A Genomic Island Defines Subspecies-Specific Virulence Features of the Host-Adapted Pathogen Campylobacter fetus subsp. venerealis

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The pathogen Campylobacter fetus comprises two subspecies, C. fetus subsp. fetus and C. fetus subsp. venerealis. Although these taxa are highly related on the genome level, they are adapted to distinct hosts and tissues. C. fetus subsp. fetus infects a diversity of hosts, including humans, and colonizes the gastrointestinal tract. In contrast, C. fetus subsp. venerealis is largely restricted to the bovine genital tract, causing epidemic abortion in these animals. In light of their close genetic relatedness, the specific niche preferences make the C. fetus subspecies an ideal model system to investigate the molecular basis of host adaptation. In this study, a subtractive-hybridization approach was applied to the genomes of the subspecies to identify different genes potentially underlying this specificity. The comparison revealed a genomic island uniquely present in C. fetus subsp. venerealis that harbors several genes indicative of horizontal transfer and that encodes the core components necessary for bacterial type IV secretion. Macromolecular transporters of this type deliver effector molecules to host cells, thereby contributing to virulence in various pathogens. Mutational inactivation of the putative secretion system confirmed its involvement in the pathogenicity of C. fetus subsp. venerealis.

Campylobacter species are Gram-negative epsilonproteobacteria highly adapted to mucosal surfaces. The majority are human and/or animal pathogens (19, 61). The 18 species comprising the genus Campylobacter display a high degree of host and tissue specificity, which makes them excellent models to study host-pathogen relationships (25). The most prominent member, Campylobacter jejuni, is a commensal of the chicken intestine and the major cause of human bacterial diarrhea (74). Comparative analysis of Campylobacter genomes has revealed a process of genome decay—supported by a small genome size (about 1.5 Mb) and the loss of metabolic genes—consistent with successful adaptation to a specific niche (41). Campylobacter genomes are among the densest bacterial genomes known, with about 95% coding sequence. Despite this evidence of reduction, plasticity in genetic composition remains evident, as strain-specific genes comprise a substantial proportion of the entire repertoire of 1,500 to 1,800 genes (16, 23, 25, 56).

This study focuses on the species Campylobacter fetus, which is represented by the two subspecies C. fetus subsp. fetus and C. fetus subsp. venerealis. Although the two taxa are genetically closely related, they exhibit striking tissue and host specificity. C. fetus subsp. fetus is a human, as well as animal, pathogen. Human infection results in serious systemic disease, especially in immunocompromised people. C. fetus subsp. fetus is the Campylobacter species most often isolated from human blood (75), and it is considered an emerging pathogen (9). The infection mode shares similarities with that of Salmonella enterica serovar Typhi. Orally acquired C. fetus subsp. fetus penetrates the intestinal mucosa, leading to bacteremia, and subsequent excretion via the biliary tract leads to secondary colonization of the intestine (9). Colonization of reproductive organs induces abortion in sheep and to a lesser extent in cattle, and very rarely in humans (11). C. fetus subsp. fetus can also be isolated from the intestinal tracts of birds and reptiles (78, 80). In contrast, C. fetus subsp. venerealis is host restricted. It is isolated primarily from the bovine genital tract and causes the epidemic disease bovine venereal campylobacteriosis (BVC). The reservoir of C. fetus subsp. venerealis is the penile prepuce of the bull. Transmission to cows occurs at coitus or during artificial insemination, and infection leads to endometritis, abortion, and infertility (28). Since BVC is a worldwide problem with substantial economic consequences, diagnosed cases must be registered (75) and import and export of bovine semen and embryos for cattle breeding requires statutory preclusion of C. fetus infection (2). Despite the distinct niche preferences of the C. fetus subspecies, they show high genetic relatedness, complicating the task of correct subspecies identification (46, 62, 81). Their population structure is clonal, and C. fetus subsp. venerealis is thought to represent a bovine clone of C. fetus (81).

In this study, we employed the C. fetus subspecies to investigate the genetic basis for their host and tissue specificities. A genomic subtractive-hybridization approach was taken to identify subspecies-specific genomic fragments. This led to the discovery of a genomic island exclusively present on the chromosome of the host-adapted subspecies C. fetus subsp. venerealis. This island harbors a type IV secretion system (T4SS), as well as mobility genes (insertion sequence [IS] transposases and
phage integrases) and shares substantial homology and similar structure with resistance plasmids found in other Campylobacter species. These features are indicative of a horizontally acquired genetic element. Finally, mutational analysis of genes within the island substantiates its involvement in C. fetus subsp. venerealis virulence.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and identification of bacterial isolates. Campylobacter and Escherichia coli strains were grown as previously described (48). The 112 Campylobacter strains used in this study are listed in Table S1 in the supplemental material. Subspecies were identified biochemically according to growth in the presence of 1% (wt/vol) glycine and the reduction of 0.1% sodium selenite in liquid culture (82). Additionally, a subspecies-specific virulence. The oligonucleotides used are described in Table 2. PCR-based genomic

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Description*</th>
<th>Source or reference</th>
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<tbody>
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<td>Type strain; vaginal mucus of heifer</td>
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<tr>
<td>C. fetus subsp. venerealis 3-18</td>
<td>Km2, Cfv ATCC 19438, virB9- Pc, -aphA-3</td>
<td>This study</td>
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<tr>
<td>C. fetus subsp. venerealis 1a (V1)</td>
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<td>31</td>
</tr>
<tr>
<td>C. fetus subsp. venerealis 3 (V3)</td>
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<td>C. fetus subsp. venerealis D96 (V4)</td>
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<td>C. fetus subsp. venerealis G91 (V5)</td>
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<tr>
<td>C. fetus subsp. venerealis TH24 (V7)</td>
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<td>46</td>
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<td>C. fetus subsp. venerealis 80/4172 (V8)</td>
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<td>C. fetus subsp. venerealis 108/4111 (V9)</td>
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<td>46</td>
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<tr>
<td>C. fetus subsp. venerealis 121/4401 (V10)</td>
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<tr>
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<td>This study</td>
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<td>C. fetus subsp. fetus H88 (F7)</td>
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<td>C. fetus subsp. fetus S88 (F8)</td>
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<td>C. fetus subsp. fetus 133/4369 (F11)</td>
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<td>86</td>
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<tr>
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<tr>
<td>E. coli DH5α</td>
<td>Tp' Sm'; recA thi pro hsdR3 M' RP4-2-Tc:Cm::Km Tn7 ϰpir</td>
<td>21</td>
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</table>

* Additional strains used in analysis of gene distribution are listed in Table S1 in the supplemental material.

b Strain abbreviations used in the figures are shown in parentheses.

* Km1, kanamycin resistance phenotype; Sm1, streptomycin resistance phenotype; Tp1, trimethoprim resistance phenotype; Cfv. C. fetus subsp. venerealis.
### Library construction and DNA sequencing.

A genomic DNA library of *C. fetus* subsp. *venerealis* ATCC 19438 was constructed according to standard procedures (4). Briefly, bacterial DNA was isolated following lysozyme lysis, partially digested with Sau3AI, and subsequently cloned into pBluescript II KS(+) Reference and/or application (88) with a cutoff value of 50 bp. Additional annotation criteria were the presence of a probable initiation codon, allowing for alternative bacterial initiation codons, as well as appropriate spacing to a ribosome-binding site (88). Annotation of the identified ORFs was accomplished on the basis of similarity searches using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/) against the nonredundant database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Sequence comparisons were made using MegAlign from Lasergene (DNAStar Inc., Madison, WI).

### Gene annotation.

Open reading frames (ORFs) were predicted using ARTEMIS (68) with a cutoff value of 50 bp. Additional annotation criteria were the presence of a probable initiation codon, allowing for alternative bacterial initiation codons, as well as appropriate spacing to a ribosome-binding site (88). Annotation of the identified ORFs was accomplished on the basis of similarity searches using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/) against the nonredundant database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Sequence comparisons were made using MegAlign from Lasergene (DNASTar).

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#### Table 2. Oligonucleotide primers

<table>
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<td>R Bgl12</td>
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<td>RDA (45)</td>
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<td>J Bgl24</td>
<td>ACCGACGTGCACTATCCGATGAGC</td>
<td>RDA (45)</td>
</tr>
<tr>
<td>J Bgl12</td>
<td>GATCTGCCCTGC</td>
<td>RDA (45)</td>
</tr>
<tr>
<td>N Bgl24</td>
<td>AGGGCACTGGTGATCCGAGG</td>
<td>RDA (45)</td>
</tr>
<tr>
<td>N Bgl12</td>
<td>GATCTGCCCTGC</td>
<td>RDA (45)</td>
</tr>
<tr>
<td>Int 11</td>
<td>CCTTTAACACGTCCGCC</td>
<td>nt 6453 to 6470 (virB3/4)</td>
</tr>
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<td>VirB4-6</td>
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<td>nt 24353 to 24330 (virB3/4)</td>
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<td>VirB8-3</td>
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<td>VirB8-4</td>
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<td>VirB8-14</td>
<td>CACCGTAATGAGAAGGCC</td>
<td>nt 11699 to 11716 (virB8)</td>
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<td>VirB11-1</td>
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<td>UP3</td>
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<td>TraE7</td>
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<td>TaxB2</td>
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<td>nt 17424 to 17490 (virD4)</td>
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<td>nt 16668 to 16686</td>
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<td>VirD4_Xhol_f</td>
<td>ACTCGGAGTTGTCATGGCATGATC</td>
<td>nt 17416 to 17390</td>
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<td>PstI_cdt KO-fwd</td>
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<td>NotI_cdt KO-rev</td>
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<td>Cff 82-40 (nt 26916 to 26937); NC_00859b</td>
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<td>ATCTGCACTGGTTTTTTGATTGGAAGGAAATTATTGTGTCATC</td>
<td>nt 6081 to 6057</td>
</tr>
</tbody>
</table>

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a Nucleotide positions correspond to GenBank accession no. EU443150 if not otherwise noted; Cff, *C. fetus* subsp. *fetus*.

b Nucleotide position relative to the indicated GenBank accession number.
Analysis of secondary structural properties and detection of specific domains or motifs in the predicted proteins was carried out with SMART (http://smart.embl-heidelberg.de), SCANNPROSITE (http://www.expasy.org/tools/scansite/), and MOTIF (http://motif.genome.ad.jp). The tandem repeats finder program (http://tandem.bu.edu/) was applied, as well as ARAGORN (http://bioinf.charite.de/ARAGORN), to search for the sequence for tRNA genes.

Survey of genomic island gene prevalence. Southern hybridization and/or PCR analyses were performed with an extended C. fetus strain collection (see Table S1 in the supplemental material). For Southern analysis, DNA from a random selection of 22 C. fetus subsp. fetus strains and 38 C. fetus subsp. venerealis strains was subjected to Southern analyses (see Fig. 2; see Table S1 in the supplemental material). Probes specific to the genomic island were constructed by PCR amplification with the oligonucleotide primers 3D1 and 3D3 (orf21; Top/traE4 and TraE7 [orf27]; VirB8-1 and VirB9-1, spanning from virB8 to virB9; VirB10-1 and VirB10-2 [virB10]; and TaxB2 and TaxB3 [virB4]) (Table 2). The DNA fragments were radiolabeled internally and hybridized as described previously (48). Additionally, PFGE was used to resolve SmaI-macrorestricted DNA originating from 10 C. fetus subsp. fetus and 11 C. fetus subsp. venerealis strains according to a recently described protocol (30) (see Fig. 4). Southern hybridizations were performed with the orf21 probe.

All strains were also tested with PCR for the presence of at least two different island-specific regions. In addition to the primer combinations mentioned above, the screen applied Trans4 and Top/traE1, spanning from orf26 to orf27; Int 11 and VirB4-6 (virB8 and virB4); VirB8-1 and VirB8-3 (virB8 and virB9); VirB9-2 and U41 (virB9); U41 and U41-2 (virB10); VirB11-1 and VirB11-2 (virB11); and TaxB2 and TaxB3 (virB4) (Table 2).

Mutational analysis. A virB9 mutant of C. fetus subsp. venerealis ATCC 19438 was constructed by insertion mutationagenesis. A 1.2-kb fragment carrying the entire gene was amplified with VirB9-PstI-fwd and VirB9-HindIII-rev. The products were digested, purified, and cloned into PstI/HindIII-cut pBluescript II KS(−) (Stratagene), to create the cloning intermediate. The gatC promoter–aphA-3 fusion was excised via NotI+EcoRI from pRYBM5, blunted, and ligated into the blunted XbaI site of pBlue-oriT-cdt. To create mutant strains, the resulting suicide vector, pL1, was conjugatively mobilized from C. coli S17-1 to the mutant or wild-type strain.

Expression analysis of vir genes. Wild-type and C. fetus strains were grown on Columbia blood agar (CBA) plates for 72 h in a microaerobic atmosphere (48). Total RNA was isolated using RNAeasy (Qiagen). One µg of total RNA was treated with 2 U of RQ1-DNase (Promega) according to the manufacturer’s specifications. For first-strand cDNA synthesis, 200 ng template was mixed with 2 µmol of antisense oligonucleotide and 0.1 mM dNTP and incubated at 65°C for 5 min. After being chilled on ice, 4 µl of 5-fold first-strand buffer and 2 µl of 0.1 M dithiothreitol (DTT) were added, and the mixture was warmed to 37°C prior to the addition of 200 U of SuperScript II reverse transcriptase (Invitrogen, Paisley, United Kingdom). The 20-µl mixture was incubated at 37°C for 60 min and then inactivated at 70°C for 5 min. For second-round synthesis, 1 µl of the first-strand product was incubated with 2.5 µl of 5-fold second-strand buffer and 0.5 µl of a 10 mM dNTP mixture, 10 pmol of each primer, and 0.5 µl of Advantage DNA polymerase (Clontech) in a final volume of 25 µl.

Cell culture and virulence assays. HeLa cells were routinely grown in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS). Caco-2 cells were grown in Eagle’s minimum essential medium (EMEM) supplemented with 10% FCS and 1% nonessential amino acids (Invitrogen). All cells were cultured without antibiotics at 37°C and 5% CO2.

A continuous coculture assay was used to assess bacterial cytotoxicity. HeLa cells were seeded in 6-well plates (5 × 104 cells/well in 3 ml). At 24 h, the cell counts of 3 wells were determined. In the remaining wells, the medium was replaced with standard medium plus 10% brucella broth (1) and then exchanged with bacteria at a multiplicity of infection (MOI) of 100. A triplicate set of cultures was harvested every 24 h over at least 4 days. Media were collected and diluted, and the bacterial cell counts were determined on CBA plates. HeLa cells were washed twice with 2 ml of phosphate-buffered saline (PBS), and the monolayers were trypsinized. The cells were enumerated with the CASY cell counter.

### Table 3. Plasmids used in this study

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<th>Plasmid</th>
<th>Relevant features</th>
<th>Reference or source</th>
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<td>pBlueI2 KS(−)</td>
<td>bla lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pBlueKS−/VirB9</td>
<td>virB9 cloned in the MCS′ of pBlueI2 KS(−)</td>
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<td>aphA-3 from pILL600 inserted in virB9 of pBlueKS−/VirB9</td>
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<td>This study</td>
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<td>pRYVL1</td>
<td>(P&lt;sub&gt;psapA&lt;/sub&gt;−aphA-3) in pRYGG1, BamHI, and PstI linker between P&lt;sub&gt;psapA&lt;/sub&gt; and aphA-3</td>
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</tr>
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<td>pRYBM3</td>
<td>(P&lt;sub&gt;psapA&lt;/sub&gt;−aphA-3); P&lt;sub&gt;psapA&lt;/sub&gt; of pRYVL1 replaced by P&lt;sub&gt;psapA&lt;/sub&gt;</td>
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<td>pRYVM5</td>
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* MCS, multiple-cloning site.
system (Innovatis AG, Reutlingen, Germany). For each experiment, cell counts were compared with those of 2 independent mock-infected controls.

Gentamicin protection assays were performed with cultured Caco-2 cells; 5 × 10^6 cells were seeded per well in 24-well tissue culture plates and incubated for 2 days to reach 80% confluence. Each well was washed once with medium before inoculation with a bacterial suspension (MOI = 1,000). The number of living bacteria was verified at infection by growth on CBA plates. Infection progressed for up to 5 h before the medium containing the bacteria was removed. The wells were washed once with 500 µl medium containing 400 µg/ml gentamicin and then incubated with 1 ml medium plus 400 µg/ml gentamicin for 3 h to kill extracellular bacteria. The medium was removed, pooled, and centrifuged at 5,000 rpm for 15 min. The pelleted material was washed with PBS and then spread on CBA plates to detect surviving extracellular bacteria. To release intracellular bacteria, eukaryotic cells were washed with 500 µl medium without drug and then incubated with 500 µl PBS containing 0.1% Triton X-100 for 20 min. Fresh medium was added. Suspensions were diluted, spread on CBA plates, and incubated for 4 days. For each measurement, cell counts were determined in triplicate, and assays were repeated on 3 different days. The values shown represent a summary of all counts of the 3 independent experiments. Statistical analysis used SigmaStat software (version 2.03; SPSS Inc., Chicago, IL). The paired t test or the Mann-Whitney rank sum test was used to assess the significance of variation exhibited in cell killing or invasion levels.

**Nucleotide sequence accession number.** The sequence of the *C. fetus* subsp. *venerealis* ATCC 19438 genomic island was deposited in GenBank under the accession number EU443150.

**RESULTS**

**Identification of subspecies-specific gene fragments.** RDA was applied to the genomes of the *C. fetus* subspecies. Two independent RDA reactions, each comprising three rounds of selective enrichment, were performed. Ultimately, the recombinant DNA of 800 clones was isolated and analyzed by hybridization. Pairs of duplicate dot blots representing all clones were differentially probed with either radioactively labeled *C. fetus* subsp. *venerealis* ATCC 19438 DNA or *C. fetus* subsp. *fetus* ATCC 27374 DNA (Fig. 1). A total of 46 clones that were apparently unique to a subspecies were identified (34 specific for *C. fetus* subsp. *venerealis* and 12 specific for *C. fetus* subsp. *fetus*). Clones showing weak differences in hybridization signals between the two reference strains were not further analyzed. Those appearing to be different for one of the subspecies were sequenced. The 34 clones specific for *C. fetus* subsp. *venerealis* ATCC 19438 corresponded to six independent DNA fragments, whereas the 12 clones that were different for *C. fetus* subsp. *fetus* ATCC 27374 represented a single gene region (Table 4). To survey the prevalence of these genomic fragments within different *C. fetus* strains, each isolated fragment was radioactively labeled and hybridized to blotted genomic DNA from selected *C. fetus* reference and field strains (Table 4 and Fig. 2).

The fragment in clone 4Ch was detected in 8 of 10 *C. fetus* subsp. *fetus* strains and absent in all tested *C. fetus* subsp. *venerealis* strains (Fig. 2B). Sequence homology implied that 4Ch encodes GDP-mannose 4,6-dehydratase, an important enzyme for lipopolysaccharide (LPS) biosynthesis. Conversely, the other six fragments identified in the initial screen were confirmed by Southern hybridization to harbor gene regions predominantly or exclusively detectable among *C. fetus* subsp. *venerealis* strains (Table 4). For example, all of the *C. fetus* subsp. *venerealis* strains tested (n = 10) and none of the 10 *C. fetus* subsp. *fetus* strains harbor gene fragment 3Dv (Fig. 2A). This apparently unique locus was chosen for detailed analysis.

**Characteristics of the *C. fetus* subsp. *venerealis*-specific genomic island.** (i) General features. DNA sequence flanking the subspecies-specific fragment 3Dv was obtained by chromosome walking within a *C. fetus* subsp. *venerealis* ATCC 19438 genomic library. A total of 45,627 bp of continuous sequence with an average 5.75-fold coverage was produced. The overall G+C content of the sequence was 32.8%, and it contained 57 ORFs, all but six of them oriented for transcription in the clockwise direction on the DNA plus strand. A linear representation and an annotation of the ORFs are presented in Fig. 3 and Table 5.

(ii) The chromosomal core and site of insertion. The first three ORFs of the analyzed chromosomal locus are similarly arranged and nearly identical to homologs from *C. fetus* subsp. *fetus* 82-40 (GenBank accession number NC_008599). orf1 and orf2 are presumably part of an ABC transporter dedicated to histidine and glutamine uptake (43), and orf3 is an apparent homolog of a sodium/proline symporter. orf3 may be a pseudogene, as an additional guanosine inserted within a poly(G) tract (nucleotide [nt] 2141) creates a premature stop codon. Nucleotides 3381 to 3459 encode a methionyl-tRNA, which is also evident in *C. fetus* subsp. *fetus* 82-40.

Pathogenicity islands (PAIs) are typically inserted into the backbone genome at specific sites that are frequently tRNA genes followed by direct repeats. The latter serve as recognition sites for the integration of bacteriophages (36, 70). Our sequence showed a 2.4-fold tandem repeat (nt 3471 to 3517) shortly after the methionyl-tRNA gene, followed by orf4, which is homologous to a bacteriophage P4-like integrase gene. Integrase genes of this type are also found in other campylobacters and on different integrative plasmids and pathoge-
nicity islands. Two additional mobility genes are present about 15 kb downstream. orf24 and orf25 are highly homologous to the Helicobacter pylori transposable element ISHp608 (47). This type of IS is always comprised of the consecutive elements orfA and orfB. A well-conserved DDEK motif, which has been suggested to be the catalytically active site, is evident in orf25 (47) and is characteristic of the IS family of IS elements, typically found in epsilonproteobacterial species and in retroviral integrases (35). Interestingly, orf24 and orf25 are absent in the partially analyzed strain C. fetus subsp. venerealis (Fig. 3). The 3′ boundary of the island shows a 19-bp remnant of a tRNA gene (nt 45138 to 45156). The chromosomal core starts with orf18, which encodes a methyl-accepting chemotaxis protein with an identical sequence in Campylobacter jejuni (GenBank accession no. NC_009714.1).

(iii) Type IV secretion system. Sequences distal to orf6 exhibit strong homology to genes commonly required for assembly of a T4SS. This class of macromolecular transfer systems functions to translocate proteins and nucleoprotein complexes from donor to recipient cells in processes related to bacterial conjugation. In pathogenic bacteria, T4SSs transmit effector proteins to eukaryotic hosts via a T4SS (67, 72). Indeed, the T4SSs exhibit the most apparent synteny and homology with the resistance (R) plasmids pCC31 of C. coli and pTet of C. jejuni (8, 26), as well as an uncharacterized locus within the Campylobacter hominis genome (GenBank accession no. NC_009714.1).

Importantly, the C. fetus subsp. venerealis locus contains two putative effector molecules, possibly transported by the T4SS. Both orf38 and orf39 encode a Fic (filamentation induced by cyclic AMP [cAMP]) domain (HPFXXGNG) (87). Bacterial Fic domain proteins disrupt host cell processes after their transmission into eukaryotic cells. In Legionella pneumophila, Coxiella burnetii, and Bartonella henselae, Fic domain-containing proteins are translocated via a T4SS (67, 72). Indeed, fusion of the putative effector proteins to Cre recombinase for analysis in CRAF (Cre recombinase reporter assay for translocation) (72) provides evidence that recognition and secretion of the product of orf39, at least, belong to the functional repertoire of this T4SS (S. Kienesberger, G. Gorkiewicz, and E. L. Zechner, unpublished data).

(iv) Plasmid-borne genes and genes of unknown function. Besides the T4SS noted above, several ORFs show homology to components typically carried by plasmids. Among them are
homologs to cpp genes found on the C. coli plasmid pCC31 (cpp45 [orf21], cpp46 [orf22], cpp49 [orf27], cpp17 [orf41], cpp18 [orf42], and cpp50 [orf44]), orf28 shows significant homology to a gene encoding a hypothetical protein of the Campylobacter kari plasmid pCL46, and orf37 matches a gene encoding a hypothetical protein found on pCU110 from C. upsaliensis. Moreover, orf33 matches an Enterococcus faecalis transcriptional regulator of the two-component response regulator class. The amino acid sequence contains a short helix-turn-helix motif also found in DeoR-like transcriptional regulators, which are known to regulate genes localized on pathogenicity islands (37). orf36 encodes a putative nucleotidyltransferase, which is a plasmid-encoded enzyme responsible for resistance to aminoglycosides also found in Campylobacter (59), and orf50 encodes an EcoRI methylase. A detailed annotation of the genes found on the genomic locus is given in Table 5.

In conclusion, the characteristics of this subspecies-specific sequence, as well as the organizations and putative functions of the genes carried, imply that the chromosome of C. fetus subsp. venerealis bears a genomic island. The 5' and 3' borders flanking the island are identical to those of C. fetus subsp. fetus 82-40. The genomic island harbors a conjugation-related T4SS and bears homology to several plasmid-borne genes, and it displays high homology and synteny to the R plasmids pCC31 (Fig. 3) and pTet found in C. coli and C. jejuni, respectively.

The genomic island is uniquely present in C. fetus subsp. venerealis. The distribution of the genomic island was surveyed in a collection of 112 C. fetus isolates (see Table S1 in the supplemental material). To this end, Southern hybridization analysis was performed with chromosomal DNA from a random selection of C. fetus strains. The gene-specific probes corresponding to orf21, orf27, virB10, and virD4, as well as a region spanning from virB8 to virB9. To expand the analysis, genomic DNA from all strains of the collection was subjected to PCR analyses with a minimum of two different primer combinations targeting different loci from the island and adding information about the presence of virB4 and virB11. In every case where the hybridization and PCR approaches were applied to a particular gene in the same strain, consistent results were obtained with both tests. All results are summarized in Table S1 in the supplemental material. None of the test genes were detected in any of the 45 C. fetus subsp. fetus strains investigated. In contrast, of the 67 C. fetus subsp. venerealis strains, 51 (76%) harbored all assessed genes, and 10 scored positively for at least one of the analyzed genes. Hybridization and/or PCR were negative for all genes tested in only 6 C. fetus subsp. venerealis strains (9%). To assess whether these strains harbor a remnant of the island, PCR primers were designed to hybridize upstream (orf3) and downstream (orf57) of the insertion site (Inter_1 and Inter_2 (Table 2)). Amplification of C. fetus subsp. fetus 82-40 DNA, which harbors no genomic island, and five of the six “negative” C. fetus subsp. venerealis strains gave the same 721-bp product, indicating that no additional DNA was inserted at the site (not shown). In contrast, DNA from one strain (V13) did not support amplification across this position (not shown). The quality of the V13 template DNA was verified by a control amplification of a gene outside of the genomic island. This finding implies that a remnant of the island may exist in this strain, but the remaining region lacks the genes investigated thus far. Alternatively, DNA of a different origin may have been integrated at this site.

To gain complementary data about whether the site of island integration is conserved in “positive” C. fetus subsp. venerealis strains, 21 C. fetus strains were examined by SmaI macrorestriction and PFGE, followed by hybridization with the island-specific orf21 probe (Fig. 4). Hybridization was observed for 7 of the 11 C. fetus subsp. venerealis strains, as expected, and was again completely absent in all C. fetus subsp. fetus strains. Six of the seven positive samples exhibited a band about 240 kb in size, while one isolate displayed a hybridization signal of about 140 kb. This finding implies that the genomic island is located at similar chromosomal positions in the majority of C. fetus subsp. venerealis strains. Moreover, comparison of the sequence of the C. fetus subsp. venerealis ATCC 19438 island
with a 33.7-kb sequence newly generated from the *C. fetus* subsp. *venerealis* 84-112 (V81) genome revealed that the site of insertion is conserved. Indeed, the loci were nearly identical. Although the *C. fetus* subsp. *venerealis* 84-112 genome lacks the IS transposase (*orf24* and *orf25*) (Fig. 3), only 3 base changes were detected within the remaining shared sequence (data not shown).

Taken together, our findings indicate a complete (100%) absence of homologous sequences from the *C. fetus* subsp. *fetus* strains surveyed and a high frequency of detection for at least some genes in *C. fetus* subsp. *venerealis* isolates (91%). We conclude that within the species *C. fetus* this locus is apparently subspecies specific. Evidence for genetic insertion at a conserved position is absent in a minority of strains, however, suggesting that the presence of these genes is not universal for *C. fetus* subsp. *venerealis*. These data also suggest that the genomic island may maintain the capacity for lateral transmission.

**vir** genes are expressed in *C. fetus* subsp. *venerealis*. In the agrobacterial paradigm T4SS, and in all similar systems studied thus far, the conserved block of *virB*-*virD* genes is organized as a transcriptional unit (15, 17). We identified a region upstream of the **vir** genes with an increased A+T value and similarity to other *Campylobacter* promoters (49, 88). Functional promoter activity was confirmed when this putative regulatory region 5' to **orf7** (nt 5760 to 6081) replaced the existing promoter regulating **aphA-3** in vector pRYVL1 (48). The test construction, pRYSK8, was introduced into *C. fetus* subsp. *venerealis* ATCC 19438 via conjugation. Derivatives of this vector backbone, which do not provide a *C. fetus*-specific promoter upstream of **aphA-3**, do not support an antibiotic-resistant phenotype (48). Kanamycin-resistant transconjugants were obtained at high frequencies after transconjugation of pRYSK8, confirming promoter activity for the putative **vir** regulatory sequence under laboratory conditions (data not shown). Moreover, application of reverse transcriptase-PCR generated cDNA of **virB8**, **virB9**, **virB10**, and **virD4** from the mRNA pool of *C. fetus* subsp. *venerealis* ATCC 19438 (Fig. 5). Transcripts were isolated following growth on CBA plates, again demonstrating expression under laboratory conditions. Notably, the insertion of a Km' cassette in **virB9** (mutant Cfv3-18) had polar effects on the transcription of downstream genes, consistent with the hypothesized operon organization (Fig. 5).

**Mutation of **virB9** reduced bacterial killing of eukaryotic cells.** To test for the putative virulence functions conferred by the island, we used a basic coculture experiment that measured the toxic effects of pathogenic bacteria on cultured human cell lines (1). HeLa cells were infected with the wild-type strain (*C. fetus* subsp. *venerealis* ATCC 19438) or the isogenic **virB9** mu-
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### TABLE 5. Annotation of genes found on the C. fetus subsp. venerealis ATCC 19438 chromosome

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2% of the cells survived until day 4. By comparison, infection of eukaryotic cells and bacteria were enumerated daily. Compared to the wild-type strain, only 30.3% of viable HeLa cells (wild type versus Cfv 3-18; Fig. 6). A significant difference between the two strains was observed at day 2 and continued to the end of the experiment. At day 2 of cocultivation with the wild-type strain, only 0.42% of wild-type-infected HeLa cells remained viable compared to the uninfected control. Thus, the virB9 mutant killed about 30% fewer cells than the wild-type strain at day 4 of cocultivation, and this result was reproducible in three independent experiments with statistical significance (wild type versus Cfv 3-18; P = 0.001; paired t test) (Fig. 6).

Disruption of virD4, but not cdnB, attenuates bacterial invasion of eukaryotic cells. The initial indication gained by the coculture experiments that mutational inactivation of vir genes attenuates the virulence of C. fetus subsp. venerealis was analyzed further. Invasion of eukaryotic cells is a hallmark property of bacterial pathogens. The reference strain, C. fetus subsp. venerealis ATCC 19438, tested as described above for cytotoxic effects, is not invasive when used in eukaryotic cell culture experiments (data not shown). For this analysis, therefore, we used the bovine isolate C. fetus subsp. venerealis 84-112 (VS1), which exhibits invasion levels comparable to those of C. jejuni (44). A standard gentamicin protection assay was applied.

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a aa, amino acids.
b As predicted by Blast analyses.
c NA, not applicable.
d CAT, tRNA with anticodon CAT.
to Caco-2 cells, as this in vitro invasion model is established for Campylobacter (44). We chose to disrupt virD4, since the gene is important to the virulence of various pathogens harboring pathogenicity-associated T4SSs (24, 71), and as it is the last gene in the predicted transcriptional unit, complementation of the mutation would not need to include downstream genes. Caco-2 cells were infected with the wild-type strain (V81) and an isogenic virD4 mutant (V81_SK1). Five hours postinfection, the culture was treated with gentamicin to kill extracellular bacteria. Intracellular bacteria were protected from the antibiotic and were enumerated following eukaryotic cell lysis. Wild-type V81 cells that survived gentamicin treatment were recovered at a ratio of 0.38 per Caco-2 cell, meaning that a maximum of 38% of these cells harbored intracellular bacteria. A similar invasion frequency was achieved over a range of 2 to 7 h of cocultivation before the addition of antibiotic (not shown), in good agreement with prior results for Campylobacter (44). Carriage of the vector control did not alter the invasion proficiency of V81. Accordingly, this wild-type value was set to 100% for comparison to the mutant strains (Fig. 7A). The isogenic virD4 mutant harboring the vector control lost 42% of the wild-type invasiveness, as bacteria were recovered at the significantly reduced ratio of 0.22 per Caco-2 cell (wild type versus virD4 mutant; \( P = 0.012 \)).

To confirm the specific involvement of VirD4 in this model of host cell invasion, we performed a similar mutational analysis of an independent virulence gene not located in the C. fetus genomic island. C. fetus, like other campylobacters, produces a cytolethal distending toxin (CDT), which is thought to be a major virulence factor but does not influence invasion ability (3, 38, 53, 65, 66). The exotoxin is composed of three subunits, including the active component CdtB. The cdtB gene of V81 was inactivated by insertion mutagenesis, and the capacity of the resulting mutant (V81_JL1) to invade Caco-2 cells was compared to that of the parent strain, V81. Intracellular bacteria were recovered from 23% and 26% of Caco-2 cells following infection with the cdtB mutant or the isogenic parent strain, respectively. This difference was not statistically significant (\( P = 0.362 \))
It is important to note that the invasiveness of the wild-type strain, V81, was not limited to the established human colonic epithelial cell line Caco-2 but was similarly exhibited by the cultured human trophoblast cell line ACH-3P (39 and data not shown).

In summary these experiments identified two virulence properties of *C. fetus* subsp. *venerealis* that are manifest in vitro: cytotoxicity against HeLa cells and the ability to invade human epithelial and placenta cells. These virulence attributes were functionally linked to expression of the putative T4SS components VirB9 and VirD4. In contrast, mutational inactivation of CdtB had no effect on the invasiveness of strain V81, validating the specificity of our mutant phenotypes. In conclusion, we propose that vir components of the T4SS encoded by the *C. fetus* subsp. *venerealis* genomic island contribute to the virulence properties of the pathogen.

**DISCUSSION**

A genomic subtractive-hybridization approach was used to investigate the genetic basis potentially underlying the distinct niche preferences displayed by *C. fetus* subsp. Species. In this report, we focused on the characteristics of a genomic island that is uniquely present in *C. fetus* subsp. *venerealis* and the potential virulence functions contributed by the island to the sub-

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**FIG. 6.** The virB9 mutant Cfv 3-18 shows an impaired cytotoxic phenotype in continuous co-culture with HeLa cells. HeLa cells were grown to confluence and then infected in triplicate at an MOI of 100 with either *C. fetus* subsp. *venerealis* ATCC 19438 (CfvT) or Cfv 3-18. (A) The number of surviving HeLa cells relative to uninfected control cultures is indicated. (B) The number of viable bacteria in the cell culture medium was determined daily. No statistical difference between CfvT and Cfv 3-18 was observed. The values shown are averages of 3 independent experiments. Statistical significance is indicated: *, P < 0.05; **, P < 0.005 (CfvT versus Cfv 3-18). The error bars indicate standard deviations.

**FIG. 7.** virD4 mutation attenuates bacterial invasion. (A) Caco-2 cells were infected with *C. fetus* subsp. *venerealis* 84-112 harboring the vector control (left) (V81[pRYSS1]), the isogenic virD4 mutant harboring the vector control (middle) (V81_SK1[pRYSS1]), or the mutant strain harboring virD4 in trans (right) (V81_SK1[pRYVL2]). Extracellular bacteria were killed with gentamicin after 5 h, and the relative number of intracellular bacteria per Caco-2 cell was compared to that of the wild type (WT) (set to 100%). (B) For a negative control of the invasion phenotype, Caco-2 cells were infected with wild-type *C. fetus* subsp. *venerealis* 84-112 (V81) or the isogenic mutant in cdtB (V81_JL1) for 3 h. The values shown are the means of three independent experiments plus standard deviations; *, P < 0.02.  

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species. An extended survey of our collection of diverse C. fetus strains from all over the world using gene hybridization and PCR revealed that none of the C. fetus subsp. fetus isolates harbored the island, whereas the set of genes we tested was conserved in the majority of the C. fetus subsp. venerealis isolates. The island seems to represent a major fraction of the genomic difference between the two subspecies as revealed by a recently sequenced genome of C. fetus subsp. venerealis 84-112 (32). Moreover, a recent paper reporting the genome analysis of C. fetus subsp. venerealis AZUL-94 also found that a major difference between the two subspecies was genes encoding type IV secretion components (57).

In general, genomic islands increase the fitness of their hosts by providing new functions. PAIs, a subset of genomic islands, have recently been recognized as a repository of virulence genes in many organisms. Acquisition of PAIs by horizontal gene transfer is clearly responsible for the conversion of many weakly pathogenic or nonpathogenic organisms into major pathogens. Consistent with this model, the island specific to C. fetus subsp. venerealis carries homologs of genes known to be involved in virulence. Notably, the island carries conserved sequence and structural homology to the virB2-virB11 and virD4 loci, named for the prototypical T-DNA transfer system of Agrobacterium tumefaciens, which are typically required for bacterial type IV secretion (15). Secretion systems of this type are complex machineries evolutionarily related to bacterial conjugation systems (20, 90). Many operate in pathogenic bacteria, such as A. tumefaciens, Helicobacter pylori, Bartonella spp., Brucella spp., Bordetella pertussis, and Legionella pneumophila, to translocate effector molecules across the Gram-negative envelope and into bacterial, plant, or mammalian cells (15, 17). The translocated effectors generally have a central function in host-pathogen interactions, e.g., they induce inflammatory responses and cytoskeletal alterations or facilitate the intracellular lifestyle of the bacterial pathogen (6). Experimental evidence has unequivocally established the importance of the T4SS as a pathogenicity factor in each of the systems mentioned. In contrast, identifying the protein substrates of the systems in question has been difficult in most organisms. Accordingly, it has been surprising to identify some systems, such as the Bartonella T4SS, in which genes for the protein effectors are tandemly arrayed immediately downstream of the virB-virD4 operon (72). In the C. fetus genomic island, two putative effector molecules downstream of the virB-virD4 operon were identified based on the presence of a Fic domain. Fic domain-containing proteins translocated by T4S or T3S systems have been identified in several pathogens, including Legionella pneumophila, Campylobacter jejuni, and Vibrio parahaemoliticus (67). Moreover, this domain is conserved in the translocated effectors BepA-BepC of Bartonella (72). Fic domain proteins disrupt host cell processes following delivery into eukaryotic host cells. Preliminary data suggest that the protein encoded by orf39 is recognized as a factor for T4 secretion (Kienesberger et al., unpublished). Its potential contribution to virulence is the focus of current investigation.

The species C. fetus is currently divided into two subspecies based on their habitats and biological and phenotypic properties (e.g., glycine tolerance), as well as the diseases they cause (81). C. fetus subsp. fetus strains may be either serotype A or serotype B, whereas C. fetus subsp. venerealis strains are restricted to serotype A (58, 64). These serotypes are associated with differences in LPS structures and also with the type of the surface layer protein (SLP) (76). The C. fetus SLPs form a proteinaceous capsule on the cell surface, which mediates resistance to complement C3b binding. SLPs further undergo antigenic variation to protect against antibody-mediated opsonization, thus contributing to both persistence and systemic infection (12–14, 33, 34, 63, 75, 79). The SLP is a constituent of both C. fetus subspecies, and it represents an ancient, highly conserved constituent of the C. fetus genome, not a pathogenicity island (76). Recent multilocus sequence typing (MLST) data have indicated that C. fetus is genetically homogeneous, in contrast to other Campylobacter species. It displays a highly clonal population structure, wherein C. fetus subsp. venerealis represents a bovine clone (81). The clonality within C. fetus suggests that genetic change accumulates slowly by vertical transmission of point mutations. Additionally, genetic diversity may be limited by the lack of natural competence in C. fetus (77). Other campylobacters, like C. jejuni or H. pylori, show extensive genetic variation, vertically as well as horizontally, and these continuous genetic alterations are important traits of the pathogens for colonizing and persisting in their habitats (10, 18, 85).

Given the phylogenetic perspective that C. fetus subsp. venerealis represents a bovine clone of C. fetus subsp. fetus with limited genetic variation, it is intriguing to speculate that the island discovered in this study contributes to the host tropism and therefore the unique pathogenicity of C. fetus subsp. venerealis. The stringent subspecies prevalence of this island is consistent with this hypothesis. Interestingly, in Bartonella, it was shown that a T4SS originating from a conjugation system was acquired by the pathogen, ultimately serving as a virulence trait (73). The absence of the island in some C. fetus subsp. venerealis isolates underscores its probable origin as a mobile genetic element. Several well-characterized T4SSs have been shown to exhibit dual functionality by maintaining the capacity to mobilize plasmids conjugatively to other bacterial cells (6, 15, 22). Mutagenic analysis of C. fetus subsp. venerealis isolate 84-112 (V81) verified that this system has also retained conjugative functions (Kienesberger et al., unpublished). Consistent with that observation, the structure of the C. fetus genomic island is highly syntenic to resistance plasmids found in other Campylobacter species, pCC31 and pTet (8, 26). Some T4SS components also mediate DNA uptake, thus conferring a natural competence on organisms such as C. jejuni or H. pylori (7, 42). The C. fetus subspecies are refractory to DNA uptake by transformation, and therefore, competence is not a function linked to the identified locus (48, 77). High-level homology to an uncharacterized genomic island of C. hominis also exists. The question of whether this genome region represents a symbiosis island conferring increased fitness on this nonpathogenic species remains open (36, 37). Other genomic islands with similarity to plasmids are also present in certain campylobacters, for example, the 71-kb Helicobacter hepaticus ATCC 51449 genomic island HHG1 shares high homology with the C. coli plasmid pCC178, and the Wolinella succinogenes DSM 1740 genomic island, WsuGI, is syntenic to the C. jejuni plasmid pVir (5, 23, 25). Mobility genes, like IS elements or prophages, present on such islands facilitate gene shuffling between plasmids and the core genome and contribute to

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