

# Global Gene Expression Profiling of Asymptomatic Bacteriuria *Escherichia coli* during Biofilm Growth in Human Urine<sup>∇</sup>

Viktoria Hancock and Per Klemm\*

Microbial Adhesion Group, Center for Biomedical Microbiology, BioCentrum-DTU,  
Technical University of Denmark, DK-2800 Lyngby, Denmark

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Urinary tract infection (UTI) is an important health problem worldwide, with many millions of cases each year, and *Escherichia coli* is the most common organism causing UTI in humans. Also, *E. coli* is responsible for most infections in patients with chronic indwelling bladder catheter. The two asymptomatic bacteriuria (ABU) *E. coli* strains 83972 and VR50 are significantly better biofilm formers in their natural growth medium, human urine, than the two uropathogenic *E. coli* isolates CFT073 and 536. We used DNA microarrays to monitor the expression profile during biofilm growth in urine of the two ABU strains 83972 and VR50. Significant differences in expression levels were seen between the biofilm expression profiles of the two strains with the corresponding planktonic expression profiles in morpholinepropanesulfonic acid minimal laboratory medium and human urine; 417 and 355 genes were up- and down-regulated, respectively, during biofilm growth in urine of 83972 and VR50. Many genes involved in transcription and stress were up-regulated in biofilm-grown cells. The role in biofilm formation of four of the up-regulated genes, i.e., *yceP*, *yqgA*, *ygiD*, and *aaeX*, was investigated by creating single-knockout mutant versions of 83972 and VR50; all mutants showed reduced biofilm formation in urine by 18 to 43% compared with the wild type ( $P < 0.05$ ). Also, the expression profile of strain 83972 in the human urinary tract partially overlaps with the biofilm expression profile.

Bacteria generally live attached to surfaces rather than as planktonic isolated cells. Often adhered bacteria form sessile communities, also referred to as biofilms, and these are commonly associated with many health problems (10). Biofilms can form on virtually any type of surface. In the medical field bacterial biofilms have attracted particular attention, because many persistent and chronic bacterial infections, including periodontitis, otitis media, biliary tract infection, and endocarditis, are now believed to be linked to the formation of biofilms. Also, virtually all medical implants are prone to colonization by bacteria, and the resultant biofilms often serve as a source for recurrent infections. Bacterial biofilm infections are particularly problematic, because sessile bacteria can withstand host immune defense mechanisms and are extremely resistant to antibiotics, biocides, and hydrodynamic shear forces that can efficiently clear corresponding planktonic bacteria (9, 10).

Urinary tract infection (UTI) is a serious health problem that affects millions of people each year (49). The recurrence rate is high, and often the infections are particularly troublesome and become chronic, with multiple episodes. UTI usually starts as a bladder infection but often ascends to affect the kidneys and ultimately can result in renal failure or dissemination to the blood. UTI is the most common infection in patients with indwelling bladder catheters, and bacteriuria is essentially unavoidable in this patient group, with an infection rate of ~100% within 1 month (20). UTI is classified into disease categories by the site of infection: cystitis (the bladder), pyelonephritis (the kidney), and bacteriuria (the urine). The

colonization of urine in the absence of clinical symptoms is called asymptomatic bacteriuria (ABU). ABU occurs in up to 6% of healthy individuals and 20% of elderly individuals. As the name implies, ABU strains generally do not cause symptoms; most patients with ABU do not need treatment, and in many cases the colonizing organism actually helps to prevent infection by other more virulent bacteria (12, 24).

*Escherichia coli* is responsible for more than 80% of all UTIs and causes both ABU and symptomatic UTI (22, 51). *E. coli* 83972, a clinical isolate capable of long-term bladder colonization, has become a model ABU organism. It was originally isolated from a girl with ABU who had carried it for at least 3 years without symptoms (1, 31). The strain is well adapted for growth in the human urinary tract (UT), where it establishes long-term bacteriuria (1, 24). Strain 83972 has been used for prophylactic purposes in numerous studies; as such it has been used as an alternative treatment in patients with recurrent UTI who are refractory to conventional therapy (12, 24). The mechanisms for bladder colonization used by ABU *E. coli* are not known. Low abundance of adhesins could explain to a large degree why ABU strains do not cause symptoms in the host; however, it does not explain how these strains are capable of efficient bladder colonization. Meanwhile, since *E. coli* strains are responsible for most infections in patients with chronic indwelling bladder catheter (56), we suspected that biofilm formation could contribute to this.

DNA microarray-assisted functional genomics provides a genome-wide portrait of the transcriptome of an organism and discloses how a bacterium adapts to a particular environmental niche at the transcriptional level. Here we analyze the global expression profile of two *E. coli* ABU strains, 83972 and VR50, during biofilm formation on abiotic surfaces in human urine.

\* Corresponding author. Mailing address: Microbial Adhesion Group, Center for Biomedical Microbiology, BioCentrum-DTU, Building 301, Technical University of Denmark, DK-2800 Lyngby, Denmark. Phone: 4545252506. Fax: 4545932809. E-mail: pkl@biocentrum.dtu.dk.

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## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* 83972 (OR:K5:H<sup>-</sup>) is a prototype ABU strain and lacks defined O and K surface antigens (1). VR50 (OR:K1:H<sup>-</sup>) is a recently isolated ABU *E. coli* strain which grows very well in urine and is capable of expressing type 1 fimbriae (41). Creation of the fluorescently tagged versions of 83972 and VR50, 83972yfp and VR50yfp, has been described previously (21).

**Growth conditions and stabilization of RNA for microarray experiments of *E. coli* grown in planktonic cultures.** Human urine was collected from four healthy men and women volunteers who had no history of UTI or antibiotic use in the prior 2 months. The urine was pooled, filter sterilized, stored at 4°C, and used within the following 2 days. Overnight cultures of VR50 were grown in triplicates in pooled human urine or morpholinepropanesulfonic acid (MOPS) minimal media (supplemented with 0.2% glucose and 1 mg/liter pantothenic acid) until reaching exponential phase and then used for inoculation of 25 ml urine or MOPS to an optical density at 600 nm (OD<sub>600</sub>) of 0.05. The cultures were grown at 37°C and 130 rpm, and 5-ml samples for isolation of RNA were extracted from three individual cultures at mid-exponential phase (corresponding to an OD<sub>600</sub> of approximately 0.4 and 0.5 in urine and MOPS, respectively). Extracted samples were immediately mixed with two volumes of RNeasy Protect Bacteria Reagent (QIAGEN AG), incubated for 5 min at room temperature to stabilize RNA, and centrifuged. The pellets were then stored at -80°C. The samples for *E. coli* 83972 grown in MOPS-glucose and human urine were grown similarly, with the exception of no addition of pantothenic acid to the MOPS cultures, and have been analyzed and described previously (43).

**Growth conditions and stabilization of RNA for microarray experiments of *E. coli* grown in biofilms.** Freshly grown urine cultures of *E. coli* 83972 and VR50 were used for inoculation of 25 ml pooled human urine in 94-mm petri dishes (Greiner Bio-One), each strain in triplicate. The cultures were grown statically at 37°C for 42 h, and the medium was carefully poured off and replaced by fresh preheated medium twice during incubation. After 42 h the bacterial lawn on each petri dish was quickly washed twice with 20 ml urine preheated to 37°C, and immediately thereafter 2 ml of a 1:2 mixture of phosphate-buffered saline (PBS) and RNeasy Protect Bacteria Reagent (QIAGEN AG) was poured on the lawn of attached cells and incubated for 5 min at room temperature to stabilize RNA. The stabilized mixture was then centrifuged, and pellets were stored at -80°C. The same batch of urine was used for growth of 83972 and VR50 in petri dishes and for growth of VR50 in planktonic cultures (see the above paragraph).

**RNA isolation and cDNA labeling.** Total RNA was isolated using the RNeasy Mini kit (QIAGEN AG), and on-column DNase digestion was performed using an RNase-Free DNase set (QIAGEN AG). The quality of the total RNA was examined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm to ensure intact high-quality RNA. Purified RNA was precipitated with ethanol and stored at -80°C until further use. Conversion of RNA (10 µg per sample) to cDNA and microarray analysis were performed according to GeneChip Expression Analysis Technical Manual 701023, Rev. 4 (Affymetrix, Inc., Santa Clara, CA).

**DNA microarray hybridization.** GeneChip *E. coli* Genome 2.0 arrays (Affymetrix) were used for hybridization of the labeled cDNA. In total, 18 samples were hybridized to 18 chips, i.e., 9 biologically independent samples from each of the two strains were hybridized to 1 chip each. For each strain, i.e., 83972 and VR50, three chips were hybridized with samples grown in three individual flasks in MOPS, three chips were hybridized with samples from cells grown in pooled human urine in three individual flasks, and three chips were hybridized with samples from biofilm cells grown in pooled human urine in three individual petri dishes. No pooling was performed, and all samples were treated separately. Hybridization, washing, and staining were performed according to GeneChip Expression Analysis Technical Manual 701023, Rev. 4 (Affymetrix), and the microarrays were scanned using a GeneChip Scanner 3000.

**Data analysis.** Array normalization and expression value calculation were performed using the DNA-Chip Analyzer (dChip) 1.3+ software program (<http://www.dchip.org/>) (29). The Invariant Set Normalization method (30) was used to normalize arrays at the probe cell level to make them comparable, and the model-based (PM-only) method was used for probe selection and computing expression values. These expression levels were attached with standard errors as measurement accuracy, which were subsequently used to compute 90% confidence intervals of fold changes in two-group comparisons. All 18 chips (12 chips generated in this study and 6 chips [83972 grown in MOPS and urine] described earlier [43]) were normalized together, and a number of different comparisons were performed in order to filter out genes differently expressed during biofilm growth in urine compared with planktonic cultures grown in both MOPS and urine. (i) The three arrays of each strain (83972 and VR50) hybridized with

samples grown in MOPS were used as the baseline for calculation of fold changes in each strain grown in biofilms in urine. (ii) Calculation of fold changes in each strain grown in biofilms in urine was performed in the same way as in the first comparison but with the three arrays hybridized with samples grown planktonically in pooled human urine as the baseline. (iii) In one comparison, the fold changes in biofilm were calculated on all 6 biofilm arrays from both strains using all 12 planktonic arrays as the baseline. The comparison criteria were carefully chosen to make sure that the estimation of the percentages of genes identified by chance, the empirical false discovery rate, was kept low ( $\leq 0.6\%$  for all comparisons in our study). Permuting our samples randomly 200 times resulted in false discovery rates between 0.0% and 0.6%, or 0 to 11 false-positive genes, for the different comparisons.

**RT-PCR.** Reverse transcription-PCR (RT-PCR) was performed to confirm DNA microarray gene expression data. RNA, from the DNase-treated RNA samples used for microarray hybridization and analysis, was converted to cDNA using SuperScript II (Invitrogen). cDNA was used directly as template for PCR, and a negative control on the RNA sample (not converted to cDNA) was run in parallel to confirm that all DNA had been removed in the earlier step. The total number of cycles used in PCR ranged from 15 to 40. RT-PCR products were examined by agarose gel electrophoresis.

**Motility assay.** The motility ability was tested for the two ABU strains. Two microliters of biofilm urine cultures were stabbed into urine plates (4:1 ratio of human urine and 0.9% NaCl) containing 0.3% agar and incubated at 37°C for 16 h. The assay was repeated thrice in different batches of urine.

**Biofilm formation in flow-cell chambers in human urine.** Flow-chamber experiments were performed at 37°C in human urine, essentially as described previously (8). Briefly, biofilms were grown on a microscope glass coverslip (Knittel 24 × 50 mm st1; Knittel Gläser) in three-channel chambers (channel dimension, 1 by 4 by 40 mm). Two hundred fifty microliters of an overnight culture standardized to an OD<sub>600</sub> of 0.05 was inoculated in each channel, and the cells were allowed to attach to the substratum for 1 h before the flow was turned on at a rate of approximately 3 ml/h. Biofilm formation was followed by microscopic observations with an LSM510 scanning confocal laser microscope (Carl Zeiss). Images were obtained using a 40×/1.3 Plan-Neofluar oil objective.

**Construction of knockout mutants of 83972 and VR50.** Mutants of 83972 and VR50 were constructed using the λRed recombination gene replacement system (13). Briefly, the *npt* gene from plasmid pKD4 was amplified using four different primer sets containing 40-nucleotide homology extensions of the four target genes (unpublished data), i.e., *yceP*, *yqgA*, *ygiD*, and *aaeX*. The PCR products were purified from agarose gels and transformed into 83972(pKD46) and VR50(pKD46), and kanamycin-resistant colonies were selected. The λRed helper plasmid pKD46 was cured by growth at 37°C, and the correct double-crossover and recombination event was confirmed by PCR.

**Biofilm formation in microplates in human urine.** Cells were grown in pooled human urine for 5 to 6 h, and 10 µl was used for inoculation of 1.0 ml urine in 24-well flat-bottom microplates (Iwaki). The microplates were incubated statically at 37°C for 16 h. Unbound cells were removed by inversion of the microplate and tapping on absorbent paper; adhered cells were then stained with 0.1% crystal violet for 15 min. Excess stain was removed by washing three times with PBS. Crystal violet was then solubilized by the addition of ethanol-acetone (80:20, vol/vol), and the A<sub>590</sub> was measured (Ultrospec III; Pharmacia). Each strain (wild-type and deletion mutant strains) was assayed in four wells on each plate, and the whole experiment was repeated three times in different batches of urine. In each plate, four wells were used as blanks containing sterile urine and four wells were inoculated with the wild-type strain (83972 or VR50) for reference. For the longer incubation time of 42 h used in the experiment comparing wild-type ABU and uropathogenic *E. coli* (UPEC) strains, the medium was changed twice during incubation, mimicking the growth conditions used for biofilm microarray analysis.

**Microarray data accession number.** The supporting microarray data have been deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) under accession number E-MEXP-926.

## RESULTS

**Biofilm formation of ABU strains 83972 and VR50.** We have recently found that many ABU strains form significantly more biofilm when grown in pooled human urine on abiotic surfaces than UPEC strains (21). Also, ABU strains *E. coli* 83972 and VR50 form about 10- to 20-fold better biofilm in polystyrene microtiter plates after 42 h than UPEC strains CFT073 and 536

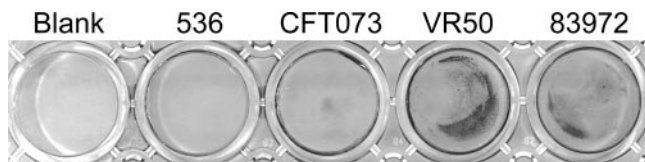


FIG. 1. Biofilm formation in polystyrene microplates in human urine of two UPEC isolates, 536 and CFT073, and two ABU strains, VR50 and 83972. The cells were incubated for 42 h at 37°C, and the urine was changed twice during incubation; the plate was scanned after staining with crystal violet and washing three times with PBS. The two ABU strains formed significantly more biofilm than the two UPEC strains (paired two-tailed *t* test,  $P < 0.001$ ).

(paired two-tailed *t* test,  $P < 0.001$ ) (Fig. 1). To study the gene expression in ABU strains during growth in biofilm, we used DNA microarray technology. The two ABU strains were grown in polystyrene petri dishes in pooled human urine for 42 h to allow biofilm formation. The bacterial biofilm was washed twice to remove planktonic cells before immediate stabilization of RNA using RNAProtect Bacteria Reagent (QIAGEN AG).

#### Global gene expression of two ABU *E. coli* strains in biofilm.

The genomic expression profiles of *E. coli* 83972 and VR50 obtained during biofilm formation in urine were compared with those during exponential growth in MOPS-glucose and pooled human urine (all in triplicates). The GeneChip *E. coli* Genome 2.0 Array was employed, which contains probe sets for all genes present in *E. coli* strains MG1655, CFT073, EDL933, and O157:H7-Sakai. In total, of 8,716 *E. coli* transcripts, 2,296 and 2,183 were significantly changed during biofilm growth for 83972 and VR50, respectively, compared with MOPS. The corresponding numbers of genes compared with planktonic cells in urine were 2,221 and 2,020, respectively. Of these significantly changed genes, about half (51 to 53%) were up-regulated. Approximately half of the differentially expressed genes during biofilm growth compared with the gene profile during planktonic growth were shared between the two ABU strains (Fig. 2). We have chosen mainly to focus on these genes.

In total, 815 genes were found to be significantly changed during biofilm formation, in both *E. coli* 83972 and VR50, compared with the gene profile of planktonic growth in MOPS and urine (Fig. 3). Expression of the majority of the genes (94.7%) was affected in the same way, i.e., 417 and 355 genes were up- and down-regulated, respectively, during biofilm growth in both 83972 and VR50. Taken together, a large number of genes was found significantly changed during biofilm growth compared with the gene profile of planktonic growth, revealing a large similarity of the expression profiles in the two strains and indicating very different gene expression profiles during the two growth conditions.

**Functional analysis of MG1655 transcripts of ABU *E. coli* in biofilm.** Classification of the significantly changed MG1655 genes into functional groups (52, 53) reveals which groups, categories of genes, are most affected during biofilm formation in urine compared to the gene profile during planktonic growth in MOPS and urine. Of a total of 417 and 355 up- and down-regulated genes in biofilm, 327 and 310, respectively, were MG1655 transcripts and are classified in Table 1. The functional analysis revealed that the group containing genes in-

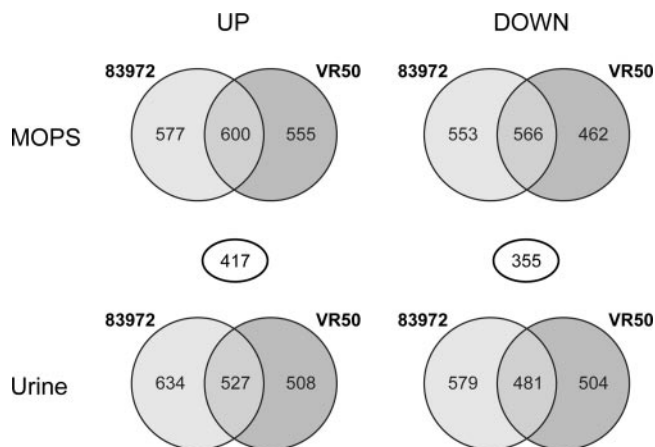


FIG. 2. Venn diagrams showing numbers of significantly up- and down-regulated genes in *E. coli* 83972 and VR50 during biofilm growth in urine compared with that during planktonic growth in MOPS-glucose and urine. The numbers displayed in circles between the Venn diagrams show the number of genes shared between the two overlapping areas; 417 and 355 genes were up- and down-regulated, respectively, during biofilm growth in both strains compared to that during planktonic growth in both MOPS and urine.

involved in transcription was by far the most up-regulated during growth in biofilm. The most down-regulated groups were those including genes involved in “translation” and “cell cycle control.” Other *E. coli* biofilm array studies have shown no change in expression of ribosomal genes during biofilm growth compared with exponentially grown cells (5, 39, 44) and up-regulation compared with stationary-phase cells (44). Of 54 ribosomal genes, 63% were significantly down-regulated, up to 8.9-fold, in biofilm in both ABU strains compared to that of exponentially grown cells. This indicates that our biofilm cells have a slower growth rate than exponential-phase cells, reflecting a growth rate somewhere between that of stationary and exponential phases. Interestingly, the least affected group was “cell motility,” where only 1 of 91 genes was found to be changed during biofilm growth compared to that during planktonic growth.

**Biofilm-related genes.** Of particular interest are the genes that have previously been suggested to be involved in *E. coli* biofilm formation, albeit with different strains and growth conditions (5, 39, 44). Among these are genes encoding cell surface structures, e.g., Ag43, type 1 fimbriae, extracellular polysaccharides, curli, and flagella, as well as genes found differently expressed in other biofilm microarray studies, e.g., genes involved in quorum sensing (*luxS* and *tqsA*) and regulation (*rfaH* and *rpoS*). The *flu* gene, encoding Ag43, has been shown to promote biofilm formation and has been reported to be up-regulated in *E. coli* K-12 biofilms (44). In the present study, the *flu* gene showed low expression and no change between the different growth conditions in either 83972 or VR50. Neither the antigen 43 precursor genes c1273 and c3655 nor any of the other known and putative autotransporter genes (b2647, b1169, b1170, *ycgV*, and *aidA*) showed any up-regulation during biofilm growth of the two strains. The *sat* gene, encoding the secreted autotransporter toxin (Sat) of uropathogenic *E. coli*, was down-regulated 4.3-fold in VR50 and showed no change in 83972.

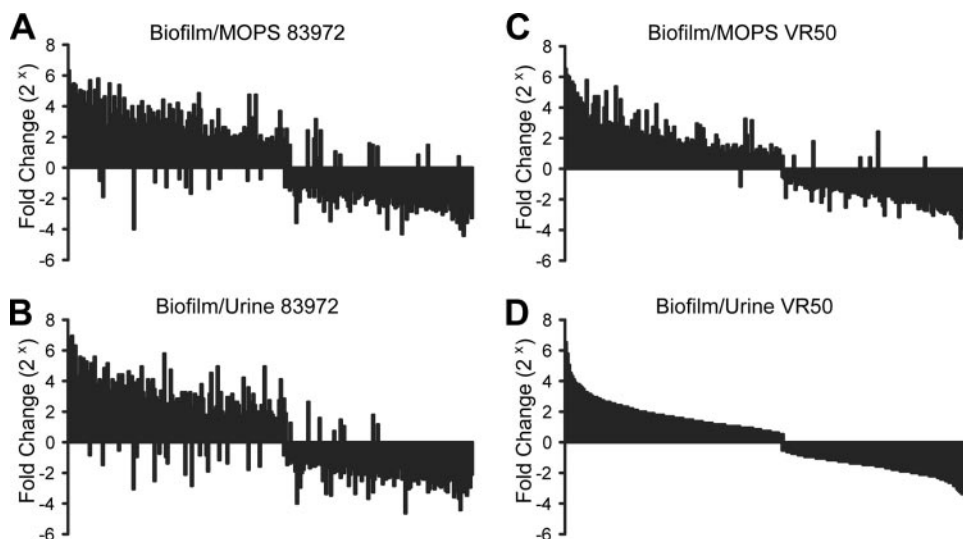


FIG. 3. Comparison of significantly changed genes during biofilm growth of *E. coli* 83972 and VR50 compared with the gene profile during planktonic growth. The 815 genes, which were significantly changed during biofilm growth in urine in both strains compared to that during planktonic growth in both MOPS and urine, have been ordered from the most up-regulated to the most down-regulated gene in VR50 compared with that in planktonic urine (D). The diagrams display the fold change of the genes in 83972 and VR50 during biofilm growth in urine compared with that during planktonic growth in MOPS (A and C, respectively) and urine (B and D, respectively).

**Down-regulation of fimbriae.** The *fim* genes, encoding type 1 fimbriae, have been reported to be up-regulated in several *E. coli* biofilm studies (5, 39, 44). Strain 83972 is not able to express type 1 fimbriae, due to a large deletion affecting the *fimEAIC* region (26), while VR50 is capable of expressing type 1 fimbriae (41). All the remaining *fim* genes present in 83972, *fimBDFGH*, were down-regulated (2.2- to 9.3-fold) during biofilm growth compared to that with MOPS. In VR50 the regulatory *fimB* gene, encoding a site-specific recombinase capable of conferring off-to-on inversion of the phase switch, was down-regulated 3.3-fold compared to growth in MOPS, while the other *fim* genes showed low expression levels and no change between the different growth conditions.

P and F1C fimbriae are two other fimbriae associated with UPEC strains and UTI (27, 34). All *pap* genes, encoding P fimbriae, showed low expression levels and no change in both strains. The *foc* genes, encoding F1C fimbriae, showed low expression and no change in VR50, while *focACG* genes were down-regulated (1.8- to 6.9-fold) during biofilm growth in 83972 compared with that during planktonic growth in MOPS and urine. The *aufA* gene of the recently characterized Auf fimbriae associated with UPEC (7) was up-regulated 2.7- and 11.3-fold in 83972 during biofilm growth compared with that of planktonic growth in MOPS and urine, respectively. The *yadC* gene, encoding a putative fimbria-like protein, was up-regulated in 83972 6.6- and 10-fold in biofilm compared to that with MOPS and urine, respectively. Other putative fimbrial genes (*sfmACDHF*, *yaH*, *yadKLMN*, *ygiL*, *iaeH*, and *c4424*) showed no change between the different growth conditions.

Taken together, no fimbria-encoding genes were seen up-regulated in VR50 biofilm, while 83972 biofilm showed up-regulation of two fimbriae-associated genes, *aufA* and *yadC*, encoding a major fimbrial subunit and a putative fimbria-like protein, respectively.

**Down-regulation of extracellular matrix components.** Production and secretion of polysaccharide polymers, such as colanic acid and cellulose, into the extracellular biofilm matrix is often recognized as important in the development of bacterial biofilms (6). It has been shown that colanic acid production is required for development of *E. coli* biofilm architecture (11). In our strains the genes involved in biosynthesis of colanic acid were all down-regulated (*galEKTU*) or showed no change (*cpsBG*, *fcl*, *wca*, *wza*, *rcaABC*, *gmd*, and *ugd*) in biofilm. The genes involved in lipid A and 2-keto-3-deoxyoctulosonic acid biosynthesis were down-regulated (*lpxABD* and *kdsABD*) or showed no change. The *murACDEFG*, *mraY*, and *ddlAB* genes involved in peptidoglycan biosynthesis were all down-regulated 2.1- to 6.2-fold. The genes involved in biosynthesis of enterobacterial common antigen, *rffACDEHGMT*, were down-regulated 2.3- to 4.6-fold in biofilm growth of both strains. The *kpsEDCSMT* genes, involved in transport of polysaccharide to the cell surface, were all down-regulated 2.7- to 7.0-fold during biofilm growth.

Cellulose synthesis has recently been shown to be involved in biofilm formation in the commensal *E. coli* strain 1094 (36). The *bcsABZC* and *bcsEFG* genes, involved in cellulose synthesis, were all down-regulated in 83972 biofilm, while the *bcsEFG* genes were down-regulated in VR50 biofilm.

Curli expression has also been shown to affect biofilm formation in *E. coli* (57). None of the curli genes, *csgABCDEFG* or *crl*, were differentially expressed in the two ABU biofilms, and all *csg* genes showed very low expression levels.

Motility has been shown to influence biofilm architecture in *E. coli* (58), and other biofilm array studies of *E. coli* have reported no change in expression of genes encoding motility (5, 39, 44). In this study, some genes encoding flagellar proteins were down-regulated in VR50, while all flagellar biosynthesis genes showed no change and low expression levels in 83972. In

TABLE 1. Numbers of significantly up- and down-regulated genes in each functional group of *E. coli* 83972 and VR50 during biofilm growth in urine compared to that of planktonic growth in MOPS-glucose and urine

Description	Up-regulated genes in biofilm		Down-regulated genes in biofilm		No. of genes
	No.	%	No.	%	
Amino acid transport and metabolism	5	2	36	12	296
Carbohydrate transport and metabolism	13	4	23	8	298
Cell cycle control, mitosis, and meiosis	3	10	6	19	31
Cell motility	1	1	0	0	91
Cell wall/membrane biogenesis	4	2	25	13	200
Coenzyme transport and metabolism	9	8	14	13	108
Defense mechanisms	1	3	0	0	35
Energy production and conversion	12	5	34	14	240
Function unknown	31	13	15	6	247
General function prediction only	26	10	16	6	269
Inorganic ion transport and metabolism	14	9	7	4	160
Intracellular trafficking and secretion	2	6	6	17	36
Lipid transport and metabolism	3	4	8	11	71
Nucleotide transport and metabolism	6	8	8	10	78
Posttranslational modification, protein turnover, chaperones	13	11	11	9	119
Replication, recombination, and repair	17	10	14	8	165
Secondary metabolites biosynthesis, transport, and catabolism	2	4	7	13	54
Signal transduction mechanisms	12	10	2	2	116
Transcription	44	19	8	3	235
Translation	12	8	45	29	156
Not in COGs <sup>a</sup>	97	9	25	2	1,065
MG1655 (Total)	327	8	310	8	4,070
CFT073	67	3	37	1	2,486
EDL933	15	1	7	0	1,787
SAKAI	8	2	1	0	373
Total	417	5	355	4	8,716

<sup>a</sup> COGs, clusters of orthologous groups of proteins.

agreement with this, the two ABU strains proved nonmotile on low-agar urine plates.

Taken together, no genes associated with production of the extracellular matrix components or flagella were up-regulated in the two biofilm-forming ABU strains, suggesting that the biofilms formed by these strains might be smooth and flat, lacking a highly structured morphology.

**Down-regulation of the AI-2 quorum-sensing system.** LuxS is involved in biosynthesis of autoinducer 2 (AI-2), a hormone-like signal that mediates cell-cell communication during quorum sensing, the response to increased cell density (50). Furthermore, LuxS is involved in regulation of pathogenicity genes in enterohemorrhagic and enteropathogenic *E. coli* strains (47). The *luxS* gene was down-regulated 7.9- and 3.7-fold in 83972 and VR50, respectively. Consistent with this, the *yhbH* gene, a sigma 54-modulating protein that is induced by AI-2 (14), was also down-regulated in both strains, 4.7- and 2.0-fold. Furthermore, deletion

of *luxS* has been shown to affect methionine metabolism and regulation in K-12 by decreased expression of *metE* (55); *metE* was repressed (11-fold) in 83972 and VR50 together with *metB-CHKL*, which were down-regulated 2.3- to 4.1-fold, while the major repressor of the *met* genes, *metJ*, was up-regulated 2.0-fold. YdgG (TqsA) has recently been shown to control biofilm formation in *E. coli* through transport of AI-2: deletion of *tqsA* resulted in a major increase in biofilm thickness and cell motility of *E. coli* K-12 (23). The *tqsA* gene was up-regulated 4.0-fold in 83972 (the 2.2-fold up-regulation in VR50 was not significant). YceP and YliH have recently been shown to regulate biofilm formation through quorum sensing in *E. coli* K-12; deletion of either *yceP* or *yliH* increased biofilm formation and extracellular AI-2 concentrations 50-fold (18). The *yceP* gene was up-regulated 6.3- and 18.6-fold, and the *yliH* gene 5.8- and 94-fold, in 83972 and VR50 biofilms, respectively. AaeR is thought to be a virulence regulator that is activated by quorum sensing and to be involved in activation of expression of locus of enterocyte effacement pathogenicity genes in enterohemorrhagic and enteropathogenic *E. coli* (46). It has been shown that AaeR is a positive transcription factor for the divergently transcribed *aaeXAB* operon, and more recently it has been shown that the *aaeAB* genes encode an aromatic carboxylic acid efflux pump whose physiological role may be as a metabolic relief valve to alleviate toxic effects of imbalanced metabolism (54). The *aaeR* gene showed no change in our biofilm cells, while the *aaeXAB* operon belonged to one of the most up-regulated in 83972 and VR50 biofilms (5.5- to 21.2-fold compared with planktonic growth).

Taken together, down-regulation of genes involved in synthesis of AI-2 and up-regulation of *tqsA*, *yceP*, and *yliH*, which all negatively affect biofilm formation through AI-2, indicate that the AI-2 system is repressed in our biofilm-grown cells.

**Regulation of genes involved in transcription.** The most up-regulated group was the group including genes involved in transcription, and many genes encoding transcriptional regulators were significantly up-regulated in biofilm-grown cells (Table 2) and among the most highly expressed (Table 3). The two regulatory genes *rfaH* and *rpoS* are believed to play a role in biofilm formation. The transcriptional activator RfaH participates in controlling several genes involved in the biosynthesis, assembly, and export of the lipopolysaccharide core. Inactivation of *rfaH* was reported to result in increased initial adhesion and biofilm formation by UPEC strain 536 (4). It is therefore interesting that this gene was significantly up-regulated in 83972 and VR50 biofilms. Although many genes involved in transcription were up-regulated, eight were down-regulated (Table 1), and five of these encoded RNA polymerase and sigma factors. Thus, the *rpoS* and *rpoN* genes, encoding the sigma S and 54 factors involved in general stress response and quorum sensing, respectively, were down-regulated (44).

**Up-regulation of genes involved in stress response.** The most up-regulated genes in biofilm included genes encoding both cold shock and heat shock proteins (Table 2). The three cold shock genes *cspG*, *cspH*, and *cspA* were up-regulated 81-, 57-, and 16-fold, respectively, and the heat shock genes *ibpB* and *ibpA* were up-regulated 34- and 8.7-fold, respectively. The *pphA* gene, involved in induction of the heat shock response, was up-regulated 6.7-fold during biofilm growth. The gene encoding the transcriptional activator SoxS, which participates

TABLE 2. Top 30 most up-regulated genes during biofilm growth in urine of *E. coli* 83972 and VR50 compared to that during planktonic growth in MOPS and urine

Gene	Code	Function and/or product	FC in BF <sup>a</sup>	Signal intensity in BF <sup>b</sup>
<i>cspG</i>	b0990	Homolog of <i>Salmonella</i> cold shock protein	80.7	4,659
<i>cspH</i>	b0989	Cold shock-like protein	56.5	2,532
<i>ibpB</i>	b3686	Heat shock protein	33.6	5,787
<i>marR</i>	b1530	Multiple antibiotic resistance protein; repressor of <i>mar</i> operon	31.5	4,859
<i>yqiJ</i>	b3050	Putative oxidoreductase	30.6	1,522
<i>oxyS</i>	b4458	Global regulatory RNA OxyS	30.5	3,916
<i>c2422</i>	c2422	Putative inner membrane ABC transporter	28.1	1,096
<i>ddg</i>	b2378	Palmitoleoyl-acyl carrier protein-dependent acyltransferase, cold-induced gene	26.2	1,859
<i>ygiD</i>	b3039	Putative enzyme with dioxygenase domain	23.4	3,863
<i>c3685</i>	c3685	Hypothetical protein	22.5	791
<i>aaeX</i>	b3242	Hypothetical protein	21.2	3,905
<i>grxA</i>	b0849	Glutaredoxin 1 redox coenzyme for glutathione-dependent ribonucleotide reductase	21.1	4,675
<i>c2436</i>	c2436	Putative pesticin receptor precursor, homologous to <i>fyuA</i>	19.8	1,196
<i>c2424</i>	c2424	Putative peptide synthetase	19.6	1,408
<i>yfiP</i>	b2583	Hypothetical protein	19.0	1,863
<i>yqjF</i>	c3859	Hypothetical protein	18.9	3,664
<i>mdtL</i>	b3710	Drug/chloramphenicol transport protein (MFS family)	18.3	1,862
<i>yhcN</i>	b3238	Hypothetical protein	17.3	5,790
<i>yqgA</i>	b2966	Putative transport membrane protein	17.2	1,010
<i>rydB</i>	b4430	Small toxic membrane polypeptide	17.1	1,167
<i>sgaB</i>	b4194	Putative PTS family enzyme II component, possibly ascorbate-specific	16.5	2,167
<i>pflE</i>	b0824	Putative pyruvate formate lyase activating enzyme	16.4	891
<i>marB</i>	b1532	Multiple antibiotic resistance protein	16.3	2,173
<i>sgaE</i>	b4198	L-ribulose 5-phosphate 4-epimerase	16.2	1,405
<i>ybaO</i>	Z0555	Putative transcriptional regulator	15.9	2,505
<i>ytfH</i>	b4212	Putative transcriptional regulator	15.9	2,383
<i>cspA</i>	b3556	Cold shock protein 7.4, transcriptional activator of <i>hns</i>	15.9	6,387
<i>yjyY</i>	b4199	Hypothetical protein	15.2	960
<i>c3865</i>	c3865	Hypothetical protein	14.7	676
<i>fixA</i>	b4140	Suppress F exclusion of bacteriophage T7	13.4	2,475

<sup>a</sup> Fold change (FC) in biofilm (BF) was calculated from all 6 biofilm arrays using all 12 planktonic arrays as the baseline.

<sup>b</sup> Signal intensity was calculated from all six biofilm arrays treated as one single sample group.

in controlling several genes involved in the response to oxidative stress and the transcription of which has been seen induced upon biofilm formation (39), was up-regulated 3.4-fold in VR50 biofilm while it was repressed in 83972. The transcriptions of *fldA*, *fur*, *fpr*, *fumC*, *inaA*, and *marRAB* are all activated by SoxS and were all up-regulated during biofilm growth by 2.0- to 31-fold in both 83972 and VR50. Transcription of SoxS is activated by SoxR, which also was up-regulated 6.8-fold in biofilm growth in both strains. Transcription of *hha*, encoding a hemolysin expression-modulating protein involved in stress response, is induced upon biofilm formation (39), and the Hha protein of virulent *E. coli* strains affects production of some virulence factors (17). *hha* was up-regulated 3.3-fold during biofilm growth in the two ABU strains. Furthermore, the stress-induced YfiD protein involved in stress resistance was also up-regulated 7.0-fold during biofilm growth in both strains. Taken together, many genes involved in stress response were highly up-regulated in biofilm of both strains, which is in agreement with previous *E. coli* biofilm array studies (5, 39, 44).

**Up-regulation of virulence-associated genes.** *E. coli* 83972 carries the virulence-associated *fyuA* gene, encoding Yersinia-bactin receptor protein (15). The *c2436* gene, encoding a pu-

tative pesticin receptor protein homologous to *fyuA*, was up-regulated in the urinary tract in vivo (40) and was one of the most up-regulated genes in biofilm of the two ABU strains (Table 2). Interestingly, all the genes flanking the *fyuA* homologue, *c2418* to *c2440* (altogether 22 genes), were significantly up-regulated in VR50 biofilm (2.2- to 63.4-fold), and nine of them were up-regulated in 83972 (2.8- to 13.1-fold). This cluster was by far the largest contiguous up-regulated gene cluster in VR50 biofilm; several of the genes in this cluster were among the most up-regulated in both VR50 and 83972 biofilms (Table 2). The cluster is part of the pathogenicity island PAI IV<sub>536</sub> of UPEC strain 536, which is a high-pathogenicity island encoding a siderophore system initially identified in pathogenic *Yersinia* spp. (16). This result indicates that both ABU strains contain PAI IV<sub>536</sub> and that genes in this pathogenicity island may play a role during biofilm growth.

YliI has recently been identified as an outer membrane protein (32) and was expressed during human infection by *E. coli* O157:H7 (25). The *yliI* gene was up-regulated 8.0- and 5.9-fold during biofilm growth in 83972 and VR50, respectively. Another virulence-associated gene, *ygdP*, was up-regulated in biofilm growth. This gene is associated with the inva-

TABLE 3. Top 50 most expressed genes during biofilm growth in urine of *E. coli* 83972 and VR50 compared to that during planktonic growth in MOPS and urine

Gene	Code	Function and/or product	Signal intensity <sup>a</sup>	FC in BF <sup>b</sup>
<i>rffH</i>	b0205	5S rRNA	11,223	1.2
<i>rrlG</i>	Z3875	23S rRNA	9,568	-1.0
<i>rrsG</i>	b2591	16S rRNA	8,062	1.1
<i>ssrA</i>	b2621	10Sa RNA (tmRNA); tags incomplete translation products for degradation	7,924	1.1
<i>ssrS</i>	b2911	6S RNA	7,601	<b>4.6</b>
<i>mmpB</i>	b3123	RNase P, RNA component; M1 RNA	7,559	1.2
<i>csrB</i>	b4408	CsrB regulatory RNA	6,638	1.2
<i>ahpC</i>	b0605	Alkyl hydroperoxide reductase, detoxification of hydroperoxides	6,622	1.3
<i>rhoL</i>	b3782	<i>rho</i> operon leader peptide	6,476	<b>1.8</b>
<i>cspA</i>	b3556	Cold shock protein 7.4, transcriptional activator of <i>hns</i>	6,387	<b>15.9</b>
<i>ibpA</i>	b3687	Heat shock protein	6,120	<b>8.7</b>
<i>t44</i>	b4414	Small toxic membrane polypeptide	6,025	1.0
<i>folE</i>	b2153	GTP cyclohydrolase I	5,942	1.2
<i>ybiJ</i>	b0802	Hypothetical protein	5,817	<b>8.2</b>
<i>yefR</i>	b1112	Hypothetical protein	5,804	<b>9.3</b>
<i>yhcN</i>	b3238	Hypothetical protein	5,790	<b>17.3</b>
<i>ibpB</i>	b3686	Heat shock protein	5,787	<b>33.6</b>
<i>bfd</i>	b3337	Regulatory or redox component complexing with Bfr, in iron storage and mobility	5,582	<b>5.2</b>
<i>rplU</i>	b3186	50S ribosomal subunit protein L21	5,548	1.1
<i>rplN</i>	b3310	50S ribosomal subunit protein L14	5,323	-1.0
<i>dps</i>	b0812	Global regulator, starvation conditions	5,274	<b>2.6</b>
<i>trxC</i>	b2582	Putative thioredoxin-like protein	5,235	<b>8.1</b>
<i>marA</i>	b1531	Multiple antibiotic resistance; transcriptional activator of defense systems	5,164	<b>8.2</b>
<i>fur</i>	b0683	Negative regulator	5,145	<b>2.7</b>
<i>asnA</i>	b3744	Asparagine synthetase A	5,129	<b>5.5</b>
<i>yhaK</i>	b3106	Predicted pirin-related protein	5,103	<b>12.1</b>
<i>dnaK</i>	b0014	Chaperone Hsp70 in DNA biosynthesis/cell division	5,101	<b>1.7</b>
<i>csrC</i>	b4457	CsrC regulatory RNA	5,014	1.3
<i>fnr</i>	b1334	Transcriptional regulation of aerobic, anaerobic respiration, osmotic balance	4,981	<b>2.1</b>
<i>rplM</i>	b3231	50S ribosomal subunit protein L13	4,955	-1.1
<i>marR</i>	b1530	Multiple antibiotic resistance protein; repressor of <i>mar</i> operon	4,859	<b>31.5</b>
<i>rho</i>	b3783	Transcription termination factor Rho; polarity suppressor	4,768	<b>1.9</b>
<i>ffs</i>	b0455	4.5S RNA; component of ribonucleoprotein particle	4,752	<b>5.9</b>
<i>rpoD</i>	b3067	RNA polymerase, sigma 70 factor	4,751	<b>2.1</b>
<i>glgS</i>	b3049	Glycogen biosynthesis, <i>rpoS</i> dependent	4,747	<b>5.3</b>
<i>groE</i>	b4142	GroES, binds to Hsp60 in presence of Mg-ATP, suppressing its ATPase activity	4,731	1.2
<i>nlpI</i>	b3163	NlpI lipoprotein involved in cell division	4,698	<b>3.7</b>
<i>infC</i>	Z2747	Initiation factor IF-3	4,677	-1.1
<i>grxA</i>	b0849	Glutaredoxin 1 redox coenzyme for glutathione-dependent ribonucleotide reductase	4,675	<b>21.1</b>
<i>cspG</i>	b0990	Homolog of <i>Salmonella</i> cold shock protein	4,659	<b>80.7</b>
<i>rpmH</i>	b3703	50S ribosomal subunit protein L34	4,648	<b>1.8</b>
<i>bfr</i>	b3336	Bacterioferrin, an iron storage homoprotein	4,644	<b>4.5</b>
<i>yhhW</i>	c4228	Protein YhhW, putative pirin protein	4,644	<b>12.3</b>
<i>pdhR</i>	b0113	Transcriptional regulator for pyruvate dehydrogenase complex	4,590	<b>5.5</b>
<i>rplK</i>	b3983	50S ribosomal subunit protein L11	4,576	-1.2
<i>iscR</i>	b2531	Fe-S cluster-containing transcription factor	4,564	<b>2.3</b>
<i>rseA</i>	b2572	Sigma E (sigma 24) factor, negative regulator	4,530	<b>1.9</b>
<i>yceD</i>	b1088	Hypothetical protein	4,522	-1.0
<i>ryhB</i>	b4451	Regulatory RNA mediating Fur regulon	4,509	<b>2.4</b>
Z5401	Z5401	Hypothetical protein	4,485	1.3

<sup>a</sup> Signal intensity was calculated from all six biofilm arrays treated as one single sample group.

<sup>b</sup> Fold change (FC) in biofilm (BF) was calculated from all 6 biofilm arrays using all 12 planktonic arrays as the baseline. Boldface indicates significant change.

sion of brain microvascular endothelial cells by *E. coli* K1 (2) and was significantly induced in both 83972 and VR50.

**Comparison with expression in vivo.** Recently, we investigated the global gene expression profile of 83972 in vivo in

three patients and compared it with those in MOPS and in vitro urine (40). Normalization of the 9 patient arrays together with the 18 arrays presented in this study revealed that a majority of the genes that were significantly changed in the

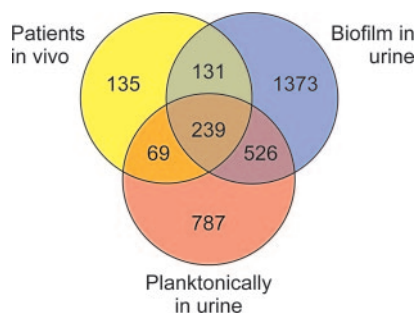


FIG. 4. Venn diagram showing numbers of significantly changed genes in *E. coli* 83972 during in vivo growth in three patients, biofilm growth, and planktonic growth in urine compared with that in MOPS. The diagram reveals that gene regulation of 83972 grown in patients more closely resembles that in biofilm than that in planktonic growth in urine.

patients were also regulated in the same way during biofilm growth. Of a total of 574 significantly changed genes in the patients compared with expression in MOPS, 64% (370 genes) were regulated in the same way (induced or repressed) during biofilm growth of 83972, and about half of these (177 genes) showed the same regulation during biofilm growth of VR50 as well. Of the 43 genes that were up-regulated in all three patients compared with growth in both MOPS and in vitro urine (Table 1 in reference 40), 23 were up-regulated in biofilm growth of 83972. Comparison of the regulation of 83972 grown in the patients with that of grown in vitro planktonically in urine and in biofilms revealed that the gene expression in biofilm growth more closely resembled expression of 83972 genes in patients than that in planktonically grown cells (Fig. 4); of the total of 574 significantly changed genes in the patients, 131 genes showed the same regulation as that during biofilm growth but no change compared with that of planktonically grown cells in urine, while only about half as many (69 genes) showed the same regulation as planktonic cells in urine but no change during biofilm growth. Interestingly, this could indicate that strain 83972 lives a partial biofilm-like existence in the human urinary tract.

**Verification of microarray results.** RT-PCR was performed to verify the transcript levels of selected genes. *aaeX*, *yqgA*, *ygiD*, and *c2422*, which all were highly up-regulated in biofilm of 83972 and VR50 (Table 2) and have not been reported to be up-regulated in other *E. coli* biofilm studies, were selected for RT-PCR. After 20 to 40 cycles of PCR, products of the four genes were visualized on agarose gels. The strength of the gel bands from each sample corresponded well to the actual signal intensities recorded from the corresponding microarray (unpublished data). 16S was used as a normalizing internal standard and was detected with the same intensity in all samples.

The expression levels of the *fim* genes reveal the sensitivity of the microarrays. The *fim* gene cluster of *E. coli* 83972 has a 4.25-kb deletion between *fimB* and *fimD*, resulting in the complete absence of *fimEAIC*, leaving only *fimF*, *fimG*, and *fimH* unaffected (26). The signal from *fimEAIC* was very low (absent call) and varied between 15 and 63 on the arrays hybridized with 83972, while the signals of *fimBDFGH* varied between 95 and 935. The signal levels (i.e., absent/present call) of the

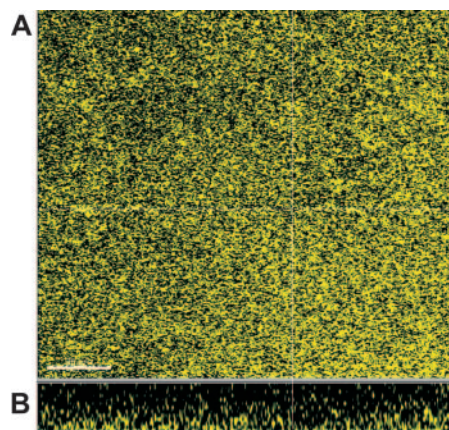


FIG. 5. Top view (A) and side view (B) of biofilm formation by ABU strain VR50 grown in human urine in a flow chamber. VR50 tagged with yellow fluorescent protein was grown in flow chambers in human urine at 37°C for 40 h, and biofilm formation was monitored by a scanning confocal laser microscope. The strain showed a flat biofilm structure lacking a highly structured matrix. The scale bar represents 30  $\mu$ m.

different *fim* genes on the arrays corresponded well to the actual presence of *fim* genes in *E. coli* 83972.

The down-regulation and low expression levels of motility genes were supported by results from motility testing on low-agar urine plates showing that 83972 and VR50 were non-motile. The down-regulation of genes encoding extracellular components, indicating a lack of highly structured biofilm morphology, was supported by examination of the biofilm structure using scanning confocal laser microscopy. Fluorescently tagged versions of 83972 and VR50 grown in urine for 40 h revealed biofilms with a smooth and flat structure; Fig. 5 shows the

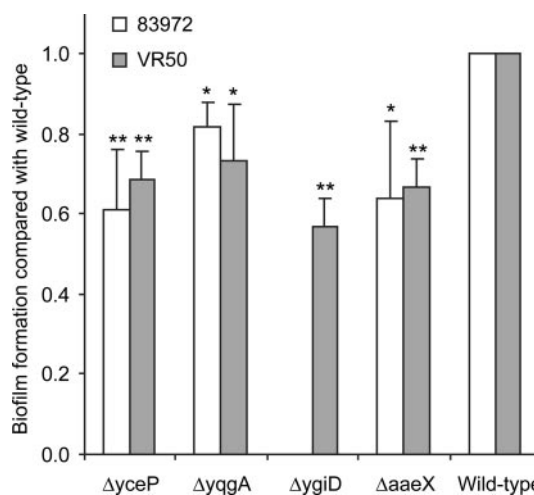


FIG. 6. Biofilm formation in polystyrene microplates in human urine of knockout mutants of the two ABU strains, 83972 and VR50. The cells were incubated for 16 h at 37°C, and  $A_{590}$  was measured after staining with crystal violet and washing three times with PBS. Values are shown relative to those of the wild type and are means of at least three separate experiments. Error bars indicate standard deviation ( $\delta_{n-1}$ ). All mutants formed significantly less biofilm than the corresponding wild-type strain (paired two-tailed *t* test; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).



biofilm formation of VR50 $yfp$  after 40 h in the flow-chamber system.

**Functional analysis of selected up-regulated genes.** To further verify the microarray results, four genes, which were highly up-regulated during biofilm formation in both 83972 and VR50, were selected for knockout mutagenesis. Three and four positive knockout mutants of 83972 and VR50, respectively, were obtained and subsequently analyzed for biofilm formation in microtiter plates in pooled human urine (Fig. 6). All knockout mutants showed significantly reduced biofilm formation in human urine compared to the wild type (paired two-tailed  $t$  test,  $P < 0.05$ ). The three mutants of 83972, i.e., 83972 $\Delta yceP$ , 83972 $\Delta yqgA$ , and 83972 $\Delta aaeX$ , reduced biofilm formation by 18 to 39%, and the four mutants of VR50, i.e., VR50 $\Delta yceP$ , VR50 $\Delta yqgA$ , VR50 $\Delta ygiD$ , and VR50 $\Delta aaeX$ , reduced biofilm formation by 26 to 43%.

## DISCUSSION

More than 50% of all microbial infections have been associated with the formation of biofilms (10). Notably, many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilms. Moreover, virtually all medical implants are prone to bacterial colonization and biofilm formation (19). Urinary tract infections often tend to become chronic, with many recurrent episodes. Moreover, patients with indwelling urinary catheters invariably get urinary tract infections within a few weeks (20). Arguably, biofilm formation could well be associated with the infectivity of UT-colonizing *E. coli* strains. Indeed, previous studies have advocated the importance of bacterial biofilm formation in urinary tract infections, notably in chronic cystitis and infections associated with catheters (33, 37).

The bacterial transcriptome is a dynamic entity that reflects the organism's immediate, ongoing response to its environment. DNA microarray-assisted functional genomics provides the global expression profile of the genome. Microarray expression profiling therefore provides a comprehensive snapshot of the transcriptome. The expression profile discloses how the bacterium adapts to an environmental niche. Adaptation to a given host environment is an extremely important parameter and underlies the capacity of an infectious agent to persist in a host. In nature, biofilms usually comprise multiple species; however, UTI in humans by *E. coli* are almost always caused by single strains (clones). We believe that our experimental approach, with surface-associated biofilm formation in human urine, mimics *in vivo* scenarios considerably, notably that of catheterized patients. Both our *E. coli* ABU strains, 83972 and VR50, were good biofilm formers under these conditions and provided ample material for global gene expression profiling. The microarray data revealed that the gene expression profiles of the two ABU *E. coli* strains, 83972 and VR50, during biofilm formation differ significantly from those observed during planktonic growth. Thus, expression of 815 genes was significantly changed in both strains during biofilm growth in urine compared with the gene profile in planktonic growth, and the large majority of these genes were affected in the same way (Fig. 3). The down-regulated genes were mainly involved in translation, metabolism, and energy production, while transcription was the largest up-regulated group, suggesting that

the two strains responded in much the same way to biofilm conditions.

Comparison of previous studies on gene expression profiles in bacterial biofilms has suggested that there may not be biofilm-specific genes. Instead, biofilms have a unique pattern of gene expression, where subsets of genes that are expressed in biofilms are also expressed under various planktonic conditions (28). A few other studies have addressed global gene expression in *E. coli* biofilms (5, 39, 44). However, these studies employed laboratory strains (*E. coli* K-12) and were performed in artificial growth media like minimal medium. Also, in all cases cells were harvested prior to stabilization of RNA. As expected, relatively few genes were affected in the same manner when comparing the individual studies. Nevertheless, in spite of the very different strains and experimental conditions, some of the biofilm-associated genes from previous studies were also affected in our ABU strains during biofilm formation in urine. For example, 8 out of 45 genes found to be up-regulated in *E. coli* K-12 strain MG1655 during biofilm formation in flow cells in minimal medium were also induced in both 83972 and VR50 during biofilm growth in urine, i.e., b2146, *cydA*, *frmR*, *hyaA*, *hybO*, *hypA*, *ndh*, and *ybfA* (44). Of the 24 genes up-regulated in *E. coli* K-12 strain TG1 during biofilm formation in minimal medium compared with both exponential and stationary growth phases (5), 7 and 13 were up-regulated in 83972 and VR50 biofilm, respectively, i.e., *rpoE*, *rseA*, *sixA*, *tatE*, *yceP*, *yebE*, and *yqcC* were induced in both strains, whereas *cpxP*, *pspABCD*, and *yjbO* were induced in VR50. Interestingly, the biofilm-related *yceP* and *yliH* genes (18) were highly up-regulated in our two ABU strains and have been reported to be up-regulated in all three preceding *E. coli* biofilm array studies (5, 39, 44). The exact roles of these two genes have not been fully revealed, and mutants show different biofilm-forming capacity depending on growth medium; they appear to be global regulators of several genes involved in catabolite repression and stress response and regulation of the uptake and export of signaling pathways (18). In our study,  $\Delta yceP$  mutants of 83972 and VR50 reduced biofilm formation in microplates in human urine by 39% and 31%, respectively.

It is interesting to note that some genes previously reported to be important for biofilm formation in *E. coli* were not affected in our ABU strains. Expression of fimbriae, notably type 1 fimbriae, has been observed to enhance biofilm formation in K-12 strains and is thought to promote attachment in the initial stages of biofilm formation (35). Our two ABU strains differ in their abilities to express fimbriae; strain VR50 can express functional type 1, whereas strain 83972 cannot (26, 41, 42). Yet they show similar growth characteristics and ability to form biofilm (Fig. 1), and they have similar gene regulation during biofilm growth (Fig. 3). Interestingly, in both strains neither intact nor residual fimbria-encoding gene clusters were activated during biofilm formation in urine. Human urine does not seem to promote expression of type 1 fimbriae; the *fim* gene cluster has not been reported up-regulated in human urine, neither in the UPEC isolate CFT073 (45) nor in 83972 grown *in vitro* or *in vivo* (40), and ABU strains expressing functional type 1 fimbriae in Luria-Bertani medium failed to do so in human urine (41). Our results suggest that type 1 fimbriae are not crucial for establishment of biofilm by UTI isolates in urine. Despite strain 83972's inability to express type

1 fimbriae, it forms significantly more biofilm than UPEC isolates CFT073 and 536 (Fig. 1), and even though VR50 is capable of expressing these surface elements, the *fim* genes are not expressed in urine biofilm. Several other genes encoding reportedly biofilm-associated products, such as Ag43 and extracellular matrix components, were not up-regulated either. Meanwhile, several virulence-associated genes, such as the *fyuA* gene, encoding Yersiniabactin receptor protein, and its adjacent genes belonging to the PAI IV<sub>536</sub> as well as *yliI* and *ygdP*, associated with virulence of *E. coli* O157:H7 and K1, respectively, were found to be induced in our strains during biofilm growth. This suggests that many virulence-associated genes are expressed during biofilm growth.

In a recent study (40), we investigated the global gene expression profile of 83972 in vivo in three patients. Comparison of the regulation of 83972 grown in the patients with those grown in vitro planktonically in urine and in biofilms revealed that the gene regulation during biofilm growth more closely resembled the regulation in patients than that in planktonically grown cells did (Fig. 4). Notably, about half of the genes up-regulated in 83972 in all three patients (Table 1 in reference 40) were induced in biofilm growth of both 83972 and VR50; the exceptions (i.e., genes up-regulated in patients but not in biofilm) were genes involved in nitrate/nitrite metabolism, the regulation of which probably reflects the specific in vivo environment in the patients. Interestingly, the fact that nearly all genes up-regulated in vivo, with exceptions for the nitrate/nitrite-related genes, were up-regulated in biofilm as well could indicate that strain 83972 partially lives a biofilm-like life in the human urinary tract.

There seems to be multiple mechanisms by which bacteria can form biofilms, employing combinations of various extracellular matrix molecules, such as surface proteins and sugar polymers and even DNA, for the establishment and build-up of the biofilm, combined with biofilm-associated changes in metabolism (6, 28, 48). The identification of key factors in biofilm formation has proven a difficult task, and the existence of a universal biofilm gene expression pattern has been questioned (3). Genetic and environmental factors greatly influence the biofilm formation; it has been demonstrated that diverse *E. coli* isolates respond very differently to changing environmental conditions (38), and many reports studying the influence of a specific "biofilm gene" have seen different, sometimes even reverse, effects on biofilm formation depending on growth media and strains used (4, 18, 23, 36, 55). In the present study, we used two genetically different clinical ABU isolates and studied these in their natural growth medium. From the present study it is not possible to identify the exact mechanism underlying the biofilm-forming capacity of the two ABU strains. However, the gene expression data indicate that the biofilms formed by these two strains lack a highly structured matrix, which was supported by microscopic analysis of the strains in the flow-cell system. Also, the results suggest that there must be other important determinants in biofilm formation that need to be discovered in order to explain why these two ABU strains are good biofilm formers and are superior to the UPEC isolates CFT073 and 536. The gene expression profiling also reveals that the biofilm life represents a very different growth mode, with many genes similarly expressed in the two strains. Down-regulation of translational genes and up-regulation of genes

encoding transport systems for drugs and metabolites, together with induction of many transcriptional and stress-related genes, indicate that the biofilm growth mode exerts great pressure on the cells, forcing them to slow down their growth rate and leading to a metabolic upset due to an intracellular stress condition.

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