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Temporal gene-expression in *Escherichia coli* K-12 biofilms

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Summary

Analysis of the temporal development of *Escherichia coli* K-12 biofilms in complex medium indicates the greatest differential gene expression between biofilm and suspension cells occurred in young biofilms at 4 and 7 h (versus 15 and 24 h). The main classes of genes differentially expressed (biofilm versus biofilm and biofilm versus suspension cells) include 42 related to stress response (e.g. *cspABFGI*), 66 related to quorum sensing (e.g. *ydgG*, *gadABC*, *hdeABD*), 20 related to motility (e.g. *flgBCEFH*, *fliLMQR*, *motB*), 13 related to fimbriae (e.g. *sfmCHM*, *fimZ*, *csgC*), 24 related to sulfur and tryptophan metabolism (e.g. *trpLBA*, *tnaLA*, *cysDNCJH*), 80 related to transport (e.g. *gatABC*, *agaBC*, *ycjJ*, *ydfJ*, *phoU*, *phnCJKM*), and six related to extracellular matrix (e.g. *wcaBDEC*). Of the 93 mutants identified and studied, 76 showed altered biofilm formation. Biofilm architecture changed from thin and dense to globular and dispersed to dense and smooth. The quorum-sensing signal AI-2 controls gene expression most clearly in mature biofilms (24 h) when intracellular AI-2 levels are highest. Sulfate transport and metabolism genes (*cysAUWDN*) and genes with unknown functions (*ymgABCZ*) were repressed in young (4, 7 h) biofilms, induced in developed biofilms (15 h), and repressed in mature (24 h) biofilms. Genes related to both motility and fimbriae were induced in biofilms at all sampling time points and colanic acid genes were induced in mature biofilms (24 h). Genes related to dihydroxyacetone phosphate synthesis from galactitol and galactosamine (e.g. *gatZABCDR*, *agaBCY*) were highly regulated in biofilms. Genes involved in the biosynthesis of indole and sulfide (*tnaLA*) are repressed in biofilms after 7 h (corroborated by decreasing intracellular indole concentrations in biofilms). Cold-shock protein transcriptional regulators (*cspABFGI*) appear to be positive biofilm regulators, and deletions in respiratory genes (e.g. *hyaACD*, *hyfCG*, *appC*, *narG*) increased biofilm formation sevenfold.

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Introduction

Microbiology began with Leeuwenhoek's discovery of free-floating organisms with his precisely made lenses (de Kruif, 1996). Today we know these bacteria do not live a solitary life but exist primarily in biofilms (Costerton *et al*., 1995). To investigate this dominant lifestyle, we conducted a temporal study of *Escherichia coli* biofilm gene-expression patterns as well as observed its development in a flow system using the modern observation tool, confocal microscopy (Heydorn *et al*., 2000). The confocal microscope allows rigorous characterization of biofilms yielding its architecture and quantifying surface coverage, thickness and roughness.

To date, three groups have performed single time point analyses of *E. coli* biofilms using DNA microarrays (Schembri *et al*., 2003; Beloin *et al*., 2004; Ren *et al*., 2004a). Schembri and colleagues (2003) found 4.8% of the genome was induced and 0.63% was repressed in biofilms grown for 32 h in continuous flow chambers relative to exponential growth of a planktonic culture while 9% of the genes were induced and 4.5% were repressed in biofilms relative to a stationary-phase culture (Schembri *et al*., 2003). Beloin and colleagues (2004) reported 1.9% of the genome was differentially expressed in 5- to 8-day biofilms relative to exponential cultures; 64 of these genes were induced more than twofold relative to exponential cultures, but only 24 genes in this subset were again induced relative to stationary-phase cultures (Beloin *et al*., 2004). Results by our group show approximately 5.5% of the genome was differentially expressed (22 genes upregulated and 201 genes repressed) (Ren *et al*., 2004a). From this initial microarray study, by exploring differential gene expression of isogenic mutants in biofilms, we have determined that direct addition of autoinducer two (AI-2) stimulates biofilm formation 30-fold through previously uncharacterized MqsR that subsequently controls chemotaxis via QseB (González Barrios *et al*., 2006a). Also, previously uncharacterized TqsA

represses biofilm formation by enhancing AI-2 export (Herzberg *et al*., 2006) and previously uncharacterized BssS represses biofilm formation by increasing intracellular and extracellular indole concentrations (Domka *et al*., 2006). Finally, Hha and YbaJ increase biofilm formation by increasing conjugation and repressing motility in the presence of conjugation plasmid R1*drd*19 (González Barrios *et al*., 2006b). By discerning how biofilms respond to these signals (e.g. indole, AI-2), it is hoped they may be manipulated for engineering and medicine.

Similar single time point studies have also been conducted to analyse differential gene expression or protein expression in biofilms of *Pseudomonas aeruginosa* (Whiteley *et al*., 2001), *Bacillus subtilis* (Stanley *et al*., 2003; Ren *et al*., 2004b), *Thermotoga maritime* (Pysz *et al*., 2004), and *Pseudomonas putida* (Sauer and Camper, 2001). These studies provide insights into the genetic basis of biofilm development by identifying groups of differentially expressed genes including motility (Whiteley *et al*., 2001; Stanley *et al*., 2003), adhesion (Ren *et al*., 2004a), phage (Whiteley *et al*., 2001; Stanley *et al*., 2003), ribosomes (Schembri *et al*., 2003) and respiratory genes (Schembri *et al*., 2003; Beloin *et al*., 2004; Ren *et al*., 2004a). However, some of the differential gene expression patterns in biofilms may be due to differences in growth of the planktonic cells used in the comparison (Lazazzera, 2005); hence, temporal studies of genes differently expressed in biofilms may be more informative than studying individual time point expression profiles (Beloin and Ghigo, 2005). Additionally, many other factors such as stress response through stationary-phase sigma factor (RpoS) (Corona-Izquierdo and Membrillo-Hernández, 2002), sulfur metabolism (Sturgill *et al*., 2004; Ren *et al*., 2005), carbon flux (Jackson *et al*., 2002) and cell signalling through both AI-2 (González Barrios *et al*., 2006a) and the putative stationary-phase signal indole (Di Martino *et al*., 2003; Ren *et al*., 2004c) have been shown to influence biofilm formation (Domka *et al*., 2006).

Temporal studies are also important as biofilm formation is a dynamic (sequential) process involving reversible and irreversible attachment, maturation and dispersion (Sauer *et al*., 2002). The only temporal transcriptome study performed to date compares exponential- and stationary-phase *P. aeruginosa* (Waite *et al*., 2005) planktonic cultures with separately grown agar colonies at 8, 14, 24 and 48 h; most changes in gene expression occurred in 8 h biofilms with approximately 14% of the genome differentially expressed relative to 14, 24 and 46 h biofilms, while expression in developed biofilms (14, 24, 48 h) was less than 1% of the genome (Waite *et al*., 2005). Twenty genes were induced in the 8 h biofilms including type IV pilus production genes, growth and metabolism-related genes, transport and hypothetical proteins, while 14, 24 and 48 h biofilms induced many genes related to AI-1 quorum sensing and RpoS. Additionally, a protein with a GGDEF domain was identified, which is believed to play a role in the switch between the planktonic and attached cell lifestyle (Waite *et al*., 2005).

Similarly, a temporal proteomic analysis of *P. aeruginosa* planktonic cells grown in a chemostat compared with separate cultures of biofilm cells attached to silicone tubing for 8 h or 1, 6, 9 and 12 days showed protein expression patterns that varied based on the stage in biofilm development (Sauer *et al*., 2002). Based on microscopic analysis, biofilm development consists of at least five stages including reversible and irreversible attachment, two stages of maturation and dispersion (Sauer *et al*., 2002). The differential protein expression between these stages and planktonic cultures was greatest in 6 day biofilms. Expression of flagella genes was important for initial attachment of the cells (motility was not observed during maturation) and was important during dispersion. *N*-3-oxododecanoyl-homoserine lactone quorum sensing was important during irreversible attachment, and *N*-butyryl-homoserine lactone quorum sensing was activated during the first maturation stage, but its effect on protein expression was not evaluated. Proteins related to oxidative damage, exopolysaccharide production and aerobic/anaerobic respiration were also differentially expressed (Sauer *et al*., 2002). Southey-Pilling and colleagues (2005) analysed protein expression in *P. aeruginosa* biofilms at two maturation stages and the dispersion stage relative to a separate culture of planktonic cells. Proteins involved in adhesion, antibiotic resistance, adaptation, stress, virulence and denitrification were first expressed during the first maturation stage, while antibiotic resistance, quorum sensing and chemotaxis were expressed in the second maturation stage. No proteins were expressed at the onset of the dispersion stage and many of the metabolic and housekeeping proteins such as arginine deiminase system and succinate semialdehyde dehydrogenase were expressed during all stages of biofilm development (Southey-Pilling *et al*., 2005).

Here, biofilm and suspension gene expression are compared after 4, 7, 15 and 24 h. Differential gene expression at all of the time points in the biofilm samples, gene expression between biofilm and suspension cells, and gene expression between individual suspension cell samples were compared. This allowed us to determine the changes that were biofilm-specific. Only the genes induced or repressed relative to suspension cells or with significant changes in expression profile over time in the biofilms were considered for further analysis. We also studied biofilm formation in continuous flow chambers, analysing the development patterns and changes in archi-

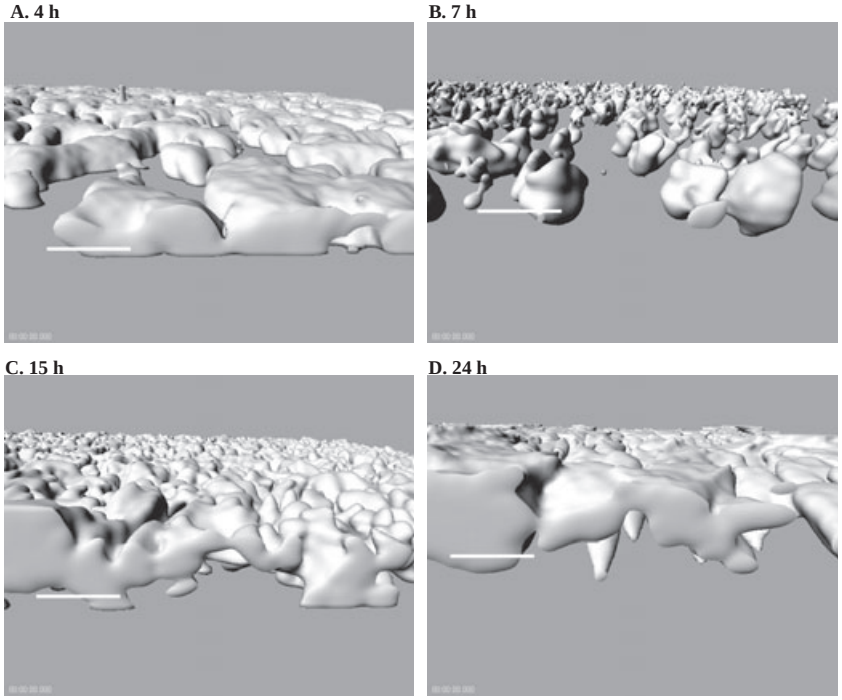

Fig. 1. *Escherichia coli* BW25113 biofilm formation in flow chambers with LB medium after 4 h (A), 7 h (B), 15 h (C) and 24 h (D). Images were analysed with IMARIS, and the scale bar is 10 μm.

tecture based on images obtained from confocal microscopy. Biofilm and suspension cells were grown in the same reactor to eliminate errors associated with growing biofilms and suspension cells in separate cultures (Ren *et al*., 2004a). It was discovered that intracellular AI-2 and indole concentrations in biofilms and suspension cells fluctuate, which lead to dramatic changes in the transcriptome; these changes are corroborated with *in vivo* measurements of these metabolites. In addition, the predictions of the microarrays are corroborated through the studies with 93 isogenic mutants and through the addition of compounds like galactitol.

Results and discussion

Biofilm temporal architecture

To study the changes in biofilm formation and architecture as a function of time, a continuous flow system was used with LB medium. Four independent flow cells were used to study biofilm formation of the *E. coli* wild-type strain at 4, 7, 15 and 24 h (Fig. 1). The changes were quantified with COMSTAT to avoid bias (Heydorn *et al*., 2000) (Table 1). Total biomass was similar for the first three time points, with nearly the same readings at 4, 7 and 15 h (14 ± 5–15 ± 2 $\mu m^3\ \mu m^{-2}$), but it more than doubled at the 24 h time point to 37 ± 11 $\mu m^3\ \mu m^{-2}$. The average thickness of the biofilm increased slightly from 20 ± 3 to 30 ± 10 μm between 4 and 7 h, decreased to 20 ± 2 μm at 15 h (even though the total biomass stayed constant at these three time points so the biofilm became more dense, Fig. 1C), and the thickness increased more than threefold at 24 h (73 ± 11 μm) similar to the trend observed with total biomass. Substratum coverage at the surface of the glass slide and average coverage over the thickness of the biofilm were monitored; these did not give the same trend as many dynamic changes in architecture were observed. The substratum coverage fluctuated with time with the highest substratum coverage (54 ± 9%) observed at 4 h because of the thin, dense, smooth biofilm with a roughness coefficient of 0.5 ± 0.1 (Fig. 1A) that formed on the surface of the glass slide. After 7 h, a more scattered, globular biofilm (Fig. 1B) with a higher roughness coefficient (0.7 ± 0.3) and decreased surface

Table 1. COMSTAT analysis of the *E. coli* K-12 BW25113 flow cell system in LB after 4, 7, 15 and 24 h.

Time (h)	Biomass ($\mu m^3\ \mu m^{-2}$)	Substratum coverage (%)	Average substratum coverage (%)	Mean thickness (μm)	Roughness coefficient
4	15 ± 2	54 ± 9	42 ± 5	20 ± 3	0.5 ± 0.1
7	14 ± 5	10 ± 3	20 ± 5	30 ± 10	0.7 ± 0.3
15	14 ± 3	33 ± 12	42 ± 12	20 ± 2	0.3 ± 0.1
24	37 ± 11	20 ± 10	47 ± 10	73 ± 11	0.2 ± 0.2

Table 2. Percentage of genes induced (above diagonal) and repressed (below diagonal) more than 2.5-fold (A) and more than fivefold (B) in the biofilms formed in LB after 4–24 h.

	S4		S7		S15		S24		B4		B7		B15		B24	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
S4			4.0	0.8	10.8	2.3	19.6	4.1	1.9	0.6	–	–	–	–	–	–
S7	3.5	0.4			–	–	12.5	2.39	–	–	2.2	0.8	–	–	–	–
S15	7.4	2.3	–	–			3.2	0.62	–	–	–	–	1.0	0.2	–	–
S24	7.6	1.8	4.0	1.4	1.9	0.4			–	–	–	–	–	–	1.0	0.2
B4	1.3	0.6	–	–	–	–	–	–			6.2	1.7	11.4	3.6	27.3	8.2
B7	–	–	0.3	0	–	–	–	–	2.5	0.5			2.3	0.4	10.3	1.2
B15	–	–	–	–	0.05	0.05	–	–	4.7	1.2	2.2	0.8			6.4	1.5
B24	–	–	–	–	–	–	0.7	0.05	4.9	1.1	3.7	1.2	2.3	0.2		

coverage (10 ± 3%) was observed, even though the total biomass stayed constant. At 15 h the surface coverage increased to 33 ± 12%; hence, a flatter denser smooth biofilm formed (Fig. 1C) with a significant decrease in roughness (0.3 ± 0.1). After 24 h, the biofilm formed a dense smooth mass with a threefold increase in thickness and the highest average substratum coverage of 47 ± 10% (Fig. 1D). The observed trend between these time points shows an increase in both biomass and thickness with the biofilm architecture changing from a smooth dense mass to a more globular structure (Table 1 and Fig. 1).

Biofilm temporal gene expression

In order to study the temporal development of biofilm, eight independent DNA microarray experiments were performed by taking samples of both biofilm and suspension cells (each from the same flask) after 4, 7, 15 and 24 h of culturing. The expression profiles were compared between each biofilm (B4, etc.) relative to the other biofilm and suspension samples and suspension experiments (S4, etc.) relative to other suspension and biofilm time points. The results of this analysis are shown in Table 2 where the percentage of both the induced and repressed genes is shown. In this study 27.3% of the genome was significantly induced and 4.9% was repressed more than 2.5-fold for B24 compared with B4. Also 19.6% of the genome was induced and 7.6% was repressed with S24 relative to S4. This indicates that during the 4–24 h period, approximately 7.7% more genes are induced in the biofilms and 2.7% more genes are repressed in suspension cells, which suggests that, overall, the cells are more metabolically active in biofilms than in suspension under these conditions.

Additionally, comparisons between the biofilm and suspension cells at each of the time points were analysed and similar to previously published results for *P. aeruginosa* (Waite *et al*., 2005), most of the differentiation between biofilm and suspension occurred early at 4 h (1.9% induced and 1.3% repressed) and 7 h (2.2% induced and 0.3% repressed). In developed biofilms, these changes were more subtle with 1% of genome induced at 15 h and 24 h and only 0.05% and 0.7% of the genome repressed respectively. These values are also consistent with the observed trend that biofilm gene expression is more closely related to stationary-phase planktonic growth (Lazazzera, 2005).

Because of the large number of differentially expressed genes, a fivefold cut-off ratio was used for the remaining biofilm analysis and a 2.5-fold cut-off ratio was used for the analysis of biofilm relative to suspension cells. The percentage of genes induced and repressed more than fivefold were also calculated and the results are shown in Table 2. The genes chosen for further investigation were based on three criteria: (i) induction or repression more than fivefold relative to other time points in biofilm development; (ii) induction or repression more than 2.5-fold in biofilms relative to suspension cells at any one of the time points (4, 7, 15, or 24 h); or (iii) the temporal expression pattern in biofilm cells was significantly different from the temporal expression pattern in planktonic cells. This ensured that the genes were both biofilm-specific and had interesting expression patterns as a function of time. Note that many of the genes that were differentially expressed were part of operons, which indicates the data are reliable. The gene functions were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) and from the EcoCyc database (http://biocyc.org/ECOLI/) (Keseler *et al*., 2005) and were categorized into 19 main groups based on the Clusters of Orthologous Groups (COG) database (Tatusov *et al*., 2001).

Based on the microarrays, biofilm formation with 93 isogenic mutants was analysed using the microtitre assay. Of 93 mutants, 80% (74 mutants) had increased biofilm formation and 2% (two mutants) reduced biofilm formation in either LB or LB glu and are shown in Table 3. More detailed analysis is presented in subsequent sections regarding these mutations.

Table 3. *Escherichia coli* strains used and biofilm formation of mutant strains relative to wild-type strain in LB and LB glu after 24 h.

Strains/plasmids	Genotype	Fold LB	Fold LB glu	Product	Source
Wild-type strain					
E. coli K-12 BW25113	*lacI*^{q}, *ΔlacZ*$_{WJ16}$, *ΔaraBAD*$_{AH33}$, *ΔrhaBAD*$_{LD78}$, *hsdR514* and *rrnB*$_{T14}$				Yale CGSG Stock Center
Plasmids					
pCM18	Em^{R}, pTRKL2-P$_{CP25}$RBSII-*gfp*3*-T$_{o}$-T$_{1}$				Hansen *et al.* (2001)
pLW11	Amp^{R}, *lsrACDBFG::lacZ* in pFZY1				Wang *et al.* (2005)
Mutant strains					
Information storage and processing					
E. coli K-12 *BW25113 envR*	Km^{R}, K-12 Δ *envR* Ω	wt	1.9 ± 1.2	EnvR transcriptional regulator	Baba *et al.* (2006)
E. coli K-12 *BW25113 cpxP*	Km^{R}, K-12 Δ *cpxP* Ω	–2.1 ± 0.3	wt	Regulator, chaperone involved in resistance to extracytoplasmic stress	Baba *et al.* (2006)
E. coli K-12 *BW25113 gyrB*	Km^{R}, K-12 Δ *gyrB* Ω	wt	5 ± 1.3	DNA gyrase subunit B, type II topoisomerase, ATPase activity	Baba *et al.* (2006)
Cellular processes					
E. coli K-12 *BW25113 ybiG*	Km^{R}, K-12 Δ *ybiG* Ω	wt	2.2 ± 0.4	Outer membrane protease, receptor for phage OX2	Baba *et al.* (2006)
E. coli K-12 BW25113 *wcaD*	Km^{R}, K-12 Δ *wcaD* Ω	–1.9 ± 1.2	wt	Putative colanic acid polymerase	Baba *et al.* (2006)
E. coli K-12 *BW25113 yjfG*	Km^{R}, K-12 Δ *yjfG* Ω	wt	3.7 ± 0.7	UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	Baba *et al.* (2006)
E. coli K-12 *BW25113 tfaD*	Km^{R}, K-12 Δ *tfaD* Ω	wt	3.1 ± 1.2	DLP12 prophage; putative tail fibre assembly gene	Baba *et al.* (2006)
E. coli K-12 *BW25113 stfR*	Km^{R}, K-12 Δ *stfR* Ω	wt	3.7 ± 1.0	Rac prophage; putative tail fibre protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 ydeQ*	Km^{R}, K-12 Δ *ydeQ* Ω	wt	3.9 ± 1.1	Putative adhesin; similar to FimH protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 yhcA*	Km^{R}, K-12 Δ *yhcA* Ω	wt	1.9 ± 0.7	Putative chaperone	Baba *et al.* (2006)
E. coli K-12 *BW25113 tauA*	Km^{R}, K-12 Δ *tauA* Ω	2.2 ± 0.4	3.9 ± 1.1	Taurine transport protein (ABC transporter)	Baba *et al.* (2006)
E. coli K-12 *BW25113 tauD*	Km^{R}, K-12 Δ *tauD* Ω	wt	3.2 ± 1.1	Taurine dioxygenase, 2-oxoglutarate-dependent	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysW*	Km^{R}, K-12 Δ *cysW* Ω	wt	3.5 ± 1.0	Sulfate transport system permease W protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysU*	Km^{R}, K-12 Δ *cysU* Ω	wt	4.0 ± 0.8	Sulfate, thiosulfate transport system permease T protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysP*	Km^{R}, K-12 Δ *cysP* Ω	wt	2.5 ± 0.6	Thiosulfate binding protein, ABC transporter	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysN*	Km^{R}, K-12 Δ *cysN* Ω	wt	1.8 ± 0.7	ATP sulfate adenylyltransferase	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysH*	Km^{R}, K-12 Δ *cysH* Ω	wt	3.5 ± 0.8	3′-phosphoadenosine 5′-phosphosulfate (PAPS) reductase	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysI*	Km^{R}, K-12 Δ *cysI* Ω	wt	2.9 ± 0.7	Sulfite reductase, alpha subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 cysJ*	Km^{R}, K-12 Δ *cysJ* Ω	wt	6 ± 1.4	Sulfite reductase flavoprotein subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 phoU*	Km^{R}, K-12 Δ *phoU* Ω	wt	6.4 ± 1.3	Negative regulator for *pho* regulon, enzyme in phosphate metabolism	Baba *et al.* (2006)
E. coli K-12 *BW25113 prpR*	Km^{R}, K-12 Δ *prpR* Ω	wt	3.6 ± 0.8	Regulator for *prp* operon	Baba *et al.* (2006)
E. coli K-12 *BW25113 citA*	Km^{R}, K-12 Δ *citA* Ω	1.8	3.9 ± 0.8	Putative sensor-type protein	Baba *et al.* (2006)
Metabolism					
E. coli K-12 *BW25113 citD*	Km^{R}, K-12 Δ *citD* Ω	1.9 ± 0.5	1.7 ± 0.7	Citrate lyase acyl carrier protein (gamma chain)	Baba *et al.* (2006)
E. coli K-12 *BW25113 citC*	Km^{R}, K-12 Δ *citC* Ω	wt	4.6 ± 1.2	Citrate lyase synthetase	Baba *et al.* (2006)
E. coli K-12 *BW25113 sdhC*	Km^{R}, K-12 Δ *sdhC* Ω	wt	1.8 ± 0.7	Succinate dehydrogenase membrane protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 sdhA*	Km^{R}, K-12 Δ *sdhA* Ω	wt	3.6 ± 0.8	Succinate dehydrogenase flavoprotein	Baba *et al.* (2006)
E. coli K-12 *BW25113 sucA*	Km^{R}, K-12 Δ *sucA* Ω	wt	2.7 ± 0.6	2-Oxoglutarate decarboxylase/dehydrogenase complex, thiamin-binding	Baba *et al.* (2006)
E. coli K-12 *BW25113 hyaA*	Km^{R}, K-12 Δ *hyaA* Ω	wt	2.8 ± 0.8	Hydrogenase-1 small subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 hyaC*	Km^{R}, K-12 Δ *hyaC* Ω	1.8 ± 0.4	6.7 ± 1.7	Hydrogenase I, *b*-type cytochrome subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 hyaD*	Km^{R}, K-12 Δ *hyaD* Ω	2.2 ± 0.3	6.2 ± 1.6	Processing of HyaA and HyaB proteins	Baba *et al.* (2006)
E. coli K-12 *BW25113 appC*	Km^{R}, K-12 Δ *appC* Ω	wt	2.3 ± 0.7	Cytochrome oxidase *bd*-II, subunit I	Baba *et al.* (2006)
E. coli K-12 *BW25113 torC*	Km^{R}, K-12 Δ *torC* Ω	1.8 ± 0.5	6.6 ± 1.8	Trimethylamine N-oxide reductase, negative regulator of *tor* operon	Baba *et al.* (2006)
E. coli K-12 *BW25113 narG*	Km^{R}, K-12 Δ *narG* Ω	2.1 ± 0.4	4.6 ± 0.9	Nitrate reductase 1, alpha subunit	Baba *et al.* (2006)

E. coli K-12 *BW25113 fumC*	Km^R, K-12 Δ *fumC* Ω	2.2 ± 0.4	7.1 ± 1.4	Fumarase C (fumarate hydratase Class II)	Baba *et al.* (2006)
E. coli K-12 *BW25113 hyfC*	Km^R, K-12 Δ *hyfC* Ω	wt	2.7 ± 0.6	Hydrogenase 4 membrane subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 hyfG*	Km^R, K-12 Δ *hyfG* Ω	wt	3.5 ± 1.1	Hydrogenase 4 subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 dctA*	Km^R, K-12 Δ *dctA* Ω	wt	4.5 ± 1.1	Citrate and C4-dicarboxylic acids transport protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 aceA*	Km^R, K-12 Δ *aceA* Ω	wt	2.0 ± 0.5	Isocitrate lyase monomer	Baba *et al.* (2006)
E. coli K-12 *BW25113 aceK*	Km^R, K-12 Δ *aceK* Ω	2.0 ± 0.4	1.8 ± 0.5	Isocitrate dehydrogenase phosphatase/isocitrate dehydrogenase kinase	Baba *et al.* (2006)
E. coli K-12 *BW25113 frdA*	Km^R, K-12 Δ *frdA* Ω	2.0 ± 0.4	5.3 ± 1.2	Fumarate reductase, anaerobic, catalytic and NAD/flavoprotein subunit	Baba *et al.* (2006)
E. coli K-12 *BW25113 prpD*	Km^R, K-12 Δ *prpD* Ω	wt	2.4 ± 1.0	2-Methyl citrate dehydratase	Baba *et al.* (2006)
E. coli K-12 *BW25113 torT*	Km^R, K-12 Δ *tort* Ω	1.8 ± 0.5	2.0 ± 0.9	Part of regulation of *tor* operon, periplasmic	Baba *et al.* (2006)
E. coli K-12 *BW25113 gatR*	Km^R, K-12 Δ *gatR* Ω	wt	6.5 ± 1.5	GatR transcriptional repressor	Baba *et al.* (2006)
E. coli K-12 *BW25113 gatC*	Km^R, K-12 Δ *gatC* Ω	wt	6.3 ± 1.7	PTS family enzyme IIC, galactitol-specific	Baba *et al.* (2006)
E. coli K-12 *BW25113 gatB*	Km^R, K-12 Δ *gatB* Ω	wt	2.3 ± 0.7	PTS family enzyme IIB, galactitol-specific	Baba *et al.* (2006)
E. coli K-12 *BW25113 gatZ*	Km^R, K-12 Δ *gatZ* Ω	wt	2.9 ± 0.6	Tagatose-1,6-bisphosphate aldolase 2	Baba *et al.* (2006)
E. coli K-12 *BW25113 agaY*	Km^R, K-12 Δ *agaY* Ω	1.8 ± 0.2	3.5 ± 0.8	Tagatose-1,6-bisphosphate aldolase 1	Baba *et al.* (2006)
E. coli K-12 *BW25113 agaB*	Km^R, K-12 Δ *agaB* Ω	wt	2.0 ± 0.5	PTS family enzyme IIB component 1, *N*-acetylgalactosamine-specific	Baba *et al.* (2006)
E. coli K-12 *BW25113 agaC*	Km^R, K-12 Δ *agaC* Ω	wt	2.6 ± 0.8	PTS family enzyme IIC component 1, *N*-acetylgalactosamine-specific	Baba *et al.* (2006)
E. coli K-12 *BW25113 ybaS*	Km^R, K-12 Δ *ybaS* Ω	2.3 ± 0.5	4.0 ± 0.6	Putative glutaminase	Baba *et al.* (2006)
E. coli K-12 *BW25113 aspC*	Km^R, K-12 Δ *aspC* Ω	wt	3.8 ± 0.9	Aspartate aminotransferase	Baba *et al.* (2006)
E. coli K-12 *BW25113 ydeD*	Km^R, K-12 Δ *ydeD* Ω	2.0 ± 0.4	3.9 ± 0.7	Amino acid metabolite efflux pump, *O*-acetylserine and cysteine export	Baba *et al.* (2006)
E. coli K-12 *BW25113 astE*	Km^R, K-12 Δ *astE* Ω	wt	3.8 ± 1.0	Succinylglutamate desuccinylase	Baba *et al.* (2006)
E. coli K-12 *BW25113 astD*	Km^R, K-12 Δ *astD* Ω	wt	3.8 ± 1.2	Succinylglutamic semialdehyde dehydrogenase	Baba *et al.* (2006)
E. coli K-12 *BW25113 astA*	Km^R, K-12 Δ *astA* Ω	wt	2.2 ± 0.7	Arginine succinyltransferase	Baba *et al.* (2006)
E. coli K-12 *BW25113 astC*	Km^R, K-12 Δ *astC* Ω	wt	6.1 ± 1.0	Acetylornithine transaminase/succinylornithine transaminase	Baba *et al.* (2006)
E. coli K-12 *BW25113 acrF*	Km^R, K-12 Δ *acrF* Ω	wt	4.4 ± 1.1	Indole transport, integral transmembrane, Drug Efflux System	Baba *et al.* (2006)
Acid resistance					
E. coli K-12 *BW25113 gadE*	Km^R, K-12 Δ *gadE* Ω	1.8 ± 0.3	7.3 ± 1.4	Transcriptional activator, acid-responsive regulator of *gadA* and *gadBC*	Baba *et al.* (2006)
E. coli K-12 *BW25113 hdeB*	Km^R, K-12 Δ *hdeB* Ω	wt	2.3 ± 0.6	Protein involved in acid resistance	Baba *et al.* (2006)
E. coli K-12 *BW25113 hdeD*	Km^R, K-12 Δ *hdeD* Ω	wt	1.9 ± 0.6	Protein involved in acid resistance	Baba *et al.* (2006)
E. coli K-12 *BW25113 gadW*	Km^R, K-12 Δ *gadW* Ω	wt	2.3 ± 1.2	Transcriptional repressor of the *gadAX* and *gadBC* operons	Baba *et al.* (2006)
E. coli K-12 *BW2511 gadA*	Km^R, K-12 Δ *gadA* Ω	wt	6.0 ± 1.8	Glutamate decarboxylase A, isozyme	Baba *et al.* (2006)
Poorly characterized					
E. coli K-12 *BW25113 htgA*	Km^R, K-12 Δ *htgA* Ω	wt	3.1 ± 1.2	Positive regulator for sigma 32 heat-shock promoters	Baba *et al.* (2006)
E. coli K-12 *BW25113 yafU*	Km^R, K-12 Δ *yafU* Ω	wt	4.5 ± 1.0	Hypothetical protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 ykfI*	Km^R, K-12 Δ *ykfI* Ω	wt	1.8 ± 0.5	Toxin of the YkfI–YafW toxin–antitoxin pair	Baba *et al.* (2006)
E. coli K-12 *BW25113 ykfA*	Km^R, K-12 Δ *ykfA* Ω	wt	3.9 ± 0.9	Putative GTP-binding factor	Baba *et al.* (2006)
E. coli K-12 *BW25113 ybcV*	Km^R, K-12 Δ *ybcV* Ω	wt	2.8 ± 0.9	Putative envelope protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 adhP*	Km^R, K-12 Δ *adhP* Ω	wt	3.1 ± 1.2	Alcohol dehydrogenase	Baba *et al.* (2006)
E. coli K-12 *BW25113 ydeM*	Km^R, K-12 Δ *ydeM* Ω	wt	3.8 ± 1.0	Putative enzyme	Baba *et al.* (2006)
E. coli K-12 *BW25113 yedM*	Km^R, K-12 Δ *yedM* Ω	wt	6.3 ± 1.0	Hypothetical protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 ais*	Km^R, K-12 Δ *ais* Ω	wt	2.3 ± 0.6	Hypothetical protein induced by aluminum	Baba *et al.* (2006)
E. coli K-12 *BW25113 yfiD*	Km^R, K-12 Δ *yfiD* Ω	wt	2.6 ± 0.8	Hypothetical protein stress-induced	Baba *et al.* (2006)
E. coli K-12 *BW25113 yfiI*	Km^R, K-12 Δ *yfiI* Ω	wt	4.6 ± 1.0	Hypothetical protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 ypjF*	Km^R, K-12 Δ *ypjF* Ω	1.8 ± 0.4	6.8 ± 1.2	Toxin of the $Y_{pj}F$-$Y_{pj}Z$ toxin–antitoxin pair	Baba *et al.* (2006)
E. coli K-12 *BW25113 yjiD*	Km^R, K-12 Δ *yjiD* Ω	2.0 ± 0.5	wt	Hypothetical protein	Baba *et al.* (2006)
E. coli K-12 *BW25113 ygaR*	Km^R, K-12 Δ *ygaR* Ω	wt	3.4 ± 1.0	Hypothetical protein	Baba *et al.* (2006)

Km^R and Em^R indicate kanamycin and erythromycin resistance respectively; wt indicates wild-type (no significant change). Standard deviations for the changes in biofilm formation relative wild-type are shown. Isogenic mutations as*tB*, *cyoB*, *elbA*, *nuoJ*, *paaF*, *prpC*, *sdhB*, *trpA*, *trpB*, *yceO*, *ycgZ*, *ydeO*, *ydeR*, *ydjO*, *yecF*, *yhaE* and *ylbE* were also studied but did not show differences in biofilm formation in these two media.

Quorum sensing genes

As AI-2 (González Barrios *et al*., 2006a) increases biofilm formation, we examined the transcriptional profile of AI-2-related genes. Sixty-six of the genes previously shown to be regulated by AI-2 (26 known to be repressed, 35 known to be induced and five with unknown effects) (DeLisa *et al*., 2001a; Sperandio *et al*., 2001; Ren *et al*., 2004d) were differentially expressed in our temporal data. Interestingly, 57% of the genes repressed by AI-2 were also repressed in B24 relative to B4, B7 and B15. Similarly, 82% of the AI-2-induced genes were also induced in B24 relative to B4, B7 and B15 (Table S1, *Supplementary material*). These results suggest that AI-2, which has the highest concentration during the exponential stage of growth and degrades thereafter in extracellular fluids (Xavier and Bassler, 2005), is present at significant concentrations in developed biofilm cells (24 h) where it controls gene expression.

To test this hypothesis, we measured intracellular AI-2 concentrations in biofilm and suspension cells via plasmid pLW11 (Wang *et al*., 2005) with a *lacZ* fusion containing the *lsrACDBFG* promoter region; transcription of this operon increases because of internal AI-2 (Taga *et al*., 2003). Our results show intracellular AI-2 concentrations increase up to 6.4 $\pm$ 0.44-fold in biofilms and up to 6.8 $\pm$ 0.51-fold in suspension cells after 7, 15 and 24 h of culturing relative to the 4 h time point; these results confirm the microarray predictions that showed increased transcription of AI-2-related genes at 24 h. Additionally, *ydgG* (*tqsA*), which was previously shown to be induced sixfold in 7 h biofilms (Ren *et al*., 2004a) and which was subsequently found to encode the exporter of AI-2 (Herzberg *et al*., 2006), was induced 26-fold in B24 relative to B4. *lsrA,* coding for the protein involved in AI-2 uptake (Xavier and Bassler, 2005), was also induced threefold in B24 relative to B15, supporting the hypothesis that AI-2 has a large impact on mature biofilms while AI-2 concentrations in suspension cells peak in the exponential stage (approximately 5 h after inoculation), and decrease thereafter (Xavier and Bassler, 2005).

Motility and fimbriae genes

Four operons associated with flagella and motility were differentially expressed including *flgBCEFH*, *fliJ*, *fliLMQR* and *cheW-motB*. The flagellar biosynthesis genes *fliM* and *fliQ* were induced in B7 relative to S7, and the temporal expression profile showed the partial operon *fliLMQR* is induced up to 12-fold in B7, B15 and B24 relative to B4 (Table S1, *Supplementary material*). The expression profile of another flagellar biosynthesis operon *flgBCEFH* was induced 10-fold in B15 and B24 relative to B7, and *flgB* was induced in B4 and B24 relative to suspension cells. Other flagella and motility genes *fliJ* and *cheW-motB* had similar temporal expression profiles. In total, 20 motility-related genes were induced at different stages in biofilm formation. These results suggest that in *E. coli* flagella genes are important throughout all stages of biofilm development from early attachment as well as in developed biofilms; these are the first results showing that flagellar genes are induced at all stages of biofilm formation. This may indicate the involvement of these genes in the detachment and movement of biofilm during later stages of development. This is consistent with the previously proposed hypothesis that motility is important for initial attachment as well as movement along the surface in *E. coli*, which was based on microscopic analyses of mutant alleles *fli*, *flh* and *mot* (Pratt and Kolter, 1998). Our results differ from other reports in *P. aeruginosa* suggesting that flagella are only important during initial stages of attachment and are turned off in mature biofilms (Sauer and Camper, 2001; Sauer *et al*., 2002).

Fimbriae genes are critical in adhesion as well as in development of cell clusters in *E. coli* biofilms (Pratt and Kolter, 1998; Prigent-Combaret *et al*., 2001). Here, fimbriae-encoding *fimA* was induced up to ninefold in B4, B7 and B15 relative to suspension cells here and in 5 day biofilms relative to exponential cultures in a previous report (Beloin *et al*., 2004). The temporal transcription profile of *fimA* based on our microarray data shows highest transcription in B4, as the genes were repressed ninefold to 16-fold in B15, and B24 relative to B4. Alternatively, all other fimbriae-related genes including *ydeQRS*, *fimZ*, *yhcA*, *ppdD* and *sfmF* were induced up to 30-fold at all stages in biofilm development, but most significantly in B24 relative to B4, B7 and B15. In total, 13 fimbriae-related genes were differentially expressed in the temporal profile. Similarly to motility, this is the first result that shows fimbriae-related genes are induced at all stages in *E. coli* biofilm formation relative to suspension cells (most induced in B4 and B24). The induction of fimbriae genes in biofilms is consistent with other reports that show fimbriae genes are induced in *E. coli* after 7 h (Ren *et al*., 2004a), 32 h (Schembri *et al*., 2003) and 5 day biofilms (Beloin *et al*., 2004).

Indole and sulfur genes

Tryptophan biosynthesis and degradation genes have been shown to be both induced and repressed in biofilms (Schembri *et al*., 2003; Ren *et al*., 2004a) and indole has been shown to both reduce (Domka *et al*., 2006) and increase (Di Martino *et al*., 2003) biofilm formation in *E. coli*. Here, 24 genes related to sulfur and tryptophan metabolism were differentially expressed in our data. Genes involved in tryptophan synthesis (*trpLAB*) were

induced threefold to 11-fold in B4 relative to S4, which corroborates our previous results that showed *trpE* (encodes anthranilate synthase) was induced fourfold in a 7 h biofilm relative to suspension cells (Ren *et al*., 2004a). The temporal profile in biofilms shows transcription of these genes in the biofilm was highest in B4 because the Trp synthesis genes *trpLBA* were repressed as a function of time up to 13-fold in B7 and B15 relative to B4 (Table S1, *Supplementary material*). This means that tryptophan biosynthesis may be important in early biofilm formation steps, but is repressed in older biofilms after 7, 15 and 24 h of culturing. The genes (*tnaLA*) for converting tryptophan to indole and cysteine to hydrogen sulfide were repressed 7.5- to 15-fold in B15 and B24 relative to B7, but were induced twofold to 3.5-fold in B7 and B15 relative to suspension cells. Previously, *tnaA* was repressed 12-fold in biofilms grown in 6 day continuous steady-state reactors (Ren *et al*., 2004a), but *tnaL* was induced in 32 h biofilms (Schembri *et al*., 2003).

These temporal expression profiles show diminished conversion of tryptophan to indole and cysteine to sulfide as the biofilm matures as well as decreased synthesis of tryptophan, suggesting decreased indole concentrations in developing (15 h) and mature (24 h) biofilms. To test this hypothesis, we measured the temporal intracellular concentration of indole in both biofilm and suspension cells after 4, 7, 15 and 24 h. Our results support the predictions by the microarray data, showing intracellular indole is reduced up to 60% in biofilm and 90% in suspension cells after 7, 15 and 24 h of culturing relative to the 4 h time point. The repression of indole concentrations in mature biofilms and suspension cells presumably allows for the development of the biofilm, corroborating our previous result that indole inhibits biofilm formation in the indole-deficient *E. coli* K-12 *yceP* mutant (Domka *et al*., 2006). Additional evidence that indole concentrations are repressed in mature biofilm is that the indole efflux genes *acrEF* are significantly induced (up to 24-fold) in B24 relative to B4, B7 and B15 whereas the gene encoding indole uptake (*mtr*) is repressed (up to fourfold) in B7 and B24 relative to B4.

Genes of an operon coding for a sulfate and thiosulfate ABC transporter (*cysPUWA*) were induced threefold to 11-fold in B7 and B15 relative to B4, but were repressed twofold to threefold in B24 relative to B15. Both sulfate and thiosulfate are reactants in the sulfate assimilation pathway leading to hydrogen sulfide. Similarly, operons involved in sulfate assimilation *cysDNC* and *cysJIH* were also induced up to 27-fold in B7, B15 and B24 biofilms relative to B4. Deletion of *cysP*, *cysU*, *cysW cysH*, *cysI* and *cysJ* increased biofilm formation up to sixfold in these mutants when grown in LB glu, but the mutations had no effect when grown in LB (Table 3); hence, sulfur-related genes are important for biofilm formation in LB glu. Previously, deletion of the positive transcriptional regulator *cysB* for these operons increased biofilm formation 10-fold in microtitre plates (Ren *et al*., 2005) and deletion of *cysE*, which codes for serine acetyltransferase, enhanced biofilm formation (Sturgill *et al*., 2004).

To test the impact on biofilm formation due to the addition of sodium sulfate and sodium sulfide (0–750 μM) to LB and LB glu media (96-well assay), biofilm formation of the *E. coli* wild-type strain and mutants *acrE*, *acrF*, *cysP*, *cysU*, *cysW*, *cysA*, *cysN*, *cysJ*, *cysI*, *cysH*, *tnaA*, *trpA*, *trpE* and the transcriptional regulator of phosphate uptake *phoU* was measured. Unfortunately no conclusions could be drawn based on this study because both chemicals inhibited cell growth (data not shown).

Stress genes

Bacteria within a biofilm matrix often express genes associated with a stress response (Schembri *et al*., 2003; Beloin *et al*., 2004; Ren *et al*., 2004a). This was also observed in our temporal data where several cold-shock-related proteins were induced twofold to 23-fold in B4 and B7 relative suspension including the transcriptional regulators *cspA*, *cspB*, *cspF*, *cspG* and *cspI*. This is the first report of these genes being induced in biofilms. The temporal analysis shows these proteins are important in biofilms at 4 and 7 h, but their expression is repressed up to 30-fold in B15 and B24 relative to B4 and B7 (Table S1, *Supplementary material*); hence, these genes may regulate expression of genes in 4 and 7 h biofilms, but are not used in mature biofilms. An oxidative stress response gene *soxS*, previously observed to be induced 63-fold in 7 h biofilms (Ren *et al*., 2004a), was induced 15-fold in B4, and fourfold in B7 relative to suspension cells. The temporal profile of this gene showed highest expression at 4 h in biofilm, as it was repressed 13-fold in B7 and B15 relative to B4. The transcription of genes coding for the heat-shock promoter transcriptional regulator *htgA* was induced twofold in B15 relative S15, which is also where its transcription appeared to be highest based on temporal analysis as the gene was induced fourfold to ninefold in B15 and B24 relative to B4 and B7. In total, 42 genes previously reported as regulated by stress (DeLisa *et al*., 2001b) or the stress-related stationary-phase sigma factor (RpoS) (Patten *et al*., 2004) were differentially expressed in our temporal biofilm analysis including *gadAB* (glutamate decarboxylase) and *gadC* (L-glutamate transporter) and *hdeABD* (acid resistance). Many of the stress-related genes such as *sodAB* (removal of superoxide radicals) were expressed at very similar levels in biofilm and suspension cells; hence, both biofilm and suspension cells are undergoing stress.

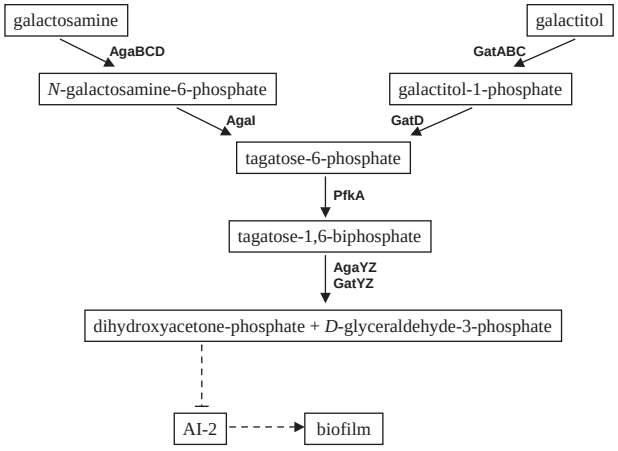

Fig. 2. Galactosamine and galactitol pathways. Solid lines are enzymatic reactions, the dashed line with an arrow shows positive regulation, and the dashed line terminating with a bar shows negative regulation (Brinkkötter *et al*., 2000; Xavier and Bassler, 2005).

Transport genes

Eighty transport-related genes were differentially expressed. Operons associated with the galactitol phosphotransferase system (PTS) and DHAP synthesis (*gatZ-ABCDR*) were some of the most regulated genes as they were repressed up to 42-fold in B15/B24 relative to B4, and were repressed significantly, 10- to 90-fold, in S15 relative to S4 (Tables S1 and S2, *Supplementary material*). Our data showed *gatD* was induced in biofilms relative to suspension cells threefold in B15, which is consistent with other sources that observed *gatD* is induced in 32 h biofilms (Schembri *et al*., 2003), but repressed in 6 day biofilms (Ren *et al*., 2004a) and *gatA* is induced in biofilms after 32 h (Schembri *et al*., 2003). This indicates that genes associated with galactitol transport are important during early biofilm development stages, and as DHAP represses AI-2 uptake (Xavier and Bassler, 2005), this indicates AI-2 uptake is enhanced in mature biofilms, which corroborates the induction and repression of AI-2-controlled genes seen at this point in biofilm development. The connection between the *gat* operon and AI-2 has been seen previously as *gatCR* are induced by AI-2 (Ren *et al*., 2004d).

Another operon associated both with PTS import of galactosamine and the synthesis of DHAP (*agaBC*, Fig. 2) was induced up to 16-fold in B24 relative to B4, B7 and B15, but showed no difference in transcription in suspension cells at these same time points. As both the *gat* and *aga* pathways lead to DHAP, the opposite regulation of these pathways was surprising but these results indicate the importance of this pathway. Previously, gene *agaC* was induced in 6 day continuous biofilms (D. Ren *et al.*, unpubl. results) and *agaY* has been reported to be repressed by AI-2 (Ren *et al*., 2004d)

To study the galactitol/galactosamine/DHAP effect, we measured the biofilm formation of isogenic mutants. The deletion of *gatB*, *gatC*, *gatZ*, *agaB*, *agaC* and *agaY* (all of which should decrease DHAP, Fig. 2) increased biofilm formation up to 6.3-fold in LB glu (Table 3). Also, the gotC and agaY mutations increased intracellular AI-2 concentrations by 20 to 60% in LB. Hence, in LB glu, decreasing DHAP probably led to more AI-2 uptake, which led to increased biofilm formation as AI-2 stimulates biofilms (González Barrios *et al*., 2006a).

Unexpectedly, the *gatR* mutation (repressor of *gat* operon) also increased biofilm formation 6.5-fold in microtitre plates relative to the wild-type strain in LB glu (Table 3). To investigate this further on a genome-wide scale, we determined the differential gene expression in biofilm cells for a *gatR* strain versus the wild-type strain in LB glu. The *gatR* mutation repressed significantly *agaZ* (–6.5-fold), *agaC* (–1.9-fold), *agaW* (–3.5-fold) and *gatD* (–3.2-fold); hence, less DHAP should be generated and biofilm formation increases. Interestingly, the cold-shock regulators *cspABFG* were all downregulated upon *gatR* deletion (roughly twofold) as were 29 phage genes.

Galactitol was added and biofilm was measured with the wild-type strain and *agaY* and *gatR* mutants in LB and LB glu (Fig. 3). Adding galactitol to the wild-type strain in LB glu decreased biofilm formation (Fig. 3B, note the cell density was also decreased up to 70%) as DHAP concentrations probably were increased and AI-2 uptake decreased. In addition, adding extracellular galactitol to the *gatR* and *agaY* strains had no effect on biofilm formation because galactitol was probably not converted to DHAP due to these pathway deletions so AI-2 concentrations and biofilm were not reduced in LB glu (Fig. 3B). In LB, galactitol addition increased biofilm formation for the wild-type and mutant strains without affecting cell density (Fig. 3A) indicating the complexity of this pathway.

Two operons involved with peptide and dipeptide transport *dppABCDF* and *oppABCDF* (http://biocyc.org/ECOLI/) were significantly repressed twofold to eightfold and fourfold to 16-fold, respectively, in B15 and B24 relative to B4 and B7. The suspension cell temporal profile shows these genes are repressed more significantly in planktonic cultures over time; hence, our temporal profile data are consistent with previous reports showing gene *oppA* was induced in biofilms after 32 h relative to both exponential and stationary-phase cells (Schembri *et al*., 2003) and *oppA* and *oppC* were induced by AI-2 (Ren *et al*., 2004d).

Genes encoding proteins involved in phosphate transport and metabolism, *phoU* and *phnCJKM*, are significantly induced in mature biofilms. *phoU* is induced 17- and 26-fold at 15 h and 24 h, respectively, relative to young (4 h) biofilms. *phnC* is induced up to 10-fold in 24 h

biofilms relative to other time points, and *phnJKM* has a similar expression profile with increased expression up to 23-fold in 15 h biofilms and 20-fold in 24 h biofilms relative to 4 h biofilm sample.

To investigate this regulation more fully, we measured differential gene expression in biofilm cells in LB glu for a *phoU* mutant and a *phnD* mutant versus wild-type. PhoU is the negative regulator of phosphate uptake (Surin *et al*., 1986). The *phnD* mutant was chosen because it encodes PhnD that along with PhnC and PhnE controls the uptake of phosphonate (Metcalf and Wanner, 1991). As expected, the *phoU* mutation induced the phosphorous uptake genes *phoB* (4.9-fold), *pstABCS* (4.3- to 12.1-fold) and *phnCDEL* (1.9- to 2.3-fold). The cold-shock regulators *cspADEG* were upregulated upon *phoU* deletion (roughly twofold), while 14 cell motility and curli genes, *fliILRP*, *fimAF* and *csgE*, were repressed roughly twofold. Interestingly, *gatYZ* were induced (roughly 2.0-fold), while *agaW* (–2.3-fold) and *gatR* (–1.6-fold) were repressed, which indicates a possible relationship between galactitiol, AI-2 and phosphate.

Unexpectedly, the *phnD* mutation did not significantly change gene expression. The *thrABC* leader peptide gene (*thrL*) was induced 4.3-fold upon *phnD* mutation, and indole-related genes, *yliH* (1.5-fold), *trpL* (1.5-fold) and *tnaL* (1.5-fold) were slightly induced, while two flagellar genes, *fliI* (–2.0-fold) and *fliP* (–1.6-fold) were repressed.

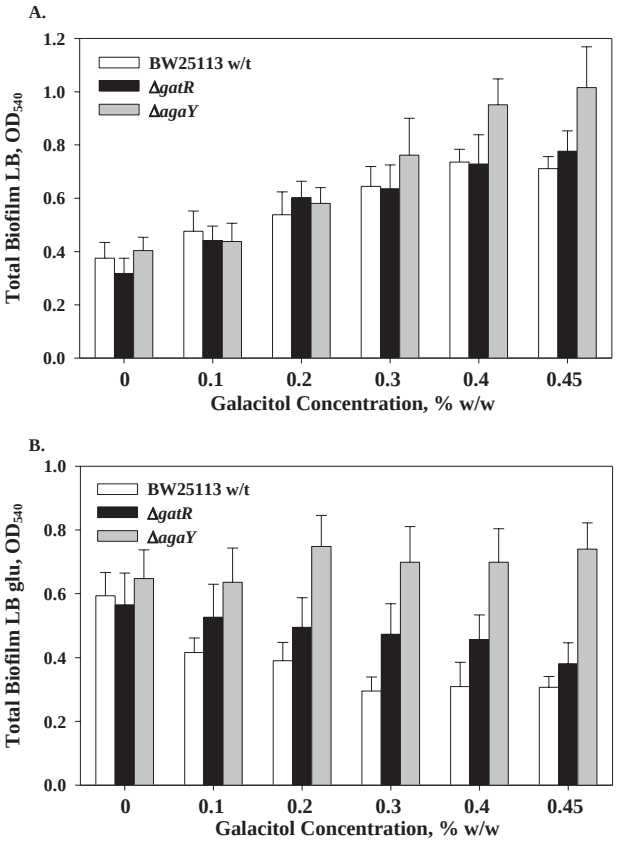

Fig. 3. Effects of galactitol on biofilm formation of *E. coli* wild-type strain and mutants *agaY* and *gatR* in LB (A) and LB glu (B). Biomass measured after 24 h. Experiments were performed in duplicate.

Extracellular matrix genes

Colanic acid synthesis operons *wcaDEC* and *wcaB* were induced up to 26-fold in B15 and B24 relative to B4. The temporal profile for these genes, unlike the other genes discussed in this paper, was very similar in suspension and biofilm cells suggesting that increased transcription of these genes is not biofilm-specific. In previous reports the formation of colanic acid was important to three-dimensional architecture of a biofilm, but not to biofilm formation (Danese *et al*., 2000; Prigent-Combaret *et al*., 2000); consistent with these results, the deletion of *wcaD* only decreased biofilm formation 1.9-fold based on our microtitre assay (Table 3). Transcription of gene *galU*, which plays a role in colanic acid biosynthesis, was repressed threefold in B7 relative to suspension cells and sixfold in B7 relative to B4. In developed biofilms, this gene was induced threefold in 24 h biofilms relative to suspension and threefold in B15 and B24 relative to B7, indicating that its expression is induced in later stages of biofilm development after 15 and 24 h of biofilm growth. Therefore, colanic acid is important in developed biofilms, but not at the 4 and 7 h time point. In a previous study we found deletion of *ydgG* (*tqsA*) increased biofilm thickness 7000-fold relative to the *E. coli* wild-type strain and was accompanied by induced expression of the colanic acid operon *wcaABCDEFG* twofold to sevenfold after 24 h of culturing (Herzberg *et al*., 2006). This is consistent with the increased transcription of this operon only after mature biofilm is established as observed in our data.

Respiratory genes

Deletion in genes coding for respiratory-related enzymes such as *hyaACD* (hydrogenase-1 subunits and processing), *hyfCG* (hydrogenase-4 subunits), *appC* (cytochrome oxidase) and *narG* (nitrate reductase) all increased biofilm formation by up to sevenfold in LB glu (Table 3). *hyaABCDE* and *narG* were induced in B7 relative to B4 biofilms, but were repressed at all other time points. Other respiration-related operons were induced throughout biofilm formation such as *hycFB*, *hyfB* and *yggP*, while *hycBC* was repressed at all time points (Table S1, *Supplementary material*). Genes *hyaABD* and *appB* (cytochrome terminal oxidase) were induced in B4 relative to suspension cells in this study (Table S1, *Supplementary material*). We found previously that the respiratory enzymes encoded by *alsD*, *lctEP*, *narGIJK*, *nasDF*, *feuA*

and *ydjL* were repressed up to 100-fold in *B. subtilis* wild-type and *spoIIGB* mutant biofilms (Ren *et al*., 2004b), and several studies found respiratory enzymes are induced in biofilms of *E. coli* (Schembri *et al*., 2003), *P. aeruginosa* (Whiteley *et al*., 2001) and *B. subtilis* (Stanley *et al*., 2003). These results indicate their putative importance to biofilm formation specifically during early stages of attachment as shown by this study.

Cyclic di-guanosine monophosphate genes

The role of aminoglycoside antibiotics, in particular tobramycin, in increasing biofilm production of *P. aeruginosa* and *E. coli* suggests the putative role of the aminoglycoside response regulator gene *arr* in regulating biofilm formation. Gene *arr* encodes cyclic di-guanosine monophosphate (c-di-GMP) (Hoffman *et al*., 2005). c-di-GMP mediates extracellular polysaccharide formation, and proteins that catalyse the synthesis of c-di-GMP have a conserved GGDEF amino acid sequence and enzymes that degrade c-di-GMP, called phosphodiesterases, have a conserved EAL amino acid motif sequence (O'Toole and Stewart, 2005). The *P. aeruginosa* genome has approximately 38 proteins with one or both of these motifs (Hoffman *et al*., 2005), and the *E. coli* K-12 genome (http://www.ncbi.nlm.nih.gov) has 10 proteins with the GGDEF motif and seven proteins with the EAL amino acid motif. Six of the genes with the GGDEF motif, *yaiC* (*b0385*, putative membrane protein), *yliF* (*b0834*, hypothetical protein), *yciR* (*b1285*, RNase II modulator), *yddV* (*b1490*, hypothetical protein), *yeaJ* (*b1786*, putative membrane protein) and *yjiU* (*b4486*, hypothetical protein) were induced twofold to fourfold ($P < 0.05$) in B24 relative to B4 and B7 based on our microarray data (Table S1, *Supplementary material*), indicating the putative role of these genes in biofilm formation (although this should be confirmed with more experiments).

Uncharacterized genes

Two hundred and thirty-three uncharacterized genes were differentially expressed in our microarray data. A subset of these genes (22) was tested for their impact on biofilm formation in microtitre biofilm assay, including *htgA* (encodes a 196 aa, positive regulator for heat-shock promoters), *ykfA* (288 aa, putative GTP-binding factor), *ybcV* (150 aa, putative envelope protein), *ydeM* (390 aa, putative enzyme), *adhP* (1041 aa, alcohol dehydrogenase), *hdeB* (339 aa, protein involved in acid resistance), *hdeD* (573 aa, protein involved in acid resistance), *yafU* (112 aa), *ykfI* (342 aa), *yedM* (116 aa), *ais* (200 aa), *yfiD* (127 aa), *yfjI* (469 aa), *ypjF* (109 aa), *yjiD* (402 aa) and *ygaR* (228 aa). Mutations in all of these genes increased biofilm formation in LB glu by twofold to 6.8-fold, and *yjiD* increased biofilm formation in LB by twofold relative to the wild-type strain (Table 3). Confirming the predictive power of our microarray results in determining altered biofilm phenotypes, 12 of the 22 uncharacterized genes showed an increase in biofilm formation (Table 3), four of these mutants had a greater than 4.4-fold increase in biofilm formation, and three of them are putative regulators (*ypjF*, *yedM* and *yafU*) while *yfjI* is a putative enzyme. Of the 93 mutants studied (based on differences in their temporal gene expression), 74 increased biofilm formation in either LB or LB glu and only two reduced biofilm formation relative to the *E. coli* wild-type strain (few strains were seen with less biofilm because *E. coli* K-12 BW25113 forms a relatively poor biofilm in 96-well plates under these conditions). Clearly the microarray data identified biofilm-relevant genes.

In summary, it is shown in this paper that *E. coli* biofilm development on glass wool occurs in stages with changes in both gene expression patterns (Table S1, *Supplementary material*) as well as changes in architecture (thickness and surface coverage) (Fig. 1, Table 1). The temporal expression of genes known to be associated with biofilm formation such as those related to quorum sensing (Davies *et al*., 1998; González Barrios *et al*., 2006a), flagella, motility, fimbriae and colanic acid biosynthesis genes was elucidated; for example, both fimbriae and motility genes were found to be upregulated at all time points. Furthermore, regulatory elements have a temporal expression pattern; for example, the quorum-sensing signal AI-2 induces and represses gene expression the most in mature biofilms. In addition, indole synthesis is repressed in ageing biofilms to facilitate biofilm development and maturation, and galactitol transport via DHAP regulates biofilm formation. For the first time, we identified genes encoding cold-shock protein transcriptional regulators (*cspA*, *cspB*, *cspF*, *cspG* and *cspI*) are induced consistently at 4 and 7 h in biofilms but are repressed up to 30-fold in 15 and 24 h biofilms. Another major group of genes differentially expressed in our data were ABC transport genes. We also identified 22 novel genes involved in biofilm formation. Genes involved in the synthesis of extracellular matrix such as colonic acid biosynthesis, *wcaBCDE*, were significantly induced in 24 h biofilms, suggesting that the extracellular matrix genes are not induced until biofilms mature.

Experimental procedures

Bacterial strains and growth media

The *E. coli* strains and plasmids are listed in Table 3. Wild-type *E. coli* K-12 BW25113 (Yale University CGSC Stock Center) and isogenic deletion mutants were used [Table 3, Genome Analysis Project in Japan (Baba *et al*., 2006)]. The

kanamycin (Kan) gene insertion and the deletions were corroborated with five primers each in polymerase chain reactions (three primers homologous to sequences internal to the Kan gene and two primers homologous to the sequence upstream and downstream of each deletion) (Baba *et al*., 2006).

Luria–Bertani (LB) medium (Sambrook *et al*., 1989) and LB supplemented with 0.2% glucose (LB glu) were used to study biofilm formation in the 96-well biofilm assay, and LB was used in the temporal biofilm flow chamber experiment. For plasmid and strain selection, 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin and 300 μg ml^{-1} erythromycin were used.

Flow cell biofilm experiments and image analysis

LB medium supplemented with 300 μg ml^{-1} erythromycin (Er) to maintain the green fluorescent protein (GFP) plasmid pCM18 (Hansen *et al*., 2001) was used to form biofilms at 37°C in a continuous flow cell (BST model FC81, Biosurface Technologies, Bozeman, MT) with *E. coli* K-12 BW25113 (Table 3). The flow cell contains a standard glass microscope slide on one side and a plastic cover slip on the other side, and the flow channel has dimensions of 47.5 mm × 12.7 mm with a 1.6 mm gap between the glass plates. The constitutive GFP plasmid pCM18 allowed visualization of the biofilm with a TCS SP2 scanning confocal laser microscope with a 40× N Plain L dry objective with correction collar and numerical aperture of 0.55 (Leica Microsystems, Heidelberg, Germany). Overnight cultures were diluted to an OD_{600} of 0.05 and used to inoculate the flow chamber for 2 h at 10 ml h^{-1} to establish the biofilm. Flow cells were inoculated with 1.82×10^7 cells for the 4 h flow cell, 1.29×10^7 cells for the 7 h flow cell, 1.02×10^7 cells for the 15 h flow cell and 1.4×10^8 cells for the 24 h flow cell. Fresh media were introduced at the same flow rate, and circulated for 4, 7, 15 and 24 h respectively. Colour confocal flow cell images (25–40 planar images for each position, nine positions for each time point) were analysed using COMSTAT image-processing software (Heydorn *et al*., 2000) as described previously (Ren *et al*., 2005), and simulated three-dimensional images were obtained using IMARIS (Bitplane, Zurich, Switzerland).

Microarray analysis

To isolate RNA from the biofilm and suspension cells, four independent *E. coli* BW25113 LB cultures (250 ml) with 10 g of untreated glass wool (Corning Glass Works, Corning, NY) were inoculated from LB overnight cultures. The cultures were incubated for 4, 7, 15 and 24 h at 37°C and 250 r.p.m. during which time the cells formed a biofilm on the surface of the glass wool. The RNA of the biofilms was isolated from the glass wool and from the suspension cell samples as described previously (Ren *et al*., 2004a). To study the highly regulated transport genes, three additional sets of arrays were conducted for glass wool biofilm cells versus wild-type biofilm cells using *gatR*, *phoU* and *phnD* isogenic mutants (24 h, LB glu).

The Affymetrix *E. coli* GeneChip antisense genome array (Affymetrix, P/N 900381), which contains probe sets for all 4290 open reading frames, rRNA, tRNA and 1350 intergenic regions, was used as described previously (González Barrios *et al*., 2006a); hybridizations were for 16 h. Using the GeneChip® Operating Software (Affymetrix), individual strain reports for both the biofilm and suspension cell cDNA samples were obtained. For the temporal work, comparison reports of differential gene expression were also generated between all of the time points (4, 7, 15 and 24 h) comparing biofilm with biofilm, biofilm with suspension, and suspension with suspension. Total cell intensity was scaled automatically in the software to an average value of 500. To ensure the reliability of the induced and repressed gene list, genes were identified as differentially expressed if the *P*-value was less than or equal to 0.05 and if the expression ratio was greater than 2.5-fold based on average of standard deviations for the expression ratios, which were between 2 and 4. Because of the large number of differentially expressed genes, a cut-off of fivefold was chosen for comparisons of biofilm samples relative to other biofilm samples for the temporal arrays, which resulted in 573 differentially expressed genes (Table S1, *Supplementary material*). The fold-changes for suspension samples relative to other suspension samples (Table S2, *Supplementary material*) were then analysed for this subset of genes. Data quality was assessed based on the manufacturer's guidelines (*Affymetrix GeneChip® Expression Analysis: Data Analysis Fundamentals*, Affymetrix, 2004) such as the visual inspection of B2 oligo positive hybridization controls on each scanned chip, comparable average background noise values between all experiments in the acceptable range from 20 to 100, correct ratios and comparable intensities of poly-A RNA controls (*B. subtilis* genes *lys*, *phe*, *thr* and *dap*) used to monitor the labelling process, and comparable scaling factors (less than threefold difference) between all experiments (2.4–6.8). In addition, the expected signals based on the *E. coli* K-12 BW25113 genotype (Table 3) were obtained (e.g. low signal for all microarrays for the deleted genes *araA* and *rhaA*). The expression data for biofilm and suspension cells samples after 4, 7, 15 and 24 h have been deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession Number GSE3905 (Edgar *et al*., 2002; Barrett *et al*., 2005).

Indole assay

To determine intracellular indole in biofilm and suspension wild-type cells, four flasks with 250 ml of LB medium with 10 g of glass wool (Corning Glass Works, Corning, NY) were inoculated from overnight LB cultures. Biofilm and suspension cells were isolated after 4, 7, 15 and 24 h of incubation at 37°C and 250 r.p.m. Intracellular indole was measured as described preciously (Domka *et al*., 2006). The experiment was repeated with a second independent culture for each time point.

Intracellular AI-2 assay

To measure intracellular AI-2 concentrations in biofilm and suspension wild-type cells, four flasks containing 250 ml of LB with ampicillin (100 μg ml^{-1}) and 10 g of untreated glass wool were inoculated from overnight LB, 100 μg ml^{-1} ampicillin cultures with the *lsrACDFG::lacZ* fusion plasmid pLW11

(Wang *et al*., 2005). Biofilm and suspension cells were isolated as for the microarray and indole assays after 4, 7, 15 and 24 h of incubation at 37°C and 250 r.p.m. Both biofilm and suspension cells were re-suspended in 0.75 ml of cold TEP buffer (100 mM Tris pH 7.4, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride) and assayed as reported previously (Wood and Peretti, 1991). The β-galactosidase activities were calculated based on a protein concentration of 0.24 mg protein per millilitre per OD_{600} (Tao *et al*., 2004). The experiment was repeated with a second independent culture for each time point.

96-well biofilm assay

Biofilm formation was quantified in 96-well polystyrene plates as reported previously (Ren *et al*., 2005). *Escherichia coli* strains were grown in LB and LB glu at 37°C for 24 h without shaking, and cultures were diluted to a turbidity of 0.05 at 600 nm before inoculating the plates. Before measuring the biofilm mass in each well, the turbidity was taken to quantify the growth of *E. coli* K-12. Total biofilm mass was measured at both the air–liquid interface and the liquid–solid interface, and five replicate wells were averaged to obtain each data point. Two independent cultures were used.

Additionally, the effect of five chemicals on biofilm formation was studied: galactitol at concentrations 0–0.045% w/w (ACROS Organics, Morris Plains, NJ), *N*-acetylgalactosamine at 300 μM (ACROS Organics, Morris Plains, NJ), sodium sulfate at concentrations 0–750 μM (Fisher Chemical, Fairlawn, NJ) and sodium sulfite at concentrations 0–750 μM (J.T. Baker, Phillipsburg, NJ). Chemicals were added to LB and LB glu media inoculated from overnight cultures (initial turbidity 0.05).

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Gene expression patterns in biofilms over 24 h in LB. Col. 1–6: greater than fivefold changes ($P = 0.05$) between biofilm samples relative to other biofilm samples (e.g. B7 versus B4). Col. 7–8: time points (4, 7, 15, or 24 h) when genes are induced or repressed in biofilms relative to suspension cells, and references (in parenthesis) to previous single time point studies, which showed these genes are induced or repressed in biofilms relative to suspension cells. Col. 9: references indicating genes linked to AI-2, stress, or the stationary-phase signal. Genes with $P > 0.05$ are omitted in the table. These expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3905).

Table S2. Fold-changes ($P = 0.05$) in gene expression between suspension cells relative to other suspension cells over 24 h in LB (columns 1–5). Only the subset of genes with a greater than fivefold change in biofilms relative to other biofilm time points (Table S1, *Supplementary material*) are analysed here. Genes with $P > 0.05$ are omitted in the table. These expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3905).

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