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Genomic Investigation into Strain Heterogeneity and Pathogenic Potential of the Emerging Gastrointestinal Pathogen Campylobacter ureolyticus

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Introduction

Within the last decade a growing number of atypical Campylobacter species have been reported as emerging human pathogens [1]. Traditionally, C. jejuni and C. coli have been the main species associated with human illness, however advances in molecular diagnostics coupled with the development of novel culture techniques have facilitated the detection and isolation of a range of under reported and highly fastidious Campylobacter species [2,3], including C. concisus and more recently C. ureolyticus [4,5].

C. ureolyticus (previously Bacteroides ureolyticus) has only recently been classified within the Campylobacter genus [6]. Although in 1991, Vandamme et al. proposed that B. ureolyticus be reclassified as a member of the Campylobacter genus [7], its fatty acid profile and hydrolysis of gelatin and casein differentiated this organism from other Campylobacter species and B. ureolyticus remained as ‘species incertae sedis’[6,8,9]. Almost two decades later, employing a polyphasic approach, Vandamme and colleagues reported that B. ureolyticus shared (i) respiratory quinone content, (ii) DNA base ratio, and (iii) phenotypic characteristics with Campylobacter species, including Campylobacter jejuni and resulted in the reclassification of Bacteroides ureolyticus as Campylobacter ureolyticus [6].

Historically, C. ureolyticus has been associated with a range of diseases, including superficial ulcers, gangrenous lesions, nongonococcal urethritis, bacterial vaginosis, and of late, male infertility [6,10,11,12]. Furthermore, analogous to several other emerging and atypical Campylobacter species, C. ureolyticus has been linked with periodontal lesions, including gingivitis and periodontitis [2,13,14].

Recent work has led to the detection and subsequent isolation of C. ureolyticus as the sole pathogen from faecal samples of diarrhoeic patients [4,15,16]. Using a species specific PCR (targeting the hsp60 gene), C. ureolyticus is now believed to be the second most common Campylobacter species detected in diarrhoeic patients surpassing the established pathogen C. coli and exceeded only by C. jejuni [13]. Additionally, analysis of infectivity data reveals a predominance of C. ureolyticus in patients at extremes of age (<5 years and >70 years) suggesting an opportunistic nature for the pathogen [17]. Furthermore, we have noted a seasonal prevalence and have identified potential reservoirs of infection [18].

Following our initial report, C. ureolyticus has been detected at significantly higher rates in patients with Ulcerative Colitis (21.7%) in comparison to healthy controls (3.1%) [19]. In support of this, a New South Wales study [20], report the detection and isolation of C. ureolyticus from biopsy specimens and faecal samples from children with newly diagnosed Crohn’s disease (CD). This group later report on the pathogenic potential of C. ureolyticus: observing that their strain C. ureolyticus UNSWCD was capable of colonizing and adhering to intestinal cells - resulting in cellular damage and microvillus degradation [21]. As such, the recent emergence of C. ureolyticus in patients with gastrointestinal illness, at higher levels than the healthy controls, provides a compelling case that C.
ureolyticus is likely to be an emerging gastrointestinal pathogen of some importance.

Despite the growing evidence to suggest that non-*C. jejuni/C. coli* species are significant contributors to human disease [2,15,22], our existing understanding of *Campylobacter* pathogenesis is essentially restricted to *C. jejuni*. Furthermore, the literature regarding the mechanisms of *C. jejuni* invasion is highly controversial, whereby some groups report the transcellular route and others described the transcellular model or a mix of both [23,24,25,26,27]. In general, the past decade has provided us with substantial findings, revealing many of the virulence components of *C. jejuni* [28], however the exact mechanism of its pathology is as yet still unclear [29,30]. The small, curved shape of this Gram-negative bacterium, coupled with flagella-mediated motility, allow *C. jejuni* to penetrate intestinal mucus [31], where it can then adhere to epithelial cells via various surface associated adhesions, such as CadF and FlpA, which mediate binding to host tissue fibronecin [32]. Once attached, the bacterium then employs a range of secretion systems including the flagellar type III, the type IV and the recently identified type VI [33,34,35,36], through which it secretes invasion antigens, such as CiaB, which may promote cellular invasion of the intestinal epithelial cells [37] . Furthermore, *C. jejuni* produces various toxins including CdtA-C, which have been reported to promote cellular cytotoxicity and apoptotic cell death [31].

More recently, whole genome investigation followed by *in vitro* analysis of the emerging gastrointestinal pathogen *C. concisus* revealed potential components contributing to the organism’s pathogenesis; including several toxins, invasins in addition to colonisation, and adhesion factors [5,31,38,39]. Studies by Man *et al.* [26] report that in addition to a transcellular route of invasion, *C. concisus* UNSWCD preferentially attaches to intercellular junctional spaces facilitating translocation across the epithelium, thus promoting a paracellular route of invasion [20,40].

A likely reason for our current lack of knowledge regarding pathogenic mechanisms of *C. ureolyticus* is the lack of genomic data: until now the potential virulence apparatus of *C. ureolyticus* has remained unknown. Herein, we provide the first whole genome analysis of two *C. ureolyticus* strains. A comparative bioinformatics based approach was performed to identify putative virulence factors, secreted proteins and genomic heterogeneity of the two *C. ureolyticus* isolates in an investigation of the pathogenic mechanisms of this emerging pathogen.

### Materials and Methods

#### Strains used in this study

The *C. ureolyticus* strains used in this study are outlined in Table 1.

#### Genome assembly

Sequence data for *Campylobacter ureolyticus* ACS-301-V-Sch3b were obtained from the Sequence Read archive (SRA), having been collected by the Broad institute as part of the Human Microbiome Project for use as a reference genome for the *Campylobacter ureolyticus* species, but which had yet to be assembled. The sample was isolated from the female vaginal tract and had been sequenced using Illumina HiSeq with 100 bp pair end reads. The accession number to the SRA raw data is SRX115248.

*C. ureolyticus* has not been previously sequenced so no reference genome is currently available for assembly. Additionally, *Campylobacter* strains of the same species have previously been shown to display large variation within the overall gene content of their genomes whereby distinct genomospecies have been identified, thought to be mainly attributable to horizontal gene transfer and gene loss. For strains with genomes divergent from their closest references or in the absence of a reference genome, reference-sequence guided assembly methods can provide limited genome definition; therefore a *de novo* assembly method was used.

The Velvet assembly tool [41] was used due to its compatibility with Illumina data and having been shown to be one of the best performing assembly programs available for paired end data [42]. Using Velvet a range of k-mer values from 39 to 59 (which determine the minimum read overlap) was tested to find the optimum hash length for assembly of the data. Velvet is based on a directed graph representation called de Bruijn graphs which uses non-redundant sets of k-mers or word length rather than sequence reads as its primary data structures. For the paired end reads a k-mer value of 53 was seen to be the optimal hash length. This information was subsequently used to carry out the assembly using Velvet, giving an N50 of 60,555 and a maximum contig length of 227,136 bp with a total of 115 contigs and a genome size of 1639961 bp, having removed contigs with a length of less than 100 bp.

(Note: The Accession Number to access *C. ureolyticus* DSM 20703 scaffolds is KB947430–KB947464 The location of the *C. ureolyticus* ACS-301-V-Sch3b scaffolds is: https://olive.broadinstitute.org/genomes/camp_ureo_acs-301-v-sch3b.1)

| Strain     | Gender | Age (Years) | Sample Source | Medical Summary/Additional Information | Availability of whole genomic data |
|------------|--------|-------------|---------------|----------------------------------------|-----------------------------------|
| CIT001     | Male   | 83          | Faeces        | Long stay psychiatry unit              | In process (Our group)            |
| CIT002     | Female | 84          | Faeces        | End stage chronic renal disease        | In process (Our group)            |
| CIT004     | Female | 3           | Faeces        | Admitted to hospital overnight with D/V and fever. Had Cryptosporidium oocysts | In process (Our group)            |
| CIT005     | Female | 3           | Faeces        | D/V. Had Cryptosporidium oocysts       | In process (Our group)            |
| CIT007     | Female | 84          | Faeces        | End stage chronic renal disease        | In process (Our group)            |
| CIT009     | Female | 83          | Faeces        | Nursing home resident                  | In process (Our group)            |
| DSM 20703  | Female | Unknown     | Amniotic Fluid| Type Strain; originally isolated in 1978| Assembled                        |
| ACS-301-Sch-V-3b | Female | Unknown     | Vagina        | A reference genome for the Broad Institute as part of the ‘The Human Microbiome Project’ | Assembled                        |

Table 1. Strains used in this study.
Degree of diversity within Campylobacter ureolyticus

In addition to using the comparative genomics modules in RAST [43] and IMG/ER [44], we also conducted customized homology searches using BLAST (blastp, tblastn) and STRING to determine probable orthologs of genes conserved between the two C. ureolyticus strains and other species within the Campylobacter genus.

Bidirectional homology searches were performed on the two C. ureolyticus strains whereby cut off values set at 25% amino acid identity plus a minimum of 85% coverage were employed to identify the percentage of unique protein between both C. ureolyticus strains. A cut off a maximum 25% identity was employed to exclude homologues. The results obtained were compared with that of searches between the validated genomes of 4 C. jejuni strains within the RAST database; C. jejuni NCTC 11168, C. jejuni RM1221, C. jejuni 260.94 and C. jejuni 81-176. Cut-off values of 70% identity with a minimum of 85% gene length coverage were employed. A cut off value of a minimum of 70% identity was employed to identify highly conserved genes. It is important to note that in this study we considered protein sequences with >25% identity to be homologous, however those with >70% identity we conserved them to be highly conserved. Using the same parameters with C. jejuni NCTC 11168 set as the reference genome and 3 C. jejuni strains RM1221, 260.94 and 81-176 were individually and jointly compared to determine degree of identity at the amino acid level.

Furthermore, to identify the protein coding genes that are conserved between Campylobacter species, the genome of both C. ureolyticus strains DSM 20703 and ACS-301-V-Sch-V-3b were each set as reference genomes and compared to the protein coding genes within the genomes of all the available Campylobacter species in the RAST database; C. jejuni NCTC 11168, C. jejuni subsp. diolica strain 269.97, C. coli RM2228 [B], C. lan RM2100, C. putativus RM1915, C. fetus subsp. fetus 82-40, C. curvus 525.92, C. concisus 13826 and C. hominis ATCC BAA-381. The minimum cut off limit for highly conserved genes was set to 70% amino acid identity using the RAST sequence based comparison tool. To identify genes unique to C. ureolyticus (ie: no homologues in the other Campylobacter species analysed) the maximum cut off identity value was set to 25% for the Campylobacter species mentioned above, additionally C. ureolyticus ACS-301-Sch-3b was included as a comparison genome where by the cut off identity value was set at a minimum of 70%. This allows for the identification of proteins conserved across the two C. ureolyticus strains but absent in other Campylobacter species.

Gene prediction, identifying orthologs and syntenic associations

C. ureolyticus strains DSMZ 20703 (type strain) and ACS-301-V-Sch-3b isolated from the female vaginal tract are the first Campylobacter ureolyticus strains to have been sequenced.

The C. ureolyticus DSMZ 20703 genome (IMG submission ID 11117, NCBI project ID 174981) and fasta sequences for 1799 protein coding genes were downloaded from the IMG/ER website. The RAST [43] web application server was used for gene predictions using the Glimmer3 [45] program.

Furthermore, functional domain analysis was conducted with Pfam and the Kyoto Encyclopedia of Genes and Genomes, available at [http://www.genome.jp/kegg] [46], was employed to determine the biochemical pathways to which genes were assigned.

The Search Tool for the Retrieval of Interacting Proteins (STRING), a database of known and predicted protein–protein interactions available at http://string.embl.de/, was employed to examine functional relationship between proteins across multiple species [47]. BlastP (NCBI database) and Atlas T4SS (http://www.t4ss.hncr.br/) were employed to identify similarity between the type IV secretion systems within in C. ureolyticus ACS-301-V-Sch3b to other organisms. Atlas T4SS is a database describing a large number of proteins related to the type IV secretion system reported in both Gram-negative and Gram-positive bacteria, as well as in Archaea.

Comparative bioinformatic analyses

Homology searches were performed using the blastp and blastn tools through the National Centre for Biotechnology Information (NCBI) website (available at [http://www.ncbi.nlm.nih.gov/]) using the default settings. Comparative bioinformatic analyses on the genomes of C. ureolyticus strains DSM 20703 and ACS-301-V-Sch3b were performed using the RAST database. Both functional and sequence based analysis were performed using default settings.

Secretome Prediction

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 Server 4.1 [48] (http://www.cbs.dtu.dk/services/SignalP/). Non-classically secreted proteins were predicted using the SecretomeP 2.0 Server [49] (http://www.cbs.dtu.dk/services/SecretomeP/). SecretomeP predicts the possible secretion of proteins following signal peptide independent secretion pathways.

The statistical cut off was the default setting for both SignalP4.1 and the SecretomeP2.0 servers.

As a comparison, using identical parameters set for the C. ureolyticus analysis, the total percentage of C. jejuni NCTC 11168 secreted proteins were predicted using both the SignalP4.1 and SecretomeP2.0 servers. The amino acid sequences of C. jejuni NCTC 11168 protein coding genes were extracted from the IMG/ER annotation pipeliner server.

Culturing of bacterial strains

C. ureolyticus strains were inoculated on to blood agar plates (Columbia Blood Agar base; Sigma Aldrich, with 5–7% defibrinated horse blood; Thermoscientific) supplemented with 2.5 g/L of Sodium Formate (Sigma Aldrich), 2.5 g/L Sodium Fumarate (Sigma-Aldrich) and 20 μg/mL of vancomycin (Sigma-Aldrich) [6]. The strains were grown under anaerobic conditions (AnaeroGen Gas Generating Systems Oxoid) at 37°C for 48 hours. The identity of the cultures were confirmed by) the presence of flat, spreading colonies on the blood agar plates, positive urea slants (Christensen’s Urea Agar, Sigma-Aldrich) and the presence of slender Gram-negative rods under a light microscopy.

Validations of syntenic associations

Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. The concentration and quality of DNA was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Fischer Scientific, Ireland). To confirm the presence of C. ureolyticus DNA in the samples following DNA extraction, C. ureolyticus specific PCR targeting the hsp60 gene was conducted as described by Bullman et al. [4]. The confirmation of the presence/absence of gene that were shown to be present in C. ureolyticus DSM 20703 but absent in ACS-301-V-Sch3b by next-generation sequencing was performed on the DSM 20703 strain along with 6 C. ureolyticus clinical isolates using PCR.
Primer pairs were designed to amplify regions (2,861, 1,625, 1,469, 311 bp) within several ORF clusters found in either DSMZ 20703 or ACS-301-V-Sch3b (Table 2). All PCR amplifications were performed in a 50 μl reaction volume, containing 3 μl DNA template, 1 U HotStarTaq DNA Polymerase (Qiagen, West Sussex, UK; 202035); 5 μl of 10× PCR buffer and 1 μl 25 mM MgCl₂ (provided with HotStarTaq DNA Polymerase); 0 μl of dNTPs mixture (1.25 mM of each dNTP; Sigma-Aldrich Ireland Ltd. Arklow, Ireland), and 2 μl of each primer (25 pmol/μl; Eurofins MWG Operon, London, UK), 29 μl molecular grade water. The thermal cycling conditions for all 4 reactions were: 94°C for 5 min, 35 cycles of 94°C for 30 s, Annealing temperature as specified in Table 1 for 30 s, and 72°C for 1 min per kb to be amplified, followed by 72°C for 5 min. PCR primer products were electrophoresed through 1.5% agarose gels at 100 V for 40–60 min. The products were then purified using the QiAquick® PCR Purification Kit (Qiagen, Manchester, UK) according to manufacturer’s instructions. PCR positive products were sequenced by MWG (Eurofins, Germany) and analysed by Clustal W [50].

Expression of these genes under standard conditions (as described previously) was investigated by reverse transcriptase PCR (rt-PCR). Briefly total RNA was extracted by Roche high purity RNA extraction kit (Roche Diagnostics, Mannheim, Germany) as per the manufacturer’s guidelines and the purity and concentration of the RNA was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies). The RNA was then purified by QiAquick® PCR Purification Kit (Qiagen, Manchester, UK) according to manufacturer’s instructions. PCR positive products were sequenced by MWG (Eurofins, Germany) and analysed by Clustal W [50].

Table 2. Primers used in this study.

| Target | Primer | Sequence 5’ – 3’ | Annealing (°C) | Product (bp) | Source |
|--------|--------|------------------|----------------|--------------|--------|
| rtx    | 71RTX F | CCT TAG CTC TTT TAT CAA GCG ATG | 58             | 2,861        | This study |
|        | 2932RTX R | CAC TCT TAT CGA TGG TAA TAA AGC C | 57             | 1,625        | This study |
| virD4  | 262VirD4F | GAA ATG CAA GAT TGG CAA ACT CAG C | 57             | 1,625        | This study |
|        | 188VirD4R | CTC AGC ATC TTC TCT TAT TGG C | 56             | 1,469        | This study |
| citB   | 266CitB F | TAC ATG AAA ACT CTC ATA GAA ATT TAA TC | 56             | 1,469        | This study |
|        | 1735 CitB R | AGT AGT AGA TAA CAA ACT CTT TTT CAT C | 56             | 1,469        | This study |
| doc    | 32Doc1 F | GTT TGC ATG ATG ATA TAA TGG ATG | 54             | 311          | This study |
|        | 343Doc1R | TGA GTT AAA TCA TCT TTT GTT ATC TC | 54             | 311          | This study |

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**Results and Discussion**

Confirmation of bacterial pathogenesis and virulence potential requires a multi-factorial approach encompassing epidemiological data, candidate gene identification and functional analysis. The recent deposition of two *C. ureolyticus* genome sequences (strains DSM 20703 and ACS-301-V-Sch3b) has for the first time facilitated the genetic analysis of the pathogenic potential of this species. The type strain DSM 20703, originally isolated in 1978 from human amniotic fluid [9], was sequenced as part of ‘The one thousand microbial genomes (KMG)’ project. Strain ACS-301-V-Sch3b was isolated from the human vaginal cavity and sequenced by the Broad Institute as a reference genome for ‘The Human Microbiome Project’.

**Campylobacter ureolyticus diversity**

The complex taxonomy of typical and emerging *Campylobacter* species is well established [53], whereby certain isolates, although conforming to the phenotypic description of a particular species, may exhibit a large degree of heterogeneity at the genomic level [54,55]. For instance the emerging pathogen *C. concisus* is composed of several genemospecies that are likely to have varying impacts on human health and disease [31,39,56]. In the current study, bidirectional homology searches between the type strain DSM 20703 and the vaginal isolate ACS-301-V-Sch3b revealed that 75–79.5% of proteins were highly conserved (70% identity). Using the same parameters with *C. jejuni* NCTC 11168 set as the reference genome, individual and multiple comparisons to 3 other *C. jejuni* strains, revealed that 92% and 87% of proteins respectively are highly conserved. Such data suggest that substantial variation exists within these two *C. ureolyticus* genomes. Interestingly, average percentage identities for all homologs revealed that *C. ureolyticus* strains had a higher variation when compared to the phylogenetically related species *C. jejuni* (94 vs. 98%, respectively).

Furthermore, whole genome comparison of the protein coding genes of the two *C. ureolyticus* strains against other members of the *Campylobacter* genus revealed that 9–22% of proteins were highly conserved across different *Campylobacter* species (Table 3). The greatest number of highly conserved homologs were present in *C. hominis* followed by *C. concisus* then *C. curvus* and *C. fetus* subsps. *fetus*. 

**Table 2. Primers used in this study.**

**Campylobacter ureolyticus Virulence Potential**
There are approximately 128 protein coding genes that were determined to be highly conserved across all Campylobacter species test when using *C. ureolyticus* DSM 20703 as the reference genome. These proteins have functions ranging from stress response, membrane transport, respiration and the metabolism of macromolecules (Table S1).

On the other hand *C. xylosgenius* RM3195 and *C. lari* RM2100 shared the lowest percentage of highly conserved protein coding genes and up to 40% of *C. ureolyticus* proteins did not contain homologs within the genomes of these species (Table 3). A total of 65 proteins were highly conserved between the two *Campylobacter* species (>70% identity) yet had no homologs (<25% identity) in any of the other *Campylobacter* species tested (Table S2). The majority of these proteins (n = 45) were classed as hypothetical and miscellaneous (n = 9). Additionally, the urease operon was also presented within this group (Table S2); however it is important to note that *C. spirotrihum* biovar. *parasuromycicus* and subset of *C. lari* UPTC (urease positive thermophilic *Campylobacter*) also contain this operon [57,58]. As the whole genomes of these species are currently unavailable, they were not included in the comparison analysis.

While the genomes of *C. ureolyticus* DSMZ 20703 (1.74-Mb) and ACS-301-V-Sch3b (1.66-Mb) have a similar estimated size, analysis indicated that 18.8% (341/1810) and 17.1% (290/1700) of their protein coding genes are unique (Fig. 1, Table S3 and S4). Based upon studies by Goris and colleagues [59], the variation we observe between the *C. ureolyticus* strains suggests species delinea-

In addition to the high degree of genomic variation observed between DSMZ 20703 and ACS-301-V-Sch3b strains, protein profiles of a further 6 *C. ureolyticus* isolates, whereby strains are clustered based on their banding patterns (Fig. 2), further confirms the significant degree of heterogeneity that exists between strains. A large scale whole genome analysis project including 12 *C. ureolyticus* strains, isolated from patients with diarrhoeal illness, asymptomatic patients and animal reservoirs is currently under way within our lab. Comparisons of whole genome coding sequences between these strains support our initial observations of substantial heterogeneity between *C. ureolyticus* strains. Paired genome comparison of the coding genes of 14 *C. ureolyticus* strains revealed that 2–20% of their proteins are unique. Furthermore, individual comparisons of the protein coding genes of the 12 *C. ureolyticus* isolates against *C. ureolyticus* DSM 20703 and *C. ureolyticus* ACS-301-Sch-V-3b revealed that 13–19% (average 16%) and 9–16% (average 12%) of proteins are unique respectively. Such heterogeneity between the *C. ureolyticus* type strain and isolates raises caution regarding its suitability as the type strain for this species.

Our preliminary data suggest that as with *C. concisus*, *C. ureolyticus* is likely composed of several genomospecies; however, additional large scale investigations will be required to determine the extent of genomic variations between strains of different origin and the consequences that such differences may have on their pathogenesis and virulence potential (this is the subject of continuing work in our lab).

The *C. ureolyticus* secretome

A significant variant in contributing to bacterial pathogenic potential is the secretome – the totality of secreted proteins - characterised by its dynamic nature, undergoing variations and adjustments to match that required by the prevailing environmental conditions [60]. The secretome accounts for a significant proportion of the total bacterial proteome and is likely to contain a number of important virulence or virulence associated factors including colonization and stress survival factors [61]. As such, *in silico* based predictions of the *C. ureolyticus* secretome can assist in cataloguing the strain’s pathogenic potential.

A total of 288 proteins were predicted to be secreted by *C. ureolyticus* DSMZ 20703 (Table S3) including at least 25 proteins with putative virulence roles (Fig. 3a). Additionally, the secretome of ACS-265 was predicted to contain 269 proteins (Table S6), 28 of which have proven roles in virulence (Fig. 3b).

Although these results are slightly lower than has been reported for *C. concisus* 13826 when using SignalP3.0 [38], our figures align closely to the predicted secretome of *C. jejuni* NCTC 11168. A combination of SignalP4.1 and SecretomP2.0 analysis, predicted that a total of 256 proteins (13.5%) were secreted by either classical or non-classical pathways by *C. jejuni* NCTC 11168. SignalP4.1 predicted 115 proteins were classically secreted and SecretomP predicted 200 proteins were secreted by a signal peptide.
independent manner, however 59 of these proteins overlapped between the two servers.

These putative secreted virulence factors are of particular interest in light of a recent report by Burgos-Portugal et al [21], which showed that the secretome of *C. ureolyticus* is toxic to host cells, significantly reducing cell viability in epithelial cell lines. It is important to note that these proteins are only predicted to be secreted and remain to be imperially proven.

**Genomic variations unique to *C. ureolyticus* DSMZ 20703; a virulence perspective**

Given that less than 83% of proteins are homologs between the two *C. ureolyticus* strains (Table S7 and S8), we identified 341 genes unique to DSMZ 20703 when compared to ACS-301-V-Sch3b, encoding proteins with a variety of functions; including capsular and extracellular polysaccharide formation, iron acquisition, metabolism, transport systems, phage components, stress response, 16 putative virulence factors subcategorised into toxins and adhesions, as well as 242 hypothetical proteins (Table S4).

**Toxins**

We identified 13 haemolytic cytotoxins and cytolytin related proteins, of which 8 were predicted to be secreted (Table 4). Owing to their ability to increase the availability of iron during the process of infection [62], such pore-forming toxins represent an important component of a pathogen’s virulence repertoire. Indeed, cell-associated and secreted haemolysins/cytolysins in the well-studied *C. jejuni* and several of the emerging *Campylobacter* species have previously been noted as potential contributors to Campylobacter gastroenteritis [62,63].

Within this group, at least 8 repeats-in-toxins (RTX) related proteins, 6 of which were predicted to be secreted were identified. Various numbers of such repeats are found in the RTX domains of several cytotoxins/leukotoxins involved in the virulence of a number of different Gram-negative genera [64]. A characteristic feature of these exoproteins is the use of type I secretion systems to facilitate protein export across the bacterial envelope into the extracellular space [65]; their functionality being limited by Ca^{2+} ion availability, which serves to sequester activity until outside of the bacterial cell [65].

Furthermore, 3 of these secreted RTX haemolysins appear to be iron-regulated, with homology (up to 52% identity over the entire amino acid sequence) to the FrpC RTX protein of pathogen *Neisseria Meningitis*. Intriguingly, Osićka et al [64], reported the detection of FrpC-specific antibodies in the sera of patients recovering from invasive meningococcal disease, demonstrating that the FrpC-like protein is produced in vivo during infection, though its exact role in the infectious process remains unknown.
Campylobacter ureolyticus Virulence Potential

[Image: SDS-PAGE of C. ureolyticus Soluble Proteins. (A) Protein profiles of 7 Campylobacter ureolyticus strains. Phoretix 1D pro was employed to cluster the lanes based on banding patterns. The Dendrogram is an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Dice coefficient distance tree. (B) Original SDS-PAGE gel, with variable regions highlighted. Lane 1: Page Ruler Plus Ladder, lane 2: CIT001, lane 3: CIT002, lane 4: CIT004, lane 5: CIT005, lane 6: CIT007, lane 7: CIT009, lane 8: DSM 20703. doi:10.1371/journal.pone.0071515.g002]

[66,67]. Akin to the emerging pathogen C. concisus, the presence of iron-regulated haemolysins in C. ureolyticus suggests a potential function for such haemolysins as an important component in promoting human disease [62].

Additionally, a secreted S-layer RTX protein exhibiting 79% identity over the entire amino acid sequence of that of C. concisus was detected. Similarly, a study by Burgos-Portugal [68], which focused on the secreted proteins of C. ureolyticus UNSWCD, isolated from an intestinal biopsy of a child with Crohn’s disease, identified an S-layer RTX protein. Such S-layer proteins are thought to render resistance to complement while also providing haemolysins as an important component in promoting human disease [62].

aptly to DS0 20703, two genes encoding the HecA protein, a member of the filamentous hemagglutinin (FHA) family, were detected. Furthermore, directly upstream of one of the hecA genes, the hecB locus, coding for a haemolysin activation protein was also identified. The HecA/B proteins make up a two-partner secretion (TPS) system, whereby a TpsA family exoprotein containing a specific conserved secretion signal is recognised by a TpsB family channel-forming transporter allowing it to cross the membrane [72]. Rogers and colleagues [73] report that HecA homologs are found in both animal and plant pathogens and interestingly within animals appear to be restricted only to pathogenic species. Furthermore, HecA has been identified as an adhesion contributing to the virulence of C. jejuni [74].

Interestingly, the 5.8 kb hecA gene within the HecA/B operon has a G+C content 17% lower than the rest of the C. ureolyticus genome (G+C content 29%). Given that lateral gene transfer of hecA genes has previously been noted in other species [75], it is not unreasonable to suggest that this adhesion may have been acquired from a pathogen outside the Campylobacter genus. For example, Cardona and colleagues [76] identified an S-layer RTX protein. Such S-layer proteins are then expressed to the cell surface, where they can interact with host cells or other pathogens. The specific role of the HecA/B system in the pathogenesis of C. ureolyticus remains to be elucidated. Further studies are needed to determine the exact function of the HecA/B system in the pathogenesis of this emerging species.

Finally, a 30 kb region of the DSM 20703 genome contains genes involved in N-linked glycosylation including pgpG, pgpA, pgpB, and pgpI, genes coding for flippase enzymes along with those implicated in exopolysaccharide and lipopolysaccharide biosynthesis and modification (Table S2). Strikingly within this region, we have identified genes involved in Sialic Acid (N-acetylgalactosamine) metabolism. Of particular interest is a cluster of genes required for de novo synthesis of Sialic Acid: siaC (NeuB superfamily), siaD (CMP-Neu5Ac-synthase) and siaA (NeuC superfamily). Furthermore, analysis indicates these genes are homologous to those of N. meningitidis with 96–100% coverage and 50–76% identity over the entire amino acid sequences. Such genes are pivotal virulence factors to N. meningitidis, by studding its capsular polysaccharide and LOS with sialic acid it facilitates their ability to colonize, persist, evade host immune response and cause disease in mammalian species [83,84].

Similarly, C. jejuni has three sets of neu genes involved in sialic acid biosynthesis, whereby it’s thought a complete cluster (neuB1, C1, A1) has a role in LOS sialylation [85]. Sialic acid is an uncommon component of bacterial surface structures and,
Figure 3. Bioinformatics based predictions of the *C. ureolyticus* secretome. (A) 288 proteins were predicted to be secreted by *C. ureolyticus* DSMZ 20703 using the SignalP 4.1 server for classically secreted proteins and the SecretomeP 2.0 server for non-classically secreted proteins. Of the 288 proteins, 187 proteins were predicted to be secreted by the SecretomeP 2.0 server alone, 57 proteins by the SignalP 4.1 alone and 44 proteins were identified by both servers. Additionally, 117 proteins of 28 were hypothetical, the remaining 171 proteins were associated with a diverse range of functions relating to membrane transport, protein metabolism, respiration, stress response and at least 25 proteins with putative virulence roles. (B) SignalP4.1 and SecretomeP2.0 servers predicted 269 proteins are secreted by *C. ureolyticus* ACS-301-V-Sch3b. 165 proteins were predicted to be secreted by the SecretomeP 2.0 server alone, a further 65 proteins were identified by the Signal 4.1 server and 39 proteins were identified by both.
through molecular mimicry, may be crucial for evasion of host immunity and in post-infection autoimmune diseases such as Guillain–Barré syndrome [83,86].

Genomic variations unique to C. ureolyticus ACS-301-V-Sch3b: a virulence perspective
We identified 290 genes unique to ACS-301-V-Sch3b (Table S5), comprising proteins involved in cell wall and capsule biosynthesis, membrane transport, phage replication, stress response, DNA and protein metabolism, 20 putative virulence factors subcategorised into type IV secretion and toxin-antitoxin systems as well as 193 hypothetical proteins.

Type IV Secretion systems (T4SS)
We identified 16 genes, 8 of which are predicted to be secreted, which appear to be involved in the archetypal VirB/D4 Type IV secretion apparatus (Table 5). Type IV secretion systems (T4SS) can be regarded as multi-subunit, molecular syringes, spanning the cell envelope to inject their specific substrate into the cytosol of target cells [87]. They are involved in conjugative DNA transfer in prokaryotes [88] and are exploited by several mammalian pathogens [89] for toxin secretion and targeted delivery of virulence factors into eukaryotic host cells during infection [90], contributing directly to pathogenicity [91].

The 16 putative C. ureolyticus virB/D4 genes, appear to contain all of the necessary components for a structurally functional T4SS. Additionally, it is worth noting that C. ureolyticus has two copies each of the virB genes: virB8, virB9, virB10 and virB11. A total of 15/16 and 10/16 of the VirB/D4 proteins in DSM 20703 were homologs to the virB/D4 genes of C. jejuni and C. fetus subspecies venerateis with an average Identity of 55% and 60% respectively. Kienesberger et al [41], reported that mutational inactivation of the virD4 and virB9 components in virulent C. fetus subsp. venerateis isolates resulted in attenuated invasion and cell-killing phenotypes in cultured human cell lines, concluding that the VirB/VirD4 T4SS is necessary for efficient invasion and cytotoxic damage [90,92]. Additionally, C. ureolyticus, like a number of other pathogens [92], contains a FIC (filamentation induced by cyclic AMP) domain-containing protein downstream of the virB-virD4 genes. FIC proteins disrupt host cell processes through AMPylation reactions on target proteins [93], and in C. fetus have been shown to be potential effector proteins translocated by the T4 machinery to mammalian cell [90], where they regulate host processes important to pathogen survival and replication [90].

Furthermore, the VirB/D4 genes in pathogen C. jejuni, located on the pVir plasmid, are likely to play an important role in bacterial invasion [94,95,96]. In support of this proposal, mutation analysis of the C. jejuni virB11 gene, carried out by Bacon et al [94], resulted in a 6-fold reduction in adherence and an 11-fold reduction in invasion leading to reduced virulence in a ferret model of infection. Subsequent studies from the same group reported that modifications to the virB9 gene resulted in a significant reduction in C. jejuni invasion [95].

Additionally, it is worth noting that the ACS-301-V-Sch3b strain also contains 3 tra genes; traL, traF and traJ in addition to the tra operon, composed of at least 10 tra genes; traB, traC, traD, traE, traM, traF, traH, traF, traG and traH. The products of these genes form part of a F-T4SS [97], however unlike the VirB/D4 genes associated with the P-T4SS, F-T4SS are most likely concerned with bacterial conjugation and DNA exchange [91].

Toxin-Antitoxin system
Within the unique proteins of the ACS-301-V-Sch3b strain we detected a Zeta toxin. This kinase is usually co-expressed as part of a toxin-antitoxin (TA) module consisting of labile antitoxin (Epsilon) and a stable toxin (Zeta) in several pathogenic bacteria [98]. As Zeta toxins may provoke an autolytic phenotype, Meinhart and colleagues [98], speculate that the suicide of a few bacteria in a rapidly growing population may promote the release of other toxins that can attack their host cells or competing bacteria, thereby protecting their own. On the other hand, Lioy and colleagues [99], demonstrate that the Zeta toxin initially induces a set of protective responses, with selective up and down-regulation of particular genes to promote entry into dormancy rather than showing bactericidal behaviour. As the ability of TA systems to induce cell lysis or cell stasis has also been linked to biofilm and persister cell formation in pathogens [98], it would be interesting to investigate the prevalence of the Zeta toxin amongst other C. ureolyticus strains [90,99,100].

Virulence determinants conserved between C. ureolyticus DSMZ 20703 and ACS-301-V-Sch3b
Amongst the 1,469 homologous proteins (Tables S5 and S6) shared between these two strains include a minimum of 35 putative virulence factors (Table 4 and 5) associated with motility and biofilm formation, adhesion and invasion.

Type IV pili (TFP): twitching motility
Bacterial motility is an important factor in survival and pathogenesis [101]; however unlike most other Campylobacter species C. ureolyticus employs a flagellum-independent motility with the aid of type IV pili (TFP) [102,103,104]. At least 10 proteins associated with the formation of TFP were identified in both strains (Table 4 and 5). TFP are thin, flexible fibres displayed by a wide variety of Gram-negative bacteria whereby they use a modified version of the type II secretion system for their biogenesis. Such bacteria may employ TFP as linear actuators to enable directional crawling known as “twitching” motility, bearing resemblance to a grappling hook [6,105,106,107,108]. To negotiate significantly long distances (several microns) and orientate direction [100], multiple type-IV pili undergo cycles of repeated extension-adhesion and retraction-release using a “tug-of-war” mechanism [108,109] driven by an ATP motor [110]. TFPs are known bacterial virulence factors supporting adhesion to host cells and abiotic surfaces, biofilm formation, motility, and horizontal gene transfer [105,111,112].

Adhesion and Invasion
Amongst common features of many pathogenic microorganisms is their ability to utilize host cell factors to facilitate attachment and invasion. Of particular interest in the C. ureolyticus genome are 3 fibronectin associated proteins, 2 of which, Campylobacter adhesion to fibronectin (CadF) and Fibronectin-like protein A (FbpA), are predicted to be secreted. Fibronectin, a large glycoprotein, is a component of the extracellular matrix (ECM) of the human intestinal epithelium, serving as an adhesion molecule for many bacteria pathogens [113]. The CadF and FbpA proteins are
Table 4. Putative virulence associated genes in *C. ureolyticus* DSM 20703.

| Gene Function                          | Accession No. | Homolog in ACS-301-V-Sch3b | Secreted |
|----------------------------------------|---------------|-----------------------------|----------|
| **Type IV pili (T4P) Formation**       |               |                             |          |
| Twitching motility protein PilT        | KC907219      | Yes                         | No       |
| Type II secretion envelope pseudopilin protein (PulG, guides folded protein to PulD in outer membrane) | KC907220 | Yes | No |
| Type II secretion envelope pseudopilin protein (PulG, guides folded protein to PulD in outer membrane) | KC907221 | Yes | No |
| Type IV pilus biogenesis protein PilQ  | KC907222      | Yes                         | No       |
| Type IV pilus biogenesis protein PilQ  | KC907223      | Yes                         | No       |
| Type IV pilus biogenesis protein PilQ  | KC907224      | Yes                         | Yes      |
| Leader peptidase (Prepilin peptidase)/N-methyltransferase | KC907225 | Yes | No |
| Type II secretory pathway, ATPase PulE/Tfp pilus assembly pathway, ATPase PilB | KC907226 | Yes | No |
| putative prepilin-type N-cleavage/methylation domain protein | KC907227 | Yes | Yes |
| Type II secretion envelope pseudopilin protein (PulG, guides folded protein to PulD in outer membrane) | KC907228 | Yes | Yes |
| Twitching motility protein PilT        | KC907229      | Yes                         | No       |
| **Factors involved in colonisation and adhesion** |               |                             |          |
| Autotransporter adhesin with YadA-like domain | KC907230 | Yes | Yes |
| exoprotein with autotransporter precursor | KC907231 | Yes | Yes |
| FliA; Type III fibronectin domain-containing lipoprotein | KC907232 | Yes | Yes |
| Fibronectin/fibrinogen-binding protein | KC907233 | Yes | No |
| CadF; Outer membrane fibronectin-binding protein | KC907234 | Yes | Yes |
| Death-on-curing family protein         | KC907235      | Yes                         | No       |
| Death-on-curing family protein         | KC907236      | Yes                         | No       |
| (HecA) Filamentous Hemagglutinin/Putative large exoprotein involved in heme utilization or adhesion | KC907237 | No | No |
| (HecA) homolog Filamentous Hemagglutinin/Putative large exoprotein, heme utilization or adhesion | KC907238 | No | No |
| Two-component system response regulator RacR | KC907239 | Yes | No |
| Major outer membrane protein (Cmp/ParA) | KC907240 | Yes | Yes |
| PAL; Peptidoglycan associated lipoprotein Omp 18 | KC907241 | Yes | Yes |
| CjaC homolog | KC907242 | Yes | Yes |
| Amino acid ABC Transporter/PEB1 (surface antigen) homolog | KC907243 | No | Yes |
| Outer membrane lipoprotein omp18 precursor/CjaD | KC907244 | Yes | Yes |
| Capsule biosynthesis protein capA | KC907245 | Yes | No |
| CjaA surface adhesion | KC907246 | Yes | Yes |
| PEB4; Major antigenic peptide | KC907247 | Yes | Yes |
| **Hemolysins**                         |               |                             |          |
| Putative hemolysin | KC907248 | Yes | No |
| Probable hemagglutinin/S-type Pyocin/Colicin E3 (cytotoxic domain) | KC907249 | No | Yes |
| TlyC; Hemolysins and related proteins containing CBS domains | KC907250 | Yes | No |
| TlyC; Hemolysins and related proteins containing CBS domains | KC907251 | Yes | No |
| Channel-forming transporter/hemolysin activation protein (HecB) TVSS | KC907252 | No | No |
| Filamentous haemagglutinin family; VacA-like (serine protease) | KC907253 | No | Yes |
| **RTX related cytotoxins**             |               |                             |          |
| RTX haemagglutinin, iron-regulated protein FrpC (N. meningitidis) related to hly | KC907254 | No | Yes |
| RTX haemagglutinin, iron-regulated protein FrpC (N. Meninfitidis) related to hly | KC907255 | No | Yes |
| RTX-Hemolysin-type calcium-binding region:FrpC related | KC907256 | No | Yes |
reported as major virulence factors in *C. jejuni* and facilitate adhesion and colonization to host epithelial cells [113,114]. Interestingly, a functional analysis of the *C. ureolyticus* secretome led Burgos-Portugal et al [21], to report the presence of a ‘CadF homologue’ which they suggest to be a significant contributing factor to the pathogenic potential of *C. ureolyticus* UNSWCD, presumably allowing for adhesion to and subsequent colonization of host cells.

Furthermore, CadF and FlpA have also been reported to be involved in the activation of the small Rho GTPases Rac1 and Cdc42 (by an as yet uncharacterised mechanism), enabling host cell entry [30,115]. The fibronectin host cell-surface receptor is the α5β1 integrin which is located on the luminal surface of M cells in the gastrointestinal tract and may promote preferential binding [116]. However, in intact epithelia, this is restricted to the basolateral surface and as such is not readily available for interaction with luminally positioned microbial pathogens [117,118]. Monteville et al. [119] demonstrated that adherence and internalization of *C. jejuni* were significantly increased by exposure of cellular basolateral surfaces, and that Fn was the receptor; suggesting that *C. jejuni* invasion may preferentially occur via a paracellular rather than an intracellular route.

Intriguingly, *in vitro* studies of *C. ureolyticus* UNSWCD indicate that this organism is capable of translocating across the cell monolayer, proposing that, as with *C. jejuni*, *C. ureolyticus* might also invade via a paracellular route [21]. In support of this hypothesis, in addition to the fibronectin binding proteins, we have also identified the zona occludens toxin (Zot) in both *C. ureolyticus* strains. The apical domain of epithelial cells is separated from the lateral domain by the zona occludens, which forms the tight junction [120]. Zot is known to mimic a physiological modulator of intercellular tight junctions, and is used by virulent pathogens such as *Vibrio cholerae* and *Neisseria meningitidis* to induce a reversible opening of tight junctions between cells and increase the paracellular permeability in a non-toxic manner [121,122]. Analysis indicates that Zot of *C. ureolyticus* bears greatest resemblance to that of *C. concisus* (Fig. 4), forming paraphyletic groups and thus are likely to have shared a common ancestor. As proposed for the emerging pathogen *C. concisus* [5] and in agreement with *in vitro* studies [21], it is likely that *C. ureolyticus* is capable of attaching to and invading the host paracellularly. We suggest *C. ureolyticus* likely targets the host cell tight junctions, by expressing Zot, and binds to the basolateral surface of the cell via fibronectin binding proteins such as the secreted CadF and FlpA.

Additional factors which may promote *C. ureolyticus* adhesion, colonisation and invasion include the 4 secreted proteins CjaA, CjaC, PEB4 and Pal/Omp18. The CjaA, CjaC and Pal/Omp18 represent a surface-exposed protein, an ABC-transporter protein and a cell membrane-associated protein respectively. All 3 proteins are known to be highly immunogenic during human infection with the foodborne pathogen *C. jejuni* [123]. Moreover, the PEB4 protein is an antigentic virulence factor implicated in host cell adhesion, invasion, and colonization in *C. jejuni* [100].

| Gene Function | Accession No. | Homolog in ACS-301-V-Sch3b | Secreted |
|---------------|---------------|----------------------------|----------|
| RTX related Ca binding hemolysin (FrpC homolog)outer membrane adhesin-like protein, partial | KC907257 | No | Yes |
| Calcium binding hemolysin protein (Not in other Campy spp)/LeukotoxinA (lktA)/hlyA | KC907258 | No | No |
| Haemolysin type calcium binding protein RTX related | KC907259 | No | Yes |
| Toxins | | | |
| Vacuolating cytotoxin paralog VacA | KC907260 | Yes | Yes |
| Addiction module toxin, RelE/StbE family | KC907261 | Yes | No |
| Zona occludens toxin | KC907262 | Yes | No |
| Zona occludens toxin | KC907263 | Yes | No |
| S-layer RTX protein | KC907264 | No | Yes |
| Invasion | | | |
| Campylobacter invasion antigen B (CiaB) | KC907265 | Yes | No |
| PldA; Phospholipase A1 precursor; Outer membrane phospholipase A | KC907266 | Yes | Yes |
| U32 Pepsidase; collagenase family | KC907267 | Yes | No |
| Cell wall-associated hydrolases (invasion-associated proteins) | KC907268 | Yes | Yes |
| T6SS | | | |
| IcmF-related protein | KC907269 | No | No |
| Type II secretion system | | | |
| MSHA biogenesis protein MshL | KC907270 | Yes | Yes |
| MSHA biogenesis protein MshM | KC907271 | Yes | No |
| Type I Secretion system | | | |
| Type I secretion membrane fusion protein, HlyD/LktD (Hemolysin secretion protein) | KC907272 | No | No |
| Hemolysin secretion protein HlyB/Leukotoxin translocation; LktB | KC907273 | No | No |

Table 4. Cont.

doi:10.1371/journal.pone.0071515.t004
| Gene Function | Accession No. | Homolog in DSM 20703 | Secreted |
|---------------|--------------|----------------------|----------|
| **Type IV secretion system (T4SS)** | | | |
| Minor pilin of type IV secretion complex (VirB5) | KC465892 | No | Yes |
| Inner membrane protein forms channel for type IV secretion of T-DNA complex, VirB4 | KC465888 | No | No |
| ATPase provides energy for assembly of type IV secretion complexes & secretion of T-DNA complex (VirB11) | KC465883 | No | No |
| Type IV secretion/competence protein (VirB10) | KC465893 | No | Yes |
| Type IV secretion/competence protein (VirB9) | KC465894 | No | Yes |
| VirB8 | KC465897 | No | No |
| VirB4 | KC465896 | No | No |
| Bore hole in peptidoglycan layer allowing type IV secretion complex assembly to occur (VirB1) | KC465885 | No | Yes |
| Type IV secretion system protein VirD4 | KC465895 | No | No |
| Integral inner membrane protein of type IV secretion complex (VirB6) | KC465891 | No | Yes |
| Inner membrane protein forms channel for type IV secretion of T-DNA complex, VirB8 | KC465890 | No | No |
| Forms the bulk of type IV secretion complex that spans outer membrane and periplasm (VirB9) | KC465887 | No | No |
| Inner membrane protein forms channel for type IV secretion of T-DNA complex (VirB10) | KC465889 | No | Yes |
| ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex, VirB11 | KC465884 | No | No |
| Coupling protein VirD4, ATPase required for T-DNA transfer | KC465886 | No | No |
| IncQ plasmid conjugative transfer DNA nicking endonuclease TraR (pTi VirD2 homolog) | KC907204 | No | Yes |
| Type IV secretion/conjugative transfer protein TrbC (VirB2; pilins) | KC907205 | No | Yes |
| **Adhesion and colonisation** | | | |
| Death-on-curing family protein | KC465877 | Yes | No |
| Death on curing protein, Doc toxin | KC465876 | Yes | No |
| Cell filamentation-like protein (Fic domain) | KC465874 | No | No |
| Cell Surface Protein (YadA Domain protein) | KC465875 | Yes | Yes |
| CadF; Outer membrane fibronectin-binding protein | KC465879 | Yes | Yes |
| Fibronectin/fibrinogen-binding protein | KC465878 | Yes | No |
| FlpA; Type III fibronectin domain-containing lipoprotein (String) | KC465881 | Yes | Yes |
| Two-component system response regulator RacR | KC465882 | Yes | No |
| Outer membrane lipoprotein omp18 precursor/CjaD | KC907206 | Yes | Yes |
| Capsule biosynthesis protein capA | KC465850 | Yes | No |
| CjaA; surface antigen | KC907207 | Yes | Yes |
| CjaC | KC907208 | Yes | Yes |
| PEB4; Major antigenic peptide | KC907209 | Yes | Yes |
| Major outer membrane protein (Cmp/PorA) | KC907210 | Yes | Yes |
| PAL; Peptidoglycan associated lipoprotein Omp 18 | KC907211 | Yes | Yes |
| **Invasion and intracellular resistance** | | | |
| Campylobacter invasion antigen B (CiaB) | KC465873 | Yes | No |
| Cell wall-associated hydrolases (invasion-associated proteins) | KC907212 | Yes | Yes |
| PltA; Phospholipase A1 precursor; Outer membrane phospholipase A | KC465880 | Yes | Yes |
| U32 pepsidase; collagenase family | KC465849 | Yes | No |
| **Toxins** | | | |
| Putative vacuolating cytotoxin (VacA) paralog | KC465870 | Yes | Yes |
| Zona occludens toxin | KC465872 | Yes | No |
| Zeta toxin | KC465871 | No | Yes |
| Hypothetical protein; addiction module RelE/StbEtoxin | KC465869 | Yes | No |
| MSHA biogenesis protein MshL pilus (Type ii and iii secretion system) | KC907213 | Yes | Yes |
| MSHA biogenesis protein MshM | KC907214 | Yes | No |
A further two proteins, the Campylobacter invasive antigen (CiaB) and the secreted phospholipase A (PldA), were detected and are likely to contribute to the pathogenic potential of C. ureolyticus. PCR confirmed that the ciaB gene is common among C. ureolyticus strains (Fig. S1) and ClustalW alignment revealed that the nucleotide sequence of this gene is highly conserved between strains. Both CiaB and PldA have been linked to an invasive phenotype in C. jejuni [124]. Indeed, disruption of the C. jejuni ciaB gene, which encodes a protein that is translocated into the cytoplasm of eukaryotic cells, results in a non-invasive phenotype [125].

**Table 5.** Cont.

| Gene Function | Accession No. | Homolog in DSM 20703 | Secreted |
|---------------|---------------|----------------------|----------|
| Hemolysins and Haemaglutinins |              |                      |          |
| Putative hemolysin | KC465868 | Yes | No |
| Hemolysins and related proteins containing CBS domains (TlyC) | KC465866 | Yes | No |
| Hemolysins and related proteins containing CBS domains (TlyC) | KC465867 | Yes | No |
| Hemaglutinin/autotransporter | KC907215 | No | Yes |
| Hemaglutinin/autotransporter | KC907216 | Yes | Yes |
| **Type IV pili** | | | |
| Type II secretory pathway, ATPase PulE/Tfp pilus assembly pathway, ATPase PilB | KC465857 | Yes | No |
| Type IV pilus biogenesis protein PilQ | KC465858 | Yes | No |
| Leader peptidase (Prepilin peptidase)/N-methyltransferase | KC465851 | Yes | No |
| Putative prepilin-type N- cleavage/methylation domain protein | KC465854 | Yes | Yes |
| Twitching motility protein PiIT | KC465855 | Yes | No |
| Twitching motility protein PiIT | KC465856 | Yes | No |
| Type II secretion envelope pseudopilin protein (PulG) | KC907217 | Yes | No |
| Type II secretion envelope pseudopilin protein (PulG) | KC907218 | Yes | No |

doi:10.1371/journal.pone.0071515.t005

**Figure 4. Evolutionary relationships of taxa Zona Occluden Toxin (ZOT).** The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. DSMZ 1 and DSMZ 2 correspond to the ZOT paralogs in C. ureolyticus DSM20703. ACS-301-V-Sch-3: C. ureolyticus ACS-301-V-Sch-3b. doi:10.1371/journal.pone.0071515.g004

**Autotransporters**

A protein family of particular interest, predicted to be present in both C. ureolyticus strains, are the autotransporters. These proteins represent an extensive and rapidly growing family contributing to bacterial virulence in Gram-negative bacteria such as C. jejuni and H. pylori [126,127]. Although autotransporters share a common mode of secretion (similar translocation units), the passenger domains at the N-terminal of the protein are highly diverse [128]. Almost without exception, all characterized passenger protein domains of autotransporters have been implicated in bacterial virulence relating to bacterial motility, adhesion, host immunomodulation, toxigenicity and intracellular spread [127,128].
Although we have identified several autotransporter related genes (Table 4 and 5), the products of which have all been predicted to be secreted, of particular interest were the adhesion related autotransporters (2 copies of which are found in ACS-301-V-Sch3b) containing a YadA domain. The YadA protein is a major adhesin of Yersinia pseudotuberculosis which is noted to promote tight adhesion to mammalian cells by binding to extracellular matrix proteins [100]. Eitel et al. [108] reports the expression of YadA promotes both bacterial adhesion and high-efficiency invasion. Moreover, the YadA protein has been detailed to mediate the initial adhesion, uptake, and transfer of the bacteria through M cells of the intestine in addition to establishing extracellular colonization of the liver, spleen and underlying lymphatic tissue [100].

Toxin-Antitoxin systems

Interestingly, two bacterial toxin-antitoxin (TA) systems RelE/StbE and the death on-curing (Doc) toxin were conserved in both C. ureolyticus strains. TA modules are highly abundant in opportunistic pathogens such as Mycobacterium tuberculosis, as mentioned previously when discussing the Zeta toxin, the ability of TA systems to induce cell lysis or cell stasis has been linked to biofilm and persist cell formation in pathogens [98,129]. RelE for example, is a global inhibitor of translation during nutrient stress, and its expression reduces the chance of starvation by lowering the cell’s nutrient requirements [130]. Doc, which appears to be quite conserved amongst strains as indicated by PCR (Fig. S1) and expressed under standard conditions, resembles members of another family of bacterial proteins called Fic. Bacterial toxin-antitoxin (TA) systems typically facilitate cell survival during intervals of stress allowing cells to switch to a reversible quasidormant state [131] and expression of such genes under standard conditions may be reflective of a subset of older or stressed cells in the culture. It is likely that these systems may be of particular importance relating to the viable but non-cultivable (VBNC) phenomenon observed within several Campylobacter species [15,132].

Conclusion

The pathogenic mechanisms responsible for acute intestinal infections by Campylobacter spp, although still poorly understood, are thought to involve adherence, cellular invasion, and toxin production. With the aid of whole genome analysis, comparative bioinformatics and secretome prediction we have identified a minimum of 106 potential virulence related factors, encompassing each of the known virulence tactics of pathogenic Campylobacter spp. Furthermore, similar to the emerging pathogen C. concisus, using genome comparisons and proteins profiles we propose the possibility of genospecies within C. ureolyticus; a taxonomic continuum comprised of several species that are likely to have different impacts on human health and disease. Campylobacter species tend to be specialists not generalists, thus the presence of such a diverse number of virulence homologs warrants functional investigation. This study provides the first whole genome analysis of C. ureolyticus and a catalogue for the investigation and confirmation of this pathogen’s virulence gene arsenal.

Supporting Information

Figure S1 PCR analyses of the RTX 2861 bp (A), VirD4 1,625 bp (B), CiaB 1469 bp (C) and Doc 311 bp (D) genes in seven Campylobacter ureolyticus strains. Lane 1: Molecular weight marker Hyperladder I (A-C, Hyperladder II (D), lane 2: CIT01, lane 3: CIT02, lane 4: CIT04, lane 5: CIT 05, lane 6: CIT07, lane 7: CIT09, lane 8: DSM 20703 and lane 9: negative control. It is important to note that the absence of an amplicon does not confirm the absence of the gene. The primers are specific to the nucleotide sequences of C. ureolyticus DSM 20703 and/or C. ureolyticus ACS-301-V-Sch3b and does not account for single nucleotide polymorphisms or variable regions between strains within the primer annealing regions. This PCR should be seen as a confirmatory rather than exclusionary test. Also, rt-PCR indicated that of theses 4 genes, doc was the only protein expressed under standard conditions (in all 5 strains as above).

Table S1 Sequence based comparison: 128 protein coding genes of C. ureolyticus DSM 20703 and ACS-301-V-Sch3b highly conserved (>70% amino acid identity) across 8 different Campylobacter species and 1 subsp.

Table S2 Sequence based comparison: 65 protein coding genes of C. ureolyticus DSM 20703 and ACS-301-V-Sch3b highly conserved within theses strains (>70% amino acid identity) but no homologs (<25% amino acid identity) within 8 different Campylobacter species and 1 subspecies.

Table S3 Secretome of C. ureolyticus DSM 20703.

Table S6 Secretome of C. ureolyticus ACS-301-V-Sch3b.

Table S7 Secretome of C. ureolyticus ACS-301-V-Sch3b.

Table S8 Secretome of C. ureolyticus ACS-301-V-Sch3b.

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