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Possible Involvement of *Mycoplasma hominis* in Inhibiting the Formation of Biofilms by Uropathogenic *Escherichia coli* (UPEC)

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Here we examined the involvement of *Mycoplasma hominis* in the formation of biofilms by uropathogenic *Escherichia coli* (UPEC) strain CFT073. Initially, we thought that *M. hominis* does not affect the fitness of UPEC, including the growth and production of signaling molecules, such as autoinducer-2 and indole. We found, however, that the presence of *M. hominis* significantly decreased the degree of biofilm formation by UPEC CFT073 (approximately a 60% reduction for 10^5 ccu/mL of *M. hominis* as compared with UPEC alone). We also found that it had a slight effect in inhibiting the attachment and cytotoxicity of UPEC CFT073. These findings are specific to these UPEC strains rather than to enterohemorrhagic *E. coli* (EHEC) strains, found in normal intestinal flora. In addition, we performed whole-transcriptome profiling and quantitative real-time polymerase chain reaction (qRT-PCR) analysis. This indicated that the PhoPQ system and the anti-termination protein (encoded by *ybcQ*) were involved in the reduction of biofilm formation by *M. hominis* (corroborated by qRT-PCR). Furthermore, our results indicate that *M. hominis* raises the degree of transcription of toxin genes, including *hha* and *pasT*. Hence, we suggest a possible role of *M. hominis* in affecting the formation of biofilms by UPEC in the urinary tract.

Key words: biofilm formation; *Mycoplasma hominis*; uropathogenic *Escherichia coli*; whole transcriptome analysis

It has been reported that diverse microorganisms commonly interact with each other in clinically-relevant host environments, such as the oral, respiratory, intestinal, and urinary tracts.[1,2] Within the urinary tract (UT) in particular, there is a remarkable microbial community and it interacts extensively with other habitants including bacterial populations and other microorganisms. Hence it is not surprising that UT infections are among the most common bacterial infections.[3] These complex communications often lead to microbial pathogenesis. To date, however, a large number of studies have focused mainly on the relationships among bacterial strains, between bacteria and fungi, and between bacteria and hosts, but very little is known about the genetic mechanisms associated with these interactions. Hence, attempts have been made to discern the etiology of microbial diseases.

Among the microorganisms responsible for UT infections, a number of key bacterial species are commonly implicated, including *Escherichia coli*, *Proteus*, *Klebsiella*, and *Staphylococcus saprophyticus*. Viruses, fungi, and parasites can also cause UT infections. Among the bacterial sources, uropathogenic *E. coli* (UPEC) is the most prevalent cause of uncomplicated UTIs, and it is one of the most common human pathogens.[4] Maintaining a certain level of persistence and virulence in dynamic microbial UT environments, several microorganisms are able to colonize surfaces and grow as communities, known as biofilms. These are commonly embedded within the extracellular polymeric substance (EPS).[5] UPEC have been found to form biofilms within the superficial umbrella cells of murine bladders.[6] A more recent study suggested that the ability to form biofilms plays a key role in protecting UPEC against host defenses, while it aids in resistance to antibiotics.[7] It is well established that bacterial biofilm formation follows a five-stage process: i) initial (reversible) attachment of bacteria to host cell surface, ii) firmly adhered (irreversible) attachment and production of EPS, iii) early development of biofilm architecture, iv) maturation of three-dimensional biofilm architecture, and v) dispersion of single cells from the biofilm.[8] Within the biofilm, bacterial dynamics interact extensively with the surrounding matrix, and there is regional variation in the expression of biofilm-associated factors. In recent, years it has been established that fimbrial genes[4] and autotransporter genes[9] (involved in reversible or irreversible attachment) as well as signaling molecule genes including cyclic di-guanylate monophosphate (c-di-GMP)[10] (involved in irreversible attachment and the maturation of biofilm) are critical in the development of UPEC biofilms, but the exact mechanisms of UPEC biofilm production remain unclear.

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Mycoplasmas are the smallest free-living microorganisms without a cell wall. They are widespread throughout nature as commensal organisms, but with potential pathogenicity in animal, plant, and human hosts.[11] Among these mycoplasmas, *M. hominis*, in particular, is deemed a causative agent of UT infection. Its etiological involvement in extragenital (non-UT) infections has also been described, particularly in immuno-compromised patients.[12] To date, however, no studies have reported interaction between mycoplasmas and bacterial flora in human organ systems, including the UT.

We conducted this study to determine the novel hypothesis that mycoplasma, a normal urinary flora, is involved in the formation of biofilm by UPEC (rather than EHEC). In addition, we performed DNA microarray and qRT-PCR studies to clarify the roles of novel genes that might be involved in the formation of biofilms by UPEC, depending upon the presence of mycoplasma.

Material and Methods

Mycoplasma, bacteria, and cell line. *M. hominis* type strain PG21 (ATCC 23114) was cultured in a Hayflick modified medium supplemented with 5 mM arginine at 37 °C for 48 h.[13] Numbers of bacteria were evaluated in color-changing units (CCU) in broth media. UPEC strain CFT073,[14] EHEC O157:H7 strain EDL933, and ATCC 43894[15] were grown in Luria-Bertani (LB) medium at 37 °C. The HeLa cell line was routinely cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, NY). The cells were cultured at 37 °C in 5% CO_2 in a humidified atmosphere.

Cytotoxicity assay. The cytotoxicity of UPEC CFT073 with and without *M. hominis* PG21 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described.[16] Briefly, HeLa cells were seeded in a 96-well plate (1×10^4 cells per well). After 24 h, the wells were washed 3 times with phosphate-buffered saline (PBS, to remove nonattached cells. UPEC CFT073 (1×10^6 cfu/mL) in the presence of *M. hominis* (10^3 and 10^5 ccu/mL) was added to the wells, which were then incubated at 37 °C for 24 h. Following MTT treatment, the absorbance was measured at a wavelength of 540 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA). PBS was used as negative control. The percentage of viable cells was calculated by the following formula:

$$\text{Cell viability (\%)} = (\text{OD of the experimental group} / \text{OD of the control group}) \times 100.$$

Attachment assay. The attachment of UPEC was tested, as previously described, with some modifications.[17] Prior to the attachment assay, the HeLa monolayers were washed 3 times with PBS to remove culture medium and non-attached cells. The UPEC strains (1×10^6 cfu/mL) with *M. hominis* (10^3 and 10^5 ccu/mL) were added to the monolayers at 37 °C in an atmosphere of 5% CO_2. After the passage of 6 h for attachment, the monolayers were washed 6 times with PBS to remove non-attached bacteria. This was followed by collection of the attached cells with a cell scraper. Serial dilutions of the mixture were plated onto LB agar and this was incubated at 37 °C for 24 h.

Cell signaling assays. Autoinducer-2 (AI-2) production and indole production were measured previously.[18] Cell-free supernatants from UPEC strain with *M. hominis* (10^3 and 10^5 ccu/mL) were collected after 3 and 9 h of incubation and added at a concentration of 10% (v/v) to reporter strain *Vibrio harveyi* BB170, which was grown in AB medium overnight and diluted 1:5,000 into fresh AB medium. After culturing at 30 °C for 4 h, bioluminescence was measured with a luminometer (Wallac Model 1420 Multilabel Counter; Perkin Elmer, Boston, MA). In addition, the indole concentration of the UPEC strain with and without *M. hominis* were measured at 3, 6, 9, and 12 h.

Crystal violet biofilm assay. A biofilm formation assay was done in 96-well polystyrene plates as previously described,[18] with slight modifications. Briefly, UPEC or EHEC cells were inoculated in a 1/5 diluted LB medium at an initial turbidity at 600 nm of 0.05 in the presence and the absence of *M. hominis* (10^3 and 10^5 ccu/mL) for 24 h without shaking. This was followed by measurement of cell density (the turbidity at 620 nm) and total biofilm (the absorbance at 540 nm) by 0.1% crystal violet staining. Normalized biofilm was calculated by dividing total biofilm with bacterial growth for each strain.

RNA isolation and DNA microarrays. The RNA isolation and microarray assay were performed by previously described methods,[19] with slight modifications. UPEC biofilms were formed on glass wool as previously described[18] with and without *M. hominis* (10^5 ccu/mL) using overnight cultures to inoculate 250 mL of 1/5 diluted LB medium with 10 g of glass wool (Corning Glass Works, Corning, NY). After incubation for 24 h with shaking (250 rpm), biofilm cells were prepared by rinsing and sonicating the glass wool in sterile 0.85% NaCl solution at 0 °C. Total RNA was isolated from the biofilm cells as described previously[18] and then purified with an RNeasy Mini Kit (Qiagen, Valencia, CA), including on-column DNase digestion with RNase-free DNase (Qiagen). Synthesis, fragmentation, and hybridization of target cRNA probes were done with Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, Santa Clara, CA), following the manufacturer's instructions. The fragmented cRNA was resuspended in 2× hybridization buffer and then applied with a pipette directly onto am *E. coli* CFT073 Microarray chip (MYcroarray, Ann Arbor, MI). The array chips were hybridized at 65 °C for 17 h in an Agilent Hybridization oven and then washed following the manufacturer's protocol (Agilent). They were analyzed by GenePix Pro 6.0 (Axon Instruments, Foster City, CA). The average fluorescence intensity for each spot was calculated and the local background was subtracted. All data normalization and the selection of fold-changes were done by GeneSpring 7.3.1 (Agilent). Intensity-dependent normalization (Lowess) was done and the ratio was reduced to the residual of the Lowess fit of intensity *vs*. the ratio curve. The averages of the normalized ratios were calculated by dividing the average normalized signal channel intensity by the average normalized control channel intensity. A gene was considered differentially expressed when the p value for comparison of two chips was lower than 0.05 to assure that the change in gene expression was statistically significant and that false positives were less than 5%. The differential gene expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible under accession no. GSE43376.

qRT-PCR. qRT-PCR was done by the CFX96 real-time PCR system (Bio-Rad). *pasT* and *hha* transcript levels were analyzed after growth to a turbidity of 0.5 at 600 nm in LB with and without *M. hominis* (10^5 ccu/mL). After RNA isolation, 50 ng of total RNA was used for the qRT-PCR reaction using an iScript™ One-Step RT-PCR Kit with SYBR (Bio-Rad). Primers (see Table 1) were designed using Primer3-Input Software (v0.4.0). Relative expression levels were calculated by the $2^{\Delta\Delta Ct}$ method.[20] Housekeeping gene *gapA* was used to normalize the gene expression data.[21]

Statistical analysis. Differences in number, for the various experiments were determined by Student's t-test. Results are representative of at least two independent biological replicates. A p-value of <0.05 in all replicate experiments was considered statistically significant.

Results

M. hominis *slightly affected the virulence of UPEC*

To determine whether mycoplasmas would affect the growth of UPEC CFT074, we plotted growth curves by co-culture with *M. hominis*. As expected, there was no

Table 1. Oligonucleotides Used in This Study

Genes	Sequence
ybcQ	f: 5′-AGATGGTTCTTGAGCGTTGG-3′
	r: 5′-CGTCATCGTCACAACATTGC-3′
phoP	f: 5′-GCAGAAGATGCCAAAGAAGC-3′
	r: 5′-ACCAGAATCGGCAGTGAAAC-3′
hha	f: 5′-CGTTGCCAGACAATTGACAC-3′
	r: 5′-TGAGGAAGGGATCTTGTCGT-3′
pasT	f: 5′-CAGATGACTGCTGCGGTAGA-3′
	r: 5′-ACGGTCCATCAACCAGACTC-3′
gapA	f: 5′-CGTTAAAGGCGCTAACTTCG-3′
	r: 5′-ACGGTGGTCATCAGACCTTC-3′

f indicates forward primer and r indicates reverse primer.

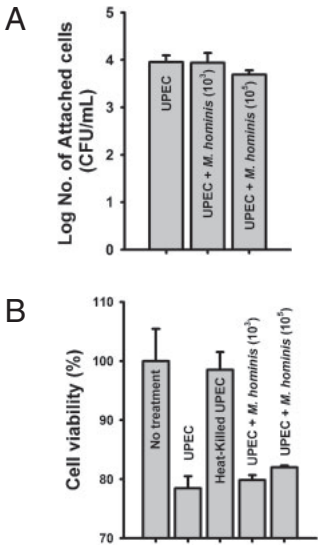

Fig. 1. Attachment to (A) and Cell Cytotoxicity of (B) UPEC Strain CFT073 as to HeLa Cells in the Presence and the Absence of *M. hominis* PG21 (10^3 or 10^5 ccu/mL).

Means and standard deviations of two independent experiments are shown.

significant difference in the growth curves of UPEC exposed to *M. hominis* as compared to UPEC alone (data not shown). Hence we examined the effects of *M. hominis* on UPEC virulence factors, such as cytotoxicity and attachment to HeLa cells. Both the attachment ability of UPEC on HeLa cells and HeLa cell cytotoxicity were slightly decreased when the HeLa cells were exposed to high concentrations of *M. hominis* (10^5 ccu/mL) as compared with UPEC alone (Fig. 1A and B). This indicates that *M. hominis* had a slight effect on the physiological features of UPEC (the planktonic state, not the sessile state), including degree of attachment and cytotoxicity.

M. hominis *specifically inhibited the formation of biofilm by UPEC rather than by the EHEC strains*

After 24 h, biofilm formation on the polystyrene surface decreased in a dose-dependent manner, due to the presence of *M. hominis* (Fig. 2). However, this reduction in biofilm formation due to UPEC disappeared when heat-killed *M. hominis* was exposed (data not shown).

Based on reports that *M. hominis* is strongly associated with the occurrence of urinary tract infections,[22] we also examined whether *M. hominis* might affect the

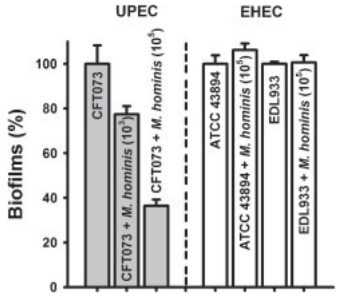

Fig. 2. Normalized Biofilm Formation (total biofilm/growth) by UPEC or EHEC Strains with and without *M. hominis* (10^3 or 10^5 ccu/mL) on 96-Well Plates of Polystyrene after 24 h of Incubation in a 1/5 Diluted LB Medium at 37 °C.

Data are averages for six replicate wells from two independent cultures, and one standard deviation is shown.

degree of biofilm formation by EHEC strains, which also form biofilms commonly associated with certain intestinal tract infections. After 24 h, there was no significant difference in the degree of biofilm formation as between the two EHEC strains in spite of the presence of *M. hominis* (10^5 ccu/mL) co-existence (Fig. 2). These results indicate that the live *M. hominis* type strain PG21 might play a key role in the formation of biofilms by UPEC in a target-organ-specific manner in the urinary tract.

In addition, it has been found that cell signaling, including AI-2 (the quorum sensing signaling molecule referred to the exponential phase) and indole (biofilm-associated signaling molecule referred to the stationary phase) had an effect on the formation of biofilms by *E. coli*.[18,23] To determine whether *M. hominis* might cause a change in the cell signaling, we assayed the concentrations of AI-2 and indole, but there were no significant differences as between the absence and the presence of *M. hominis* (data not shown). This indicates that *M. hominis* might play a role in the formation of biofilms by UPEC without any effect or association with cell signaling.

M. hominis *had an effect on YbcQ and PhoPQ expression in UPEC*

To explore further the role of *M. hominis* in the formation of biofilms by UPEC, we performed a whole transcriptome analysis of biofilm cells grown on glass wool with and without *M. hominis* after 24 h. Of the whole genome, only 14 genes were induced (2.5-fold) by the presence of *M. hominis* in cells forming the UPEC biofilm (Table 2). Moreover, there were no genes whose expression was decreased by more than 2.5-fold. Of the up-regulated genes, *ybcQ* (which encodes anti-termination protein Q homolog) and *phoP*/*phoQ* (which encodes DNA-binding response regulators in a two-component regulatory system) were significantly induced by the presence of *M. hominis*. This was confirmed by qRT-PCR. Consistently with the microarray data, transcription of *ybcQ*, *phoP*, and *phoQ* was induced by 3.2 ± 0.5, 2.7 ± 0.4, and 2.9 ± 0.2 fold respectively.

PasT and Hha toxins in TA systems were involved in UPEC biofilm fitness

We have found recently that TA systems can affect the formation of biofilms by *E. coli*.[18] Toxin genes such

Table 2. Genes Differentially Expressed in Cells Forming Biofilms Due to UPEC CFT073 in the Presence and the Absence of *M. hominis* PG21 in a 1/5 Diluted LB Medium after 24 h Incubation at 37 °C

Gene	Number	Fold change	Description
ydhT	c2062	2.5	Hypothetical protein YdhT
bcr	c2719	2.5	Bicyclomycin resistance protein
ilvD	c4693	2.5	Dihydroxy-acid dehydratase
yccU	c1102	2.5	Protein YccU
—	c2119	2.5	Hypothetical protein
fhiA	c0377	2.5	FhiA protein
—	c5065	2.5	Hypothetical protein
—	c4207	2.5	Putative fimbrial adhesin precursor
phoQ	c1508	2.5	Sensor protein PhoQ
phoP	c1509	2.5	Sensor protein PhoP
—	c0973	2.5	Hypothetical protein
—	c0394	2.6	Hypothetical protein
—	c1610	2.6	Conserved hypothetical protein
ybcQ	c1559	2.6	Anti-termination protein Q homolog from lambdoid

as *hha* (a toxin gene in Hha-TomB TA system) and *pasT* (a toxin that take part in PasT-PasI TA system) are directly involved in the formation of biofilms by *E. coli*. Our microarray data indicated that transcriptions of *hha* and *pasT* were induced by 2.4- and 2.2-fold respectively. Hence we examined by qRT-PCR to determine whether *hha* and *pasT* are directly involved in the formation of biofilms by UPEC depending on the presence of *M. hominis*. In the presence of *M. hominis* PG21, the *hha* and *pasT* transcripts were constantly induced (3.1 ± 0.2 and 2.9 ± 0.4 fold respectively) in UPEC CFT073. This indicates that *hha* and *pasT* might be involved in the formation of biofilms by UPEC, depending on the presence of *M. hominis*.

Discussion

Our results indicate that *M. hominis* inhibited the formation of biofilms by UPEC irrespective of specific bacterial physiology, including growth, production of cell signaling molecules, attachment, and cytotoxicity of UPEC in planktonic status. *M. hominis* type strain PG21 had an effect on the formation of biofilms by UPEC as opposed to by EHEC (Fig. 2). A species of *M. pneumonia* is normally present in the respiratory environment, and that the population of *M. hominis* is a normal urinary microbiota.[11] This enables them to face diverse friends and foes in the habitats. It is therefore plausible that *M. hominis*, a normal flora in the urinary tract, has an effect on the formation of biofilms by UPEC, one of the causative agents of UT infections. This suggests that the strategies of species of mycoplasma in fighting other microorganisms are dependent on the specific habitat.

By whole transcriptome analysis, we found that *ybcQ* and *phoPQ* transcripts were induced by the presence of *M. hominis* (Table 2). YbcQ is a predicted anti-termination protein associated with prophage Q. In a previous study,[24] we found that the prophage Q genes negatively regulate the biofilms produced by the *E. coli* K-12 strain, suggesting that YbcQ, prophage-linked factors, are also directly involved in the formation of biofilms by UPEC. Equally importantly, Beloin *et al.*[25] have reported that inactivation of the *rfaH* gene, encoding an anti-terminator, led to an increase in the formation of biofilms by the UPEC strain. Based on our bioinformatics analysis, there are no homology genes matching *ybcQ* in the genome of EHEC O157:H7 EDL933, a bacterium in which biofilm formation remained unaffected by the presence of *M. hominis* (Fig. 2)

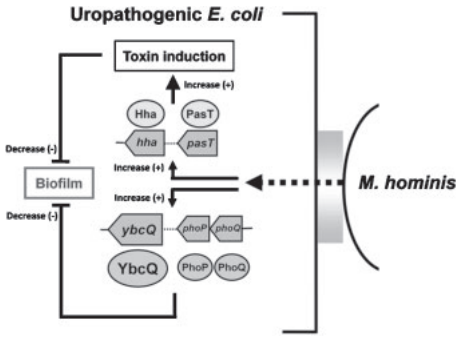

Fig. 3. Schematic of the Mechanism of the Formation of Biofilms by UPEC Depending on the Presence of *M. hominis*.
This illustrates that Hha and PasT are directly involved in the formation of biofilms by UPEC and that YbcQ and PhoPQ negatively regulate it [→, increase (+); ⊥, decrease (−)].

Hence we assume that YbcQ potentially plays a key role in the formation of biolfilms by UPEC, depending on the presence of mycoplasma. In addition, our results indicate that PhoPQ system-encoding genes were significantly induced upon exposure to *M. hominis* (Table 2). Consistently with our findings, an *in vivo* model with *Caenorhabditis elegans* showed that the formation of biofilms by *Yersinia spp.* was induced in the absence of *phoP*.[26] Taken together, these observations suggest that YbcQ and PhoPQ are associated with decreased formation of biofilms by UPEC, depending on the presence of *M. hominis*.

Recent studies suggest the biological functions of TA systems, which include growth control, persister formation, antiphage measures, and the general stress response.[27] This lends credence to the idea that the TA system can also affect biofilm formation.[18] A small transcriptional hemolysin repressor, Hha, is a toxin in the Hha-TomB TA system.[28] We have suggested not only that deletion of it induced the formation of biofilms,[28] but also that it is a global regulator of individual toxins that is involved in the suppression of biofilm formation, as by MazF, RelE, ChpB, YoeB and YafQ.[18] It is also worth noting that *pasT* is a toxin gene in the novel PasT/PasI TA pair, and it critically promotes stress resistance and persister formation by UPEC CFT073 strain.[29] Thus our findings can provide evidence that *M. hominis* selectively induces the regulation of specific toxins, such as Hha and PasT, possibly leading to inhibition of biofilm formation in the urinary tract.

To our knowledge, this is first report that a cell-wall deficient *M. hominis* species inhibits biofilm formation by urinary pathogens with direct regulation of *ybcQ* and *phoPQ* transcription (Fig. 3). Considering that *M. hominis* is also associated with Hha and PasT as toxin in TA system directly involved in the formation of biofilms, our results confirm the idea that *M. hominis* interacts with other microorganisms and thereby controls their biofilm fitness in the urinary tract.

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