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Original Article: Laboratory Investigation

Genome-wide transcriptome analysis of fluoroquinolone resistance in clinical isolates of *Escherichia coli*

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Abbreviations & Acronyms
cDNA = complementary deoxyribonucleic acid
DNA = deoxyribonucleic acid
FQN = fluoroquinolones
LB = Luria-Bertani
MIC = minimum inhibitory concentration
NS = not significant
psp = phage shock protein
pspC = phage shock protein C
RNA = ribonucleic acid
RT–PCR = reverse transcription polymerase chain reaction
TF = transformants
UTI = urinary tract infections

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Objectives: Coincident with their worldwide use, resistance to fluoroquinolones in *Escherichia coli* has increased. To identify the gene expression profiles underlying fluoroquinolone resistance, we carried out genome-wide transcriptome analysis of fluoroquinolone-sensitive *E. coli*.

Methods: Four fluoroquinolone-sensitive *E. coli* and five fluoroquinolone-resistant *E. coli* clinical isolates were subjected to complementary deoxyribonucleic acid microarray analysis. Some upregulated genes' expression was verified by real-time polymerase chain reaction using 104 *E. coli* clinical isolates, and minimum inhibitory concentration tests were carried out by using their transformants.

Results: A total of 40 genes were significantly upregulated in fluoroquinolone-resistant *E. coli* isolates ($P < 0.05$). The expression of phage shock protein operons, which are involved in biofilm formation, was markedly upregulated in our profile of fluoroquinolone-resistant *E. coli*. One of the phage shock protein operons, *pspC*, was significantly upregulated in 50 fluoroquinolone-resistant *E. coli* isolates ($P < 0.0001$). The expression of type I fimbriae genes, which are pilus operons involved in biofilm formation, were markedly downregulated in fluoroquinolone-resistant *E. coli*. Deoxyribonucleic acid adenine methyltransferase (*dam*), which represses type I fimbriae genes, was significantly upregulated in the clinical fluoroquinolone-resistant *E. coli* isolates ($P = 0.007$). We established pspC- and dam-expressing *E. coli* transformants from fluoroquinolone-sensitive *E. coli*, and the minimum inhibitory concentration tests showed that the transformants acquired fluoroquinolone resistance, suggesting that upregulation of these genes contributes to acquiring fluoroquinolone resistance.

Conclusions: Upregulation of *psp* operones and *dam* underlying pilus operons down-regulation might be associated with fluoroquinolone resistance in *E. coli*.

Key words: *Escherichia coli*, fluoroquinolone resistance, microarray.

Introduction

FQN are potent antimicrobial agents used to treat UTI. The worldwide use of FQN has caused a remarkable emergence of resistance among bacteria.[1] In particular, FQN resistance in *Escherichia coli* has become a serious problem in therapy for UTI. The mechanisms of FQN resistance in various bacteria include biofilm formation, overexpression of multidrug efflux pump related genes and chromosomal mutations of DNA gyrase and topoisomerase IV.[2–6] Phage shock protein (psp) response and pilus operons play an important role in acquiring pathogenesis of UTI and biofilm formation in *E. coli*.[7–9] Many investigators are making every effort to overcome FQN resistance by investigating novel mechanisms.[6,10] Gene expression profiling by cDNA microarray analysis has become a powerful tool for simultaneously determining the presence of a wide diversity of genes within given *E. coli* strain conditions. This method has been used in various studies involving taxonomy,[11] heat shock,[12] biofilm formation,[13,14] genotyping of microbial strains[15] and detection of environmentally important genes.[16,17] Recently, cDNA microarray analysis was used to detect

Table 1 Patient characteristics

	FQN-sensitive *E. coli* isolates ($n = 52$)	FQN-resistant *E. coli* isolates ($n = 50$)	*P*-value†
Age (years)			NS
Median	51	64	
Range	1–82	11–90	
Sex, *n* (% total)			NS
Male	10 (19)	15 (30)	
Female	42 (81)	35 (70)	

Susceptibility distribution of FQN-sensitive *E. coli* isolates

	≤0.03	0.06	0.13	0.25	0.5	4	8	16	32	64	128	>128‡
Ciprofloxacin	16	26	5	2	3							
Levofloxacin	17	25	5	2	3							

Susceptibility distribution of FQN-sensitive *E. coli* isolates

	≤0.03	0.06	0.13	0.25	0.5	4	8	16	32	64	128	>128
Ciprofloxacin						7	4	6	8	8	6	11
Levofloxacin						7	6	13	10	3	6	5

†*P*-value was determined by the Mann–Whitney *U*-test. ‡MIC (μg/mL).

antimicrobial resistance genes[18] and virulence genes[19–21] of *E. coli* and other strains. However, there is as yet no study on FQN resistance that has used cDNA microarrays with clinical *E. coli* isolates.

In the present study, we established the gene expression profiling specific to FQN resistance. We carried out the cDNA microarray screening test for the expression of 10 208 genes in four FQN-sensitive *E. coli* and five FQN-resistant *E. coli* clinical isolates. We validated the expression levels of some genes, which were upregulated in the FQN-resistant *E. coli* isolates from the screening test, in a larger number of clinical isolates from UTI by using real-time quantitative RT–PCR. In addition, we carried out MIC tests to determine whether transformation of FQN-sensitive *E. coli* with those upregulated genes could change their FQN susceptibility phenotype.

Methods

Bacterial strains and determinations of MIC

A total of 102 clinical *E. coli* isolates from urine of patients with UTI at Kagoshima University Hospital between 2007 and 2010 were used. Table 1 summarizes the patients' backgrounds and the antimicrobial susceptibility of each sample. All isolates of both FQN-sensitive *E. coli* and FQN-resistant *E. coli* groups were cultured under the same conditions. MICs were determined by using the microdilution method in cation-adjusted Mueller-Hinton broth according to Clinical and Laboratory Standards Institute (CLSI) guidelines, and these strains were defined as antibiotic susceptible or resistant to ciprofloxacin and levofloxacin.[22,23]

Total RNA extraction

Total RNA was extracted from the 102 *E. coli* isolates with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), as described previously.[24] RNase-free DNase (Qiagen) was added to the columns to eliminate DNA contamination according to the manufacturer's protocol. The quality of the RNA was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan).

cDNA microarrays and hybridization

Among the 102 *E. coli* isolates, 10 (five FQN-sensitive *E. coli* and five FQN-resistant *E. coli* isolates), which showed the top five lowest or highest susceptibility distribution of both ciprofloxacin and levofloxacin, were subjected to microarray analysis. Finally, one FQN-sensitive *E. coli* was omitted from the analysis as a result of the low quality of total RNA. Antimicrobial susceptibilities and the MIC of ciprofloxacin and levofloxacin with these nine isolates are shown in Table 2. GeneChip *E. coli* Genome 2.0 Array (Affymetrix, Santa Clara, CA, USA) was used to analyze the

Table 2 Antimicrobials susceptibility in samples for microarray analysis

Antimicrobials	FQN-sensitive *E. coli* ($n = 4$) MIC range (μg/mL)	FQN-resistant *E. coli* ($n = 5$)
Ciprofloxacin	≤0.03	32–>128
Levofloxacin	≤0.03	16–>128
Meropenem	≤0.03	≤0.03
Ceftazidime	0.25–0.5	0.25–0.5
Cefepime	0.06–0.25	0.125–0.25
Piperacillin	0.5–1	0.5–4
Amikacin	1–4	2–4

Susceptibility distribution of FQN-sensitive *E. coli* isolates ($n = 4$)

	≤0.03	0.06	0.13	0.25	0.5	4	8	16	32	64	128	>128†
Ciprofloxacin	4											
Levofloxacin	4											

Susceptibility distribution of FQN-sensitive *E. coli* isolates ($n = 5$)

	≤0.03	0.06	0.13	0.25	0.5	4	8	16	32	64	128	>128
Ciprofloxacin									1	1	2	1
Levofloxacin									2	2		1

†MIC (μg/mL).

complete *E. coli* transcriptome. Each microarray contained approximately 10 000 probe sets for all 10 208 genes present in four strains of *E. coli* (K12, CFT073, O157: H7-EDL933, O157: H7-Sakai). cDNA synthesis and labeling was carried out by following the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, RNA was denatured at 70°C with random primer and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA) at 42°C for 1 h. cDNA fragmentation was carried out by incubating first-strand cDNA with DNase I at 37°C for 10 min. Terminal labeling was carried out by incubating the cDNA product with GeneChip DNA Labeling Reagent (Affymetrix) and terminal deoxynucleotidyl transferase (Promega, Tokyo, Japan) at 37°C for 1 h. After hybridization of the fragmented- and biotinylated-cDNA to the chips, the hybridization signals were detected and quantified with a GeneChip Scanner 3000 (Affymetrix). The gene expression data were preprocessed and analyzed with GeneChip Operating Software (Affymetrix) from Bio Matrix Research, Chiba, Japan.

Real-time RT–PCR

To validate the differentially expressed genes in the microarray analysis, all RNA samples were subjected to real-time RT–PCR. As described previously,[25] we used a One Step PrimeScript RT–PCR Kit (Takara Bio, Otsu, Japan) for each primer set with PrimeScript RT enzyme Mix I (Takara Bio) for the RT reactions (+RT reactions) using 100 ng of total RNA. Control reactions were simultaneously carried out under the same conditions without RT (–RT reactions). Both reaction mixtures were aliquoted into 384-well ABI reaction plates. Gene-specific PCR products were continuously assayed by using a 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: stage 1 consisted of 42°C for 5 min; stage 2 consisted of 95°C for 10 s; and stage 3 consisted of 40 cycles of 95°C for 5 s, followed by 60°C for 30 s. The real-time RT–PCR data were analyzed with sodium dodecylsulfate 2.0 software (Applied Biosystems). Each set of +RT versus –RT reactions was compared against a standard curve generated for each primer set using *E. coli* linear DNA as a standard. The calculated quantities for each +RT or –RT reaction were standardized to each individual primer-set-generated standard curve, as described previously.[26] Data are expressed as the quantitative value of the +RT reaction minus that of the –RT reaction. Primer pairs to amplify *pspC* were 5′-GTCGATTTT CTTCGGTCTGG-3′ (forward) and 5′ACGTTCCATCTCGCGTAA AC-3′ (reverse) and primers for DNA adenine methyltransferase (*dam*) were 5′-TCAGTTCCGCGAAGAGTTCA-3′ (forward) and

5′-ACAGTACCGCCCGACGG-3′ (reverse). These primers were designed with the ABI PRISM Primer Express 2.0 software (Applied Biosystems). All reactions were carried out in duplicate, and a negative control lacking cDNA was included.

Cloning of the *pspC* and *dam* genes, construction of the expression vectors, and generation of pspC and dam-expressing *E. coli* transformants

Fragments of 1139 bp and 1227 bp containing full-length *pspC* and *dam* genes were amplified from cDNA extracted from the FQN-resistant *E. coli* isolate with marked expression of these genes using primers, as follows: *pspC*, CGACTCTAGAGGATCCCGCGCTATTTCTGGCTATTC (forward) and TACCGAG CTCGAATTCATCTCTTTTG CTTGCCCTGA (reverse); and *dam*, ACCGTCCAGCCA TTACACTC (forward) and TACCGAGCTCGAATTCTG TTTCATCCGCTTCTCCTT (reverse). These primers were designed so as to combine the original sequences with sequences of the restriction enzymes at their 3′ end. To construct the pspC and dam vector, full-length cDNA of *pspC* and *dam* were respectively inserted in the BamHI/EcoRI and PstI/EcoRI restriction sites of the pSTV29DNA vector, which has a chloramphenicol-resistant gene and *lac* promoter region (Takara Bio). DNA sequences for all constructs were verified by DNA sequencing (BIO MATRIX RESEARCH, Tokyo, Japan). Four FQN-sensitive *E. coli* isolates, which showed very low expression levels of *pspC* and *dam*, were treated with the rubidium-chloride method, as described elsewhere,[27] to deliver the vectors into competent cells. Then, the isolates were incubated on ice with the vectors followed by brief heat shock (42°C for 2 min) in order to allow the vectors to enter the isolates. Non-targeting vector (vehicle) was used as the control. The isolates were split and grown in LB broth supplemented with 50 μg/mL of chloramphenicol. To investigate whether FQN susceptibility is affected by *pspC* and *dam* expression, we selected the one with the highest expression of *pspC* and *dam* among the cultured isolates for the subsequent MIC tests. The isolates were precultured in LB broth supplemented with 12 μg/mL of lac operon inducer isopropyl-β-D-1-thiogalactopyranoside (*IPTG*) to activate the *pspC* and *dam* promoters.

Statistical analysis and annotation of gene function

The relationship between two groups and the numerical values obtained by the microarray analysis was analyzed with the Welch's *t*-test and that by real-time RT–PCR was analyzed with the Mann–Whitney *U*-test. The analysis software was Expert StatView (version 4; SAS Institute, Cary, NC, USA); differences of $P < 0.05$ were considered statistically significant.

The molecular functions of the up- and downregulated genes were classified into 11 groups, as referenced in the BLAST program available at the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/blast/) and EcoCyc (http://ecocyc.org): transcription, metabolism, transport, phage-related, DNA replication, carbon compound catabolism, pathogenesis, proteolysis, RNA-related, translation and open reading frames of unknown function.

Results

Antibiotic susceptibility of *E. coli* isolates from UTI patients

Our cohort included 52 FQN-sensitive *E. coli* and 50 FQN-resistant *E. coli* isolates. Most of the strains resistant to ciprofloxacin showed cross-resistance to levofloxacin (Table 1). Among these, nine isolates, which were selected as the most susceptible or resistant isolates to ciprofloxacin and levofloxacin, were subjected to microarray analysis. There was no cross-resistance to other microbials in these nine isolates, and the MIC distributions of ciprofloxacin and levofloxacin are summarized in Table 2.

Identification of genes differentially expressed between FQN-sensitive *E. coli* and FQN-resistant *E. coli* isolates by cDNA microarray analysis

Microarray analysis of nine *E. coli* isolates (four FQN-sensitive *E. coli* and five FQN-resistant *E. coli* isolates) identified 40 genes in FQN-resistant *E. coli* (12 with unknown functions) that were more than 2.5-fold upregulated compared with FQN-sensitive *E. coli* isolates ($P < 0.05$), as shown in Table 3. In contrast, just six genes in FQN-resistant *E. coli* were identified as downregulated by less than 0.5-fold relative to FQN-sensitive *E. coli* isolates (Table 4).

Molecular function of up- or downregulated genes in FQN-resistant *E. coli*

Figure 1 shows functional annotations of the 40 upregulated genes that were involved in transcription (18%), metabolism (12%), transport (12%), phage-related (7%), DNA replication (7%), other functions (15%) and open reading frames of unknown function (29%). The genes related to each category were as follows: transcription (*araC, fhlA, rseC, ybbI, ycgE, ygaV, yhhP*), metabolism (*crcA, tdcB, ycjM, hemN, mraW*), transport (*cycA, gltJ, nikB, ybeJ, ybhF*), phage-related (*pspC, pspD, pspE*), DNA replication (*dam, mutM, sulA*), carbon compound catabolism (*dhaL*), pathogenesis

Table 3 Frequently upregulated genes in FQN-resistant *E. coli* relative to FQN-sensitive *E. coli*

Genes	Molecular function	Ratio†	Signal intensity‡
ycjM	Predicted glucosyltransferase	4.31	4.27 ± 0.68
pspC	DNA-binding transcriptional activator	3.76	3.59 ± 1.75
yebE	Hypothetical protein	3.38	3.38 ± 1.09
rseC	SoxR and Sigma-E factor Regulatory protein	3.37	3.51 ± 0.61
yodA	Conserved metal-binding protein	3.31	2.81 ± 0.22
yqfA	Predicted oxidoreductase, inner membrane subunit	3.17	2.85 ± 0.26
c0583	Hypothetical protein	3.08	3.20 ± 0.81
ybeQ	Hypothetical protein	3.06	2.82 ± 0.21
pspE	Thiosulfate:cyanide sulfurtransferase	3.05	2.95 ± 0.34
fhlA	Formate hydrogenlyase transcriptional activator	3.04	2.34 ± 0.39
vacB/rnr	Exoribonuclease R, RNase R	3.00	2.94 ± 0.08
hemN	Coproporphyrinogen III oxidase	2.98	3.08 ± 0.46
ybhF	Fused predicted transporter subunits of ABC superfamily: ATP-binding components	2.96	2.97 ± 0.63
ygaV	Predicted DNA-binding transcriptional regulator	2.94	2.95 ± 0.74
crcA	Palmitoyl transferase for Lipid A	2.93	2.48 ± 0.34
yebA	Predicted peptidase	2.92	2.89 ± 0.32
pspD	Peripheral inner membrane phage-shock protein	2.91	3.02 ± 1.09
araC	DNA-binding transcriptional dual regulator	2.91	2.99 ± 0.62
dam	DNA adenine methylase	2.90	2.96 ± 0.74
mraW	S-adenosyl-methyltransferase	2.89	2.71 ± 0.30
yihE	Predicted kinase	2.79	3.13 ± 0.69
ybeJ/gltI	Glutamate and aspartate transporter subunit	2.76	2.72 ± 0.86
rygC	Unknown RNA	2.75	2.47 ± 0.45
yfeY	Hypothetical protein	2.74	2.77 ± 0.30
yncJ	Hypothetical protein	2.74	2.77 ± 0.62
ycgE	Predicted DNA-binding transcriptional regulator	2.69	2.42 ± 0.55
spr	Predicted peptidase, outer membrane lipoprotein	2.68	2.81 ± 0.30
ybbI/cueR	DNA-binding transcriptional activator of copper-responsive regulon genes	2.67	2.67 ± 0.38
dhaL	Dihydroxyacetone kinase, C-terminal domain	2.66	2.81 ± 0.89
sulA	SOS cell division inhibitor	2.65	3.17 ± 0.55
ytfP	Hypothetical protein	2.64	2.75 ± 0.41
cycA	D-alanine/D-serine/glycine transporter	2.63	2.19 ± 0.36
mutM	Formamidopyrimidine-DNA glycosylase	2.63	3.07 ± 0.55
ydhF	Predicted oxidoreductase	2.63	2.58 ± 0.25
yhhN	Conserved inner membrane protein	2.59	2.60 ± 0.52
yhhP/sirA	Cell developmental protein SirA	2.52	2.71 ± 0.40
ygaP	Predicted inner membrane protein with hydrolase activity	2.52	2.37 ± 0.65
gltJ	Glutamate and aspartate transporter subunit	2.51	2.51 ± 0.76
nikB	Nickel transporter subunit	2.51	2.33 ± 0.66
tdcB	Threonine dehydratase	2.50	2.46 ± 0.65

Welch's *t*-test, $P < 0.05$. †Ratio refers to expression in FQN-resistant *E. coli* relative to FQN-sensitive *E. coli* isolates. ‡Signal intensity was measured of the abundance of a transcript in FQN-resistant *E. coli* isolates.

(*vacB*), proteolysis (*yebA*), RNA-related (*rygC*), translocation (*yihE*) and 12 genes with unknown functions (*yebE*, *yodA*, *yqfA*, *c0583*, *yebQ*, *yfeY*, *yncJ*, *spr*, *ytfP*, *ydhF*, *yhhN*, *ygaP*). The annotations of the six downregulated genes were as follows: transport (*ynfF, ynfE*), metabolism (*cutF*), transcription (*hydG*) and unknown functions (*ygaW, ymgB*). We found that the following subsets of genes, which seem to be involved in FQN resistance, were simultaneously upregulated: the multidrug efflux system, which might confer multiple antimicrobial agent resistance (Table S1), and psp induced by membrane stresses (Table S2). In contrast, type I fimbriae (*fimAICDFGH*), virulent genes that are necessary for adhesion and biofilm formation, were downregulated in FQN-resistant *E. coli* (Table S3).

Table 4 Frequently downregulated genes in FQN-resistant *E. coli* relative to FQN-sensitive *E. coli*

Genes	Molecular function	Ratio†	Signal intensity‡
hydG	Transcriptional Regulatory protein zraR	0.21	0.21 ± 0.02
ygaW	Predicted inner membrane protein	0.30	0.30 ± 0.05
ymgB	Hypothetical protein	0.34	0.35 ± 0.12
ynfF	Oxidoreductase subunit	0.35	0.37 ± 0.10
ynfE	Putative dimethyl sulfoxide reductase chain ynfE precursor	0.45	0.45 ± 0.06
cutF/nlpE	Copper homeostasis protein cutF precursor	0.47	0.43 ± 0.05

Welch's *t*-test, $P < 0.05$. †Ratio refers to expression in FQN-resistant *E. coli* relative to FQN-sensitive *E. coli* isolates. ‡Signal intensity was measured of the abundance of a transcript in FQN-resistant *E. coli* isolates.

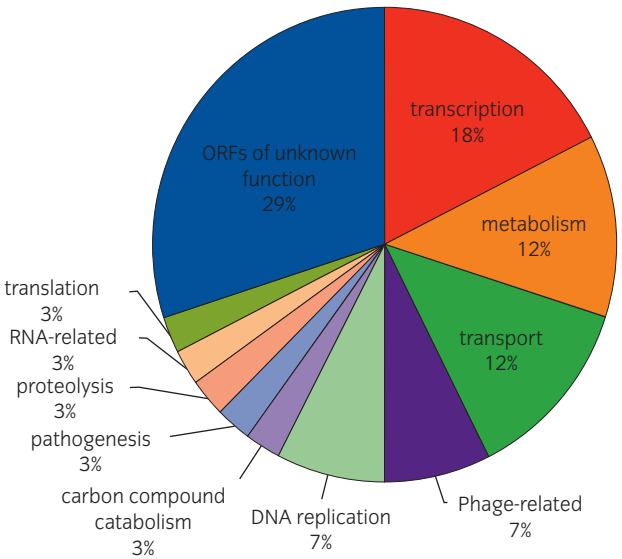

Fig. 1 Distribution of molecular-functional categorized genes in FQN-resistant *E. coli* isolates by microarray analysis. The functional features of upregulated genes were classified into 11 groups.

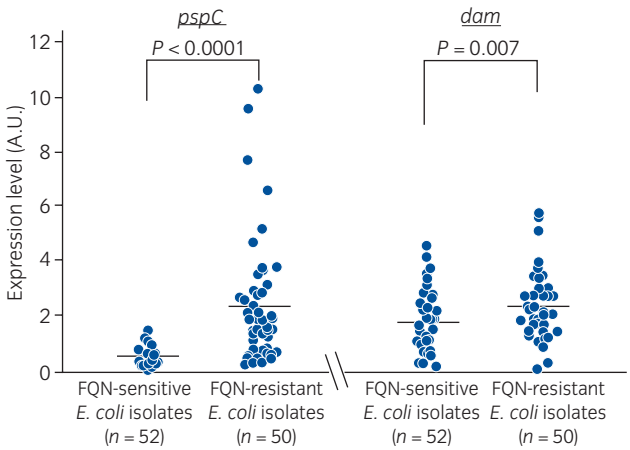

Fig. 2 Verification of the two upregulated genes (*pspC* and *dam*) from the microarray in 52 FQN-sensitive *E. coli* and 50 FQN-resistant *E. coli* clinical isolates by real-time RT–PCR. Statistical significance was determined by the Mann–Whitney *U*-test.

Real-time RT–PCR validation of microarray results

We focused on two genes (*pspC* and *dam*) among the genes that were upregulated in FQN-resistant *E. coli* in our microarray analysis because *pspC*, a psp, was the most upregulated among the *pspABCDE* genes in FQN-resistant *E. coli* isolates (Table S2), and *dam* is known to be involved in DNA replication, DNA repair, transcription and control of the expression of virulent genes.[28] Subsequently, we subjected these genes to real-time RT–PCR using 102 *E. coli* isolates and found that the genes shown by microarray analysis to be upregulated did indeed show higher expression in FQN-resistant *E. coli* than in FQN-sensitive *E. coli* isolates (*pspC*: 2.177 ± 0.292 [FQN-resistant *E. coli*] *vs* 0.389 ± 0.064 [FQN-sensitive *E. coli*], $P < 0.0001$; *dam*: 2.380 ± 0.177 [FQN-resistant *E. coli*] *vs* 1.680 ± 0.152 [FQN-sensitive *E. coli*], $P = 0.007$), as shown in Figure 2.

FQN susceptibility in pspC- and dam-expressing *E. coli* transformants

To determine whether FQN susceptibility is affected by *pspC* and *dam* expression, we constructed pspC- and dam-expressing *E. coli* transformants and subjected them to MIC tests. The MICs of ciprofloxacin and levofloxacin were respectively elevated from 0.03 to 16 μg/mL and 0.03 to 8 μg/mL through transformation of *pspC* and *dam* to the FQN-sensitive *E. coli* isolates (Table 5).

Discussion

To investigate new candidate genes involved in FQN resistance, we looked at the conventional mechanisms of antimicrobial-resistant *E. coli*, such as biofilm formation

Table 5 Antimicrobial susceptibility in transformants

	MIC (μg/mL) for			
	Wild type	Vehicle	*pspC*-TF	*dam*-TF
Ciprofloxacin	0.03	0.03	16	16
Levofloxacin	0.03	0.03	8	8

and multidrug efflux transport systems. Previous studies showed that the expression of pspABCDE operons, which are involved in biofilm formation, were strongly induced by membrane-altering stresses, such as heat shock, osmotic shock and exposure to organic solvents.[7,29] In our microarray data, *psp* family genes were upregulated in FQN-resistant *E. coli* isolates (Tables 3 and S2). We confirmed that the expression level of *pspC*, which was the most upregulated gene among the *psp* family genes, was substantially higher in a larger number of FQN-resistant *E. coli* isolates than it was in FQN-sensitive *E. coli* isolates. Furthermore, the transformation of the FQN-sensitive *E. coli* isolate with *pspC* resulted in acquired FQN resistance against ciprofloxacin and levofloxacin. These results suggested that psp might be induced by exposure of FQN to membranes and play an important role in FQN resistance in *E. coli*. There are multiple promoters, which activate entire expressions of the pspABCDE operon.[30] Because overexpressions of *pspABCDE* were found in FQN resistance in *E. coli* isolates that had no cross resistance to other antimicrobials, it is plausible that these promoters might be activated in FQN resistant isolates. Further examinations are necessary to elucidate the mechanism of the pspABCDE operon overexpression in FQN resistance. Previous reports showed that *pspABCDE* expression was induced during biofilm formation and development of bacteriophage filaments, such as type I fimbriae in *E. coli* cells.[31,32] Hence, we looked at the expression levels of type I fimbriae genes (*fimAICDFGH*), one of the pilus operons, which plays a role in the pathogenesis of acute UTI and the initial attachments of biofilm formation.[8,9] However, in our microarray data, all of the type I fimbriae genes were downregulated in FQN-resistant *E. coli* in comparison with FQN-sensitive *E. coli* isolates (Table S2), suggesting that the acquisition of FQN resistance in *E. coli* might not be associated with development of bacteriophage filaments.

To identify the new candidate genes contributing to FQN resistance, we focused on *dam*, of which the expression ratio was the highest (2.9-fold) of the genes in the DNA replication category (Table 3), because there have been no reports on the role of *dam* expression in FQN resistance. Previous studies showed that *dam* regulates various processes, such as DNA replication, repair and transcription.[33] Interestingly, *dam* represses pilus operons (including type I fimbriae genes) through promoter hypermethylation in UTIs,[28] and type I fimbriae genes were downregulated in our microarray data (Table S3). Because *dam* controls the expression of specific genes in response to environmental stimulation, it is plausible that bacterial strains might repress virulent gene expressions, such as type I fimbriae genes, and protect themselves from attack and detection by their host immune response, otherwise host cells would immunologically detect *dam* (–) strains easily because of the continuous virulent gene expression.[34,35] FQN-resistant *E. coli* might upregulate *dam* genes to repress the expression of virulent genes in order to protect itself from exposure to FQN. We confirmed that the expression level of *dam* was substantially upregulated in a larger number of *E. coli* isolates. In addition, the transformation of the FQN-sensitive *E. coli* isolate with *dam* resulted in acquiring FQN resistance against ciprofloxacin and levofloxacin. Thus, *dam* might play an important role in FQN resistance in *E. coli*. In contrast, a recent report showed that just four genes (*cynT*, *marA*, *gale*, *yfdO*) were overexpressed after exposure to FQN, and that they might be associated with FQN resistance. The data was derived from ASKA (A Complete Set of *E. coli* K-12 open reading frame archive), and *pspC* and *dam* were not listed as overexpressed genes in their study.[36] Because we used clinical *E. coli* isolates for the genome-wide expression analyses, these discrepancies might be caused by the samples subjected to the analyses. Another concern is that the *pspC* and the *dam* gene might contribute for cross-resistance to other antibiotics, such as penicillins, cephems or aminoglycoside. Therefore, further studies are necessary to elucidate the precise role of the *pspC* and the *dam* gene.

Multidrug efflux transport systems have been reported to be important mechanisms of FQN resistance in bacteria.[2–6] In *E. coli* cells, FQN can be extruded from the outer or inner membrane through the action of various efflux pumps. Previous studies have reported that *acrAB*, *mdfA* and *norE* are involved in FQN resistance.[37] *acrAB* encode multicomponent efflux pumps of the resistance-nodulation-division family that function in association with an outer membrane protein, TolC.[38] Consistent with these studies, our microarray data demonstrated that *acrA* or *acrB* showed a more than 2.5-fold enhancement of the expression ratio in FQN-resistant *E. coli* isolates in comparison with FQN-sensitive *E. coli* isolates (Table S1), even though they were not listed in the top 40 upregulated genes (Table 3). However, *MdfA*, which encodes MdfA MFS multidrug transporter and *norE*, which encodes NorE multidrug efflux MATE transporter,[39] were slightly upregulated in FQN-resistant *E. coli* in comparison with FQN-sensitive *E. coli* isolates, suggesting that these transporters might not be associated with FQN resistance in *E. coli*. Another major FQN resistance is chromosomal mutations of DNA gyrase, which is caused by quinolone resistance-determining region modifications.[3,6] It might be interesting to know how quinolone resistance-

determining region modifications interact with *pspC* and *dam* overexpressions. Further examination might be necessary to elucidate this matter.

In conclusion, upregulation of *psp* operones and *dam* underlying pilus operons downregulation might be critical for *E. coli* to acquire FQN resistance. Our gene expression profile of FQN-resistant *E. coli* might be useful for understanding the potential mechanisms of how *E. coli* acquires FQN resistance, and might yield new insights into potential antimicrobial therapies for UTI patients with FQN-resistant *E. coli* infection.

Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Multidrug efflux transport system related genes in FQN-resistant *E. coli* isolates
Table S2 Transcription unit: *pspABCDE* in FQN-resistant *E. coli* isolates
Table S3 Type I fimbriae: fimAICDFGH in FQN-resistant *E. coli* isolates

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Editorial Comment

Editorial Comment to Genome-wide transcriptome analysis of fluoroquinolone resistance in clinical isolates of *Escherichia coli*

Researchers studying antibiotic resistance use either defined laboratory strains or clinical isolates. The genetic backgrounds of laboratory strains are characterized, and various genetic permutations can be introduced, allowing direct comparisons among isogenic strains. The biggest disadvantage, however, is that these laboratory strains are not pathogenic, and therefore might fail to represent important variables found in the strains that cause disease. The obvious advantage of studying patient isolates is that these bacteria possess the genes and the transcriptional regulation necessary to cause infections, but they are so genetically diverse that direct comparisons are difficult. Therefore, these approaches are often combined; genetic alterations identified in clinical isolates are explored in isogenic laboratory strains, and genes discovered to affect antibiotic resistance in defined laboratory strains are then looked for in the clinic. Usually results from these two approaches agree, but when they do not, things get interesting.

The study by Yamane *et al*. shows an example of an interesting difference.[1] Using a genome-wide approach, they identified several novel genes that were upregulated in fluoroquinolone-resistant clinical isolates compared with fluoroquinolone-susceptible isolates. They focused on two of these genes, *pspC* and *dam*, and found that either of these genes cloned from a fluoroquinolone-resistant isolate increased fluoroquinolone minimal inhibitory concentration when introduced into a drug-susceptible *Escherichia coli* clinical isolate. These genes are not encoded on a pathogenicity island or a plasmid, but are highly conserved in the bacterial genome. This result raises the question of why