Appl Microbiol Biotechnol (2004) 64: 515–524
DOI 10.1007/s00253-003-1517-y

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Gene expression in *Escherichia coli* biofilms

Received: 28 July 2003 / Revised: 31 October 2003 / Accepted: 21 November 2003 / Published online: 16 January 2004

Abstract DNA microarrays were used to study the gene expression profile of *Escherichia coli* JM109 and K12 biofilms. Both glass wool in shake flasks and mild steel 1010 plates in continuous reactors were used to create the biofilms. For the biofilms grown on glass wool, 22 genes were induced significantly ($p \leq 0.05$) compared to suspension cells, including several genes for the stress response (*hslS*, *hslT*, *hha*, and *soxS*), type I fimbriae (*fimG*), metabolism (*metK*), and 11 genes of unknown function (*ybaJ*, *ychM*, *yefM*, *ygfA*, *b1060*, *b1112*, *b2377*, *b3022*, *b1373*, *b1601*, and *b0836*). The DNA microarray results were corroborated with RNA dot blotting. For the biofilm grown on mild steel plates, the DNA microarray data showed that, at a specific growth rate of 0.05/h, the mature biofilm after 5 days in the continuous reactors did not exhibit differential gene expression compared to suspension cells although genes were induced at 0.03/h. The present study suggests that biofilm gene expression is strongly associated with environmental conditions and that stress genes are involved in *E. coli* JM109 biofilm formation.

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Introduction

Bacterial biofilms—sessile microbial communities formed on solid surfaces—are ubiquitous in natural environments as well as those related to medicine and engineering (Elvers and Lappin-Scott 2000). Due to their high resistance to antibiotics (Nickel et al. 1985), biofilms cause serious problems to human health such as lung infections, dental disease, and urinary tract infections (Potera 1999). It is estimated that biofilms are involved in 65% of human bacterial infections (Potera 1999). Biofilms are also problematic in industry since they can stimulate microbial-induced corrosion on the surfaces of pipes, reduce the efficiency of heat exchangers, and cause food spoilage (Elvers and Lappin-Scott 2000).

Biofilm formation is a dynamic process including attachment of the cells to the surface, increase in cell population, and maturation of the biofilm (Elvers and Lappin-Scott 2000; Kuchma and O'Toole 2000). The fully developed biofilm has a three-dimensional structure made up of a polysaccharide matrix that contains water channels for the transfer of nutrients and for the removal of wastes (Elvers and Lappin-Scott 2000).

Biofilms have attracted extensive research; however, the understanding of biofilm formation at the genetic level lags behind what is known of their physical properties (Kolter and Losick 1998). Recently, random insertion mutagenesis and screening has been used successfully to show that motility and type I fimbriae are important for *Escherichia coli* early biofilm formation (Pratt and Kolter 1998), and that flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* early biofilm formation (O'Toole and Kolter 1998). It has also been suggested that the sporulation gene *spo0A* is important for biofilm formation of *Bacillus subtilis* (Hamon and Lazazzera 2001) and that the quorum-sensing system (bacterial gene expression controlled by sensing their population; Bassler 1999) *luxI*/*luxR* is important for biofilm formation with *P. aeruginosa* (Davies et al. 1998); however, this is controversial as several reports dispute the importance of quorum sensing in biofilm

formation of Gram-negative strains (Sauer and Camper 2001; Heydorn et al. 2002; Purevdorj et al. 2002).

Compared to the traditional methods of studying individual genes, proteomics provides a global study of gene expression and has been used successfully to study biofilm formation of *Bacillus cereus* (Oosthuizen et al. 2001, 2002; Steyn et al. 2001). By using 2-D electrophoresis, it was found that 15 proteins were uniquely expressed in 2-h *B. cereus* biofilms and 7 proteins in 18-h biofilms (Oosthuizen et al. 2002). Moreover, due to the dynamic character of biofilms, the green fluorescent protein system has been used to study gene expression in vivo and in three dimensions (Kievit et al. 2001; Heydorn et al. 2002). With this approach, it was found that the *P. aeruginosa* quorum sensing genes *lasI* and *rhlI* were most expressed at the attachment surface of a liquid-solid interface biofilm, and the expression of *lasI* decreased with time while *rhlI* was more consistent during biofilm development (Kievit et al. 2001).

DNA microarrays provide a means to monitor the global gene expression profile in response to different stimuli (Whiteley et al. 2001). They have been widely used to study microbial physiology, including response to heat shock and other stresses (Wilson et al. 1999; Helmann et al. 2001; Zheng et al. 2001), quorum sensing (DeLisa et al. 2001; Sperandio et al. 2001), anaerobic metabolism (Ye et al. 2000), sporulation (Fawcett et al. 2000), and biofilm formation (Whiteley et al. 2001; Schembri et al. 2003; Stanley et al. 2003). DNA microarrays have advantages for understanding biofilm formation because this mode of growth requires a significant change in gene expression for cell attachment and structure development (Kuchma and O'Toole 2000). Recently, Schembri et al. (2003) studied the early stages of *E. coli* MG1655 biofilm formation (on glass slides in flow chambers) with DNA microarrays, and reported that the biofilm cells have 5.4% or 13.5% of genes differentially expressed compared to exponentially growing suspension cells or stationary suspension cells, respectively.

While DNA microarrays are a promising approach for studying biofilms, there are some challenges, including the short half-life of *E. coli* mRNA (3–5 min; Lodish et al. 1999); hence, sampling and cell lysis should be rapid so that transcription can be stopped before significant mRNA degradation occurs. This is very important for biofilm experiments because harvesting biofilm cells takes longer than harvesting regular suspension cells. In the present study, transcription was terminated within seconds to 1 min. Also, a rigorous criterion of 23S rRNA/16S rRNA >2 was used to check RNA integrity as prescribed by the Qiagen RNeasy Mini Kit. Since biofilm formation is a dynamic procedure and is sensitive to many environmental factors, growth conditions were optimized to ensure adequate yields of total RNA from the biofilm and suspension cells in each individual reactor. Hence, gene expression in the biofilm was not compared to separately grown suspension cells and no combined parallel samples were analyzed as in a previous study (Schembri et al. 2003).

Compared to the genes involved in cell attachment and biofilm development, little is known about the genes involved in maintaining biofilms. However, this information is important since biofilm infections are strongly associated with the antibiotic resistance of mature biofilms (Nickel et al. 1985; Potera 1999). In the present study, both 7-h batch and 5-day continuous *E. coli* biofilms were studied for differential gene expression, and it is one of the first reports biofilm formation studied with DNA microarrays. Of the 22 genes found as important biofilm genes of *E. coli* JM109, 17 were identified for the first time.

Materials and methods

Bacterial strains and growth medium

E. coli JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 thi Δ(*lac-proAB*) F′[*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15]) (Yanisch-Perron et al. 1985) and *E. coli* K12 (F^{-} λ^{-} *ilvG rfb50 rph1*) (ATCC 25404) were used to grow biofilms on glass wool and metal surfaces, respectively. LB medium (Sambrook et al. 1989) containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl was used to grow the strains and develop the biofilms.

E. coli JM109 biofilm formation on glass wool in batch reactors

E. coli JM109 was grown in LB medium overnight, and 1 ml overnight culture was inoculated into a 250 ml shake flask containing 100 ml fresh LB and 0.5 g untreated glass wool (Corning Glass Works, Corning, N.Y.). The cells were incubated with shaking (250 rpm in a C25 incubator shaker; New Brunswick Scientific, Edison, N.J.) at 37°C to form the biofilm on the glass wool. Seven hours after inoculation, the glass wool was taken from the culture and quickly and gently washed two times in 100 ml 0°C 0.85% NaCl buffer (within 30 s). Then the biofilm cells were removed from the glass wool by sonication in 200 ml 0°C 0.85% NaCl buffer for 2 min. The buffer containing biofilm cells was centrifuged (10,000 *g* in a J2-HS centrifuge; Beckman, Palo Alto, Calif.) for 3 min at −2°C to precipitate the cells. The biofilm cells were then resuspended in 6 ml 0°C 0.85% NaCl buffer, transferred to mini bead beater tubes, and centrifuged (10,000 *g*) for 15 s at room temperature; the cell pellets were flash-frozen in a dry ice-ethanol bath. While sonicating the biofilm cells, suspension cells with an optical density at 600 nm (OD) of 2 were harvested by centrifugation (10,000 *g*) at room temperature for 15 s in mini bead beater tubes. The cell pellets were then flash-frozen in a dry ice-ethanol bath and kept at −80°C until RNA isolation.

E. coli K12 biofilm formation on metal in continuous reactors

Biofilms were developed on mild steel 1010 plates in continuous reactors that contained 150 ml LB medium and in which the temperature was controlled at 34°C. Each autoclavable reactor consists of a 5.5 cm conical glass cell, a mild steel 1010 plate at the bottom, and a Teflon top (Örnek et al. 2002). Air was filtered and supplied to the reactors at 200 ml/min. The reactors were inoculated with a 1:150 dilution of an overnight culture of *E. coli* K12. Continuous nutrient addition commenced 1 day after inoculation at 8 ml/h LB medium [dilution rate (μ)=0.05 h^{-1}]. Biofilm and suspension cells were sampled 5 days after inoculation. After opening the reactor quickly, the metal plate was washed quickly and gently in 0°C 0.85% NaCl buffer to remove the contaminating suspension cells and sonicated (FS3 sonicator; Fisher, Hanover Park, Ill.) for 2 min in 0°C 0.85% NaCl buffer to remove the biofilm

cells from the metal surface. The buffer containing biofilm cells was centrifuged for 3 min at −2°C (10,000 *g* in a Beckman J2-HS centrifuge), and the precipitated biofilm cells were resuspended in 6 ml 0°C 0.85% NaCl buffer, transferred to cold mini bead beater tubes (Biospec, Bartlesville, Okla.), and centrifuged (10,000 *g* in a Hermle microcentrifuge; Labnet, Woodbridge, N.J.) for 15 s at room temperature. The cell pellets were frozen immediately by putting the tubes in a dry ice-ethanol bath. Cell samples were kept at −80°C until RNA isolation. During the sonication of the metal plates, suspension cells were taken by pipetting into mini bead beater tubes. Cells were centrifuged (10,000 *g* in a Hermle microcentrifuge) for 15 s at room temperature. The cell pellets were then frozen in a dry ice-ethanol bath and kept at −80°C until RNA isolation.

Total RNA isolation

To lyse the cells, 1.0 ml RLT buffer (Qiagen, Valencia, Calif.) and 0.2 ml 0.1 mm zirconia/silica beads (Biospec) were added to the frozen bead beater tubes containing the cell pellets. The tubes were closed tightly and beat for 30 s at the maximum speed in a mini bead beater (Cat. No. 3110BX, Biospec). Total RNA was isolated following the protocol of the RNeasy Mini Kit (Qiagen), including an on-column DNase digestion with RNase-free DNase (Qiagen). An OD reading at 260 nm was used to quantify the RNA yield. OD_{260}/OD_{280} and 23S/16S rRNA were measured to check the purity and integrity of RNA (RNeasy Mini handbook, Qiagen).

DNA microarrays

E. coli DNA microarrays were prepared as described previously (Wei et al. 2001). Each gene probe was synthesized by polymerase chain reaction (PCR) as the full open reading frame, 200–2,000 nt. The double-strand PCR products were denatured in 50% dimethyl sulfoxide and spotted onto aminosilane slides (Full Moon Biosystems, Sunnyvale, Calif.). Total RNA isolated from the experimental samples was converted by reverse transcription to complimentary DNA (cDNA), which was hybridized to the denatured DNA probes on the microarray slides to quantify the expression level of each gene. It has been shown that each array can detect 4,228 of the 4,290 *E. coli* ORFs (Wei et al. 2001). Each gene has two spots per slide.

Synthesis of Cy3- or Cy5-labeled cDNA

To convert the total RNA into labeled cDNA, reverse transcription was performed in 1.5 ml microcentrifuge tubes (Fisher) to which 6 μg total RNA and 6 μg random hexamer primers (Invitrogen, Carlsbad, Calif.) were added; the volume was adjusted to 24 μl with RNase-free water (Invitrogen). The mixture was incubated for 10 min at 70°C followed by 10 min at room temperature for annealing, then the reaction components were added, consisting of 8 μl 5× SuperScript II reaction buffer (Invitrogen), 4 μl 0.1 M dithiothreitol (DTT) (Invitrogen), 1 μl deoxynucleoside triphosphates (dNTPs) mix (2 mM each of dATP, dGTP, dTTP and 1 mM dCTP), 1 μl 0.5 mM Cy3- or Cy5-labeled dCTP (Amersham Biosciences, Piscataway, N.J.), and 2 μl SuperScript II reverse transcriptase (10 U/μl, Invitrogen). cDNA synthesis was conducted at 42°C for 2 h and stopped by heating at 94°C for 5 min. After cDNA synthesis, the RNA template was removed with 2 μl 2.5 M NaOH. The pH was neutralized with 10 μl 2 M HEPES buffer, and the cDNA was purified with a Qiaquick PCR Mini Kit (Qiagen). The efficiency of labeling was checked via absorbance at 260 nm for the cDNA concentration, 550 nm for Cy3 incorporation, and 650 nm for Cy5 incorporation.

Hybridization and washing

The suspension and the biofilm cDNA samples (6 μg of each) were each labeled with both Cy3 and Cy5 dyes to remove artifacts related to different labeling efficiencies; hence, each experiment needed two slides. The Cy3-labeled suspension sample and Cy5-labeled biofilm sample were hybridized on the first slide. Similarly, the Cy5-labeled suspension sample and Cy3-labeled biofilm sample were hybridized on the second slide. Since each gene has two spots on a slide, the two hybridizations generated eight data points for each gene (four points for the suspension sample and four points for the biofilm sample).

The DNA microarrays were incubated in prehybridization solution [3.5× SSC (Invitrogen), 0.1% SDS (Invitrogen), 0.1% bovine serum albumin (Invitrogen)] at 45°C for 20 min. The arrays were rinsed with double-distilled water (ddH_2O) and spun dry by centrifugation. Labeled cDNA (6 μg) was concentrated to 10 μl total volume and mixed with 10 μl 4× cDNA hybridization solution (Full Moon Biosystems) and 20 μl formamide (EM Science, Gibbstown, N.J.). The hybridization mix was heated to 95°C for 2 min and added to the DNA microarrays; each array was covered with a coverslip (Corning) and incubated overnight at 37°C for hybridization. When the hybridization was finished, the coverslips were removed in 1× SSC, 0.1% SDS at room temperature, and the arrays were washed once for 5 min in 1× SSC, 0.1% SDS at 40°C, twice for 10 min in 0.1× SSC, 0.1% SDS at 40°C, and twice for 1 min in 0.1× SSC at 40°C. The arrays were quickly rinsed by dipping in ddH_2O at room temperature and then spun dry by centrifugation.

Image and data analysis

The hybridized slides were scanned with a Generation III Array Scanner (Molecular Dynamics, Sunnyvale, Calif.); 570 nm and 670 nm were used to quantify the probes labeled with Cy3 and Cy5, respectively. The signal was quantified with Array Vision 4.0 or 6.0 software (Imaging Research, Ontario, Canada). Genes were identified as differentially expressed in the biofilm if the expression ratio was greater than 2.5 and the *P*-value (*t*-test) was less than 0.05. *P*-Values were calculated on log-transformed, normalized intensities. Including the *P*-value criterion ensures the reliability of the induced/repressed gene list. Normalization was relative to the median total fluorescent intensity per slide per channel. The gene functions were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/).

RNA dot blotting

Digoxigenin (DIG)-labeled DNA probes of six genes, *b3022*, *hha*, *soxS*, *yhaJ*, *hslT*, and *b0753*, were synthesized using the PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany). PCR was performed in 30 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec. The final extension was at 72°C for 7 min. The probes have lengths of between 172 bp and 400 bp (see Table 3 for specific primers). Total RNA (1.25, 2.5, or 5 μg) from independent cell cultures (different experiments than those used for the DNA microarrays but identical culture conditions) was blotted on positively-charged nylon membranes (Boehringer Ingelheim, Ridgefield, Conn.) using a Bio-Dot Microfiltration Apparatus (Bio-Rad, Richmond, Calif.). Total RNA was fixed by baking for 2 h at 80°C. DNA probes (about 400 ng, a serial dilution of RNA samples was tested to ensure excess of the DNA probes) were denatured in boiling water for 5 min before hybridizing to RNA. Hybridization (50°C, 16 h) and washing were conducted by following the protocol for DIG labeling and detection (Roche Applied Science). To detect the signal, disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2-(5-chloro)tricycle [3.3.1.1,7] decan}-4-yl) phenyl phosphate (Roche Applied Science) was used as a substrate to give chemiluminescence, and the light was recorded by Biomax X-ray film (Kodak, Rochester, N.Y.).

Microscopy observation of *E. coli* JM109 biofilm on glass wool

The biofilm was developed as for the microarray experiments. The glass wool containing the biofilm was gently washed two times in 0.85% NaCl buffer and stained with 0.1% crystal violet for 20 min, and the extra dye was washed off with 0.85% NaCl buffer (twice). The cells on the surface of glass wool were imaged using optical microscopy at 400× total magnification (Zeiss Axioskop, Zeiss, Oberkochen, Germany).

Confocal laser scanning microscopy

Metal plates with attached *E. coli* K12 biofilms were immersed in 0.85% NaCl buffer to remove the bulk supernatant cells. The Live/Dead Baclight bacteria viability assay kit (L-7007; Molecular Probes, Eugene, Ore.) was used to stain the biofilm at a concentration of 1.125 μl/ml (for both kit components A and B). The staining process was performed in the dark at room temperature for 30 min. The stained plates were protected with a cover glass and observed with confocal microscopy (MRC 600, Bio-Rad, Hercules, Calif.), which was conducted using the dual channel mode (K1/K2 filter block combination); the sample was excited at 488 nm and biofilm thickness was measured by focusing through the biofilm from the solid-liquid interface to the metal surface and averaging 5–6 positions. The images were processed with National Institutes of Health Image 1.6 and Adobe Photoshop 5.5 (Adobe, San Jose, Calif.). Z-section images (single image of the sectioning plane parallel to the metal surface) and vertical section images (a collection of images orthogonal to the metal surface at one position) were obtained.

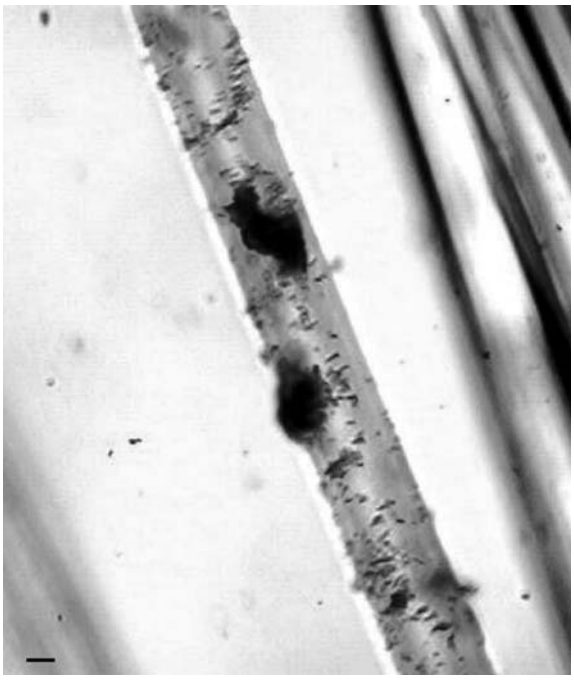

Fig. 1 *Escherichia coli* JM109 biofilm formed on glass wool in shake flasks 7 h after inoculation. Total magnification 400×. *Bar* 10 μm

Table 1 Genes induced in the *Escherichia coli* JM109 biofilm grown on glass wool. Each sample set contained one biofilm RNA sample and one suspension RNA sample from the same independent reactor. Genes consistently induced in both data sets are in *bold*

Gene	b-number	Expression ratio (set 1)	Expression ratio (set 2)	Description
Known genes				
hslS	b3686	48	16	Heat shock protein
soxS	b4062	35	63	Regulation of superoxide response regulon, global regulator
hha	b0460	20	30	Haemolysin expression modulating protein, regulator
glnA	b3870	12	1.5	Glutamine synthetase
rhoL	b3782	5	6	Leader, RNA synthesis, modification, DNA transcription
hslT	b3687	4	4	Heat shock protein
fimG	b4319	3	1.4	Fimbrial morphology
rho	b3783	3	5	Transcription termination factor Rho, polarity suppressor
tus	b1610	3	4	Factor, DNA-replication, repair, restriction/modification
metK	b2942	2	3	Methionine adenosyltransferase 1 (adomet synthetase), methyl and propylamine donor, corepressor of met genes
trpE	b1264	2	4	Anthranilate synthase component I
Unknown genes				
ybaJ	b0461	37	35	Unknown
b2377	b2377	21	4	Unknown
b1112	b1112	17	7	Unknown, possible stress response (Zheng et al. 2001)
b3022	b3022	8	8	Unknown (protein 36% identical to *Sinorhizobium meliloti*Lrp, leucine-responsive regulatory protein, for LPS synthesis)
b1601	b1601	5	6	Unknown
ygfA	b2912	5	8	Putative ligase
b1060	b1060	4	5	Unknown
b1373	b1373	3	1.3	Unknown
b0836	b0836	3	3	Putative receptor
ychM	b1206	2	3	Unknown
yefM	b2017	2	3	Unknown

Results

E. coli JM109 biofilm formation on glass wool in batch reactors

E. coli JM109 was tested and found to yield good biofilms when grown on glass wool for 7 h (about 40 μg total RNA was obtained from biofilm cells in each culture). Observation with a microscope at 400× indicated the cells attached well to the glass wool and cell clusters were clearly seen (Fig. 1). This condition was therefore used for further study.

Two independent sets of samples (each set had a suspension RNA sample and a biofilm RNA sample harvested from the same individual flask) were harvested and analyzed with DNA microarrays. In the first set, 13 genes (7 with unknown functions) were induced in the biofilm (expressed more than 2.5-fold over suspension cells, *P*-value <0.05), while 252 genes were repressed more than 2.5-fold. Hence, there are more repressed genes than induced genes in the biofilm. Similarly, in the second set, 19 genes (9 with unknown functions) were induced in the biofilm, while 718 genes were repressed more than 2.5-fold. These two sets of samples agreed well except that the second set has more repressed genes. Consistently, 9 genes (5 with unknown functions) were induced and 201 genes were repressed in both sets of samples. Hence, 69% of the induced genes and 79% of the repressed genes from the first set were identified in the second set. Interestingly, although there were only 9 induced genes shared by these two sets of samples, the other 4 induced genes from the first set sample and 9 of the other 10 induced genes (except for *insB_6*) from the second set were also up-regulated in the other sample set (either the levels of induction were lower than the 2.5 threshold or the *P*-value was higher than 0.05). Hence, we purpose that all of these 22 consistently up-regulated genes are candidates for biofilm-formation genes and they are shown in Table 1. Table 2 lists the 20 consistently most-repressed genes.

Induction of stress genes in the glass wool biofilm

The microarray results from the glass wool biofilms identified several induced genes related to the stress response (*hslS*, *hslT*, *hha*, and *soxS*), type I fimbriae (*fimG*), metabolism (*metK*), and some genes with unknown functions (*ybaJ*, *ychM*, *yefM*, *ygfA*, *b1060*, *b1112*, *b2377*, *b3022*, *b1373*, *b1601*, and *b0836*) (Table 1). *hslT* (alternate name *ibpA*) and *hslS* (alternate name *ibpB*) encode small proteins for response to heat shock and superoxide stresses (Kitagawa et al. 2000), and *soxS* encodes the regulator SoxS in response to superoxide (Zheng et al. 2001; Michán et al. 2002). *E. coli* cells overexpressing *hslT* and *hslS* are more resistant to different stresses including heat, ethanol, and superoxide (Kitagawa et al. 2000). Three genes found induced in the glass wool biofilm in the present study, *b1112*, *soxS*, and *hslS*, were also found induced in *E. coli* in response to hydrogen peroxide (Zheng et al. 2001). Hence, upregulation of *hslT*,

Table 2 Genes repressed in the *E. coli* JM109 biofilm grown on glass wool (the 20 consistently most-repressed genes). Each sample set contained one biofilm RNA sample and one suspension RNA sample from the same independent reactor

Gene	b-number	Expression ratio (set 1)	Expression ratio (set 2)	Description
Known genes				
gabD	b2661	−9	−7	Succinate-semialdehyde dehydrogenase, NADP-dependent activity
lacZ	b0344	−8	−7	Enzyme, degradation of small molecules: carbon compounds
ygaF	b2660	−7	−6	Unknown
artI	b0863	−7	−5	Transport, transport of small molecules: amino acids, amines
artP	b0864	−7	−5	Transport, transport of small molecules: amino acids, amines
hyaA	b0972	−7	−5	Enzyme, energy metabolism, carbon: aerobic respiration
fruK	b2168	−6	−5	Enzyme, energy metabolism, carbon: glycolysis
Unknown genes				
b0725	b0725	−14	−3	Unknown
yhfG	b3362	−9	−4	Unknown
b0753	b0753	−8	−6	Putative regulator, not classified
b1444	b1444	−7	−6	Putative aldehyde dehydrogenase
yhiD	b3508	−7	−3	Putative transport ATPase
b1836	b1836	−7	−4	Unknown
b0334	b0334	−7	−6	Unknown
yceK	b1050	−7	−8	Unknown
ybgF	b0742	−7	−4	Unknown
yhiM	b3491	−6	−5	Unknown
b1747	b1747	−6	−5	Unknown
b1824	b1824	−6	−8	Unknown
b0333	b0333	−6	−6	Putative enzyme, not classified

Table 3 Confirmation of gene expression in the *E. coli* JM109 biofilm grown on glass wool with RNA dot blotting. Each sample set contained one biofilm RNA sample and one suspension RNA sample from the same independent reactor

Gene	Primers used for probe synthesis	Expression ratio (DNA microarray)		Expression ratio (RNA dot blotting)
		Set 1	Set 2	
b3022	5′-ATGGAAAAACGCACACCACATACAC-3′ 5′-AAGCCTGGGTCTGTAAACATCCTGC-3′	+8	+8	+5
hha	5′-GTCCGAAAAACCTTTAACGAAAACC-3′ 5′-TTTATTCATGGTCAATTCGGCGAGG-3′	+20	+30	+10
soxS	5′-TTCAAAGTGGTACTTGCAACGAATG-3′ 5′-TAATCGCTGGGAGTGCGATCAAACT-3′	+35	+63	+20
ybaJ	5′-ATGGATGAATACTCACCCAAAAGAC-3′ 5′-TCCATTTCTGAAGATCCTGCATATT-3′	+37	+35	+10
hslT	5′-CGATTTATCCCCACTGATGCGTCAA-3′ 5′-ACGTTCGCTGATAGCGATACGCTGC-3′	+4	+4	+10
b0753	5′-ACTGGCCACATTATTTCTGACTGCC-3′ 5′-TTACTGCGTGGTACCGTCGGTTTTG-3′	−8	−6	−5

hslS, *soxS*, and *b1112* here suggests the *E. coli* JM109 biofilm on glass wool needs expression of these stress genes.

Also induced in the biofilm are the regulator *hha* and the downstream gene *ybaJ* with unknown function. The *hha* gene plays a role in a temperature and osmolarity-dependent regulation of expression of *E. coli* virulence factors including hemolysin and Vir antigen (Mourino et al. 1996).

Induction of genes with unknown functions in the glass wool biofilm

Of the 22 candidate biofilm genes (Table 1), 11 have unknown functions. One of these, *b3022*, was induced in both sets of *E. coli* JM109 glass wool biofilm samples compared to suspension samples. Also, it was expressed in all three sets of the *E. coli* K12 biofilm samples grown in continuous reactors (Table 4, discussed later). A BLAST search (NCBI database, http://www.ncbi.nlm.nih.gov/) indicates it encodes a protein that has homology to proteins from other strains such as a hypothetical protein from *Yersinia pestis* (67% identity) and a hypothetical protein from *Ralstonia eutropha* (60% identity). Interestingly, it has 36% identity to Lrp of *Sinorhizobium meliloti*, which is a leucine-responsive regulatory protein used for lipopolysaccharide (LPS) synthesis (Lagares et al. 2001). Further study with knockout mutation of this gene may generate information for understanding *E. coli* biofilms.

Repression of genes in the glass wool biofilm

Previous reports showed *E. coli* CsrA is a repressor of biofilm formation and expression of *csrA* is decreased during biofilm formation (Jackson et al. 2002). In agreement with this, *csrA* was found repressed in our glass wool biofilm cells compared to suspension cells (−1.6× in sample set 1 and −2.9× in sample set 2). Table 2 shows the 20 consistent, most-repressed genes including those with functions for metabolism, transport, and some with unknown functions.

Validation of the DNA microarray results with RNA dot blotting

To corroborate the gene expression results of the glass wool biofilm, total RNA was isolated from independent reactor samples, prepared as for the DNA microarray experiments, and the expression level of six representative genes of interest were quantified with RNA dot blotting: *b3022*, *hha*, *soxS*, *yhaJ*, *hslT*, and *b0753*. In the microarrays, the first five genes were induced in the glass wool biofilm and *b0753* was repressed. The expression of all six of these genes was confirmed with RNA dot blotting (Table 3). For example, *b3022* was induced 8-fold in the microarray experiments and induced 5-fold in RNA dot blotting experiment. Hence, the DNA microarray results provided reliable information about *E. coli* JM109 biofilm grown on glass wool.

E. coli K12 biofilm formation on metal plates in continuous reactors

One objective of the present study was to discover the genes required for maintaining biofilms under continuous flow conditions. For this purpose, *E. coli* K12 was grown in continuous reactors to form a biofilm on the submerged mild steel plates. Total RNA from mature biofilms (5-day-old) and corresponding suspension cultures (in the same reactor) was isolated for microarray analysis. However, it was surprising to find that no genes were consistently induced compared to the suspension cells (genes were expressed in the biofilms, but not expressed more than the suspension cells). To confirm that this low level of differential gene expression is physiologically real rather than an artifact caused by poor labeling or hybridization,

Table 4 Genes consistently expressed in the *E. coli* K12 biofilm grown on mild steel 1010 plates in a continuous reactor for 5 days at 0.05/h specific growth rate (μ). Each sample set contained the biofilm and suspension cells from the same independent reactor

Genes expressed in both set 1 and set 2	Genes expressed in both set 1 and set 3	Genes expressed in both set 2 vs 3
rpsH,*smg*, *yhbT*,*yhgF*, *b0837*, *b0838*, *b1598*, *b3022*, *deoB*, *fadB*, *purA*, *rmf*	*rpoD*, *rpoE*, *rseA*, *wrbA*, *ybeD*, *yccD*, *yccJ*, *ydbA_1*, *ygjG*, *yhbM*, *yhcP*,*yhdA*, *yheG*, *yhjD*, *yi22_2*, *yi22_3*, *yjfO*,*b0221*, *b0561*, *b0645*, *b0662*, *b0686*, *b1578*, *b1598*, *b1783*, *b1964*, *b2016*, *b2475*, *b3022*, *b3814*, *deoB*, *fadB*, *purA*, *rmf*	*rmf*, *smg*, *tufB*, *ycbB*, *ydaA*, *yfiA*, *b0485*, *b0926*, *b1598*, *b1678*, *b1725*, *b1955*, *b3022*, *bola*, *clpA*, *deoB*, *gatY*, *hslT*, *lpxC*, *nrdD*, *ompA*, *purA*

this experiment was repeated three times and the integrity of RNA and the quality of cDNA were carefully checked. Each time, it was found that the RNA was of high quality, with a 23S/16S rRNA ratio greater than 2 (RNeasy Mini Handbook, Qiagen), and the labeling reactions were successful, with the quality of cDNA (both size and yield) very similar to those that yielded good signals. Hence, it was tested whether there was any ingredient in the RNA or cDNA samples that affected the hybridization; RNA from other experiments that gave good hybridization results was labeled with Cy3, and the problematic RNA was labeled with Cy5. The two cDNA samples were then spiked and hybridized to the same microarray slide. Each time, the Cy3-labeled RNA (positive control) gave good images and the problematic RNA did not. Hence, there was no contaminant affecting hybridization and the low signals from hybridization are physiologically true.

The *E. coli* K12 biofilm grown in continuous reactors (4-day old) was examined for live cells using confocal laser scanning microscopy and found to contain water channels (clearly seen, image not shown) that had a thickness of 8±0.5 μm. Also, the high integrity of RNA suggests that the RNA we studied was from healthy cells. The continuous reactors used for the present study contained 150 ml LB with a feeding rate of 8 ml LB/h (μ=0.05 h^{-1}), which is comparable to a previous report involving *P. aeruginosa* biofilms, in which the chemostat contained 100 ml defined medium with a feeding rate of 20 ml/h (μ=0.2 h^{-1}) (Whiteley et al. 2001). Similar growth conditions have been widely used in our lab for different strains and good biofilms were obtained (Jayaraman et al. 1997). Hence, the mature *E. coli* K12 biofilm has a low level of differential gene expression under these conditions.

Three sets of independent samples were analyzed with the DNA microarrays for the continuous K12 biofilms. Although no genes were induced in the *E. coli* K12 biofilm compared to suspension cells, there were genes significantly expressed in the biofilm for each set of samples [about 12% of the total 4,228 tested genes have *P*-values <0.05 and average signal (from biofilm samples) >0.2]. By selecting genes with expression *P*-values <0.05 and average signal (from biofilm samples) >0.5, 150, 98, or 117 genes were identified that were significantly expressed in the three sets of biofilm samples, respectively (the consistently expressed genes are shown in Table 4). Among these genes, six were consistently expressed in all of the biofilm samples: *b1598*, *b3022*, *deoB*, *purA*, *rmf*, *smg*. Around 800 genes were repressed in these three data sets.

The expressed genes may be associated with stationary growth. When *E. coli* cells enter stationary phase, the 70S ribosomes become 100S (Wada et al. 1990). *rmf* encodes the ribosome modulation factor (RMF), which is associated with 100S dimers and is important for survival in stationary phase (Ishihama 1997). Moreover, *purA* encodes adenylosuccinate synthetase and is induced when *E. coli* infects eukaryotic cells (Hoffman et al. 2001); *deoB* encodes an enzyme for salvage of nucleosides and nucleotides (NCBI database). The other three genes (*b1598*, *b3022*, *smg*) consistently expressed in biofilm have unknown functions. Interestingly, the unknown gene *b3022* was also found induced in the glass wool biofilm.

Different results were obtained using the DNA microarrays when there was a more turbid suspension culture (3.4 OD vs 2 OD) as a result of a lower dilution rate (μ=0.03 h^{-1}). As shown from the single data set (set 4, two DNA microarrays), a 6-day *E. coli* K12 biofilm had 33 genes induced and 15 genes repressed—the most induced and repressed genes are shown in Table 5—and they largely consist of uncharacterized genes. The induced genes include the unknown operon *b1565* and *b1566* (7.7- and 8.3-fold, respectively) as well as a putative cold-shock protein *cspI* (12.3-fold), which agrees well with the *E. coli* JM109 glass wool data where stress genes were also discovered. The repressed genes include *tnaA* (encodes tryptophanase), which was repressed 12.5-fold.

Discussion

In this paper we clearly showed that *E. coli* JM109 has differential gene expression in biofilms compared to suspension cultures, identified many previously uncharacterized genes that are induced in the biofilm, discovered that the stress response was involved in biofilms, and confirmed that type I fimbriae are induced in biofilms under these conditions. Recently, Schembri et al. (2003) used DNA microarrays to study gene expression for the early stages (42 h after inoculation) of biofilm formation of an *E. coli* K12 derivative in flow chambers. The data confirmed some previous reports, such as the roles of type I fimbriae (Pratt and Kolter 1998) and Antigen 43 (Kjaergaard et al. 2000) in biofilm formation, although the induced and repressed gene lists were obtained by comparing biofilm cells with separately grown suspension

Table 5 Representative genes induced and repressed in the *E. coli* K12 biofilm grown on mild steel 1010 plates in a continuous reactor for 6 days at 0.03/h specific growth rate (μ)

Gene set 4	b-number	Expression ratio	Description
Representative induced genes			
yohH	b2139	14.2	ORF, unknown
phnD	b4105	11.3	Transport of small molecules: anions
yadD	b0132	10.8	ORF, unknown
cspI	b1552	12.3	Cold shock-like protein
yohF	b2137	11.3	Putative enzyme, not classified
b1448	b1448	9.7	Putative transport, drug/analog sensitivity
b2583	b2583	9.0	ORF, unknown
b1566	b1566	8.3	ORF, unknown
mesJ	b0188	8.0	ORF, cell division
yfiG	b2582	8.0	Putative enzyme, not classified
b1565	b1565	7.7	ORF, unknown
Representative repressed genes			
hdeB	b3509	−21.1	ORF, unknown
tnaA	b3708	−12.5	Tryptophanase
b2881	b2881	−10.8	Putative enzyme, not classified
exbD	b3005	−9.1	Transport of small molecules: cations
gatD	b2091	−9.0	Enzyme, degradation of small molecules: carbon compounds
b2872	b2872	−9.0	Putative enzyme, not classified
gadB	b1493	−8.7	Glutamate decarboxylase isozyme
hdeA	b3510	−8.7	ORF, unknown

cells (either exponential or stationary), which may not be optimum for comparison. In our study, biofilm and suspension cells were harvested from the same reactor to eliminate artifacts. The biofilm-induced gene list in the present study (for the *E. coli* JM109 biofilm grown on glass wool) is very different from that in the report of Schembri et al. (2003). While there were 22 genes found to be potential biofilm genes in our study, Schembri et al. found that 206 or 389 genes were induced in biofilms compared with exponential or stationary suspension cells, respectively (both studies use 2.5-fold as the cut off ratio). However, for the top 50 induced genes listed for each comparison (only the genes listed in the publication were compared, the complete data is not available) (Schembri et al. 2003), only 4 (*hslS*, *b0836*, *hslT*, and *b1060* when compared to exponential suspension cells) or one (*glnA* when compared to stationary suspension cells) in that study were in common with those found in our study (induced more than 2.5-fold in at least one data set for the *E. coli* JM109 biofilm on glass wool and up-regulated in the other data set). Moreover, to minimize the effect of the growth phase, Schembri et al. (2003) listed all the 45 genes consistently induced in biofilms compared to both exponential and stationary suspension cells; none of these genes were found induced in the *E. coli* JM109 biofilm on glass wool in our study. These differences are likely due to the different growth conditions of the suspension cells (minimum glucose medium vs LB used here), the different flow conditions (flow chamber vs shake flasks used here), and the different age of the biofilms (42 h vs 7 h here).

Type I fimbriae (or pili), encoded by gene cluster *fimABCDEFGH*, are involved in attachment of *E. coli* cells to the surface or specific receptors of eukaryotic cells (Moat and Foster 1995). Also, type I fimbriae have been shown to play a role in initial attachment in biofilm formation (Pratt and Kolter 1998; Schembri et al. 2003). In the microarray results of the present study, *fimG* was found up-regulated 1.4- to 3-fold in the *E. coli* JM109 biofilm cells grown on glass wool (Table 1). Also, the other seven genes in *fimABCDEFGH* were slightly up-regulated in biofilm cells compared to suspension cells (*fimA* 2.4×, *fimB* 1.3×, *fimC* 1.2×, *fimD* 1.6×, *fimE* 1.4×, *fimF* 1.2×, *fimH* 1.6×). Although the induction of the other seven genes was less significant (less than the 2.5× threshold), it appears that type I fimbriae are necessary for early biofilm formation on glass wool.

That the mature *E. coli* K12 biofilm grown in a continuous reactor is somewhat inactive in terms of relative gene expression was a surprising discovery in the present study. During the preparation of this manuscript, two studies have been published that showed that the conjugative F factor stimulated biofilm formation of F^- *E. coli* strains including *E. coli* K12 (Ghigo 2001; Reisner et al. 2003). Therefore, the better biofilms formed by *E. coli* JM109 (F^+) compared to *E. coli* K12 (F^-) in this study were due to the absence of the F factor in *E. coli* K12. Upon adding the R1*drd19* plasmid (Ghigo 2001), we found K12 produced ten times more biofilm (results not shown), which is consistent with the induction of the *fim* operon in the JM109 glasswool biofilm (Table 1) since the F plasmid encodes F fimbriae (Ghigo 2001). Hence, the

microarray data of *E. coli* K12 in this study should be considered as preliminary and further study with *E. coli* K12 carrying F factor may be necessary.

In contrast to the K12 biofilm formed in the continuous reactors, the JM109 glass wool biofilm was grown in the presence of a fluid sheer force (shaking) in batch, which is a more challenging environment for maintaining the biofilm. Hence, stress genes were induced in the glass wool biofilm. The induction of these genes indicates that bacteria have different strategies for maintaining biofilms under different conditions. Drugs with these genes as targets may have potential in biofilm control.

The sigma factor σ^E, encoded by *rpoS*, controls the synthesis of more than 50 proteins responsible for different stresses such as starvation, heat shock, cold shock, and transition from exponential phase to stationary phase (Loewen et al. 1998). The recent study by Schembri et al. (2003) showed 46% of the genes controlled by *rpoS* (30 out of 65 genes listed by Loewen et al. 1998) were differentially expressed in an *E. coli* biofilm compared to exponentially growing suspension cells (28 were up-regulated and 2 were down-regulated) or stationary (OD=1.3) suspension cells (14 were up-regulated and 16 were down-regulated). In our *E. coli* JM109 study, none of these 65 genes was significantly induced (*P*-value <0.05, expression ratio >2.5). However, 19 of the *rpoS*-related genes were significantly repressed in our glass wool biofilm compared to the suspension culture (*P*-value <0.05, expression ratio <−2.5). The expression of all the other genes (46 out of 65 genes listed by Loewen et al. 1998) was not significantly different between our suspension and glass wool biofilm cells, either because of the high *P*-value (>0.05) or because of small expression ratios (<2.5 fold). Furthermore, *rpoS* itself was repressed 2.9- and 3.8-fold, respectively, in the two independent glass wool data sets in our study (both have a *P*-value <0.05). Among these 19 *ropS*-related repressed genes in our glass wool biofilm, 18 are known to be positively affected by *rpoS* (Loewen et al. 1998). Hence, for our studies, *rpoS* and the genes positively affected by it were repressed in the *E. coli* JM109 glass wool biofilm.

In the 30 *rpoS*-affected genes listed in the report of Schembri et al. (2003), 28 were up-regulated when compared with exponential cells. However, only 14 were up-regulated when compared with stationary cells (OD=1.3). Hence, the higher cell density in the suspension cultures appears to be responsible for more repression of the *rpoS*-affected genes. Given that the suspension culture in the present study had an even higher cell density (OD=2), it is not surprising to see more *rpoS*-affected genes repressed. It should be noted, however, that the repression of *rpoS* in our experiments does not rule out its possible role in biofilm formation. In the study of Schembri et al.(2003), an *rpoS* mutant was constructed and found to be unable to form biofilm in a flow chamber for 42 h. Also, an earlier study by Adams and McLean (1999) had shown that an *rpoS* mutant had 50% less biofilm compared to the wild type and the mutation had no apparent effect on planktonic growth. Further study to investigate which stage of biofilm formation is affected by *rpoS* will be necessary.

By using random insertion mutagenesis, Prigent-Combaret et al. (1999) have shown that up to 38% of genes are differentially expressed in *E. coli* biofilms compared to suspension cells; however, 5.5% of genes (22 up-regulated genes and 201 repressed genes) were differentially expressed in the present study (JM109 glass wool biofilm). In comparison, we recently found that 14% of genes are induced or repressed in *B. subtilis* air-liquid interface biofilm compared to suspension cells (Ren et al. 2004). The numbers of induced and repressed genes in other reports are smaller. For example, a recent study of a *P. aeruginosa* biofilm using DNA microarrays showed only 1% of genes were differentially expressed in biofilms compared to suspension cells (Whiteley et al. 2001).

Moreover, there are also disagreements regarding the genes required for biofilm formation. Besides the quorum-sensing genes mentioned in the Introduction, twitching genes have also been reported to be either important (O'Toole and Kolter 1998) or not important (Heydorn et al. 2002) in microcolony formation in a *P. aeruginosa* biofilm. This discrepancy can be attributed partly to the condition of biofilm growth and the stage of biofilm formation being studied. Biofilm formation is a dynamic process with different genes expressed at different stages (Kuchma and O'Toole 2000). For example, flagellar genes are involved in early biofilm formation but are not needed in mature biofilm (Pratt and Kolter 1998; Heydorn et al. 2002). Consistently, in the present study, flagellar genes were repressed in *E. coli* K12 biofilms grown in continuous reactors (such as *fliCDG*, data not shown), but were neither induced nor repressed in the glass wool biofilm (data not shown). Hence, the environmental conditions and the stage of biofilm development should be carefully considered in explaining biofilm results.

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