292) was used.

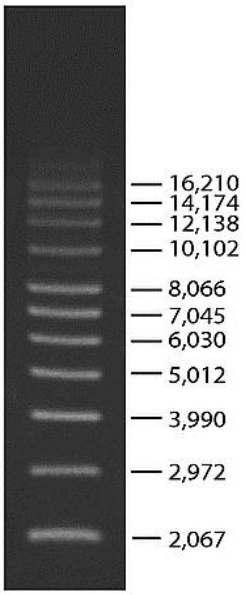


Figure 2.1. Supercoiled DNA Ladder (Sigma, USA) as a marker. The size of each supercoiled DNA indicated as base pairs

2.2.3 Touchdown Polymerase Chain Reaction

Using a toothpick, some bacterial cells from a single colony were scraped and transferred on the bottom of a PCR tube. After cells were suspended into ddH_2O, they were incubated at 100°C for 5 minutes. The template DNA was next obtained**.**

After PCR products were ran on a 1% agarose gel, they were visualized under UV light. After checking the size of the DNA bands, respective colonies were stored at -20°C or – 80 °C.

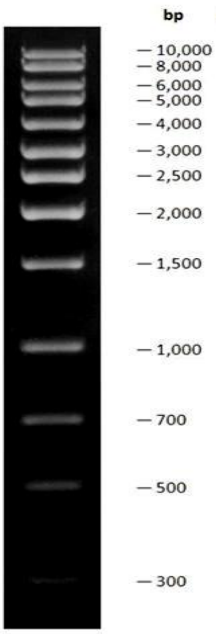


Figure 2.2. 1 kb molecular DNA standards (Fermentas, EU)

Table 2.4 Touchdown Polymerase Chain Reaction (50µL) ingredients

Ingradients	**µl**
ddH_2O	36,75
10 x NH_4SO_4 Buffer (Promega, USA)	5
dNTP (10 mM) (Fermentas, England)	1
Forward (F) primer (10 µM)	1
Reverse (R) primer (10 µM)	1
$MgCl_2$ (25 mM) (Promega, USA)	3
Go Taq DNA Polimerase (5 U/µl) (Promega,USA)	0.25
Templete plasmid DNA (100ng/µL)	2

Table 2.5 Touchdown Polymerase Chain Reaction temperature cycles

Step	Temperature (^{0}C)	Time (sn.)	Cycle Number
Denaturation	94	120	1
Denaturation	94	20	20
Annealing	70-->60*	45	
Elongation	72	90	
Denatuation	94	20	20
Annealing	60	45	
Elongation	72	90	
Elongation	72	300	1

The PCR products were purified by using Roche High Pure purification kit as recommended by the manufacturer. The purified products were treated with the *DpnI* restriction endonuclease enzyme for 2 hours at 37^{0}C (Table 2.6). After enzyme cleavage, the purification was repeated.

Table 2.6 *DpnI* restriction endonuclease reaction mixture

	Reaction mixture (µl)
ddH_2O	11.9
10X Tango Buffer	2
DpnI	0.5
1000 ng/µl DNA	5.6

2.2.4 Cloning of *marT* gene into PBAD$_{24}$ vector

To understand the role of *marT* gene on expression of 14 selected genes in this study, *marT* gene was cloned downstream of the arabinose inducible BAD promotor. The genetic map of pBAD$_{24}$ vector is presented in Figure 2.3.

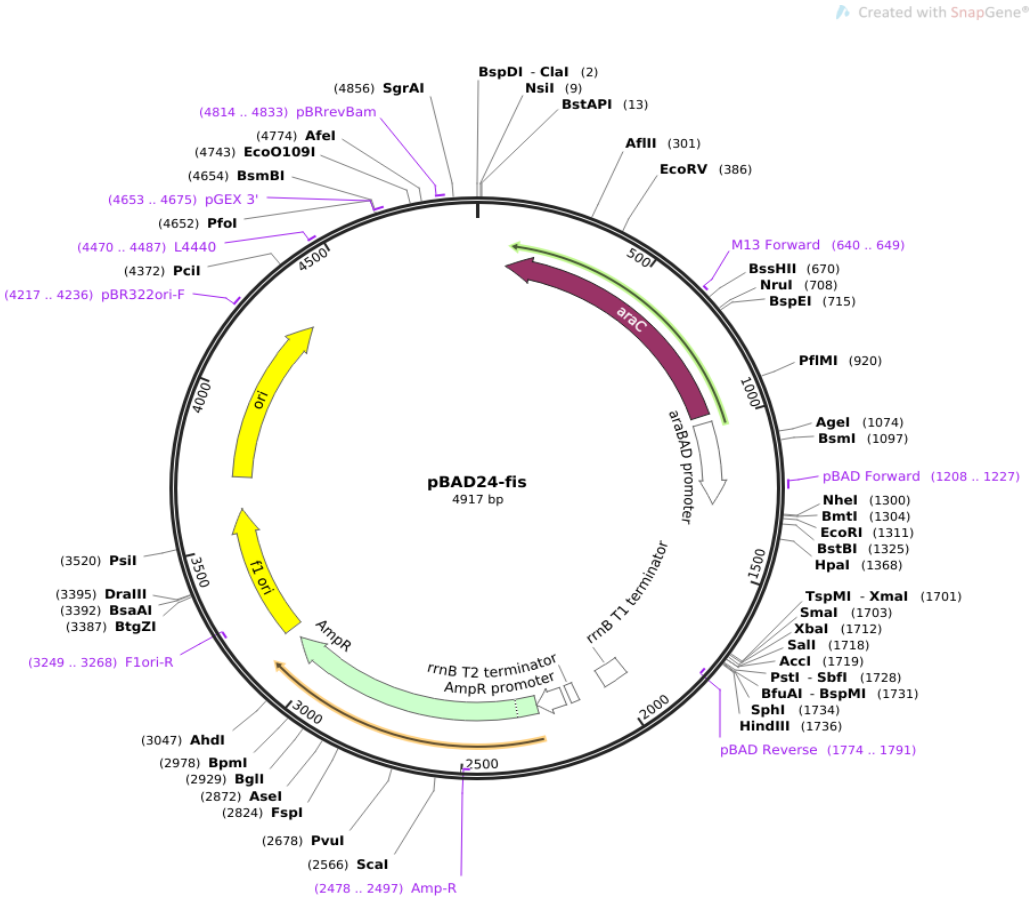


Figure 2.3. The plasmid vector pBAD$_{24}$ (https://www.addene.org 1995)

2.2.4.1 Amplification of *marT* gene with PCR

The genomic DNA was isolated from *S*. Typhimurium ATCC14028 which was grown in LB at 37 °C, by shaking at 200 rpm for 18 hours. The primer pairs were designed for the amplification of *marT* gene by adding *EcoRI* and *PstI* recognition sites (Table 2.9). In this study, Fermentas' Taq DNA polymerase was used and the PCR reactions were performed as using GeneAmp9700 Thermocycler (Applied BioSystems, USA).

The reaction mixture used for amplification and the amplification conditions are outlined in Tables 2.7 and 2.8, respectively.

Table 2.7 PCR ingredients used for amplification of *marT* gene.

PCR ingredients	**50 µL**
ddH_2O	27,5µL
Buffer(10X)	10 µL
dNTP(10mM)	1 µL
Forward Primer(µM)	2,5 µL
Reverse Primer(µM)	2,5 µL
$MgCl_2$	4 µL
Taq DNA polimerase	0,5 µL
DNA	2 µL

Table 2.8 The temperature cycle of Polymerase Chain Reaction for *marT* gene amplification

Step	**Temperature**	**Time**	**Cycle Number**
Denaturation	98 ^{0}C	30 sec	1
Denaturation	98 ^{0}C	10 sec	30
Annealing	61^{0}C	30 sec	
Elongation	72 ^{0}C	90 sec	
Elongation	72 ^{0}C	7 min	1

After PCR, the DNA samples were run in 1% of agarose gel, at 100 V for 1 hour, then stained with ethidium bromide for 30 minutes. The stained gels were scanned by using a Gel Logic 200 Imaging System (Kodak, USA) and photographed.

2.2.4.2 Restriction enzyme cleavage

In order to confirm the presence of restriction endonuclease cleavage sites in the target gene, Webcutter 2.0 (http://bio.lundberg.gu.se/cutter2/) programme was used.

marT gene PCR products were purified by using High Pure PCR Product Purification Kit (Roche Chem. Co., USA). For creating sticky ends in circular $pBAD_{24}$ vector and purified gene products, the molecules were cut with *EcoRI* and *PstI* enzymes. For all the cleavage reactions, the reaction mixtures were used as recommended by the manufacturer (Table 2.9).

Table 2.9 The RE enzyme cleavage reaction ingredients

	***marT* (75ng)**	**$pBAD_{24}$ (24ng)**
Distilled water	17 µL	7µL
O Buffer (10X)	3 µL	2µL
Product	10 µL	10µL
PstI	1 µL	0,5µL
EcoRI	1 µL	0,5µL

The enzyme cleavage reactions were performed at 37 °C for 1 hour. At the end of each cleavage reaction, the tube was incubated at 65 °C for 20 minutes for inactivation of the enzyme. The linearized vector and the fragments to be cloned were next purified by using High Pure Product Purification Kit (Roche Chem. Co., USA).

1 kb marker (Fermentas, EU) was used in 1% agarose gel to control the results of vector cleavage. Supercoiled marker (Sigma, USA) was used to control the DNA samples to be used in the ligation experiments. Their concentrations and purity were detected in a Nanodrop 2000 spectrophotometer device (Thermo).

2.2.4.3 Ligation

After purification of the linearized vector as well as the insert, the ligation reactions were performed by using T4 DNA ligase and the respective buffer solution (Fermentas, EU) in a total volume of 20 µL (Table 2.11). The ligation mixtures having an insert (i) to vector (v) ratio of 1:3, 1:1, 3:1, respectively, according to their molar concentrations, were tested for ligation efficacy. The mixtures were incubated at 22°C for 4 hours. At the end of incubation, T4 DNA ligase enzyme was inactivated at 65°C for 10 minutes.

The ligation products were stored at -20°C till transfer to recipient cells by electroporation. The insert to vector molarity ratios were calculated according to the below equation:

$$\textbf{DNA Concentration (ng)} = \frac{\textbf{DNA size (bp)} \times \textbf{Vector Concentration (ng)} \times \textbf{Ligation rate}}{\textbf{Vector size (bp)}}$$

Table 2.10 Ligation mixtures

	3i :1v	**1i:1v**	**3v :1i**
ddH_2O	14,30 µL	14,95 µL	11,55 µL
T4 DNA ligase buffer (10X)	2 µL	2 µL	2 µL
marT (22 ng/µL)	1 µL	0,35 µL	0,35 µL
$pBAD_{24}$ (30 ng/µL)	1,7 µL	1,7 µL	5,1 µL
T4 DNA ligase	1 µL	1 µL	1 µL

In the construction of mutants, the Quick & Easy *E. coli* Gene Deletion kit (K006, GeneBridges, Germany) was used as recommended by the manufacturer. The target gene-specific primer sequences are shown in and the PCR conditions performed to obtain the amplicons to be used in homologous recombination are given in Table 2.12 and Table 2.13 respectively. The target-specific primers were designed to obtain the PCR products with homologue gene regions. All primers were provided by the Heliks Biotek (Ankara, Turkey) Co. Lyophilized primers were diluted to 100µM with ddH_2O and to be used in PCR reaction, re-diluted to a concentration of 10µM. These primers were next used in Touchdown PCR reaction.

Table 2.11. Target gene-spesific primer sequences used in the construction of mutants.

Gene	**Primers**
csgA	F:5'-CCAGGGTGCGGATAACAGTACTATTGAACTGACTCA GAATGGTTTCAGAAAATTAACCCTCACTAAAGGGCGG-3'
	R:5'-CCAACCTGACGCACCATTACGCTGGAATCAGATGC GGTCTGATTAACCAG TACGACTCACTATAGGGCTCG-3'
csgD	F:5'-CGATGAGTGAGTAATGCGGACTCGGTGCTGTTGTAGC GGTAATTTCCTGAATTAACCCTCACTAAAGGGCGG-3'
	R:5'-GCCTCATATTAACGGCGTGTTTTACGCTACTGAAGAC CAGGAACACGTGAATTAACCCTCACTAAAGGGCGG-3'
fimA	F:5'GACGCTGGGTCAATACCGTACCGCCAGCTTTACG GCGATTGGTAATACGAAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'- CACTTTCGGATCGCAGTCATTCAGGACGATGGAGAAA GGCACCTGCGCAGTACGACTCACTATAGGGCTCG-3'
fimD	F:5'-GGTAATCATCAGCCTCCCGGTATTTACCGGGTGGATA TCTGGCGTAACGAATTAACCCTCACTAAAGGGCGG-3'
	R:5'-CAGCCGTTTAATCCACTCCGGTGTAAAACAAGGCAT CAGGCCGCCGGATTTACGACTCACTATAGGGCTCG-3'

Table 2.11. (continued)

fimF	F:5'-GATCAACGTCGATTGCCACTTGAACAGGCCAGCAAG GCCGTCGATATTGAAATTAACCCTCACTAAAGGGCGG-3'
	R:5'-TGATCAGGAAGGTCGCATCCGCGTTAGCAAGTCCG GGCTGCACGCCATCGTACGACTCACTATAGGGCTCG-3'
fimH	F:5'-ATCGGCGCGTCGTTATTTAGTCAGGCGGGGGCGGGT AATCGTCCGCAAGGAATTAACCCTCACTAAAGGGCGG-3'
	R:5'-ATTATCGGACACCATCGCCTGCCCTGAGGCCT TTTCGGCTTCAAGCCGCA TACGACTCACTATAGGGCTCG-3'
fliZ	F:-5'-TCAGAACTGGCGGTAAAGGGGGATTTCTGTCTG GGCGCAATCTGCTGATGAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-GCAATTACCTCAGCGAGCAAAACATTTCCCACGATC TGCTGCAGGACGGTACGACTCACTATAGGGCTCG-3'
ompC	F:5'-CGTAACCGTTGCTGATGTCCTTACCTTTAGACTGCAGGT AAGCCACAGACAATTAACCCTCACTAAAGGGCGG-3'
	R:5'-GCAGCGCAGTATTCTCAGACCTATAACGCAACCCGTTTT GGTACCTCTATACGACTCACTATAGGGCTCG-3'
rmbA	F: 5'-TGCAATGGGCTGACAGTCTGGCAGACAGTGGCATGCA TATTGTCCTGATAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-TGCTTTTCAAAATCCAGTAATTGGCCAGCGGAGTT AAACTTCTGTCAGAATACGACTCACTATAGGGCTCG-3'
sthB	F: 5'-TATCGCTAACCACATCGCCTGCTTTAATTTTTGAC CATGAGTTGGTGACG AATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-GAACTTCGCTGAACTGACGGCCTTATACGGATTAGCAGG CGGCATAACCGTACGACTCACTATAGGGCTCG-3'

Table 2.11. (continued)

sthE	F: 5'-GCGACTACAGCCATCCATTGTGACTTCGGAGGTGGAAA CCCAGCCCGATTAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-TGGCGCTTATTCTGCTGTTCGCGTATTCCGGTCATAGT TACGCCTCCTGTTACGACTCACTATAGGGCTCG-3'
stjB	F: 5'-AAGTCTAATTTCCCCGGGCAGCGCCATGCTGGCGCGT AGTTCACTTTTCGAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-GGCACAAGACTATCAAGCGGCAGGAGGGATGCTGCAA TGGCTTCCAACCATACGACTCACTATAGGGCTCG-3'
stjC	F: 5'-TCACACCATTGTCGTCAGGTAAATCAACGACGGTGGTATA TAACAAAGCCAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-TACTCAGCGTCCCCTTTGTTTTTTCAACACTCTTTTCACAG GCTAACGCTTACGACTCACTATAGGGCTCG-3'
yaiC	F:5'-GAGCATGAATCTGATGGGCGCTGGCGGGCGGCGGCTATTC ACTGTCGGAAAATTAACCCTCACTAAAGGGCGG-3'
	R: 5'-ACTTACCCAGGCGAACAGAAGCGGATACAACATAATG ACGGGCAAAGAGA TACGACTCACTATAGGGCTCG-3'

Table 2.12 PCR conditions for the production of amplicons used in homologous recombination

Reaction	**Temperature (°C)**	**Time (s)**	**Circle**
Denaturation	98	30	1
Denaturation	98	10	30
Annealing	60	30	
Elongation	72	90	
Elongation	72	60	1

The chromosomal mutants of all the genes used in the study were obtained using the Red Recombinase system based-Quick & Easy *E. coli* Gene Deletion Kit (GeneBridges, Germany). Kanamycin gene cassette was used to knock out the genes. *S.* Typhimurium 14028 mutants were first selected on LB agar media containing 50 µg/mL kanamycin (due to the resistance cassette) and 100 µg/mL ampicillin (due to the pRED / ET_{amp} plasmid). Then, the entered kanamycin cassette to the target genes was detected by amplifying the kanamycin gene cassette on chromosomal DNA which was isolated from the mutant strains by using specific primers.

The mutations were done by using bacteriophage lambda Red recombinase system-based "Quick & Easy *E.coli* Gene Deletion" kit (K006). The principle of this method is based on induction of Red operon in pRED/ET_{amp} plasmid by the addition of L-arabinose. First of all, pRED/ET_{amp} plasmid was transformed into the wild type, competent *S.* Typhimurium 14028 cells by electroporation (Thermo Electron Corporation, Ohio, 0.1 cm cuvettes). After induction of Red operon, the PCR

product carrying the homology arms is electroporated. RED/ET recombination inserts the functional cassette into the target locus on the bacterial chromosome. Finally, the cell suspension was removed and transferred into selective LB agar medium and only colonies carrying this inserted modification can be selected on kanamycin included agar plates. pRED/ETamp plasmid has a temperature sensitive origin so, it can be propagated only at 30^0C. At the time the cells were transferred into growth media and incubated at 37 ^{0}C, plasmid is automatically removed.

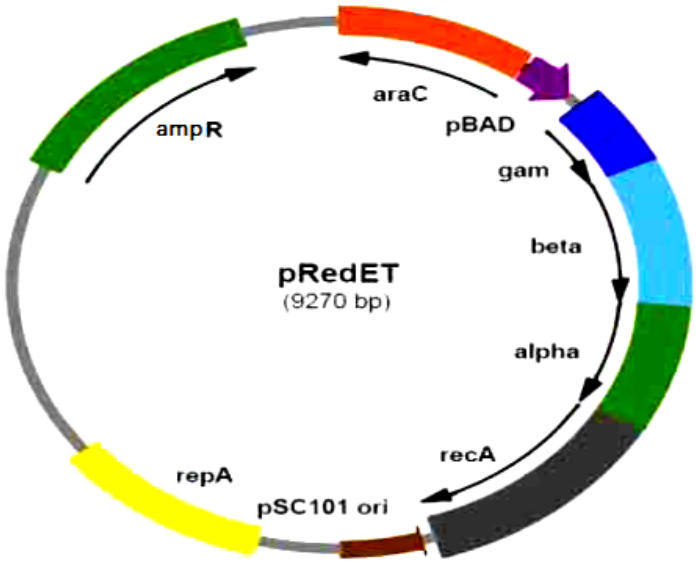


Figure 2.4. pRED/ET plasmid vector's genetic map (https://www.addene.org 1995)

In the following step, expression of target genes was examined by inducing the *marT* gene. For inducing the *marT* gene, 0.01% of L-arabinose was added into growth media of the strains planned to be tested and incubated for 30 minutes at 37 °C at agitated condition. In the next step, PCR products were transformed into the cells where recombinase system is expressed. For this purpose, 400-800 ng of PCR products were mixed with electrocompetent cells (as recommended by the Kit's manufacturer) and the electroporation was performed according to the protocol. After electroporation, the cells were transferred into selective LB agar medium containing kanamycin (15 µg/mL).

At the last step, kanamycin-resistant electrotransformants were selected and transferred into antibiotic selective media for storage.

2.3 Competent cell preparation and plasmid transformation

The cells were artificially made competent for transformation. *S*. Typhimurium strains were grown overnight in LB as described earlier. A 100µL sample was taken from the culture and inoculated into a fresh 10mL of LB broth and grown by shaking until the bacterial density (OD_{600}) reaches to 0,25 to 0,35 (approximately 2.5 hours). 1mL of the culture was transferred into a microcentrifuge tube and the cells were precipitated by centrifuging at 10000 rpm at $+4^0$C for 2 minutes and cooled in a 100 µL of TSS solution carefully. The cell suspension was kept on ice for 30 minutes and then 60 ng/µL of recombinant plasmid was transferred into the tube. The cell suspension and the plasmid mixture were mixed slowly, and the mixture was kept on ice for another 30 minutes. After incubation on ice, the heat shock was performed at 42-45 °C in a water bath for 45 seconds then the mixture was taken on ice immediately where it was held for 2 minutes. The mixture was then centrifuged, the supernatant (900 µL) was removed, the pellet was dissolved in 100 µL of remained liquid and the sample was transferred to LB broth including ampicillin (100 µL/mL). It was then incubated at 30^0C for 18 hours before plating out for the selection of transformants/recombinants.

2.4 Preparation of the electrocompetent cells

For preparing electrocompetent cells, the method defined by Sambrook *et al*., 1989 was used. After *S*. Typhimurium strains were grown overnight in 5 mL of LB broth by shaking at 200 rpm at 37°C, then the cells were transferred to LB plates where they were incubated overnight.at 37°C, to form single colonies. The colonies were next transferred into LB broth and grown overnight by shaking at 200 rpm at 37°C. The resulting culture was then transferred into a new tube of LB broth and

reincubated for 2 hours. The aliquots were taken at 15 minutes' intervals and optical density (OD_{600}) was measured. After the optical density of the culture was reached to 0.4 to 0.6, the incubation was terminated and the culture was taken on ice. The aliquots of cell suspension were then transferred into previously cooled centrifuge tubes and centrifuged at 4°C, 10000 rpm for 20 minutes. The supernatant from each tube was removed and the pellet was dissolved with the cold sterile distilled water. The suspension was rewashed and centrifuged at 4°C, 10000 rpm for 20 minutes for 2 more times and finally dissolved in 50 µL of sterile distilled water. The competent cell preparations were stored at -80°C until they were used.

2.5 The electroporation of the recombinant plasmids

After ligation, the recombinant plasmids were transformed into the competent cells in a CelljecT Uno electroporator device (Thermo, USA) using the electroporation cuvettes (Thermo, USA) which have sample reservoirs of 2 mm. Before this process, the competent cells, the plasmids and the electroporation cuvettes were kept on ice. A 2 µL of the recombinant plasmid solution were added into 50 µL of the competent cells which were then transferred into electroporation cuvettes. The electrical current was adjusted 1800 V for a second.

1 mL of LB broth was added to each cuvette containing electroporated cells and the cuvette contents were transferred into microcentrifuge tubes where they were incubated for 2 hours at 37°C at agitating condition. After incubation, the samples were transferred to LB agar plates containing 100 µg/mL of ampicillin and incubated at 37°C overnight (Sambrook *et al*., 1989). From the selected recombinants, the plasmid DNA isolation was done as using Genejet Miniprep plasmid isolation kit (Fermentas, EU) according to the manufacturer.

2.6 Total RNA isolation

mRNA isolation was performed using High Pure RNA Isolation Kit (Roche/Germany), according to the instructions of the manufacturer. The 18 hours incubated cells were added to 5 mL of LB broth and incubated at 37°C by shaking until their OD was reached to 0,1. 0.01% of arabinose then was added and incubation was continued for 30 minutes after which they were transferred into microcentrifuge tubes and centrifuged at 4°C, 12000 rpm for 2 minutes. The pellet was dissolved in 200 µL of peptone buffer solution (PBS). It was then mixed for 15 seconds with 400 µL of lysis buffer and then, after adding 100 µL of lysozyme (50mg/ml) it was incubated at 37 °C for 15 minutes. At the end of incubation, the samples were transferred into spin colon tubes provided with the kit, and centrifuged at 4°C, 12000 rpm for 1 minute. The supernatant was removed and 90 µL of DNase incubation buffer and 10 µL of DNaseI were mixed and added onto the filter of the spin tube. At the end of incubation at 20°C for 20 minutes.

500 µL of wash buffer was added onto the filter and centrifuged at 4 °C, 12000 rpm for 1 minute. Then the sample was washed with 500 µL and 200 µL of wash buffer 2, respectively, and recentrifuged at the same conditions. At the last step, 50 µL of elution buffer was added, and the mixture was recentrifuged at 4 °C, 12000 rpm for 1 minute. After the determination of the purity and the concentration of the resulting RNAs (Nanodrop – 2000 Spectrometer, Thermo, USA), the purity was controlled by agarose gel (2%) electrophoresis. The cDNA synthesis was performed immediately after controlling mRNAs.

2.7 cDNA synthesis

2000 ng/mL of RNA samples were used for cDNA synthesis performed by using cDNA Synthesis Kit (Roche, Germany). The reaction mixtures and the temperature cycles are given in Table 3.5 and 3.13, respectively. Each obtained cDNAs were diluted to 1:10 with ddH_2O to be used in real time PCR studies and stored at -20°C.

Table 2.13 cDNA synthesis protocol

Ingredient	**Concentration**	**Final concentration**	**µL**
Distilled water			
Random hexomere primer	600pmol/µL		2
RNA		2000 ng/mL	

*Incubated at 65 °C for 10 minutes for denaturation of RNA

Reverse Transcriptase Buffer	5X	1X(800 mM $MgCl_2$	4
RNase inhibitory	40 U/µL	20 U	0,5
dNTP mix	10mM (each nucleotide)	1 mM (each nucleotide)	2
Reverse transcriptase	20U/µL	10U	0,5
Total volume			20

Table 2.14 Reverse Transcription Temperature Cycle

Step	**Temperature (°C)**	**Time (min)**	**Cycle Number**
Elongation 1	25	10	1
Elongation 2	50	60	1
Inactivation	85	5	1

2.8 Determination of the gene expression levels with Q-RT PCR

The genes and gene-specific primers used in Q-RT PCR assays are shown in Table 2.3 and Table 2.4. Light Cycler 480 (Roche, Germany) was used for Q RT PCR. The amplification mixtures were prepared according to the instructions of the manufacturer (Table 2.15). The program was performed according to the protocol suggested in the Light Cycler 480, SYBR Green I kit (Roche, Germany) (Table 2.16).

Table 2.15 The amplification mixture for quantitavive real time PCR

Ingredient	**Concentration**	**Final Concentration**	**µL**
SYBR Green Master Mix	2X	1X	5
Forward Primer (F)	10 pmol	10 pmol	1
Reverse Primer(R)	10 pmol	10 pmol	1
cDNA template	1:10 diluted	50 ng/mL	1
ddH_2O			2
			10

Table 2.16 The program of quantitative real time PCR used in Light Cycler device

Step	Process	Temperature (°C)	Time	Increase (°C/sn)	Data acquisition
First denaturation	First denaturation	95	10’	4,8	-
Amplification (45 cycle)	Denaturation	95	10’’	4,8	-
	Annealing	*57	10’’	2,5	-
	Elongation	72	08’’	4,8	Single
Melting curve	Denaturation	95	30’’	4,8	-
	Annealing	72	30’’	2,5	-
	Denaturation	99	0	0,11	Sustained
Cooling	Cooling	40	30’’	2,5	-

2.9 Quantification in Q-RT PCR

To evaluate Q RT-PCR data, our relative quantification strategy was proportioning the changes in the level of RNA of a particular gene in a sample to the changes in another target gene's RNA level in another sample. The calculation is based on comparison of differences in cycle threshold (Ct) by taking amplification yield in consideration which is an indication of how close the PCR reaction to the perfection. In the study, PCR amplification productivity (E) was calculated for each of the primer pairs. For this, 1/10 serial dilutions of the wild type cDNA sample (calibrator sample) were made (1 - 1: 10000) and the graph plotted against the Ct values (threshold cycle values) of the $\log_{10}$ copy number obtained from the dilution series was created. The exponential amplification (equation 3.1) and the productivity (equation 3.2) were calculated from the slope of the amplification peak obtained from the graphic. The perfection of the PCR increases when exponential amplification comes close to 2 and the amplification productivity comes close to 1 (Rassmussen, 2001). If productivity is equal to 1 (=100%), it means that the reaction number is doubled in each cycle.

Equation 1.1: Exponential amplification (10 (-1/slope)

Equation 1.2: Productivity (E): (10(-1/slope))-1

Equation 1.3: $R=2^{-\Delta\Delta Ct}$

At the end of calculations of exponential amplification, it was seen that the PCR reactions were actualized almost close to perfection. For this reason, the evaluation of the results of PCR (quantification) was done with the unaccounted reaction productivity approach method (Equation 3.3) (Livak and Schmittgen, 2001).

2.10 Q-RT PCR normalization and statistical analysis

For actualized relative quantification, a normalization step is required for which a housekeeping gene showing no difference in its expression level (minimum effect) must be used as a reference gene (Bustin, 2002; 2005). At the end of reaction, the expression value (treshold cycle; Ct) of the target gene is obtained separately for each sample and normalized by proportioning the housekeeping gene (16S in this study) to the expression level of each sample.

The results of qRT-PCR were normalized according to the given formula as using the approach method selected for quantification.

$2^{-\Delta\Delta Ct} = 2^{(CT,X - CT,R\ control) - (CT,X-CT,R\ test)}$

CT,X : Ct value of the target gene
CT,R : Ct value of the reference gene
Control: Ct value of the wild type strain
Test: Ct value of the *marT* mutants

Ct (threshold cycle; it was determined according to the peak profiles obtained at the end of analysis. In the formula, as a reference; the 16S gene's Ct value, for control; the wild type *S*. Typhimurium 14028 and for the test; the *marT* mutants' Ct values were used.

In order to actualize the statistical analysis, the "R" statistical program was used. The results from the experiments were controlled by using ANOVA test. The results based on "F" values obtained from the variant analysis were read as "statistically meaningful" or "meaningless". The evaluation was done between the meaningful groups by using the Tukey accuracy test (Neupane *et al*, 2014).

2.11 The isolation and the transformation of the pET-6xHis (-30) GFP vector expressing GFP (Green Fluorescent Protein)

E. coli BL21 Star (DE3) cells containing pET-6XHis-(-30) GFP plasmid (Figure 2.5) was supplied by Addgene. The plasmid isolation and transformation protocols were as in Section 2.3.

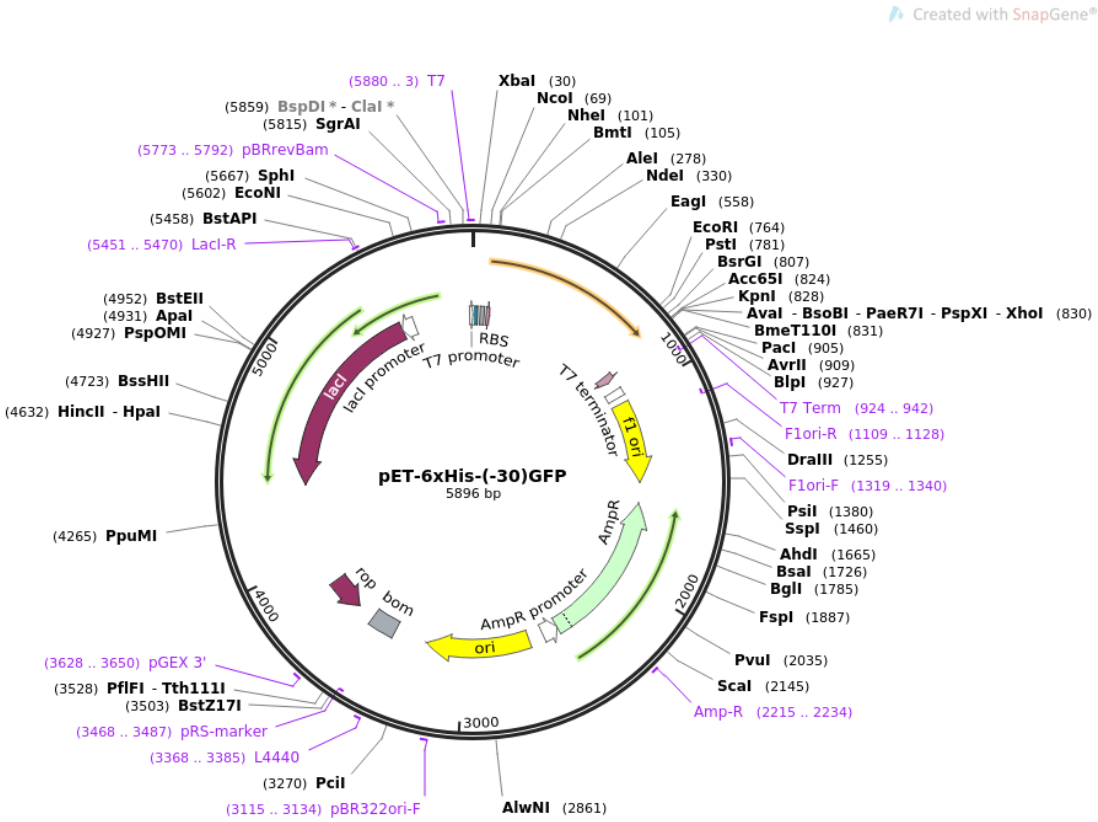


Figure 2.5. pET-6xHis (-30) GFP plasmid vector's genetic map (https://www.addgene.org 2015)

2.12 Construction a new vector (pET28aGFP) for GFP expression under the control of *lac* promoter

Due to the lack of expression of pET-6XHis-(30) GFP plasmid in our *Salmonella* strains (while there was expression in *E. coli* host), we constructed a new vector having GFP expression under the control of *lac* promoter.

A GFP coding series were already obtained in our laboratory and by using *EcoRI* cleavage region, the gene was transformed into pET28a (+) vector. In this way, a new vector, namely pET28aGFP (a pET28a vector, expressing GFP gene originally contained in pET- 6XHis-(-30) GFP was constructed.

In order to provide a vector which expresses high level of green fluorescence protein under the control of ***lac***, instead of T7 promotor (in order to be used in other bacterial cells which do not express T7 RNA polymerase, pUC19 P*lac* series partially replaced T7 promotor in pET28aGFP by transforming it between *XbaI – NcoI* restriction cleavage regions in the target vector.

As a gene resource for P*lac* the pUC19 vector DNAs were used and the region between 1-213 bases in this vector was amplified by using lac2pet-f (*Xba*I): 5'-acgtTCTAGAGCGCCCAATACGCAAACCGCC-3', lac2pet-r (*Nco*I): 5'-acgtCCATGGCTGTTTCCTGTGTGAAATTGT-3' oligonucleotide primers. For 75 mM Tris-HCl (pH 8.8, 25°C), 20 mM (NH4)2SO4, 0.01% of (v/v) Tween 20, 1.5 mM MgCl2, 0.2 µM dNTP, 0.2 µM forward and reverse primers, 1 U Taq DNA polymerase and 100 ng DNA template were used. The PCR mixture was incubated at 94°C for 2 minutes during the first denaturation, and for denaturation; 30 seconds at 95°C, for annealing; 60 seconds at 65°C, and for elongation; 60 seconds at 72°C.

The last elongation was completed at 72°C for 5 minutes. The products were run in an agarose gel and examined under UV light. For cloning of PCR into pET28aGFP vector, both molecules were cut with *NcoI and XbaI* enzymes. With this purpose, 5 µg of plasmid DNA or purified 1 µg of PCR product was incubated for 2 hours at 37°C with reaction mixture containing 10 U *Nco*I and 10 U *Xba*I, 50 mM potassium acetate, 20 mM Tris acetate pH 7.9 (25°C), 10 mM magnesium acetate 1 mM DTT, 0.1 mg/ml BSA. The enzyme inactivation was made at 80°C for 20 minutes.

For ligation, 40 mM Tris HCl (pH 7.8, 25°C), 10 mM MgCl2, 10 mM DTT ve 5 mM ATP, 5 U T4 DNA ligase, 0,1 pmole vector DNA and 0,4 pmole PCR product were incubated at 22 °C for 2 hours.

For transforming ligation products to *Escherichia coli* DH5α, a chemical method was used. An overnight was culture diluted for 250 times and grown until the cell density reaches to 0,6 (OD_{600}) at 37°C and centrifuged. The cells were resuspended in 2xYT medium that contained 10% (w/v) PEG-6000, 5% (v/v) DMSO, 10 mM $MgCl_2$ and 10 mM $MgSO_4$ and incubated on ice.

At the end of 1-hour incubation, 100 µl of cell suspension was mixed with 5 µl of ligation product and incubated on ice for another hour. After heat shock, the cells mixed with 900 µl of 2xYT, and incubated for one more hour at 37°C. For the selection of transformants, the LB agar plates containing 50 µg/ml kanamycin were used.

For the control of recombination, colony PCR was performed with T7-5': 5'-AATTAATACGACTCACTATAGGG-3'and T73':'ATGCTAGTTATTGCTCAGCGG3' oligonucleotide primers. For this purpose, colonies were suspended in 5 µl of ddH_2O containing 75 mM Tris-HCl (pH 8.8, 25°C), 20 mM (NH4)2SO4, 0.01% (v/v) Tween 20, 1.5 mM $MgCl_2$, 0.2 µM dNTP, 0.2 µM forward and reverse primers, 1 U Taq DNA polymerase. The first denaturation; at 94°C for 2 minutes, during 35 cycle for denaturation; 30 seconds, 95°C, for annealing; 60 seconds, 50°C and for elongation; 60 seconds, 72°C were the PCR conditions. The last elongation was completed at 72 °C, by incubating for 5 minutes. The PCR products were analyzed in a 1,6% of agarose gel. After the final controls, the vector was transformed to our mutants as described in section 2.5. Final confirmation of GFP expression was made under a fluorescence microscope (Zeiss AX10), and the after this, the samples were prepared for examination under a Zeiss Ism 510 Confocal Laser Microscope (CLSM).

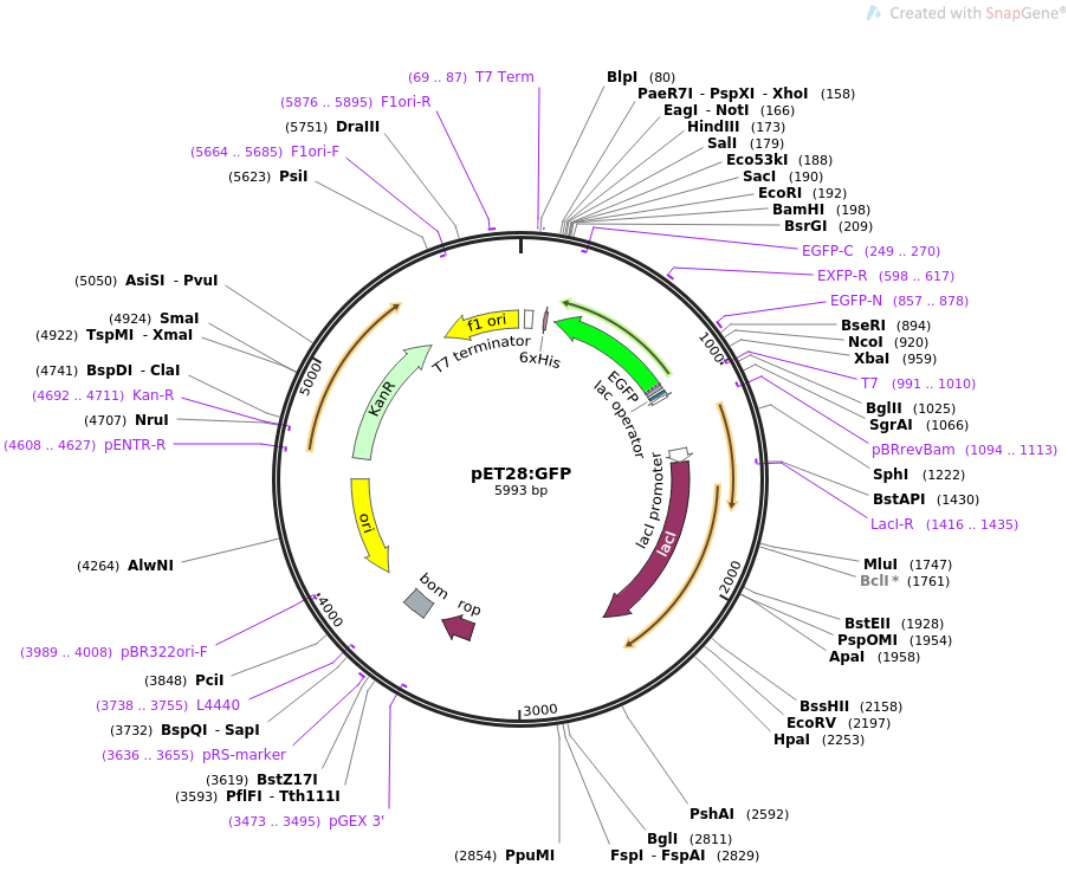


Figure 2.6. pET28a: GFP plasmid vector's genetic map (Addgene plasmid # 60733 ; http://n2t.net/addgene:60733 ; RRID:Addgene_60733)

2.13 Biofilm quantification

The mutants and the wild type S. Typhimurium were grown at 37°C with shaking at 200 rpm for 18 hours in a 5 mL of LB broth without NaCl. The medium contained the antibiotics (100 μg/ml ampicillin and 50 μg/ml kanamycin) whenever required. When the OD_{575} reached to 0.2, 30 μL of the culture was mixed with 100 mL of LB broth without NaCl and transferred to a 96 wells polystyrene micro-dilution plate. The microdilution plates were allowed to incubate for 24, 48 and 72 h for mature biofilm formation at 20 °C. The plates were then stained with crystal violet (CV) staining (130 μl per well from a 1% CV solution) and dried at room temperature. After washing the plates three times with sterile ddH2O, 130 μl of ethanol: acetone solution (70:30% w:w) was added into each well. After incubation at room temperature for 30 min, biofilm quantifications were performed at OD_{595} using an Elisa reader (Molecular Devices SpectraMax M2 Microplate Reader, USA) (Vestby et al., 2009). The experiments were performed to include 3 biological parallels and 2 technical replicates.

2.14 Attachment to Caco-2 and Hep-2 cell cultures

HEp-2 and Caco-2 cells were incubated for 4-6 days at T75 flasks respectively at 37°C, in the presence of 5% of CO_2 in the environment in DMEM (LONZA-12-614F) with high glucose including 10% and 20% inactivated fetal calf serum, 1% of L-glutamine, 1X non-essential amino acid (Biological Industries, 013401B), 20µg/mL of penicillin-streptomycin and 5 µg/mL of plasmocin (Invivogen, France).

The adhesion of the bacterial strains to Caco-2 and HEp-2 cells was performed as described by Hancox *et al.* (1997). Shortly, Caco-2 and HEp-2 monolayers in 6-well tissue culture plates were washed 3 times with sterile PBS (Phosphate Buffered Saline) to get rid of the medium and then, bacterial cultures (10^9 CFU/ml) were transferred onto the cells at a 1000:1 ratio (bacteria/cell). These plates were incubated at 37 °C under static conditions for 30 min, 60 min and 90 min, respectively, in an environment of 5% CO^2. At the end of the incubation period, the washing with PBS was carried out to remove the non-attached bacterial cells and 1 ml of Tween 80 was added to the each well of the plate. Following the incubation for 30 min, the bacterial suspensions in the wells were taken into sterile microcentrifuge tubes, diluted to 10^{-7} and inoculated onto LB agar plates. The number of bacteria adhering to Caco-2 and HEp-2 cells was determined by counting the colonies at the end of 18 h incubation under static conditions at 37 °C. Experiments were carried out in 3 biological parallels and 2 technical replicates. The attachment and colonization of bacteria tested for Caco-2 and HEp-2 cells have also been visualized by confocal laser microscopy (Zeiss lsm 510, Germany) as described before. For this purpose, the plasmid pET28aGFP that provides green fluorescent protein expression was transformed to the wild type and mutant strains tested in the study. Flow chambers were seeded with Caco-2 and HEp-2 cells and the cells were stained with 1 µM cell tracker orange (CMTMR, Invitrogen, C2927). CLSM images were captured by using a Zeiss Ism 510 device at Bilkent University, UNAM facility.

After incubation, the cells were washed with PBS (Biological Industries, 020231A) and then treated with 0,25% / 0,02% of Trypsin-EDTA (Biological Industries,030501B) at 37 °C for 4 to 5 minutes. On the top of the lifted cells, 10 mL of cell specific medium was added for inactivating trypsin. After that, the transferred cells to new falcon tubes were centrifuged at 1500 rpm for 5 minutes. After centrifuging, the supernatant was removed and the pellet was dissolved in an appropriate medium without antibiotic.

100 µl of cell was taken from the suspension and transferred to a clean micro-centrifuge tube, 100 µl of Trypan Blue dye (HyClone, C838W29) was added on it. 10 µl of the dye used for separating the viable and dead cells from each other, a lamella was placed on it then it was transferred to Thoma slide and the cell counting was completed with hematocytometer.

For this purpose, in the 10X objective, the viable and dead cells which were in the four corner squares and in one middle square including 10^{-4} of suspension were counted. For determination of the cell number, the required calculation was done by this way;

Total viable cells: **A**

Total dead cells: **B**

(A/B) x 100 = % viable cells

Mean cell per square **A/5**

Dilution factor: 100µl dye + 100µl cell =200µl so, 200/100= **2**.

Cell concentration (cell/mL): mean cell per square x dilution factor x 10^4

In this way, the cell number in 1 mL was determined and from this knowledge the cell inoculation was done to 4 wells tissue culture slides (BD Falcon, 354112) as having 1-2x10^5 of cell number (Figure 2.6) The cells were incubated at 37°C in the presence of 5% of CO_2 for 24 hours. After the developed cells were washed with

PBS for 2 times and dyed as treated with 1µM of CellTracker™ Orange (CMTMR Invitrogen, C2927) for 30 minutes.

After dying process, the culture including 10^7 CFU/ml of bacteria induced with IPTG (Bioshop, IPT001.10) for 16 hours added to cells. The slides added bacteria incubated again at 37°C in the presence of 5% of CO_2 for 24 and 48 hours. After stated incubation period of time completed, the slides were washed with PBS for 2 times for removing the unattached cells.

After washing process, the Vectashield® Mounting Medium (Vector laboratories, H-1200) was added to samples and the slides were covered with object slides and it was ready to be visualized. To prevent fading of GFP light, the slides were placed in a box and kept in dark till visualization.

Figure 2.7. Tissue culture slides for confocal laser microscopy testing

CHAPTER 3

3 RESULTS AND DISCUSSION

3.1 Obtaining 14 target gene mutants in wild type *Salmonella* strains

By using Red/ET recombination system, mutations were done in the 14 target genes. The genes and the sequences of target gene-specific primers are given in Table 2.3. The confirmation of the recombination of the kanamycin gene cassette into the target genes was done by amplifying 193 bp fragments from each mutant (Figure 3.1).

3.2 The transformation of $pBAD_{24}$ expression vectors having arabinose inducible promoter into *Salmonella* Typhimurium 14028 kanamycin-resistant, target gene mutants

marT gene was downstreamed to the arabinose- inducible *E. coli* BAD promotor which was already cloned in $pBAD_{24}$ vector. The purpose of this cloning was to control *marT* expression by using L-arabinose. The resulting $pBAD_{24}$ vector carrying ampicillin resistance gene was transformed into kanamycin-resistant, target gene inactivated mutants and for the further experiments, the transformants were selected in LB agar media containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL). Plasmid isolation verified the transformation of the mutant strains.

3.3 The cleavage of pBAD$_{24}$ plasmid with restriction endonuclease enzymes (*EcoR1* Fermentas EU)

S. Thyphimurium mutant strains containing PBAD$_{24}$/*marT* and pBAD$_{24}$ vectors were cleavaged with endonuclease restriction enzyme *EcoR1*. The restriction enzyme that cleaved within or very close to its recognition sequence is active at single-sites.

3.4 Examining target genes' expressions transcriptionally after inducing *marT* gene through arabinose

On the 14 target genes and on *marT* gene itself, the regulatory effect of *marT* was examined through stimulating MarT protein production as adding arabinose into media at a last concentration of 0.01% after transforming pBAD$_{24}$ plasmid having *marT* gene previously downstreamed to arabinose promoter into the *S*. Typhimurium 14028 *marT* gene mutant strains. For the next step, the total RNA isolation was materialized from the wild type *S*. Typhimurium, MZ1627 (Δ*marT*/ pBAD$_{24}$+*marT*) and MZ1629 (Δ*marT*/ pBAD$_{24}$) mutant strains. They were stimulated by the 0.01% of arabinose concentration. RNA isolates subjected to cDNA synthesis, the primers belong to target genes were used and the obtained cDNAs were multiplied through QRT-PCR.

To examine the *marT* gene's regulatory effect on the 14 target genes and on itself MarT protein production was activated through adding arabinose (0.01%) into media after transforming $pBAD_{24}$ plasmid having *marT* gene downstreamed to arabinose promoter into the *marT* gene mutant strains (MZ1627).

Followed by induction of *marT*, the increased and decreased expression levels of the target genes were agreed to be regulated positively and negatively respectively. After acquiring the expression values of each target gene through qRT-PCR study, their Ct (threshold cycle) values were normalized by proportioning the expression level of the housekeeping gene (16S was used as a housekeeping gene).

The qRT-PCR findings showed that, in the presence of arabinose induction (0.01%), all of the target genes' expressions increased significantly (figure 3.12). The collected data pointed out that the increased expression levels of the genes are being regulated upon the arabinose induction of *marT*. The data shows that, the five of the target genes (*rmbA, stjC, fimF, fliZ* and *fimH*) have higher impact in their expression depending on the changes in *marT* gene expression.

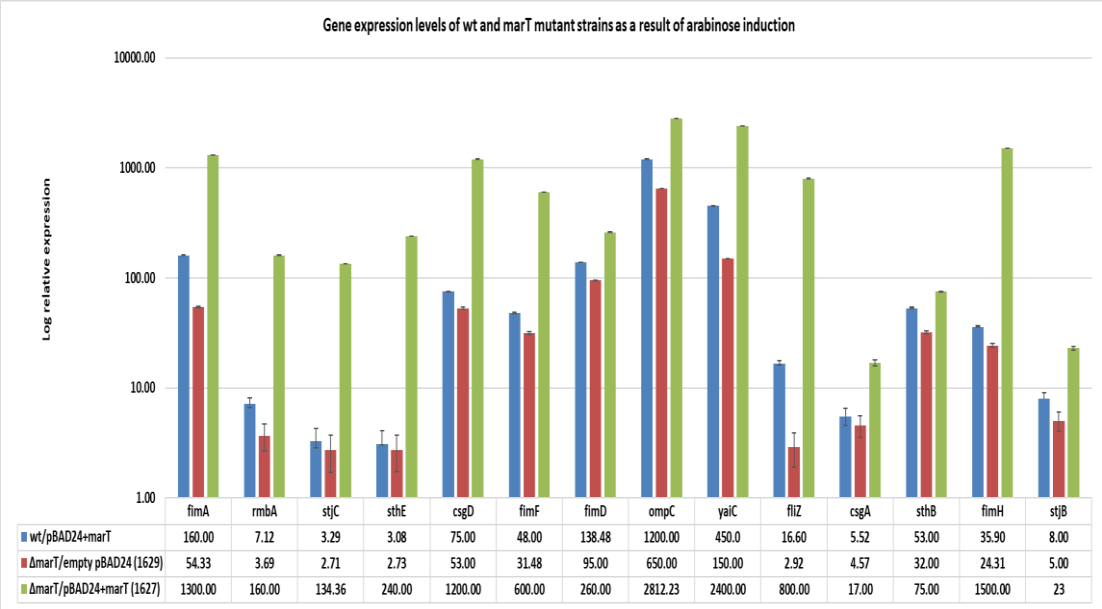


Figure 3.1. Comparison of the gene expression in *S*. Typhimurium wild type and *marT* mutant (*S*. Typhimurium 1629) and *S*. Typhimurium 1627 strain in the presence and absence of arabinose induction, as determined by QRT-PCR

3.5 Abilities of biofilm production of wild type and target gene mutant strains

The confirmation of amplification of 193 bp fragments of recombined kanamycin Fgene cassette in the target genes was done as using the site-specific primers provided from the Quick & Easy *E. coli* Gene Deletion kit. The amplified PCR product sizes verified the presence of kanamycin gene cassette into the target genes (figure 3.1). The goal is controlling the transcriptional expression through gene specific primers.

As considering wild type *S.* Typhimurium 14028 strains, the studied 15 genes (including *marT* gene) showed early expression in the growth cycle however, this result was unexpected in mutant strains. The results showed that, the reason why the gene specific primers were used in amplification, there were no mutant genes amplified, yet in *S*. Typhimurium 14028 wild type strains, these genes expressed early (figure 3.13). It was clear that the mutations have worked productively.

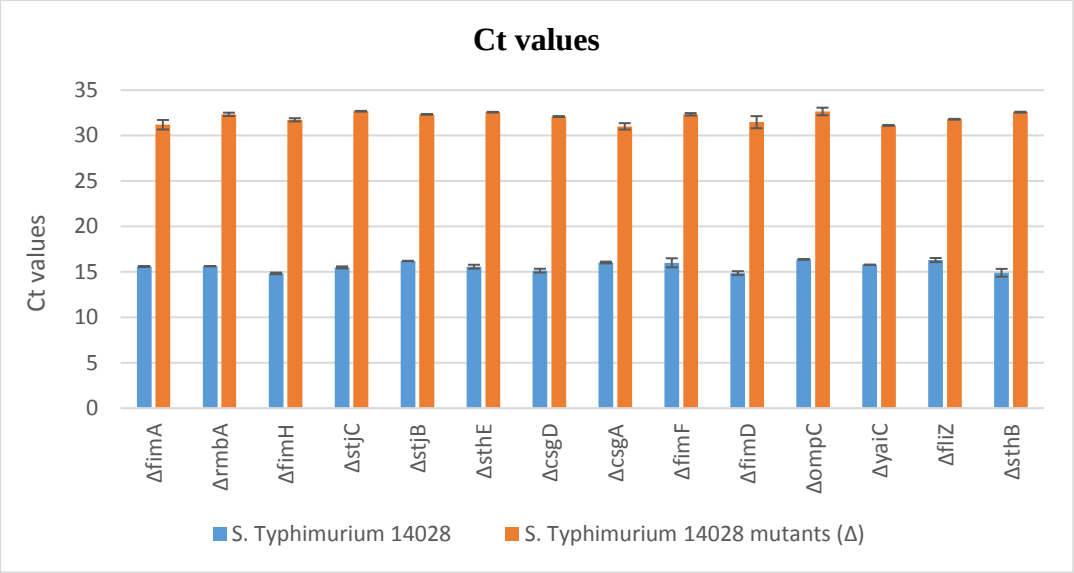


Figure 3.2. The values of cycle threshold (Ct) of target genes in wild type *S.* Typhimurium 14028 and its mutants.

The kanamycin resistant mutants and the wild type strain of *S.* Typhimurium 14028 were examined with regard to their biofilm production characteristics on polystyrene surfaces and the results showed that, in the absence of the target genes which have an individual role in biofilm production examined through microarray studies previously, there was a noteworthy decrease in the capacity of biofilm production after 24 and 48 hours of incubation.

In the absence of two of the target genes, *csgA* which encodes a large subunit of curli fimbria and *csgD* which is accepted as main regulator of biofilm forming in *Salmonella*, a remarkable reduction was seen when the results were compared with the wild type *S.* Typhimurium 14028. The results clearly illustrated how important these two genes in biofilm production. When they were blocked, a significant reduction occurred in biofilm forming.

When the comparison between the mutants and the wild type strain was done about their biofilm production capacities depending on incubation hours, a great reduction was recorded in 24 and 48 hours (figure 3.14).

Furthermore, as it is seen in figure 3.14, after 72 hours of incubation there is a decrease in biofilm production capacity in wild type *S*. Typhimurium 14028. The biofilm cells appear starting breaking down at this time. Because of that, the characteristics of biofilm production of all the mutants and the wild type strain were specifically examined at 24 and 48 hours of incubation.

Table. 3.1. Biofilm production characteristics of wild type S. Typhimurium 14028 and its mutants.

Biofilm production characteristics of wild type *S.* Typhimurium 14028 and its mutants															
Avarage-NC															
	ΔsthB	*ΔfimD*	*ΔstjB*	*ΔcsgA*	*ΔyaiC*	*ΔompC*	*ΔfimA*	*ΔrmbA*	*ΔfimF*	*ΔsthE*	*ΔfimH*	*ΔstjC*	*ΔfliZ*	*ΔcsgD*	*WT 14028*
24 hrs	0.15375	0.290625	0.293	0.1165	0.304875	0.19125	0.398	0.366	0.338375	0.19075	0.387375	0.30475	0.487375	0.139625	0.56625
48 hrs	0.4265	1.296875	1.05475	0.139375	1.444125	1.22	1.33225	1.655875	0.7605	1.0505	1.457875	1.50175	1.840375	0.1515	2.1935
72 hrs															1.535375

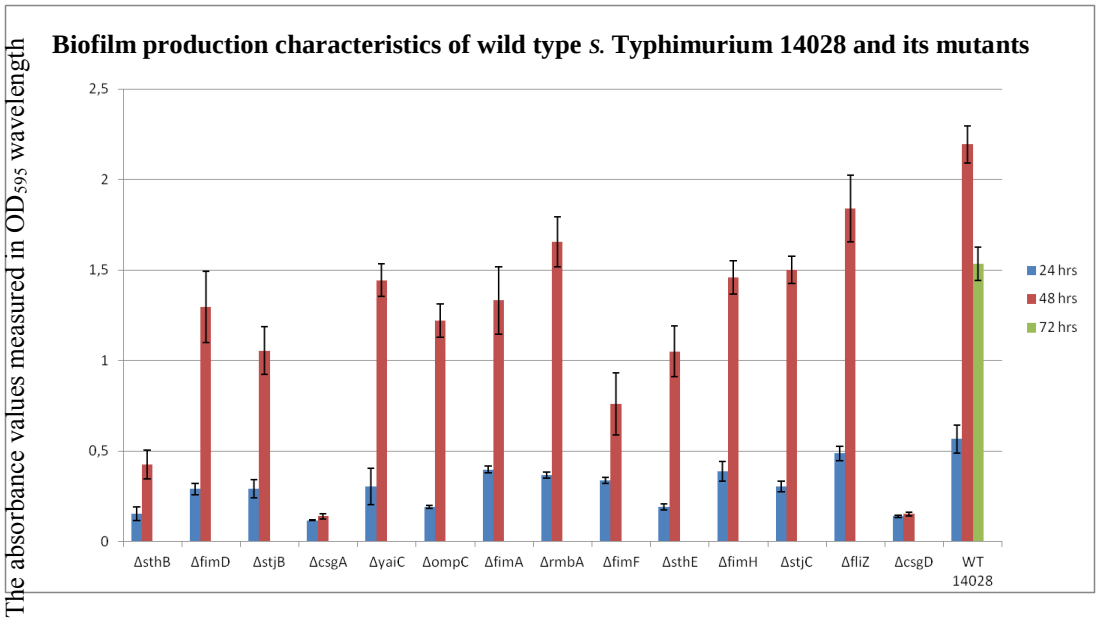


Figure 3.3. Biofilm production characteristics of wild type *S*. Typhimurium 14028 and its mutant strains.

3.6 Adhesion characteristics of wild type and mutant strains on HEp-2 and Caco-2 cells

Adhesion ability to the epithelial cells of wild type and mutant strains were examined as using Caco-2 and HEp-2 monolayer cells. Firstly, the cells were examined microscopically and then the culture plates were covered with a single film. Finally, the bacterial experiment was completed (figure 3.15 and figure 3.16). Adhesion characteristics were verified after 30, 60 and 90 minutes of incubation for each sample.

When the results were examined, it was clear that binding to Caco-2 and HEp-2 cells were unavoidable for both wild type and mutant strains within very short incubation times (30,60,90 minutes respectively). Also, the results showed that, with the increase of incubation time, the number of attached cells increased. In addition to this, the level of adhesion which is directly related to virulence capacity of wild type and mutant strains did not show any notable difference (table 3.2 and 3.3). Figures 3.15 and 3.16 show the cell density of Caco-2 and HEp-2 cells under light microscope. It is important to examine the cell density right before using them for any study. The optimal density of the cells should be between 50-60%.

Caco-2

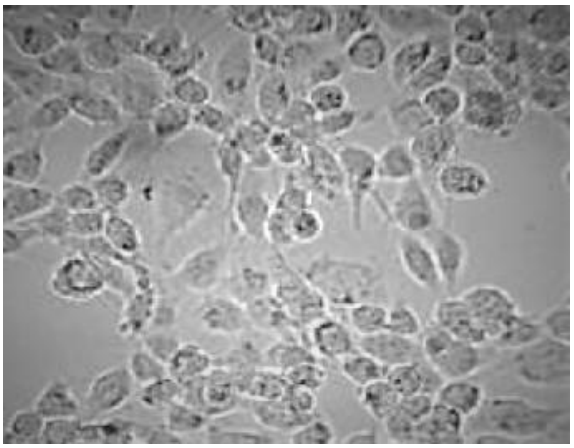


Figure 3.4. Light microscope image of Caco-2 monolayer (Leica, Type 090-135.001, X40).

HEp-2C

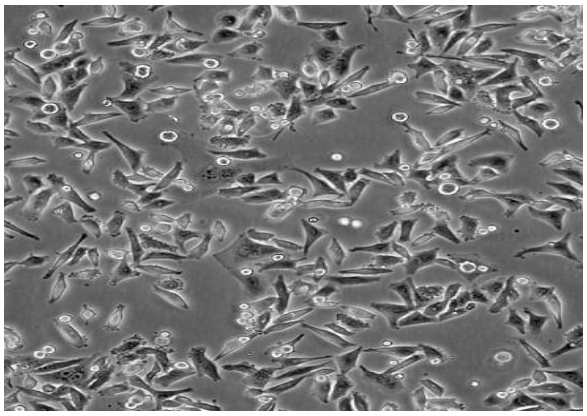


Figure 3.5. Light microscope image of Caco-2 and HEp-2 monolayers (Leica, Type 090-135.001, X40).

Table 3.2. Numbers of attached bacterial cells to Caco-2 monolayer cell lines

Wild Type Strai And Mutants	**Initial Bacterial Cell Numbers**	**Bacterial Cell Numbers After Adsorption to Caco-2 Cells (Cfu/mL)**		
		30 min	**60 min**	**90 min**
***S.Typhimurium* 14028**	$3{,}5 \times 10^7$	$1{,}5 \times 10^3$	$6{,}3 \times 10^3$	$4{,}4 \times 10^4$
Δ***ompC***	$2{,}2 \times 10^7$	$1{,}8 \times 10^3$	$3{,}9 \times 10^3$	$4{,}3 \times 10^3$
Δ***fimD***	$2{,}5 \times 10^7$	$2{,}1 \times 10^4$	$2{,}7 \times 10^4$	$3{,}6 \times 10^4$
Δ***csgD***	$3{,}5 \times 10^7$	1×10^3	$6{,}7 \times 10^3$	$5{,}3 \times 10^4$
Δ***fliZ***	$2{,}2 \times 10^7$	$4{,}1 \times 10^3$	5×10^3	$2{,}7 \times 10^4$
Δ***sthB***	$3{,}2 \times 10^7$	$1{,}3 \times 10^4$	$1{,}7 \times 10^4$	$3{,}6 \times 10^4$
Δ***yaiC***	$5{,}7 \times 10^7$	1×10^3	$4{,}2 \times 10^3$	1×10^4
Δ***fimF***	$2{,}6 \times 10^7$	$6{,}6 \times 10^3$	7×10^3	$6{,}5 \times 10^4$
Δ***csgA***	$2{,}2 \times 10^7$	$2{,}5 \times 10^3$	$8{,}5 \times 10^3$	$3{,}2 \times 10^4$
Δ***stjC***	$2{,}6 \times 10^7$	$5{,}3 \times 10^3$	$3{,}5 \times 10^4$	4×10^4
Δ***fimH***	$3{,}3 \times 10^7$	$3{,}1 \times 10^4$	$3{,}2 \times 10^4$	$3{,}4 \times 10^4$
Δ***rmbA***	$2{,}5 \times 10^7$	$2{,}5 \times 10^3$	4×10^3	$5{,}3 \times 10^3$
Δ***sthE***	3×10^7	$1{,}3 \times 10^3$	7×10^3	8×10^3
Δ***fimA***	$3{,}3 \times 10^7$	$2{,}4 \times 10^4$	$4{,}6 \times 10^4$	$5{,}4 \times 10^4$
Δ***stjB***	$2{,}1 \times 10^7$	$3{,}2 \times 10^3$	1×10^4	$8{,}6 \times 10^4$
Δ***marT***	$4{,}7 \times 10^7$	$2{,}8 \times 10^3$	9×10^3	7×10^4

Table 3.3. Numbers of attached bacterial cells to HEp-2 monolayer cell lines

Wild Type Strain And Mutants	**Initial Bacterial Cell Numbers**	**Bacterial Cell Numbers After Adsorption to HEp-2 Cells (Cfu/mL)**		
		30 min	**60 min**	**90 min**
***S.Typhimurium* 14028**	8×10^8	$3{,}6 \times 10^3$	$5{,}4 \times 10^3$	$4{,}4 \times 10^4$
Δ***ompC***	$1{,}7 \times 10^7$	$1{,}7 \times 10^3$	$5{,}5 \times 10^3$	$9{,}2 \times 10^3$
Δ***fimD***	2×10^7	2×10^4	$3{,}42 \times 10^4$	$6{,}1 \times 10^4$
Δ***csgD***	$1{,}5 \times 10^7$	$1{,}5 \times 10^4$	$2{,}2 \times 10^4$	$2{,}7 \times 10^4$
Δ***fliZ***	$3{,}1 \times 10^7$	$3{,}4 \times 10^3$	$3{,}2 \times 10^4$	$5{,}7 \times 10^4$
Δ***sthB***	$2{,}2 \times 10^7$	$1{,}3 \times 10^3$	7×10^3	$5{,}8 \times 10^4$
Δ***yaiC***	$2{,}7 \times 10^7$	$5{,}1 \times 10^3$	$3{,}3 \times 10^4$	$7{,}3 \times 10^4$
Δ***fimF***	$2{,}8 \times 10^7$	$2{,}2 \times 10^4$	$4{,}8 \times 10^4$	1×10^5
Δ***csgA***	$1{,}8 \times 10^7$	2×10^4	$2{,}3 \times 10^4$	$5{,}4 \times 10^4$
Δ***stjC***	6×10^7	$6{,}4 \times 10^3$	$4{,}7 \times 10^3$	$3{,}8 \times 10^4$
Δ***fimH***	$2{,}2 \times 10^7$	$1{,}4 \times 10^4$	4×10^4	5×10^4
Δ***rmbA***	5×10^7	$1{,}5 \times 10^3$	$4{,}4 \times 10^3$	$9{,}8 \times 10^3$
Δ***sthE***	$4{,}3 \times 10^7$	$1{,}8 \times 10^3$	4×10^3	2×10^4
Δ***fimA***	$1{,}7 \times 10^7$	$2{,}2 \times 10^3$	6×10^3	$1{,}8 \times 10^4$
Δ***stjB***	$1{,}5 \times 10^7$	$1{,}2 \times 10^3$	5×10^3	$9{,}3 \times 10^3$
Δ***marT***	$6{,}9 \times 10^8$	$1{,}5 \times 10^3$	$7{,}4 \times 10^3$	$3{,}2 \times 10^4$

This adhesion experiment was also supported with additional experiment with transformation of pET28aGFP vector into wild type and mutant strains. Through this experiment, the cell attachment was able to be visualized as using confocal laser microscopy. The visual results showed that, mutants did not show any remarkable attachment characteristics due to single gene mutations (figure 3.22 and 3.23).

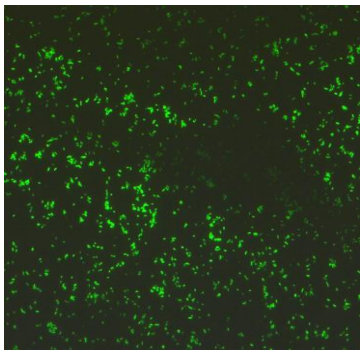


Figure 3.6. *E. coli* BL21 Star (DE3) cells pET28aGFP as control (Zeiss AX10)

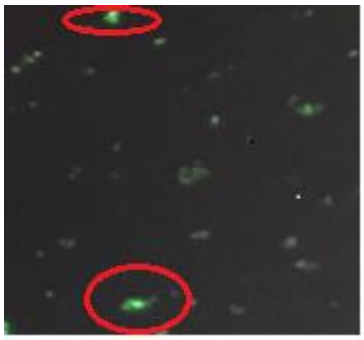
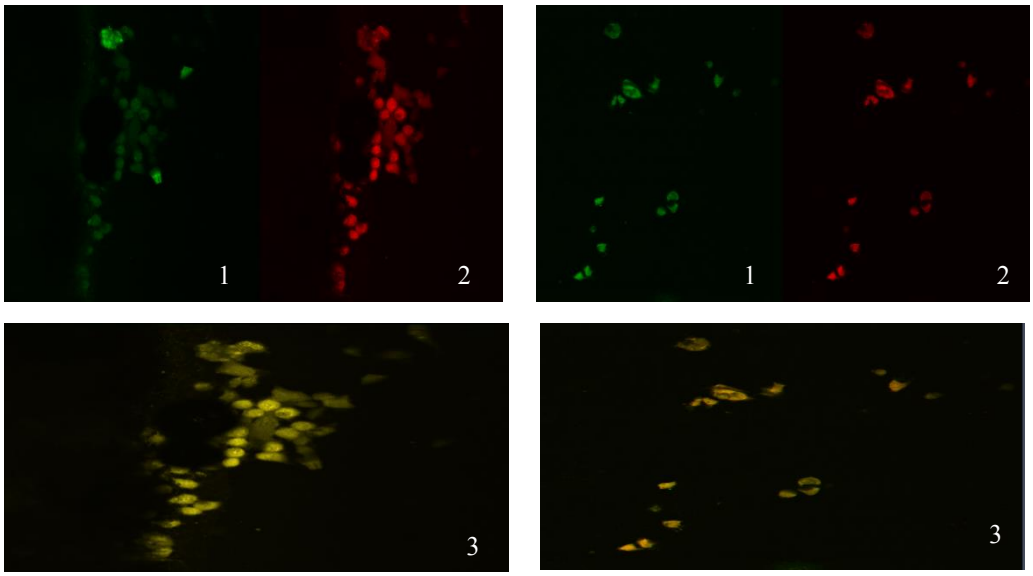


Figure 3.7. Δ*fimD* (pBAD24+ *marT*) cells including pET28aGFP (Zeiss AX10)

24 hours of attachment

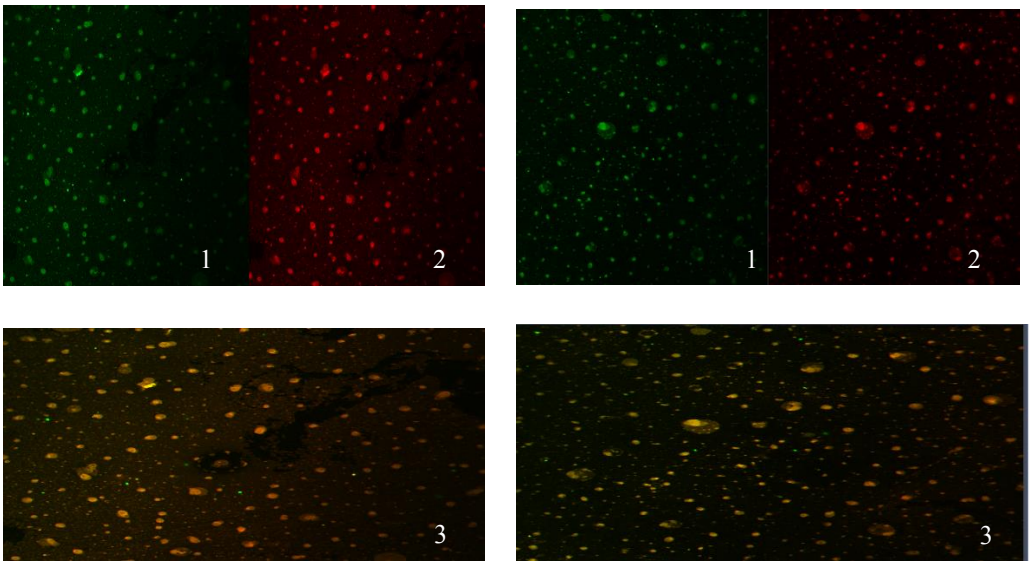
48 hours of attachment



(a) *S.* Typhimurium 14028

(b) *S.* Typhimurium 14028

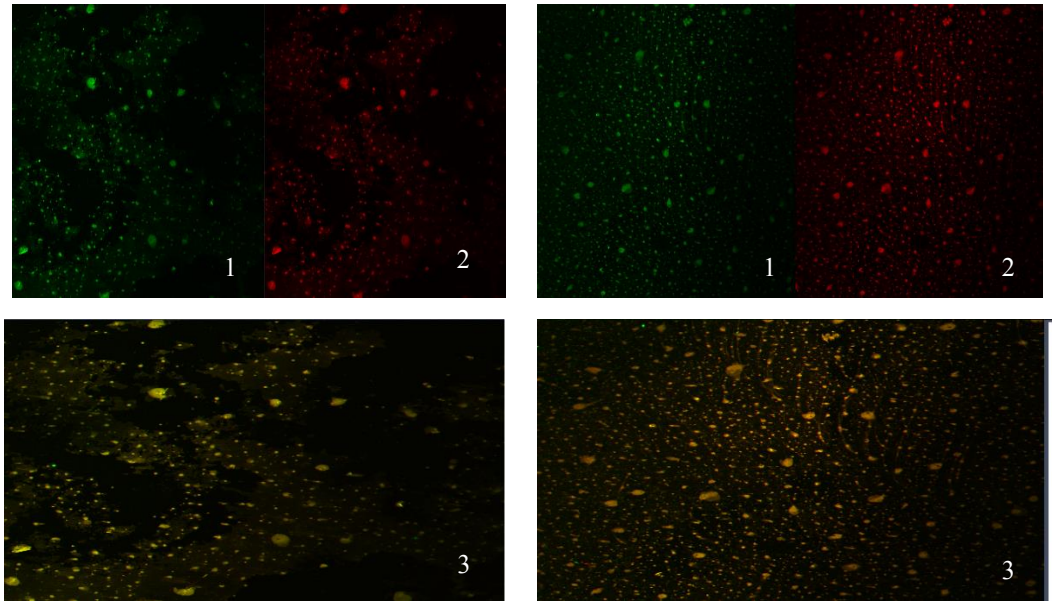
Figure 3.8 Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 (a,b) to Caco-2 cell line. (1: bacteria with GFP expression 2: cell monolayer 3: bacterial attachment to cell line)



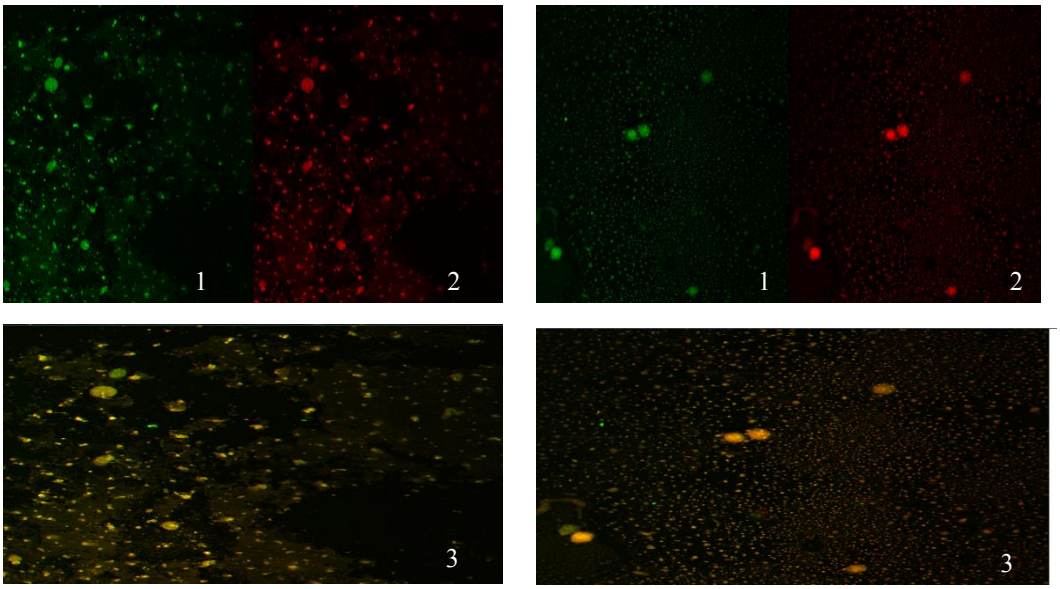
(c) Δ*csgD* (d) Δ*csgD*

Figure 3.22. (continued) Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 mutant (c,d) to Caco-2 cell line.

24 hours attachment 48 hours of attachment



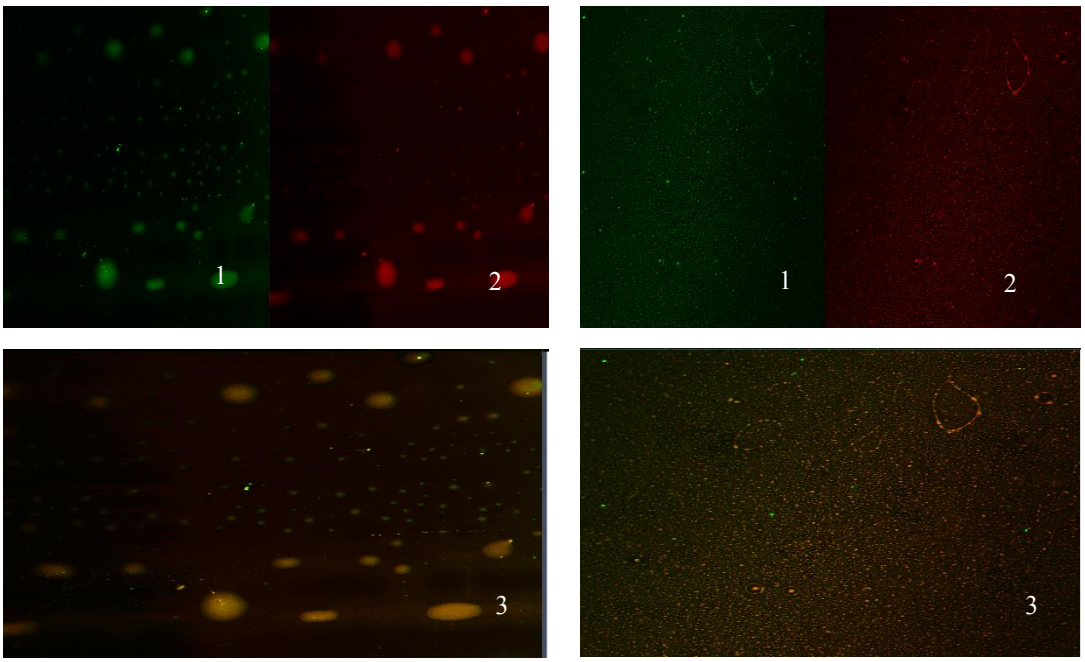
(e) Δ*fimD* (f) Δ*fimD*



(g) Δ*stjC* (h) Δ*stjC*

Figure 3.8. (continued) Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 mutants (e,f,g,h) to Caco-2 cell line.

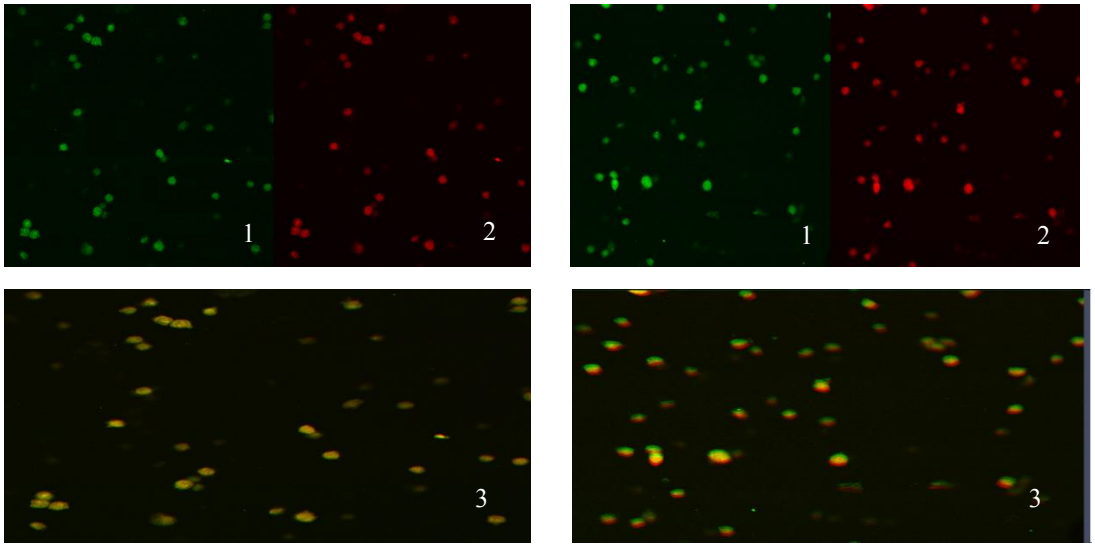
24 hours of attachment 48 hours of attachment



(i) Δ*marT* (j) Δ*marT*

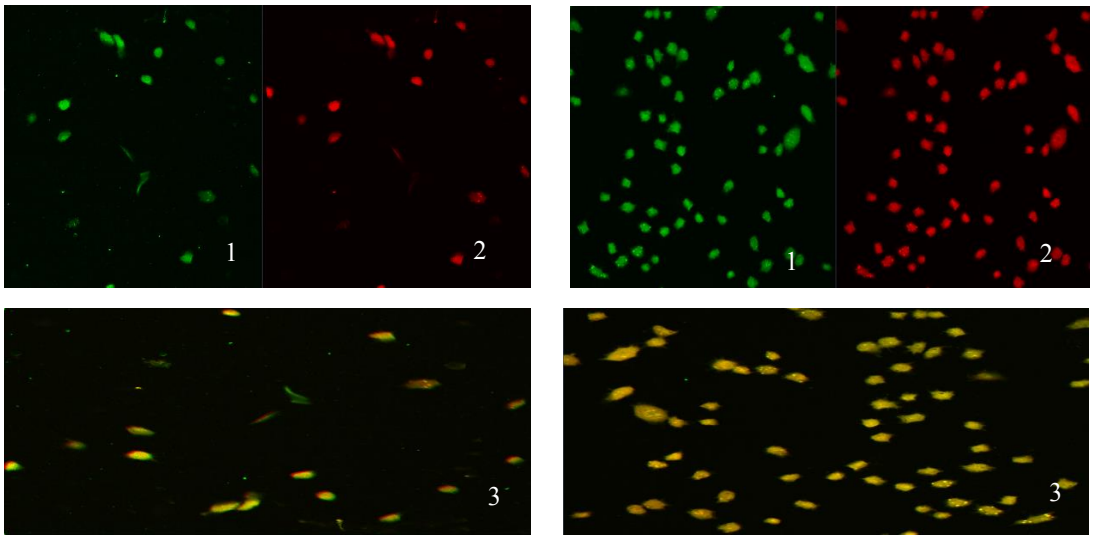
Figure 3.8. (continued) Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 mutant (i,j) to Caco-2 cell line.

24 hours of attachment 48 hours of attachment



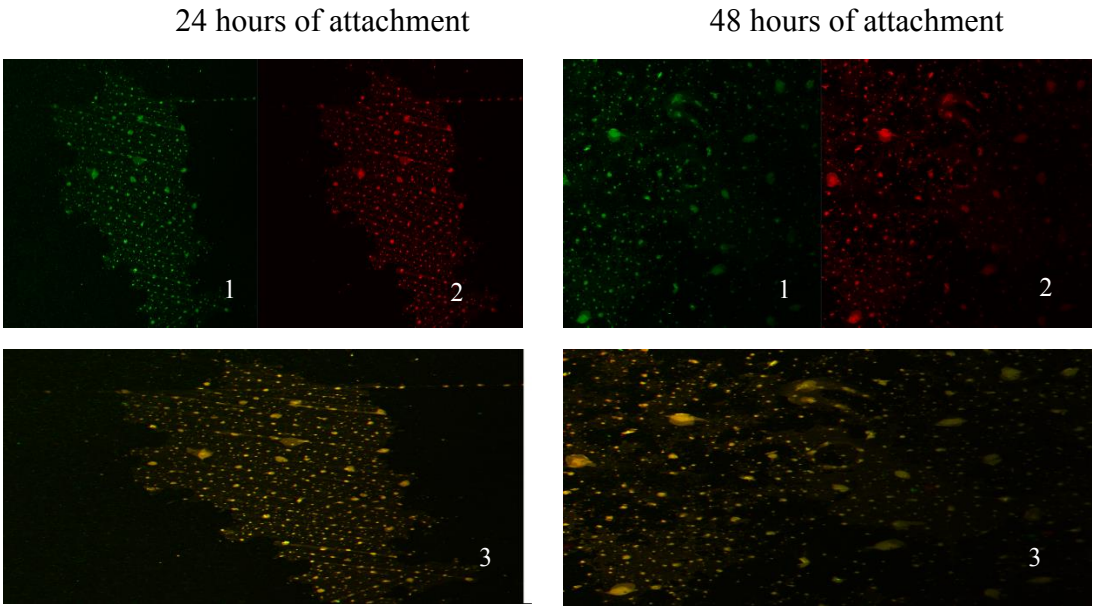
(a) *S.* Typhimurium 14028 (b) *S.* Typhimurium 14028

Figure 3.9 Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 (a,b) to HEp-2 cell line. (1: bacteria with GFP expression 2: cell monolayer 3: bacterial attachment to cell line)

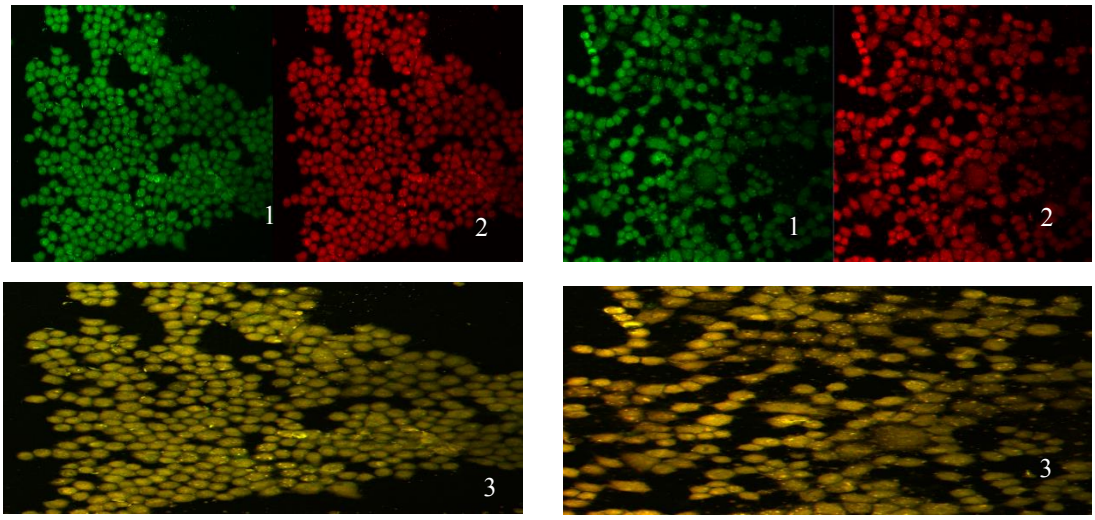


(c) Δ*csgD* (d) Δ*csgD*

Figure 3.9. (continued) Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 mutant (c,d) to HEp-2 cell line.



(e) Δ*fimD* (f) Δ*fimD*

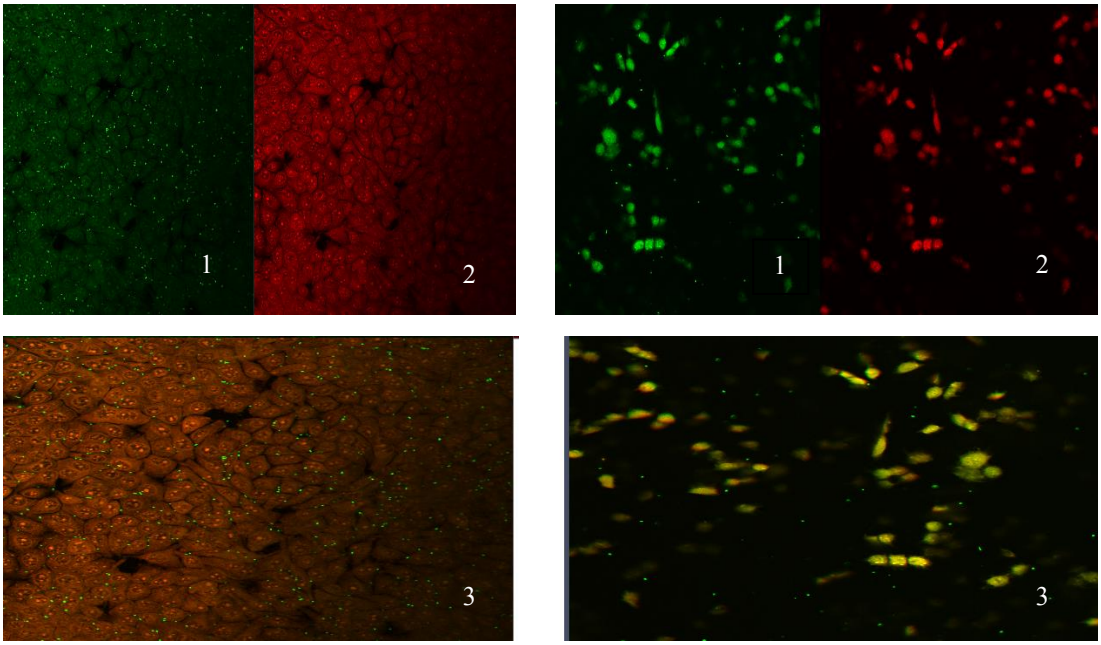


(g) Δ*stjC* (h) Δ*stjC*

Figure 3.9. (continued) Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 mutants (e,f,g,h) to HEp-2 cell line.

24 hours of attachment

48 hours of attachment



(i) Δ*marT*

(j) Δ*marT*

Figure 3.29. (continued) Confocal Laser Microscopic images of adhesion characteristics of wild type *S.* Typhimurium 14028 mutant (i,j) to HEp-2 cell line.

The positive regulation characteristics of *marT* gene for all of the target genes; *fimD, fimA, fimF* and *fimH* (all encode type I fimbrial protein in *Salmonella*) (Zeiner *et al*., 2012) *stjC* and *stjB* (encode chaperon precursors for stj fimbria in *S.* Typhimurim) (Akkoç *et al*., 2009; Yüksel *et al*., 2012), *fliZ* (encodes a regulatory protein which boosts transcriptional activity of class II fimbrial operons again in *Salmonella*), *ompC* (encodes the main outher membrane protein which is needed for attaching to macrophages in *Salmonella*) (Negm and Pistole 1999), *sthE*, *sthB* (both encode the subunits of sth fimbria) (Townsend e*t al*,. 2001; Latasa *et al*., 2005), *csgA* (encodes the protein needed for the main subunit of curli fimria) *csgD* (it is the main regulator of biofilm forming in *Salmonella*) (Liu *et al*., 2014), *yaiC* (encodes diguanylate cyclise) (Cowles *et al*. 2016), *rmbA* (encodes supposed transcriptional protein) *marT* (it is known as the positive regulator of *misL*) were identified through qRT-PCR analysis.

In the light of the study results, it has been understood that, *marT* gene has an important role in regulation of 14 target genes studied which are part of special cellular progress individually and also *marT* acts as their auto-regulator. Some of the transcriptional activities describe that, *marT* gene has multiple effects on phenotypes as taking a part in large number of transcriptional activities which are effective in many metabolic paths (Gottesman 1984; Perez-Rueda and Collado-Vides 2000; Gutierrez-Rois *et al*., 2003; Ishihama 2010).

Positively regulated genes by *marT* which encode proteins have enormous role in *Salmonell*a pathogenicty in fimbria forming, biofilm regulation so on. This knowledge leads us to *marT*, the global regulator in *Salmonella* so; it is needed to be reconsidered the list of the regulators having role in pathogenicity in *Salmonella*.

The experiment results showed that in mutant strains, there was a remarkable decrease in their biofilm production levels after 24 and 48 hours of incubation when compared to wild type strain. The maximum decrease was seen in *csgA* and *csgD* single gene mutants as supposed which are accepted as major positive regulators of biofilm formation in *Salmonella*.

Previously, the roles of the target genes (*csgA, csgD, fimA, fimD, fimE, fimF, sthB, sthE, yaiC and marT*) on biofilm production in *Salmonella* were described (Morgan *et al*., 2004; Ledeboer *et al*., 2006; Crawford *et al*., 2010; Gonzales-Escebedo and Gunn 2013; İlçe and Akçelik 2015; El Hag *et al*., 2017; MacKenzie *et al*., 2017) but in consequence of ongoing works such as mutational assays and biofilm forming experiments it was understood for the first time that, *fliZ, ompC, rmbA, stjB* and *stjC* genes have a straight relation with biofilm production in *Salmonella*. On the other hand, even though there have been some earlier studies about the cellular tasks of the *fliZ* and *ompC* genes, no adequate data about the exact role of the *rmbA* gene in *Salmonella*. It was thought that, this gene (*rmbA*) is a hypothetical regulator in transcription after some of the DNA sequence analyses and bioinformatic studies done (Blanc-Potard *et al*., 1999).

Moreover, in this study, the role of stj fimbria was detected in *Salmonella enterica* serovar Typhimurium biofilm formation. There has been one study reported earlier about the role of stj fimbria in *Salmonella* and based on the mouse model systems, the results showed that stj fimbria has a significant role in boosting the persistence of *S*. Typhimurium (Akçelik *et al*., 2011). In this study, another vital characteristic of the stj fimbria on the virulence of *Salmonella* was portrayed.

With the biofilm forms bacteria can be more persistent than their planktonic forms. They can manage with the competitive intestinal flora and tough conditions effortlessly. After entering the host, bacteria no longer produce flagella; instead, aggregation is started for forming the structure of biofilm. That's how the organism protects itself from rough conditions of the host's systems. Moreover, as it was specified in some earlier studies that, the structures of flagella have an impact on cell invasion in *Salmonella*. In the earlier studies done with Bacillus, Pseudomonas, Vibrio and *E. coli*, it was concluded that, the rotation of flagella is restrained in the beginning of the biofilm production process through blockage of the gene expression (Guttenplan and Kearns 2013). Importantly in our study, *marT* gene has been found as the main regulator of the operons of flagella which takes us to reconsidering the behavioural diversity of the pathogen among in vivo and in vitro phases.

In this study it was also important to understand whether the target genes' activity is significant or not in the attachment of *Salmonella*. That's why Caco-2 and HEp-2 cells were used and the binding of the mutant and the wild type strains to these cells were examined to see whether that target genes have a direct role or not in the attachment. In the earlier studies it was discovered that, there were many factors such as fimbrial adhesins effect to attachment to *Salmonella*. In the outgoing experiments, 35 individual fimbrial operons have been discovered in *Salmonella* serotypes. They may have numerous combinations in *Salmonella* serotypes and they can replace each other. Besides, for colonization and attachment to the host cell, many of the functions needed are unidentified and there is no data about when the expression is generated (De Massi *et al*., 2017).

Another attachment group is built by non fimbrial adhesins. There are some very important adhesins in ABC transporter system such as BapA, SadA proteins, MisL autotransporter protein, EPS (extracellular polysaccharide), LPS (lipopolysaccharides). The fimbrial and non fimbrial adhesisns have no particular role in leading the attachment to the host cell; besides, they can replace each other which imply the great importance of attachment for pathogen. Furthermore, if any of the fimbrial adhesins disappear, the same mission is taken over by others to avoid the harm to the process (Berne *et al*., 2018). With the light of the given literature and the results of the confocal laser microscopy, it was seen that, there is no difference in the attachment of the mutant and the wild type strains of *Salmonella* to Caco-2 and HEp-2 cells.

If we consider the study in respect to its contribution to science and practicability, two important discoveries needed to be told here; firstly, *marT* gene has a role as a positive transcriptional regulator on 14 individual genes/operons in *Salmonella* specifically needed for biofilm production and pathogenicity and secondly, *marT* gene can act as a positive auto-regulator. This data might lead to numerous studies to understand the physiology of *Salmonella*, the development of biofilm forming and the regulation process.

One of the most important findingdescribed through this study is that, *marT* gene is *csgD* gene's regulator. *csgD* is well-known as the major regulator of biofilm formation in *Salmonella*. This is the proof that, to give physiological and metabolic responses to the environmental circumstances, organism goes to the biofilm creation path and it is controlled by numerous systems but only some of them are identified.

Identification of the roles of *marT* gene in this work paves the way for development of new approaches such as finding novel chemical control agents for the avoidance of the biofilm formation. Rather than the discovery of specific inhibitors to interfere with the formation of each of the biofilm related elements, biofilms can better be controlled through a single specific inhibitor targeting the global regulator MarT.

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APPENDICES

A. Composıtıon and Preperatıon of Culture Medıa

LB (Luria-Bertani) Broth and Agar

Tripton	10 g
Maya Extract	5 g
NaCl	10 g
Destiled water	1000mL
Agar	15 g

The ingredients were solved with the addition of distilled water (ddH_2O) and the volume completed to 1000 ml. Before sterilization pH was arranged to 7.0 ± 0.2. After the liquid media distributed to the glass tubes and the rigid media prepared after agar addition, they were sterilized in the autoclave.

LB (Luria-Bertani) Broth without NaCl

Tripton	10 g
Maya Extract	5 g
Distilled water	1000mL

The ingredients were solved with the addition of distilled water (ddH_2O) and the volume completed to a L.. Before sterilization pH was arranged to 7.0 ± 0.2.

B. Buffers and Stock Solutıons

Tris (Tris Hydroxymethyl Amino Methane)/ EDTA
(Ethylene Diamine Tetra Acetic Acid) Solution

Tris	1.21 g
EDTA	0.37 g
Distilled water	1000 mL

The ingredients were solved with the addition of distilled water (ddH_2O) and the volume completed to 1000 ml. Before sterilization pH was arranged to 7.0 ± 0.2.

PBS (Phosphate Saline Buffer)

NaCl	8 g
KaCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled water	1000 mL

The ingredients were solved with the addition of distilled water (ddH_2O) and the volume completed to a L. Sterilized in an autoclave..

Saline Buffer

NaCl	8.5 g
Distilled water	1000 mL

Sterilized in an autoclave.

Glacial Acetic Acid Solution

33 % of Glacial Acetic Acid	100 mL

CTAB/NaCL	g/100mL
NaCl	4,1 g
CTAB	10 g
ddH_2O	100 mL

NaCl was dissolved in distilled water and 10 g of CTAB was added onto it slowly. For dissolving CTAB, the temperature was increased to 65 °C and the total volume was completed to 100 mL using distilled water.

Stock Tris-Acetate-EDTA (TAE) Buffer (50X) Acid) Solution

Tris. HCL	242g
Glacial Acetic Acid	57,1 mL
EDTA (0,5 M)	100 mL

Phenol/Chloroform/Isomyl alcohol solvent	mL
Phenol	25 mL
Chloroform	24 mL
Isomyl alcohol	1 mL

Tris/HCl (0.5 M EDTA pH:8)	g/L
Tris	1,21 g
EDTA	0,37g

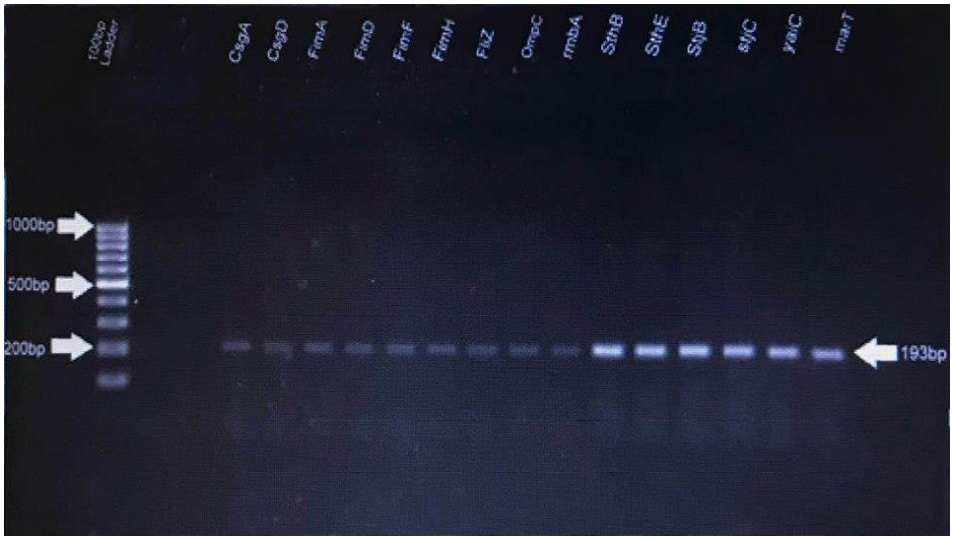
After gradients were dissolved with the addition of distilled water the total volume was completed to 1000 mL. pH 7.0 ± 0.02 (before sterilization) was arranged. Autoclave sterilized.

C. Icons and Chemıcals

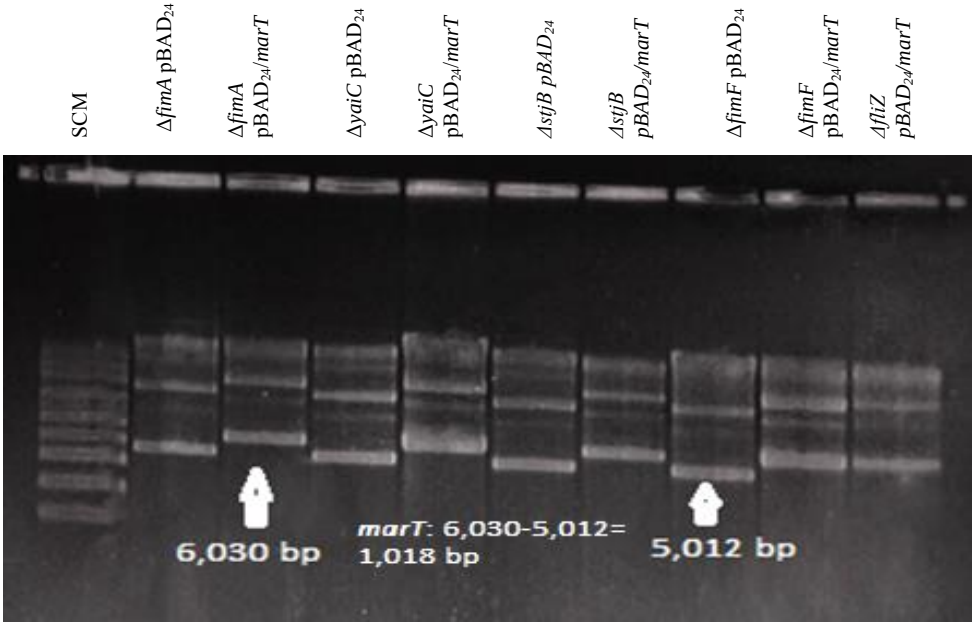
amp	Ampicillin
c-di-GMP	Cyclic-di-guanosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Ct	Treshold cycle
E	Efficiency
-f	Forward primer
-r	Reverse primer
LB	Luria Bertani
log	Decimal logarithm
OD	Optical density
OmpR	Extracellular membrane protein
PBS	Potassium buffer saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time PCR
rpm	Rotation per munite
S.	*Salmonella*
TAE	Tris - Acedic acid - EDTA
Tm	Melting temperature
UV	Ultraviolet
V	Volt
Δ	Mutation
µg	Microgram
µL	Microlitre

Chemicals	**Supplier**
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulfate	Merck
β- mercaptoethanol	Merck
$CaCl_2$ H_2O	Merck
EDTA	Sigma
Ethanol	Merck
Glucose	Merck
Glycerol	Merck
HCL	Merck
NaCl	Merck
NaOH	Merck

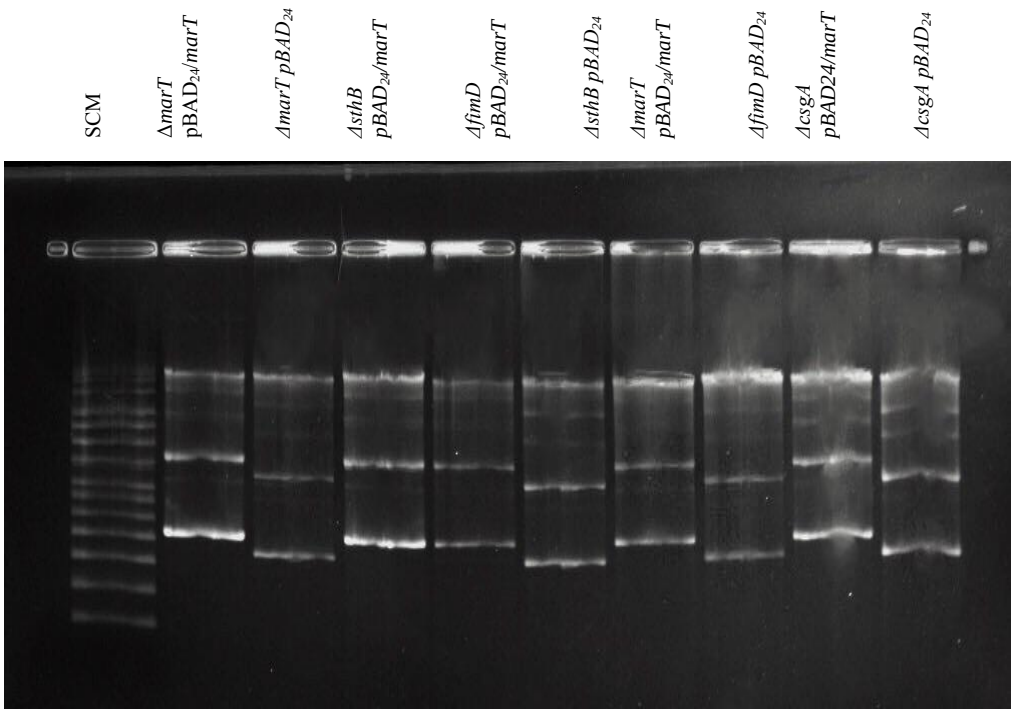
D. Gel Images



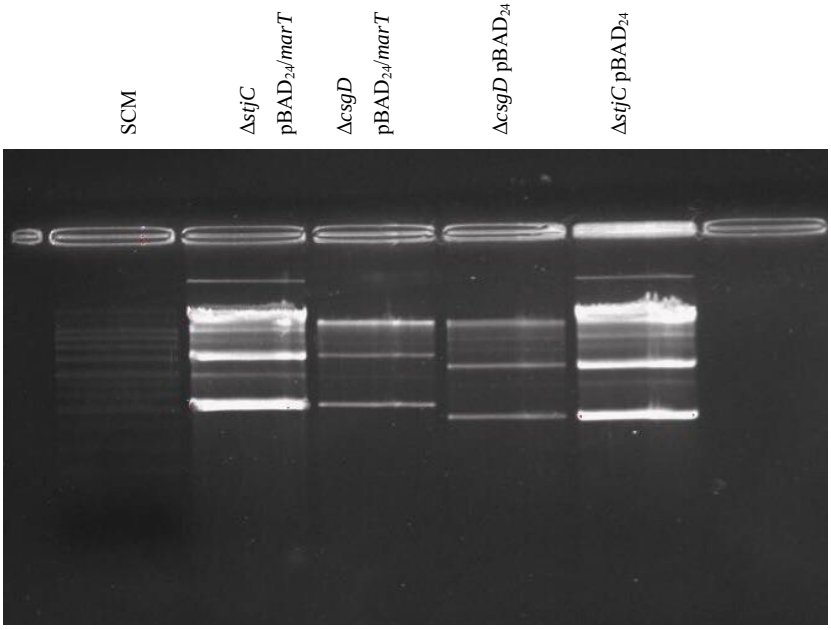
D.1. PCR results (193 bp amplicon) confirming the presence of the kanamycin cassette within the target genes.



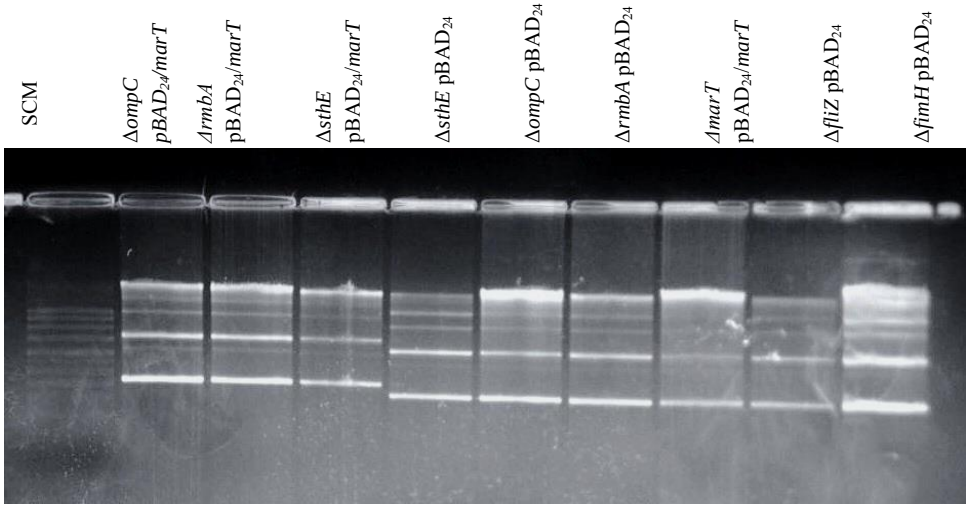
D.2. $pBAD_{24}$+*marT* and the empty $pBAD_{24}$ vectors in *S*. Typhimurium mutant strains.



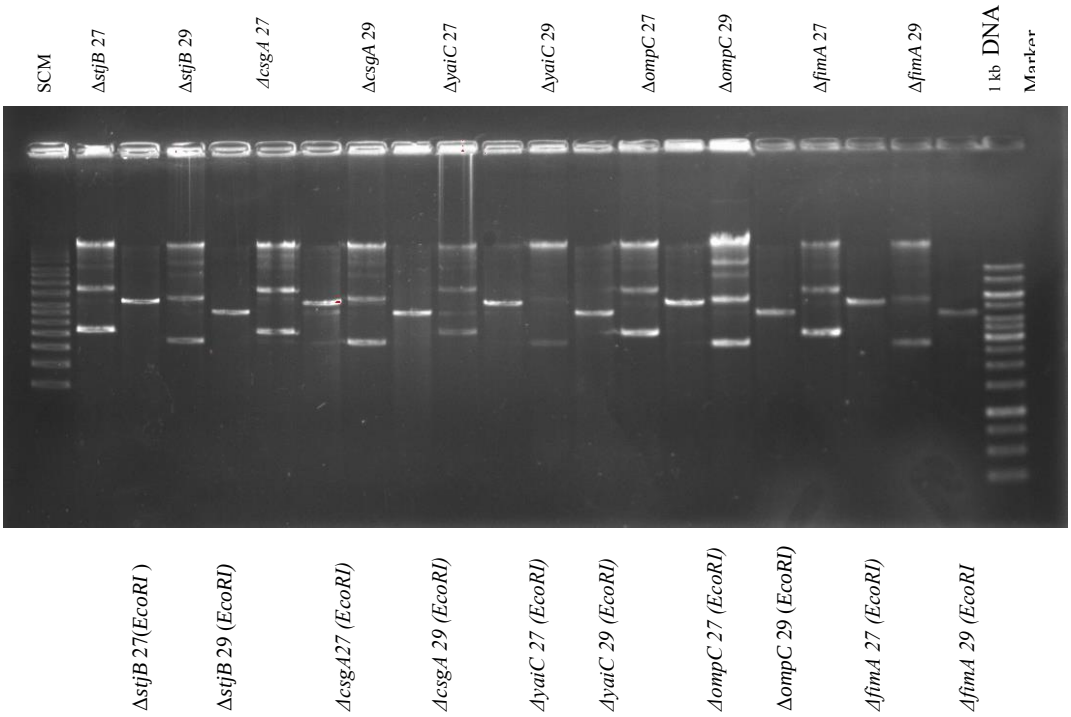
D. 3. pBAD$_{24}$+*marT* and the empty pBAD$_{24}$ vectors in *S.* Thyphimurium mutant strains



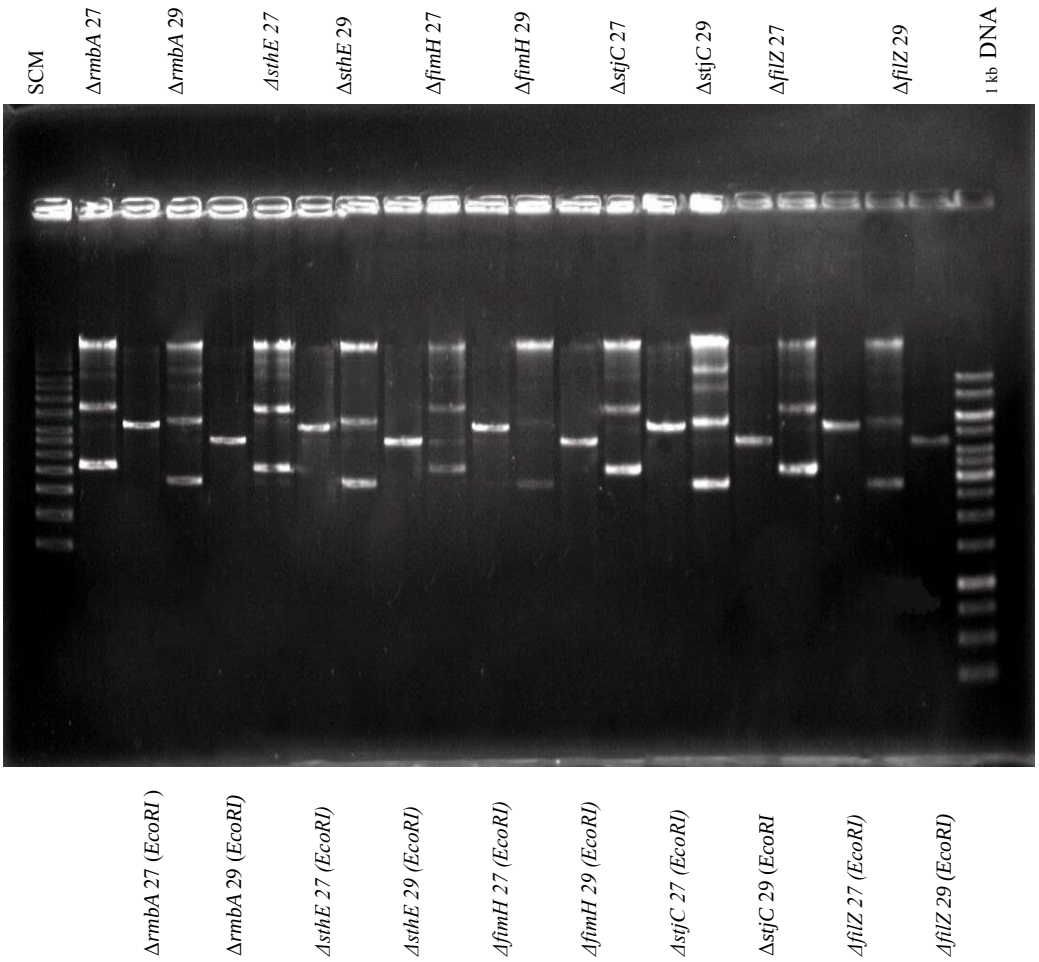
D.4. pBAD$_{24}$+*marT* and the empty pBAD$_{24}$ vectors in *S.* Thyphimurium mutant strains.



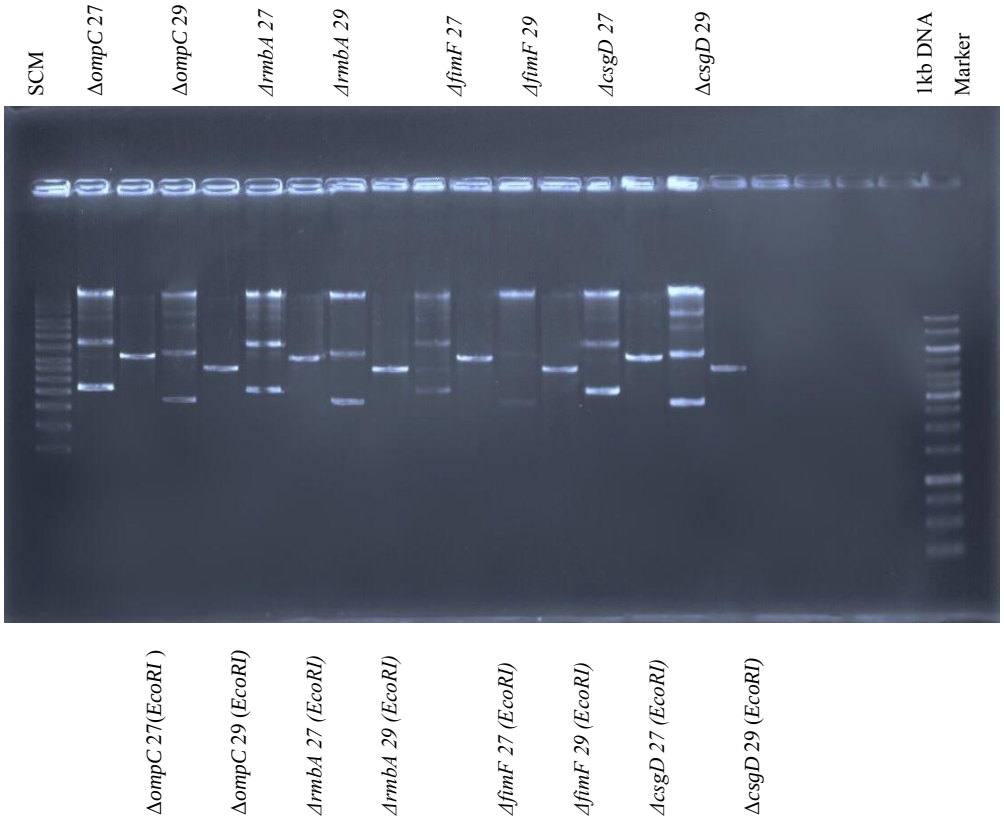
D.5. pBAD$_{24}$+*marT* and the empty pBAD$_{24}$ vectors isolated from *S.* Thyphimurium mutant strains



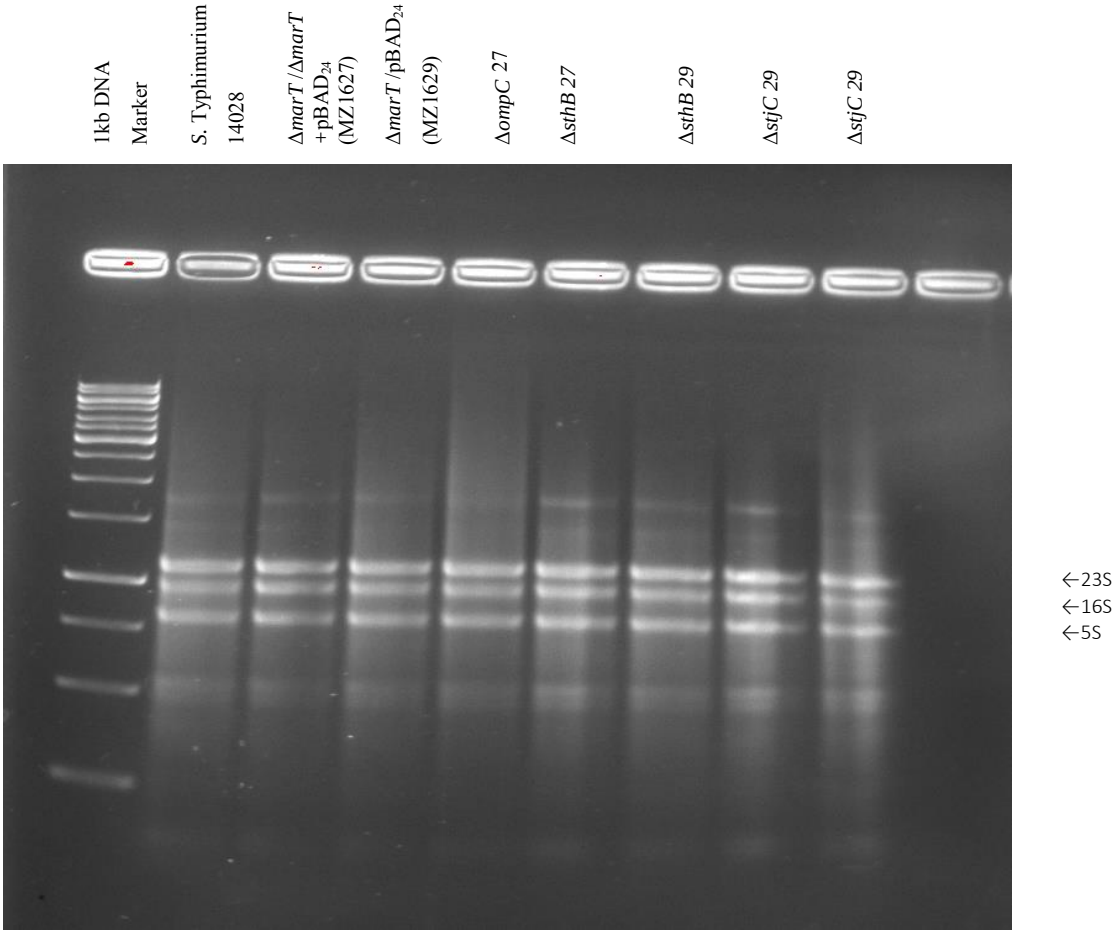
D.6. *S*. Thyphimurium kanamycin resistant, MZ1627 (ΔmarT-pBAD$_{24}$/*marT*) and MZ1629 (ΔmarT-pBAD$_{24}$) mutant strains and the vector cleavage with endonuclease restriction enzyme *EcoR1*.



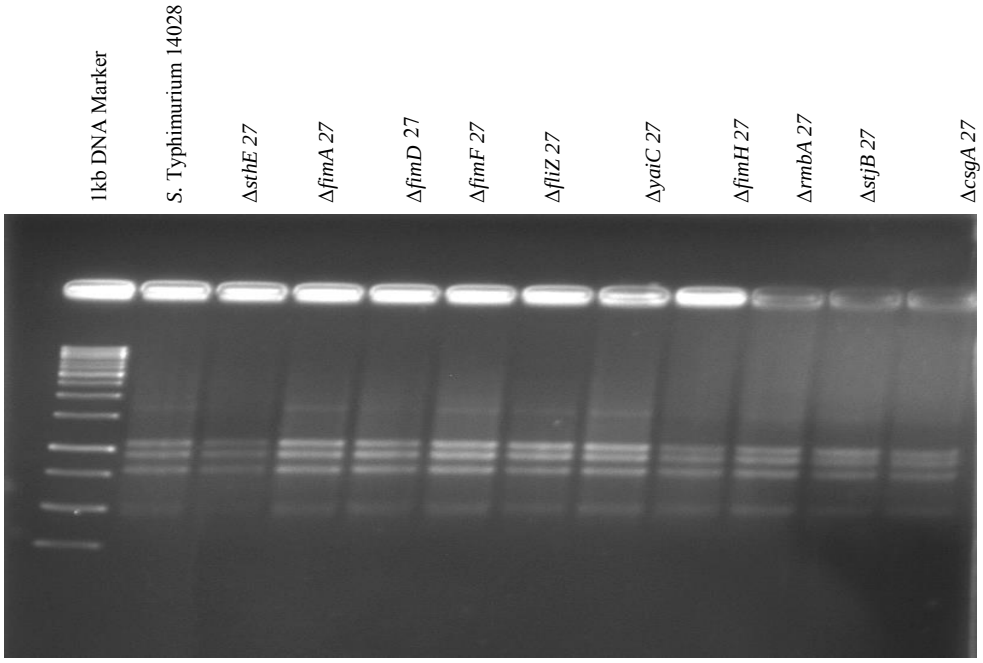
D.7. *S*. Thyphimurium kanamycin resistant, MZ1627 (ΔmarT-pBAD$_{24}$/*marT*) and MZ1629 (ΔmarT-pBAD$_{24}$) mutant strains and the vector cleavage with endonuclease restriction enzyme *EcoR1*.



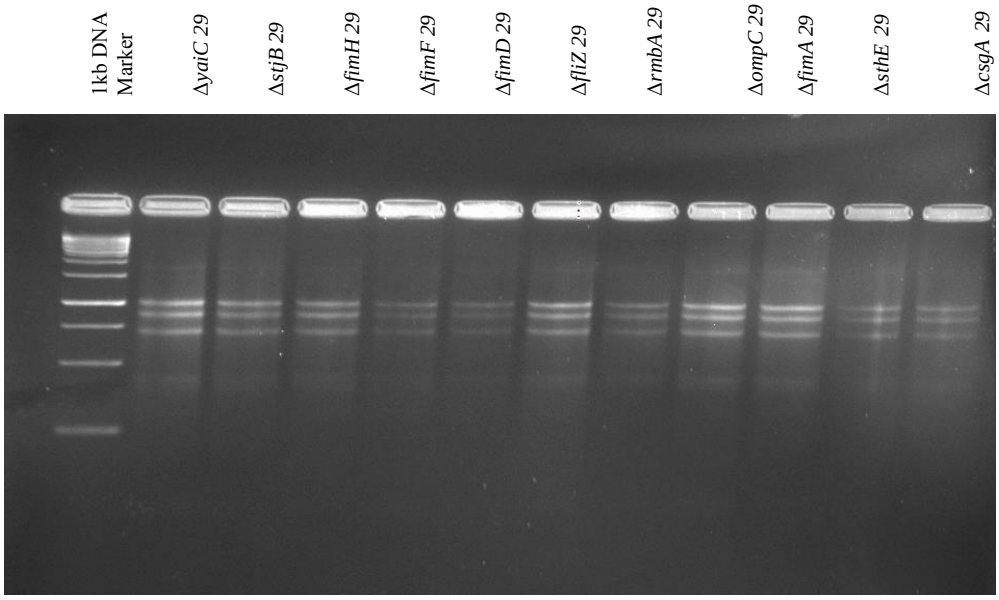
D.8. *S*. Thyphimurium kanamycin resistant, MZ1627 (ΔmarT-pBAD$_{24}$/*marT*) and MZ1629 (ΔmarT-pBAD$_{24}$) mutant strains and the vector cleavage with endonuclease restriction enzyme *EcoR1*.



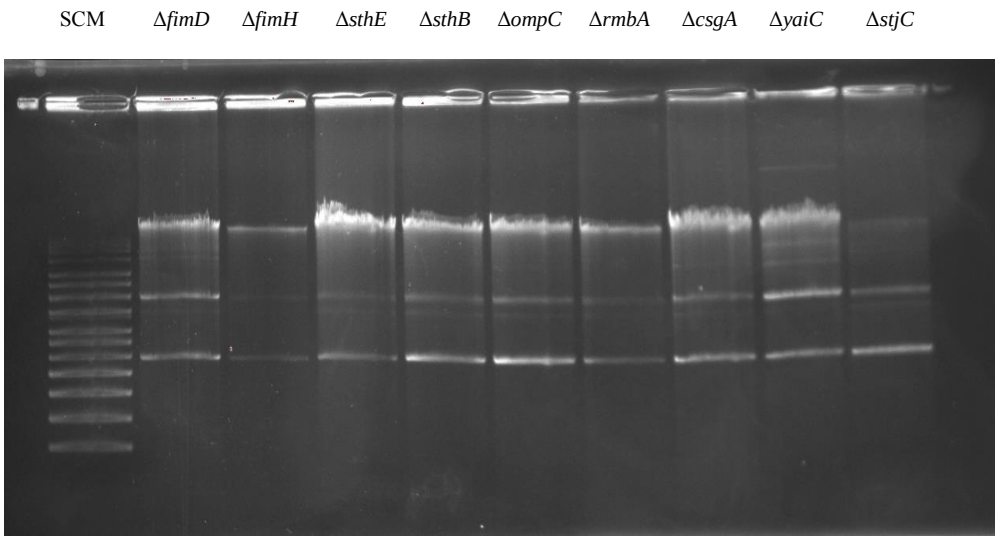
D.9. Gel image of mRNA isolated *S.* Typhimurium derivative, *marT* gene disrupted MZ1627 strains with *marT* gene included plasmid vector $pBAD_{24}$ (Δ*marT*/ $pBAD_{24}$+*marT*) and *S.* Typhimurium derivative, *marT* gene disrupted MZ1629 strains without *marT* gene in the plasmid vector $pBAD_{24}$ (Δ*marT*/ $pBAD_{24}$).



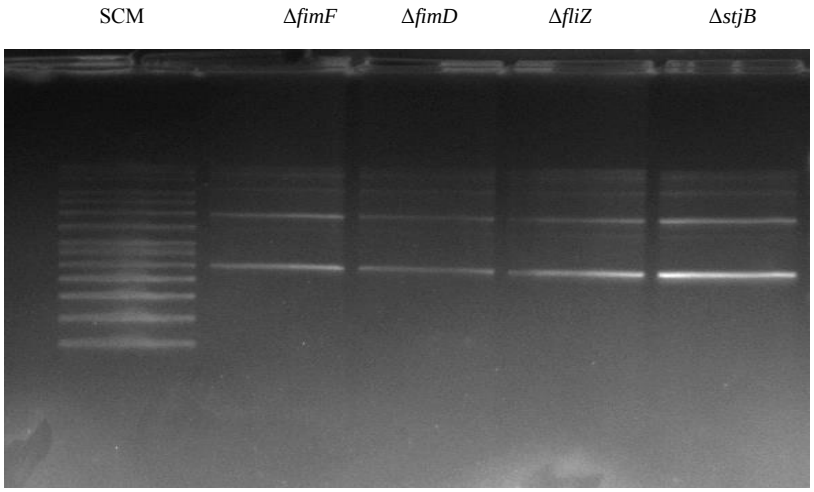
D.10. Gel image of RNA isolated *S*. Typhimurium mutant strains



D.11. Gel image of RNA isolated *S*. Typhimurium mutant strains

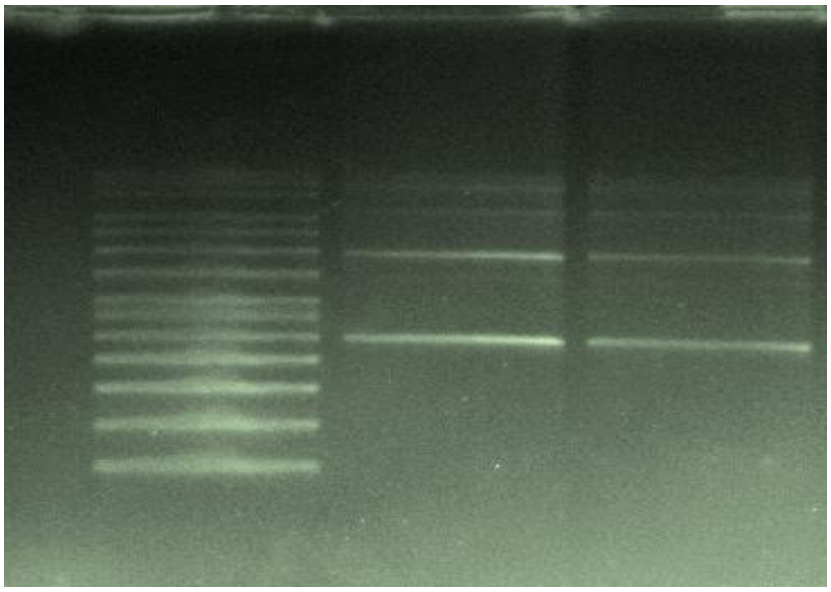


D.12. Gel image of transformed pET28aGFP vector into *S*. Typhimurium mutant strains.



D.13. Gel image of transformed pET28aGFP vector into *S*. Typhimurium mutant strains.

SCM *S.* Typhimurium 14028 *S.* Typhimurium 14028



D.14. Gel image of transformed pET28aGFP vector into wild type *S.* Typhimurium

CURRICULUM VITAE

PERSONAL INFORMATION

Date of birth 26.05.1982
Place of birth Istanbul-TURKEY
Telefon (GSM) +90 5366439169

LANGUAGE

- English (advanced)

EDUCATION

2012- 2020 **PhD**

Middle East technical University Ankara, TÜRKİYE

Biological Sciences/ Molecular Biology and Genetics

(GPA: 3.21/4.00)

- PhD thesis topic: *marT* as a regulatory gene for biofilm formation in *Salmonella* Typhimurium .

Adviser: Prof. Dr. Gülay Özcengiz (Middle East Technical University, Ankara)
Co-adviser: Ass. Prof. Dr. Nefise Akçelik (Ankara University/Biotechnology Institute) Project manager: Dr. Mustafa Akçelik (Ankara University)

2009-2011 **Master Degree**

Royal Melbourne Institute of Technology Melbourne,AUSTRALIA

Applied Sciences/Biotechnology and Clinical Microbiology

- Research study topic: *Trichophyton rubrum* in immunosuppressed patients.

Adviser: Prof. Dr. Peter Smooker (RMIT,/Department of Biosciences and Food Technology,Melbourne)

2007 **Postgraduation Degree**

Eskisehir Osmangazi University of Arts and Science Eskisehir, TURKEY

Biological Sciences/Pedagogic Formation

2002-2006 **Bachelor Degree**

Eskisehir Osmangazi University of Arts and Sceince Eskisehir, TURKEY

Biological Sciences

- **Bachelor thesis topic**: Cancer genetics

Adviser: Ass. Prof. Dr. Ayşe Mercangöz (ESOGU/Biological Sciences, Eskisehir)

PROJECTS AND RESEARCH EXPERIMENTS

1. Examination of the effectiveness of existing drug active ingredients in silico, in vitro and in vivo against COVID 19. TUBITAK T1004 (18AG020)- Doctorate fellow student (TÜBİTAK scholarship).

(Advisers: Prof. Dr. Can Akçalı, Dr. Mehmet Altay Ünal, Ankara University Stem Cell Institute, Ankara, TURKEY).

2. Determination Of Biofilm Regulation Caharacteristics Of *marT* Gene At *Salmonella* Typhimurium. TUBITAK 1001 – Doctorate project (TÜBİTAK scholarship) 2016-2020 (Advisers: Prof. Dr. Gülay Özcengiz, Prof. Dr. Mustafa Akçelik, Ass.Prof.Dr. Nefise Akçelik, METU, Ankara University, Ankara, TURKEY)

3. The article named "Regulation of biofilm formation by *marT* in *Salmonella* Typhimurium" has been published. (Advisers: Prof. Dr. Gülay Özcengiz, Prof. Dr. Mustafa Akçelik, Ass.Prof.Dr. Nefise Akçelik, METU, Ankara University, Ankara, TURKEY).

4. Project Biofilm Producing Capacities of Wild Type *Salmonella* Typhimurium 14028 Strain and Mutants At Different Incubation Periods was presented orally in the International Conference on Food Science and Technology in Rome,Italy in November 13-15, 2017 (Advisers: , Prof. Dr. Mustafa Akçelik, Ass.Prof.Dr. Nefise Akçelik Ankara University, Ankara, TURKEY).

5. The article named " Determination of the effect of four different *seqA* and *dam* genes in *Salmonella* serotypes on their biofilm forming characteristics" is waiting to be published (Advisers: Prof. Dr. Mustafa Akçelik, Ass.Prof.Dr. Nefise Akçelik Ankara University, Ankara, TURKEY).

6. I participated in a project named “Biofilm Producing Capacities of Wild Type *Salmonella* Typhimurium 14028 Strain and Mutants At Different Incubation Periods” as a researcher in Ankara University Biotehnology Institute 2017-2019 (Advisers: Prof. Dr. Mustafa Akçelik, Ass.Prof.Dr. Nefise Akçelik, Ankara University, Ankara, TURKEY).

7. I was a part of a Tubitak project entitled “Determination of protectivity of the recombinant chaperonin 10 protein from *Bordetella pertussis”* 2014-2015 (Adviser: Prof. Dr. Gülay Özcegiz, METU, Ankara, TURKEY).

8. I studied on *Mycobacterium tuberculosis* and *Mycobacteria* other than tuberculosis (MOTT) in the Department of Tuberculosis in Duzen Laboratories 2011-2012 (Adviser: Prof. Dr. Görkem Yaman, Maltepe University Faculty of Medical School Hospital, Istanbul, TURKEY).

9. Completed research study entitled :*Trich ophyton rubrum* in immunosuppressed patients, 2009-2011. (Adviser: Prof. Dr. Peter Smooker (RMIT,/Department of Biosciences and Food Technology,Melbourne, AU).

10. Participation in the field trip to South Melton Treatment Plant to examine the stages of waste water treatment process in Melbourne, Australia,2009-2011 (Adviser: Prof. Dr. Margaret Deighton, RMIT, Melbourne, AU).

11. Participation in the field trip to Dingley Composting Plant to examine the stages of green waste composting process in Melbourne, Australia, 2009-2011 (Adviser: Prof. Dr. Margaret Deighton, RMIT, Melbourne, AU).

CERTIFICATES

- Certificate of Participation, TS EN ISO (International Organization for Standardization) 9001:2000 Quality Management Education Certificate,2006

LABORATORY TECHNIQUES

- PCR/QPCR
- 2D SDS PAGE and Western Blot
- Gene Clonning and Expression studies
- Cell Culture Technique
- Biofilm Detection Techniques
- MALDI-TOF Technique
- Identification Techniques of Bacteria Biochemically
- Fluoresence Microscopy Techniques
- Bacterial cDNA synthesis, mRNA and plasmid isolation techniques
- Plasmid transformation, electroporation tehniques
- Techniques of creation of gene specific mutations with the insertion of gene cassettes
- Electrocompetent bacterial cell preparation
- Agarose gel techniques
- MTT assay