ROLE OF *yjfO, agaB* AND *atoS* IN *ESCHERICHIA COLI* BIOFILM FORMATION AND STRESS RESPONSE

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San Marcos, Texas
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ABSTRACT

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In nature, bacteria exist predominantly as a surface adherent community referred to as a biofilm, which differs phenotypically from planktonic cells. Alterations in phenotypic expression are essential not only for transition through the various stages of biofilm formation, but also for maintenance. In this study, we sought to elicit differences in phenotypic expression in *E. coli* biofilms, as well as to characterize several mutants to determine if expression is essential for biofilm formation. Of the numerous differentially expressed genes identified by gene array analysis, *yjfO*, *agaB*, and *atoS* were selected for further characterization Results from both epifluorescence microscopy and microtiter assays confirmed that all mutant strains displayed a reduction in biofilm formation in comparison to the wild-type *E. coli*. Additionally, we sought to elicit differences in the

viability of both planktonic and biofilm cells following exposure to hydrogen peroxide, pH shifts and elevated temperature Planktonically grown mutant strains exhibited a decreased viability following exposure to acidic pH and hydrogen peroxide, whereas the *atoS* mutant exhibited an increased viability at elevated temperatures compared to that of the wild-type. Exposure of four day biofilms to hydrogen peroxide resulted in an insignificant decrease in biofilm mass for all strains, whereas exposure to acidic pH resulted in a decreased biofilm for only the wild-type and the *yjfO* mutant. Overall, our results suggest that mutations affecting *yjfO*, *agaB*, or *atoS* lead to a decreased ability to form a biofilm and a decreased ability to survive exposure to hydrogen peroxide and acidic pH when grown planktonically.

I. INTRODUCTION

In nature the majority of microorganisms exist as a fully surface adherent community known as a biofilm Members of these communities differ significantly in phenotypic expression in comparison to their planktonic counterparts Alterations in the microorganism's genetic profile facilitate the transition from free-floating planktonic cells to fully adherent biofilm communities.

Bacterial biofilms have been noted to form on a variety of surface types including polystyrene, plastic and glass (18, 24). Biofilms are also capable of developing on catheters, contacts and intrauterine devices (6), which if untreated can progress to a chronic nosocomial infection In patients undergoing short-term catheterization, 10-50% is likely to develop a urinary tract infection, whereas almost all patients undergoing long term catheterization will develop a UTI (32) In addition, biofilms have been noted to accumulate in plumbing systems, which if left untreated leads to biofouling. Insight into the genetics of biofilm formation is necessary to develop a novel approach to minimize biofilm formation on both medical devices as well as plumbing systems. Several studies have demonstrated that genetic differences exist between planktonic and biofilm cells of *E. coli* (30), *P. aeruginosa* (7, 40), *V. cholerae* (38) and *S. aureus* (27). During the initial stages of biofilm formation, expression of both flagella and pili are essential for movement toward the surface, as well as for initial reversible attachment (24, 38). Following initial attachment, motility ceases and the cells become irreversibly attached

(28) Approximately six days following initial colonization, cell layering becomes apparent, which averages 100μm in thickness (28). During this stage of biofilm formation, microcolonies are formed within the biofilm, which are stabilized by the increased production of exopolysaccharides (37). In *P. aeruginosa* biofilms, production of biosurfactants (22) is essential for stabilization of the microcolonies Within the three dimensional biofilm, channel formation has also been observed, which allows for liquid and nutrient flow throughout the system (33). After approximately nine days, the biofilm begins to disperse and the cells regain motility (29). Overall, biofilm formation facilitates gene transfer between members of the community (37), allows for delayed penetration of antimicrobials and disinfectants (6) as well as an increased efficiency at evading the host's immune response (6).

In the present study, we sought to elicit differences in gene expression between planktonic and biofilm cells of *E. coli* using gene array analysis. Numerous genes were determined to be significantly differentially expressed more than 2 fold in the biofilm. Of the differentially expressed genes identified by array analysis, our study focused on *yjfO, agaB,* and *atoS.* It was determined that a deficiency in any of afore mentioned genes results in a reduction of biofilm formation in comparison to the wild-type Additionally, mutants were determined to have altered efficiency at surviving a variety of naturally stressors commonly encountered in the bacteria's natural environment Results obtained from our study can be further applied to target these specific genes in an attempt to reduce biofilm formation on medical implants and plumbing sources.

II. MATERIALS AND METHODS

Bacterial Strains and Media:

The strains used for the completion of this study are listed in Table 1. All strains were stored at -80°C in 50% glycerol and Luria-Bertani (LB) broth. Cultures were routinely transferred on Luria agar supplemented with 50μg/ml of kanamycin for the appropriate strains. All strains were cultivated in morpholinepropanesulfonic acid (MOPS) minimal media supplemented with serine (1mg/ml) as the carbon source and amino acids required by auxotrophic strains (Ile, Arg, Gly, His, Leu, Met, Phe, Val Thr each at 0.04mg/ml) (19). Minimal media used for the growth of B3138 was supplemented with 1.5mg/ml of serine, due to a 12hr lag period compared to that of the wild-type. Supplementation with additional serine resulted in a growth curve complementary to the wild-type.

Table 1: *E. coli* strains used for the completion of this study.

Strain	Characteristics	Source
DS291	Wildtype CF 1648=MG1655	D.A. Siegele at Texas A&M University
B4149	yjfO::Tn5(KAN-I-SceI) at position 167 in Minus orientation	F. R. Blattner at University of Wisconsin-Madison
B3138	agaB::Tn5KAN-I-SceI at position 304 in Minus orientation	F. R. Blattner at University of Wisconsin-Madison
B2219	atoS::Tn5KAN-2 at position 923 in Minus orientation	F. R. Blattner at University of Wisconsin-Madison

Biofilm Cultivation:

The chemostat apparatus used in this study was assembled as previous described by Whiteley et al (41). A chemostat was filled with MOPs minimal media and inoculated with 1ml of *E. coli* DS291. The culture was subsequently incubated for 24 h at 37°C, after which continuous culture was initiated at a dilution rate of $0.025h^{-1}$. The culture was allowed to equilibrate for one full generation (40 h), at which point a second pump set to a flow rate of 100 ml/h was turned on The culture was allowed to continuously flow through 4 m laboratory tubing (Dow Corning #515-014) for 96 h (42)

Planktonic Cell Cultivation:

For planktonic cell cultivation, 200 ml of cells were extracted from the chemostat apparatus and divided into 50 ml centrifuge tubes containing ice cold stop solution (5% phenol/water-saturated in ethanol). The cells were centrifuged at 4000 rpm for 20 min at 4°C The resulting pellets were resuspended and consolidated into one tube and recentrifuged as conducted previously (2).

Biofilm Cell Cultivation:

For biofilm cell cultivation, media in the tubing was drained and 25 ml of ice-cold stop solution was added to the tubing. The tubing was cut into 3 cm pieces and placed into 200 ml ice-cold stop solution. Biofilm cells were scraped from the tubing into ice-cold stop solution using a scalpel. Remaining attached cells were removed from the tubing by sonicating at 60 htz for 10 min (Sonicor Instrument Corporation, Copiague, New York). The cells were subsequently centrifuged at 4000 rpm, 4°C for 20 min (2).

RNA Processing.

RNA was extracted from both planktonic and biofilm cells using a modified hot-phenol extraction (2) Cells were lysed with lysozyme and RNA was subsequently isolated by phenol extraction at 65°C. Chloroform was subsequently added to remove the phenol. DNA was precipitated using 95% ethanol and subsequently washed with 80% ethanol. The nucleic acid pellet was resuspended in 200 µl RNAse-free diethyl pyrocarbonate-treated deionized water (DEPC). DNA was removed using RNAse-free DNase (Boehringer Mannheim; #776185). Resulting RNA was purified using one phenol extraction, one phenol/chloroform, two chloroform extractions and an ethanol precipitation and wash. The resulting RNA was resuspended in 100 µl DEPC water. RNA was quantified at 260 nm.

Gene Array Analysis:

Gene array analysis was conducted as previously described by Arnold et al. (2) ^{33}P-labeled cDNA probes were prepared using *E. coli* gene-specific primers (Sigma-Genosys, The Woodlands, TX). Resulting cDNA was subsequently hybridized to sequential Panorama *E. coli* gene arrays (Sigma-Genosys). Uninduced *lac* operon was used to establish background. Results were obtained by exposing the filters to a Fujix BAS2000 phosphorimager. Results were obtained using Visage HDG Analyzer software (R.M. Lupton, Inc. Jackson MI) on a Sun Microsystems ULTRA10 workstation. Subsequent analysis was conducted using Microsoft Excel.

For differential expression patterns to be considered significant, a two fold change in expression level (compared to the background) during both the original and replicate run was needed.

Microtiter Assay:

Microtiter biofilm assays were conducted as previously described (21). Overnight cultures of each strain were diluted 1:10 into fresh MOPs minimal media and 200 µl of dilute culture and 100 µl fresh media was aliquoted into each well of the microtiter plates (Becton Dickinson Labware, Franklin Lakes, N J.) in triplicate. All plates were incubated at 37°C. Four wells for each corresponding strain were sampled in 24 h increments. Cell turbidity was evaluated using a microtiter plate reader (BioTek Instruments Inc., Winooski, V.T.) at an optical density of 570 nm for both planktonic and biofilm growth. Following initial turbidity measurements, media was removed from each well and the remaining cells were stained for 20 min at room temperature with 100 µl 1% crystal violet. Cells were then washed three times with sterile water to remove unbound crystal violet. Each well was then destained with 200 µl 95% ethanol and biofilm formation was quantified at 570 nm Results were obtained by taking the average of the four samples from the three replicates minus the average of the control wells.

Epifluorescence Microscopy:

To compare biofilm formation of the mutants and wild-type, each strain was grown in a chemostat coupled to a flow cell (Stovall Life Science Inc, Greensboro, N C) in duplicate as outlined above. After 96 h of circulation through the flow cell, the flow cell was removed and each chamber was washed with 5 ml of sterile water Cells were stained with 20 µM syto 9 (invitrogen) for 30 min. The flow cell was then washed with an additional 5 ml sterile water Biofilms were viewed using a Nikon Eclipse 80 I microscope at 100X. Images were obtained using a Nikon DXM 1200F digital camera and images were analyzed using Image-Pro Plus (Version 5 1).

Heat Sensitivity Assay:

The heat sensitivity assay was performed as previously described (42). All strains were incubated in MOPs minimal media at 37°C with shaking at 100 rpms to OD_{600} of 0.3. To assess strains sensitivity to heat, 5 ml of each strain was removed and heated for 20 min at 65°C. Following heat treatment, cultures were serially diluted and plated on Luria-Bertani (LB) and incubated at 37°C for 24 h. To determine the viability of the culture following exposure to elevated temperature, the log function for the controls and experimentals were calculated and averaged. The temperature sensitivity assay was conducted in duplicate.

pH Sensitivity Assay:

The pH sensitivity assay was performed with modifications as previously described (42). All strains were cultivated in MOPS minimal media at 37°C with shaking at 100 rpm. Once all cultures were in early log (OD_{600} of 0.3) 2ml of culture was incubated for an additional hour at 37°C without shaking in MOPs pH 2.5 or MOPs pH 12 Following incubation, cultures were serially diluted and plated on Luria-Bertani (LB) and subsequently incubated at 37°C for 24 h. To determine the viability of the culture following exposure to acidic or alkaline pH, the log function for the controls and experimentals were calculated and averaged. The pH sensitivity assay was conducted in duplicate.

Hydrogen Peroxide Sensitivity Assay:

Hydrogen peroxide sensitivity was assessed as previously described (42). All strains were incubated at MOPs at 37°C with shaking at 100 rpm to OD_{600} of 0.3. Following incubation, 1ml of culture was incubated with 20 mM hydrogen peroxide at

37°C without shaking for 15 min Following the 15 min incubation, cultures were serially diluted and plated on LB and subsequently incubated at 37°C for 24 h. To determine the viability of the culture following exposure to hydrogen peroxide, the log function for the controls and experimentals were calculated and averaged The hydrogen peroxide assay was conducted in duplicate.

Microtiter Stress Assay:

In order to elicit differences between mutants and wild-type biofilms at surviving exposure to acid and hydrogen peroxide the methods outlined by Zhang et al. were employed (42). An overnight culture for each strain was diluted 1:10 into MOPs minimal media. Eight microtiter wells were inoculated with 200 µl of dilute culture and 100 µl of fresh media. Plates were incubated at 37° for 96 h, 10 mM HCl or 20 mM H_2O_2 was added to four of the wells, and the plates were incubated for an addition 18 h at 37°C. Following incubation, biofilm formation was quantified as outlined above. Each microtiter assay was conducted in duplicate and sensitivity to acid and peroxide were accessed by taking the average of the control and experimental samples.

III. RESULTS

Gene Array:

Comparison of genes differentially expressed in biofilms of *E. coli* identified numerous genes significantly differentially expressed (more than 2 fold in comparison to that of the planktonic cells). The majority of the genes identified by the array analysis were unclassified, involved in transport or involved in a variety of metabolic process. For the purpose of this study, we sought to do further characterization of *yjfO, atoS* and *agaB*. Both *agaB* and *atoS* were neutrally expressed in the array and were thus chosen to determine how the lack of differential expression affects the organism's ability of form a biofilm. Additionally, *yjfO* was selected due to significant up regulation in both replicates as well as it's lack of characterization.

Table 2: Array results for genes selected for further phenotypic characterization.

	Gene Type	Replicate 1	Replicate 2
yjfO (B4189)	Unknown	3.6	3.0
agaB (B3138)	Transport protein	1.0	1.0
atoS (B2219)	Regulatory Protein	0.55	1.02

Epifluorescence Microscopy:

To compare the mutant's abilities to form biofilms comparable to that of the wild-

type, all four strains were grown in a chemostat connected to a flow cell All strains were allowed to circulate through the flow cell for 96 h before image processing. Based on the images obtained from all strains (Figure 1), it can be concluded that all three mutants had a decreased ability to form biofilms when compared to the biofilm forming capabilities of the wild-type. As shown in Figure 1, the biofilm formed by the wild-type strain resulted in large areas of cell clumping with minimal areas of attached individual cells. Large cell clumping was noted in almost all areas of the flow cell, with the largest overall mass noted near the entry and exit points of the flow cell as well as along the outer edges. In contrast, all mutant strains lacked the large overall clumping that predominates in the wild-type biofilm; however sparse individual cell attachment was still noted in all mutant strains. Some clumping was noted near the outer edges of the flow cell, but the overall mass of clumping was still significantly diminished in comparison to that of the wild-type Furthermore, in all images obtained for B3138, cell alignment was noted in several areas on the flow cell, which lead to the formation of long bacterial chains.

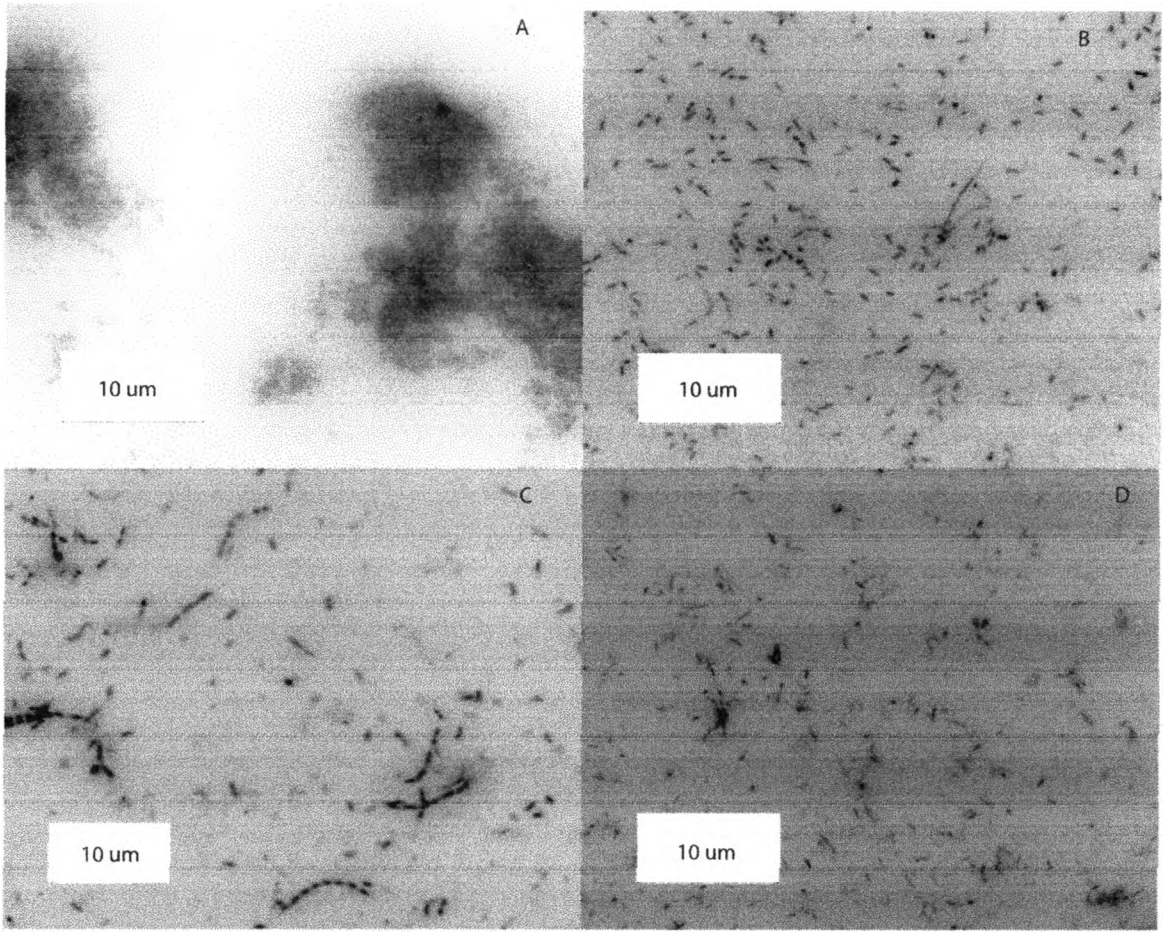

Figure 1: Representative epifluorescence microscopy images of biofilms formed by *E. coli* DS291 (A), B4189 (B), B3138 (C) and B2219 (D).

Microtiter Assay:

To quantitatively compare biofilm formation of the mutant strains of *E. coli*, all four strains were grown in a 96-well microtiter plate and sampled in 24 h increments to assess biofilm forming capabilities. At each sample point, the cells were stained with crystal violet and subsequently washed to remove unbound stain. Each well was then destained using 95% ethanol and results were obtained by reading the absorbance at 570 nm. As shown in Figure 2, all three mutants exhibited a reduced biofilm at all four sample points when compared to the wild-type strain. All strains exhibited an increase in biofilm mass between 24 h and 48 h of incubation. Following 48 h incubation, the

biofilm formed by all strains began a steady decrease in mass such that the 96 h biofilm resembled a similar overall mass to the biofilm formed at 24 h.

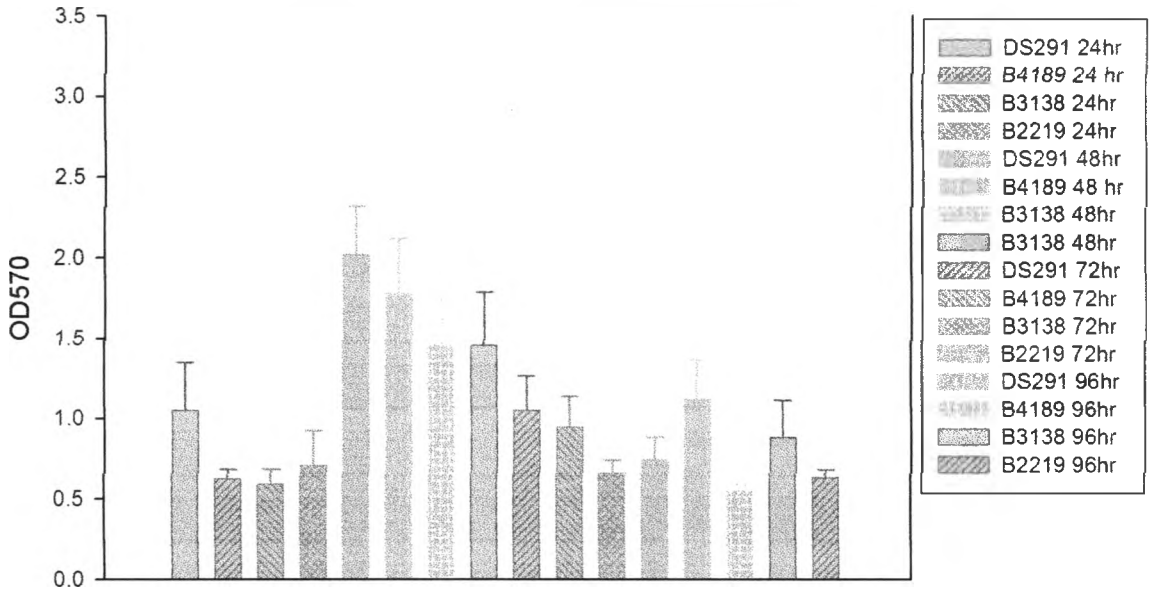

Figure 2: Average biofilm formation for various *E. coli* strains using a microtiter assay. *E. coli* DS291, B4189, B3138 and B2219 were sampled in 24 h increments over a 96 h period. Error bars represent standard error.

pH Sensitivity Assay:

To compare the ability of wild-type and mutants to survive acidic and alkaline pH, each strain was exposed to pH 2.5 or pH 12 MOPs minimal media for 1 h. As shown in Figure 3, significant reduction in cell viability was noted for all strains in comparison to the controls upon exposure to an acidic pH. All mutant strains showed approximately a two log reduction in cell numbers in comparison to that of the wild-type strain. Exposure to alkaline pH resulted in an insignificant change in viability for all strains in comparison to the control (Figure 4). Furthermore, no significant difference in viability was noted between the wild-type and any of the mutants. pH testing of 96 h cultures inoculated into

pH 7.4 MOPs minimal media showed an increase in pH to 8.5 following 96 h of incubation.

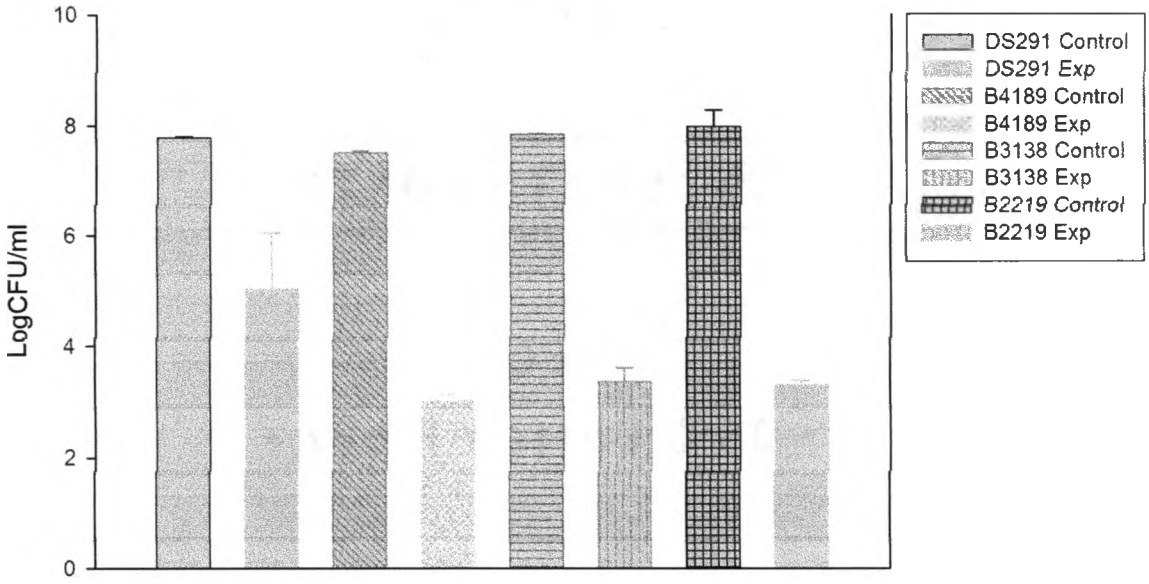

Figure 3: Average survival for planktonically grown *E. coli* following exposure to acidity. Cell survival is expressed as $\log_{10}$ CFU/ml. Error bars represent standard error.

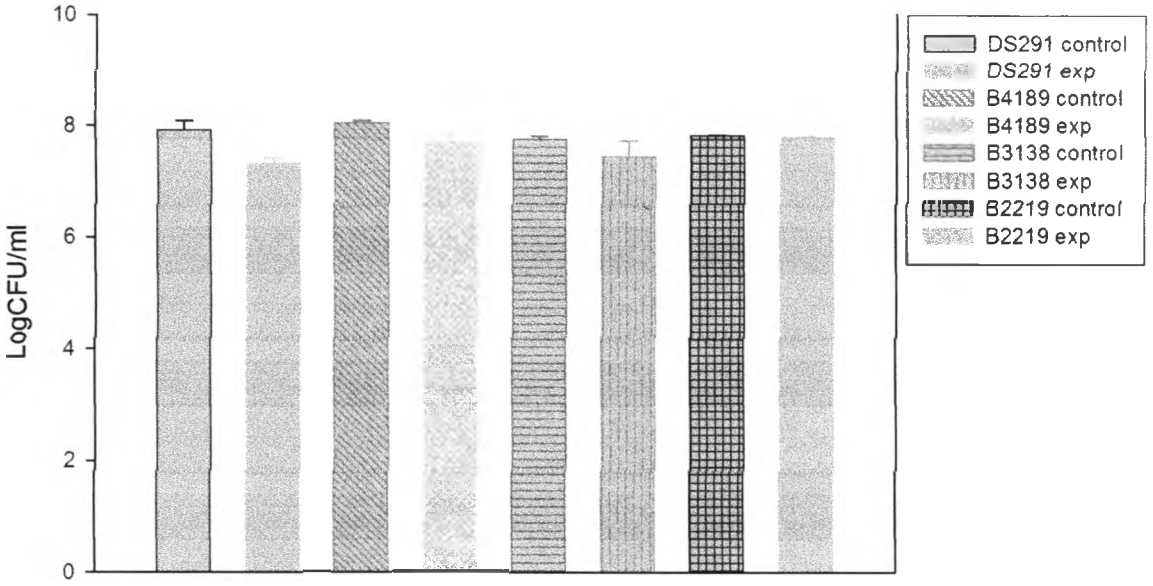

Figure 4: Average survival for planktonically grown *E. coli* following exposure to basic pH. Cell survival is expressed as $\log_{10}$ CFU/ml. Error bars represent standard error.

Hydrogen Peroxide Sensitivity Assay:

In order to compare the ability of the wild-type and mutants to survive hydrogen peroxide, all strains were exposed to 20 mM hydrogen peroxide for 15 min. As shown in Figure 5, significant reduction in cell viability occurred following exposure to hydrogen peroxide for all strains except for the wild-type. Incubation with hydrogen peroxide resulted in an insignificant decrease of 0.77 logs, whereas the most significant decrease was noted for B4189 which had a 2.3 log decrease. B3138 and B2219 also had a significant decrease of 2.51 and 1.53 respectively. Additionally, B4189 had a total reduction of 2.17 logs when compared to the wild-type DS291. Both B3138 and B2219 also had a 1.81 and 0.85 decrease respectively in comparison to DS291.

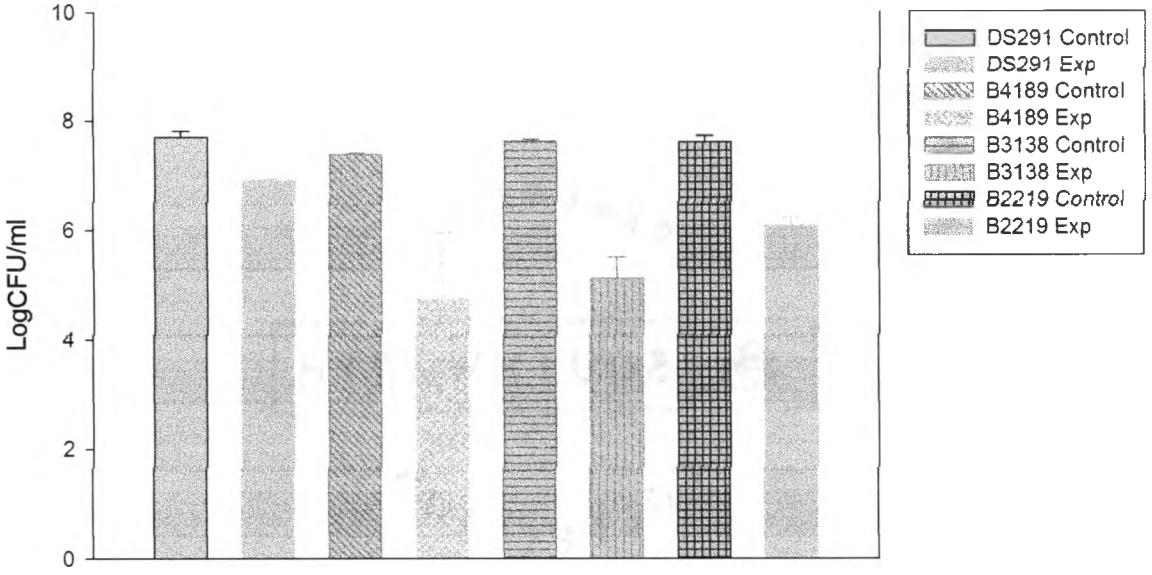

Figure 5: Average survival for planktonically grown *E. coli* following exposure to H_2O_2. Cell survival is expressed as $\log_{10}$ CFU/ml. Error bars represent standard error.

Heat Sensitivity Assay:

To compare the ability of the wild-type and mutants to survive elevated temperatures all strains were incubated at 65°C for 20 min. A significant decline in overall cell numbers was noted for all strains in comparison to the controls. The wild-type and both B4189 and B3138 all had a similar decrease in viability of approximately 2.03 logs. In contrast, B2219 had a decrease of 0.57 logs compared to the controls, but an increase in viability of 1.44 logs in comparison to the other strains.

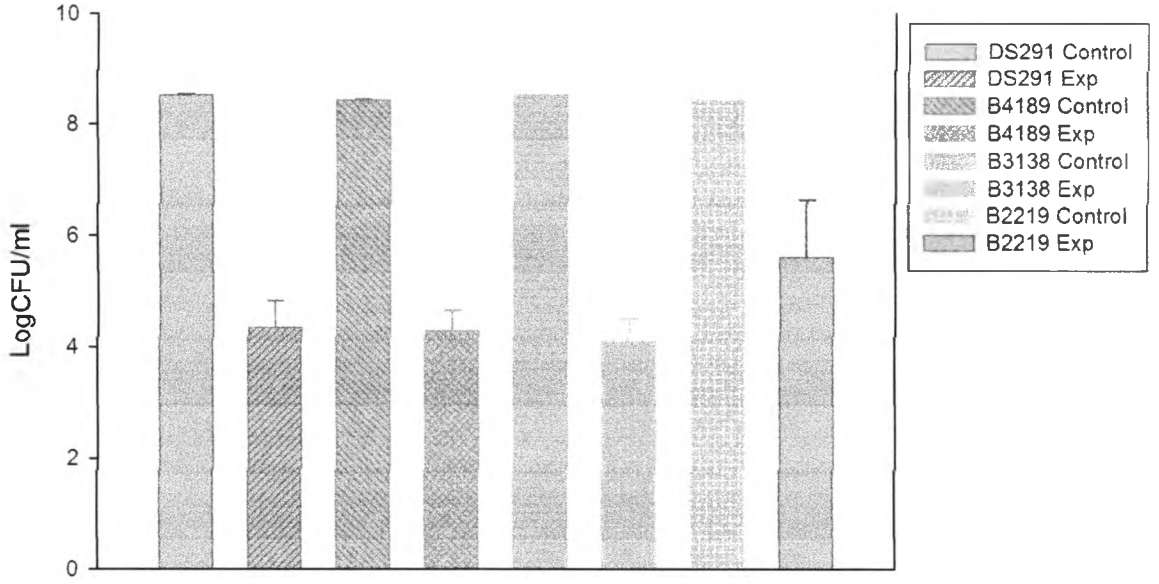

Figure 6: Average survival for planktonically grown *E. coli* following exposure to 65°C. Cell survival is expressed as $\log_{10}$ CFU/ml. Error bars represent standard error.

Microtiter Stress Assay:

To compare the effect of acidic pH and hydrogen peroxide on biofilms, all strains were inoculated into a microtiter plate and subsequently incubated for 96 h. Following incubation, four wells of each were inoculated with 10 mM HCl or 20 mM H_2O_2. All plates were incubated for an addition 18 h and subsequently stained to compare the effects of acid or peroxide on the established biofilms. As shown in Figure 6, addition of 10 mM HCl to 96 h biofilm resulted in a varying degree in reduction in biofilm mass. Exposure to 10 mM HCl for 18 h resulted in a significant reduction in overall biofilm mass for the wild-type biofilm and a minimal decrease in biofilm for both B4189 and B2219. Incubation of all strains for 18 h with 20 mM H_2O_2 resulted in an insignificant difference in biofilm mass for all strains (Figure 7). A slight increase of 0.05 and 0.06 in

biofilm mass was noted for DS291 and B4189 respectively, whereas B2219 exhibited only a 0.02 decrease.

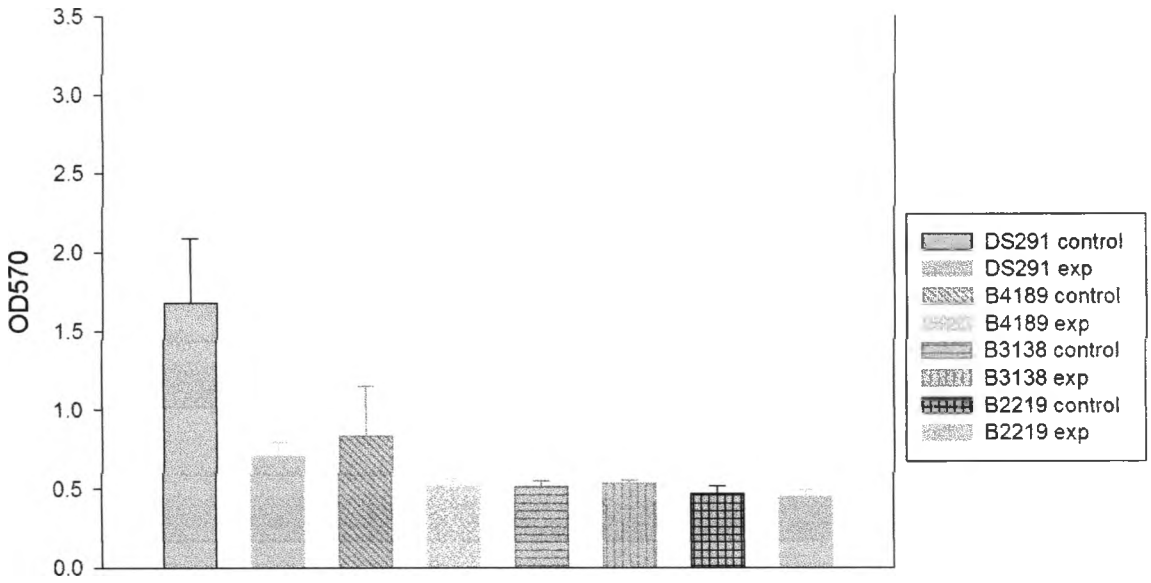

Figure 7: Average biofilm remaining following 18 h incubation with 10mM HCl. Biofilms were grown for 96 h prior to the addition of 10mM HCl. Biofilm growth is expressed as a function of the average planktonic growth for all replicates. Error bars represent standard error.

Figure 8: Average biofilm remaining following 18 h incubation with 20mM H_2O_2. Biofilms were grown for 96 h prior to the addition of 20mM H_2O_2. Biofilm growth is expressed as a function of the average planktonic growth for all replicates. Error bars represent standard error.

IV. DISCUSSION

Upon ingestion of contaminated food, potential invading pathogens must first be able to survive the acidic pH of gastric juice, which acts as the first line of defense against invading pathogens (4). Fluctuations in the pH of the gastric juice correlates with lowering the infective dose (ID) required to cause disease (4). After traversing the stomach, the organisms pass through to the intestines where the local pH increases to between 4 and 6. It is here that invading pathogens must be able to overcome weak acids that are produced as metabolic byproducts by the resident flora (14). Weak acids are capable of traversing into the bacterial cell were they freely dissociate in the cytoplasm, thus inducing acid stress (4, 14). Several studies have demonstrated that the presence of weak acids decreases species viability, yet certain species of *E. coli* are capable of combating weak acids (4, 14) It is this resistance to acidity that allows *E. coli* to efficiently colonize and inhabit the intestines (4, 10, 14). In addition to exposure to acidic pH, *E. coli* must also combat alkaline pH in pancreatic secretions, which it encounters as it passes through the pyloric sphincter (31).

In the present study, we sought to evaluate differences in phenotypic expression between planktonic and biofilm cells of *E. coli*. Of the numerous differentially expressed genes identified by array analysis, *yjfO, agaB,* and *atoS* were selected to determine if differential expression is essential to biofilm formation. Furthermore, we sought to determine if expression of these genes results in differential survival rates upon exposure

of planktonic cells or biofilms to common environmental stressors such as elevated temperature, alterations in pH or hydrogen peroxide. Bacteria residing within a biofilm encounter a wide range of environmental stressors that differ from those encountered by their planktonic counterparts. Several studies evaluating differential gene expression in bacterial biofilms have demonstrated that genes involved in the stress response are differentially expressed in cells located within the biofilm (3, 26, 27). In addition to its role as the central regulator of the general stress response, RpoS has been implicated in biofilm formation (7). A study conducted by Adams and McLean (1) demonstrated that deletion of *rpoS* leads to a decrease in overall biofilm formation, but has negligible effects on planktonic cells. Additionally, cells residing within the biofilm encounter differential oxygen availability patterns than those encountered by planktonic cells. These cells commonly encounter decreased oxygen availability due to decreased diffusion rates as well as a buildup of CO_2, which results from bacterial metabolism (7, 9, 25). Several studies have demonstrated that expression of genes involved in the oxygen stress response are essential to biofilm formation (15) and that anaerobic growth conditions may in fact promote biofilm formation (7, 28). In addition to combating oxygen availability, cells residing in a biofilm encounter pH gradients (9). As an organism approaches a surface for colonization it encounters an acidic environment due to proton accumulation at the liquid-solid interface (9). Differential gene expression profiling also has shown that alkaline shock genes increase in expression in biofilms (27). Furthermore, in nature the majority of bacterial biofilms consist of multiple species. The presence of another species within the community can lead to a lowering of the overall pH, due to

byproducts produced during metabolism, which further facilitates antagonism between the community members (5).

The formation of a bacterial biofilm is a complex dynamic process that requires alterations in gene expression to facilitate the transition not only from planktonic to biofilm but also for the maintenance of the biofilm. Several studies have noted that flagella (24), pili (20) and curli (35) are necessary for initial surface colonization as well as microcolony formation. Furthermore, the expression of species specific factors such as Ag43 (13) and alginate (8) are crucial to biofilm formation. Similarly, the results of our study suggest that *yjfO*, *agaB* and *atoS* are essential for biofilm formation. Results from both epifluorescence microscopy as well as the microtiter assay suggest that defects in any of these genes causes a significant decrease in overall biofilm formation. Maximum biofilms were formed at 48 h, after which a decrease in biofilm was noted for all strains. Reductions in overall biofilm mass was noted at both the 72 h and 96 h incubation points, which can most likely be attributed to a buildup of metabolic byproducts and diminished oxygen availability, which lead to cell detachment and cell death. The need for differential expression of *yjfO* is further supported by results obtained in several other studies that also found *yjfO* to be differentially expressed in biofilm cells (26, 30). Additionally, results from the epifluroescence imaging of the *agaB* mutant revealed large chain formation, which could possibly be attributed to an error in cell division which results in insufficient cell separation following division, however further evidence is needed to support this theory. A similar phenomenon is noted in *S. mutans ropA* mutants, in which longer chains consisting of shorter cells formed (39). Growth curve comparisons of all *E. coli* strains revealed that the *agaB* mutant had a significant lag period of 12 h in

comparison to the 4 h lag period exhibited by the other *E. coli* strains used in this study. These results further support the idea that mutations in *agaB* result in defects in growth.

In addition to defects in biofilm formation, all three mutant strains exhibited decreased viability when grown planktonically and exposed to acidic pH and hydrogen peroxide. In addition to the present study, several other studies have noted a strong correlation between biofilm formation and stress response (26, 39). A recent study conducted by Wen et al. (39) found that several *com* mutants exhibited a diminished biofilms in addition to an increased sensitivity to acidic pH. In our study, we sought to determine the effects of temperature, alteration of pH and hydrogen peroxide on cells grown both planktonically and in a biofilm. Planktonic cells exposed to all stressors showed significant decreases upon exposure to acidic pH and hydrogen peroxide, whereas only the *atoS* mutant had an increased viability when exposed to elevated temperature in comparison to the other strains. Exposure to alkaline pH resulted in an insignificant difference for all strains in comparison to the controls. This is in contrast to the findings of other studies that showed *yjfO* to be differentially expressed upon exposure to alkaline pH in both aerobic and anaerobic environments (12, 17). pH testing of the cultures revealed that the overall pH increases to 8.5 following 96 h of incubation. The overall increase in media pH as well as the viability of the cells when exposed to alkaline pH suggests that the particular strains used in this study may have adapted mechanisms to survive the resulting alkaline pH that is generated due to build-up of byproducts in the environment. All strains used in this study had a decreased viability when exposed to acidic pH for 1 h, with the decrease being more significant for all mutants than the wild-type. Several studies have shown that several strains of *E. coli* are

able to survive exposure to acidic pH with no significant decrease in viability for several hours. (4, 10, 14) The discrepancy in our strains viability could possiblly be attributed to differences in growth phase. In *E. coli*, entrance into stationary phase leads to induction of *rpoS*, which has been implicated in enhancing culture viability in extreme acidic and alkaline environments (31). It has been proposed that pH homeostasis is achieved in bacteria through buffering, decreased membrane permeability or proton pumps. The difference noted in viability between the mutants and wild-type when grown in an acidic environment could possibly be attributed to a defect in one or more of the afore mentioned systems.

In addition to increased sensitivity to acidic pH, all mutant strains exhibited an increased sensitivity to hydrogen peroxide when compared to the viability of the wild-type, with the most increased sensitivity noted for the *yjfO* mutant. In their natural environment, microorganisms encounter hydrogen peroxide in numerous different ways Macrophages produce reactive oxygen intermediates such as hydrogen peroxide and superoxide to combat invading pathogens (11). During colonization of the upper respiratory tract *Streptococcus* produces hydrogen peroxide, which allows for more efficient colonization by the *Streptococcus.* The hydrogen peroxide that is generated is toxic to numerous bacterial species and thus allows for more efficient colonization by *Streptococcus,* due to the antagonistic interactions generated against the normal flora. (23). Organisms such as *E. coli* encode catalase, which allows for the breakdown of hydrogen peroxide into oxygen and water (16). In *E. coli*, *katG* which encodes the catalase enzyme (16) is regulated by OxyR (34). Induction of the OxyR regulon confers resistance to hydrogen peroxide (34) and thus enhanced viability when competing with

another pathogen or the immune response. The wild-type *E. coli* used in this study had a minimal decrease in viability when exposed to the hydrogen peroxide. All the mutants strains, however exhibited a significant reduction in cell numbers following exposure to hydrogen peroxide These results suggest that mutations in these genes may lead to a defect in breakdown of peroxide.

In addition to evaluating the effects of pH and peroxide on the mutant strains, we also sought to evaluate the effects of elevated temperature on all mutant strains. All strains resulted in a decrease in cell numbers following heat treatment. Comparison of resulting cell numbers following heat treatment showed that the *yjfO*, *agaB* and the wild-type exhibited a similar reduction in cell number, whereas the *atoS* exhibited a slight increase in viability compared to the other strains.

In the present study, we also sought to evaluate the effects of acidic pH and hydrogen peroxide on established biofilms for all strains Cells residing within a biofilm exhibit an increased resistance to antibiotics and disinfectants (6). Our results indicate that incubation with 20mM hydrogen peroxide and 10mM HCl had a minimal effect on the overall biofilm of the mutants. All strains showed negligible effects except for on the biofilm formed by the wild-type, which had a decreased biofilm following incubation with 10mM HCl. The lack of decreased biofilm for all strains when incubated with hydrogen peroxide is most likely due to decreased penetration into the biofilm; however the exact mechanism of resistance is under further evaluation.

The results of our study suggest that a mutation affecting *yjfO*, *agaB* or *atoS* leads to a decreased ability to form a biofilm and a decreased ability to survive acidity and oxidative stress when grown planktonically, however mutations in the afore mentioned

genes have minimal affect on four day old biofilms exposed to acidity or oxidative stress. Further research is needed to fully access the roles of differential expression of each of these genes in biofilm formation and maintenance Further study is needed to determine if the mutants can withstand exposure to acidity and oxidative stressors if the stressor is added before the biofilm reaches maturity as well as to determine the effect of environmental stressors on planktonic cells of the mutants once they have reached stationary phase to determine if mutations in these genes affects the activity of *rpoS*.

APPENDIX

Table 3: Genes differential expressed more than 2 fold in a *E. coli* biofilm

Gene	Description	Function	Replicate One Expression above Planktonic	Replicate Two Expression above Planktonic
b1317 (o219)			3.21	2.00
yjbF	hypothetical 25.0 kD lipoprotein in pgi-xylE intergenic region	Unknown	2.14	2.01
yigK			2.78	2.01
yecC	f222; This 222 aa ORF is 48 pct identical (0gaps) to 208 residues of an approx 232 aa protein YCKA_BACSU SW: P42399	Not classified	2.10	2.03
yhhA			3.92	2.04
dead	f646; two frameshifts relative to ECODEAD: 99 pct identical amino acid sequence and equal length to DEAD_ECOLI SW.P23304	RNA synthesis, modification. DNA transcription	3.37	2.05
b1904 (o107)			2.27	2 05
b0331 (o296)	o296, 37 pct identical (5gaps) to 293 residues of approx. 296 aa protein BCPA_STRHY SW.P11435	Not classified	2.03	2.06

b1360 (o248)	o248; This 248 aa ORF is 50 pct identical (3gaps) to 241 residues of an approx 248 aa protein DNAC_ECOLI SW: P07905	Not classified	2 12	2.08
B1586 (o115)	o115. This 115 aa ORF is 30 pct identical (2gaps) to 62 residues of an approx 2032 aa protein LAR_DROME SW: P16621	Unknown	2.18	2.10
yheF	putative general secretion pathway protein d precursor	Not classified	2.43	2 11
b2371 (f394)			3.89	2.14
gntR			2 87	2.14
yggZ			2.99	2.14
b2627 (f729)			2.01	2 15
yecI	Ferritin-like protein	Transport of small molecules. Cations	2.58	2.20
b1678 (f334)			2.21	2 20
b2270 (f575)			2.22	2.20
yjfR	Hypothetical 40.3 kD protein in aidB-rpsF intergenic region	Unknown	3.00	2.22
b2986 (o230)	o230; This 230 aa ORF is 39 pct identical (9gaps) to 64 residues of an approx 200 aa protein KAD1_PIG SW: P00571	Unknown	2.22	2.24
accB	biotin carboxyl carrier protein	Biosynthesis of cofactors, carriers: biotin carboxyl carrier protein (BCCP)	2.48	2.24
yjfV			3.33	2.25
b0557 (bacteriophage)			2.60	2.29

hisL	his operon leader peptide	Amino acid biosynthesis: Histidine	3.46	2.29
infA			4.35	2.30
b1364 (o93)	o93; 30 pct identical (6gaps) to 75 residues from flagella biosynthetic protein, FLIP_BACSU SW: P35528 (221 aa). UUG start	Unknown	2.30	2.30
hemL	glutamate-1-semialdehyde 2,1-aminomutase	Biosynthesis of cofactors, carriers. Heme, porphyrin	2.94	2.38
b1625 (o71)			3.31	2.40
rpoH	RNA polymerase sigma-32 subunit	Global regulatory functions	2.66	2.44
yacL			2.51	2 44
B1567 (f49)	f49; This 49 aa ORF is 35 pct identical (3gaps) to 40 residues of an approx. 440 aa. protein YK12_YEAST SW: P36080	Unknown	3.51	2.51
b2084 (f88)			2.20	2.51
csrA			2.31	2.56
cspE	cold shock-like protein cspE	Not classified	3.20	2.57
ytfM			2 63	2.59
b2969 (f286)			2.50	2.64
b2245 (f267)			2.41	2.65
b1953 (o80)			2.62	2.71
yiaB			8.09	2 74
ytfJ	18 2 kD protein in cysq-msra intergenic region precursor (f18)	Unknown	2 44	2.78
b1425 (f67)			3.15	2.85
yjfO			3.60	3.00
b3533 (hypothetical)			2 32	3.08
nusG	Transcription antitermination	RNA synthesis, modification,	2.37	3.30

	protein nusg	DNA transcription		
yjfN			*4 68*	3.45
B1858 (o251)	o251; This 251 aa ORF is 54 pct identical (0gaps) to 239 residues of an approx 272 aa protein Y408_HAEIN SW: P44692	Not classified	3.93	3.71

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