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ORAL MICROBIOLOGY
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Identification of novel genes in the oral pathogen *Campylobacter rectus*

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Introduction: A poorly described bacterium, *Campylobacter rectus*, has been implicated as an etiological agent of periodontal disease. The aim of this study was to use a comparative genomics approach to identify genes that contribute to the lifestyle of *C. rectus* as an oral pathogen.
Methods: Suppressive subtractive hybridization was used to identify genes encoded by *C. rectus* ATCC 33238, but not present in the genome of a related *Campylobacter* species, *Campylobacter jejuni* ATCC 11168.
Results: Suppressive subtractive hybridization identified 154 unique DNA sequences from the *C. rectus* genome. Ninety-two of the 154 clones were classified as *C. rectus*-specific, as they did not show significant sequence homology to genes identified in any strain of *C. jejuni* (BLAST E-value >1E-3). BLAST analysis predicted that the 92 *C. rectus*-specific gene fragments play a role in a variety of biological processes including signal transduction mechanisms (histidine kinase, response regulators, diguanylate cyclases, chemotaxis receptor) and potentially virulence (S-layer RTX and cysteine desulfhydrase). Further analysis of the *C. rectus*-specific clones showed that 10 genes had *Campylobacter* homologues that were only found in species that commonly reside within the oral cavity of humans and 10 other fragments shared homology only with non-campylobacter organisms.
Conclusions: These data provide the first substantial insights into the genomic content of *C. rectus*, a significant oral pathogen. The genes identified in this study are a valuable resource for initiating new research on the virulence of *C. rectus* during periodontitis.

Key words: *Campylobacter rectus*; gene discovery; periodontitis; subtractive hybridization

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The most common type of periodontitis is a microbe-induced, inflammatory disorder of the connective tissue and alveolar bone that surrounds and supports the teeth (21). Periodontitis is a common disease, affecting up to 35% of adults between the ages of 30 and 90 years in the United States alone (1). Approximately 13% of patients with periodontitis will develop severe forms of the disease, which, if untreated, may result in tooth loss and systemic complications including increased blood sugar levels, an increased risk of heart attack and stroke, and an increased risk of pregnancy complications (21, 24).

Campylobacter rectus is a poorly characterized, motile, anaerobic, gram-negative bacterium that has been implicated as an etiological agent of periodontal disease (9, 11, 15). Interestingly, recent studies indicate that women with periodontal disease are up to seven times more likely to experience preterm labor than their healthy counterparts (4, 18). Serological data from human studies have implied that *C. rectus*, as part of the polymicrobial community, plays a significant role in the preterm labors of mothers with periodontitis (16). In addition, a pregnant mouse model has shown the association of *C. rectus* with fetal growth restriction and decreased survival of neonatal pups (19, 30), including type strain ATCC 33238 (30).

The genome of *C. rectus* has not been sequenced, and therefore the suite of genes important to the survival of *C. rectus* within the oral cavity has not been identified. In this study, suppressive subtractive hybridization (SSH) (29) was used to identify genes encoded by *C. rectus* but not present in the sequenced genome of a related *Campylobacter* species, *Campylobacter jejuni*. We hypothesized that, since *C. jejuni* is a human gastrointestinal pathogen (31), genes identified by SSH with

C. jejuni as the driver and *C. rectus* as the tester will include genes that play a role in allowing *C. rectus* to adapt to a lifestyle within the oral cavity of humans.

Materials and methods

Bacterial strains and growth conditions

C. jejuni (NCTC 11168, ATCC 700819) and *C. rectus* (ATCC 33238 and clinical isolate 314; 5) were grown under standard conditions. Briefly, *C. jejuni* was grown on Mueller Hinton agar plates (Oxoid, Lenexa, KS) in a MACS-VA500 Workstation (Don Whitley Scientific, Frederick, MD) under microaerophilic conditions at 37°C for 24 h. The microaerophilic gas mixture was 83% nitrogen, 8% oxygen, 4% hydrogen, and 5% carbon dioxide. *C. rectus* 33238 and 314 were grown on tryptic soy blood agar plates (Anaerobe Systems, Morgan Hill, CA) supplemented with sodium formate–fumarate (1 : 1 ratio; pH 7.0) to 0.3%. Plate cultures were grown for 48 h at 37°C under anaerobic conditions (Thermo Forma 1250, Waltham, MA).

Genomic DNA extraction

Three agar plates (each with confluent growth of *Campylobacter* species) per strain were harvested with a sterile cotton swab into cold sterile phosphate-buffered saline, pH 7.0, and centrifuged at 800 × g (4°C) in a swinging bucket rotor for 15 min. The supernatants were removed, and the pellets were immediately frozen at −70°C. Bacterial genomic DNA was isolated from frozen pellets using the cetyltrimethylammonium bromide procedure (3).

Suppressive subtractive hybridization

Suppressive subtractive hybridization was performed using the PCR-Select bacterial genome subtraction kit (Clontech, Mountain View, CA). For SSH using *C. rectus* 33238 as the tester and *C. jejuni* 11168 as the driver, a total of 2 μg of tester and driver genomic DNA was digested for 16 h at 37°C with *Alu*I. Digested genomic DNA samples were then purified using a phenol–chloroform extraction step as described in the manufacturer's instructions (Clontech). Purified, digested DNA from *C. rectus* (tester) was then divided into two aliquots, and each was ligated at 16°C for 16 h to a different adapter oligonucleotide (Table 1; adapter 1 or adapter 2R), using T4 DNA ligase (Clontech). Adapter-ligated *C. rectus* DNAs were denatured (98°C for 1.5 min) and each (adapter 1 or adapter 2R) was separately combined with a 50-fold excess of denatured (98°C for 1.5 min), *Alu*I-digested *C. jejuni* (driver) DNA, and the mixture was incubated at 60°C for 1.5 h (hybridization one). In this step, DNA sequences common between the tester and driver species are allowed to hybridize, leaving enriched tester-specific (*C. rectus*) single-stranded DNA. Next, the two aliquots of *C. rectus* adapter DNAs (from hybridization one) were combined and incubated at 60°C overnight with a 50-fold excess of freshly denatured (98°C for 1.5 min) driver DNA. During this stage, tester-specific DNAs are enriched by single-stranded, tester DNA ligated to adapter 1 hybridizing to single-stranded, tester DNA ligated to adapter 2R (hybridization two). The recessed ends of adapters were then filled in with DNA polymerase (Advantage 2 Polymerase; Clontech) by incubation at 72°C for 5 min. Polymerase chain reaction (PCR) was then carried out to amplify the tester-specific DNA selectively using a primer that is complementary to sequences found in both adapter 1 and adapter 2R (Table 1; PCR primer 1). Each 25-μl PCR contained 2.5 μl 10× reaction buffer (Advantage 2 PCR buffer; Clontech), 0.2 mM dinucleotide triphosphate (dNTPs), 0.4 μM primer, 19.5 μl nuclease-free water, 1 μl template from hybridization two, and 0.5 μl Advantage® 2 Polymerase mix (Clontech). The thermal cycling conditions for this PCR were one cycle of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The resulting PCR products were diluted 1 : 40 in nuclease-free water and used as a template in a secondary PCR. In the secondary PCR, nested primers complementary to adapters 1 or 2R were used (Table 1; Nested primer 1 and Nested primer 2) to further enrich the *C. rectus*-specific DNA. In the secondary PCR, each 25-μl PCR contained 2.5 μl 10× reaction buffer (Advantage 2 PCR buffer; Clontech), 0.2 mM dNTPs, 0.4 μM primer, 18.5 μl nuclease-free water, 1 μl diluted primary PCR product, and 0.5 μl

Table 1. Oligonucleotides used in this study

Oligonucleotide (reference)	Sequence (5′ → 3′)	Description
Adapter 1 (http://www.clontech.com)	CTAATACGACTCACTATAGGGCTCGAGCG GCCGCCCGGGCAGGT	SSH adapter
Adapter 2R (http://www.clontech.com)	CTAATACGACTCACTATAGGGCAGCGTGG TCGCGGCCGAGGT	SSH adapter
PCR primer 1 (http://www.clontech.com)	CTAATACGACTCACTATAGGGC	SSH first-round PCR primer
Nested primer 1 (http://www.clontech.com)	TCGAGCGGCCGCCCGGGCAGGT	SSH second-round PCR primer
Nested primer 2 (http://www.clontech.com)	AGCGTGGTCGCGGCCGAGGT	SSH second-round PCR primer
M13 F (http://www.invitrogen.com)	CGCCAGGGTTTTCCCAGTCACGA	DNA Sequencing primer
M13 R (http://www.invitrogen.com)	TCACACAGGAAACAGCTATGAC	DNA Sequencing primer
SapC-F1 (this study)	CCGGGCAGGTCTAAATTTGAAAGTG	SSH clone 43 specific PCR primer
SapC-R1 (this study)	TGGAAGTTGCCCAAACTGCTTAAATGTA	SSH clone 43 specific PCR primer
RTX-F1 (this study)	GCGTCTTTTATTTCCTCTTCGCTCTTTC	SSH clone 137 specific PCR primer
RTX-R1 (this study)	CGCTCTAACTCCTACCGCAAAGAAAGA	SSH clone 137 specific PCR primer
84-F1 (this study)	CTGCTAAACAAAAATTCGGAAATACCTCAG	SSH clone 84 specific PCR primer
84-R1 (this study)	CTCAAACGATGAGCGCTAACATCTTTACTA	SSH clone 84 specific PCR primer
148-F1 (this study)	TATAAACCCGACCTGCGGTAAAATTTCAG	SSH clone 148 specific PCR primer
148-R1 (this study)	TGGAAGTGTCGCGTGAAGGAGTTT	SSH clone 148 specific PCR primer
98-F1 (this study)	TCTCACGAGCCTAAATTTAGCGAAGGTAGA	SSH clone 98 specific PCR primer
98-R1 (this study)	GATAGAGCCAAACCTCGCCTCTTTCT	SSH clone 98 specific PCR primer
14-F1 (this study)	CTCGGTGACGGTTTTGTCGGTATTT	SSH clone 14 specific PCR primer
14-R1 (this study)	AAACACACCGACATAGAGAGCGCTAAA	SSH clone 14 specific PCR primer
92-F1 (this study)	TACTTGATACAACGTCTATTCGGAGATTCG	SSH clone 92 specific PCR primer
92-R1 (this study)	CGATTTTTGTTACGGTTCGATACACGATTT	SSH clone 92 specific PCR primer

PCR, polymerase chain reaction; SSH, suppressive subtractive hybridization.

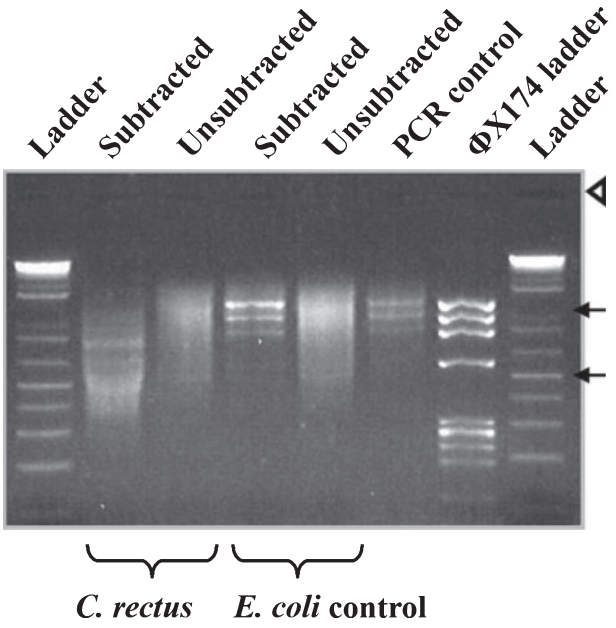

Fig. 1. Electrophoresis of secondary polymerase chain reaction (PCR) products following suppressive subtractive hybridization (SSH) using *Campylobacter rectus* 33238 as a tester and *Campylobacter jejuni* 11168 as a driver. The *Escherichia coli* (strain K-12) control consisted of a SSH reaction using *E. coli* DNA spiked with ΦX174 (*Hae*III-digested) as a tester and *E. coli* K-12 DNA as a driver. The PCR control is a previously subtracted *E. coli* K-12 control (Clontech). ΦX174 ladder = *Hae*III-digested ΦX174 DNA (New England Biolabs; Ipswich, MA); DNA ladder = 1 kb Plus DNA ladder (Invitrogen); arrows mark the position of 500 and 1000 base pairs and an arrowhead denotes the location of gel loading wells.

Advantage® 2 Polymerase mix (Clontech). The thermal cycling conditions for this PCR were one cycle of 94°C for 3 min, followed by 12 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min. In the secondary PCR, only double-stranded (formed in hybridization 2) DNAs specific to the tester that have different adapters on each end are amplified.

In parallel with the *Campylobacter* SSH, a control SSH reaction was run using DNA from *Escherichia coli* K-12. In the control reaction, *Alu*I-digested *E. coli* DNA was subtracted from *Alu*I-digested *E. coli* DNA spiked with *Hae*III-digested ΦX174 DNA. After subtraction of the of the *E. coli* tester DNA with *E. coli* driver DNA, the amplicons produced in the secondary PCR should correspond to the fragments making up the digested ΦX174 DNA (Clontech). Secondary amplicons of *E. coli* and *Campylobacter* SSH reactions were analyzed on 2.0% agarose gels (Fig. 1).

SSH library construction and DNA sequencing

Secondary PCR products were ligated into pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA), and transformed into *E. coli* TOP10F′. Transformed *E. coli* were grown at 37°C for 16 h on Luria–Bertani (LB) agar plates supplemented with 100 μg/ml carbenicillin and 40 μg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) to screen for plasmids with inserts. Individual, white colonies with inserts were grown in LB broth supplemented with 100 μg/ml carbenicillin at 37°C for 16 h, with shaking (250 r.p.m.). Plasmids from individual, recombinant *E. coli* colonies were then isolated using the GeneJET™ Plasmid Miniprep kit (Fermentas, Glen Bernie, MD). Putative *C. rectus*-specific inserts were sequenced by the University of North Carolina at Chapel Hill Genome Analysis Facility using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence reactions were performed with the ABI PRISM™ BigDye™ Terminator Version 1.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems).

PCR analysis of SSH clones

Representative SSH plasmids were verified as *C. rectus*-specific, and therefore lacking in *C. jejuni*, using PCR. Specifically, primers specific to sequenced inserts were designed using Vector NTI (Invitrogen), and used to amplify genomic DNA from *C. rectus* (33238 and 314) or *C. jejuni* 11168 using PCR (Table 1 and Fig. 2). For the PCR, each 50-μl reaction contained 10 μl 10× QIAGEN PCR buffer (Qiagen, Valencia, CA), 0.2 mM dNTPs, 0.2 μM forward and reverse primers, 2.0 mM $MgCl_2$, 50 ng genomic DNA and 2.5 U *Taq* polymerase (Taq PCR Core kit; Qiagen). The thermal cycling conditions were one cycle of 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Amplicons were analyzed on 2.0% agarose gels (Fig. 2).

Sequence analysis

DNA chromatographs were edited manually and contigs were assembled using VECTOR NTI CONTIG EXPRESS software (Invitrogen). In particular, the nucleotide sequences were analyzed to determine the boundaries of the insertions. Insert sequences were used as templates for sequence homology searches using the BLAST suite of programs (http://www.ncbi.nlm.nih.gov) on the BLAST

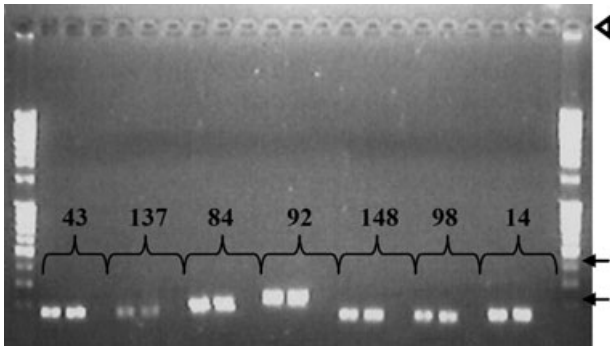

Fig. 2. Polymerase chain reaction (PCR) confirmation of select *Campylobacter rectus*-specific clones (1.0% agarose gel shown). Numbers indicate clone numbers (Table 4), and each bracket denotes three PCR reactions: *C. rectus* 33238 genomic DNA, *C. rectus* 314 genomic DNA, and *Campylobacter jejuni* 11168 genomic DNA. Amplicons were of the expected sizes. Arrows mark the position of 200 and 400 base pairs and an arrowhead denotes the location of gel-loading wells.

Table 2. Summary of *Campylobacter rectus*-specific clones identified by suppressive subtractive hybridization

Description	Number of clones	Average insert (bp)	Average G + C %	Total (bp)	% of *C. rectus*-specific clones
C. rectus specific	92	586	40.0	53,884	100.0
No homology[1]	41	560	37.0	22,927	45.0
Significant homology[2]	51	607	43.0	30,957	55.0

[1]BLASTn or BLASTx expected value of >1E-3; [2]BLASTn or BLASTx expected value of <1E-3.

network server of the National Center for Biotechnology Information. Searches were performed using the default parameters for the BLASTn, BLASTp, and BLASTx algorithms (2). Both the non-redundant and unfinished microbial databases included in GenBank™ were queried.

Results and Discussion

SSH between *C. rectus* 33238 and *C. jejuni* 11168

The SSH was performed to identify *C. rectus* genes that were absent from *C. jejuni*. In this study, *C. rectus* 33238 was used as a tester and *C. jejuni* 11168 was used as a driver. Electrophoresis of secondary PCR products (see Materials and methods) indicated a positive subtractive hybridization between *C. rectus* and *C. jejuni* genomic DNA. As expected, differential amplicon patterns were visualized upon comparing unsubtracted with subtracted *C. rectus* DNA. The *E. coli* control genomic DNA spiked with *Hae*III-digested ΦX174 yielded amplicons corresponding to the ladder alone (ΦX174) and the PCR control (previously subtracted *E. coli* control; Clontech), upon subtraction with identical *E. coli* DNA (Fig. 1).

Cloning of subtracted *C. rectus* DNA (secondary PCR products) yielded a total of 200 colonies with inserts. To characterize the subtracted library, inserts from 180 randomly selected colonies were sequenced, yielding 154 unique clones. BLAST analysis showed 92 of the 154 clones (60.0%) contained DNA fragments that did not share significant homology to DNA from the sequenced genomes of 10 *C. jejuni* strains, including NCTC 11168 (BLASTn or BLASTx E-value of >1E-3), which was comparable to the 50.0% of clones obtained from SSH that are predicted to be tester specific (29). Forty-one (45.0%) of the 92 *C. rectus*-specific clones did not share significant homology to any known sequences. Significant homology was defined as yielding a BLAST expected value of <1E-3. The average insert size of the 92 *C. rectus*-specific clones was 586 base pairs, with an average G + C content of 40.0% (Table 2). The average G + C content of the clones was similar to the G + C content of the *C. rectus* genome, which is estimated to be 45.4% (27). The 92 *C. rectus*-specific clones represented a total of 53,884 base pairs of DNA from the genome of *C. rectus* 33238. Assuming the genome size of *C. rectus* is similar to the estimated genome size of a related oral species, *Campylobacter curvus* 525.92 (∼ 2000 kilobase pairs, http://www.tigr.com), 53,884 base pairs would represent 2.7% of the total *C. rectus* genome.

Seven representative *C. rectus*-specific clones identified by SSH and subsequent sequencing were confirmed as being present in the *C. rectus* genome and lacking from the *C. jejuni* genome by PCR. PCR products of the expected sizes were amplified from *C. rectus* genomic DNA, but not from *C. jejuni* DNA (Fig. 2). In addition to strain 33238, the presence of the representative DNA fragments in a second *C. rectus* strain, clinical isolate 314, was confirmed (Fig. 2). Strain 314 was included to test the conservation of *C. rectus* sequences in an independent isolate.

In silico analyses of *C. rectus*-specific clones

General sequence features

The 51 genes showing significant homology to sequences deposited in GenBank™ were grouped according to categories of the cluster of orthologous groups (COG) database. The DNA sequences of the 51 *C. rectus*-specific clones have been deposited in the publicly available genome survey sequences database (http://www.ncbi.nlm.nih.gov/dbGSS). As shown in Table 3, the genes are predicted to play a role in a variety of biological processes, ranging from amino acid metabolism to signal transduction mechanisms. Individual BLASTx analyses of the 51 clones are summarized in TABLE 4. The majority of clones (51.0%) shared greatest sequence identity with genes identified in the related, primarily oral campylobacters, *C. curvus* and *Campylobacter concisus*.

Genes related to signal transduction

Five of the *C. rectus*-specific clones (Table 3) contained gene fragments predicted to be involved in two-component signal transduction systems (TCST). Further analysis of the TCST-related clones revealed a potential histidine kinase (clone 150), two genes likely to play a role in the synthesis of the bacterial second messenger cyclic-di-guanosine monophosphate (GMP) (clones 7 and 14), a putative transcriptional regulator that contained a CheY-like receiver domain and a helix-turn-helix DNA-binding domain (clone 98), and a putative methyl-accepting chemotaxis receptor (clone 189). Of the TCST-related clones, 14 and 98 were of particular interest. Clone 14 shared greatest homology with diguanylate cyclases identified in the genomes of *Campylobacter* species that typically reside outside the gastrointestinal tract of mammals. Proteins containing the amino acid domain GGDEF, denoting a diguanylate cyclase, in conjunction with proteins containing the

Table 3. Functional classification of 51 *Campylobacter rectus*-specific genes according to categories of the cluster of orthologous groups (COG) database[1]

Function	Number of *C. rectus*-specific genes
Function unknown	17
General function prediction only	11
Amino acid transport and metabolism	1
Carbohydrate transport and metabolism	1
Cell motility	1
DNA replication, repair, and recombination	2
Energy production and conversion	2
Inorganic ion transport and metabolism	3
Membrane biosynthesis	2
Secondary metabolites biosynthesis, transport, and catabolism	4
Signal transduction mechanisms	5
Transcription	1
Translation, ribosome structure, and biogenesis	1

[1]http://www.ncbi.nlm.nih.gov/COG

Table 4. Summary of BLASTx analysis of *Campylobacter rectus*-specific clones

Clone no.	Length (bp)	E-value[1]	Highest BLASTx hit	Gene (GenBank™ no.)	Identity[2] (%)	*Campylobacter* species[3]
137	684	6.00E-22	*Campylobacter rectus*	S-layer RTX (AAD02003)	59	Cr
52	595	3.00E-24	*Campylobacter rectus*	S-layer RTX (AAD02003)	46	Cr
170	958	3.00E-89	*Campylobacter rectus*	S-layer RTX (AAD02003)	100	Cr
148	525	1.00E-26	*Campylobacter concisus*	Tartrate dehydratase (YP_001466330)	98	Ccon, Ccur
184	294	1.00E-06	*Campylobacter concisus*	Hypothetical protein (YP_001467671)	82	Ccon, Ch
127, 146	345	6.00E-09	*Campylobacter concisus*	Integral membrane protein (YP_001467597)	90	Ccon, Ch
35	540	1.00E-15	*Campylobacter concisus*	Crispr-protein (YP_0014066979)	78	Ccon, Ccur
69	556	3.00E-17	*Campylobacter concisus*	Hypothetical protein (YP_001407112)	39	Ccon, Ch
8	942	3.00E-09	*Campylobacter concisus*	Putative transporter (YP_001408122)	65	Ccon, Ccur
99	656	1.00E-12	*Campylobacter concisus*	NAD (FAD)-dehydrogenase (YP_001466199)	59	Ccon, Ccur, Ch
7	295	3.00E-06	*Campylobacter concisus*	Diguanylate cyclase (YP_001467480)	28	Ccon, Cf
143, 188	680	1.00E-45	*Campylobacter concisus*	UV-repair protein (ABW74736)	80	Ccon, Cl
60	1058	1.00E-52	*Campylobacter concisus*	Na^+/H^+ antiporter (YP_001466432)	79	Ccon, Ccur, Cl
46	857	4.00E-53	*Campylobacter concisus*	UV-repair protein (ABW74736)	80	Ccon, Cl
189	692	4.00E-25	*Campylobacter concisus*	Chemotaxis receptor (YP_001466399)	58	Ccon, Ccur, Cf
138	525	2.00E-21	*Campylobacter concisus*	G-specific endonuclease (YP_001467479)	75	Ccon, Ch
181	586	6.00E-54	*Campylobacter curvus*	Putative aminotransferase (YP_001409172)	67	Ccur, Ccon
139, 179	619	2.00E-39	*Campylobacter curvus*	Sugar efflux transporter (YP_001466148)	70	Ccon
82	320	6.00E-34	*Campylobacter curvus*	Pilus Assembly (YP_001408882)	61	Ccur, Ccon, Cf, Ch
119	448	5.00E-13	*Campylobacter curvus*	Hypothetical protein (YP_001407812)	75	Ccur, Ccon
108	479	2.00E-17	*Campylobacter curvus*	Hypothetical protein (YP_001408159)	80	Ccur, Ccon
78	945	1.00E-12	*Campylobacter curvus*	Hypothetical protein (YP_001408122)	91	Ccur, Ccon, Cf
129	465	1.00E-08	*Campylobacter curvus*	Mannonate dehydratase (YP_001407560)	53	Ccur
155	593	1.00E-23	*Campylobacter curvus*	NADPH oxidoreductase (YP_001408949)	58	Ccur, Cf, Ccon
123	774	2.00E-54	*Campylobacter curvus*	Peptidase M50 (YP_001407942)	60	Ccur, Ccon, Cf
160	733	2.00E-47	*Campylobacter curvus*	Metalloid reductase (YP_001408713)	52	Ccur
187	303	2.00E-27	*Campylobacter curvus*	TonB-dependent receptor (YP_001409006)	56	Ccur, Ch
124	334	4.00E-23	*Campylobacter curvus*	Polyketide biosynthesis (YP_001409035)	50	Ccur, Cl
98	505	7.00E-33	*Campylobacter curvus*	Response regulator (YP_001408651)	55	Ccon, Ccur
80, 100	535	1.00E-51	*Campylobacter fetus*	Endonuclease IV (YP_892797)	69	Cf, Ccon, Ccur
33	671	1.00E-75	*Campylobacter fetus*	FlavoCytochrome C subunit (YP_891346)	83	Cf, Ccon, Ccur
43	181	5.00E-13	*Campylobacter fetus*	SapC (YP_891659)	59	Cf
162, 171	508	2.00E-39	*Campylobacter fetus*	Glycosyl transferase (YP_891685)	52	Cf, Ch
14	450	6.00E-42	*Campylobacter fetus*	Diguanylate cyclase (YP_892811)	52	Cf, Ccur, Ccon, Cl
55	1176	2.00E-20	*Campylobacter hominis*	Hypothetical protein (YP_001407277)	48	Ch
117	939	4.00E-10	*Campylobacter hominis*	Hypothetical protein (YP_001406739)	25	Ch
157	801	2.00E-14	*Campylobacter lari*	Hypothetical protein (ZP_00368940)	80	Cl
75, 94	619	5.00E-42	*Wolinella succiongenes*	Sulfurtransferase (NP_906857)	45	None
150	274	7.00E-29	*Wolinella succiongenes*	Histidine kinase (NP_906625)	62	Ccon, Cf
36	666	1.00E-03	*Methanosarcina acetivorans*	Hypothetical protein (NP_616963)	34	None
54	526	3.00E-31	*Listeria welshmeri*	Subtilisin-like serine protease (YP_848974)	55	None
2, 45	905	7.00E-95	*Listeria monocytogenes*	AAA -Superfamily ATPase (ZP_00229692)	63	Cl, Ccon, Cf
77	673	3.00E-18	*Clostridium phytofermentas*	*N*-acetyltransferase (YP_01558073)	33	None
20	772	8.00E-17	*Idiomarina baltica*	Hypothetical protein (ZP_01042784)	33	None
142	770	1.00E-19	*Idiomarina baltica*	Hypothetical protein (ZP_01042784)	32	None
147, 154	1158	2.00E-09	*Escherichia coli*	Hypothetical protein (ABE05728)	58	Ch
96, 136	544	5.00E-03	*Streptococcus mutans*	Putative acetyltransferase (NP_722347)	31	None
32	221	7.00E-05	*Bacillus species*	Hypothetical protein (ZP_01725140)	55	None
42	316	2.00E-16	*Neisseria meningtidis*	Transposase (AAP44503)	40	Ch
110	214	7.00E-03	*Thermosinus carboxydiverans*	Anaerobic antiporter (ZP_01665172)	50	None
131	732	6.00E-05	*Trichomonas vaginalis*	Hypothetical protein (XP_001305067)	29	None

[1]BLASTx value of <1E-3 was considered significant; [2]relative to the highest BLASTx hit, amino acid identity; [3]*Campylobacter* species with a gene sharing significant homology (BLAST E-value of <1E-3) with each SSH clone.
Ccon, *C. concisus*; Ccur, *C. curvus*; Cl, *C. lari*; Ch, *C. hominis*; Cf, *C. fetus* ssp. *fetus*; Cr = *C. rectus.*

EAL domain (phosphodiesterase), are responsible for the synthesis and degradation of the bacterial second messenger cyclic-di-GMP. Intracellular cyclic-di-GMP concentrations have been shown to influence various bacterial processes including virulence gene expression, biofilm formation, adhesion, and motility (10). Interestingly, the number and type of genes containing GGDEF and/or EAL domains encoded in bacterial genomes is highly variable, ranging from 0 to 100 per genome; and so might be related to the adaptability of a particular species to different ecological niches (13). *C. jejuni* lacking a functional copy of *cbrR*, which contains a GGDEF domain, has a reduced ability to colonize chickens compared to wild-type *C. jejuni* (22). Although both clone 14 and *C. jejuni cbrR* contain GGDEF domains, they do not share significant sequence homology. More specifically, BLASTx analysis of the available *C. jejuni* genomes did not identify *cbrR* (GenBank™ Accession no. CAL34788) as a gene sharing significant sequence identity or similarity with clone 14. The identification of genes in *C. curvus* (GenBank™ Accession no. YP_001407438), *Campylobacter fetus* ssp. *fetus* (GenBank™ Accession no. YP_892811), and *C. concisus* (GenBank™ Accession no. YP_001465990) sharing significant sequence homology to clone 14 (BLASTx E-values of 2E-36, 6E-42 and 6E-34; respectively) suggests that the gene represented

by clone 14 may play a role in allowing *C. rectus* to occupy a niche outside the mammalian gastrointestinal tract.

Clone 98 shares greatest sequence homology with putative transcriptional regulators that contain a CheY-like receiver domain and a helix-turn-helix DNA-binding domain. Among the 17 genome sequences (covering eight species) available for the genus *Campylobacter* (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), genes sharing significant sequence identity and similarity to clone 98 are only found in the genomes of *C. curvus* (GenBank™ Accession no. YP_001408651; BLASTx E-value of 7E-33) and *C. concisus* (GenBank™ Accession no. YP_001466535; BLASTx E-value of 8E-31). In addition, BLASTx analysis showed that genes from the oral bacterial pathogens *Treponema denticola* (GenBank™ Accession no. AAS12843; BLASTx E-value of 3E-06) and *Streptococcus mutans* (GenBank™ Accession no. AAN58233; BLASTx E-value of 1E-04) shared significant sequence homology to clone 98. Despite the potential conservation of this gene among these three distantly related oral pathogens, the function of this gene has not been elucidated.

Genes potentially related to virulence and/or host colonization

The virulence of *C. rectus* is poorly understood despite its association with periodontal disease. That is, only a limited number of gene products (*csxA*, *csxB*, *crsA*, and *groEL* genes) have been proposed as potential virulence factors (6, 12, 23, 28). The *csx* genes share significant homology with RTX-type bacterial toxins, *groEL* encodes for a heat-shock protein that stimulates the production of interleukin-6 and interleukin-8 by human gingival cells, and *crsA* encodes for the major protein constituting the S-layer of *C. rectus.* The S-layer is a paracrystalline protein structure external to the bacterial outer membrane and can have several functions related to host immune avoidance, including conferring resistance to complement-mediated killing and causing the downregulation of proinflammatory cytokines (25).

Three *C. rectus*-specific clones identified in this study (52, 137, and 170) shared significant sequence homology to previously identified *csx* genes. Clone 170 perfectly matched the previously identified *csxB* gene (100% DNA and amino acid sequence identity; GenBank™ Accession no. AAD02003). A SSH clone containing a fragment of *csxB* might be expected in this study, as the sequenced genome of *C. jejuni* 11168 lacks *csx* genes. Clones 52 and 137 were not identical to previously identified *csx* genes in *C. rectus*, but did share significant sequence identity with *C. rectus csxB* (Table 4). Hence, clones 52 and 137 are likely to represent newly identified RTX-related genes, which appear to be uniquely found in *C. rectus*.

In *C. fetus* ssp. *fetus*, a pathogen of ungulates and immunocompromised humans, secretion of the protein subunits of the S-layer depends on the presence of the *sapCDEF* genes (26). Clone 43 shared greatest sequence homology with *sapC* from *C. fetus* ssp. *fetus* (GenBank™ Accession no. AAC97196; BLASTx E-value of 5E-13). The presence of *sapC* in *C. rectus* was expected because *C. fetus* ssp. *fetus* and *C. rectus* are the only *Campylobacter* species that contain an S-layer.

Genes in common with C. curvus *and/or* C. concisus

C. rectus-specific clones 8, 35, 98, 108, 119, 129, 139, 148, 160 and 181 shared highest sequence homology to genes previously identified in sequenced members of the genus *Campylobacter*, but only in the primarily oral species *C. curvus* and/or *C. concisus*. The majority of these clones (60.0%; 8, 35, 108, 119, 129, 160) shared highest sequence homology to genes with undefined biological functions. Of the remaining clones belonging to this group (181, 139, and 148), clone 181 was of particular interest. Clone 181 contained 586 base pairs of *C. rectus* genomic DNA, which included a predicted partial open reading frame of 151 amino acids. A conserved domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.html) analysis of the 151-amino-acid sequence identified a domain annotated as MalY (COG1168), which is conserved among bifunctional pyridoxal-5′-phosphate (PLP)-dependent enzymes with β-cystathionase activities. Interestingly, a potential virulence factor, cystalysin, encoded by the oral pathogen *T. denticola* (ATCC 35405), also contains the conserved MalY domain (7, 8, 14). According to BLASTp, the 151 amino acids contained within clone 181 shared significant homology to cystalysin from *T. denticola* (GenBank™ Accession no. 1C7N_A; BLASTp E-value of 2E-11). In addition to removing sulfhydryl and amino groups from selected sulfur-containing compounds (e.g. L-cysteine) to produce hydrogen sulfide, ammonia, and pyruvate; cystalysin functions to lyze erythrocytes and hemoxidize hemoglobin (27–29). Interestingly, in addition to sharing homology with cystalysin, clone 181 shared significant homology with a putative hemolysin from *T. denticola* (GenBank™ Accession no. AAS12185; BLASTp E-value of 4E-13), which itself shares significant homology to cystalysin.

In common with *T. denticola*, *C. rectus* is found within subgingival pockets during periodontitis in humans (17) and produces detectable levels of volatile sulfur compounds *in vitro* (20). It is believed that cystalysin supports the anaerobic growth of *T. denticola* by producing pyruvate, provides a source of iron by the lysis of erythrocytes, and functions to alter the periodontal ecology by producing hydrogen sulfide (a common volatile sulfur compound). Hydrogen sulfide is highly toxic for mammalian cells so enzymes participating in its production in periodontal pockets might contribute to the progression of periodontitis (7, 8, 14). Although the function of the gene identified in clone 181 is unknown; the shared homology of the gene fragment with *T. denticola* cystalysin raises the possibility that this gene plays a similar role in *C. rectus.*

Genes previously not identified in the Campylobacter genus

A number of *C. rectus* genes identified by SSH have not been previously identified in the sequenced genomes of any *Campylobacter* species. The SSH clones meeting this criterion included clones 20, 32, 36, 54, 75, 77, 96, 110, 131, and 142. More specifically, this group of clones did not show significant sequence homology to available sequences from *Campylobacter* species (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Interestingly, the majority (70.0%) of these 10 clones shared highest sequence homology with bacterial genes with unknown functions. The remaining three clones (110, 75, and 77) shared greatest sequence homology with genes believed to play roles in anaerobic energy production (clone 110), sulfur metabolism, (clone 75), and protein translation (clone 77; Table 4). Although these clones contained gene fragments with ill-defined biological functions, their identification in a representative *Campylobacter* species suggests that the spectrum of genomic diversity among members of the *Campylobacter* genus has yet to be fully appreciated.

Conclusions

Using suppressive subtractive hybridization as a comparative genomics approach,

this study provides the first substantial insights into the genomic content of *C. rectus*, an ill-defined etiological agent of human periodontitis. In particular, the data collected here have identified genes encoded by *C. rectus* that are lacking in the related, gastrointestinal species *C. jejuni*. Most significantly, the genes identified here included those potentially related to niche adaptation, virulence, and/or host colonization. The further characterization of *C. rectus* genes identified in this study, including genes sharing significant homology with a response regulator (clone 98) and a putative virulence factor previously identified in oral, pathogenic bacteria (clone 181), will lead to a greater understanding of the mechanisms that contribute to the lifestyle of *C. rectus* as an etiological agent of periodontal disease.

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