Pathogenic Potential of *Campylobacter ureolyticus*

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The recent detection and isolation of the aflagellate *Campylobacter ureolyticus* (previously known as *Bacteroides ureolyticus*) from intestinal biopsy specimens and fecal samples of children with newly diagnosed Crohn’s disease led us to investigate the pathogenic potential of this bacterium. Adherence and gentamicin protection assays were employed to quantify the levels of adherence to and invasion into host cells. *C. ureolyticus* UNSWC was able to adhere to the Caco-2 intestinal epithelial cell line with a value of 5.341% ± 0.74% but was not able to invade the Caco-2 cells. The addition of two proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), to the cell line did not affect attachment or invasion, with attachment levels being 4.156% ± 0.61% (*P* = 0.270) for TNF-α and 6.472% ± 0.61% (*P* = 0.235) for IFN-γ. Scanning electron microscopy visually confirmed attachment and revealed that *C. ureolyticus* UNSWC colonizes and adheres to intestinal cells, inducing cellular damage and microvillus degradation. Purification and identification of the *C. ureolyticus* UNSWC secretome detected a total of 111 proteins, from which 29 were bioinformatically predicted to be secretory proteins. Functional classification revealed three putative virulence and colonization factors: the surface antigen CjaA, an outer membrane fibronectin binding protein, and an S-layer RTX toxin. These results suggest that *C. ureolyticus* has the potential to be a pathogen of the gastrointestinal tract.

*Bacteroides ureolyticus*, an aflagellate Gram-negative bacterium which may exhibit twitching motility, has recently been reclassified as a member of the *Campylobacter* genus (27, 28). In 1991, Vandamme et al. first proposed that *Bacteroides ureolyticus* should be reclassified as a member of the *Campylobacter* genus (29). According to Vandamme et al., *B. ureolyticus* fell into the rRNA cluster that contains members of the *Campylobacter* genus (29). Follow-up studies by the same group showed through polyphasic taxonomic analyses that *B. ureolyticus* shared (i) respiratory quinone content, (ii) DNA base ratio, and (iii) phenotypic characteristics with *Campylobacter* species, including *Campylobacter jejuni* (26). In a subsequent study, however, Vandamme et al. reported differences with respect to (i) fatty acid composition, (ii) proteolytic metabolism, (iii) hydrolysis of gelatin and casein, and (iv) ability to hydrolyze urea (28). In 2010, a publication by Vandamme et al. (27) finally acknowledged and verified the reclassification of *B. ureolyticus* to *Campylobacter ureolyticus* comb. nov., a newly designated member of the *Campylobacter* genus.

Prior to reclassification, the isolation of *C. ureolyticus* was reported from patients with a range of diseases, including superficial ulcers, soft tissue infections, nongonococcal urethritis, perianal abscess, and gangrenous lesions. The bacteria has also been reported to be present in amniotic fluid and the genital tract of humans (5–8). In addition, *C. ureolyticus* is commonly isolated from patients suffering from periodontal disease, a disease which involves inflammatory and destructive conditions of the tissues surrounding the teeth (6).

*C. ureolyticus* has also been isolated from biopsy samples of children with newly diagnosed Crohn’s disease (CD) (32), one of two categorical subtype diseases within inflammatory bowel diseases (IBD). The fact that such a bacterium was successfully isolated from these biopsy samples raises the question as to whether *C. ureolyticus* could potentially play a role in intestinal diseases or, alternatively, whether it is simply part of the normal flora of the gastrointestinal tract. Given this, we recently conducted studies to investigate the pathogenic potential of *C. ureolyticus* and showed that the bacterium when cultured with human monocytes and primary macrophages initiated the production of inflammatory cytokines (18). While this suggests that *C. ureolyticus* may have the potential to cause intestinal disease, it does not identify the bacterial factors involved or what effect contact with this bacterium has on human intestinal cells.

In this study, we examined the pathogenic potential of *C. ureolyticus* by quantifying and visualizing the adherence and invasive abilities of this bacterium through adherence and gentamicin protection (invasion) assays and scanning electron microscopy (SEM). Considering that the two prime proinflammatory cytokines associated with IBD include tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (30, 31), we investigated the pathogenic potential of *C. ureolyticus* under the effect of pre-existing inflammation. In addition, to establish a direct measure of the pathogenic potential of *C. ureolyticus*, its secretome was determined by proteomics coupled with mass spectrometry.

**MATERIALS AND METHODS**

**Bacterial cultures.** *Campylobacter ureolyticus* UNSWC isolated from an intestinal biopsy specimen of a child with CD and *C. ureolyticus* UNSWE and UNSWR isolated from fecal samples of children with no pathology were cultured on horse blood agar (HBA) (blood agar base number 2, supplemented with 6% sterile defibrinated horse blood [Oxoid, Hampshire, United Kingdom]) for 48 h at 37°C under microaerobic conditions, generated by an atmospheric gas generating system (*Campylobacter* Gas Generation Kit, BR0056A; Oxoid). *Salmonella enterica* serovar Typhimurium LT2 and *Escherichia coli* K-12 (University of New South Wales Institute of Microbiology) were used as control bacteria.

**Materials.** Materials were obtained from standard suppliers unless noted otherwise.

**Molecular biological techniques.** Total cellular RNA was isolated from *Campylobacter ureolyticus* cultures using the RNeasy Microkit (Qiagen) according to the manufacturer’s instructions. After DNase treatment, complementary DNA was synthesized using the SuperScript II First-Strand Synthesis System (Invitrogen) following the manufacturer’s instructions. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the primers listed in Table 1. PCR products were separated on 2% agarose gels and visualized after ethidium bromide staining. Semi-quantitative RT-PCR was performed with the primers listed in Table 1.

**Protein analysis.** A total of 111 proteins were isolated from the *C. ureolyticus* secretome, and were identified by mass spectrometry. The proteins were excised from a 2D gel and digested with trypsin. The resulting peptides were analyzed by a hybrid linear ion trap mass spectrometer (G2iFE, Micromass) and identified using the ProteinLynx Global Server. The identified proteins were classified into functional categories using the online MIPS tool (http://mips.gsf.de/). A total of 29 proteins were identified as secretory proteins using the MASCOT search engine (http://www.matrixscience.com/). The proteins were further classified into three functional categories: (i) proteolytic enzymes, (ii) cytoskeletal associated, and (iii) signal transduction proteins.

**Functional classification of the *C. ureolyticus* secretome.** The functional classification of the *C. ureolyticus* secretome revealed three putative virulence and colonization factors: the surface antigen CjaA, an outer membrane fibronectin binding protein, and an S-layer RTX toxin. These results suggest that *C. ureolyticus* has the potential to be a pathogen of the gastrointestinal tract.
[UNSW] culture collection) were grown on nutrient agar (Oxoid) for 24 h at 37°C under aerobic conditions.

Cultivation of human intestinal cell lines Caco-2 and HT-29. The cell lines used in this study were the human adenocarcinoma intestinal epithelial lines Caco-2 (American Type Culture Collection HTB-37) and HT-29 (American Type Culture Collection HTB-38). Cells were cultured using the protocols outlined by Man et al. (19). To seed the wells and establish a confluent cell monolayer, a procedure similar to that used by Man et al. (19) was employed.

Adherence and gentamicin protection (invasion) assays. Prior to infection, cell monolayers were washed three times with antibiotic-free cell culture medium. Where applicable, the cell monolayers were treated with 40 ng/ml TNF-α (Sigma) or IFN-γ (Sigma) for 1 h prior to the commencement of the adherence and invasion assays. The monolayers were then infected at a multiplicity of infection (MOI) of 200 for *C. ureolyticus* strains and an MOI of 100 for *S. Typhimurium* LT2 (positive control). Due to the highly invasive nature of *S. Typhimurium* LT2, a lower MOI was employed. The 24-well tissue culture plate was centrifuged at 232 x g for 5 min using a 4K15C centrifuge (Sigma, St. Louis, MO) in order to promote bacterial-mammalian cell contact. Infected monolayers were then incubated for 6 h at 37°C and 5% CO₂. The number of viable *C. ureolyticus* bacteria was determined after the 6-h incubation period in order to avoid any bias that may arise due to bacterial growth or death during the incubation period.

For the adherence assays (*n* = 6 per cell line), the monolayers were washed four times with antibiotic-free medium and then lysed using 500 μl of 0.5 M 1% Triton X-100 (Sigma) for 5 min. After collection of the cell lysate, cell counts were performed in quadruplicate on the appropriate agar plates. For the invasion assays (*n* = 6 per cell line), the monolayers were washed three times in antibiotic-free cell culture medium, after which 1 ml of medium (minimal essential medium [MEM] or McCoy’s 5A) with gentamicin (Invitrogen, Mulgrave, Australia) at a concentration of 200 μg/ml was added to kill extracellular bacteria. Following the 1 h of incubation, the medium was collected and plated to determine whether any viable extracellular bacteria remained. Subsequently, cells were lysed by addition of 500 μl of 1% Triton X-100 for 5 min, and cell lysates were plated in quadruplicate on the appropriate agar plates.

The percentages of adherence and invasion for each bacterium were determined using the following formulas: (i) percent adherence = [(number of adhering bacteria – number of number of invading bacteria)/total number of bacteria added to Caco-2 cells] x 100; (ii) percent invasion = (number of invading bacteria/total number of bacteria added to Caco-2 cells) x 100.

Translocation assays. Transwell filter units (0.4 μm; Nunc) were collagen coated and seeded with 5.0 x 10⁵ cells/ml. HT-29 cells were incubated for 14 days with the apical and basolateral media changed every 48 h. Inserts were washed three times with McCoy’s 5A antibiotic-free cell culture medium and inoculated with *C. ureolyticus* UNSWCD at an MOI of 200. Monolayers were incubated for 6 h, after which 100 μl was removed from the basal compartment and serially diluted, and cells were counted via drop plate count method.

Statistical analysis. The statistical significance of the differences in the levels of adherence achieved by *C. ureolyticus* UNSWCD with and without the addition of both proinflammatory cytokines was determined using a two-tailed paired *t* test. Statistically significant values were defined as *P* values < 0.05. The statistical software used was Prism GraphPad, version 5.0 (GraphPad Software; San Diego, CA).

Scanning electron microscopy. Caco-2 cells were grown on polyl-lysine-coated coverslips for 48 h in cell culture medium in 24-well plates at a cell density of 5 x 10⁵ cells per well and incubated at 37°C and 5% CO₂. *C. ureolyticus* UNSWCD was harvested and added at an MOI of 200 to the corresponding wells for 1 h, 3 h, and 6 h. To examine the effect of preexisting inflammation on attachment and invasion, 40 ng/ml of TNF-α or IFN-γ was added to the corresponding wells 1 h prior to coincubation with *C. ureolyticus* UNSWCD. Samples were fixed and ethanol dehydrated following a procedure similar to that outlined by Man et al. (19). The critical-point drying stage was performed over 3 h employing a CPD 030 critical point dryer (Bal-Tec AG, Balzers, Liechtenstein). Coverslips were mounted onto carbon tabs. A sputter gold coat was cast on top of these coverslips using an Emitech K550X gold coater (Emitech, Ashford, United Kingdom). SEM was performed using a Hitachi S3400-X model scanning electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan).

Measurement of mammalian cell viability. Cells were seeded at 5.0 x 10⁵ cells onto 24-well tissue culture plates and incubated for 48 h. Cell monolayers were washed three times with antibiotic-free medium and detached using 0.25% trypsin-EDTA (Invitrogen) for 5 min, after which fetal bovine serum (PBS; Invitrogen) was added to deactivate the trypsin. Cell viability was determined by performing a cell count on the trypsinized cells using a hemocytometer and 0.4% trypan blue (Sigma).

IL-8 production. To study the effects of *C. ureolyticus* UNSWCD and *E. coli* K-12 on the secretion of cytokines, HT-29 cells were grown with and without bacteria (*C. ureolyticus* UNSWCD, MOI of 200; *E. coli* K-12, MOI of 100) at a density of 5 x 10⁵ cells/ml. The supernatants were collected after 24 h, and the levels of interleukin-8 (IL-8) were measured using a human IL-8 enzyme-linked immunosorbent kit (ELISA) kit (Invitrogen) according to the manufacturer’s instructions.

Purification of secreted proteins. The secreted proteins were prepared as described previously by Bumann et al. (2). Logarithmic cultures of *C. ureolyticus* UNSWCD were centrifuged at 7,230 x g for 15 min at 4°C. The supernatant was filtered through a 0.22-μm-pore-size membrane filter to remove residual bacteria. The secreted proteins were precipitated using a previously described trichloroacetic acid (TCA) method (14). Briefly, 300 ml of filtrate was mixed with 95 ml of prechilled TCA and incubated on ice water for 15 min. The mixture was then centrifuged at 7,230 x g for 10 min at 4°C. The protein pellet was resuspended in 10 ml of acetone and centrifuged at 7,230 x g for 10 min. The acetone washing step was repeated twice, and the final protein pellet was air dried. The protein pellet was then dissolved in 0.5 ml of TSSU buffer (0.1% SDS, 2.5 M urea, and 50 mM Tris-HCl, pH 8.0) or phosphate-buffered saline (PBS) for cell viability assays and stored at −80°C.

SDS-PAGE and linear trap quadrupole (LTQ)-liquid chromatography-tandem mass spectrometry (LC/MS-MS). The protein concentration of the isolated *C. ureolyticus* UNSWCD secreted proteins was determined using a bicinchoninic acid (BCA) method microtiter protocol according to the manufacturer’s instructions (Pierce, Rockford, IL). Forty micrograms of the secreted proteins was resuspended in an equal volume of SDS-PAGE sample buffer (0.375 M Tris, pH 6.8, 0.01% SDS, 20% glyceral, 40 μg/ml SDS, 31 mg/ml dithiothreitol [DTT], 1 μg/ml bromophenol blue), and the proteins were further denatured by heating at 100°C for 5 min. Proteins were separated on 12% SDS-PAGE gels by electrophoresis for 1.5 h at 100 V. Gels were stained using Coomassie brilliant blue G-250 (Bio-Rad).

Protein bands were excised from the gels and digested according to a previously method described by Kaakoush et al. (15). Digests (1 μl) were separated by nano-LC using an Ultimate 3000 high-performance liquid chromatography (HPLC) and autosampler system (Dionex, Amsterdam, Netherlands) and were analyzed using a procedure similar to that described by Kaakoush et al. (15). Peak lists were generated using Mascot Daemon/extract_msn (Matrix Science, London, United Kingdom) using the default parameters and submitted to the database search program Mascot (version 2.2; Matrix Science). Search parameters were the following: the precursor tolerance was 4 ppm, and the product ion tolerance was ±0.4 Da; Met(O) was specified as variable modification; enzyme specificity was trypsin; one missed cleavage was possible; and the NCBI nonredundant (nr) database (accessed December 2010) was searched. A false-positive significance threshold was set at a P value of < 0.01 to eliminate any false-positive identification.

Bioinformatics analyses. BLASTP searches against the proteome of *Campylobacter concisus* BAA-1457 were performed using complete-
tein sequences available from the NCBI database (http://www.ncbi.nlm.nih.gov) to remove repetitive proteins since the genome of *C. ureolyticus* UNSWCD has not been sequenced. Proteins that were absent in *C. concisus* BAA-1457 were standardized against *Campylobacter hominis* BAA-381. The presence and location of signal peptide cleavage sites in the amino acid sequences were predicted using the default settings for Gram-negative bacteria on the SignalP, version 3.0, server (http://www.cbs.dtu.dk/services/SIGNALP/). Nonclassically secreted proteins were predicted by using the SecretomeP, version 2.0, server (http://www.cbs.dtu.dk/services/SecretomeP/). The Kyoto Encyclopedia of Genes and Genomes (KEGG [http://www.genome.jp/kegg]) was employed to determine the pathway values of 6.1% 

RESULTS AND DISCUSSION

Adherence, invasive, and translocation abilities of *C. ureolyticus* UNSWCD. As a result of the isolation of *C. ureolyticus* UNSWCD from a patient with CD (32) and of the observation that other *Campylobacter* species associated with intestinal disease including *C. jejuni*, *Campylobacter coli*, and *C. concisus* are able to colonize and invade intestinal epithelial cells, *in vitro* adherence, invasion, and translocation assays were used to determine the extent of *C. ureolyticus* UNSWCD adherence to and invasion into Caco-2 and HT-29 cells. No invasion by *C. ureolyticus* UNSWCD, UNSWE, or UNSWR was observed at an MOI of 200 for either cell line. As expected, the positive control, *S. Typhimurium* LT2 (MOI of 100), invaded cells at 1.3% ± 0.1%. No *S. Typhimurium* LT2 was recovered from the supernatant, suggesting that the *S. Typhimurium* LT2 invasion was not due to the growth of extracellular bacteria resistant to killing by gentamicin. Moreover, we have recently shown that strains from the closely related species *C. concisus* can internalize into both Caco-2 and HT-29 cells (19), indicating that the lack of infection was not due to the human cells. *C. ureolyticus* UNSWCD was capable of translocating across the cell monolayer (0.018% ± 0.002%), indicating that the bacterium could invade through a paracellular route. The results of the adherence assay showed that *C. ureolyticus* UNSWCD was able to attach to Caco-2 and HT-29 cells with percentage attachment values of 5.3% ± 0.7% and 1.4% ± 0.2%, respectively. Similar attachment levels were observed for *C. ureolyticus* UNSWE, which was able to attach to Caco-2 and HT-29 cells at percentage attachment values of 6.1% ± 0.4% and 1.2% ± 0.2%, respectively. Moreover, *C. ureolyticus* UNSWR also attached with values of 5.4% ± 0.3% and 1.0% ± 0.4%, respectively. Attachment of the positive control, *S. Typhimurium* LT2, was shown to be 16.6% ± 1.0%. The level of attachment observed for *S. Typhimurium* LT2 was approximately three times higher than that of *C. ureolyticus* UNSWCD for Caco-2 cells.

Scanning electron microscopy (SEM) was used to complement the *in vitro* assay procedure by visually confirming the manner in which *C. ureolyticus* UNSWCD interacts with the Caco-2 cell line. Following infection of Caco-2 human intestinal cells with *C. ureolyticus* UNSWCD for 1 h, 3 h, and 6 h, the process of attachment was viewed using SEM. Upon analysis it was revealed that the aflagellate *C. ureolyticus* UNSWCD (Fig. 1A) appeared to employ a “sticky end” as one mechanism of attachment to the microvilli and to the Caco-2 cells directly (Fig. 1E and F). Interestingly, the same mode of attachment has been observed in another aflagellate *Campylobacter* species, *C. hominis* UNSWCD, as well as in flagellated members of the *Campylobacter* genus, including *Campylobacter showae* UNSWCD and *C. concisus* ATCC 51562, which have all been shown to attract neighboring microvilli onto the bacterial surface (18, 19).

The addition of *C. ureolyticus* UNSWCD at an MOI of 200 to Caco-2 cells appeared to cause a degradation of the filamentous structure of microvilli (Fig. 1C and D and insets, showing results for 6 h of incubation, and 2A to D, showing results for 1 h and 3 h of incubation). Cellular debris of these remnant microvillus structures was observed (Fig. 1G and insets). As a result, the Caco-2 cell apical membrane surface appeared to lack the highly dense accumulation of microvilli compared with the negative-control sample (no addition of *C. ureolyticus* UNSWCD), which showed highly dense microvilli (Fig. 1B).

Interestingly, Ganan et al. (11) reported that *C. jejuni* strain 118, at an exponential phase of 18 h, adhered to Caco-2 cells at 2.56%. Given that *C. jejuni* is an established gastrointestinal pathogen, the fact that *C. ureolyticus* UNSWCD has a higher percentage attachment than *C. jejuni* would support the pathogenic potential of this bacterium. However, unlike *C. jejuni*, *C. ureolyticus* does not have the ability to invade intestinal epithelial cells, which could suggest that their pathogenic mechanisms differ.

Aggregation of *C. ureolyticus* UNSWCD was observed on both an inert material (Fig. 1A) and on a living surface (Fig. 1C). This behavior is a typical feature seen in biofilm formation, which results in an exopolymERIC matrix surrounding the bacteria, forming a structured community of cells that are adherent to each other and/or to the inert or living surface. Biofilms are known to assist in cell-to-cell communication and also function in virulence.

Effect of preexisting inflammation on the adherence and invasive ability of *C. ureolyticus* UNSWCD. To determine the effect of preexisting inflammation on the ability of *C. ureolyticus* UNSWCD to adhere to and invade host intestinal cells, Caco-2 and HT-29 cells were treated with proinflammatory cytokines (TNF-α and IFN-γ) prior to infection with *C. ureolyticus* UNSWCD. The choice of TNF-α and IFN-γ was based on the fact that a Th helper 1 (Th1) immune response is observed in the gastrointestinal tract of patients suffering from CD (25, 31). In the presence of the proinflammatory cytokines TNF-α and IFN-γ, the levels of attachment of *C. ureolyticus* UNSWCD to Caco-2 and HT-29 cells were observed to be 4.2% ± 0.6% and 6.5% ± 0.6% with TNF-α and 1.3% ± 0.4% and 1.4% ± 0.4% with IFN-γ, respectively. These values were not significantly different from the attachment levels recorded for *C. ureolyticus* UNSWCD without the addition of the proinflammatory cytokines (5.3% ± 0.7% and 1.4% ± 0.2%, respectively) compared to the addition of TNF-α and IFN-γ (for Caco-2, P = 0.270 and P = 0.235, and for HT-29, P = 0.52 and P = 0.60, for the addition of TNF-α and IFN-γ, respectively).

The effect of preexisting inflammation on the attachment of *C. ureolyticus* UNSWCD to Caco-2 cells was also observed using SEM. Examination of Caco-2 cells using SEM showed that the presence of *C. ureolyticus* UNSWCD on the apical membrane surface of Caco-2 cells once again induced cellular damage, causing degradation of microvilli observed around the aggregated mass of *C. ureolyticus* UNSWCD (Fig. 3D and E). *C. ureolyticus* UNSWCD was seen in aggregated masses attaching to microvilli, which appeared to be at a lower density than observed in the negative control (Fig. 3F to I). While the presence of inflammation has been shown to increase the ability of other *Campylobacter* species such as *C. concisus* UNSWCD
to facilitate invasion into intestinal cells (18), *C. ureolyticus* UNSWCD does not appear to take advantage of possible inflammation-induced morphological changes in host cells to exert its ability to attach to or invade Caco-2 cells. As a result, it would appear that the mechanism by which *C. ureolyticus* UNSWCD exerts its pathogenic effect is not affected by preexisting inflammation.

The observation of Caco-2 microvillus degradation by *C. ureolyticus* UNSWCD is supported by an early molecular study reported by Fontaine et al. (9). This study showed that cell-free filtrates of *C. ureolyticus* cause a loss of ciliary activity through disruption and sloughing of the epithelial cells from the mucosal epithelium of human fallopian tubes and bovine oviduct organ cultures (9). Subsequently, studies by Fontaine et al. suggested that these resultant cytopathic effects may be due to the existence of an unidentified membrane-bound endotoxin in the form of a lipopolysaccharide (LPS) (8–10). Interestingly, a number of studies have suggested that the proteolytic activity from proteases could be a contributing factor in the *C. ureolyticus* tissue damage observed in ulcerative and gangrenous lesions such as genital and perineal ulcers, decubitus and varicose lesions, and diabetic gangrene (4, 21). However, even though the effect of proteolytic enzymes as described in these studies has been speculated to induce this form of tissue damage, no study has identified proteolytic enzymes in *C. ureolyticus*.

**Effect of *C. ureolyticus* UNSWCD infection and cytokines on interleukin-8 production.** To confirm that the addition of TNF-α and IFN-γ induced inflammation in host cells, we measured the secretion of IL-8 in cells exposed to these cytokines. Cells exposed to TNF-α (687.5 ± 3.1 pg/ml; *P* < 0.0001) and IFN-γ (58.2 ± 2.0 pg/ml; *P* < 0.01) produced significantly greater quantities of IL-8 than the negative control (33.8 ± 1.5 pg/ml), indicating that the addition of these cytokines resulted in an inflamed state. Cells infected with *C. ureolyticus* UNSWCD (58.2 ± 2.8 pg/ml; *P* < 0.01) produced significantly higher levels of IL-8 than the negative control. Interestingly, heat-killed *C. ureolyticus* UNSWCD produced levels similar to those observed for viable bacteria (62.3 ± 3.5 pg/ml). Upon addition of TNF-α, cells infected with *C. ureolyticus* UNSWCD produced a level of IL-8 (681.7 ± 6.2 pg/ml; *P* = 0.12) similar to that observed in nonin-
ected cells exposed to TNF-α. In contrast, addition of IFN-γ to cells infected with *C. ureolyticus* UNSWCD produced a significantly higher level of IL-8 (249.0 ± 9.1 pg/ml; *P* = 0.002) than that observed in noninfected cells exposed to IFN-γ. Infection of cells with commensal *E. coli* K-12 resulted in the production of significantly lower levels of IL-8 (12.8 ± 1.1 pg/ml; *P* < 0.01) than those of the negative control. However, heat-killed *E. coli* K-12 induced significantly higher levels of IL-8 (78.7 ± 3.4 pg/ml; *P* < 0.001), possibly due to the presence of endotoxins that are released upon cellular lysis. These results suggest that *C. ureolyticus* UNSWCD elicits a mild inflammatory response from epithelial cells; however, this response is substantially elevated upon exposure to IFN-γ.

**Effect of *C. ureolyticus* UNSWCD infection on Caco-2 cell viability.** As a result of observing cellular damage and microvillus degradation on the apical membrane surface of Caco-2 cells, we quantified the extent of Caco-2 cell damage upon *C. ureolyticus* UNSWCD infection. Caco-2 cells were inoculated with *C. ureolyticus* UNSWCD at MOIs of 100, 200, and 1,000, and cell viability was determined. For the noninfected cells, 95.5% ± 5.9% of cells were viable after 6 h; in comparison, upon inoculation with *C. ureolyticus* UNSWCD (MOI of 100, 88.8% ± 2.8%; MOI of 200, 83.6% ± 5.1%; and MOI of 1,000, 61.5% ± 2.2%), the viability of Caco-2 cells decreased. Paired *t* test analysis revealed that cell viability did significantly decrease upon infection with *C. ureolyticus* UNSWCD compared with the negative control (MOI of 100, *P* = 0.047; MOI of 200, *P* = 0.0014; and MOI of 1,000, *P* = 0.0001). Additionally, an investigation was carried out to determine if the effect of cellular damage increased in the presence of preexisting inflammation induced by TNF-α and IFN-γ. The addition of pro-inflammatory cytokines prior to infection with *C. ureolyticus* UNSWCD (MOI of 200) resulted in cell viabilities of 82.5% ± 0.6% (TNF-α) and 85.6% ± 0.6% (IFN-γ). These results confirm that *C. ureolyticus* does indeed damage Caco-2 cells, as observed by SEM. In addition, the viabilities of the Caco-2 cells infected at an MOI of 200 with and without cytokines were relatively similar and, thus, consolidated the observation that preexisting inflammation had no severe effect on the bacterium’s pathogenic potential.

**Secretome of *C. ureolyticus* UNSWCD.** The observed degradation of Caco-2 cell microvilli led to the hypothesis that *C. ureolyticus* UNSWCD may potentially have proteolytic enzymes or degradative toxins capable of causing this effect. The secretome is a vital part of any bacteria’s pathogenic repertoire and can ultimately reflect a measure of a bacterium’s pathogenic potential (15). The secreted proteins of *C. ureolyticus* UNSWCD were purified and run on an SDS-PAGE gel (Fig. 4), trypsin digested to peptides, and analyzed using an LTQ-Fourier transform (LTQ-FT) Ultra MS/MS mass spectrometer. Following bioinformatic analyses, 111 proteins were identified from a BLASTP analysis to the *C. concisus* 13826 and *C. hominis* ATCC BAA-381 genomes (see Table S1 in the supplemental material). The genome of *C. concisus*.
ureolyticus is as yet unknown; thus, the genomes of *C. concisus* 13826 and *C. hominis* ATCC BAA-381 were used as the reference genomes. Additionally, SignalP and SecretomeP bioinformatic analysis resulted in the identification of 29 proteins predicted to be secreted (Table 1), as well as 82 putatively nonsecretory proteins. Functional classification resulted in the identification of three putative virulence and colonization factors: the surface antigen CjaA, the outer membrane fibronectin binding protein, and the S-layer RTX (for repeats in toxin) toxin.

The predicted nonsecretory proteins identified in the *C. ureolyticus* UNSWCD secretome consisted of enriched fractions involved in amino acid metabolism (22/82, or 26.8%), nucleotide metabolism (18/82, or 22.0%), and carbohydrate metabolism (18/82, or 22.0%). The finding of nonsecreted proteins in the secretome may relate to the fact that these metabolic proteins are highly abundant in the cell and may actually be present as contaminants within the secretome. Indeed, it has been reported that cellular lysis and degradation during the growth of bacterial cultures may result in contamination by high-abundance nonsecretory proteins (15). This is supported by other studies on the secretome of *Helicobacter pylori* that have also identified proteins such as thioredoxin, cochaperone GroES, 3-keto acyl carrier acyl synthase, and fumarate reductase flavoprotein subunit (2, 14). However, owing to their high stringency, the bioinformatic prediction processes employed may have also overlooked true positives. As a result, this does not conclusively rule out the possibility that some of these nonsecretary proteins (n = 82) may actually be secretory proteins.

The surface antigen, CjaA protein, found to be secreted by *C. ureolyticus* UNSWCD (Table 1) is a surface-exposed protein which is homologous to ABC transport proteins and is highly immunodominant in *C. jejuni* (23). The ABC transport system transports substrates across the periplasm to maintain lipid asymmetry, which is critical to the cell membrane in Gram-negative
bacteria (12). In addition, the surface antigen CjaA is one of two outer membrane substrate-binding proteins, alongside CjaC, involved in amino acid transport (15). According to a study by Muller et al. (20), CjaA has been characterized as an extracytoplasmic solute receptor in a putative ATP-binding cassette-type cysteine transporter, which has been shown to be a cysteine binding protein component of an ABC transport system (18).

An outer membrane fibronectin binding protein (CadF homolog) was also identified. This particular protein is widely known to mediate adhesion to the host cell (16). Fibronectin is a large glycoprotein, which is a component of the extracellular matrix (ECM) of the human intestinal epithelium. A study by Konkel et al. (16) reported that C. jejuni binds to fibronectin on the basolateral surface of human colonic cells. The secretion of an extracellular binding protein that is specific to receptors in the intestinal epithelium is significant in terms of C. ureolyticus UNSWCD potentially playing a pathogenic role in adhesion to and subsequent colonization of host cells (16). According to Patti et al. (22) the capability of bacteria to effectively attach to ECM components is a vital phenomenon as in some bacterial species it may be directly related to virulence.

In addition, the S-layer RTX protein (for repeats in toxin) was also found to be secreted by C. ureolyticus UNSWCD. RTX proteins are characterized by a domain located generally in the C-terminal part of the protein, consisting of a variable number of highly conserved glycine rich repeats. This domain has a high Ca$^{2+}$ binding capacity and is involved in binding of the toxin to the target cell (1). A study by Lally et al. (17) revealed that RTX proteins are pore-forming toxins synthesized by a diverse group of Gram-negative pathogens. For instance, uropathogenic E. coli (UPEC) strains secrete an α-hemolysin toxin (HlyA) belonging to the RTX protein family, which contributes to this bacterium’s role as a genitourinary pathogen (24). Primarily the two forms of host cell death associated with this type of toxin include apoptosis and necrosis (17). RTX toxins act by creating pores in eukaryotic cell membranes. Binding of calcium by the toxin is necessary for pore formation. High levels of toxin lyses cells as the pores formed by the toxin allow cytoplasmic contents to leak out (24).

**TABLE 1** Functional classification of *C. ureolyticus* UNSWCD secreted proteins that were bioinformatically predicted to be secreted (*n* = 29)

<table>
<thead>
<tr>
<th>ORF</th>
<th>GI no.</th>
<th>Protein name</th>
<th>Functional classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCC13826_0021</td>
<td>157164945</td>
<td>DNA-binding protein HU 1</td>
<td>DNA/RNA protein</td>
</tr>
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<td>CCC13826_0029</td>
<td>157165109</td>
<td>t-Asparaginase</td>
<td>Protein synthesis</td>
</tr>
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<td>CCC13826_0131</td>
<td>157167409</td>
<td>Peptidoglycan associated protein</td>
<td>Cell wall synthesis</td>
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<td>CCC13826_0170</td>
<td>157165495</td>
<td>50S ribosomal protein L1</td>
<td>Protein synthesis</td>
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<td>CCC13826_0208</td>
<td>157165207</td>
<td>Fumarate hydratase</td>
<td>Oxidative phosphorylation</td>
</tr>
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<td>CCC13826_0425</td>
<td>157165747</td>
<td>Fumarate reductase flavoprotein</td>
<td>Glycolysis/Gluconeogenesis</td>
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<tr>
<td>CCC13826_0516</td>
<td>157164277</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Fatty acid synthesis</td>
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<tr>
<td>CCC13826_0560</td>
<td>157164063</td>
<td>3-Oxoacl-acyl carrier protein synthase II</td>
<td>Fatty acid Synthesis</td>
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<td>3-Ketoacyl-acyl carrier protein reductase</td>
<td>Lipopolysaccharide biosynthesis</td>
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<tr>
<td>CCC13826_0576</td>
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<td>ADP-glyceromanno-heptose 6-epimerase</td>
<td>Virulence</td>
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<td>CCC13826_0664</td>
<td>157164816</td>
<td>Surface antigen, CjaA</td>
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<td>CCC13826_0702</td>
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<td>NAD-FAD-utilizing dehydrogenase</td>
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<td>Soluble transport</td>
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<td>C4-dicarboxylate-binding periplasmic protein</td>
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<td>Radical SAM domain-containing protein</td>
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<td>Pyruvate metabolism</td>
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<td>DNA repair/recombination</td>
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<td>157165491</td>
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<td>154149942</td>
<td>Holo-acyl carrier protein synthase</td>
<td>Fatty acid synthesis</td>
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<tr>
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<td>157165707</td>
<td>Fructose-bisphosphate aldolase</td>
<td>Pentose phosphate pathway, fructose metabolism</td>
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<td>157165134</td>
<td>Acrystyllase</td>
<td>Peptidase</td>
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</tr>
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<td>218129864</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
</tr>
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</table>

$^a$ ORF, open reading frame.

$^b$ SAM, sterile alpha motif.

**FIG 4** SDS-PAGE of the secretome of *C. ureolyticus* UNSWCD. *C. ureolyticus* UNSWCD secreted protein bands (lane S) stained with Coomassie blue brilliant dye. Lane M, molecular mass marker.
Of interest was the identification of a hypothetical protein belonging to Bacteroides eggerthii, a member of the Bacteroides genus that has been isolated from human feces (3, 13). The relevance of identifying this protein from C. ureolyticus was that the bacterium was previously a member of the Bacteroides genus. Such proteins could provide insights into the mechanism by which C. ureolyticus is able to hydrolyze gelatin and casein, given that this particular activity is not common among Campylobacter species (27, 28).

**Effect of the secretome of C. ureolyticus UNSWCD on cell viability and interleukin-8 production.** To assess the toxicity of the secretome of C. ureolyticus UNSWCD, we quantified cell viability upon inoculation with 2.6 μg, 13 μg, 26 μg, and 130 μg of the purified secretome. For the noninfected cells, 98.7% ± 0.2% of cells were viable after 6 h; in comparison, upon inoculation with 2.6 μg, 96.5% ± 0.3%; 13 μg, 93.7% ± 0.3%; 26 μg, 87.5% ± 2.0%; and 130 μg, 81.0% ± 0.1%, the viability of host cells decreased. Paired t test analysis revealed that cell viability significantly decreased upon the inoculation of the secretome compared with the negative control (2.6 μg, P = 0.037; 13 μg, P = 0.007; 26 μg, P = 0.026; and 130 μg, P = 0.0003). These results confirm that the secretome is indeed toxic to host cells. Furthermore, we quantified the production of IL-8 in HT-29 cells exposed to the same concentrations of the C. ureolyticus secretome and found that all concentrations resulted in similar quantities of IL-8 (2.6 μg, 102.7 ± 12.9 pg/ml; 13 μg, 88.0 ± 5.8 pg/ml; 26 μg, 96.1 ± 5.9 pg/ml; and 130 μg, 90.5 ± 2.4 pg/ml) which were significantly higher (P < 0.0001) than quantities in both the negative control (33.8 ± 1.5 pg/ml) and cells exposed to C. ureolyticus (58.2 ± 2.8 pg/ml).

**Conclusions.** This study has shown that C. ureolyticus UNSWCD has the ability to attach to and translocate through but not invade the human intestinal epithelial cell lines Caco-2 and HT-29, both with and without the effect of preexisting inflammation. As a result of the characterization of the C. ureolyticus UNSWCD secretome, we have shown that this bacterium possesses putative virulence and colonization factors which may contribute to its pathogenic potential.

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