Comparative Genomics of All Three *Campylobacter sputorum* Biovars and a Novel Cattle-Associated *C. sputorum* Clade

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Abstract

Campylobacter sputorum is a nonthermotolerant campylobacter that is primarily isolated from food animals such as cattle and sheep. *C. sputorum* is also infrequently associated with human illness. Based on catalase and urease activity, three biovars are currently recognized within *C. sputorum*: bv. sputorum (catalase negative, urease negative), bv. fecalis (catalase positive, urease negative), and bv. paraureolyticus (catalase negative, urease positive). A multi-locus sequence typing (MLST) method was recently constructed for *C. sputorum*. MLST typing of several cattle-associated *C. sputorum* isolates suggested that they are members of a divergent *C. sputorum* clade. Although catalase positive, and thus technically bv. fecalis, the taxonomic position of these strains could not be determined solely by MLST. To further characterize *C. sputorum*, the genomes of four strains, representing all three biovars and the divergent clade, were sequenced to completion. Here we present a comparative genomic analysis of the four *C. sputorum* genomes. This analysis indicates that the three biovars and the cattle-associated strains are highly related at the genome level with similarities in gene content. Furthermore, the four genomes are strongly syntenic with one or two minor inversions. However, substantial differences in gene content were observed among the three biovars. Finally, although the strain representing the cattle-associated isolates was shown to be *C. sputorum*, it is possible that this strain is a member of a novel *C. sputorum* subspecies; thus, these cattle-associated strains may form a second taxon within *C. sputorum*.

Key words: sputorum, fecalis, *Campylobacter sputorum*, paraureolyticus, comparative genomics.

Introduction

An anaerobic vibrio was isolated in 1914 from a patient with acute bronchitis (Tunnicliff 1914), and was subsequently assigned the name *Vibrio sputorum* by Prévot (Prévot 1940). *V. sputorum* was originally characterized as catalase-negative (MacDonald 1953) and an anaerobe (Breed et al. 1957), but subsequent studies established that these organisms were microaerophilic (Loesche et al. 1965). Related organisms *V. bubulus* and "*V. fecalis*" were isolated from cattle (Florent 1953) and sheep (Firehammer 1965), respectively. Although *V. sputorum* and *V. bubulus* were originally proposed to be related at the subspecies level (Loesche et al. 1965), these organisms, now assigned to the genus *Campylobacter* (Véron and Chatelain 1973), and "*Campylobacter fecalis*" could not be distinguished by DNA homology, and were classified as biovars of *C. sputorum* (Roop et al. 1985). This

taxonomic division was subsequently revised (On et al. 1998); currently, three *C. sputorum* biovars are recognized, based on catalase and urease activity: bv. sputorum, bv. fecalis (catalase positive), and bv. paraureolyticus (urease positive).

C. sputorum strains have been isolated primarily from livestock, such as cattle (Atabay and Corry 1998; On et al. 1999; Oporto and Hurtado 2011) and sheep (On et al. 1998; Terzolo 1988). However, *C. sputorum* has also been isolated from feral swine (Jay-Russell et al. 2012), and healthy or diarrheic humans and dogs (Chaban et al. 2010; Inglis et al. 2011). Isolation of *C. sputorum* from human clinical samples is infrequent and only biovars sputorum and paraureolyticus have been obtained from such material (On et al. 1998).

A novel multi-locus sequence typing (MLST) method was developed that could type *C. sputorum* (Miller et al. 2012). This method was used to characterize several *C. sputorum*

Table 1

General Features of the *C. sputorum* Genomes

	bv. sputorum RM3237	bv. fecalis LMG 8532	bv. paraureolyticus LMG 17589	*C. sputorum* RM8705
General				
Source	Unknown	Cow, fecal	Human, fecal	Cow, fecal
Location	Unknown	UK	Canada	USA (Calif.)
MLST sequence type	ST-1	ST-3	ST-2	ST-8
Coverage ($\times$)(454;Illumina;PacBio; Total)	85;377;171; 633	124;1218;629;1971	73;1107;622; 1802	60;1081;842; 1983
Accession #	CP019682	CP019683	CP019684	CP019685
Chromosome				
Size (kbp)[a]	1752.3	1757.3	1725.0	1681.3
% G+C content	29.7	29.7	29.6	29.3
CDS numbers	1,664	1,685	1,662	1,586
Assigned function (% CDS)	880 (53)	874 (52)	891 (54)	858 (54)
Pseudogenes	55	46	33	56
General function (% CDS)	477 (29)	476 (28)	474 (28)	453 (29)
Hypothetical (% CDS)	307 (18)	335 (20)	297 (18)	275 (17)
Core genes (% CDS)	1,405 (84)	1,405 (83)	1,405 (85)	1,405 (89)
Taxon-specific genes[b] (% CDS)	87 (5)	62 (4)	57 (3)	91 (6)
Genetic islands	1	4	3	2
Zot islands	0	1	1	0
CRISPR/Cas loci	1 (type I-B)	1 (type I-B)	1 (type I-B)	1 (type I-B)
G:C tracts $\geq$ 8 nt				
Total;HV (# intergenic)	17;6 (3)	12;4 (3)	14;12 (9)	20;10 (5)
Signal transduction[c]				
Che/Mot proteins	11	11	11	11
MCPs	10 (6)	15 (1)	11 (4)	14 (2)
2CS response regulators	12	12	14	11 (1)
2CS histidine kinases	11 (2)	12 (2)	13 (2)	11 (2)
Diguanylate cyclases	5 (1)	5 (1)	7	7
Diguanylate phosphodiesterases	2 (1)	3	3	2 (1)
Other	6	5	6	4 (1)
Motility				
Fla genes	2	2	2	2
R/M systems[c]				
Type I (*hsd*)	1	0	0	0
Type II/IIG/III	1/1/0 (1)	0/1/0	1/1/0 (1)	0/1/0 (2)
DNA methyltransferases	2	1	2	1
Transcription				
Regulatory proteins	24	23	22	23
Catalase (*katA*)	N	Y	N	Y
Urease (*ureFECBADG*)	N	N	Y	N

[a]Size listed in kbp due to variation at the hypervariable G:C tracts.
[b]Genes observed in only one biovar or taxon.
[c]Numbers in parentheses are pseudogenes.
CRISPR: clustered regularly interspaced short palindromic repeats; HV: hypervariable; Che/Mot: chemotaxis/motility; MCP: methyl-accepting chemotaxis protein; 2CS: two component system; R/M: restriction/modification; Zot: zonula occludens toxin.

strains that were isolated from cattle in California (Miller et al. 2012). Phenotypic analysis of these strains indicated that they were mostly catalase positive, consistent with an identification of bv. fecalis. However, MLST typing clearly placed these organisms (STs 8, 13, 14, and 15) in a clade separate from the three recognized *C. sputorum* biovars (Miller et al. 2012).

In this study, we present four complete *C. sputorum* genomes: one for each *C. sputorum* biovar and a fourth genome (strain RM8705; ST-8) representing the divergent cattle-associated *C. sputorum* clade. The genomic data and analysis presented here provides further evidence that the divergent clade represents a novel taxon within *C. sputorum*.

Results

General Features

A summary of the *C. sputorum* genome features is presented in table 1. The four *C. sputorum* genomes are similar in size

(1,681–1,757 kbp.) and GC content (∼29.6%). This similarity in size also correlates with predicted coding sequences, where 1,586–1,685 CDSs were identified in the four genomes. Coding sequences were divided into three categories: assigned function (i.e., genes with a gene name and a specific function); general function; and hypothetical. Percentages of predicted coding sequences within each of these categories were also similar across the four genomes. No plasmids were identified in any of the four strains.

The *C. sputorum* genomes contain a low number of hypervariable G:C tracts and restriction–modification systems (table 1). Twelve to twenty GC tracts 8 bp or longer were identified in the *C. sputorum* genomes. However, only 4–12 of these tracts were observed to be hypervariable with many being intergenic or located within fragmented genes. Therefore, the number of contingency genes predicted in each *C. sputorum* genome ranges from one to five, with a total of nine for the four genomes. Five of these nine contingency genes were related to signal transduction, with one contingency gene encoding a restriction–modification system subunit. Very few restriction–modification systems (1–3 per genome) or nonR/M system-associated DNA methyltransferases (one to two per genome) were predicted to be encoded by the *C. sputorum* genomes.

Gene Conservation and Synteny

Comparative genomic analysis of the four closed genomes indicated that the "pan-*sputorum*" gene set contains a minimum of 1,972 genes (supplementary table 1, Supplementary Material online). Of these, 1,405 (approx. 83–89% of each genome) were identified in each *C. sputorum* genome, thus comprising the "core" gene set for the four closed genomes (table 1). Of the remaining 567 genes, 185 were associated with functional categories shown previously to be a source of variation within *Campylobacter* (e.g., genetic islands, biosynthesis of outer surface structures, restriction/modification, and signal transduction), and 186 were hypothetical genes not located within a genetic island. Additionally, 3–6% of each genome contains biovar-specific genes identified only in that genome ("Taxon-specific"; table 1), within the scope of the four *C. sputorum* strains.

The four *C. sputorum* genomes are generally collinear (fig. 1), with relatively few inversions or other syntenic breaks. Between the three biovars, the major syntenic breaks represent either genomic islands, including the zonula occludens toxin (*zot*) genetic island (Yap et al. 2014), or differences at the flagellar modification locus (fig. 1). An additional difference in bv. paraureolyticus is the presence of a *nosZDG/C1/C2/HFYL* nitrous oxide reductase locus, identified previously in other nonthermotolerant *Campylobacter* spp., for example, *C. fetus*, *C. iguaniorum*, *C. hyointestinalis, C. lanienae,* and *C. gracilis* (Miller and Yee 2015; Gilbert et al. 2016). Befitting its position within a divergent clade, further syntenic breaks
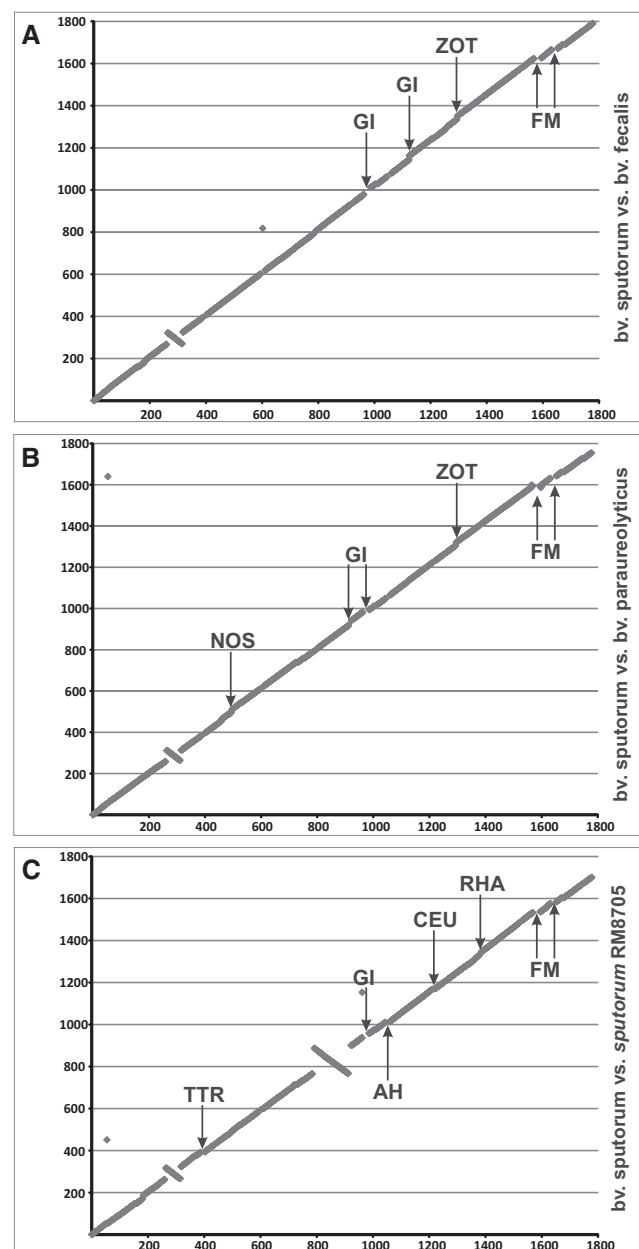

Fig. 1.—Colinearity of the *C. sputorum* genomes. The position of each core protein in the *C. sputorum* bv. sputorum genome (*x*-axes) is plotted against the genomic positions of their respective orthologs (*y*-axes) in (A) *C. sputorum* bv. fecalis, (B) *C. sputorum* bv. paraureolyticus or (C) *C. sputorum* strain RM8705. The *x* and *y* axis values represent locus tag numbers. GI: genomic island; ZOT: zonula occludens toxin island; FM: flagellar modification locus; NOS: *nosZDG/C1/C2/HFYL* nitrous oxide reductase locus; TTR: *ttrACBSR* tetrathionate reductase locus; AH: allophanate hydrolase locus; CEU: *ceuBCDE-exbBD-tonB* locus; RHA: dTDP-*β*-L-rhamnose locus.

were identified in the strain RM8705 genome, with respect to the genomes of the three *C. sputorum* biovars. These include the absences of three loci: a *ttrACBSR* tetrathionate reductase locus; the *ceuBCDE-exbBD-tonB* ferric enterobactin

transporter locus; and a locus encoding allophanate hydrolase (fig. 1). *C. sputorum* strain RM8705 contains an additional glycosylation locus not identified in the other three genomes (fig.1). This locus putatively encodes four-step pathways for the synthesis of either dTDP-β-L-rhamnose or dTDP-3-amino-3,6-dideoxy-α-D-galactopyranose from α-D-glucopyranose-1-phosphate. A glycosylation locus is present at the same chromosomal location in the other three genomes; however, the loci encode different sets of glycosyltransferases (bvs. fecalis and paraureolyticus encode one set, bv. sputorum encodes another).

Average Nucleotide Identity and *In Silico* DNA–DNA Hybridization Analysis

The four *C. sputorum* genomes were compared to themselves and to the genomes of seven other related nonthermotolerant *Campylobacter* spp. using average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (isDDH) analyses (supplementary table 2, Supplementary Material online). ANI values range from 98% to 99% among the three *C. sputorum* biovars, while ANI values between strain RM8705 and the three biovars range from 94% to 95%. ANI values between *C. sputorum* and the other *Campylobacter* spp. are 71% or less. Similarly, isDDH values between the three biovars are between 92 and 95%, whereas the isDDH values between these biovars and strain RM8705 are between 82 and 87%. isDDH values between *C. sputorum* and the related *Campylobacter* spp. are less than 15%.

Discussion

The *C. sputorum* biovars form a discrete cluster within the nonthermotolerant *Campylobacter* spp. and are most related at the 16S rRNA level to *C. corcagiensis* and *C. hominis* (supplementary fig. 1, Supplementary Material online). Although the three *C. sputorum* biovars could be distinguished phenotypically, via catalase and urease activity, their genomic relatedness was not completely understood. *C. sputorum* segregate by biovar following MLST analysis (Miller et al. 2012), but this reflects only seven loci and not the complete genome. The data presented here, following comparative analysis of complete genomes, indicate that the *C. sputorum* biovars are highly similar, with several key features in common, such as 1–2 restriction–modification systems and few potential contingency genes. Their genomes are also collinear, with syntenic breaks that correspond to those identified in comparisons of other *Campylobacter* strains: for example, genetic islands and glycosylation (Pearson et al. 2003; Parker et al. 2006; Miller and Parker 2011; Ali et al. 2012). However, substantial differences are present between the three biovars; only 83–85% of the "core" gene set is conserved in each genome (table 1). Thus, the proportion of each biovar genome that is comprised of "core" genes is likely

lower than expected, considering that these genomes represent a single species, and a similar comparison of the six-species *C. lari* group identified a core gene proportion of ~77% per genome (Miller et al. 2014a). If and how these differences correlate with variation in host range and colonization, with pathogenicity, or with other features of *C. sputorum* biology remains to be determined. Moreover, the data presented here characterize single strains from each biovar; genomic data will be needed from additional strains to form a more complete picture. Nevertheless, these genomic data will be invaluable for further research into *C. sputorum* biology.

Preliminary typing data placed strain RM8705 in a clade separate from the cluster containing the three *C. sputorum* biovars (Miller et al. 2012). Both the ANI and isDDH data presented in this study (supplementary table 2, Supplementary Material online), using the full *C. sputorum* genomes, are consistent with these results. Strain RM8705, as well as other members of this clade, is catalase positive (Miller et al. 2012). Thus, strain RM8705 would be typed as bv. fecalis, although the data presented in supplementary table 2, Supplementary Material online suggest that this strain is not bv. fecalis *sensu stricto*. isDDH data indicate that strain RM8705 is *C. sputorum*, since the isDDH values with respect to related *Campylobacter* spp. are clearly above the 70% threshold that was recommended to define novel species (Wayne et al. 1987). However, both the ANI and isDDH values are substantially lower when strain RM8705 is compared to any of the three biovars. The ANI values are at or slightly below the 95% threshold proposed for species definition (Goris et al. 2007; Richter and Rossello-Mora 2009) and are consistent with ANI values observed previously between some *Campylobacter* subspecies (Miller et al. 2016). Thus, strain RM8705 and the other cattle-associated *C. sputorum* strains may be members of a novel subspecies within *C. sputorum*. Further research will be necessary to clarify these results.

Materials and Methods

Growth Conditions, Chemicals and Biochemical Assays

All *Campylobacter* strains were cultured routinely under microaerobic conditions [1–2% O_2 + Bioblend gas (10% H_2, 10% CO_2, and 80% N_2; Praxair, Danbury, CT)] at 37 °C on Anaerobe Basal Agar (ABA; Oxoid, ThermoFisher Scientific, Waltham, MA) supplemented with 15% (v/v) lysed horse blood (Innovative Research, Novi, MI). PCR enzymes and reagents were purchased from New England Biolabs (Beverly, MA) or Epicentre (Madison, WI). All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) or ThermoFisher Scientific. DNA sequencing reagents and consumables were purchased from Applied Biosystems (Foster City, CA), Roche Life Science (Indianapolis, IN), Illumina Inc. (San Diego, CA) or Pacific Biosciences (Menlo Park, CA). PCR and sequencing oligonucleotides were purchased from

Eurofins MWG Operon (Huntsville, AL). Prior to genome sequencing, all strains were validated by *atpA* typing (Miller et al. 2014b) or determination of catalase and urease activity (Miller et al. 2012).

PCR and DNA Sequencing

PCR and Sanger sequencing was performed as previously described (Miller et al. 2014a). Shotgun and paired-end (8–12 kb) 454 reads were obtained on a Roche 454 GS-FLX+ Genome Sequencer with Titanium chemistry using standard protocols. Illumina HiSeq reads were obtained from SeqWright (Houston, TX). SMRT sequencing was performed on the Pacific Biosciences (PacBio) RSII sequencing platform using 10 kb or 20 kb SMRTbell libraries, C2/C2 sequencing chemistry, and the 90-min data collection protocols. The SMRTbell libraries were prepared from 5 to 10 μg of bacterial genomic DNA, using the standard protocol from Pacific Biosciences (Menlo Park, CA, USA), and processed for sequencing as recommended by the supplier. A FASTQ file was generated from the PacBio reads using SMRTanalysis (ver. 2.1), and the reads were error-corrected using pacBioToCA with self-correction (Koren et al. 2013). The longest 20× of the corrected reads were assembled with Celera Assembler 8.1 (Koren et al. 2012). The resulting contigs were polished using Quiver (Chin et al. 2013).

Genome Assembly

Three next-generation sequencing platforms, Roche 454, Illumina HiSeq, and PacBio, were used to complete the four *C. sputorum* genomes (table 1), as previously described (Miller et al. 2014a). Briefly, for each genome: shotgun and paired-end 454 reads were assembled into a single chromosomal scaffold; a custom Perl script, the 454 repeat contigs and PCR amplification/Sanger sequencing were used to bridge the contig gaps and create a contiguous sequence; Illumina HiSeq reads were used to validate all 454 base calls; and PacBio data were used for further base call validation and to address repeat regions that could not be resolved using 454/Illumina/Sanger sequencing. Each assembly was also verified using a bacterial optical restriction map (OpGen, Gaithersburg, MD). The final coverage, using all three next-generation sequencing platforms, was between 633 and 1983× (table 1).

Genome Annotation

Putative coding sequences (CDSs) were identified using GeneMark (Besemer and Borodovsky 2005). Annotation was accomplished by comparing the predicted proteins to the proteomes of related *Campylobacter* species [i.e., *C. concisus* (CP000792), *C. curvus* (CP000767), *C rectus* (ACFU00000000), *C. showae* (AMZQ00000000), *C. corcagiensis* (JFAP00000000), *C gracilis* (CP012196), and

C. ureolyticus (CP012195)] and to the NCBI nonredundant (nr) database using BLASTP; positive matches had an identity of ≥ 50%, and an alignment length of ≥ 75% across both the query and match sequences. Final annotation, including manual start codon curation, determination of homopolymeric G:C tract variability and identification of rRNA- and tRNA-coding genes and pseudogenes, was performed as previously described (Miller et al. 2014a). CRISPR elements were identified using CRISPRfinder (Grissa et al. 2007).

Comparative Genome Analysis

The predicted proteomes of the completed *C. sputorum* genomes were combined to create a composite proteome. Comparative genomic analysis was performed through a pairwise BLASTP analysis of this composite proteome against itself. For each protein, a custom Perl script was used to identify the top match within the other three proteomes, where present, using the match parameters described above. Additional pairwise BLASTP analyses, where the composite *C. sputorum* proteome was amended with various sets of *Campylobacter* proteomes, were also performed, but at a match identity of 35%. Proteins identified in all four *C. sputorum* genomes were assigned to the "core" protein set. The "pan-*sputorum*" proteome of 1,972 proteins was compiled using these "core" proteins and proteins identified in one, two or three of the *C. sputorum* taxa (supplementary table 1). Further comparative analyses were performed using JSpecies [v. 1.2.1; Richter and Rossello-Mora 2009] and default parameters to determine average nucleotide identity (ANI) values, and GGDC (Meier-Kolthoff et al. 2013) to calculate *in silico* DNA–DNA hybridization (isDDH) values.

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