BIOFILM-INDUCED GENE EXPRESSION IN CHEMOSTAT GROWN *ESCHERICHIA COLI* AS DETERMINED BY A GENE ARRAY

THESIS

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ABSTRACT

Biofilms are attached communities of cells encased in a polysaccharide matrix that act together to increase their survival in the environment. Biofilms are the major mode of bacterial growth in nature with approximately 99% of all microbial activity occurring in them. In society, biofilms cause a variety of problems. The Centers for Disease Control estimate that 65% of all nosocomial bacterial infections are the result of biofilm activity. Biofilms can colonize surgical prostethis and result in infections that are difficult to treat with antibiotics. In industry, biofilms colonize bridges, pipes, filters and ship hulls resulting in increased corrosion and loss of profit. Previous studies have shown genes important in the initial events of biofilm formation, but few studies have focused on gene expression in mature biofilm growths. Gene expression patterns were studied in biofilm and planktonic cultures of *Escherichia coli* MG1655 after a four-day growth period in a serine-limited chemostat. After four days of growth, both planktonic and biofilm cultures were aseptically removed and mRNA was extracted using phenol / chloroform extraction. Radiolabeled cDNA probes were then made from the mRNA by using primers of all 4290 open reading frames in the *E. coli* genome. The labeled probes were hybridized to genome wide gene arrays obtained commercially. Gene expression was measured using a phosphorimager and image analysis software. Genes differentially expressed by a factor of two fold were considered significant. Out of the 4290 open reading frames in the *E. coli* genome, 28 genes were differentially expressed by a factor of at least two fold in either biofilm or planktonic cell cultures. Of the 28 genes differentially expressed, 20 genes have no known function. As a whole, the findings of this study suggest that the two growth types are similar in mature gene expression;

however, any of the 20 genes of unknown function described in this study may prove vital to biofilm survival and could offer new ways of controlling them.

INTRODUCTION

Biofilm Formation

Biofilms are widely considered to be the major mode of bacterial growth in nature with an estimated 99% of all bacterial activity occurring in them (Costerton et al. 1995). These adherent aggregates of cells have been shown to grow on a variety of abiotic and biological surfaces (Pratt and Kolter (1998). Because of their ability to colonize and adhere to a large variety of surfaces, biofilms cause diverse problems. In health care, biofilms are a major cause of disease. The Centers for Disease Control estimates that 65% of all nosocomial bacterial infections are the result of biofilm activity. Persistent infections which biofilms have been shown to be a part include the colonization of surgically implanted prosthesis. *Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Staphylococcus aureus* have all been shown to colonize artificial heart valves and joints resulting in infections that are difficult to treat (Gristina *et al.* 1988). In industry, biofilms obstruct pipes and filters resulting in delays and increased production costs (reviewed in Costerton, 1999). Increased corrosion rates causing damage to bridges and ship hulls are also associated with biofilm growth (Zhang and Dexter (1995). While there are differences in the way different species of bacteria react in a biofilm, there are some similarities all species seem to follow.

The majority of Bacteria, Archaea and many plants and fungi are able to grow as a biofilm under appropriate conditions. General biofilm forming strategies of bacteria are described next. Using active motility or natural fluid currents as a means for travel, cells

in a fluid environment actively seek out submerged surfaces for colonization. Once on the surface, the cells migrate toward other cells using flagella, type IV pili, chemotaxis and twitching motility. The cells then attach to the surface and to other bacteria using pili and form aggregates of cells called microcolonies (Davey and O'Toole (2000). Each microcolony is subsequently enclosed in an exopolysaccharide matrix. A conglomeration of microcolonies makes up a mature biofilm (Watnick and Kolter (2000). The space between the microcolonies creates natural fluid channels that have been compared to primitive circulatory systems. The channels disseminate waste and nutrients throughout the biofilm (Stoodley *et al.* 1994). As a whole, biofilms are thought to function as primitive multicellular organisms due to the water channels acting as a circulatory system and some cells exhibiting specific functions (Costerton *et al.* 1995). Various species of bacteria also use cell-to-cell communication (quorum sensing) as a means to attract other bacterial cells and to form the biofilm (Davey and O'Toole (2000). The data in table 1 shows an overview of biofilm forming strategy, organism using the strategy, gene responsible and function.

Strategy	**Organism**	**Gene**	**Function**	**Reference**
Motility	*P. aeruginosa*	*sad*	Flagellar motility	O'Toole, *et al*, 2000
	E. coli	*fliC*	Flagella sythesis	Pratt and Kolter, 1998
		motA *motB* *motA/B*	Flagella function	Pratt and Kolter, 1998
Attachment	*E. coli*	*fim*	Type 1 pili formation	Pratt and Kolter, 1998
		csgA	Curli production	Vidal, *et al*, 1998

				1998
	V. cholerae	*mshA*	Pili formation	Watnick and Kolter, 1999
	S. parasanguis	*fap1*	Fimbrae synthesis	Froeliger and Fives-Taylor, 2001
		fimA		Oligino and Fives-Taylor, 1993
	S. gordonii	*adpA*		Loo, *et al*, 2000
		scaA		Kolenbrander and Anderson, 1990
	S. crista	*scbA*		Correia, *et al*, 1996
	S. pneumoniae	*psaA*		Sampson, *et al*, 1994
	S. sanguis	*ssaB*		Ganeshkumar, *et al*, 1991
		bacA	Peptidoglycan biosynthesis	Loo, *et al*, 2000
	S. aureus	*bap*		Cucarella, 2001
	E. faecalis	*esp*		Toledo-Arana, *et al*, 2001
Nutrient sensing	*E. faecalis*	*appC*	Nutrient sensing	Toledo-Arana, *et al*, 2001
Quorum sensing	*P. aeruginosa*	*las/rhl*	Quorum sensing	Davies, *et al*, 1998
	S. gordonii	*comD*	Histamine kinase	Lunsford and London, 1996
Unknown	*P. aeruginosa*	*gacA*	Virulence	De Kievit, *et al*, 2001
EPS	*P. aeruginosa*	*AlgC/T*	Alginate biosynthesis	Davies and Geesey, 1995
Osmoregulation	*E. coli*	*proU*	Glycine betaine transport system	Gowrishankar, 1989
	E. coli	*ompC*	Porin	Sarma and Reeves, 1977
	E. coli	*wcaB*	Colanic acid synthesis	Sledjeski and Gottesman, 1996

Quorum sensing	*E. coli*	*pepT*	Aminotripeptidase T	Prigent-Combaret, *et al*, 1999
Regulatory	*E. coli*	*csrA*	Carbon Storage Regulator	Jackson, *et al*,2002

Table 1 Previous genes for various organisms that previous research has determined are part of the early stages of biofilm development.

Figure 1 illustrates the basic steps involved in initial biofilm formation for various bacteria.

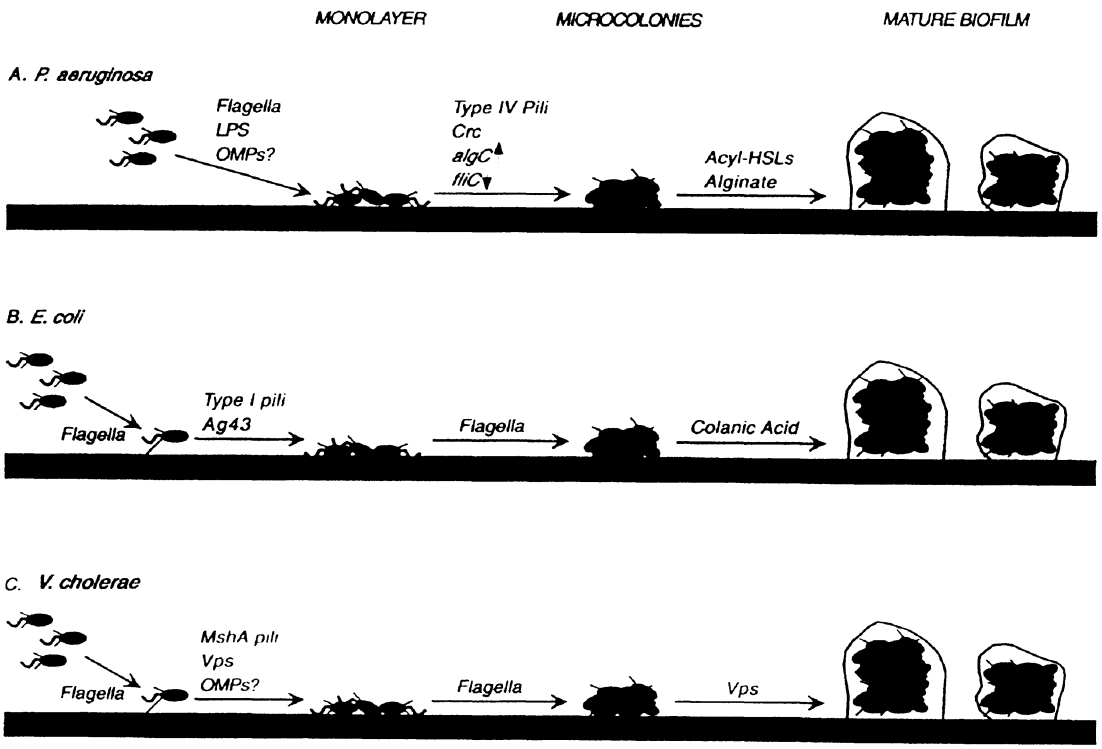

Fig 1 (Taken from Fig 5 in Davey and O'Toole (2000)

Quorum Sensing

Many species of bacteria produce diffusible low molecular weight pheromones (N-acyl homoserine lactones) as a means to communicate. The communication can occur

between the same species or between different species (Fuqua *et al.* 1994). The effect of these chemicals, often referred to as autoinducers, is associated with cell density. Each cell produces a small amount of autoinducer, as the population of cells increases, so does the concentration of the pheromone. Once the pheromone reaches a certain concentration, gene expression is effected. These chemical signals affect several aspects of bacterial physiology including bioluminescence, pathogenicity, plasmid transfer, and antibiotic biosynthesis (Fuqua *et al.* 1994). Regulating the formation of biofilm is another important function of autoinducers in some bacteria.

In *Pseudomonas aeruginosa*, quorum sensing plays an important role in biofilm formation. Two gene systems (*las* and *rhl*) normally associated with virulence factor regulation have been shown to play a role in biofilm formation. The *las* system is composed of LasR and LasI. The protein LasR is a transcriptional activator and the protein LasI is an autoinducer synthase enzyme that directs the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone. Release of this pheromone activates the *rhl* system. The *rhl* system is composed of RhlR and RhlI. As in the *las* system, RhlR is a transcriptional activator and RhiI is an autoinducer that synthesizes N-buytryl-L-homoserine lactone. Bacteria with a mutation in the *las* system form thin and unorganized biofilm compared to the wild type organism. When an exogenous supply of the autoinducer was added to the mutant media, biofilm formation was restored. It was also discovered that mature biofilms do not express *las*. As the biofilm matures, expression decreases (Davies *et al.* 1998). Although the *lasI/R* and *rhlI/R* genes were originally

associated with elastase and rhamnolipid synthesis in *P. aeruginosa* (Pearson *et al.* 1994, Pearson *et al.* 1995), there are now at least 39 genes under quorum sensing control in this organism (Whiteley and Greenberg (1999). Several of these genes are likely to be important in biofilm growth and formation.

Biofilm Formation Assay

The assay of choice for studying the initial steps in biofilm formation is the microtiter assay. In this assay, 96 well microtiter plates are used as a substrate for the development of the biofilms. Bacterial cells are mutated using Tn917 transposon mutagenisis or similar mutation technique and antibiotic resistance as a selection marker. After mutation, resistant cells are inoculated into the wells of the microtiter plate and allowed to grow. After the growth period, the wells are gently rinsed to remove unattached cells and stained. Wells that have few or no attached bacteria are deemed biofilm defective mutants. Once a mutant is discovered, the insertion site is determined as well as gene function (O'Toole and Kolter (1998). This microtiter screen is widely used to identify genes involved in the initial stages of biofilm formation (Davey and O'Toole (2000).

Gram-positive Biofilm Forming Bacteria

Oral Biofilms

Oral biofilms are a major cause of endocarditis and the predominant form of bacterial growth in oral cavities (Durach 1995). Although dental plaques are composed of a variety of bacterial species, biofilm formation begins with the colonization of the

tooth surface by primary colonizers. Three species of gram-positive organisms represent this group. *Staphylococcus gordonii, Staphylococcus songuis,* and *Streptococcus parasanguis* (Nyuad and Kiian (1987). The initial stages of their attachment and growth using the biofilm formation assay have been studied to some extent.

S. parasanguis is a primary oral colonizer and can lead to the formation of dental plaque. The study found that mutants of *fap1,* a gene encoding fimbriae, were unable to form biofilms. Cells with a mutated *fap1* gene were grown on plastic cover slips and then viewed using phase-contrast microscopy over time. These cells were seldom attached and no microcolonies were observed. Wild type strains on the other hand formed dense cell layers and microcolonies. These findings indicate the importance of surface adhesion in biofilm formation (Froeliger and Fives-Taylor (2001).

Staphylococcus gordonii is another primary colonizer of the oral cavity and has been shown to initiate biofilm formation on tooth surfaces and can ultimately lead to endocarditis. Dental plaques are multispecies biofilm, but the majority of culturable organisms from them are *S. gordonii* (Nyvad and Kilian (1990). In a study conducted by Loo *et al.*(2000), the biofilm formation assay developed by O'Toole and Kolter (1998) was used to identify mutants defective in biofilm formation. The study revealed that many of the genes required for biofilm formation are involved in signal transduction, peptidoglycan biosynthesis, and adhesion. Of the 25,000 mutants generated using Tn917 transposon mutagenesis, 18 genes were identified to be important in the initial stages of biofilm formation. Of the 18 genes described, only 8 have known functions. *comD* is a

gene that encodes a histamine kinase involved in quorum sensing. Bacteria with a mutation in this gene were unable to form biofilm. This finding is interesting because it is the first time quorum sensing has been implicated in gram-positive biofilm formation. Four genes necessary for biofilm development were found to be involved in osmoregulation or more specifically to peptidoglycan biosythesis. Penicillin binding protein 2B and 5, *glmM,* and *bacA* were all mutated in biofilm deficient strains indicating the importance of cell wall integrity and structure in biofilm formation. A gene that encodes a protein involved in adhesion, *adpA,* was also found to be involved in biofilm initiation. Mutated cells were unable to attach and could not form biofilm. *AppC* encodes a member of an oligopeptide transport system in *Bacillus subtilis* and is believed to be involved in nutrient sensing. This gene is also important in *S. gordonii* biofilm formation. In *Bacillus subtilis*, this gene is thought to be involved in cell growth-monitoring communication. It is believed to function in communication between cell wall synthesis and the cytoplasm that senses the turnover rate of cell wall peptides (Koide and Hoch, 1994). The gene may have a similar role in *S. gordonii* biofilm formation. The other nine genes isolated from biofilm-defective mutants have no known function.

Other Gram-positive Biofilm Forming Organisms

Staphylococcus aureus is a biofilm-forming organism and is involved in a variety of human diseases. It is a common cause of nosocomial infections (Van den Bergh and Verbugh (1999). A large reason for the prevelance of this organism in these infections is its ability to attach to and colonize a variety of surfaces (Foster and Hook (1998). In a

study conducted by Cucarella *et al.* (2001) a gene involved in the early formation of biofilm was identified using Tn917 mutagenisis biofilm assay (O'Toole and Kolter (1998). Of approximately 4,000 mutants screened, two were deficient in biofilm formation. Both mutants had insertions in the same gene, but at different locations. The gene, *bap*, codes for 'biofilm associated protein'. This protein is involved in primary adherence and intercellular adhesion. Mutant strains were unable to adhere to polystyrene and could not form microcolonies. Wild type strains were able to proficiently attach to the surface as well as form well-defined microcolonies. Furthermore, all biofilm-forming strains of *S. aureus* tested contained the *bap* gene.

Enterococcus faecalis is a gram-positive biofilm-forming organism found in the oral cavity and digestive tract. This organism is normally nonpathogenic to humans but has recently been implicated in 12% of all nosocomial infections due to increased antibiotic resistance (Edmond *et al.* 1996). One gene of particular interest is *esp*. This gene is present in approximately 93% of all biofilm-producing strains of *E. faecalis* and deficient in all non-biofilm producing strains. The function of the gene is unknown; however, the gene sequence has a high similarity to *bap* in *S. aureus* leading to the conclusion of similar function. Cells with a mutated *esp* gene were unable to attach to an abiotic surface (Toledo-Arana *et al.* 2001).

Common Themes in Gram-positive Organisms

All gram-positive biofilm forming organisms have one commonality that allows them to form biofilms. They all require the ability to attach to a surface for colonization.

In the case of oral biofilms, the ability to attach to other cells is also a requirement. *S. gordonii* has also been shown to require nutrient sensing function and some preliminary results indicate a connection to quorum sensing in biofilm formation. Some gram-negative biofilm forming organisms use slightly different strategies.

Gram-negative Biofilm Forming Organisms

Vibrio cholerae is the causative agent of the human digestive disease cholera. The disease spreads through fecal contaminated food and water (Blake 1994) and has the ability to cause pandemics. In order to cause wide spread disease, the organism must survive outside the human body for extended periods of time in nutrient limited environments. Biofilm formation may be an important mechanism for its survival when outside the human body. Recent studies have shown *V. cholerae* to form three-dimensional biofilms on abiotic surfaces (Watnick *et al.* 1999)

V. cholerae naturally inhabits both salt and fresh water aquatic ecosystems and has been shown to attach to a variety of surfaces including plants, filamentous green algae, zooplankton and crustaceans (Colwell 1996). Studies on the initial attachment of the bacterium to surfaces indicate similarities to *Escherichia coli*. Flagella are used to bring the organism in close enough to attach and to spread across the surface and attachment is through pili. The flagella, however; only serve to speed up biofilm formation. Mutants with a defective flagellum gene and pili gene, *mshA,* were slower in developing biofilm but developed mature biofilms that were indistinguishable from the wild type biofilm EPS is also important in *V. cholerae* biofilm development. Wild type

mature biofilms show well formed architecture similar to *P. aeruginosa* biofilms but EPS mutants formed thin unorganized biofilms (Watnick and Kolter (1999) (Yildiz and Schoolnik (1999).

P. aeruginosa is a ubiquitous environmental organism and is the causative agent of a variety of acute infections including chronic respiratory tract infections in cystic fibrosis patients (Govan and Deretic (1996). *P. aeruginosa* is one the most extensively studied organisms in terms of biofilm formation since it readily forms biofilms. Using biofilm assay, the initial events of biofilm formation have been investigated. A study conducted by O'Toole and Kolter (1998) revealed two mutant strains designated *sad* for surface attachment defective. One class had defective flagellum-mediated motility and the other was defective in type IV pili production. Flagellar motility is essential in *P. aeruginosa* biofilm formation, lack of flagella leads to poor biofilm formation. Flagellar mutation blocked the cell from interacting with the surface. Type IV pili is associate with surface movement known as twitching motility. Strains without type IV pili synthesis were able to attach and form a monolayer, but were unable to form microcolonies indicating that twitching motility is important in biofilm organization. Another important gene involved in biofilm development in *P. aeruginosa* biofilms is *crc*. Crc is a catabolite repressor protein that plays a role in sugar metabolism. This protein has also been shown to play a role in type IV pili production by regulating *pilA* and *pilB*. These two genes encode the main structural protein of type IV pili and an accessory factor

required for pilus assembly. The link to sugar metabolism indicates a necessity for nutrient availability in biofilm formation (O'Toole *et al.* 2000).

Recently, a study was conducted on gene expression in mature *P. aeruginosa* biofilms. This study utilized a once flow growth system (media was not re-circulated) to cultivate biofilm and microarrays to determine expression levels. Of the 5,570 predicted genes in the *P. aeruginosa* genome, 5,500 were looked at. Out of the 5,500, only 74 (~ 1% of the genome) showed differential expression in biofilm. 34 were up regulated in biofilm and 39 were repressed. A fraction of these genes have previously been associated with biofilm antibiotic resistance in *P. aeruginosa*. The overall conclusion in the study was that mature biofilm and planktonic cells are very similar in gene expression patterns; however, the study also concludes that because biofilms are a heterogeneous mixture of cells, some cells within the biofilm may exhibit radically different expression patterns (Whiteley, *et al.* (2001).

Another important gene involved in biofilm formation in *P. aeruginosa* is *gacA*. This gene is part of the GacA/S two component global regulatory system, and is involved in virulence. The way in which it effects biofilm formation is unknown, but cells deficient in this gene show a 10-fold decrease in biofilm formation. These cells also retain flagellar motility, type IV pili twitching motility, quorum sensing and alginate production indicating an alternative pathway for biofilm formation (Parkins *et al.* 2001).

E. coli is a gram-negative enteric bacterium and is a normal inhabitant of the gastrointestinal tract. This organism attaches to and colonizes a variety of abiotic surfaces resulting in biofilm formation. Also, *E. coli* K 12 requires casamino acid supplementation to initiate biofilm formation under laboratory conditions (Pratt and Kolter (1998). *E. coli* has been shown to require a number of genes involving motility and attachment to initiate biofilm formation.

In a study conducted by Pratt and Kolter (1998), mini Tn10 cam insertion and subsequent biofilm assay in 96 well plates was used to identify biofilm defective mutants in *E. coli* cells. The study revealed that flagellar motility and type 1 pili attachment are critical for biofilm formation. Cells with mutated genes responsible for flagella synthesis *fliC* and for flagellar function, *motA, motB, motAB,* were isolated. These cells had an inability to form flagella or were able to synthesize it but rendered it inoperative. These cells were unable to form biofilm. The study also revealed cells defective in *fim* (a gene required for type 1 pili formation) were unable to form biofilm. These cells could not attach to the surface and create microcolonies.

E. coli biofilms are encased in an exopolysaccharide (EPS) containing colanic acid and other polysaccharides. The synthesis of EPS is induced upon attachment to a surface (Prigent-Combaret *et al.* 1999). In a study conducted by Danese, *et al.* (2000) using mini-Tn10 cam transposon mutagenisis isolated a *wcf* mutant deficient in colanic acid production. When grown as a biofilm, *wcaF* mutants were able to attach to a surface but were unable to form the characteristic three-dimensional shape seen in wild type

strains. The cells in these mutants are tightly packed together and close to the surface. Attachment using curli adhesin is also important to *E. coli* biofilm formation. Vidal *et al.* (1998), found that when a gene responsible for synthesizing culi adhesin subunits, *csgA* was mutated, the cells were unable to attach and form biofilm.

A recent study looking at the role of the global regulator protein CsrA in biofilm formation found some interesting results. When the gene that codes for the protein was rendered non-functional, biofilm formation was dramatically increased. When the mutant biofilm was compared microscopically to the wild type biofilm, no visual changes could be seen in the mature biofilm but the mutant cells formed biofilm much more rapidly than did the wild type cells. Furthermore, when the gene was over-expressed using a multicopy plasmid, biofilm production was completely disrupted. The study also looked at the genes role in biofilm dispersal. A strain of *E. coli* was constructed in which the *scrA* gene could be controlled. The gene was only expressed in the presence of IPTG (isopropyl-B-D-thiogalactopyranoside). When the gene was expressed in mature biofilm, the biofilm dispersed over a period of 4 to 6 hours. The expression level of *csrA* was determined in new as well as mature biofilms. Using a B-galactosidase fusion, expression of the gene was followed throughout the biofilm formation. The study determined that the gene activity decreased during the first few hours of development and then increased to planktonic levels after 1.5 to 2 days (Jackson, *et al.* (2002).

Most data on biofilm growth focuses on initial surface interactions and genes involved in the early stages of biofilm growth. Some studies have however uncovered

some processes in established biofilms. In *P. aeruginosa, algC* is required for alginate production, which is an essential component of EPS. Using fluorogenic substrates and a *lacZ* reporter gene, Davies and Geesey (1995) revealed that *algC* is induced after attachment. In clinical strains of *P. aeruginosa* isolated from cystic fibrosis patients, Garrett *et al.* (1999) found that genes encoding alginate synthesis, *algT*, were upregulated and genes encoding flagella synthesis, *fliC*, were down regulated. These findings are consistent with the theory that upon attachment, flagella are no longer needed and large amounts of EPS are required in mature biofilm.

In *E. coli*, gene expression in biofilms has been shown to differ from that in planktonic cells. Using Mu dX transposon insertions to generate transcriptional fusions to a promoterless *lacZ* gene to allow the monitoring of promoter activity by means of a simple colorimetric assay, Prigent-Combaret *et al.* (1999) examined 885 bacterial strains. They found that approximately 38% of the genes expressed are altered when growing in biofilm. One gene, *fliC*, which encodes the flagellar structural protein was downregulated in biofilm. This finding is consistent with the findings of Gattett *et al.* (1999). They found that *fliC* was also down regulated in *P. aeruginosa* biofilms.

The conditions within the biofilm may also differ between biofilm and planktonic cells. Using the same protocol as above, Prigent-Combaret *et al.* (1999) discovered that bacteria within biofilms encounter higher-osmolarity conditions, greater oxygen limitation, and higher cell density than planktonic cells growing in a liquid medium. Measuring intracellular K^+ concentrations in planktonic and biofilm cells, the study found

biofilm cells to contain more K^+ than planktonic. Furthermore, four genes known to be osmoregulated were compared between biofilm and planktonic cells. The four genes are: the porin *ompC* gene (Sarma and Reeves (1977), the *proU* operon (encodes a high-affinity glycine betaine transport system) (Gowrishankar 1985), *wcaB* (involved in the synthesis of colanic acid in EPS) (Sledjeski and Gottesman (1996) and *fliC* (flagellin synthesis) which is down-regulated by high salt concentrations (Shi *et al.* 1993). Expression of the genes *ompC, proU,* and *wcaB*, were all up-regulated 2-3 fold in biofilms compared to planktonic cells. The *fliC* gene was also down regulated in biofilm cells as expected but was not completely abolished. One gene identified (using insertion mutagenesis described above) as the uncharacterized *f*92 ORF (ECAE000245; min 33.5), encoding a putative short protein (92 amino acids) was also down regulated in biofilm and affected by osmolarity.

Because of the environment within a biofilm, oxygen diffusion could play a role in biofilm growth and development. To determine the effects of oxygen content within the biofilm on gene expression, Prigent-Combaret *et al.* (1999) monitored a high affinity nickel transport system, *nikA*. This system is known to be highly tuned by the level of oxygen availability (Wu and Mandrand-Berthelot (1986). Using a *lacZ* fusion, expression of the gene was monitored in both biofilm and planktonic cells. After 23 and 45 h, a five-fold increase in expression was observed in biofilm cells indicating a definite difference in oxygen concentration. Prigent-Combaret (1999) also identified another gene affected by anaerobiosis, *pepT*, which encodes an aminotripeptidase T involved in

the removal of the N-terminal amino acid from tripeptides. Up regulation of *pepT* due to low oxygen concentrations has also been demonstrated in *Salmonella typhimurium* (Strauch *et al.* 1985). An important note is that unlike *nicA,* the genes *pepT, f92, ompC* and *proU* remained differentially expressed in the absence of oxygen indicating oxygen availability alone is not responsible for the differential expression of these genes in biofilms (Prigent-Combaret 1999).

To further examine the regulation of *pepT*, its expression in different growth conditions was examined. Cells with *pepT-lacZ* fusions were grown in medium containing high osmolarity and medium containing low osmolarity. Gene expression of *pepT* remained constant in both mediums indicating *pepT* is not osmoregulated. Using conditioned media (media containing signaling factors), cells containing *pepT-lacZ* fusions were examined. An over expression of *pepT* was seen at low cell density indicating a possible cell-to-cell signaling (i.e. quorum sensing regulation) connection to *pepT*.

Because the genome has been completely sequenced (Blattner *et al.* 1997) and gene arrays are commercially available, *E. coli* is an ideal organism to use in global gene expression studies. Several studies have successfully used gene array technology to analyze genome wide expression patterns in this organism. Tao *et al.* (1999) used array technology to measure gene expression patterns in *E. coli* K 12 grown in late logarithmic phase on minimal glucose medium and on Luria broth containing glucose. Recently, Arnold *et al.* (2001) used array analysis to study the effects of acetate treatment on *E. coli*

K 12. Their study revealed genome wide expression patterns and also identified genes that were both up regulated and down regulated under the experimental conditions. Using gene array technology, both studies demonstrated that genome wide expression patterns can be reliably compared in organisms grown under different conditions. In this study, we compared the transcriptional profiles of *E. coli* K12 chemostat-grown biofilm and planktonic cells. The focus of the study is on housekeeping genes in mature biofilm rather than genes involved in initial biofilm formation.

MATERIALS AND METHODS

Bacterial Strain

DS291 (Wildtype CF 1648 = MG 1655), an *E. coli* K-12 strain was obtained from D. A. Siegele at Texas A&M University and used in this study.

Media

Potassium morpholinopropane sulfonate (MOPS) buffered minimal media adapted from Neidhardt *et al.* (1974) with serine as a limited carbon source and the addition of amino acids required by amino acid auxotrophic strains was used to culture this organism. The media preparation is as follows:

(I) 1 liter of 10X MOPS concentrate is prepared by mixing in order the following solutions to prevent precipitation of various salts: 400 ml 1.0 M MOPS, freshly prepared, pH 7.4 using KOH; 40 ml 1.0 M N-Tris(hydroxymethyl)-methyl glycine (Tricine), freshly prepared, pH 7.4 using KOH; 10 ml 0.01 M $FeSO_4$, freshly prepared; 50 ml 1.90 M NH_4CL; 10 ml 0.276 M K_2SO_4; 10 ml 5.0×10^{-4} M $CaCl_2$; 10 ml 0.528 M $MgCl_2$; 100 ml 5.0 M NaCl; micronutrients{10 ml of a stock solution containing 3.0×10^{-6} M $(NH_4)_6(MO_7)_{24}$; 4.0×10^{-4} M H_3BO_3; 3.0×10^{-5} M $CoCl_2$; 1.0×10^{-5} M $CuSO_4$; 8.0×10^{-5} M $MnCl_2$; 1.0×10^{-5} M $ZnSO_4$}; and 360 ml glass distilled water for a total volume of 1000 ml. This solution is filter sterilized through a 0.22 micron filter and stored at 4° C. At this point, the carbon source, phosphate source and nutrients for amino acid auxotrophs are absent. (II) For each liter of MOPS medium, aseptically add 100 ml 10X MOPS concentrate; the carbon source, serine, (1mg/ml); uridine, (0.01 mg/ml); required

amino acids {Ile, Arg, Gly, His, Leu, Met, Phe, Val, Thr}, (each at 0.04 mg/ml), and sterile ddH_2O to bring to volume. The final pH of the medium is approximately 7.2.

Serine Limitation

Organisms form biofilm as a reaction to environmental stress (Watnick and Kolter (2000). To efficiently grow biofilm, nutrient limited media was used (see above). To determine the concentration of serine that creates a survival response for the selected organism, media containing all components except serine was made. Serine was then serially diluted in test tubes containing the described media. Each tube was inoculated with one loop full of bacteria from an overnight culture and placed in a 37° C incubator for 24 h. After 24 h, the OD 600 was taken of each sample and plotted out on a graph (Fig 2). The serine concentration used for this experiment is 1 mg/ml.

Biofilm Cultivation

Using a chemostat apparatus, which allows continual growth of bacterial cells under nutrient limited conditions (McLean *et al.* 1999), *E. coli* DS291 cells were grown at a dilution rate of $0.025h^{-1}$. The cells were taken from stocks frozen at –80°C, streaked onto nutrient agar and allowed to grow overnight at 37°C. A colony was then suspended in 1 ml of sterile phosphate buffered saline (PBS) and aseptically injected into the chemostat apparatus and allowed to grow for 24 h at 37°C. After 24 h incubation time, the feeding pumps were turned on at a dilution rate of $0.025h^{-1}$ and the chemostat was allowed to equilibrate for one full generation time or 40 h. After 40 h, a circulation pump was turned on at a flow rate of 100 ml/h. The media was allowed to flow through approximately 4 m of laboratory tubing (Dow Corning # 515-014) for a period of 4 d.

Planktonic cell counts were taken every 24 h over the 4 d period. After 4 d of biofilm growth, both planktonic and biofilm cells were collected.

Planktonic cell collection

200 ml of planktonic cells were aseptically taken directly from the chemostat culture flask and divided up between four 50 ml centrifuge tubes containing 4.5 ml ice cold stop solution (5% phenol/water-saturated in ethanol) and placed directly on ice. The cells were spun down at 4000 rpm, 4° C for 20 min. All but 5ml of the supernatant was decanted. The cells were re-suspended, combined into one 50 ml centrifuge tube and centrifuged as previously described. After centrifugation, the supernatant was poured off and the spun cells were stored at – 80° C for RNA extraction.

TEM imaging

Images were taken of lysed biofilm and planktonic cells using a transmission electron microscope (TEM). Both biofilm and planktonic samples were lysed using phenol / chloroform extraction. The samples were fixed using a 2% glutaraldehyde solution and dehydrated stepwise using acetone solutions from 10% to 100%. Grids were coated with formvar. 20 µl of dehydrated samples were then added to the grid and the acetone was allowed to evaporate. A 2% phosphotungstic acid solution was then added as a negative stain and allowed to evaporate. The samples were then viewed under the TEM.

Confocal imaging

Confocal images were taken of bacteria that were attached to the tubing used to grow biofilm. *E. coli* cells were grown as biofilm in a chemostat environment for a period of 4 days. At the end of the growth period, planktonic cells were removed by

washing the tubing out with 25 ml phosphate buffered saline. A 1 cm section of tubing was removed and placed in a solution containing 5 micro-liters of BacLite live dead stain (L-7007, Molecular Probes) in 10 ml dH_2O for 10 min. The tube piece was then placed on a microscope slide upside down and viewed under the confocal microscope.

Biofilm collection

The outside of the laboratory tubing (approximately 3 m) containing the biofilm was wiped down with 70% ethanol. All remaining media was drained out of the tubing and the lowest end (end closest to the bottom of the culture flask) was clamped off. The top end of the tube was cut and 25 ml of ice-cold stop solution was aseptically poured into the tube. The bottom clamp was released allowing the stop solution to flow through the tube and drain into the culture flask rinsing any remaining planktonic cells out. The bottom tube was immediately re-clamped to prevent media from flowing back into the tube. The tube was cut into pieces approximately 3 cm in length and placed in an 800 ml beaker containing 200 ml ice-cold stop solution. Each piece of tubing was removed, cut down the center to produce two halves and, using a scalpel, aseptically scraped into a sterile glass petri dish containing ice-cold stop solution in order to remove attached biofilm. After being cut and scraped, the pieces were put back into the beaker. The stop solution in the glass petri dish was also put into the beaker. The beaker was placed into an ice bath sonicator (Sonicor Instrument Corporation, Copiague, New York) and sonicated at 60 htz for 10 min to break up biofilm and detach remaining cells from tubing. After sonication, the supernatant was poured into 50 ml centrifuge tubes. The cells were then centrifuged and stored using the same technique as was used for the planktonic cells above.

RNA Processing

Cells were removed from the –80° C freezer and thawed on ice. Total RNA was extracted from the thawed cells using a modified hot-phenol extraction (Wonchul 2000). Briefly, cells were resuspended and lysed with lysozyme. Proteins were removed using phenol extraction (65°C). The phenol was removed using chloroform and DNA precipitated using 95% ethanol and washed with 80% ethanol. Nucleic acid pellet was suspended in 200 µl RNAse-free DEPC-treated water. Contaminating DNA was removed with RNAse-free DNAse (Boehringer Mannheim; # 776185) followed by 1 phenol extraction and 1 phenol/chloroform extraction, 2 chloroform extractions and an ethanol precipitation and wash. The RNA was then resuspended in 100 µl of DEPC water. RNA concentration was determined from the absorbance at 260 nm.

Gene Array Analysis

The gene array protocol is described in Arnold *et al.* (2001). ^{33}P-labeled cDNA probes were prepared using *E. coli* gene-specific primers (Sigma-Genosys, The Woodlands, Tex.). The cDNA was hybridized to sequential Panorama *E. coli* gene arrays (Sigma-Genosys). Background was defined as the expression level of the uninduced *lac* operon. The filters were exposed to a Fujix BAS2000 phosphorimager and results were analyzed using Visage HDG Analyzer software (R.M. Lupton, Inc,. Jackson, Mich.) running on a Sun Microsystems ULTRA10 workstation. Further analysis was done using Microsoft Excel.

The criteria used for the determination of altered expression in open reading frames (ORF's) was one, there had to be at least a two fold increase or decrease in

expression as compared to the background and two, the expression level had to change at least two fold in at least two independent experiments to be considered significant.

Results

Stability of Chemostat Grown Bacterial Cultures

In gene expression studies, it is important to have stable cell cultures to decrease variation among the cell population. To ensure chemostat culture uniformity, planktonic cell counts were taken in triplicate every 24 h. The data in table 2 shows planktonic cell counts over the entire growth period and Fig 2 is a graphical representation of the same data.

	CFU/cm	CFU in 3 Meters
Chemostat One	$9.65X10^{7}$	$2.90X10^{11}$
Chemostat Two	$8.87X10^{7}$	$2.66X10^{11}$
Surface Area	602.88 sq cm	

Table 2. Biofilm cell counts from two chemostats after a 4-day growth period.

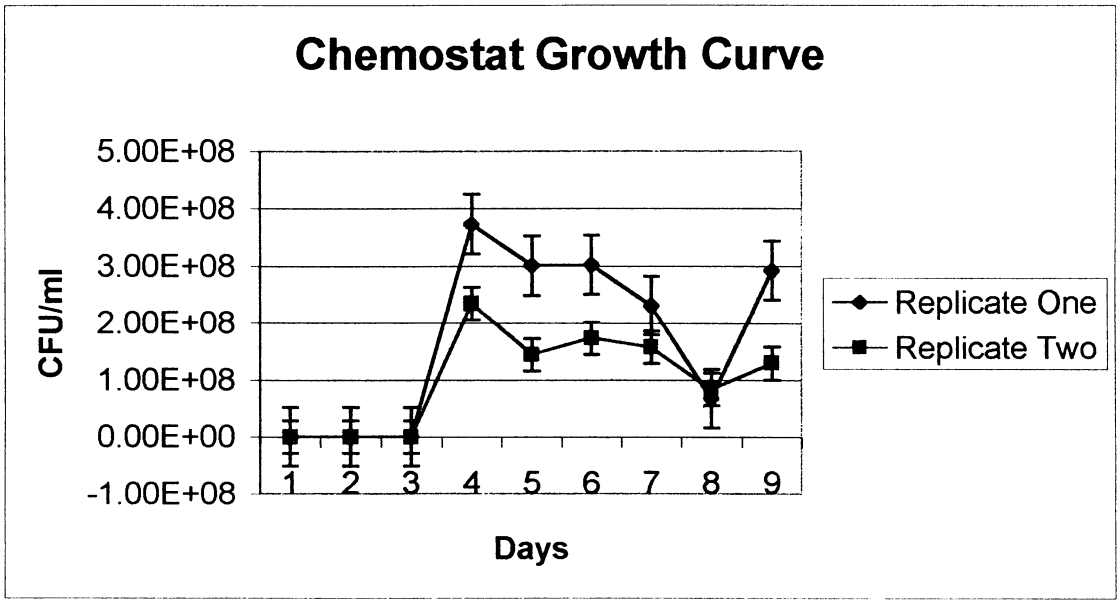

Fig 2. Graph of planktonic cells over the entire chemostat growth period. The biofilm cultivation began on day five.

Contamination was checked during the growth period by visually checking cell counts for colony uniformity and Biolog was performed before inoculation as well as

after the growth period on planktonic cells only. The data in table 3 shows the percent identity to *E. coli* as determined by Biolog on two samples. The data in table 4 shows each biochemical test performed in Biolog and the results.

Planktonic

Replicate One (pre chemostat)	100% probability
Replicate One (Post chemostat)	99% probability
Replicate Two (pre chemostat)	100% probability
Replicate Two (Post chemostat)	100% probability

Table 3. Probability of cells being *E. coli* according to Biolog.

Test	W	1	2	3	4	Test	W	1	2	3	4	Test	W	1	2	3	4
Water	A1	-	-	-	-	Xylitol	C1	-	-	-	-	L-alanyl-glycine	F7	+	+	+	+
Alpha-cyclodextrin	A2	-	-	-	-	methyl-pyruvate	C11	+	+	+	+	L-asparagine	F8	+	+	+/-	+/-
Dextrin	A3	+	+	+	+	Mono-methyl succinate	C12	+	+/-	+	+/-	L-aspartic acid	F9	+	+	+	+/-
Glycogen	A4	-	-	-	-	Acetic acid	D1	+	+	-	+	L-glutamic acid	F10	-	-	-	-
Tween 40	A5	-	-	-	-	Cis-aconitic acid	D2	-	-	-	-	Glycyl-L-aspartic acid	F11	+	+	+	+
Tween 80	A6	+/-	-	-	+/-	Citric acid	D3	-	-	-	-	Glycyl-L-glutamic acid	F12	-	-	-	-
N-acetyl-D-galactos-amine	A7	+	+/-	+	+/-	Formic acid	D4	-	-	-	-	L-histidine	G1	-	-	-	-
N-acetyl-D-glucosamine	A8	+	+	+	+	D-galactonic acid lactone	D5	+	+	+	+	Hydroxy L-proline	G2	-	-	-	-
Adonitol	A9	-	-	-	-	D-galacturonic acid	D6	+	+	+	+	L-leucine	G3	-	-	-	-
L-arabinose	A10	+	+	+	+	D-gluconic acid	D7	+	+	+	+	L-ornithine	G4	-	-	-	-
D-arabitol	A11	-	-	-	-	D-glucosaminic acid	D8	-	-	-	-	L-phenyl alanine	G5	-	-	-	-
Cellobiose	A12	-	-	-	-	D-glucuronic acid	D9	+	+	+	+	L-proline	G6	-	-	-	-
I-erythritol	B1	-	-	-	-	Alpha-hydroxy butyric acid	D10	+/-	-	-	+	L-pyroglutamic acid	G7	-	-	-	-
D-fructose	B2	+	+	+	+	Beta-hydroxy butyric acid	D11	-	-	-	-	D-serine	G8	+	+	+	+
L-fucose	B3	+	+	+	+	Gamma-hydroxy butyric acid	D12	-	-	-	-	L-serine	G9	+	+	+	+/-
D-galactose	B4	+	+	+	+	p-hydroxy phenylacetic acid	E1	-	-	-	-	L-threonine	G10	-	-	-	-
Gentiobiose	B5	-	-	-	-	Itaconic acid	E2	-	-	-	-	D,L-carnitine	G11	-	-	-	-
Alpha-D-glucose	B6	+	+	+	+	Alpha-keto butyric	E3	+/-	-	-	+/-	Gamma-amino	G12	-	-	-	-

						acid						butyric acid					
M-inositol	B7	-	-	-	-	Alpha-keto glutaric acid	E4	+/-	+	-	-	Urocanic acid	H1	-	-	-	-
Alpha-D-lactose	B8	+	+	+	+	Alpha-keto valeric acid	E5	-	-	-	-	inosine	H2	+	+	+	+
Lactulose	B9	-	-	-	-	D,L-lactic acid	E6	+	+	+	+	uridine	H3	+	+	+	+
Maltose	B10	+	+	+	+	Malonic acid	E7	-	-	-	-	thymidine	H4	+	+	+	+
D-mannitol	B11	+	+	+	+	Propionic acid	E8	-	-	-	-	phenyl ethylamine	H5	-	-	-	-
D-mannose	B12	+	+	+	+	Quinic acid	E9	-	-	-	-	Putrescine	H6	-	-	-	-
D-melibiose	C1	+	+	+	+	D-saccharic acid	E10	-	-	-	-	2-amino ethanol	H7	-	-	-	-
Beta-methyl-D-glucoside	C2	+	+/-	-	+	Sebacic acid	E11	-	-	-	-	2,3-butanediol	H8	-	-	-	-
D-psicose	C3	+/-	+	+	+	Succinic acid	E12	+	+	+	+	Glycerol	H9	+	+	+	+
D-raffinose	C4	-	+	-	-	Bromo succinic acid	F1	+	+	-	-	D,L-alpha-glycerol phosphate	H10	+	+	+	+
L-rhamnose	C5	+	+	+	+	Succinamic acid	F2	-	-	-	-	Glucose-1-phosphate	H11	+	+	+	+
D-sorbitol	C6	+	+	+	+	Glucuronamide	F3	-	+	+	+	Glucose-6-phosphate	H12	+	+	+	+
Sucrose	C7	-	-	-	-	Alaninamide	F4	-	-	-	-						
D-trehalose	C8	+	+	+	+	D-alanine	F5	-	+	-	-						
Turanose	C9	-	-	-	-	L-alanine	F6	+	+	+	+/-						

Table 4. Summary of Biolog results. Each biochemical test indicates wheather or not an organism can utiliaze the product as a carbon source. 1= Replicate one, organism before chemostat inoculation. 2 = Replicate one, organism after biofilm growth period. 3 = Replicate two, organism before chemostat inoculation. 3 = Replicate two, organism after biofilm growth period. - = negative, + = positive, +/- = undetermined result.

Confirmation of Biofilm Presence

It is important to determine the actual presence of biofilm on the tubing since both biofilm and planktonic cells share the same media. Tubing used to grow biofilm was

taken, rinsed and stained with a fluorescence dye and viewed under confocal microscopy to confirm the presence of attached cells.

Transmission Electron Microscope

To visualize the effect cell lysis has on planktonic and biofilm cell, both types of cells were lysed using the same method and visualized using a transmission electron microscope. Figures 3 thru 6 illustrate planktonic and biofilm cells at different stages in the cell lysis procedure.

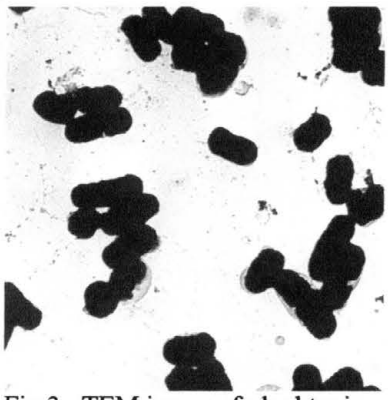

Fig 3. TEM image of planktonic cells before cell lysis. 6,000X magnification

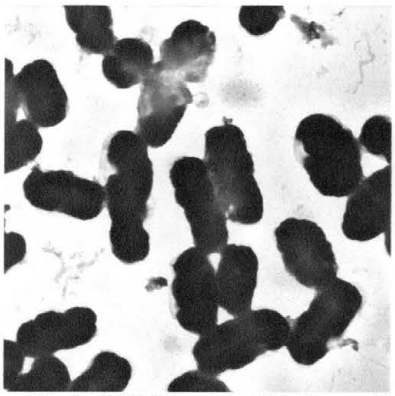

Fig 4. TEM image of biofilm cells before cell lysis. 10,000X magnification

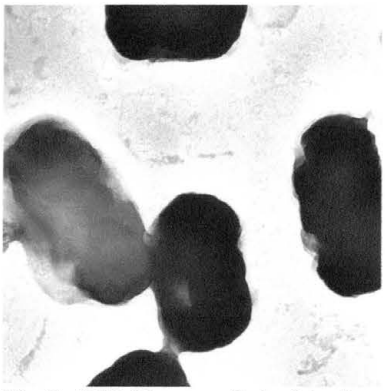

Fig 5. TEM image of planktonic cells after cell lysis. 52,000X magnification

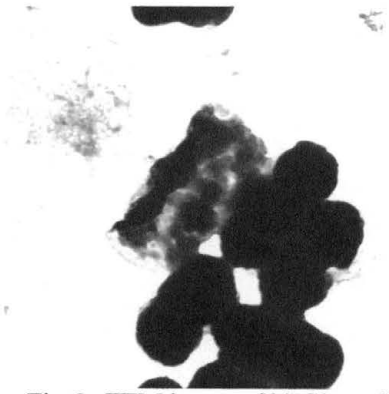

Fig 6. TEM image of biofilm cells after cell lysis. 15,000X magnification

RNA Extraction

Extracting RNA from biofilm cells is difficult. To get a visual representation of cells that have undergone phenol/chloroform RNA extraction, a transmission electron microscope was used. Cells were lysed using the same methods used in the gene array experiment, negative stained and then viewed under the microscope (figures not shown).

E. coli MG1655 cells were grown as a mature biofilm and as a planktonic cell culture under nutrient limited conditions in a chemostat environment (Blazer, 2001). After 4 days of growth, the biofilm and planktonic cells were removed and the total RNA was extracted using standard phenol/chloroform methods. The data in table 5 shows the yield of RNA extracted, protein contamination and background contamination from planktonic and biofilm cells.

	Planktonic	Biofilm
Replicate One, RNA [ ]	1.097	0.187
280/260	0.664	0.612
260/280	1.51	1.64
320	0.0003	0.0001
Replicate Two, RNA [ ]	2.10	0.428
280/260	0.658	0.697
260/280	1.52	1.43
320	0.0056	0.0003

Table 5. Amount of RNA retrieved from planktonic and biofilm cells. Amounts are shown in micrograms per µl. RNA concentration was calculated using OD260X400X40/1000 where 400 is the dilution factor, 40 is the constant for RNA concentration, and dividing by 1000 puts the concentration into micrograms per µl. 260/280 ratio was used as a protein contamination indicator. The320 absorption was used as a background correction.

All RNA samples were run out on an agarose formaldehyde denaturing gel.

Figure 7 shows both replicates.

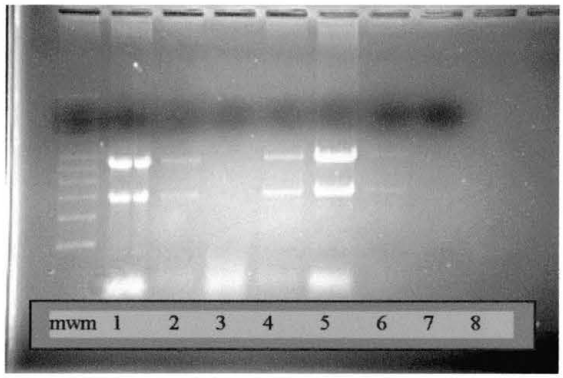

Fig 7. Denaturing formaldehyde gel showing RNA used in gene array experiments. Lanes 1&2 show replicate one. Lanes 4&5 shows replicate two. Lane 1 is planktonic, lane 2 is Biofilm. Lane 4 is biofilm and lane 5 is planktonic.

The RNA was reverse transcribed into cDNA and radiolabeled with P^{33}. Radioactive counts were taken from all samples and standardized to $2.0X10^7$ CPM. The data in table 6 shows the radiation counts of all samples.

	Planktonic		Biofilm	
Replicate One	572973 Integrated	69.5% Incorporation	690950 Integrated	81.8% Incorporation
	824600 Total		844130 Total	
Replicate Two	736950 Integrated	76.7% Incorporation	666320 Integrated	70.8% Incorporation
	959860 Total		940800 Total	

Table 6. Radiation counts for all cDNA samples. Counts are shown in counts per minute (CPM). Total counts are counts before unincorporated nucleotides are removed. Integrated counts are counts after unincorporated nucleotides are removed. Percent incorporation is calculated by dividing Incorporated counts by total counts. 70% incorporation is considered useable.

Array Hybridization and Analysis:

A phosphorimage of two gene arrays is shown in figure 8. Each gene array is arranged into three panels. Each spot on the array contains 10 ng of PCR amplified DNA and each gene on the array is spotted in duplicate. The four corners of each panel contain

genomic DNA as a control (http://www.sigmaaldrich.com.au/panorama.htm).

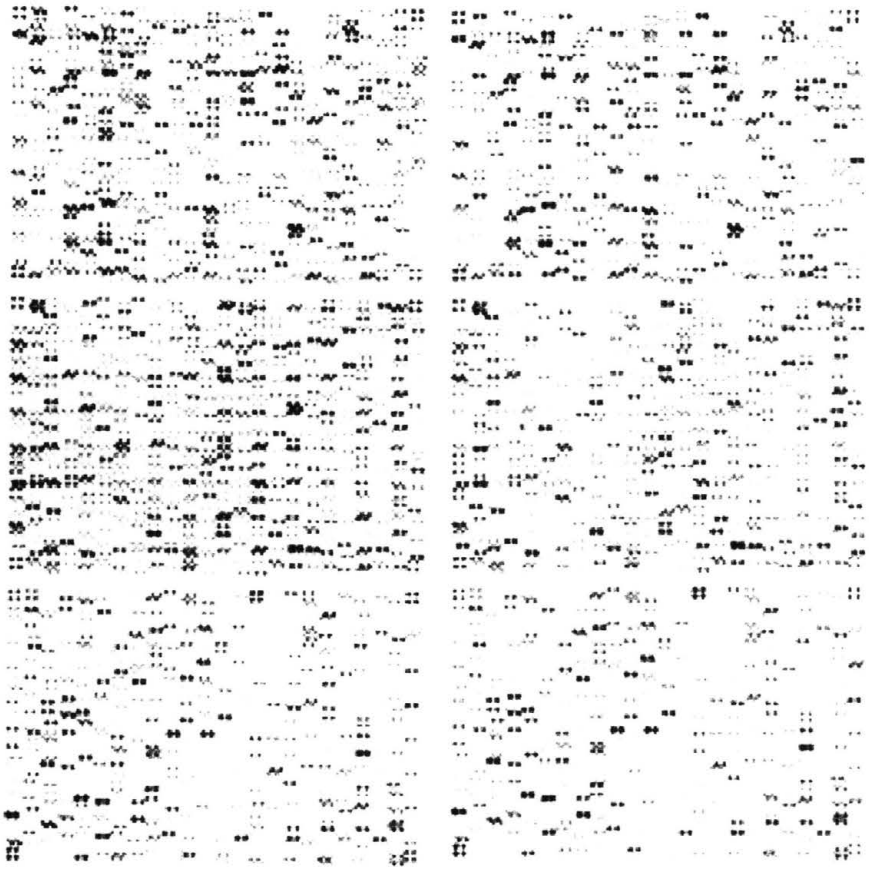

Fig 8. Example of a phosphorimage of two gene arrays. Each array is separated into three panels and each gene is duplicated twice. One array is on the left and one is on the right. (taken from http://www.sigmaaldrich.com.au/panorama.htm).

The probes were then allowed to hybridize to separate gene arrays containing all 4290 open reading frames in the *E. coli* genome (Blattner, F. R. et. al. 1997). The radioactive intensity of each gene on the arrays was analyzed using a phosphorimager. The expression level of each gene was then determined and compared to its planktonic/biofilm counterpart. Genes that had an increase in expression by a minimum of two fold when compared to their planktonic or biofilm counterpart were considered up-regulated. Genes up-regulated from greater than or equal to 1.5 to 1.9 were also determined. Genes being expressed below the background level, which is defined as the

level of expression of the uninduced *lac* operon, were discarded. A list of genes up regulated by a factor of two or greater in both biofilm and planktonic cell is shown by the data in Table 7. The data in tables 8 thru 12 shows genes being up regulated in decreasing amounts.

Biofilm	Replicate One	Replicate Two	Planktonic	Replicate One	Replicate Two
Gene	Expression above Planktonic	Expression above Planktonic	Gene	Expression above Biofilm	Expression above Biofilm
deaD	3.37	2.05	*b0795 (f332)*	2.13	2.08
cspE	3.20	2.57	*ydeA*	2.08	2.04
accB	2.48	2.24	*rbfA*	2.22	2.33
hisL	3.46	2.29	*b0788 (f318)*	2.86	2.17
nusG	2.37	3.30	*b1012 (f382)*	2.00	2.38
hemL	2.94	2.38	*b4341 (o54)*	2.22	2.70
rpoH	2.66	2.44	*yebA*	2.08	2.86
b0331 (o296)	2.03	2.06			
b1364 (o93)	2.30	2.30			
yheF	2.43	2.11			
b1360 (o248)	2.12	2.08			
ytfJ	2.44	2.78			
b2986 (o230)	2.22	2.24			
yjbF	2.14	2.01			
B1586 (o115)	2.18	2.10			
yjfR	3.00	2.22			
yecC	2.10	2.03			
yecI	2.59	2.20			
B3533 (hypo)	2.32	3.08			
B1567 (f49)	3.51	2.51			
b1858 (o251)	3.93	3.72			

Table 7. Genes up-regulated in both replicates by a factor of 2 two or greater in both planktonic and biofilm cells.

Biofilm	Replicate One	Replicate Two	Planktonic	Replicate One	Replicate Two
Gene	Expression Above Planktonic	Expression Above Planktonic	Gene	Expression Above Biofilm	Expression Above Biofilm
ilvH	1.97	2.27	*hyaF*	1.92	1.96
b1437 (f65)	1.94	2.45	yehZ	3.03	1.99
b2632 (o289)	1.94	2.68	livF	3.70	1.96
b1527 (o371)	1.99	2.27	yehU	2.38	1.96
b1393 (o255)	2.36	1.99	yjiH	1.99	1.92
b1434 (o178)	2.17	1.98	b1957 (f60)	2.94	1.99
b2987 (f252)	2.34	1.93			

Table 8. Genes up-regulated in both replicates by a factor of between 1.9 and 2 in both planktonic and biofilm cells. Genes up regulated by more than two fold are excluded from this table.

Biofilm	Replicate One	Replicate Two	Planktonic	Replicate One	Replicate Two
Gene	Expression Above Planktonic	Expression Above Planktonic	Gene	Expression Above Biofilm	Expression Above Biofilm
rplK	1.80	1.99	*b2968 (f178)*	2.04	1.85
rpsA	1.92	1.87	*gltJ*	1.85	2.38
b2043 (f464)	3.08	1.86	*cmtB*	2.33	1.82
yhbT	1.84	1.82	b3047 (o252)	3.47	1.82
b2739 (o258)	1.80	2.35	narY	2.78	1.89
B1520 (f321)	3.09	1.87	metE	1.96	1.89
yjdC	1.87	2.37			
b1606 (o240)	1.83	1.87			
b2544 (f290)	1.88	1.86			

Table 9. Genes up-regulated by a factor of between 1.8 and 1.9.

Biofilm	Replicate One	Replicate Two	Planktonic	Replicate One	Replicate Two
Gene	Expression Above Planktonic	Expression Above Planktonic	Gene	Expression above Biofilm	Expression above Biofilm
infC	1.72	1.85	*nupG*	3.57	1.72
b0458 (f169)	2.25	1.79	*ipdA*	1.82	1.79
ybeH	1.75	2.39	*pstC*	2.27	1.72
b0607 (f142)	1.72	2.07	*b3047 (o252)*	3.85	1.82
sppA	1.80	2.90	*tolQ*	1.79	1.82
B2673 (o81)	1.81	1.79	*b0617 (f98)*	1.72	1.89
b1681 (f423)	1.76	1.91	*dmsB*	1.96	1.79
b2674 (o136)	1.74	1.78	*frdA*	2.94	1.75
yifH	1.84	1.70	*b2324 (o688)*	2.27	1.79
ycjD	1.99	1.73	*b1117 (o228)*	2.33	1.69
yjgX	1.80	1.82	*ybaM*	3.33	1.79
yihD	1.70	1.90			
ycjC	2.89	1.71			
crr	1.77	1.71			
b2873 (o465)	1.73	1.75			
b3012 (o236)	1.71	1.80			
yedA	1.83	1.73			
yjaB	2.12	1.73			
B1481 (f92)	1.88	1.71			
b1554 (f177)	1.75	1.95			

Table 10. Genes up-regulated by a factor of between 1.7 and 1.8.

Biofilm	Replicate One	Replicate Two	Planktonic	Replicate One	Replicate Two
Gene	Expression Above Planktonic	Expression Above Planktonic	Gene	Expression above Biofilm	Expression above Biofilm
cysU	1.73	1.62	*b2077 (o471)*	1.64	1.69
b2556 (f496)	2.54	1.70	*napG*	1.64	1.61
rpoS	1.67	1.76	*b2299 (f180)*	2.04	1.64
cysM	1.66	1.97	*b2768 (o191)*	2.38	1.64
ppdB	3.48	1.65	*appC*	1.67	1.92
fabG	1.69	1.76	*rfaY*	1.75	1.61
tesB	1.63	3.56	*fumB*	3.85	1.67
b0659 (f155)	2.08	1.60	*relB*	2.13	1.61
fadD	1.65	1.82	*ictD*	2.17	1.61
asnS	2.70	1.62	*potF*	1.92	1.64
ybaB	1.65	1.64	*phnC*	1.69	1.69
ybcJ	1.62	1.96	*yadF*	1.69	1.61
b1378 (f1174)	1.65	1.64	*adk*	2.38	1.67
b1398 (o437)	1.70	1.65	*yiiD*	1.67	2.08
yieC	1.62	1.62	*yheD*	2.38	1.67
b1240 (f76)	1.66	1.62	*ychM*	1.79	1.69
b1330 (f343)	2.08	1.63	*ydjB*	2.94	1.61
yheH	1.64	3.19			
o149	1.61	1.84			
b2272 (o167)	2.11	1.66			
b2301 (f214)	2.18	1.61			
ydfB	1.68	1.98			
yjbH	2.36	1.69			
b1825 (f95)	3.46	1.65			
b1630 (o352)	1.66	2.44			
yijD	2.57	1.68			
yjbR	1.69	1.92			
b1843 (o218)	1.64	2.22			

Table 11. Genes up-regulated by a factor of between 1.6 and 1.7.

Biofilm	Replicate One	Replicate Two	Planktonic	Replicate One	Replicate Two
Gene	Expression Above Planktonic	Expression Above Planktonic	Gene	Expression above Biofilm	Expression above Biofilm
ybeI	2.43	1.54	*yicM*	3.13	1.56
ybiC	1.54	1.78	*proV*	1.85	1.54
b1180 (o219)	1.53	1.86	*glvG*	1.92	1.54
cynT	1.59	1.55	*flhD*	1.52	1.59
b1444 (o474)	1.56	1.66	*yhdM*	1.61	1.54
yjhP	2.25	1.59	*osmE*	1.64	1.54
yfaA	1.59	1.53	*potD*	2.22	1.59
b1651 (o135)	1.57	2.20	*glpE*	1.92	1.56
yjiA	1.66	1.58	*ykgC*	1.59	1.59
yraQ	1.55	2.70	*minE*	1.54	1.67
b3838 (hypo)	2.82	1.57	*flgM*	1.59	1.56
yjfP	1.54	2.33	*hyaD*	1.56	1.56
b2359 (o148)	1.51	1.73	*b1634 (o500)*	1.59	1.52
b2325 (f92)	1.91	1.57	*mbhA*	2.94	1.59
yjbA	2.29	1.54	*yaeI*	1.52	1.59
b2447 (o197)	1.57	1.92	*fusA*	1.67	1.59
fumA	1.52	1.56	*b0841 (f198)*	1.69	1.59
b1631 (o206)	1.57	1.82	*rfbB*	1.52	2.17
yebL	1.58	2.21	*pepQ*	1.64	1.59
			yabF	1.72	1.59
			topB	1.52	1.64
			b0371 (o486)	1.52	1.69
			tufB	1.56	1.56
			b1191 (f536)	1.59	2.17
			b1047 (f385)	1.69	1.52
			b3865 (f199)	1.64	1.56

Table 12. Genes up-regulated by a factor of between 1.5 and 1.6.

The data in table 13 shows differentially expressed genes and their respective function for biofilm up-regulated genes. The data in table 14 shows planktonic up-regulated genes and their function.

Biofilm Up-regulated Genes

Gene Name	Description	Function
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
cspE	cold shock-like protein cspE	Not classified
accB	biotin carboxyl carrier protein	Biosynthesis of cofactors, carriers: biotin carboxyl carrier protein (BCCP)
hisL	his operon leader peptide	Amino acid biosynthesis: Histidine
nusG	transcription antitermination protein nusg	RNA synthesis, modification, DNA transcription
hemL	glutamate-1-semialdehyde 2,1-aminomutase	Biosynthesis of cofactors, carriers: Heme, porphyrin
rpoH	RNA polymerase sigma-32 subunit	Global regulatory functions

B0331 (o296)	o296; 37 pct identical (5 gaps) to 293 residues of approx. 296 aa protein BCPA_STRHY SW: P11435	Not classified
b1364 (o93)	o93; 30 pct identical (6 gaps) to 75 residues from flagellar biosynthetic protein, FLIP_BACSU SW: P35528 (221 aa); UUG start	Unknown
yheF	putative general secretion pathway protein d precursor	Not classified
B1360 (o248)	o248; This 248 aa ORF is 50 pct identical (3 gaps) to 241 residues of an approx. 248 aa protein DNAC_ECOLI SW: P07905	Not classified
ytfJ	18.2 kD protein in cysq-msra intergenic region precursor (f18	Unknown
B2986 (o230)	o230; This 230 aa ORF is 39 pct identical (9 gaps) to 64 residues of an approx. 200 aa protein KAD1_PIG SW: P00571	Unknown
yjbF	hypothetical 25.0 kD lipoprotein in pgi-xylE intergenic region	Unknown
B1586 (o115)	o115; This 115 aa ORF is 30 pct identical (2 gaps) to 62 residues of an approx. 2032 aa protein LAR_DROME SW: P16621	Unknown
yjfR	hypothetical 40.3 kD protein in aidB-rpsF intergenic region	Unknown
yecC	f222; This 222 aa ORF is 48 pct identical (0 gaps) to 208 residues of an approx. 232 aa protein YCKA_BACSU SW: P42399	Not classified
yecI	Ferritin-like protein	Transport of small molecules: Cations
b3533 (hypo)	hypothetical 101.6 kD protein in dctA-dppF intergenic region	Not classified
B1567 (f49)	f49; This 49 aa ORF is 35 pct identical (3 gaps) to 40 residues of an approx. 440 aa protein YKI2_YEAST SW: P36080	Unknown
b1858 (o251)	o251; This 251 aa ORF is 54 pct identical (0 gaps) to 239 residues of an approx. 272 aa protein Y408_HAEIN SW: P44692	Not classified
ilvH	acetolactate synthase isozyme III small subunit	Amino acid biosynthesis: Isoleucine, Valine
B1437 (f65)	f65; UUG start; This 65 aa ORF is 31 pct identical (2 gaps) to 48 residues of an approx. 336 aa protein UL38_HCMVA SW: P16779	Unknown
b2632 (o289)	o289; This 289 aa ORF is 24 pct identical (3 gaps) to 131 residues of an approx. 528 aa protein MSS1_YEAST SW: P32559	Not classified
b1527 (o371)	o371; This 371 aa ORF is 31 pct identical (7 gaps) to 111 residues of an approx. 336 aa protein IPNS_STRCL SW: P10621	Unknown
b1393 (o255)	o255; This 255 aa ORF is 47 pct identical (0 gaps) to 250 residues of an approx. 296 aa protein ECHM_CAEEL SW: P34559	Not classified
b1434 (o178)	o178; residues 15-87 are 31 pct identical to aa 27-99 from GB: MTBFRA_1 Accession L26406	Unknown
B2987 (f252)	probable low-affinity inorganic phosphate transporter 2	Transport of small molecules: Anions
rplK	50S ribosomal subunit protein L11	Ribosomal proteins - synthesis, modification
rpsA	30S ribosomal protein S1	Ribosomal proteins - synthesis, modification
B2043 (f464)	f464; This 464 aa ORF is 77 pct identical (7 gaps) to 461 residues of an approx. 472 aa protein	Unknown

	YEFK_SALTY SW: P26389	
yhbT	hypothetical 19.7 kD protein in sohA-mtr intergenic region	Unknown
b2739 (o258)	o258; 40 pct identical amino acid sequence and equal length to GIP_ECOLI SW: P30147	Unknown
B1520 (f321)	f321; This 321 aa ORF is 27 pct identical (11 gaps) to 97 residues of an approx. 464 aa protein YKT8_CAEEL SW: P34319	Unknown
yjdC	f199; This 199 aa ORF is 100 pct identical to 190 residues of a 191 aa protein YJDC_ECOLI SW: P36656 but contains 8 additional N-ter aa and about 0 C-ter residues	Unknown
b1606 (o240)	o240; This 240 aa ORF is 31 pct identical (19 gaps) to 237 residues of an approx. 280 aa protein FIXR_BRAJA SW: P05406	Not classified
B2544(f290)	f290; This 290 aa ORF is 32 pct identical (4 gaps) to 105 residues of an approx. 312 aa protein YIHR_ECOLI SW: P32139	Unknown
infC	initiation factor IF-3	Proteins - translation and modification
B0458 (f169)	f169; This 169 aa ORF is 32 pct identical (0 gaps) to 53 residues of an approx. 592 aa protein ASN1_PEA SW: P19251	Unknown
ybeH	hypothetical protein in cspE-lipA intergenic region	Unknown
B0607 (f142)	f142; This 142 aa ORF is 30 pct identical (4 gaps) to 126 residues of an approx. 152 aa protein YFMU_COXBU SW: P45680	Unknown
sppA	protease IV	Degradation of proteins, peptides, glyco
B2673 (o81)	o81; This 81 aa ORF is 28 pct identical (2 gaps) to 70 residues of an approx. 104 aa protein VG56_BPML5 SW: Q05266	Biosynthesis of cofactors, carriers: Thioredoxin, glutaredoxin, glutathione
B1681 (f423)	f423; This 423 aa ORF is 29 pct identical (1 gap) to 172 residues of an approx. 488 aa protein YC24_CYAPA SW: P48260	Unknown
b2674 (o136)	o136; This 136 aa ORF is 55 pct identical (2 gaps) to 127 residues of an approx. 160 aa protein Y230_MYCGE SW: P47472	Unknown
yifH	hypothetical 19.6 kD protein in rffE-rffT intergenic region	Unknown
ycjD	hypothetical 14.0 kD protein in envM-sapF intergenic region	Unknown
yjgX	hypothetical 16.4 kD protein in leuX-fecE intergenic region	Unknown
yihD	hypothetical 10.3 kD protein in mobA 3'region (o89)	Unknown
ycjC	o185; 100 pct identical to 121 aa fragment YCJC_ECOLI SW: P38522 but has 64 additional N-terminal residues	Unknown
crr	pts system, glucose-specific IIA component	Transport of small molecules: Carbohydrates, organic acids, alcohols
b2873 (f92)	o465; This 465 aa ORF is 36 pct identical (12 gaps) to 455 residues of an approx. 576 aa protein TO64_RAT SW: P47942; UUG start	Unknown
b1554 (f177)	f177; This 177 aa ORF is 32 pct identical (15 gaps) to 166 residues of an approx. 168 aa protein LYCV_BPPA2 SW: P10439	Not classified
cysU	sulfate transport system permease protein CysT	Transport of small molecules: Anions

b2556 (f496)	f496; f496 (ttg start); ORF_f460 GB: U36841 uses atg start at 2685714; This 496 aa ORF is 30 pct identical (14 gaps) to 292 residues of an approx. 480 aa protein CREC_ECOLI SW: P08401	Not classified
rpoS	RNA polymerase sigma subunit RpoS (sigma-38)	Global regulatory functions
cysM	Cysteine synthase B	Amino acid biosynthesis: Cysteine
ppdB	prepilin peptidase dependent protein B precursor	Not classified
fabG	3-oxoacyl-[acyl-carrier protein] reductase	Fatty acid and phosphatidic acid biosynthesis
tesB	acyl-coA thioesterase II	Fatty acid and phosphatidic acid biosynthesis
b0659 (f155)	f155; This 155 aa ORF is 24 pct identical (7 gaps) to 113 residues of an approx. 336 aa protein FBP_HAEIN SW: P35755	Unknown
fadD	long-chain-fatty-acid--CoA ligase	Degradation of small molecules: Fatty acids
asnS	asparaginyl-tRNA synthetase	Aminoacyl tRNA synthetases, tRNA modification
ybaB	hypothetical 12.0 kD protein in dnaX-recR intergenic region	Unknown
ybcJ	hypothetical 7.4 kD protein in cysS-folD intergenic region	Unknown
b1378 (f1174)	f1174; This 1174 aa ORF is 55 pct identical (35 gaps) to 1161 residues of an approx. 1200 aa protein NIFJ_ANASP SW: Q06879	Not classified
b1398 (o437)	o437; 22 pct identical (2 gaps) to 106 residues from PKSJ_BACSU SW: P40806	Unknown
yieC	hypothetical 60.6 kD protein in bglB 5'region	Not classified
b1240 (f76)	f76; f76; broken; This 76 aa ORF is 42 pct identical (2 gaps) to 49 residues of an approx. 448 aa protein YI41_ECOLI SW: P03835	Unknown
b1330 (f343)	f343	Unknown
yheH	putative general secretion pathway protein i precursor	Not classified
o149	o149	Unknown
b2272 (o167)	o167	Unknown
b2301 (f214)	f214; This 214 aa ORF is 30 pct identical (3 gaps) to 130 residues of an approx. 224 aa protein GTT1_DIACA SW: P28342	Unknown
ydfB	hypothetical protein in dicA-dicB intergenic region	Unknown
yjbR	hypothetical 13.4 kD protein in tyrB-uvrA intergenic region	Unknown
b1843 (o218)	o218; This 218 aa ORF is 24 pct identical (2 gaps) to 87 residues of an approx. 2056 aa protein FAS1_YEAST SW: P07149	Unknown
ybeI	hypothetical 13.8 kD protein in cspE-lipA intergenic region	Unknown
ybiC	hypothetical 38.9 kD protein in dinG/rarB 3'region	Not classified
b1180 (o219)	o219; This 219 aa ORF is 43 pct identical (1 gap) to 187 residues of an approx. 216 aa protein YO23_CAEEL SW: P34673	Not classified
cynT	cyanate permease	Central intermediary metabolism: Pool, multipurpose conversions
b1444 (o474)	o474; This 474 aa ORF is 40 pct identical (8 gaps) to 468 residues of an approx. 528 aa protein YEQ3_YEAST SW: P40047	Not classified
yjhP	hypothetical 27.4 kD protein in fecI-fimB intergenic region	Not classified
yfaA	(243 aa) but contains 16 additional N-ter aa and 319 C-ter residues	Unknown

b1651 (o135)	o135; 74 pct identical amino acid sequence and equal length to Y323_HAEIN SW: P44638	Central intermediary metabolism: Pool, multipurpose conversions
yjiA	hypothetical 32.0 kD protein in mrr-tsr intergenic region (f2	Unknown
b2359 (o148)	o148; This 148 aa ORF is 26 pct identical (7 gaps) to 103 residues of an approx. 200 aa protein Y06Q_BPT4 SW: P39224	Unknown
b2325 (f92)	f92; This 92 aa ORF is 31 pct identical (4 gaps) to 67 residues of an approx. 624 aa protein UL32_HSVEB SW: P28952	Unknown
yjbA	o136	Unknown
b2447 (o197)	o197	Unknown
fumA	fumarate hydratase class I	Energy metabolism, carbon: TCA cycle
b1631 (o206)	o206; This 206 aa ORF is 48 pct identical (1 gap) to 200 residues of an approx. 208 aa protein YG87_HAEIN SW: P44291	Unknown
yebL	31.1 kD protein in msbB-ruvB intergenic region	Not classified

Table 13. Gene description and function of all genes up-regulated in biofilm cells (*E coli* Array Information Version 1.5 (Sigma 2000).

Planktonic Up-regulated Genes

Gene Name	Description	Function
B0795 (f332)	f332; This 332 aa ORF is 30 pct identical (7 gaps) to 294 residues of an approx. 360 aa protein YHII_ECOLI SW: P37626	Not classified
ydeA	hypothetical protein in marR 5'region	Drug/analog sensitivity
rbfA	ribosome-binding factor a (p15b protein)	Proteins - translation and modification
b0788 (f318)	f318; This 318 aa ORF is 28 pct identical (12 gaps) to 122 residues of an approx. 280 aa protein CYST_ECOLI SW: P16701	Unknown
b1012 (f382)	f382; This 382 aa ORF is 23 pct identical (14 gaps) to 295 residues of an approx. 376 aa protein YZEC_BACSU SW: P40402	Unknown
b4341 (o54)	o54	Unknown
b0795 (f332)	f332; This 332 aa ORF is 30 pct identical (7 gaps) to 294 residues of an approx. 360 aa protein YHII_ECOLI SW: P37626	Not classified
ydeA	hypothetical protein in marR 5'region	Drug/analog sensitivity
rbfA	ribosome-binding factor a (p15b protein)	Proteins - translation and modification
b0788 (f318)	f318; This 318 aa ORF is 28 pct identical (12 gaps) to 122 residues of an approx. 280 aa protein CYST_ECOLI SW: P16701	Unknown
b1012 (f382)	f382; This 382 aa ORF is 23 pct identical (14 gaps) to 295 residues of an approx. 376 aa protein YZEC_BACSU SW: P40402	Unknown
b4341 (o54)	o54	Unknown
yebA	hypothetical 46.7 kD protein in msbB-ruvB intergenic region	Unknown
metE	5-methyltetrahydropteroyltriglutamate- homocysteine methyltransferase	Amino acid biosynthesis: Methionine
nupG	nucleoside permease NupG	Transport of small molecules:

		Nucleosides, purines, pyrimidines
lpdA	dihydrolipoamide dehydrogenase	Energy metabolism, carbon: Pyruvate dehydrogenase
pstC	phosphate transport system permease protein PstC	Transport of small molecules: Anions
b3047 (o252)	o252; phage > ecoli	Not classified
tolQ	o230; 100 pct identical to TOLQ_ECOLI SW: P05828; alternate gene name fii	Colicin-related functions
b0617 (f98)	f98; residues 9-86 are 51 pct identical to 9-86 from CILG_KLEPN SW: P02903 (97 aa) a citrate lyase acyl carrier protein	Central intermediary metabolism: Pool, multipurpose conversions
dmsB	anaerobic dimethyl sulfoxide reductase chain B	Energy metabolism, carbon: Anaerobic respiration
frdA	fumarate reductase, flavoprotein subunit	Energy metabolism, carbon: Anaerobic respiration
b2324 (o688)	o688; This 688 aa ORF is 44 pct identical (5 gaps) to 337 residues of an approx. 392 aa protein YF35_HAEIN SW: P44246	Not classified
b1117 (o228)	o228; residues 10-216 are 65 pct identical to aa 15-221 from hypothetical ABC transporter ATP-binding protein YBBA_HAEIN SW: P45247 (227 aa)	Not classified
ybaM	hypothetical 6.0 kD protein in acrR-priC intergenic region	Unknown
b2077 (o471)	o471; This 471 aa ORF is 46 pct identical (11 gaps) to 457 residues of an approx. 480 aa protein YIEO_ECOLI SW: P31474	Not classified
napG	ferredoxin-type protein NapG	Energy metabolism, carbon: Electron transport
b2299 (f180)	f180; This 180 aa ORF is 32 pct identical (2 gaps) to 81 residues of an approx. 160 aa protein MUTT_STRAM SW: P32091	Not classified
b2768 (o191)	o191; This 191 aa ORF is 28 pct identical (0 gaps) to 185 residues of an approx. 200 aa protein GLPP_BACSU SW: P30300	Not classified
appC	probable cytochrome oxidase subunit I	Energy metabolism, carbon: Electron transport
rfaY	f232; 100 pct identical amino acid sequence and equal length to RFAY_ECOLI SW: P27240	Macromolecule metabolism: Lipopolysaccharide
fumB	fumarate hydratase class i, anaerobic (fumarase)	Energy metabolism, carbon: TCA cycle
relB	f79; 100 pct identical to RELB_ECOLI SW: P07007; CG Site No. 305	Global regulatory functions
lctD	o396	Energy metabolism, carbon: Aerobic respiration
potF	putrescine-binding periplasmic protein precursor	Transport of small molecules: Amino acids, amines
phnC	phosphonates transport ATP-binding protein phnC	Central intermediary metabolism: Phosphorus compounds
yadF	Hypothetical protein in hpt-panD intergenic region	Not classified
adk	adenylate kinase	Purine ribonucleotide biosynthesis
yiiD	hypothetical 37.1 kD protein in glnA-fdhE intergenic region	Not classified
yheD	putative general secretion pathway protein b	Not classified
ychM	hypothetical protein in pth-prs intergenic region	Unknown
ydjB	hypothetical 23.4 kD protein in ansA 3'region	Unknown
yicM	hypothetical 43.6 kD protein in nlpA 3' region	Not classified
proV	glycine betaine/L-proline transport ATP-binding protein ProV	Transport of small molecules: Amino acids, amines
glvG	probable 6-phospho-beta-glucosidase	Transport of small molecules:

		Carbohydrates, organic acids, alcohols
flhD	flagellar transcriptional activator FlhD	Surface structures
yhdM	hypothetical transcriptional regulator in mscL- rplQ intergenic region	Not classified
osmE	osmotically inducible protein E precursor	Global regulatory functions
PotD	spermidine/putrescine-binding periplasmic protein precursor	Transport of small molecules: Amino acids, amines
glpE	glpE protein	Energy metabolism, carbon: Anaerobic respiration
ykgC	f450; 35 pct identical (29 gaps) to 430 residues of approx. 632 aa protein MERA_BACSR SW: P16171	Not classified
minE	cell division topological specificity factor	Cell division
flgM	negative regulator of flagellin synthesis (anti- sigma factor)	Surface structures
hyaD	hydrogenase-1 operon protein HyaD	Energy metabolism, carbon: Aerobic respiration
b1634 (o500)	o500; This 500 aa ORF is 52 pct identical (4 gaps) to 475 residues of an approx. 496 aa protein YHIP_ECOLI SW: P36837	Not classified
mbhA	o211; Residues 2-211 are 100 pct identical to hypothetical protein MbhA GB: ECODINJ_10 ACCESSION: D38582; Residues 51-211 are 100 pct identical to residues 1-161 of 161 aa hypothetical protein GB: ECOTSF_38 ACCESSION: D83536	Not classified
yaeI	hypothetical protein in htrA-dapD intergenic region	Unknown
fusA	f704; CG Site No. 732; alternate name far; 100 pct identical amino acid sequence and equal length to EFG_ECOLI SW: P02996	Proteins - translation and modification
b0841 (f198)	f198; This 198 aa ORF is 31 pct identical (4 gaps) to 179 residues of an approx. 208 aa protein BCRC_BACLI SW: P42334	Unknown
rfbB	dtdp-glucose 4,6-dehydratase	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions
pepQ	Proline dipeptidase	Degradation of proteins, peptides, glyco
yabF	hypothetical NAD(P)H oxidoreductase in fixC-kefC intergenic region	Not classified
topB	DNA topoisomerase III	DNA – replication, repair, restriction/modification
b0371 (o486)	o486; This 486 aa ORF is 21 pct identical (36 gaps) to 422 residues of an approx. 537 aa protein fragment ARP_PLAFA SW: P04931	Unknown
tufB	elongation factor EF-Tu (duplicate gene)	Proteins - translation and modification
b1191 (f536)	f536; This 536 aa ORF is 26 pct identical (30 gaps) to 315 residues of an approx. 720 aa protein NAH4_RAT SW: P26434	Unknown
b1047 (f385)	f385; This 385 aa ORF is 24 pct identical (21 gaps) to 211 residues of an approx. 376 aa protein SSR2_RAT SW: P30680	Unknown
b3865 (f199)	f199; matches PS00017: ATP_GTP_A	Unknown

Table 14. Gene description and function of all genes up-regulated in planktonic cells (*E. coli* Array Information Version 1.5 (Sigma 2000).

DISCUSSION

Cell Culture Stability

The chemostat system used in this study was effective. The cell cultures remained stable over the entire culture period except for one cell count taken on the 8th day (Fig 2). This cell count was unusually low. The different results on this day can be accounted for by an apparent mistake in the dilutions since previous chemostat experiments (data not shown) have shown stable cell counts over the entire growth period and the cell culture numbers were not low the next day. Biofilm / planktonic cell mixing did not seem to be a major factor in the expression studies. A discernable difference in gene expression was seen between the two cell types. The system was also highly reproducible and can be employed at minimal cost.

A chemostat was used in this study to provide uniform cell cultures and to also grow reproducible biofilm cultures. This technique does have a few drawbacks when employed for gene expression studies. First, contamination was often a problem. While this problem was overcome, time lost due to contaminated chemostats was significant. Second, the chemostat setup is a continuous flow system which re-circulates media. The media re-circulation causes some mixing of biofilm and planktonic cells which may alter the results slightly. To prevent mixing of the cells, a system recently employed in other biofilm expression studies could be utilized. The system is a once flow system which keeps planktonic and biofilm cells completely separate. Cells are inoculated into a system and allowed to attach for a predetermined period of time. Fresh media is then circulated continuously over the attached cells rinsing away any planktonic cells

(Whiteley *et. al.*, 2001). A drawback to this method however is that the once flow system only cultures biofilm. Because all planktonic cells are rinsed away, planktonic and biofilm cells must be cultured in different environments. The separation of the two cultures could introduce an unknown variable into the system.

Biofilm cell counts were taken after the four-day biofilm growth period on two chemostats. The cell counts revealed high numbers of biofilm cells attached to the tubing. This number may or may not be accurate. In the gene expression study, after the four day biofilm growth period, the tubing the cells were growing on was rinsed with a phenol / ethanol solution. Cells, which remain attached after the wash, are defined as biofilm cells. This solution was used for two reasons. The first reason is to rinse away planktonic cells and second is to stop all gene expression and enzymatic activity within the cell. When the cell counts were taken, the tube was rinsed with phosphate buffered saline to ensure survival of attached cells. The phenol/alcohol could have removed many of the cells from the tubing resulting in lower cell counts. This problem was not pursued further since sufficient RNA was obtained.

Confocal Microscopy

Confocal images of the tubing used to grow the biofilm were taken. The images confirmed the presence of attached bacterial colonies. There were no notable problems with the procedure.

Biolog

The Biolog system was used to check for culture purity before chemostat inoculation and after the biofilm growth period. The system was easily used and no major problems were encountered. All samples resulted in a positive result for *E. coli*.

On sample (replicate one, post chemostat) resulted in only a 99% probability of the cells being *E. coli*. This result is not uncommon since, according to Biolog, each individual biochemical test does not result in a valid result 100% of the time.

RNA Yield

RNA was extracted from both planktonic and biofilm cells. Planktonic cells resulted in ample RNA for the array experiments; however, biofilm cells resulted in low RNA amounts. Two reasons for low biofilm RNA yields could apply. Biofilm cells are incased in a polysaccharide matrix, this makes them difficult to break open. Also, the phenol ethanol stop solution used could have removed some of the biofilm resulting in lower than expected cell counts.

Different RNA extraction procedures were used in an attempt to increase RNA yield (data not shown). A technique using zirconium beads for cell breakage and Tri-reagent as a nucleic acid/protein separator was used. This technique, while quicker, resulted in low RNA yield and RNAse contamination occurred frequently. Also, since phenol was used as a metabolic stop solution during cell processing and storage, phase separation with Tri-reagent was an issue. The extra phenol caused poor separation or often no separation of the different Tri-reagent layers. Because of these reasons, the technique was abandoned. The phenol / chloroform extraction method was successfully employed. This method, while loosing some RNA during extractions and washes, supplied sufficient RNA for the array experiments and RNAse contamination was rarely an issue. Also, this technique was recommended by the manufacturer of the arrays (Sigma/Genosys).

Transmission Electron Microscopy

In an attempt to better understand the low levels of RNA extraction in biofilm, transmission electron microscope (TEM) studies were done. Biofilms are encased in a polysaccharide matrix, which makes them hard to break apart. The same matrix could potentially effect how well the cell breaks open during cell lysis procedures. *E. coli* cells were grown as planktonic as well as biofilm and lysed using the same procedure used in the gene array experiment. Images were then taken of the cells before lyses as well as after lyses. No discernable difference was seen between the cells however. The cells before lysis closely resembled cells after the lysis procedure. One possible reason for this could be due to the environment the cells are placed in when viewed under the TEM. Specimens are dehydrated and placed under a vacuum. This environment could affect cell morphology.

cDNA Production

No major issues arose while making the cDNA probes used in this study. Radioactive incorporation and cDNA production were consistent and ample.

Phosphorimaging

Phosphorimaging for this study was done at a 100 μm resolution. This resolution did seem effective, but some blurring of the array spots did occur. A 50 μm resolution image was taken. The image was obtained, but not analyzed. The array spots were clearly defined compared to the 100 micron spots, but problems with the analysis software made processing of these images difficult at this time.

Gene Array Analysis

In this study sequential arrays, or arrays that were imprinted with the *E. coli* genome one after the other, were used to ensure array uniformity. Small differences in the amount of DNA added to each array can occur over the entire manufacturing process, which could result in different readings if arrays were used, that were manufactured at different points during the process.

The Sun Microsystems ULTRA10 system worked fairly well; however, because a small error in spot alignment will result in inaccurate results, each spot must be checked and realigned manually. This process was effective but very time consuming.

A total of 28 genes were differentially expressed by at least a two fold level in biofilm and planktonic cells. 21 genes were up-regulated in biofilm and 7 were up-regulated in planktonic cells. Of the 21 genes up-regulated in biofilm, 14 have no known function or are unclassified. This result indicates the possibility of novel genes being utilized in mature biofilm. In planktonic cell, 6 out of the 7 have no known function. This result indicates genes that have little function in mature biofilm but are essential for planktonic function.

The results of genes expressed with levels of between 1.9 and 1.5 above either biofilm or planktonic were also obtained. These results, while below the established cut off, are still valuable. Many of these genes were up-regulated by a factor of 2 or better in at least one replicate. The lower expression number may be inaccurate due to a variety of problems including array abnormalities or computer errors. Future studies may show these genes to be up-regulated and have important functions in biofilms. As the gene

expression amount goes down from 2 to 1.5, less significance can be given to the results but all genes are worth listing because future studies may reveal their significance.

Genes showing differential expression in this study are different than those of past expression studies on mature biofilm. Seven different genes have previously been shown to have differential expression under biofilm growth conditions. Prigent-Combaret *et al.* (1999) studied 885 bacterial strains. Approximately 38% of the genes being expressed in these strains were altered in biofilm. One gene, *fliC,* which encodes a flagellar structural protein was down-regulated in biofilm. Garrett *et al.* (1999) also found *fliC* to be down-regulated in *Pseudomonas aeruginosa* biofilms. In this study, *fliC* was not shown to be down-regulated in planktonic cells. The expression level of this gene is shown in table 15. Also, Prigent-Combaret *et al.* (1999) found bacteria existing within biofilm encounter higher osmolarity, greater oxygen limitation and higher cell density than planktonic cells. The study found four genes, known to be osmoregulated, differentially expressed in biofilm cells. These genes were *ompC, proU, wcaB, and fliC.* The gene *ompC* (Sarma and Reeves (1977) is a porin gene. The *proU* operon encodes a high-affinity glycine betaine transport system (Gowrishankar 1985). The gene *wcaB* is involved in the sythesis of colanic acid in EPS) (Sledjeski and Gottesman (1996). One uncharacterized gene, *f*92 ORF (ECAE000245; min33.5), encodes a putative short protein (92 amino acids) and has been shown to be down-regulated in biofilm and effected by osmolarity and *fliC* which is involved in flagella synthesis and is down-regulated in biofilm has been shown to be down-regulated by high salt concentrations. Table 15 shows the function, expression level in the previous studies and the expression level in this study. *nikA* is a high affinity nickel transport system, which is highly tuned

by the level of oxygen availability. The expression of this gene has been shown to increase in biofilm (Wu and Mandrand-Berthelot (1986). One possible quorum sensing gene, *pepT*, has been shown to be over-expressed at low cell density.

A recent study looked at the role of *csrA* in biofilm development. In their study, knocking out gene expression caused a dramatic increase in biofilm formation, while over expression resulted in no biofilm formation (Jackson, *et al.* (2002). In this study, the *csrA* gene was expressed below the background in both replicates meaning the gene was expressed below the un-induced lac operon.

Gene	Function	Previous Expression	Replicate One	Replicate Two
ompC	Porin	up 2-3 fold in biofilm	1.02	0.79
proU	glycine betaine transport system	up 2-3 fold in biofilm		
wcaB	Colanic acid synthesis	up 2-3 fold in biofilm	0.71	0.99
fliC	flagella synthesis	down in biofilm	1.66	0.80
nikA	nickel transport	up 5 fold in biofilm	1.10	0.84
pepT	quorum sensing	overexpressed in low cell density	0.95	1.18
f92	Unknown	down in biofilm	1.88	2.00
csrA	Global Regulatory	down in biofilm	Below Background	Below Background

Table 15. The function and expression level (in this study) of genes previously shown to be differentially expressed in biofilm when compared to planktonic cells.

In this study the genes, *ompC, wcaB, fliC, nikA,* and *pepT* all show expression levels of about one meaning that the expression level was the same in both planktonic and biofilm cells. The uncharacterized gene *f92*, which has previously been shown to be down-regulated in biofilm, showed a two fold increase in expression in biofilm in this study. Since the function of *f92* has not been determined, the disparity between the results is difficult to explain.

One possible explanation for the difference between findings in this study compared to others is the culture conditions the bacteria were grown under. Since the

bacteria were grown under different conditions and environments, the reaction they have to their environment may differ. Since many of the genes have been shown to be osmoregulated, the continuous flow of media and nutrients through the biofilm in this study could possibly keep the osmolarity similar in both planktonic and biofilm environments. The conditions the bacteria were exposed to must be taken into account when looking at the results of this expression study or any other study.

A recent study on gene expression in *Pseudomonas aeruginosa* biofilms was recently conducted. The study compared gene expression in 6 day old biofilm and planktonic cells grown in a once flow system. The study found 73 genes to be differentially expressed in biofilm compared to planktonic cells. They determined that gene expression between planktonic and biofilm cells is "remarkably similar". (Whiteley *et al.* (2001)).

Genes previously determined to be important in biofilm formation were not found to be up-regulated in this study. This is not surprising since this study looked at mature biofilm and not at the initial steps involved in their formation. Many of the genes responsible for initiating biofilm development would not be needed in mature biofilms.

In this study, 28 genes show an increase in expression of at least two fold. Many of these genes have no known function. While the unknown genes revealed in this study are of particular interest and could potentially play significant roles in biofilm physiology, the majority of the genome remained the same when compared to planktonic cells. This finding agrees with the previous *Pseudomonas* findings in that the two cell types are similar.

The use of gene arrays in gene expression studies has several strengths and weaknesses. The major strengths are that they are commercially available and they can be used to compare genome wide expression patterns under various conditions. Weaknesses are that they are currently expensive so obtaining multiple replicates can become expensive very quickly. Also, because a two fold expression difference is used to determine either up-regulation or down-regulation small differences in gene expression that may have a large impact on other genes cannot be determined. If gene arrays alone were used, these genes would be missed.

The use of arrays for expression analysis can best be used to obtain estimates of what is happening and serve as a good starting point when looking at gene expression under two different conditions. Once a gene or genes of interest are identified, other techniques must be utilized to obtain more accurate measurements of expression and to determine function.

Some possible future work on this project could include: An experiment should be done on the up-regulated genes to confirm increased expression or decreased expression. Real Time PCR, nuclease protection assay or northern analyses are all good options for the experiment.

Real Time PCR is based on detection of fluorescent signals released during the amplification process. A probe is designed which anneals to the DNA template. When the polymerase reaches the probe during amplification, the probe is released and a fluorescence signal is released. The amount of signal produced during the PCR process is proportional to the amount of product generated. The more starting material present, the sooner a signal can be detected. When standardized against a known external control,

quantitative expression data can be determined. The advantage of this technique is that it is highly sensitive and can be performed in a short time period; however, the start up costs for equipment can be expensive.

The nuclease protection assay utilizes solution hybridization for quantitative analysis of gene expression. Total RNA is suspended in a small volume of liquid. Probes of desired genes are made, radiolabeled and added to the solution. The probes bind to the RNA and unbound probe and remaining RNA is removed via digestion with nuclease. The remaining solution is run out on a denaturing polyacrylimide gel and viewed by autoradiography. When a known external standard is used along side the unknown, quantities of the amount of gene present in the sample can be determined. The advantage of nuclease protection assays is that multiple probes can be used in one experiment by making the probes different sizes. Also, RNA degradation is not a problem since the size of the probe is used to determine the presence of the gene, so even partially degraded genes can be detected and quantified.

Northern analysis is another technique that could be utilized to confirm the array results. In this technique, total RNA is separated by denaturing agarose gel electrophoresis, transferred to a nylon membrane and cross linked. A gene specific labeled probe is added to the membrane and viewed via autoradiography. The advantage to this technique is that it is simple and can be done a minimal cost. The disadvantage is that only one probe can be used per experiment, so large amounts of RNA are needed to do several genes. Also, any RNA degradation will result in inaccurate results since the gene size not probe size is used to determine expression. A small degradation in the gene, will result in decreased signal and quantification of expression will be inaccurate.

Each technique has its own advantages and disadvantages, but all can be performed at minimal cost and time if equipment is available. Also, many genes not included in the two fold up-regulation category show a greater than two fold expression increase in one replicate. These genes should be looked at further in order to determine expression under the defined conditions. Again, the experiments mentioned above would be good candidates to accomplish the experiment. Gene knockouts on the unidentified genes to determine function are another important future study. The next logical step in this study is doing gene knockouts to determine their importance in biofilm function. Multiple replicates of the gene array experiment under different condition and media use to determine if gene expression patterns found in this study are consistent in other environments is also a possible direction of study. While doing these experiments would be costly, valuable data could be obtained. These are only a few of the projects that could come out of this study; however, these are the most important directions at this point in the project.

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