Suppression of Antimicrobial Peptide Expression by *Ureaplasma* Species

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Ureaplasma* species commonly colonize the adult urogenital tract and are implicated in invasive diseases of adults and neonates. Factors that permit the organisms to cause chronic colonization or infection are poorly understood. We sought to investigate whether host innate immune responses, specifically, antimicrobial peptides (AMPs), are involved in determining the outcome of *Ureaplasma* infections. THP-1 cells, a human monocytoid tumor line, were cocultured with *Ureaplasma parvum* and *U. urealyticum*. Gene expression levels of a variety of host defense genes were quantified by real-time PCR. *In vitro* antimicrobial activities of synthetic AMPs against *Ureaplasma* spp. were determined using a flow cytometry-based assay. Chromosomal histone modifications in host defense gene promoters were tested by chromatin immunoprecipitation (ChIP). DNA methylation status in the AMP promoter regions was also investigated. After stimulation with *U. parvum* and *U. urealyticum*, the expression of cell defense genes, including the AMP genes (*DEFB1*, *DEFA5*, *DEFA6*, and *CAMP*), was significantly downregulated compared to that of *TNFA* and *IL-8*, which were upregulated. *In vitro* flow cytometry-based antimicrobial assay revealed that synthetic peptides LL-37, hBD-3, and hBD-1 had activity against *Ureaplasma* spp. Downregulation of the AMP genes was associated with chromatin modification alterations, including the significantly decreased histone H3K9 acetylation with *U. parvum* infection. No DNA methylation status changes were detected upon *Ureaplasma* infection. In conclusion, AMPs have *in vitro* activity against *Ureaplasma* spp., and suppression of AMP expression might be important for the organisms to avoid this aspect of the host innate immune response and to establish chronic infection and colonization.

Ureaplasmas belong to the class *Mollicutes*, bacteria that lack a cell wall, and have been known for decades to colonize the human urogenital tract. Ureaplasmas are adapted to life as obligate parasites in eukaryotic hosts and use urea as their sole source of energy. There are two species that occur in humans, *Ureaplasma parvum* and *Ureaplasma urealyticum*. Ureaplasmas are frequently isolated from the adult lower urogenital tract, with a prevalence reaching 70% in some populations, and they also are commonly found in the respiratory tracts of preterm neonates, in whom they occasionally cause invasive disease (1). Although they are often considered commensals, numerous studies have implicated ureaplasmas in a wide variety of disorders, including chorioamnionitis, postpartum endometritis, nongonococcal urethritis (NGU), infertility, urinary calculi, arthritis, and preterm birth, as well as pneumonia, meningitis, and chronic lung disease in neonates (1). The host immune response to *Ureaplasma* infections involves elevated proinflammatory cytokines, such as interleukin 1α (IL-1α), IL-1β, IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor alpha (TNF-α), as well as infiltration of neutrophils and macrophages at infection sites (2, 3). Very little is understood regarding how ureaplasmas cause human disease and, in particular, how they manage to establish chronic colonization and infection in the human urogenital tract.

Concerted attempts have been made to try to identify virulent strains of *Ureaplasma* as well as specific virulence factors. However, our previous studies and other reports have indicated that the virulence of *Ureaplasma* spp. is not likely associated with specific serovars (4, 5). Other studies have suggested that the various clinical outcomes, ranging from asymptomatic colonization to severe inflammation and invasive disease, might be primarily dependent on host-specific factors (6). Despite increasing recognition of the importance of these common organisms in human disease, little work has been done to characterize the interactions

between *Ureaplasma* and the host that lead to diverse outcomes. The key question of how the organism manages to evade immune surveillance during the establishment of chronic infections is unanswered.

Ureaplasmas reside primarily in the mucosa in close association with epithelial cells. Innate immunity provides the first line of defense against infecting organisms in the mucosa. Antimicrobial peptides (AMPs) play an important role in the defense against a wide range of Gram-negative and Gram-positive bacteria, fungi, and viruses (7–9). AMPs act as endogenous antibiotics in the direct inhibition of pathogens and also as modulators of innate and adaptive immune responses. The most extensively studied human AMPs are defensins and cathelicidin, which are cationic, amphipathic peptides capable of disrupting microbial membranes (8). Human defensins include six α-defensins (HNP-1 to -4 and HD5 and -6, encoded by genes *DEFA1* to -6), four major β-defensins (hBD-1 to -4, encoded by genes *DEFB1*, *DEFB4*, *DEFB103*, and *DEFB104*, respectively) and at least two other forms of β-defensins, and θ-defensins. The α-defensins HNP-1 to -4 are expressed mainly by neutrophils, while HD5, HD6, and hBDs are expressed predominantly by mucosal epithelial cells. Human cathelicidin (hCAP18/LL-37) is encoded by the single gene *CAMP* and is expressed mainly in granulocytes and epithelial cells of the

skin, lungs, and gut. Extensive studies have characterized the activities of human AMPs against a variety of microorganisms (8). However, only a limited number of studies involved the *Mollicutes* (10–12), and there have been almost none that included *Ureaplasma*. Published reports suggest that canine β-defensins and cathelicidin display low activity against *U. urealyticum* (13, 14), and two AMPs from frogs show activity against *Ureaplasma* clinical isolates from humans (15, 16). A recent study indicated that a mouse cystatin-related epididymal spermatogenetic (CRES) protein had anti-*Ureaplasma* activity (17).

The coexistence of bacterial pathogens with their eukaryotic hosts over long periods has permitted the evolution of selective mechanisms by which pathogens manipulate host cell functions for survival, replication, and escape from early innate as well as adaptive immune responses. Recent reports demonstrate that some bacterial pathogens are able to induce host cell chromatin remodeling and thus impose their own transcriptional signature onto host cells, thereby suppressing host defense responses and avoiding immune surveillance (18). For example, a family of bacterial toxins from *Listeria monocytogenes*, the cholesterol-dependent cytolysins (CDC), were reported to induce a global dephosphorylation of H3S10, correlating with repression of host immunity genes (19). Garcia-Garcia et al. reported that infection of human monocytoid THP-1 cells by the intracellular pathogen *Anaplasma phagocytophilum* led to silencing of host defense genes, a process dependent on the upregulation of histone deacetylase 1 (HDAC1) (20).

We hypothesize that *Ureaplasma* spp. are able to manipulate host AMP gene expression by epigenetic mechanisms and thus avoid killing by this important arm of the innate immune system in order to create a persistent infection. We carried out coinfection studies with the human THP-1 monocytoid tumor cell line and *Ureaplasma* spp. and examined the effect on expression of AMP genes. The antimicrobial activity of AMPs against *Ureaplasma* spp. and possible epigenetic mechanisms involved in the *Ureaplasma*-host cell interactions were also explored.

MATERIALS AND METHODS

Cell line. Human THP-1 cells (ATCC CCL-240) were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS).

Bacterial culture. *U. parvum* (serovar 3, ATCC 27815) and *U. urealyticum* (serovar 10, ATCC 33699) were grown in regular Shepard's 10B urea broth overnight at 37°C until color changed. One-milliliter aliquots were made and stored at −80°C as a regular stock for the antimicrobial activity assay. The numbers of CFU of the frozen stock were determined.

For the cell stimulation study, to eliminate the background effects from the regular Shepard's 10B urea broth, overnight cultures of *U. parvum* and *U. urealyticum* were harvested by centrifugation at $10,000 \times g$ for 90 min at 4°C and washed three times in 30 ml of phosphate-buffered saline (PBS) by centrifugation at $10,000 \times g$ for 20 min at 4°C. Bacteria were resuspended in 1 ml of PBS containing 20% FBS, and 0.1-ml aliquots were made. The aliquots were stored at −80°C, and one tube was thawed for CFU determination.

THP-1 cell stimulation. Freshly grown THP-1 cells at a concentration of 5×10^5 cells/ml were distributed into a 24-well plate (0.5 ml/well). Washed *U. parvum* or *U. urealyticum* stocks were thawed and added to the wells at a ratio of 5 CFU/cell. Phorbol myristate acetate (12-O-tetradecanoyl-phorbol-13-acetate [PMA]; Sigma, St. Louis, MO) was used (200 nM final concentration) as a stimulation control. After addition of 0.5 ml of fresh RPMI 1640 medium to each well, the plate was incubated at 37°C plus 5% CO_2 for various periods.

RNA isolation and quantitative RT-PCR. THP-1 cells cocultured with ureaplasmas were harvested at 4, 8, 24, and 48 h. Total RNA was purified using TRIzol reagent (Life Technologies). cDNA was synthesized using the SuperScript First-Strand Synthesis System for real-time PCR (RT-PCR) (Life Technologies). Real-time PCR was performed using a LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN) to quantify gene expression levels of a variety of host defense genes, including *DEFB1* and *DEFA5*, *DEFA6*, and *CAMP*, as well as *IL-8* and *TNFA*. Primer sequences were from published resources or the qPrimerDepot database (21) (Table 1). The resulting amplicons were examined by melting peaks and agarose gel, and only those primers that generated a single amplicon were chosen. The relative gene expression level of each defense gene was calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method (22). *RPLP0* was used as a housekeeping gene for normalization, and uninfected cells were used as a reference.

***In vitro* antimicrobial activity assay.** Synthetic hBD1-, hBD-2, hBD-3, hBD-4, HNP-1, HNP-2, and LL-37 peptides (all from Ana Spec, Inc., Fremont, CA, except for hBD-2, which was from Phoenix Pharmaceuticals, Burlingame, CA) were dissolved in water or dimethyl sulfoxide (DMSO) according to the manufacturer's instructions to make the stock solutions of 1 mg/ml. A flow cytometric method of viability determination using the membrane potential-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine axonal [DiBAC$_4$(3)] was adapted from the work of Nuding et al. (23). Preliminary experiments indicated that the activity of AMPs was dependent on both the bacterial and AMP concentrations and exhibited no differences with incubation times between 30 and 90 min (data not shown). Thus, a 30-min incubation time and a bacterial concentration of about 1×10^5 CFU/ml was chosen for all subsequent experiments.

Frozen stocks of *U. parvum* (serovar 3, ATCC 27815) and *U. urealyticum* (serovar 10, ATCC 33699) were quickly thawed at 37°C and diluted with 10B broth to reach the concentration of 1×10^5 CFU/ml. Ureaplasmas were incubated with AMPs at concentrations ranging from 6.25 to 100 μg/ml (serial 1:1 dilutions) in a final volume of 100 μl for 30 min at 37°C. Bacteria incubated with the same volume of corresponding AMP solvents (water or DMSO) served as negative controls. After incubation, the anionic dye DiBAC$_4$(3) (AnaSpec, Inc.) was added to the cultures to a final concentration of 1 μg/ml to label the depolarized bacteria. After incubation for 10 min at 37°C, the bacteria were centrifuged for 10 min at $6,000 \times g$ at room temperature. Pellets were resuspended in 100 μl of PBS, and the depolarized fluorescent bacteria were detected with an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA).

A total of 5,000 to 10,000 events were collected at a low flow rate (14 μl/min) from each sample. The FL1 channel was used to detect the emission of DiBAC$_4$(3) (maximum wavelength = 516 nm), and a dot plot with FL1 side scatter was generated. The gates for differentiation of viable, nondepolarized and depolarized bacteria were determined according to the untreated negative control and heat-killed positive control. The survival rates were calculated by normalizing the percentage of viable, nondepolarized cells in the experimental groups to the values for their negative controls.

ChIP. Histone modification patterns in the 1,000-bp proximal promoter regions of defense genes were analyzed by chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR). THP-1 cells (1×10^6) were stimulated with *U. parvum*, *U. urealyticum*, or 200 nM PMA as described above for 16 h and cross-linked with 1% formaldehyde. Cells were lysed with lysing buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% [wt/vol] SDS, Halt protease inhibitor cocktail [1:100 dilution from 100× stock; Thermo Scientific, IL], 1 mM phenylmethylsulfonyl fluoride [PMSF], 20 mM sodium butyrate), and nuclei were harvested by centrifugation at $4,000 \times g$ for 5 min at 4°C. Chromatin was sheared on ice with a Microson XL ultrasonic cell disruptor (Heat Systems, Farmingdale, NY) at 30% output power three times for 30 s each time, with 30-s pauses between. Sheared chromatin was incubated at 4°C for 2 h with protein A/G Dynabeads (Life Technologies) that conjugated with anti-acetyl-histone H3 (Lys9) (catalog no. 07-352), anti-trimethyl-histone H3 (Lys9) (catalog no. 07-442), or anti-trimethyl-histone H3 (Lys4) (catalog

TABLE 1 Primers for gene expression quantification, ChIP DNA quantification, and DNA methylation analysis

Gene	Primer name	Primer sequence (5′–3′)	Application	Source
ACP6	ACP6-eF ACP6-eR	ATAGCTGGGGGTTCCACTCT AAAATGGTGCAGGTCGTGTT	Gene expression quantification	qPrimerDepot
CAMP	CAMP-eF CAMP-eR	GGGCACACTGTCTCCTTCAC TCGGATGCTAACCTCTACCG	Gene expression quantification	qPrimerDepot
CAT	CAT-eF CAT-eR	ACGGGGCCCTACTGTAATAA AGATGCAGCACTGGAAGGAG	Gene expression quantification	qPrimerDepot
DEFA5	DEFA5-eF DEFA5-eR	GGACTCACGGGTAGCACAAC CCTTTGCAGGAAATGGACTC	Gene expression quantification	qPrimerDepot
DEFA6	DEFA6-eF DEFA6-eR	GACCTTCTGCAATGGCAAGT AGGACTTTGCCGTCTCCTTT	Gene expression quantification	qPrimerDepot
DEFB1	DEFB1-eF DEFB1-eR	GGGCAGGCAGAATAGAGACA TTTTGTCTGAGATGGCCTCA	Gene expression quantification	qPrimerDepot
IL-8	IL-8 F IL-8 R	CTAGGACAAGAGCCAGGAAGA AACCCTCTGCACCCAGTTTTC	Gene expression quantification	49
MPO	MPO-eF MPO-eR	TCCCGAAGTAAGAGGGTGTG CCCTGTCTCCTCACCAACC	Gene expression quantification	qPrimerDepot
RPLP0	RPLP0-F RPLP0-R	ATCTGCTTGGAGCCCACAT GCGACCTGGAAGTCCAACTA	Gene expression quantification	qPrimerDepot
TNFA	TNFa1-F TNFa1-R	CCCAGGCAGTCAGATCATCTTC GGTTTGCTACAACATGGGCTACA	Gene expression quantification	50
CAMP	CAMP-F1 CAMP-R1	GGCTTGGGAACATTTTGAGA ATCCCCTTCTGCATCCTTCT	ChIP PCR	This study
DEFA5	DEFA5-F1 DEFA5-R1	GGAGCATCAAAGGGATCTTG TGAGGAGTCAGCCTGGATTT	ChIP PCR	This study
DEFA6	DEFA6-F1 DEFA6-R1	AGCATCAAAGGGACATGGAG AGGAGCCAGCCTGGATTTAT	ChIP PCR	This study
DEFB103	DEFB103-F1 DEFB103-R1	CAGTCTGGGCAGCATAGTGA GCCTCCACACATGGCTAAAT	ChIP PCR	This study
DEFB104	DEFB104-F1 DEFB104-R1	AGAAACGATTCAGGGAAGCA GAGGTGTTGGGGCTACAGAA	ChIP PCR	This study
DEFB1	DEFB1-F1 DEFB1-R1	TCCAGAAACCCCATCAGAAC CCGCTGGATTTAGCTTTCAG	ChIP PCR	This study
TNFA	TNFA-F1 TNFA-R1	ACCACAGCAATGGGTAGGAG GTCCCCATACTCGACTTCCA	ChIP PCR	This study
DEFA6	defa6_msF defa6_msR	GGTTTTTATTAGGGGTTTTTATGTGG ACCCATTTACTCTAAAATCCAACTTT	DNA methylation	This study
DEFB1	defb1_msF1 defb1_msR1	TTAAGGAAAATTTGAGGGATATTTG CTTCCTCTAACCAAAACTACCTTCTC	DNA methylation	This study
DEFB104A	defb104a_msF2 defb104a_msR2 defb4_msF1	GTGTTTTTTTTAAGGGTAAATGTTAGG AATAACAACAAAAAATACTTAATAAC TGAGGATGGGGTTTATTATGTTTT	DNA methylation	This study
DEFB4	defb4_msR1 defb4_msF2 defb4_msR2	CCACTCACCTACTACCCACTCTATAC TTTTGAAAATATGGGGTTTTTTTATAA CACTCAAAAAATATCAACTCCACAA	DNA methylation	This study
TNFA	TNFa_msF TNFa_msR	TTTATTTTTTTATTTAAGGGGAAATG CCATATACCAAACATCCTATCTCTC	DNA methylation	This study
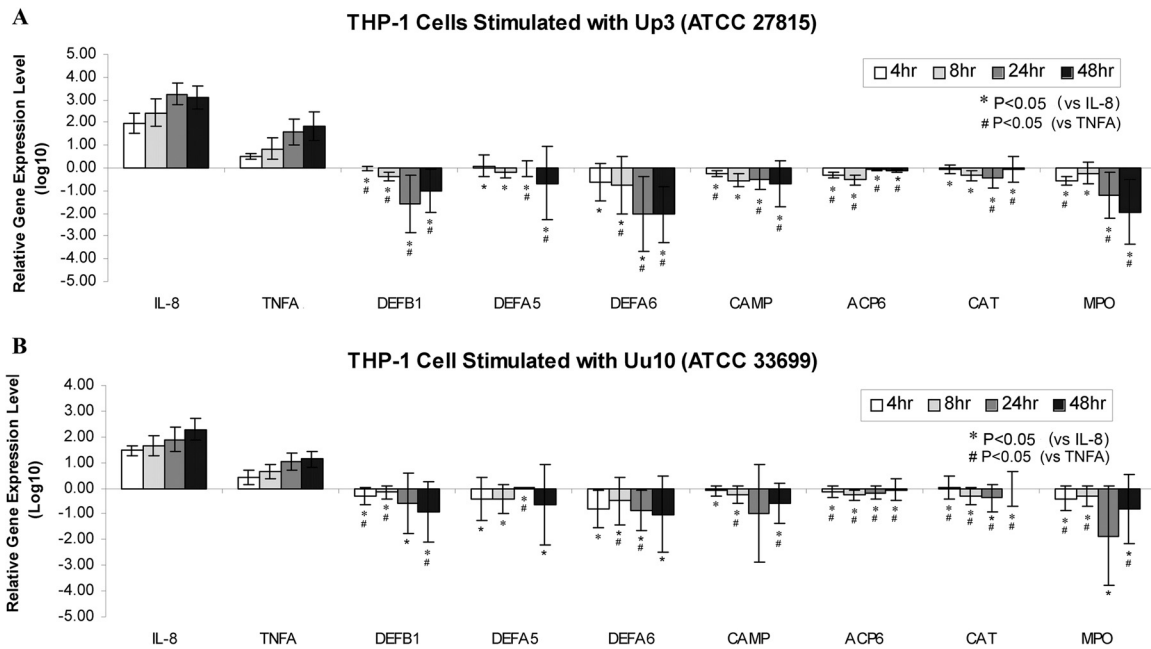
FIG 1 Host defense gene expression in THP-1 cells upon *Ureaplasma* stimulation. A total of 5×10^5 THP-1 cells were cocultured with *U. parvum* (serovar 3, ATCC 27815, 5 CFU/cell) (A) or *U. urealyticum* (serovar 10, ATCC 33699, 5 CFU/cell) (B) for 4, 8, 24, and 48 h. Gene expression levels of *DEFB1*, *DEFA5*, *DEFA6*, *CAMP*, *ACP6*, *CAT*, and *MPO* were quantified by real-time PCR using cDNA synthesized from RNA harvested from each well. *RPLP0* was used as a housekeeping gene for normalization. Untreated cells were used as a reference. The relative gene expression level of each tested gene was calculated using the $2^{-\Delta\Delta CT}$ method and is presented in $\log_{10}$ form. Data represent mean relative gene expression levels ± standard deviations ($n = 4$ independent experiments). Numbers that are <0 denote downregulation, and those that are >0 indicate upregulation.

no. 07-473) (All from Millipore, CA). Isotype-matched antibody was included as a negative ChIP control. Immunoprecipitated protein DNA was digested with proteinase K (50 μg/ml) for 30 min at 56°C and purified with QIAquick nucleotide removal columns (Qiagen, Germantown, MD). The resulted DNA was quantified by qPCR using primers specific for the gene promoter region. Primers were designed by Primer 3 (Table 1). The relative target DNA changes were calculated by comparing the percentage of precipitated DNA (percent input) in stimulated cells to that in the uninfected control.

DNA methylation analysis. Genomic DNA of infected and uninfected cells was purified with the Qiagen Blood and Tissue kit (Qiagen). Bisulfite conversion was carried out using the Epitect bisulfite kit (Qiagen) according to the manufacturer's instructions. Primers specific for the CpG-rich promoter regions were designed using the online software MethPrimer (http://www.urogene.org/methprimer/) (Table 1). PCR products were sequenced and analyzed using CLC DNA workbench 5.

Statistical analysis. The Q-Q plot was first conducted to examine data distribution and did not show obvious deviations from the normality assumption. To account for potential dependence of measurements obtained on the same experiment occasion, the paired *t* test was used to assess the statistical significance of the difference in relative gene expression levels between the tested genes (*DEFB1*, *DEFA5*, *DEFA6*, *CAMP*, *ACP6*, *CAT*, and *MPO*) and the reference genes (*IL-8* and *TNFA*) for THP-1 cells stimulated with *U. parvum* and *U. urealyticum* at 4, 8, 24, and 48 h. The paired *t* test was also conducted to compare the histone modification alterations in the defense gene promoters with the reference gene (*TNFA*) in the THP-1 cells infected by *U. parvum* and *U. urealyticum*, stratified by the types of antibodies (H3K9ac, H3K4me3, and H3K9me3). The histone modification patterns for *U. parvum*- and *U. urealyticum*-infected THP-1 cells were further compared with PMA, stratified by the defense genes. A *P* value of <0.05 was considered statistically significant.

RESULTS

Host defense gene expression is downregulated in *Ureaplasma*-infected cells. The expression of 7 genes (*DEFB1*, *DEFA5*, *DEFA6*, *CAMP*, *ACP6*, *CAT*, and *MPO*) encoding AMPs or otherwise involved in oxidative and enzymatic defense responses was quantified by RT-PCR. Results showed that upon infection with *U. parvum* or *U. urealyticum*, the expression of IL-8 and TNF-α was upregulated (Fig. 1), which is consistent with previous reports (24, 25). Interestingly, on the other hand, the expression of four AMP genes, *DEFB1*, *DEFA5*, *DEFA6*, and *CAMP*, was significantly downregulated ($P < 0.05$) compared to that of *IL-8* or *TNFA* in THP-1 cells at most time points of infection. Notably, the expression of *ACP6*, *CAT*, and *MPO* was also significantly ($P < 0.05$) downregulated compared to that of *IL-8* and *TNFA*. It is known that cellular defense genes are organized as clusters in the chromosomes; e.g., most of the defensins are localized on chromosome 8p23 (26). Thus, it is possible that the host defense gene expression is globally suppressed by infection with *Ureaplasma* spp.

***In vitro* activities of AMPs against *Ureaplasma* spp.** We next explored whether the downregulated AMPs have any adverse effect on the survival of *Ureaplasma* spp. by using an *in vitro* antimicrobial assay in which the viability of the ureaplasmas was monitored by flow cytometry (Fig. 2). The viable, nondepolarized cells were not labeled by DiBAC$_4$(3) and appeared in the low-fluorescence gate in the flow cytometry plot, while the depolarized cells were fluorescently labeled and were located in the high-fluorescence gate. In the representative examples shown, about 88% of the *U. parvum* cells were viable in the untreated culture from
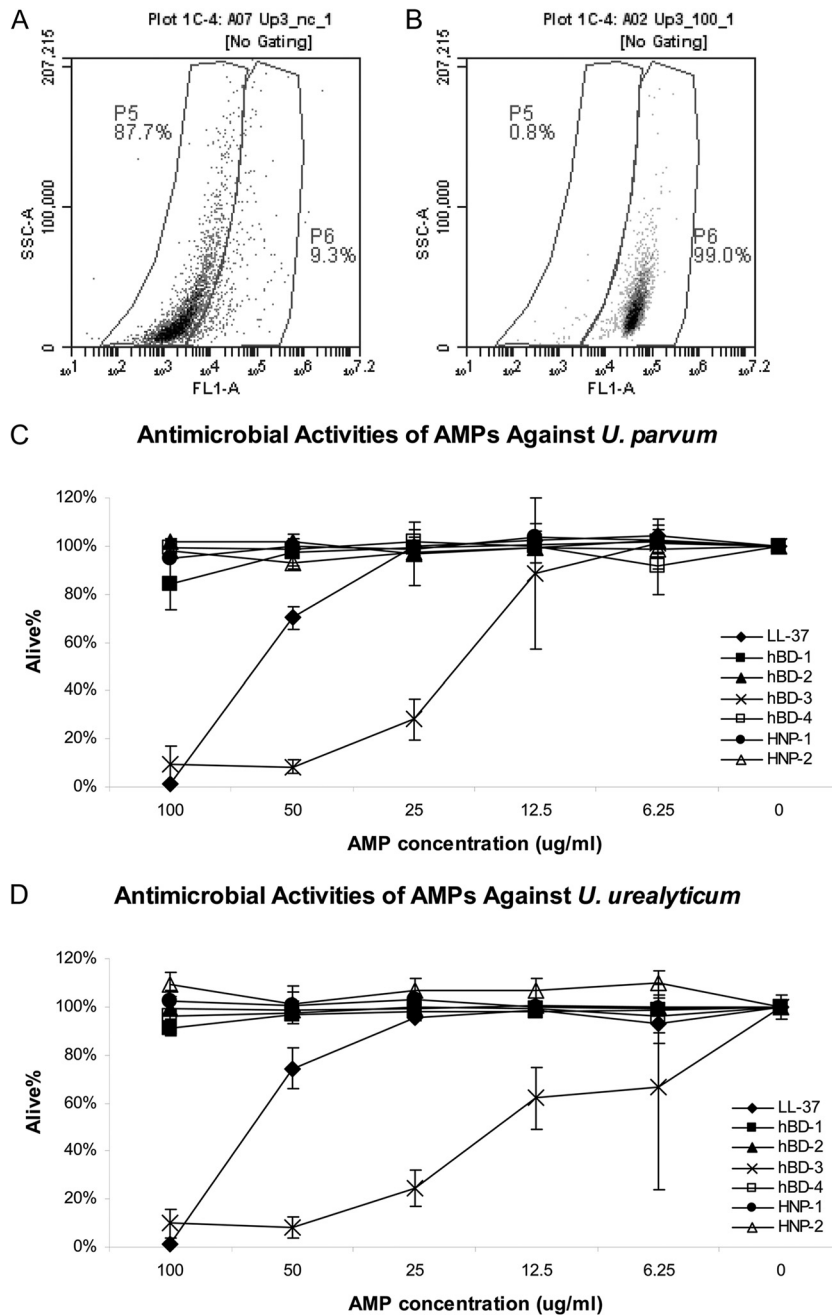
FIG 2 *In vitro* antimicrobial activity assay. Diluted stocks of *U. parvum* or *U. urealyticum* were incubated with synthetic AMP or AMP solvents at the indicated concentrations for 30 min at 37°C. After treatment, bacteria were incubated with DiBAC$_4$, to label depolarized cells. Control viable nondepolarized bacteria from freshly thawed stock (low-fluorescence gate P5 [A]) and depolarized cells after treatment with LL-37 (depolarized high-fluorescence gate P6 [B]) are shown as representative examples. A total of 5,000 to 10,000 events were collected from each experimental treatment. The survival rates were calculated by normalizing the percentage of viable, nondepolarized cells in the experimental group to the value for the negative controls (C and D). Data represent average survival rates ± standard deviations from 3 experiments.

thawed stock and were plotted in the lower-fluorescence gate P5 (Fig. 2A). Following incubation with 100 μg/ml of LL-37, the majority of the bacteria were depolarized and were plotted in the higher-fluorescence gate P6 (Fig. 2B). LL-37 and hBD-3 directly impaired the viability of *U. parvum* and *U. urealyticum* in a dose-dependent manner in the range of 6.25 to 100 μg/ml (Fig. 2B, C, and D). The activities of LL-37 against *U. parvum* and *U. urealyti-*

cum were similar, with 50% inhibitory concentrations (IC$_{50}$s) of about 65 μg/ml. At a concentration of 100 μg/ml, more than 98% of the bacteria were depolarized. hBD-3 showed higher activities than did LL-37 against the two *Ureaplasma* spp., with IC$_{50}$s of about 19 and 17 μg/ml for *U. parvum* and *U. urealyticum*, respectively. The observed activity of hBD-1 against ureaplasmas was much lower than those of LL-37 and hBD-3 under the tested con-
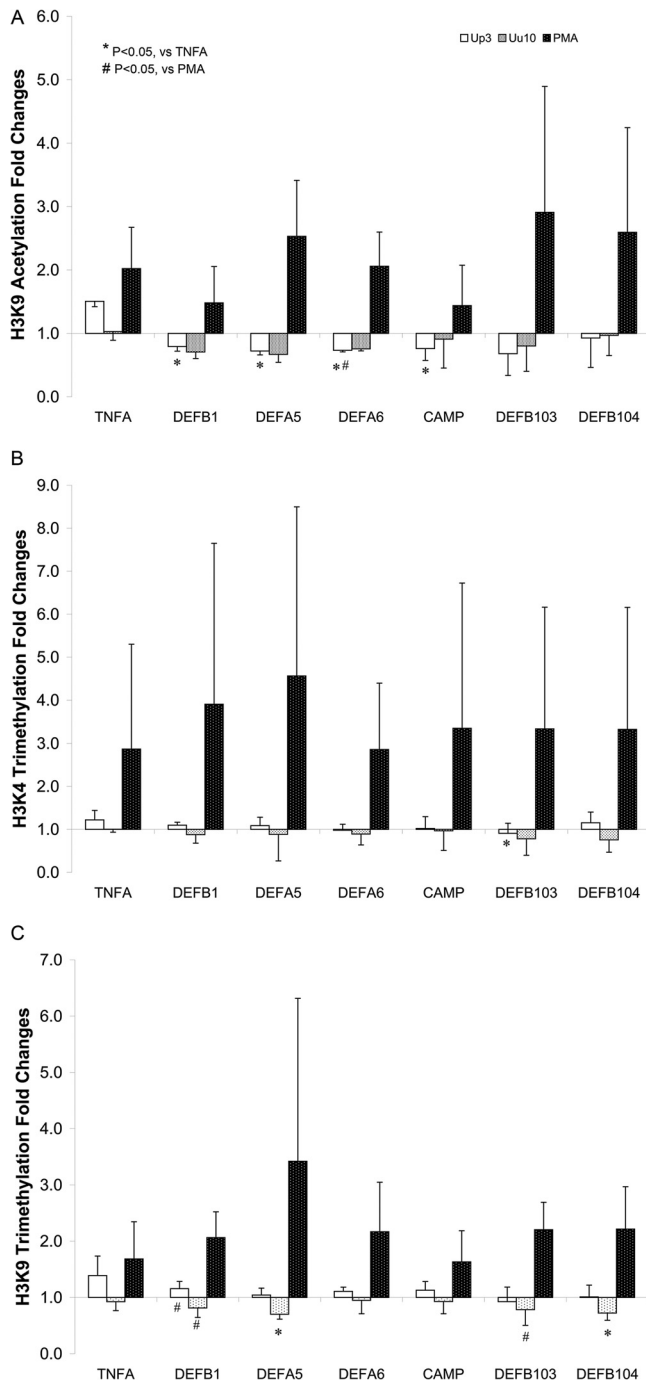
FIG 3 Histone modification alterations in the defense gene promoters in *Ureaplasma*-infected THP-1 cells. THP-1 cells (1 × 10⁶) were cocultured with *U. parvum* or *U. urealyticum* (5 CFU/cell) or PMA (200 nM) for 16 h. Formaldehyde-cross-linked, sheared chromatin was immunoprecipitated using anti-acetyl-histone H3 (Lys9 [A]) or anti-trimethyl-histone H3 (Lys9 [B] or Lys4 [C]). Immunoprecipitated DNA fragments were quantified by qPCR using primers specific for the promoter regions of the genes tested. Histone modification alterations were expressed as the ratio of immunoprecipitated chromatin target (% input) from stimulated to unstimulated cells. Data are the average fold changes of histone modifications ± standard deviations of three independent experiments.

dition. No antimicrobial activity was observed for hBD-2, hBD-4, HNP-1, or HNP-2 under the tested condition.

These data demonstrate that selected human AMPs, at least LL-37 and hBD-3, can directly damage *Ureaplasma* species, and thus suppression of the AMPs by *Ureaplasma* infection would compromise host antimicrobial defense and potentially facilitate the survival of *Ureaplasma* spp. on the mucosal surface and the establishment of chronic infection.

Ureaplasma infection induces host defense gene chromatin modifications. It is known that silencing of the genes is often associated with epigenetic modifications in the chromatin (5). To test for possible host chromatin alterations upon *Ureaplasma* infection, we employed chromatin immunoprecipition (ChIP) to look for histone modifications in the AMP gene promoters in the presence or absence of *Ureaplasma* infection (Fig. 3). The results indicate that *U. parvum* or *U. urealyticum* infection is associated with a decrease in histone H3 Lys9 (H3K9) acetylation, a modification related to gene activation, in the promoters of *DEFB1*, *DEFA5*, *DEFA6*, *CAMP*, *DEFB103*, and *DEFB104* (Fig. 3A). Compared to *TNFA* under the same infection condition, the decrease of H3K9 acetylation in *DEFB1*, *DEFA5*, *DEFA6*, and *CAMP* promoters was significant ($P < 0.05$) in cells infected with *U. parvum* but not *U. urealyticum*. On the other hand, in cells stimulated with PMA, H3K9 histone acetylation in the six genes was uniformly increased. Except for *DEFB103*, there were no significant changes in histone H3 Lys4 (H3K4) trimethylation, another gene activation modification, in cells infected with *U. parvum* or *U. urealyticum* (Fig. 3B). Infection with *U. urealyticum* caused a slight but significant ($P < 0.05$) decrease in H3K9 trimethylation (a gene repression modification) of *DEFA5* and *DEFB104* compared to that of *TNFA* (Fig. 3C). The H3K9 trimethylation alteration in genes *DEFB1* and *DEFB103* caused by *U. parvum* or *U. urealyticum* infection was also significant ($P < 0.05$) compared to the alteration in cells stimulated with PMA. Thus, the downregulation of AMP genes by the infection of *Ureaplasma* spp. correlated with host cell chromatin modifications; however, the two species exhibit differences in the ability to carry out histone modification.

No DNA methylation changes were detected in the THP-1 cells after coculture with ureaplasmas (data not shown). Thus, this mechanism does not seem to play an essential role in regulating AMP gene expression during ureaplasma infection.

DISCUSSION

Data in these experiments demonstrate for the first time in a human cell line that *in vitro* infection with *U. parvum* or *U. urealyticum* causes downregulation of host defense gene expression and that synthetic AMP peptides have bactericidal activities against *Ureaplasma* spp. *in vitro*. An epigenetic process by which *Ureaplasma* spp. suppress the host defense genes has been identified, although the molecular mechanism(s) remain to be characterized.

Our data indicate that human AMPs, LL-37 and BD-3, are active in damaging *Ureaplasma* spp. that infect humans. Previous studies in other laboratories have demonstrated that three canine β-defensins have low bactericidal activity against *U. urealyticum* (13). Canine cathelicidin also had a low activity against *U. urealyticum*, while the activity against *Ureaplasma canigenitalium*, a species that infects dogs, was higher (14), indicating possible species specificities of the AMP targets. Among the seven synthetic human AMPs tested in this study, LL-37 and BD-3 showed promising activity against both *U. parvum* and *U. urealyticum*, while the

activity of BD-1 was low and no activity was observed for BD-2, BD-4, HNP-1, or HNP-2. Looking at other *Mollicutes*, it has been found that BD-2 and BD-3, but not BD-1, possess activity against *Mycoplasma pneumoniae* (11). It is also noteworthy that *Mycoplasma fermentans* and *Mycoplasma hyorhinis* were susceptible to four membrane-active peptides: alamethicin, dernaseptin B2, gramicidin S, and surfactin (27). We noticed that the *in vitro* antimicrobial activity of synthetic LL-37 and BD-3 under the tested concentrations was mainly to damage the membrane and depolarize the ureaplasmas. Some depolarized ureaplasmas were still viable, probably because the membrane damage was not severe enough under the tested AMP concentrations and could be repaired once the AMPs were diluted (tested by a parallel CFU dilution assay; data not shown).

Because of their lack of a cell wall, ureaplasmas are naturally resistant to cell wall-active antibiotics, leaving the treatment dependent on the classes that affect protein or DNA synthesis, primarily macrolides, tetracyclines, and fluoroquinolones. However, resistance to all these classes of antibiotics has been identified in clinical isolates (28, 51). Hence, there is an increasing interest in identifying new antibiotics and treatment strategies against ureaplasmas. The AMPs are among the compounds under investigation for their general antimicrobial potency, and several of them are undergoing clinical evaluation (29, 30). Regarding the *Mollicutes*, a recombinant plasmid expressing a gene for an AMP, melittin, was introduced into cell line and animal models, and the results suggested efficacy in the treatment of infections caused by *Mycoplasma hominis* and *Mycoplasma gallisepticum* (31–34). Our study may provide another promising new strategy for treating ureaplasma infections. Two individual peptides, LL-37 and BD-3, were found to be active against ureaplasmas *in vitro*. It is known that the *in vivo* conditions are much different, i.e., that the bacteria could be attacked by multiple AMPs of different structural classes, and that the local concentrations of AMPs could be much higher (35). Because AMPs are multifunctional molecules that not only serve as gene-coded antibiotics but also influence diverse cellular processes involved in resolution of infection and repair of damaged epithelia (36), subsequent studies are needed to clarify a possible role for AMPs in treating ureaplasmal infections *in vivo*.

Although it is clear from the epidemiological studies that ureaplasmas are associated with many invasive infections, the mechanism(s) of pathogenicity of this organism is still under debate. We speculate that the persistent colonization might be associated with low virulence and an ability to suppress host innate immune responses. Data from a sheep model confirmed that chronic fetal exposure to *U. parvum* suppressed the lipopolysaccharide (LPS)-induced innate immune response with decreased lung proinflammatory and anti-inflammatory cytokine expression, fewer CD3^{+} T lymphocytes, and decreased *in vitro* monocyte responsiveness (37). In this study, we showed that *U. parvum* and *U. urealyticum* infection of THP-1 cells caused selective downregulation of the AMP genes and other cell defense genes, providing further proof of the ability of *Ureaplasma* spp. to manipulate the host defense response. This finding may have an important clinical implication. In many cases, there are coinfections of *Ureaplasma* spp. together with other microorganisms (38). It is possible that the impaired innate immune response caused by *Ureaplasma* spp. could increase the susceptibility of the host to the adverse effects of other pathogens. In studies of other mycoplasmas involved in human and animal diseases, suppression of host immune responses

has also been observed. Reports have documented that both humoral and cellular immunity was suppressed in patients with *M. pneumoniae* infection (39, 40), although a mechanism has not been identified. A global suppression of ovine peripheral blood mononuclear cells by *M. ovipneumoniae* has also been reported, again without a clear description of the pathogenic process employed by the organism (41).

Pathogen-induced epigenetic alterations in host cells have been observed in many other microbes. The protein effector OspF in *Shigella flexneri* blocks the access of transcription factor NF-κB to chromatin, resulting in attenuated expression of host genes involved in immune responses (42). An epigenetic regulatory mechanism has been described in which the histone deacetylase inhibitors butyrate and trichostatin A enhance the expression of cathelicidin in lung epithelial cells by increasing histone acetylation (43). Our results indicate that upon *Ureaplasma* infection, the decrease of histone 3 acetylation in the promoter of AMP genes, including *CAMP* (encoding cathelicidin LL-37), is associated with downregulated gene expression, similar to the reported effects of butyrate and trichostatin A. Our results also suggest that while *U. parvum* and *U. urealyticum* may regulate the host AMP expression similarly in the site of H3K9 acetylation, the histone codes, a combination of multiple histone modifications, employed by the two organisms might be different. This might influence the virulence and host adaptation of the two organisms.

In this work, we also reported a new approach in monitoring the antimicrobial activity of AMPs against *Ureaplasma* by flow cytometry. This is a fast and accurate method for counting the viability of the microorganisms compared to the traditional CFU counting method (23). The use of flow cytometry with DNA dyes and membrane-impermeant probes for determining the viability of various *Mycoplasma* species has been developed recently (44–48). We have for the first time applied this method to *Ureaplasma* spp. to identify and enumerate viable cells in the antimicrobial assay. Up to 10,000 cells can be analyzed within minutes using flow cytometry, in contrast to the traditional CFU plating method, in which several hundred colonies are counted by visual observation. However, one drawback is that this method may not differentiate membrane-damaged but potentially viable and dead bacteria.

There are limitations to this study. First, the reported results were based only on the ATCC type strains. Future studies should consider clinical strains with different patient origins for their ability to suppress the host defense response. Second, due to the availability of high-quality RT-PCR primers for quantifying the gene expression level and the ChIP DNA amount, only a limited number of AMP genes were examined in this study. To obtain a whole picture of global gene expression changes upon ureaplasma infection, other methods, such as transcriptome sequencing (RNA-seq), should be considered.

In summary, our *in vitro* study with human cells demonstrated the novel finding that *Ureaplasma* infection induces the suppression of AMP gene transcription likely involving the modulation of histone acetylation by an unknown mechanism. This impairment of cellular AMP gene activation may play a role in the ability of *Ureaplasma* spp. to avoid the host immune system and facilitate the establishment of chronic infection. Future studies to better characterize the mechanism of *Ureaplasma*-mediated AMP gene suppression are warranted.

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