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GENOMICS AND PROTEOMICS

5-Fluorouracil reduces biofilm formation in *Escherichia coli* K-12 through global regulator AriR as an antivirulence compound

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Abstract The uracil analog, 5-fluorouracil (5-FU), reduces virulence and biofilm formation for *Pseudomonas aeruginosa* PA14 without affecting its growth. As 5-FU is an approved anticancer drug, its antivirulence attributes in *P. aeruginosa* prompted us to examine the effect of this compound on three different *Escherichia coli* K-12 strains and its effect on virulence genes in *E. coli* O157:H7 (EHEC); the mechanism by which it functions was also examined. 5-FU decreased biofilm formation in a dose-dependent manner in *E. coli* K-12 and repressed the expression of virulence genes in EHEC. Five other uracil analogs were also tested for their effects on biofilm formation, and none of these compounds affected the biofilm formation in *E. coli* K-12. Whole-transcriptome analysis revealed that 5-FU induced the expression of 157 genes and repressed the expression of 19 genes. Biofilm formation with the addition of 5-FU was checked in 21 isogenic knockout mutants whose gene expression was induced in the microarray data; we found that 5-FU does not decrease biofilm formation of the cells that lack AriR, a global DNA regulator that controls acid resistance in *E. coli*. Hence, 5-FU represses biofilm formation of *E. coli* K-12 through AriR and is a novel antivirulence compound for this strain.

Keywords Biofilm · *Escherichia coli* · Antivirulence · 5-Fluorouracil

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Introduction

Bacteria prefer to live in the biofilm state (Sauer et al. 2004) in which they acquire resistance to surfactants, phagocytes, and antibiotics (Costerton et al. 1995). The slow penetration of antibiotics into the biofilms, altered chemical microenvironment within the biofilm, and the different phenotypic states of bacteria in the biofilm render biofilms antibiotic resistant (Stewart and Costerton 2001). Biofilms cause serious problems in medicine and in industry (Donlan and Costerton 2002). In the US, the annual cost of biofilms in cardiovascular and orthopedic implants is more than $3 billion (Klemm et al. 2007). Therefore, biofilm prevention and control are gaining importance (Labbate et al. 2004).

Thus far, only a few compounds have been discovered to combat *Escherichia coli* biofilms without affecting growth. Furanone, from the marine alga *Delisea pulchra* (Ren et al. 2001), indole (Lee et al. 2007b), and indole derivatives (5-hydroxyindole and 7-hydroxyindole; Lee et al. 2007a) reduce biofilm formation of *E. coli*. By screening 13,000 plant extracts, ursolic acid was also discovered to inhibit *E. coli* biofilm formation (Ren et al. 2005). Compounds that attenuate virulence without affecting growth are possibly better than antimicrobials since cells are less likely to develop resistance against them (Hentzer et al. 2002).

In *E. coli*, uracil-related genes, the genes involved in uridine monophosphate biosynthesis and uracil transport

(*carAB*, *pyrLBI*, *pyrC*, *pyrD*, *pyrF*, and *uraA*), are repressed by the addition of indole and by SdiA at 30°C and are induced by the addition of AI-2 at 37°C (Lee et al. 2008). Also, in *E. coli*, SdiA- and AI-2-based signaling are intertwined (Lee et al. 2008). Furthermore, by screening 5,850 transposon mutants for altered biofilm formation of *Pseudomonas aeruginosa* PA14, the same uracil-related mutations (*carA*, *carB*, *pyrB*, *pyrC*, *pyrD*, *pyrE*, and *pyrF*) abolish biofilm formation and quorum-sensing phenotypes (elastase activity, pyocyanin production, 2-heptyl-3-hydroxy-4-quinolone production, rhamnolipid production, and swarming motility), whereas the mutations for other pyrimidines and purine biosynthesis did not alter biofilm formation (Ueda et al. 2009). Hence, uracil or a uracil derivative appears to affect quorum sensing in both *E. coli* and *P. aeruginosa*.

These results prompted us to examine uracil analogs as biofilm effectors. By screening uracil analogs for biofilm formation of *P. aeruginosa*, 5-fluorouracil (5-FU) was discovered as a biofilm inhibitor of *P. aeruginosa* (Ueda et al. 2009). 5-FU is also a biofilm inhibitor of *Staphylococcus epidermis* (Hussain et al. 1992). Effective biofilm inhibitors could benefit many populations by impacting treatments for various diseases (Cegelski et al. 2008).

In this study, we screened uracil analogs to observe their effects on the biofilm formation of *E. coli* K-12 and found that 5-FU also inhibits the biofilm formation of *E. coli* K-12. We also investigated the genetic basis for the effects of 5-FU by looking at the whole transcriptome. Our biofilm and microarray results suggest that 5-FU works through AriR. AriR, previously known as YmgB (Lee et al. 2007c), is a regulator that inhibits biofilm formation and motility and protects cells from acid (Lee et al. 2007c).

Materials and methods

Bacterial strains, media, growth conditions, and chemicals The strains used in this study are listed in Table 1; they were grown at 37°C. Luria Bertani (LB) medium (Sambrook et al. 1989) and LB medium supplemented with 0.2% glucose were used for the crystal violet biofilm experiments and the glass wool biofilm DNA microarray experiments. LB and Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L) and 10 mM HEPES (Hyclone, Logan, UT, USA) were used for the quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) experiments. Kanamy-

Table 1 *E. coli* K-12 and *E. coli* O157:H7 strains used in this study

Strains	Genotype	Source
E. coli K-12	Wild type	ATCC 25404
E. coli K-12 MG1655	$F^- \lambda^-$ *ilvG rfb-50 rph-1*	(Blattner et al. 1997)
E. coli K-12 BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	(Datsenko and Wanner 2000)
E. coli K-12 BW25113 *hisA*	K-12 BW25113 Δ*hisA* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *hisB*	K-12 BW25113 Δ*hisB* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *hisC*	K-12 BW25113 Δ*hisC* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *hisD*	K-12 BW25113 Δ*hisD* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *hisG*	K-12 BW25113 Δ*hisG* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *hisH*	K-12 BW25113 Δ*hisH* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *hisL*	K-12 BW25113 Δ*hisL* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *ivy*	K-12 BW25113 Δ*ivy* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *malE*	K-12 BW25113 Δ*malE* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *mipA*	K-12 BW25113 Δ*mipA* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *ompT*	K-12 BW25113 Δ*ompT* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *tig*	K-12 BW25113 Δ*tig* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *trpB*	K-12 BW25113 Δ*trpB* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *yceD*	K-12 BW25113 Δ*yceD* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *ycgZ*	K-12 BW25113 Δ*ycgZ* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *yciE*	K-12 BW25113 Δ*yciE* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *yciF*	K-12 BW25113 Δ*yciF* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *yciG*	K-12 BW25113 Δ*yciG* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *ygdI*	K-12 BW25113 Δ*ygdI* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *ymgA*	K-12 BW25113 Δ*ymgA* Ω Km^r	(Baba et al. 2006)
E. coli K-12 BW25113 *ariR*	K-12 BW25113 Δ*ariR* Ω Km^r	(Baba et al. 2006)
E. coli O157:H7	EHEC $Stx1^+$ and $Stx2^+$	(Strockbine et al. 1986)

Km^r kanamycin resistance

cin (50 μg/mL) was used to select the *E. coli* K-12 BW25113 knockout mutants.

Biofilm formation Biofilm formation of the wild-type strain and the mutants was performed in 96-well polystyrene plates as indicated previously (Pratt and Kolter 1998) with 300 μL of crystal violet per well, and the absorbance was measured at 540 nm. In brief, overnight cultures were inoculated with an initial turbidity of 0.05 at 600 nm for 7 h without shaking. Twelve replicate wells were averaged to obtain each data point, and two independent cultures were used. Uracil analogs 5-aminouracil, 6-azauracil, 5-bromouracil, 5-fluorouracil (Fisher Scientific, Hanover Park, IL, USA), and 5-nitrouracil (MP Biomedical, Solon, OH, USA) were diluted in dimethylformamide (DMF). 5-(*Trans*-2-bromovinyl)-uracil (Sigma-Aldrich, St. Louis, MO, USA) was diluted in ethanol. Stock solutions (0, 5, 10, 25, and 50 mM) were added at 0.1 vol.% to *E. coli* K-12 American Type Culture Collection (ATCC) 25404, and DMF and ethanol were added at 0.1 vol.% as solvent negative controls.

Swimming motility Swimming motility assays were performed as explained previously (Sperandio et al. 2002) by measuring halos on agar plates. In brief, overnight cultures were regrown to a turbidity of 1 at 600 nm. The cultures were inoculated into the plates containing 5-FU with a toothpick, and halos were measured after 5 and 8 h. Ten identical plates (five plates from each of two independent cultures) were used to evaluate the swimming motility for each condition.

RNA isolation in biofilms To identify the genes controlled by 5-FU, *E. coli* K-12 ATCC 25404 was grown in LB medium; 1 mL of the overnight cultures (turbidity 7.5 at 600 nm) was inoculated into 250 mL of fresh LB medium supplemented with 250 μL of 10 mM 5-FU or DMF and containing 10 g of glass wool (Corning Glass Works, Corning, NY, USA; Ren et al. 2004a), and the cultures were incubated for 7 h at 37°C with shaking. Total RNA was obtained from the biofilm cells by using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) as described previously (Ren et al. 2005). The RNase inhibitor, RNAlater (Applied Biosystems, Austin, TX, USA), was used to isolate RNA for the second whole-transcriptome study.

DNA microarrays The *E. coli* GeneChip Genome 2.0 arrays containing 10,208 probe sets for open reading frames, ribosomal RNA, transfer RNA, and intergenic regions for four *E. coli* strains (MG1655, CFT073, O157:H7-Sakai, and O157:H7-EDL933) were used for the DNA microarray experiments. Complementary DNA synthesis, fragmentation, and hybridization were performed as described previously (González Barrios et al. 2006). Hybridization was performed for 16 h, and the total cell intensity was scaled automatically in the software to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA controls were used to monitor the labeling process. For each binary microarray comparison of differential gene expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 11–15 probe pairs (p value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the p value for comparing two chips was lower than 0.05 (to assure that the changes in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (1.5-fold) than the standard deviation (1.3-fold and 1.1-fold) for all the genes of the microarray (Ren et al. 2004b).

qRT-PCR To determine the effects of 5-FU on the expression of virulence genes (*eae*, *escC*, and *espA*; Table 2), *E. coli* O157:H7 (EHEC) was grown in LB medium overnight. The overnight culture was diluted to a turbidity of 0.05 at 600 nm in 10 mL of DMEM medium, and 10 μL of 0.5 mM 5-FU or 10 μL of DMF were added to 10 mL of

Table 2 Primers used for qRT-PCR experiments

Primer name	Nucleotide sequence (5′ to 3′)	Primer name	Nucleotide sequence (5′ to 3′)
eae-F	GCTGGCCCTTGGTTTGATCA	*eae*-R	GCGGAGATGACTTCAGCACTT
espA-F	TCAGAATCGCAGCCTGAAAA	*espA*-R	CGAAGGATGAGGTGGTTAAGCT
escC-F	GCGTAAACTGGTCCGGTACGT	*escC*-R	TGCGGGTAGAGCTTTAAAGGCAAT
rpoA-F	GCGCTCATCTTCTTCCGAAT	*rpoA*-R	CGCGGTCGTGGTTATGTG
ymgA-F	TGCTCCAGATACTGCGCATGAAGA	*ymgA*-R	TTCTTCGGTTTGGCCCTCCTGAAT
ariR-F	GTTAGGGCAGGCTGTCACCAATTT	*ariR*-R	GCTGTGTATCGCAACACGATTTCC
rrsG-F	ACTTAACAAACCGCCTGCGT	*rrsG*-R	TATTGCACAATGGGCGCAAG

Primer sequences for *eae*, *escC*, *espA*, and *rpoA* were obtained from Kendall et al. (2007)

cultures. Cells were incubated to a turbidity of 1.0 at 600 nm at 37°C with shaking and were centrifuged at 10,000×*g* for 2 min. RNA was obtained from the cell pellets using the RNeasy Mini Kit (Qiagen Sciences) as described previously (Ren et al. 2005). The expression of *eae* (contained within the operon of virulence genes known as the locus of enterocyte effacement 5 (*LEE5*; Sharma et al. 2005)), *escC* (*LEE2*), and *espA* (*LEE4*) was determined by using qRT-PCR. The housekeeping gene *rpoA* was used to normalize the gene expression data. qRT-PCR was performed in triplicate for each sample.

To corroborate the DNA microarray data, the transcription level of two induced genes, *ariR* and *ymgA*, was quantified using qRT-PCR by using the same RNA used for the DNA microarrays. The housekeeping gene *rrsG* was used to normalize the expression data of the interesting genes.

In vitro adhesion assays Human colonic epithelial cells HCT-8 cells were cultured and propagated in Roswell Park Memorial Institute (RPMI) medium with 10% horse serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. HCT-8 cells (ATCC) were cultured in 24-well tissue culture plates at 37°C in 5% CO_2 until 80% confluence was reached. The in vitro adhesion of EHEC cells to HCT-8 cells was performed as described previously (Bansal et al. 2007).

Microarray accession numbers The expression data for biofilm samples with and without 5-FU are summarized in Table 3 and have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through accession number GSE12750 (Edgar et al. 2002).

Results

5-Fluorouracil inhibits E. coli *biofilm formation* As uracil influences virulence and biofilm formation in *P. aeruginosa* (Ueda et al. 2009), we investigated the effects of uracil analogs on the biofilm formation of *E. coli* K-12 ATCC 25404. This strain was chosen because it is a wild-type strain. Of the examined uracil analogs, only 5-FU reduced the biofilm formation of *E. coli* K-12 ATCC 25404 (Fig. 1). We also examined the effects of 5-FU (0 to 50 μM) on the biofilm formation of three different *E. coli* K-12 strains to ensure that the effect of 5-FU was general (ATCC 25404, BW25113, and MG1655). In all three strains, 5-FU reduced the biofilm formation in a dose-dependent manner in LB medium (Fig. 2). In order to circumvent any growth effect, all the biofilm formation results were normalized by cell density. In all three strains, biofilm formation was decreased by around fivefold with the addition of 25 μM of 5-FU.

To determine the toxicity of 5-FU, *E. coli* K-12 ATCC 25404 was used. Twenty-five micromolar of 5-FU repressed the specific growth rate by 42±3%, and 10 μM of 5-FU repressed the specific growth rate by 25±5% (the specific growth rates were 1.66±0.03, 1.24±0.08, and 0.97±0.05 for 0, 10, and 25 μM of 5-FU, respectively). Hence, 5-FU reduced the biofilm formation of *E. coli* K-12 by 500% with about 42% toxicity at 25 μM. To reduce the impact of 5-FU on cell growth, 10 μM was utilized for subsequent experiments.

Differential gene expression in biofilms upon 5-FU contact To explore the mechanism by which 5-FU represses the biofilm formation of *E. coli*, two whole-transcriptome analyses of biofilm cells were performed at 7 h after the addition of 5-FU (10 μM) in LB medium. In the first whole-transcriptome analysis, 5-FU induced the expression of 157 genes and repressed the expression of 19 genes with more than 1.5-fold change (Table 3). There was good agreement between the two whole-transcriptome studies (Table 3); out of 44 important induced and repressed genes, 36 genes behaved the same way in both microarrays. Genes related to periplasmic proteins and protein folding were repressed in both microarrays, and genes related to the amino acid biosynthesis, lysozyme, maltose transport, membrane proteins, nucleotide biosynthesis, stress, and regulation of transcription were induced in both microarrays.

Unlike ursolic acid and 7-hydroxyindole, which repressed genes with functions for cysteine metabolism (Lee et al. 2007a; Ren et al. 2005), and furanone, which repressed the expression of AI-2-induced genes (Ren et al. 2004b), 5-FU did not alter the expression of these genes. 5-FU induced the expression of seven histidine genes (*hisABCDGHL*) as well as the stress-related genes *yciE*, *yciF*, and *yciG*. YciG is also necessary for swarming motility in *E. coli* (Inoue et al. 2007). Biofilm-related genes *ymgA* and *ariR* were also upregulated in the microarray; deletion of these genes increases biofilm formation (Lee et al. 2007c). qRT-PCR showed that *ymgA* and *ariR* were induced by 2.6- and 3.7-fold, respectively; hence, qRT-PCR corroborated that 5-FU induces the expression of *ymgA* and *ariR*. Therefore, YmgA and AriR reduce biofilm formation and their induction by 5-FU may explain the reduction in biofilm formation seen upon addition of 5-FU.

5-FU controls biofilm formation through AriR To determine which of the genes identified by the whole-transcriptome analysis were directly related to the reduction of biofilm by 5-FU, we examined the biofilm formation of 21

Table 3 Partial list of induced and repressed genes for *E. coli* K-12 ATCC 25404 contacted with 10 μM 5-fluorouracil versus no 5-fluorouracil in biofilms formed on glass wool in LB at 37°C after 7 h

B #	Gene name	Fold change		Descriptions
		First	Second	
Amino acid biosynthesis				
b0001	*thrL*	1.2	1.3	*thr* operon leader peptide
b3670	*ilvN*	1.2	1.3	Acetolactate synthase isozyme I small subunit
b3671	*ilvB*	1.4	1.3	Acetolactate synthase isozyme I large subunit
Acid resistance and cold shock protein				
b1164	*ycgZ*	1.6	1.4	Cold shock protein, hypothetical function
b1165	*ymgA*	1.6	−1.1	Acid resistance
b1166	*ariR*	1.6	1.0	Regulator of acid resistance
b1823	*cspC*	−1.7	−1.1	Cold shock protein
Acyl carrier protein				
b1094	*acpP*	−1.5	−1.1	Acyl carrier protein
DNA binding				
b0440	*hupB*	1.6	1.2	Histone-like protein HU-beta, HU-1
b1158	*pinE*	2.3	−1.7	DNA invertase, site-specific recombination, e14 prophage
Histidine-related				
b2018	*hisL*	1.5	1.7	*his* operon leader peptide
b2019	*hisG*	1.6	1.3	ATP-phosphoribosyltransferase
b2020	*hisD*	1.6	1.5	Histidinol dehydrogenase
b2021	*hisC*	1.7	1.4	Histidinol-phosphate aminotransferase
b2022	*hisB*	1.5	1.1	Imidazole glycerol phosphate dehydratase/histidinol phosphatase; bifunctional enzyme; HAD21
b2023	*hisH*	1.5	1.1	Amidotransferase component of imidazole glycerol phosphate (IGP) synthase
b2024	*hisA*	1.5	1.0	*N*-(5′-phospho-L-ribosylformimino)-5-amino-1-(5′-phosphoribosyl)-4-imidazolecarboxamide 636 isomerase
Initiation factor				
b1718	*infC*	−1.5	1.1	Protein chain initiation factor IF3
Iron transport				
b1252	*tonB*	1.2	1.3	TonB protein
Lysozyme inhibitor				
b0220	*ivy*	1.5	1.1	Inhibitor of vertebrate lysozyme
Maltose transport				
b4034	*malE*	1.5	1.1	Maltose-binding protein, periplasmic; MBP; substrate recognition for active transport of and chemotaxis toward maltose and maltodextrin
Membrane protein				
b1782	*mipA*	1.5	1.1	MltA-interacting protein; outer membrane; binds MrcB and MltA in a heterotrimer
Nucleotide binding				
b1713	*pheS*	1.6	1.1	Phenylalanine–tRNA ligase, beta-subunit
b1714	*pheT*	1.6	1.1	Phenylalanine–tRNA ligase, alpha-subunit
Outer membrane protein				
b0565	*ompT*	1.5	1.1	Outer membrane protease VII, DLP12 prophage; OM protein 2b; omptin
b2215	*ompC*	−1.6	1.1	Outer membrane protein C
Periplasmic protein				
b4376	*osmY*	−1.6	−1.3	Osmotically inducible periplasmic protein
Protein folding				
b3686	*ibpB*	−1.3	−1.4	16-kDa heat shock protein B
b3687	*ibpA*	−1.2	−1.3	16-kDa heat shock protein A
Ribosomal protein				
b0953	*rmf*	−1.7	−2.3	Ribosome modulation factor
b1089	*rpmF*	1.4	1.3	50 S ribosomal subunit protein L32
b3341	*rpsG*	−2.6	−1.2	30 S ribosomal subunit protein S7
RNA polymerase				
b2741	*rpoS*	−1.6	−1.1	RNA polymerase subunit
Stress-related				
b1257	*yciE*	1.5	1.1	Hypothetical protein
b1258	*yciF*	1.5	1.1	H-NS repressed; dimeric; rubrerythrin/ferritin-like putative metal-binding protein
b1259	*yciG*	1.5	1.1	Required for swarming phenotype, function unknown

Table 3 (continued)

B #	Gene name	Fold change		Descriptions
		First	Second	
Transcriptional regulator				
b1450	*yncC*	1.2	1.3	Transcriptional regulator
Trigger factor				
b0436	*tig*	1.5	−1.1	Trigger factor, protein folding chaperone; also peptidyl-prolyl *cis-trans* isomerase
Tryptophan synthesis				
b1261	*trpB*	1.5	1.1	Tryptophan synthase, beta-subunit
Unknown functions				
b1088	*yceD*	1.5	1.1	Hypothetical protein
b1550	*gnsB*	2.0	−1.2	Overexpression increases unsaturated fatty acid content of phospholipids
b1625	*ydgT*	1.4	1.3	Hypothetical protein YDGT
b2809	*ygdI*	1.6	1.3	Hypothetical lipoprotein YGDI precursor
b3469	*zntA*	−1.4	−1.3	Pb/Cd/Zn/Hg transporting ATPase (P-type ATPase family)

Complete microarray analysis has been deposited at the NCBI Gene Expression Omnibus (GSE12750)

isogenic knockout mutants of *E. coli* K-12 with 5-FU based on the whole-transcriptome results (Table 3). Three mutations (*hisA*, *ivy*, and *yceD*) decreased biofilm formation, and the other 18 mutations enhanced significantly biofilm formation in LB medium (Fig. 3); for example, deleting *tig* and *yciG* increased biofilm formation tenfold. These results indicate that 5-FU induces, in general, the expression of biofilm-repressing proteins.

Of the 21 mutants, upon addition of 10 μM 5-FU, only the biofilm formation of the *ariR* mutant was not affected by the addition of 5-FU. Hence, biofilm reduction by 5-FU requires *ariR*. Since *ariR* represses biofilm as reported previously (Domka et al. 2007; Lee et al. 2007c) and as shown here (Fig. 3), this result is consistent.

To corroborate these results, we also examined the biofilm formation of *ariR* mutant in LB glucose (0.2%) medium. In LB glu medium, the biofilm formation of the *ariR* mutant was also increased (threefold) relative to the wild-type strain and was not affected by the addition of 5-FU (Fig. 4). Hence, 5-FU consistently requires *ariR* to reduce biofilms.

To learn more about the interaction of 5-FU and *ariR*, we examined biofilm formation while overexpressed *ariR* with 5-FU. However, overexpressing *ariR* abolished *E. coli* biofilm formation (data not shown). This result could be expected since AriR reduces biofilm formation (Figs. 3 and

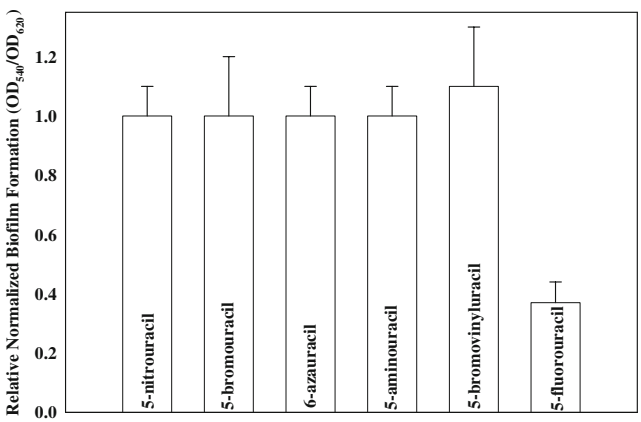

Fig. 1 Effect of uracil derivatives at 10 μM (5-nitrouracil, 5-bromouracil, 6-azauracil, 5-aminouracil, 5-bromovinyluracil, and 5-fluorouracil) on relative normalized (OD_{540}/OD_{620}) biofilm formation of *E. coli* K-12 ATCC 25404 at 37°C after 7 h in 96-well plates in LB medium. At least two independent experiments were conducted (total of 12 wells), and *error bars* indicate one standard deviation

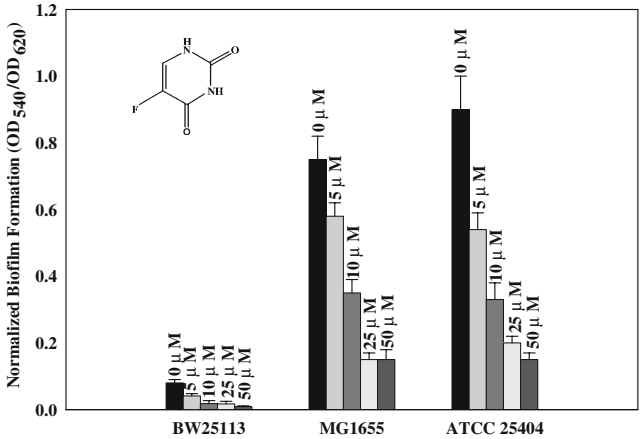

Fig. 2 Effect of 5-fluorouracil (0, 5, 10, 25, and 50 μM) on normalized (OD_{540}/OD_{620}) biofilm formation of *E. coli* K-12 BW25113, *E. coli* K-12 MG1655, and *E. coli* K-12 ATCC 25404 at 37°C after 7 h in 96-well plates in LB medium. At least two independent experiments were conducted (total of 12 wells), and *error bars* indicate one standard deviation. 5-Fluorouracil is shown as an *inset*

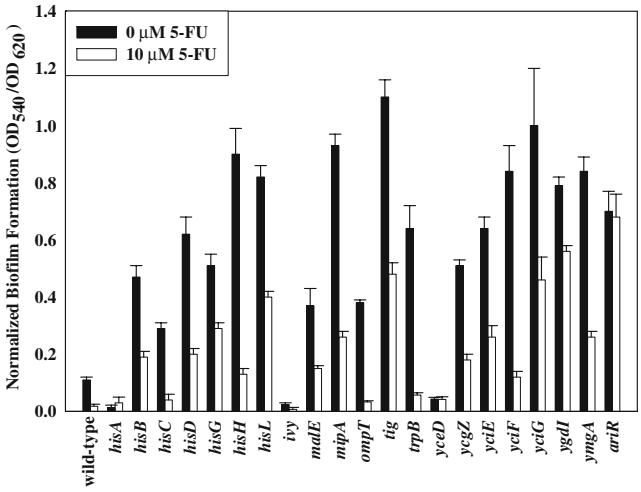

Fig. 3 Effect of 5-fluorouracil (0 and 10 μM) on normalized (OD_{540}/OD_{620}) biofilm formation of *E. coli* K-12 BW25113 and 21 mutants at 37°C after 7 h in 96-well plates in LB medium

4). Therefore, the effect of 5-FU while overexpressing *ariR* could not be measured.

5-FU does not affect swimming motility As 5-FU decreases the biofilm formation of *E. coli* K-12 ATCC 25404 and *E. coli* biofilm formation and motility are often related (Wood et al. 2006), we examined the swimming motility of ATCC 25404 by the addition of 5-FU. After 5 h, neither 5 nor 10 μM of 5-FU altered the swimming motility of this strain (swimming motilities in centimeters were 0.6±0.1, 0.6±0.1, and 0.71±0.07 with 0, 5, and 10 μM of 5-FU, respectively). After 8 h, 10 μM of 5-FU increased the swimming motility of ATCC 25404 by only 1.4-fold (swimming motilities in centimeters were 1.5±0.2, 1.8±0.3, and 2.2±0.2 for 0, 5, and 10 μM of 5-FU, respectively). Hence, the mechanism of biofilm inhibition by 5-FU in ATCC 25404 is not related to cell motility.

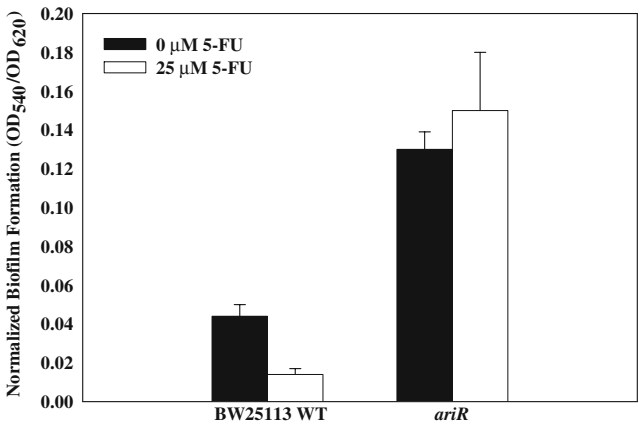

Fig. 4 Effect of 5-fluorouracil (0 and 25 μM) on normalized (OD_{540}/OD_{620}) biofilm formation of *E. coli* K-12 BW25113 and the *ariR* mutant at 37°C after 7 h in 96-well plates in LB glu medium

5-FU represses the expression of virulence genes of EHEC and reduces attachment As 5-FU inhibits virulence phenotypes in *P. aeruginosa* PA14 (Ueda et al. 2009), we examined if 5-FU affects the virulence genes of EHEC. In DMEM medium, 10 μM 5-FU inhibits completely the growth of EHEC and 1 μM inhibits the growth by 2.0±0.2-fold; hence, we tried lower concentrations (note that these results reveal that 5-FU is very potent with this pathogen). The growth rate of EHEC in DMEM medium with 0.5 μM 5-FU repressed the growth by 28±5%; therefore, we used 0.5 μM of 5-FU for the qRT-PCR experiments in DMEM medium. EHEC possesses the pathogenicity island termed LEE, which is necessary for the attachment and effacing lesions caused by EHEC (Walters and Sperandio 2006). LEE is composed of five operons (*LEE1* to *LEE5*) and contains 41 genes (Walters and Sperandio 2006). In DMEM medium, 0.5 μM of 5-FU repressed the expression of *eae*, *escC*, and *espA* genes by 2.8-, 3.4-, and 2.2-fold versus EHEC without 5-FU. *eae* gene belongs to the *LEE5* operon (Sharma et al. 2005); *escC* gene belongs to the *LEE2* operon (Roe et al. 2003), and *espA* gene belongs to the *LEE4* operon (Sharma et al. 2005). Our results indicate that 5-FU represses the expression of the examined three *LEE* operons.

As 5-FU repressed the expression of virulence genes required for attachment of EHEC in DMEM medium, we measured the in vitro adhesion of EHEC to the human epithelial intestinal cells, HCT-8, in RPMI medium. In vitro adhesion of EHEC to HCT-8 cells, normalized by colony-forming units in RPMI medium, was not altered in RPMI medium with the addition of 0.5 μM of 5-FU (results not shown).

Discussion

As *E. coli* is the best-studied microorganism (Lugtenberg et al. 2002) and its knockout mutants are readily available (Baba et al. 2006), using *E. coli* to unravel some of the mechanisms of biofilm inhibition is advantageous (Wood 2009). Here, we found that 5-FU reduces biofilm formation in *E. coli* via AriR which is known from its X-ray crystallography structure to be a biofilm repressor and global regulator (Lee et al. 2007c). The lines of evidence supporting this finding are: (1) the whole-transcriptome analysis showed that 5-FU induces the expression of a AriR (Table 3), (2) an examination of the effect of 5-FU on the biofilm formation of 21 isogenic mutants identified in the whole-transcriptome study showed that only the *ariR* mutant did not respond to 5-FU addition (Fig. 3), (3)

biofilm formation in LB glu medium showed again that 5-FU reduces biofilm formation of the wild-type but not the *ariR* mutant (Fig. 4), and (4) qRT-PCR showed that *ariR* is induced by 5-FU by 3.7-fold. Hence, 5-FU represses biofilm formation by inducing the expression of *ariR* and becomes ineffective in the *ariR* mutant.

5-FU also induced the expression of seven histidine genes, and we examined the biofilm formation of these seven histidine mutants with 5-FU addition. Except for the *hisA* mutant, the six histidine mutants induced biofilm formation vs. the wild-type strain (Fig. 3); hence, histidine biosynthesis appears important for biofilm formation.

In addition, 5-FU induced the expression of stress genes *yciEFG*; *yciE* and *yciF* are both induced in *E. coli* contacted with the antibiotics ampicillin and orfloxacin (Kaldalu et al. 2004). Mutation in these three genes induced biofilm formation dramatically by seven to ninefold (Fig. 3); hence, in this study, decreasing the ability of the cell to respond to stress increases biofilm formation. These results are consistent in that previously we showed that deletion of *ycfR*, which encodes a protein that mediates stress related to acid, heat, hydrogen peroxide, and cadmium, also increases biofilm formation (Zhang et al. 2007). Hence, stress increases biofilm formation.

When bacteria encounter growth inhibitors, they develop resistance, which makes treatment of bacterial infections more difficult (Rasmussen and Givskov 2006). Compounds controlling the expression of virulence without affecting growth are thereby superior (Lesic et al. 2007). In this study, based on our knowledge of uracil influencing biofilm formation and virulence factors (Lee et al. 2008; Ueda et al. 2009), we found that the uracil analog, 5-FU, is a potent biofilm inhibitor of *E. coli* and works through AriR.

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