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HERB-RESISTANT UPEC STRAINS HAVE DIFFERENT BIOFILM FORMATION ABILITIES AND DIFFERENT EXPRESSION OF CERTAIN BIOFILM RELATED GENES

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Abstract

Background: The mechanisms and patterns of drug resistances of E. coli strains that cause uncomplicated urinary tract infections (UTIs) vary considerably. The emerging herb-resistance of uropathogenic Escherichia coli (UPEC) has been a serious health problem, yet with unknown underlying mechanisms.
Methods: To explore the potential herb-resistance mechanisms of E. coli strains that cause uncomplicated UTIs, three clinically isolated herb-resistant UPEC strains (1351, 4996, 5028) were analyzed for their abilities to form biofilms and the expressions of the *pga ABCD* and luxS genes.
Results: We found that the expression of *pgaA* and *pgaB* are very different between 1351 and 5028, and the loss-of-function of *luxS* in 4996 has impact on biofilm formation.
Conclusions: 1. Herb-resistance of the strains is related to their abilities of biofilm formation. 2. Biofilm formation capabilities of herb-resistant strains show different responses to the presence of glucose. 3. *luxS* encoded AI-2 is not essential for biofilm formation in this strain but may help with more biofilm formation.

Key words: Herb-resistance; Biofilm formation; Uropathogenic Escherichia coli (UPEC); *pga ABCD*; *luxS*.

Introduction

Uncomplicated urinary tract infections (UTIs) are one of the most common infection diseases still occurring today, with more than half of the world women population acquire it at least once during their lifetimes (Hooton, 2012). In more than 80% of the cases, the infection is caused by *E. coli* strains (Nicolle, et al., 2006). However, both the mechanisms and patterns of antibiotic resistances of *E. coli* strains that cause the Uncomplicated UTIs vary considerably at different regions and among different populations (Aypak, et al., 2009; Gupta, et al., 2011; Wiedemann , et al., 2014), making an effective and accurate clinical antibiotic remedy more difficult. Recently, the Chinese herbs have been tested and studied for their abilities to tackle the fluoroquinolone-resistant *E. coli* strains that cause the Uncomplicated UTIs (Tong, et al., 2011a; Tong, et al., 2011b).

Naturally bacteria live in surface-associated biofilm communities that contain bacterial aggregates and extracellular polymeric matrix. The biofilm serves as a protective enclosure to shield the bacteria from environmental stresses and antimicrobial chemicals (Heilmann and G€otz, 2010; Vogeleer, et al., 2014). In addition to the exopolysaccharides that serve as the key components of the structure, the biofilm also contains proteins, lipids, metabolites and nutrients etc. Three polysaccharides, β-1,6-N-acetyl-D-glucosamine polymer (PGA), colonic acid and cellulose, have been shown to be important for biofilm formation (Beloin, et al., 2008). A *pgaABCD* operon was discovered to be responsible for the synthesis and export of PGA (Wang, et al., 2004; Itoh, et al., 2008). While *pgaC* encodes a GT-2 glycosyltransferase, *pgaD* encodes a protein without any known structure (Itoh, et al., 2008) . Protein encoded by *pgaA* and *pgaB*, however, are involved in the export of PGA, with *pgaA* forming a β-barrel (Whitney, et al., 2011) and *pgaB* bearing a deacetylase

domain (Cantarel, et al., 2009) and belongs to the carbohydrate esterase 4 family (Nishiyama, et al., 2013). The biofilm formation also depends on a quorum-sensing system, the *luxS* encoded autoinducer 2 (AI-2) acting as a signal molecule (Vidal, et al., 2013; Vidal, et al., 2011; Trappetti, et al., 2011; Joyce, et al., 2004).

Here from three clinically isolated herb-resistant uropathogenic Escherichia coli (UPEC) strains, we found they had different abilities to form biofilms, different expressions of *pgaA* and *pgaB* genes, and the function of *luxS* has impact on biofilms formation. Therefore, these three UPEC strains may have different mechanisms of drug-resistance.

Methods

UPEC strains

Three UPEC strains (1351, 4996, 5028) were isolated clinically from a hospital at the Hebei Medical University, which contained a herb-resistant plasmid and showed various resistance to a Chinese herbal extract.

Biofilm measurement

E. coli strains were grown on LB plates for overnight. Single colonies were selected and cultured in 10 ml liquid LB for 18 hr. The cultures were then diluted to 0.5 McFarland, 2 μl of culture was transferred to a well of a 96-well plate and diluted to 200 μl with LB. Each sample had three repeats in three wells. After incubating the 96-well plate at 37°C for 24 hr, the LB culture was discarded and the plate was rinsed with 0.9% NaCl three times, air dried, and stained with 1% crystal violet for 5 min. The plate was then rinsed with ddH_2O three times and air dried. The biofilm formed in the plate wells were then dissolved in 30% acetic acid and measured for their absorbance at 570 nm. The OD_{570} values were analyzed with non-paired t-test for statistical significance.

Competent cells and transformation

To make competent 4996 cells, a single colony of strain 4996 was inoculated into 10 ml LB medium and cultured for 18 hr. From this culture, 1 ml was transferred into a new 10 ml LB culture, which was cultured at 37°C until OD_{600} reached 0.6. The culture was placed on ice for 30 min before the cells were collected by centrifugation at 4°C for 10 min at 4000g. The cells were resuspended in 1X volume of pre-chilled 10% glycerol for washing and collected again under the same condition. Finally the cells were resuspended in 3 ml of pre-chilled 10% glycerol and stored in -80°C as aliquots of 100 μl. For transformation, a tube of competent cells (100 μl) was thawn on ice before 10 μl of plasmid solution was added. The cell mixture was then transferred to a 2 mm cuvette for electroporation (200 Ohm，25 uFd，2.5 KV) and 300 μl of LB was added in. After culturing at 37°C for 1 hr, cells were spread on zeiocin plates for selection.

Knock-out protocol

To knock out the *luxS* gene in strain 4996, a pET32a-zencin plasmid was constructed, which contains a 1322 bp fragment upstream of luxS, a zeocin gene, and a 1049 bp fragment downstream fragment of luxS.
luxS- upstream-F-BamHI: CGGGATCCCGCTGTATCGTGAAGGGCTGAA
luxS- upstream-R-NdeI: GGAATTCCATATGTTAGCCACCTCCGGTAATTTTTTTAAA
Zeo- upstream - NdeI: GGAATTCCATATGGTGTTGACAATTAATCATCG
Zeo- upstream - NheI: CTAGCTAGCTCAGTCCTGCTCCTCGGC
luxS- downstream-F-NheI: CTAGCTAGCTCAGTAAACTATCTTCACAATTAATTGAAAA AAG
luxS- downstream-R-XhoI: CCGCTCGAG GACACTGCAAACTCTGCGCGG

Primer designed and tested

The specific PCR primers of five key genes (*luxS*, *pgaA*, *pgaB*, *pga*C and *pga*D) were designed as following.
pgaA: TCTTGCGGCGTATATTGGTAGG, CGACCCGACAATCACCAGTACG
pgaB: CGACGAAATGCGGCAATAACAC, GCGGCGGCATATATTGTGGAAC
pgaC: TCACCATCGGGATCAGCAAAT, GCAGCAGAATACCGGGAAAGA
pgaD: GGGATCTTGCGGTTCACGTTC, AACGCACCAGGAAATCAGGGAC
luxS: TGCCGAACAAAGAAGTGATG, CTTCGTTGCTGTTGATGCGTAC
These primers and PCR conditions (95 °C 15 min, 95 °C 10 sec, 95 °C 32 sec; 40 cycles) were first tested with genomic DNA template and agarose gel electrophoresis. These five pairs of primers were used successfully to amplify the five target genes from all three UPEC strains.

qRT-PCR expression detection

A two-step qRT-PCR method was developed using the PCR primers tested. RNA was isolated from these two strains (RNAprep pure Kit, DP430, TIANGEN Biotech, Beijing) grown in LB medium with 0.5% glucose, reverse-transcribed (FastQuant RT Kit, KR106, TIANGEN Biotech, Beijing) and used as template for qPCR reactions (SuperReal PreMix Plus, FP205, TIANGEN Biotech, Beijing) using ABI 7500 fast. The dissociation curves for the amplification of these five genes indicated specific single product amplification, as shown in Figure 4.

Results

The in-vitro biofilm formation capabilities of these three strains are similar in LB medium

Three UPEC strains (1351, 4996, 5028) were isolated clinically from a hospital at the Hebei Medical University, and showed various resistance to a Chinese herbal extract (data not shown).

To gain some insight to the mechanism of herb resistance, the biofilm formation abilities of these three strains were tested. As indicated in figure 1, the herb extract-sensitive control strain Trans10 showed very low reading at OD_{570}, but all three herb extract-resistant UPEC strains showed more than five times higher of readings ($P<0.05$), indicating these three strains had much higher ability to form biofilm than the control strain. However, when compared among each other, there was no significant difference observed, indicating there is probably no significant direct correlation between the biofilm formation capability and their resistance to herbal extract, since these three strains showed different herb tolerance (Tong, et al., 2011b).

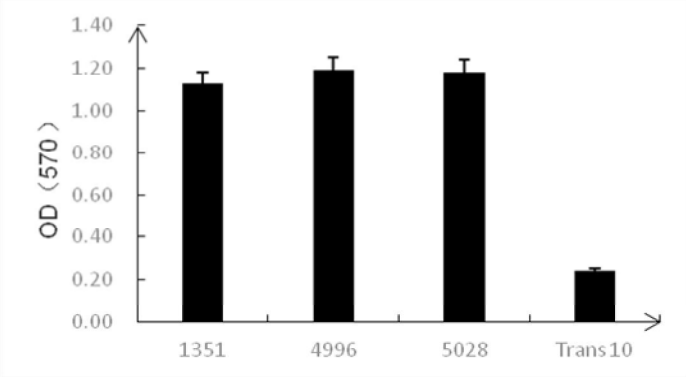

Figure 1: Biofilm formation test

Glucose in the medium has a different impact on the biofilm formation for these three UPEC strains

To see if there is any difference in their abilities to form biofilms under different culturing condition, these three UPEC strains were tested again in LB medium with 0.5% glucose. Interestingly, these three strains showed very different responses to the glucose in the medium (Figure 2). While strains 4996 and 5028 showed significantly higher abilities to form biofilm when 0.5% of glucose was included in the media, strain 1351 showed the opposite response with much reduced biofilm formation (Figure 2, Table 1). As shown in Table 1, strain 1351 showed 64% reduction of biofilm formation, but strains 4996 and 5028 showed increases of 53.3% and 143.0%, respectively.

Table 1: Effect of glucose on biofilm formation (OD_{570})

	LB	LB (0.5% sugar)	difference	P value
1351	1.042	0.375	-64.0%	0.00016
4996	0.946	1.45	53.3%	0.0004
5028	0.621	1.509	143.0%	0

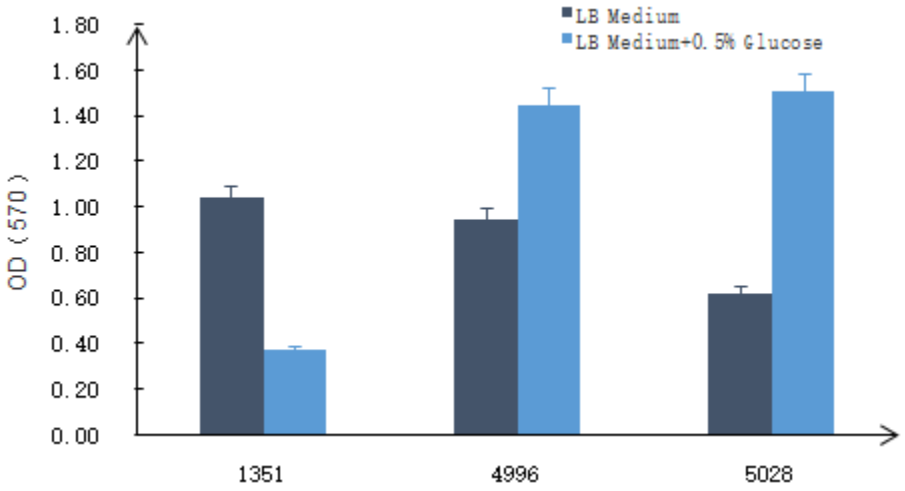

Figure 2: The effect of sugar in the LB medium on biofilm formation

Expression analysis of genes involved in the regulation of biofilm formation

To look more into the mechanisms of biofilm formation of these three UPEC strains, the expressions of five key genes (*luxS*, *pgaA*, *pgaB*, *pgaC* and *pgaD*) involved in the regulation of biofilm formation were examined, using the qRT-PCR method developed (Figure 3 and Figure 4; detail of the method see materials and methods section). Since strains 4996 and 5028 showed similar abilities of biofilm formation with or without glucose in the medium, only 5028 and 1351 were tested for the expression of these five genes.

The expression of *pgaC* between strain 1351 and 5028 are very similar, but 5028 showed 30-50% higher expression of both *pgaD* and *luxS* (Figure 5 and Table 2). However, dramatic expression differences were detected on *pgaA* and *pgaB* between these two strains. Strain 5028 showed 10 times higher expression of *pgaA* and 20 times higher expression of *pgaB* (Figure 5 and Table 2). This may be correlated with their very different responses of biofilm formation when 0.5% glucose was added into the LB medium (Figure 2 and Table 1).

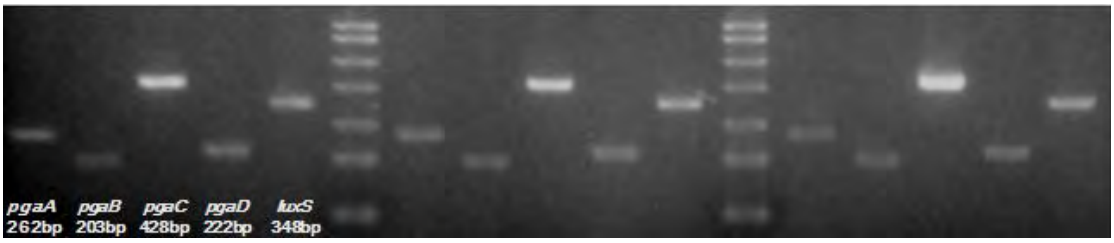

Figure 3: PCR amplification of *pgaA*, *pgaB*, *pgaC*, *pgaD* and *luxS* genes

Lane 6 and 12：DNA ladder (bp) 700, 600, 500, 400, 300, 200, 100. Lane 1, 7, 13 Amplification of pgaA gene. Lane 2, 8, 14 Amplification of pgaB gene. Lane 3, 9, 15 Amplification of pgaC gene. Lane 4, 10, 16 Amplification of pgaD gene. Lane 5, 11, 17 Amplification of luxS gene. Lane 1-5 Amplification with 1051 genome DNA as template. Lane 7-11 Amplification with 4996 genome DNA as template. Lane 13-17 Amplification with 5028 genome DNA as template.

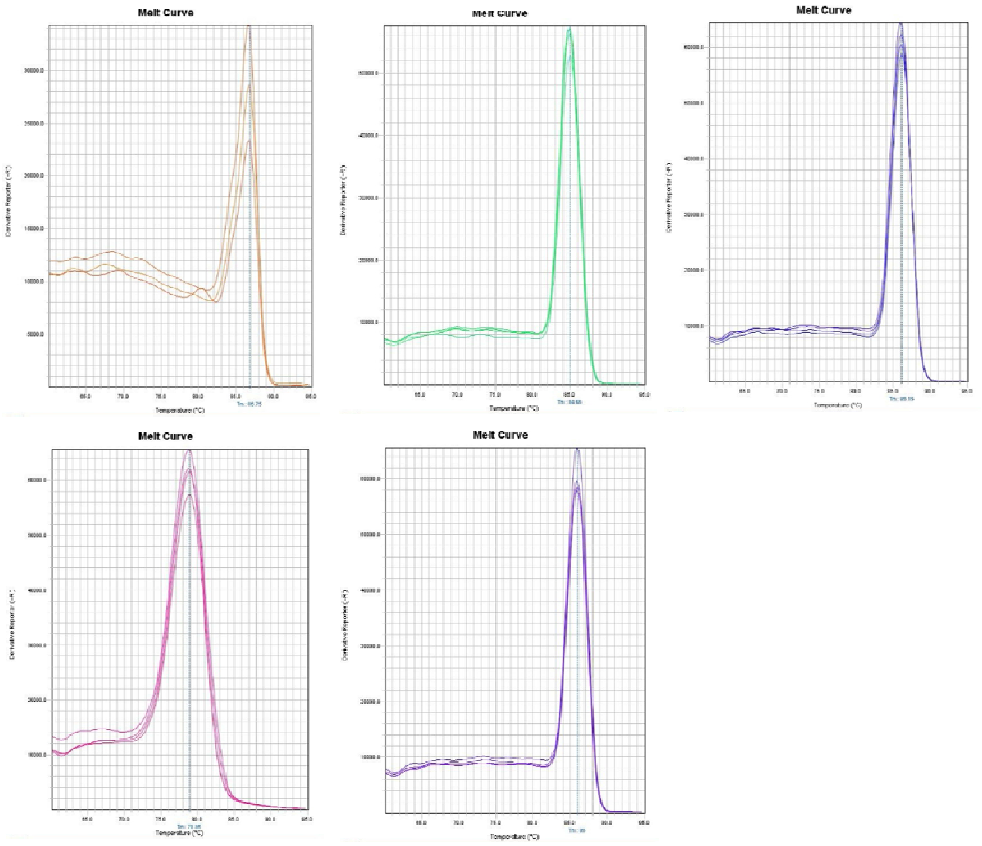

Figure 4: Dissociation curves of qRT-PCR amplification of *pgaA*, *pgaB*, *pagC*, *pgaD* and *luxS*, respectively

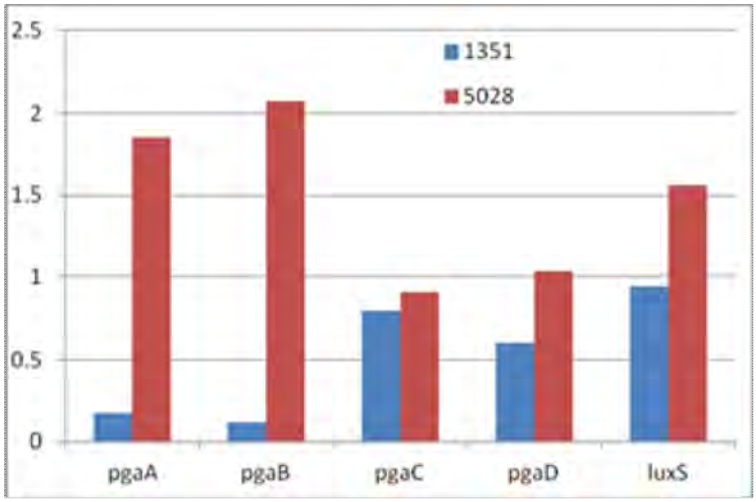

Figure 5: Expressions of *pgaA*, *pgaB*, *pgaC*, *pgaD* and *luxS* in strains 1351 and 5028

Table 2: Gene expression in 1351 and 5028 revealed by qRT-PCR

	pgaA	*pgaB*	*pgaC*	*pgaD*	*luxS*
1351	0.171	0.114	0.793	0.605	0.945
5028	1.847	2.071	0.907	1.032	1.556

Loss of luxS function had slight impact on biofilm formation

Between strain 1351 and strain 5028, their biofilm forming abilities are very different in LB medium with 0.5% glucose (Figure 2 and Table 1). However, among the five genes tested, only the expressions of *pgaA* and *pgaB* are very different (Figure 5 and Table 2). To explore further the involvement of these genes in biofilm formation, luxS knockout line was constructed in strain 4996 and tested for its ability of biofilm formation.

To knock out the *luxS* gene in strain 4996, a pET32a-zencin plasmid was constructed, which contains a 1322 bp fragment upstream of luxS, a zeocin gene, and a 1049 bp fragment downstream fragment of luxS. These three DNA fragments were PCR amplified using the primers listed in materials and methods section, cloned onto plasmid pET32a using the restriction enzymes indicated in the primer names. The final construct was confirmed by restriction enzyme digestions and sequencing. The PCR amplified fragments are shown in Figure 6a and b. The restriction enzyme digestion confirmation of the final construct is shown in Figure 6c.

Plasmid pET32a-zeocin was introduced into strain 4996 via electroporation with prepared competent 4996 cells. After transformation, cells were spread on zeiocin plates for selection. Positive clones were identified using PCR reactions showing positive on the amplification of the zeocin gene and negative on the amplification of *luxS*, as indicated in Figure 7. Both 4996 and 4996ΔluxS strains were tested for their abilities to form biofilm. As indicated in Figure 8, 4996ΔluxS showed a decrease of about 30% of biofilm formation (P=0.016). Therefore, luxS may not be playing a prominent role in biofilm formation in UPEC strains, which is consistent with the conclusion drawn from the gene expression data comparing strains 1351 and 5028 (Figure 5 and Table 2).

Discussion

These three UPEC strains (1351, 4996, 5028) with herb-resistant plasmid showed very similar biofilm formation capabilities that were much stronger than the control sensitive strain Trans 10 (Figure 1), indicating the herb-resistance of these strains is related to their abilities of biofilm formation. However, when glucose was added into the media, both strain 4996 and strain 5028 showed similar degrees of elevated biofilm formation capabilities, but strain 1351 showed a significant decrease of biofilm formation (Figure 2). This observation seems to indicate 1351 has a very different response to the presence of glucose, which presumably was used as blocks or precursors of the PGA biosynthesis. Therefore, it is reasonable to postulate that strain 1351 might have lost its ability to process the glucose for biofilm formation. For biofilm formation testing, it would be more informative to test different sugars at different concentrations for these three strains. The molecular mechanism behind the different biofilm formation abilities when glucose was added into the media may be due to the existence or missing of certain plasmid that bears the function of related genes, or certain mutations on chromosome. Whole genome sequence comparison among these three strains should yield some hints to the mechanisms behind.

When the expression of genes involved in PGA biosynthesis and export were tested, both *pgaA* and *pgaB* showed dramatic difference between strains 1351 and 5028, while the other three genes showed similar levels (Figure 5). The much higher expression of *pgaA* and *pgaB* in strain 5028 compared to 1351 seemed to be consistent with the biofilm formation abilities in the presence of 0.5% glucose (Figure 2). Based on this observation, one is almost enticed to speculate that the presence of 0.5% glucose in the media suppressed the expression of *pgaA* and *pgaB* in 1351 but not in 5028. This seems to be consistent with the fact that less biofilm was formed (Figure 2) since less PGA would have been exported from the cells without necessary expression of *pgaA* and *pgaB*. It would be nice to have control experiment with LB medium without glucose, to confirm the effect of glucose on biofilm formation. This makes one wonder if there is any sequence differences between these two strains for the *pgaA* and *pgaB* regions.

Lastly, when the luxS gene was knocked out from strain 4996, its ability to form biofilm was only reduced about 30% (Figure 8), indicating the *luxS* encoded AI-2 is not essential for biofilm formation in this strain but may help with more biofilm formation.

Conclusions

1. Herb-resistance of the strains is related to their abilities of biofilm formation. 2. Biofilm formation capabilities of herb-resistant strains show different responses to the presence of glucose. 3. *luxS* encoded AI-2 is not essential for biofilm formation in this strain but may help with more biofilm formation.

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Declaration: Authors declare that this research presents no conflict of interests.

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