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Interbacterial Macromolecular Transfer by the *Campylobacter fetus* subsp. *venerealis* Type IV Secretion System▽

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We report here the first demonstration of intra- and interspecies conjugative plasmid DNA transfer for *Campylobacter fetus*. Gene regions carried by a *Campylobacter coli* plasmid were identified that are sufficient for conjugative mobilization to *Escherichia coli* and *C. fetus* recipients. A broader functional range is predicted. Efficient DNA transfer involves the *virB9* and *virD4* genes of the type IV bacterial secretion system encoded by a pathogenicity island of *C. fetus* subsp. *venerealis*. Complementation of these phenotypes from expression constructions based on the promoter of the *C. fetus* surface antigen protein (*sap*) locus was temperature dependent, and a temperature regulation of the *sap* promoter was subsequently confirmed under laboratory conditions. Gene transfer was sensitive to surface or entry exclusion functions in potential recipient cells carrying IncPα plasmid RP4 implying functional relatedness to *C. fetus* proteins. The *virB*/*virD4* locus is also known to be involved in bacterial invasion and killing of cultured human cells *in vitro*. Whether specifically secreted effector proteins contribute to host colonization and infection activities is currently unknown. Two putative effector proteins carrying an FIC domain conserved in a few bacterial type III and type IV secreted proteins of pathogens were analyzed for secretion by the *C. fetus* or heterologous conjugative systems. No evidence for interbacterial translocation of the Fic proteins was found.

Type IV secretion systems (T4SS) are membrane-associated transporter complexes used by Gram-negative and Gram-positive bacteria to deliver substrate molecules to a variety of target cells in a contact-dependent manner (for reviews, see references 3, 5, 12, 16, 21, and 32). Secreted macromolecules are proteins or protein-DNA complexes; thus, some T4SS support horizontal gene transfer. The mechanisms involved include conjugation, oncogenic T-DNA delivery to plant cells by the phytopathogen *Agrobacterium tumefaciens*, and DNA uptake and transformation, as well as DNA release into the extracellular milieu (15). Moreover, several mammalian pathogens utilize their T4 translocation machinery for toxin secretion and the targeted delivery of virulence factors into eukaryotic host cells during infection. Accordingly, T4SS contribute to pathogenesis in various ways, including increased genome plasticity, antibiotic resistance spread, enhanced surface colonization and biofilm formation, and the specific injection of virulence proteins. In the latter case, the translocated proteins affect basic cellular functions of the host, resulting in the induction of disease (9, 23, 56, 70).

Macromolecular secretion across bacterial and host cell membranes requires the assembly of a multisubunit, cell envelope-spanning machinery comprising a secretion channel and often a pilus or cell surface filament. In paradigm conjugation systems, these components are collectively referred to as the Mating pair formation (Mpf) proteins (58). Systems related to the F-plasmid share 8 of 10 highly conserved Mpf components common in T4SS (40). Conjugative P-like (e.g., IncP, -W, and -N) and I-type (e.g., R64 and ColIb-P9) systems require distinct sets of auxiliary genes. The T4SS associated with bacterial virulence have been genetically categorized into distinct classes, T4a and T4b, based on whether they share common ancestries with the *A. tumefaciens* VirB/D4 (T4a) or ColIb-P9 (T4b) archetypal systems (19).

T4a pathways are highly conserved and well studied. The *Agrobacterium* apparatus includes 12 envelope-spanning components (VirB1-B11) expressed from a single operon. In addition, an inner membrane anchored T4 coupling protein T4CP (VirD4) governs the selective uptake of secretion substrates into the VirB translocation channel (3). T4CPs are universally required for transferring protein-DNA complexes and are typically necessary for protein translocation and virulence in pathogen-associated systems (22, 41, 54, 59). Several virulence-associated T4SS appear to have retained the capacity to mobilize plasmids by conjugation, while gaining proficiency in specific protein translocation (10, 69). Our work with the human and animal pathogen *Campylobacter fetus* recently revealed a subspecies-specific pathogenicity island (PAI) encoding a functional bacterial T4a secretion apparatus (28). This pathogen belongs to the group of epsilonproteobacteria and is highly adapted to mucosal surfaces (31). The two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, show a clonal population structure (65). Despite that level of genetic relatedness the subspecies exhibit distinct host and tissue prefer-

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TABLE 1. Bacterial strains used in this study

Strain	Description[a]	Source or reference[b]
C. fetus		
subsp. *fetus* ATCC 27374	Type strain; Nal^r	ATCC
subsp. *fetus* F12	Human isolate, septicemia; Cip^r Nal^r	37
subsp. *fetus* 82-40	Human isolate, septicemia; GenBank NC_008599	
subsp. *venerealis* ATCC 19438	Type strain; Nal^r	ATCC
subsp. *venerealis* 3-18	Cfv ATCC 19438 *virB9*::P_{Cc}-*aphA-3*	28
subsp. *venerealis* SK1	Cfv ATCC 19438 *virD4*::P_{rpsJ}-*aphA-3*	28
subsp. *venerealis* JL1	Cfv ATCC 19438 *cdtB*::P_{rpsJ}-*aphA-3*	28
subsp. *venerealis* 84-112	Bovine isolate, genital secretion; Nal^r	50
subsp. *venerealis* V81_SK1	Cfv 84-112 *virD4*::P_{rpsJ}-*aphA-3*	28
E. coli		
DH5α	*endA1 recA1 gyrA96 thi-1 hsdR17 supE44* λ$^-$ *relA1 deoR* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*ΔM15	73
MT102	*araD139* (*ara-leu*)*7697* Δ*lac thi hsdR mcr rpsL*	4
MS411	*ilvG rfb-50 thi*	M. Schembri (DTU)
SAR18	*ara* Δ(*lac-pro*) *thi attB*::*bla*-$_{PA1/04/03}$-*gfpmut3**-T$_o$	51
MS614	Sm^r; *ilvG rfb-50 thi rpsL*	M. Schembri (DTU)
CHS26Cm::LKL	CSH26 *galK*::*cat*::*loxP*-Km-*loxP*	39
MT102Cm::LKL	MT102 *galK*::*cat*::*loxP*-Km-*loxP*	39
CHS26Cm::LTL	CSH26 *galK*::*cat*::*loxP*-TetRA-*loxP*	39
S17-1 λ*pir*	Tp^r Sm^r; *recA thi pro hsdR*$^-$*M*$^+$ RP4:2-Tc::Mu:Km Tn7 λ*pir*	18

[a] Nal^r, nalidixic acid resistance; Cip^r, ciprofloxacin resistance; Sm^r, streptomycin resistance; Tp^r, trimethoprim resistance.
[b] ATCC, American Type Culture Collection; DTU, Technical University of Denmark.

ences. *C. fetus* subsp. *venerealis* is largely restricted to the bovine genital tract, causing epidemic abortion in these animals and substantial economic losses to the cattle industry (8). *C. fetus* subsp. *fetus* is an important agent in ovine abortion worldwide and can also induce severe disease in humans (7, 8, 61, 63). This subspecies is the predominant *Campylobacter* species isolated from human blood and is considered to be an emerging pathogen (7). We are interested in the contribution of the *C. fetus* subsp. *venerealis* chromosomal PAI and its resident T4SS to the host and tissue tropism of this subspecies. Mutational inactivation of the *virD4* and *virB9* components in virulent *C. fetus* subsp. *venerealis* isolates attenuated invasion and cell-killing phenotypes in cultured human cell lines (28). Nonetheless, progress in understanding the role this chromosomally encoded T4SS plays in host-pathogen interactions remains challenging; this is due to the difficulty of manipulating the organism genetically and also to the current absence of well-characterized *in vitro* models of tissue-specific infection (34).

This study aimed to identify macromolecular substrates translocated by the *C. fetus* T4SS. We demonstrate that *C. fetus* subsp. *venerealis* supports intra- and interspecies conjugative mobilization of plasmid DNA in a process requiring the T4SS. Functional overlap between *C. fetus* subsp. *venerealis* and IncP plasmid components was detected via plasmid RP4-induced fertility inhibition. Two putative secretion substrates encoded by the *C. fetus* subsp. *venerealis* PAI were investigated. The data provide evidence for conjugative DNA delivery but not interbacterial protein transfer.

MATERIALS AND METHODS

Sequence analysis and alignments. The predicted protein sequences of the *C. fetus* subsp. *venerealis* virB/D4 locus (GenBank accession number EU443150) were analyzed by using BLAST-P (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Predicted proteins were compared over the whole amino acid sequence by using the CLUSTAL W function of MegAlign from Lasergene (DNAStar, Inc., Madison, WI) with default settings. Protein domains were identified by using the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/structure/index.shtml).

Bacterial strains. Strains are listed in Table 1. Growth conditions for *Campylobacter* and *Escherichia coli* strains were as previously described (33). *C. fetus* subsp. *venerealis vir* gene mutants were described earlier (28).

Plasmids and PCR amplification. Plasmids are listed in Table 2. Plasmid DNA was purified from *E. coli* and *Campylobacter* cells with a QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Restriction endonucleases and DNA modifying enzymes, obtained from New England Biolabs, Inc. (Beverly, MA), and Fermentas GmbH (St. Leon-Rot, Germany) were used as recommended by the suppliers. Phusion High-Fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland) was used to amplify DNA fragments for cloning. All other PCR amplifications utilized DyNAzyme II DNA polymerase (Finnzymes Oy) according to the manufacturer's specifications.

Construction of plasmids. Numbered oligonucleotide primers are described in Table 3. A 1.6-kb *mobA* fragment pSK108-1 was excised with SmaI and NdeI and then blunted using T4 DNA polymerase (New England Biolabs) before ligation with the SmaI site of pRYSK3 to create pRYSK9. A 220-bp NotI/PstI fragment containing the *gatC* promoter was amplified from *C. fetus* subsp. *venerealis* chromosomal DNA with the primers 5 and 6. This regulatory region was then used to replace the *sapA* promoter of pRYVL1. A kanamycin resistance phenotype in *C. fetus* subsp. *venerealis* confirmed promoter activity for the resulting construction, pRYBM5. To generate the complementation vector pRYSK12, the *gatC* promoter fragment was amplified from pRYBM5 with primers 5 and 7, digested with NotI/BamHI, and inserted into a NotI/BamHI-digested pRYVL2. A 420-bp fragment containing the pIP1455 iteron sequence and a putative *nic* site was amplified from pRYSS1 by using the primer pair 8 and 9 and inserted into the SmaI site of pRYSK3 to generate pRYSK17. To fuse the Cre recombinase with the FIC domain-containing proteins, Fic1 and Fic2, the *fic* genes were amplified from chromosomal *C. fetus* subsp. *venerealis* DNA using the primer pairs 12/13 and 14/15, respectively. The products were inserted in the KpnI/SalI-cut plasmid CFP B to generate C-terminal fusions with the Cre coding sequence, resulting in pCreSK1 and pCreSK2, respectively. The 1,028-bp *cre* gene, the 1,860-bp *cre-fic1* fusion, and the 1,944-bp *cre-fic2* fusion were amplified from either CFP B or pCreSK1 or pCreSK2 with primers 16 and 17. PCR products were cut and ligated with SmaI-digested pRYSK3 to generate pRYSK15, pRYSK13, and pRYSK14, respectively. For Cre-TrwC, the *trwC* gene was amplified from plasmid pSU1501 using the primers 18/19 and then ligated with a KpnI/SalI-cut CFP B vector.

Plasmid mobilization from *C. fetus* to *E. coli*. *E. coli* (2×10^7 CFU) from an overnight broth culture was mixed with 2×10^9 *Campylobacter* cells harvested from Columbia blood agar (CBA) plates after 24 h growth. The suspension was spotted onto a nitrocellulose filter (25 mm, 0.22 μm; Millipore), which was

TABLE 2. Plasmids used in this study

Plasmid	Relevant features[a]	Source or reference
pRY111	P_{Cc}-*cat* mob_{IncP} $oriV_{pIP1455}$ $oriV_{pBR322}$	75
pRYGG1	P_{sapA} in the pRY111 MCS	33
pRYSS1	Fusion (Φ) (P_{sapA}-*aphA-3*) in pRYGG1	33
pRYVL1	Φ(P_{sapA}-*aphA-3*) in pRYGG1; BamHI and PstI linker inserted between P_{sapA} and *aphA-3*	33
pRYVL2	Φ(P_{sapA}-*virD4-aphA-3*) in pRYGG1	33
pRYMJ2	Φ(P_{sapA}-*Cfv-virB9-virB10-virB11-virD4*) in pRYGG1	33
pRYEL1	Φ(P_{sapA}-*gfp*) in pRYGG1	33
pSK108-1	3,207 bp of pCFV108 in pBluescript KS II(−)	33
pRYSK2	$oriV_{pIP1455}$ replaced by $oriV_{pCFV108}$	33
pBlue-oriT	mob_{IncP} in the pBluescript KS II(−) MCS	33
pRYSK3	pCFV108 iterons; Φ(P_{sapA}-*aphA-3*) in pBlue-oriT	33
pRYSK9	$mobA_{pCfv108}$ in pRYSK3	This study
pRYSK17	$nic_{pIP1455}$ in pRYSK3	This study
pRYBM5	Φ(P_{gatC}-*aphA-3*); P_{sapA} of pRYVL1 replaced by P_{gatC}	This study
pRYSK12	Φ(P_{gatC}-*virD4*); P_{sapA} of pRYVL2 replaced by P_{gatC}	This study
CFP B	Cre fusion plasmid	49
pCreSK1	Φ(*Cre-fic1*) in CFPB	This study
pCreSK2	Φ(*Cre-fic2*) in CFPB	This study
pRYSK13	Φ(*Cre-fic1*) in pRYSK3	This study
pRYSK14	Φ(*Cre-fic2*) in pRYSK3	This study
pRYSK15	Cre in pRYSK3	This study
pCreTraI R1	R1 traI encoding residues 3 to 1756 in CFP B	39
pCreTraI F	KpnI-SalI *traI* fragment from p99I+ in CFP B	39
pSU1501	R388 *trwC* in pKK223-3	29
pCreTrwC	R388 *trwC* encoding residues 2 to 966 in CFP B	This study
pJMTraD	Wild-type F *traD* in pBAD24	42
pKD46	Red recombinase expression plasmid: *araBp gam-bet-exo oriR101 repA101*(Ts) *bla*	17
RP4	IncP conjugative plasmid	48
pSU2007	IncW conjugative plasmid; Km^r	44
pAR119	IncF conjugative plasmid; Km^r	52
pLG211	IncI conjugative plasmid; Km^r	14
R1-16	IncFII conjugative plasmid; Km^r fin-	27
pOX38	IncFI, conjugative plasmid, KmR	13
pOX38*traD*411	*traD*-null derivative of pOX38	43

[a] Km^r, kanamycin resistance.

placed on an antibiotic-free CBA plate and incubated in a microaerobic atmosphere at 37 or 32°C for 18 to 24 h. *C. fetus* grows poorly at temperatures below 25°C and above 39°C, and DNA mobilization was not detected at these temperatures. In some experiments, the filters were initially soaked in buffer containing 20 mM Tris-HCl and 1 mM $MgCl_2$ (pH 8) with or without 300 μg of DNase I (Roche, Mannheim, Germany). After incubation, cells were removed from the filter by vortexing in 1 ml of phosphate-buffered saline (PBS). Serial dilutions of the bacterial suspension were plated on LB agar containing 25 μg of kanamycin and/or 25 μg of chloramphenicol/ml to select for transconjugant *E. coli* cells. Transconjugant colonies were subcultured, and the plasmid content was verified either by DNA isolation, followed by restriction mapping, or by PCR analysis. Viable cell counts were determined for donor and recipient strains by serial dilutions and subsequent plating.

Plasmid mobilization to *Campylobacter*. Conjugational DNA delivery from *C. fetus* to *Campylobacter* was performed as described for *E. coli* recipients. When *C. fetus* subsp. *fetus* served as a recipient, bacteria were mixed in a ratio of 10:1 (2×10^9 donor and 2×10^8 recipient cells, respectively, in suspension). The cell suspension was spotted onto a nitrocellulose filter and incubated under microaerophilic conditions at 37 or 32°C for 24 h either in the presence or absence of DNase I. Cells were removed from the filter by vortexing in 1 ml of PBS, and the suspension was spread over five CBA plates. Selection for transconjugant *C. fetus* subsp. *fetus* F12 combined ciprofloxacin (32 μg/ml) with selection for the newly acquired plasmid. Agar plates were incubated under microaerophilic conditions at 37°C for 5 days. In experiments with *C. fetus* subsp. *venerealis* JL1 recipients, equivalent cell numbers (2×10^9 CFU) were combined. Selection for transconjugants combined kanamycin for the chromosomally encoded *aphA-3* gene and selection for the acquired plasmid. CBA plates were incubated under microaerophilic conditions at 37°C for up to 12 days.

Cre recombinase assay for translocation (CRAfT). To generate *E. coli* MT102Cm::LKL, a *cat*::loxP-Km-loxP cassette was inserted into *galK* of *E. coli* MT102. The primer pair 10/11 was used to amplify the *cat*::loxP-Km-loxP cassette from *E. coli* CSH26Cm::LKL. The temperature-sensitive pKD46 was introduced into MT102, and the *cat*::loxP-Km-loxP cassette was integrated via homologous recombination as described previously (17).

Campylobacter strains were thawed 72 h prior to the assay and subcultured every 24 h on kanamycin-containing CBA plates. The assay for protein translocation was performed as described for DNA transfer from *Campylobacter* to *E. coli.* CSH26Cm::LKL or MT102Cm::LKL served as *E. coli* recipients. Recombinant colonies resulting from Cre-mediated reconstitution of *cat* expression were selected on LB agar containing 20 μg of chloramphenicol/ml.

To screen for Cre-Fic translocation by a heterologous T4SS *E. coli* MS614 donor strains harbored plasmid R1-16, pOX38 or pSU2007. *E. coli* S17-1 λ*pir* provided the P-type transfer system. The *E. coli* indicator strain was replaced with CSH26Cm::LTL. This strain was created with the lambda RED system as described above using targeting DNA made with primers 12 and 13 and template pAR183. All donor strains additionally contained a CFP B-based plasmid expressing the fusion protein. Drug-free LB was inoculated to an optical density at 600 nm of 0.005 with an overnight culture of the donor strain and incubated at 37°C for 60 min. A 10-fold excess of recipient strain *E. coli* CSH26Cm::LTL was added, and the mixture was incubated for 2.5 h at 37°C in liquid (pOX38 or R1-16) or after spotting onto a nitrocellulose filter (25 mm, 0.45 μm; Millipore) placed on antibiotic-free LB agar (S17-1 λ*pir* or pSU2007). Mating was stopped by vortexing the suspension or the filter in 1 ml of LB medium for 1 min and immediately chilling the sample on ice. Transconjugants were selected on LB agar containing kanamycin (25 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 50 μg/ml). Recombinant colonies resulting from Cre-

TABLE 3. Oligonucleotides used in this study

No.	Oligonucleotide[a]	Sequence[b] (5′–3′)	Description and/or accession no.[c]
1	aphA-3_f*	GA**GGATCC**GCTAAAATGAGAATATCACCG	*aphA-3* from codon 2 (nt 607 to 626); M26832†
2	aphA-3_r*	GA**GGATCC**CTTTTTAGACATCTAAATCTAGG	*aphA-3* (nt 1423 to 1401); M26832†
3	cat_BamHI_f*	TT**GGATCC**CAATTCACAAAGATTGATATA	*cat* from codon 2 (nt 312 to 332); M35190†
4	cat_BamHI_r*	G**GGATCC**TATTTATTCAGCAAGTCTTG	*cat* (nt 931 to 912); M35190†
5	1146_NotI_f	TT**GCGGCCGC**AATAGTATCCTTAACATAAAATTTT	Upstream of *gatC*; (nt 113712 to 113745); NC_008599†
6	1146_PstI_r	ATT**CTGCAG**AGAATACAACTCCATTTTTGATT	Upstream of *gatC*; (nt 1136947 to 1136925); NC_008599†
7	gatC_BamHI_r	TT**GGATCC**AGAATACAACTCCATTTTTGATT	Upstream of *gatC*; (nt 1136947 to 1136925); NC_008599†
8	nic_SmaI_f	TAT**CCCGGG**TTAGGTATAATCTCACAAAACG	pIP1455
9	nic_SmaI_r	TAA**CCCGGG**TAGCACGGAAGACGGACAAA	pIP1455
10	galK_f	TCCATCAGCGTGACTACCAT	*E. coli* K-12 *galK* (nt 787966-787985); NC_000913†
11	galK_r	TAGCCGTCGTGCCTTACT	*E. coli* K-12 *galK* (nt 789594-789577)
12	Fic1_KpnI_f	GCT**GGTACC**GATGGCGGTGTAAATTTAGGC	*fic1* (nt 31745 to 31765)
13	Fic1_SalI_r	CTA**GTCGAC**TTATCTCTCCTTTTCCTTTGAAT	*fic1* (nt 32578 to 32556)
14	Fic2_KpnI_f	GCT**GGTACC**CAAGAACAATATACGGAAATCAA	*fic2* (nt 32581 to 32603)
15	Fic2_SalI_r	CTA**GTCGAC**TTATCTTTCCTTTTCTTTTGATTTT	*fic2* (nt 33498 to 33474)
16	Cre_SmaI_f	TAA**CCCGGG**ATGTCCAATTTACTGACCGTA	Cre fusion plasmid CFP B (nt 245 to 265)
17	Cre_SmaI_r	TTA**CCCGGG**TGAAGGCTCTCAAGGGCAT	Cre fusion plasmid CFP B (nt 1295 to 1313)
18	TrwC_SFw1	CATGTA**GGTACC**CTCAGTCACATGGTATTGAC	*trwC* from codon 2 (nt 1542 to 1561); X63150.2†
19	TrwC_SRev1	GCAATC**GTCGAC**TTACCTTCCGGCCTCCATGCCG	*trwC* (nt 4439 to 4418); X63150.2†
20	Trans17	TGATCCTGGTTTTGGAGTGA	Transposase A (nt 21254 to 21273)
21	Trans8	GTATGAGCTTTATCCTTTGTTTC	Transposase B (nt 21992 to 21970)
22	Trans14	GGGGCATCAACTCTTAGAGG	Transposase B (nt 22528 to 22547)
23	Trans18	CATTGAGAATTTCGGCACCT	ORF3 (nt 23060 to 23041)‡
24	Trans4	ATCACTCAGCAGCAAGAGCG	ORF3 (nt 22739 to 22758)
25	TopTraE1	TTGCACGTGCGAGTTCAGGC	Topoisomerase (nt 23294 to 23275)
26	TraE 7c	GCAAGAGCGGAAAGCTTCTGCC	Topoisomerase (nt 25347 to 25368)
27	TraE 21	GTTGATAGCTGCTTTGCG	Chromosome segregation (nt 25844 to 25827)
28	TraE 8d	GGAGCAAAACAAAGAACCAAC	Chromosome segregation (nt 26410 to 26430)
29	TraE 9	CAGCATTTAAGGCAGAAC	Lytic transglycosylase (nt 26860 to 26843)
30	TraE 10	GCAAGTTAATTCAATACACG	Lytic transgycosylase (nt 27030 to 27049)
31	TraE 29	GGCTATCGACGTCAAAGCC	*dnaG* (nt 27330 to 27312)
32	TraE 14	GAAAGAACAGCTCAATGG	*dnaG* (nt 28322 to 28339)
33	TraE 13	CGGCGCGAGTGGGAAAG	ORF8 (nt 28616 to 28600)‡
34	TraE 32	CGCGCCGCATATATTAGAA	ORF8 (nt 28610 to 28628)‡
35	TraE 33	CGACCTTTGTCGGTTGATTT	ORF9 (nt 28940 to 28921)‡
36	TraE 20	CCAGTGGCACAATCCGC	ORF9 (nt 28864 to 28880)‡
37	TraE 34	GCAGCCTTTCATTTGGGTAA	ORF10 (nt 29279 to 29260)‡
38	TraE 28	GCCAAAATTTAGCCAAATGGTAGC	ORF10 (nt 29731 to 29754)‡
39	TraE 35	CCCAACTCGCAGTTTTTCAT	ORF11 (nt 30142 to 30123)‡
40	TraE 36	GGTTGTGGCGAAGAAGCTAA	ORF11 (nt 29985 to 30004)‡
41	TraE 24R	GAGTGCCAGCTTTATGGC	*parA* (nt 30353 to 30336)
42	TraE 24	GCCATAAAGCTGGCACTC	*parA* (nt 30336 to 30353)
43	TraE 23	GCGGTGTCTTTTGCAAAGC	Nucleotidyltransferase (nt 31112 to 31094)
44	NucTra1	CCAAAACTGCAAGAAGACGG	Nucleotidyltransferase (nt 31048 to 31067)
45	Fic1-2	TATCGTCGTCATTTTTGGCA	*fic1* (nt 32195 to 32176)
46	Fic1-1	GGCTCATCATAGCACAGCAA	*fic1* (nt 31822 to 31841)
47	Fic2-2	TTTCAAGCCCTTGTGGAAAG	*fic2* (nt 32917 to 32902)
48	Fic2-5	ATGGGAGCGATCAAACAACC	*fic2* (nt 33445 to 33461)
49	Cpp17-6	ACCGATCAAGAAGCAGTCGT	*cpp17* (nt 34120 to 34101)
50	NucTra2	GCCGTATCGCATAGATCGAC	nt 31259 to 31240
51	Cpp17-2	CGATCTCGATGATCTACGCA	nt 34442 to 34423
52	Km_screen_rev	GATCTTTAAATGGAGTGT	*aphA-3* (nt 1105 to 1088); M26832†
53	glnA_RT_fwd	GCGAGTGGAATGATGGTAAAG	*glnA* (nt 1053483 to 1053504); NC_008599†
54	glnA_RT_rev	CTCTAACGCTTTTCTCTCCTG	*glnA* (nt 1054086 to 1054066); NC_008599†

[a] Asterisks (*) indicate primers applied in PCR verification of conjugative DNA uptake.
[b] Relevant restriction sites are indicated in boldface.
[c] †, GenBank accession numbers correspond to EU443150 unless otherwise noted. ‡, ORF numbers refer to the designation used in Fig. 5B. nt, nucleotide.

TABLE 4. Comparative identities of *C. fetus* subsp. *venerealis* Vir proteins and T4SS orthologs

Organism or system[a]	Protein identity (%)[b] in:									Location[c]	Accession no.[d]
	VirB3/4	VirB5	VirB6	VirB8	VirB9	VirB10	VirB11	VirD4	Sum		
C. fetus subsp. *venerealis* (*vir*)*	100	100	100	100	100	100	100	100	800	C	EU553150
C. hominis	**56**	**22**	**29**	**56**	**59**	**45**	**62**	54	**382**	C	NC_009714
C. coli pCC31*	46	**22**	25	**56**	52	39	54	**59**	352	P	NC_006134
A. actinomycetemcomitans pVT745*	37	**22**	18	31	42	32	42	43	266	P	NC_002579
Broad-host-range plasmid pIP02T*	32	14	16	31	33	28	38	42	223	P	NC_003213
*X. axonopodis**	31	–	17	25	22	23	31	36	184	C	NC_003919
B. henselae (*vir*)	28	11	15	27	20	24	31	15	171	C	BX897699
L. pneumophila (*lvhB*)*	21	7	17	18	24	20	25	20	152	C	AE017354
H. pylori (*cag orHP*)	24	–	–	15	27	21	37	23	147	C	NC_000915
A. tumefaciens pTi*	26	1	13	17	22	20	27	20	146	P	NC_003065
R. prowazekii	30	–	–	14	21	18	29	24	135	C	NC_000963
Broad-host-range plasmid RP4*	17	2	13	–	–	18	24	19	93	P	NC_001621

[a] Organism and system were selected according to BLAST scores, the presence of a putative coupling protein, genetic organization and function (see Materials and Methods). Systems shown to be conjugative are indicated by an asterisk (*).
[b] The most similar homologs are indicated in boldface. –, No known ortholog.
[c] Genes were carried on the chromosome (C) or plasmid (P).
[d] Accession number of the genome or plasmid DNA sequence.

mediated reconstitution of *cat* expression were selected on LB agar containing 10 μg of chloramphenicol/ml. Donors were selected on streptomycin (100 μg/ml). Frequencies of conjugation and protein translocation were determined as transconjugants or recombinants per donor, respectively.

Prior to all CRAfT assays, every test protein fusion was assessed for stability and Cre recombinase activity *in vivo* by transformation of the indicator strains *E. coli* CSH26Cm:: LKL, CSH26Cm::LTL or *E. coli* MT102Cm::LKL with each expression vector. Transformants expressing the test fusion proteins, Cre alone, or the control fusions with known T4S substrates all exhibited an equivalent recombination frequency.

Isolation of RNA and preparation of cDNA. Total RNA was isolated from *C. fetus* subsp. *venerealis* strain ATCC 19438 with or without plasmid pRYSS1 after 24 or 48 h of culture by using a RNeasy Protect bacteria kit (Qiagen) according to the manufacturer's instruction. Contaminating DNA was removed by incubation with 1.5 U of DNase I per μg of total RNA as recommended by the manufacturer (Fermentas). cDNA was prepared from 0.3 to 0.5 μg of RNA using specific primers (15 pmol) for first-strand synthesis and Moloney murine leukemia virus reverse transcriptase (RT; Fermentas) in a total volume of 20 μl. Negative controls were incubated without RT. Reaction products were treated with RNase H (New England Biolabs) at 37°C for 20 min without heat deactivation.

Analysis of *sapA* promoter temperature regulation. RNA was isolated from *C. fetus* subsp. *venerealis* ATCC 19438(pRYSS1) grown at 32, 37, or 39°C. For second-strand synthesis, PCR primers were positioned to amplify a 500-kb fragment of *aphA-3* from pRYSS1 and a 600-kb fragment of *glnA* (Table 3, primers 1 and 52 to 54). PCR fragments were amplified from 2 μl of the RT product as a template using Phusion High-Fidelity DNA polymerase (Finnzymes). Plasmid DNA (for *aphA-3*) and genomic DNA (for *glnA*) were used as positive control PCR templates.

Operon analysis. RNA was isolated from *C. fetus* subsp. *venerealis* ATCC 19438 and cDNA prepared as described above. For second strand synthesis, PCR primers were positioned to amplify fragments spanning at least two open reading frames (ORFs) (Table 3, primers 20 to 51). PCR fragments were amplified from 2 μl (10%) of the RNase H-treated RT product as a template using Phusion High-Fidelity DNA polymerase (Finnzymes). Genomic DNA from *C. fetus* subsp. *venerealis* reference strain ATCC 19438 was used as a positive control PCR template.

RESULTS

***C. fetus virB/virD4* gene homology and structure predict nucleoprotein and effector protein translocation.** CLUSTAL W comparisons were performed for the mating pore formation (Mpf) proteins and the putative T4CP (VirD4) of the *C. fetus* subsp. *venerealis* T4SS to ortholog proteins of public database entries (Table 4). Reference systems were selected to obtain broad distribution within the major branches of the phylogenetic trees of the ATPases VirB4 and VirB11 created by Fernandez-Lopez et al. (20) and Frank et al. (24). Our analysis focused on well-characterized systems of known function that additionally encode a T4CP and the most closely related T4SS of *Campylobacter*. Protein identities were generally low. BLAST search revealed that the Mpf proteins of the chromosomally encoded T4SS of *C. hominis* exhibited the highest overall homology to the *C. fetus* subsp. *venerealis* components, followed by plasmid pCC31 of *C. coli* and the pVT745 plasmid of *Actinobacillus actinomycetemcomitans.* The function of the *C. hominis* system is currently unknown. pCC31 and pVT745 are conjugative plasmids. Comparison to pCC31 revealed identities greater than 50% for VirB8, VirB9, VirB11, and VirD4. The *C. hominis* Mpf proteins display the highest overall identity, while the *C. fetus* subsp. *venerealis* VirD4 overlaps most closely with CmgD4 of pCC31 (59%).

CLUSTAL W analysis of *Campylobacter* T4CPs were performed and revealed numerous stretches of amino acids with 100% identity within the protein sequences. High conservation extends into their shared C-terminal regions. The *C. fetus* protein carries additionally atypical insertions that are extremely rich in charged residues. Both the *C. fetus* subsp. *venerealis* and the *C. hominis* proteins carry long nonconserved C-terminal tails. In the case of F-like T4CPs, an atypical C-terminal extension is important for specificity of substrate recognition (55). A conserved domain database search facilitated assignment of the *C. fetus* subsp. *venerealis* VirD4 to the TraG, TraG/TraD family (pfam02534) of the P_loop_NTPase superfamily (cl09099). Presence of a *virD4* homologue in *C. fetus* subsp. *venerealis* potentially links this T4SS to an ancestral or currently functional conjugative gene transfer system.

Understanding of bacterial T4SS has advanced dramatically in recent years. Accordingly, it becomes feasible to predict potential secretion substrates based on conserved protein domains, as well as gene organization (60). We identified two open reading frames (ORFs; *orf38* and *orf39*; EU443150) downstream of the VirB/VirD4 locus that belong to the FIC (filamentation induced by cAMP) superfamily (cl00960). Re-

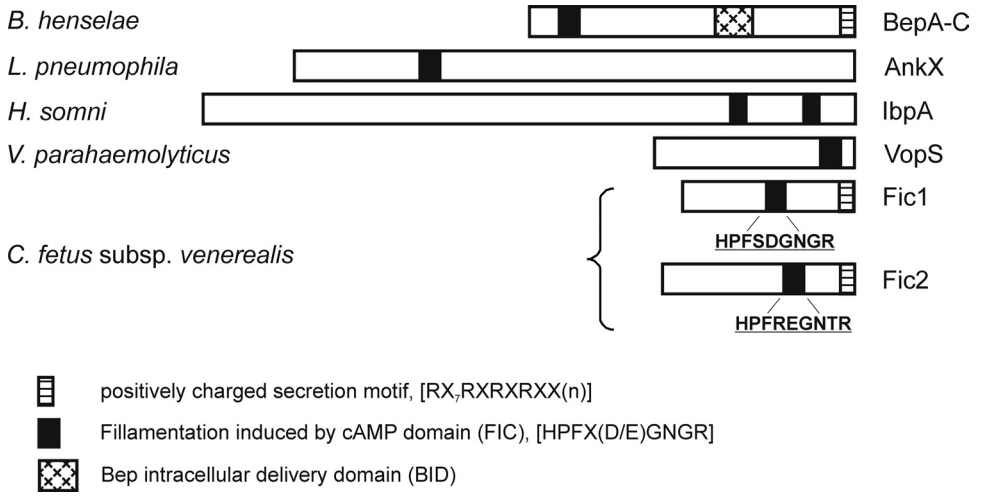

FIG. 1. The FIC domain is conserved in T3 and T4SS substrates. Known effector proteins carrying the FIC domain and conserved motif [HPFx(D/E)GN(G/K)R] that contributes to AMPylation of host proteins. Where investigated, additional protein features required for T4CP recognition and translocation are illustrated as described previously (see below). Candidate secretion substrates of the *C. fetus* T4SS harboring the FIC signature are shown.

lated proteins are involved in cell division (36) and, importantly, are conserved among effector proteins secreted by type III (T3) and T4SS of Gram-negative bacteria (Fig. 1), including *Bartonella* species, *Histophilus somni*, *Vibrio parahaemolyticus*, and *Legionella pneumophila* (11, 53, 56, 60, 74, 76, 78). Fic1 of *C. fetus* subsp. *venerealis* was assigned to the FIC protein family COG3177 of unknown function, whereas Fic2 belongs to the FIC protein family COG2184, including proteins involved in cell division and chromosome partitioning. Fic1 (*orf38*) is 278 amino acids in length and the best BLASTP match (60% identity) was the *Helicobacter cinaedi* CCUC18818 hypothetical protein HcinC1-00045 (accession number ZP_03657318). Fic2 (*orf39*) is 306 amino acids in length and interestingly has no homology to proteins from *H. cinaedi*. The best BLASTP match (41% identity) is to the hypothetical protein CLOSS21_03156 (accession number ZP_02440650) from *Clostridium* sp. strain SS2/1. The presence of ORFs containing FIC domains 3′ to the VirB/VirD4 locus as part of the *C. fetus* subsp. *venerealis* chromosomal PAI supports the hypothesis that proteins Fic1 and Fic2 are translocated by the T4SS.

***C. fetus* subsp. *venerealis* mobilizes *C. coli* plasmid pIP1455 DNA to *E. coli*.** The T4SS of the *C. fetus* subsp. *venerealis* PAI is integrated in the genome. Since it is not carried by a self-transmissible conjugative plasmid, potential DNA transfer activity was tested via plasmid mobilization. Small cryptic plasmids often represent natural substrates for conjugative dissemination, since they encode a minimum of mobilization functions (a relaxase and *nic* site) and replication features supporting their spread and maintenance in bacterial populations. The plasmid profile of 60 *C. fetus* subsp. *venerealis* isolates carrying the T4 *vir* genes within our strain collection was analyzed, but small plasmids were generally absent (not shown). Given the strong conservation observed between components of the *C. fetus* subsp. *venerealis* PAI and those of the *C. coli* conjugative plasmid pCC31, we based the analysis on the cryptic *C. coli* plasmid pIP1455 (38). Vectors carrying the pIP1455 replicon can be propagated in *C. fetus* (33). Moreover, we noted that the pIP1455 backbone carries mobility features that might support its conjugative mobilization from a heterologous T4SS (Fig. 2). Test constructions for interspecies mobilization also carried selection markers for both species and the pBR322 origin for replication in *E. coli* (Fig. 2). The pIP1455-based shuttle vector pRYSS1 (33) was introduced to *C. fetus* subsp. *venerealis* strain 84-112 via conjugation. The capacity of this host to further transfer the test plasmid to the *E. coli* K-12 strain MT102 was investigated in a surface mating experiment. Suspensions of the potential donor and recipient bacteria were mixed and then collected on a filter, which was placed on blood agar for 24 h. *C. fetus* is not naturally competent. Nonetheless, filters were first soaked in buffer containing 1 mM $MgCl_2$ without or with 300 μg of DNase I. At cell harvest, negative selection against *C. fetus* and positive selection for transconjugant *E. coli* was achieved by plating on LB agar. Presence of pRYSS1 in kanamycin- and chloramphenicol-resistant *E. coli* colonies was verified by plasmid isolation and restriction enzyme digestion (data not shown). Interspecies transfer occurred at frequencies of $3.16 \times 10^{-6} \pm 3.8 \times 10^{-7}$ transconjugants per donor without DNase I and $4.03 \times 10^{-6} \pm 3.3 \times 10^{-7}$ in the presence of DNase I; effectively ruling out transmission due to natural transformation. Parallel experiments using an alternative donor strain known to express the T4 *vir* genes, *C. fetus* subsp. *venerealis* ATCC 19438 (28), verified the transfer activity at equivalent frequencies (not shown). Conversely, substitution of the *E. coli* K-12 strain used as recipient in the experiment for *E. coli* MS411 or *E. coli* SAR18 gave rise to very low transfer frequencies ($\sim 10^{-10}$ transconjugants per donor). All transconjugant colonies were routinely analyzed to verify that these indeed carried the mobilizable pRYSS1 plasmid. Uptake of plasmid DNA was confirmed in every case. The frequency of plasmid transfer was higher at 32°C than 37°C using *E. coli* recipient MT102 and the pIP1455-derived shuttle vector pRYGG1 (Fig. 3A).

We next confirmed that mobilization of the plasmid was due to features carried by the pIP1455 backbone by measuring

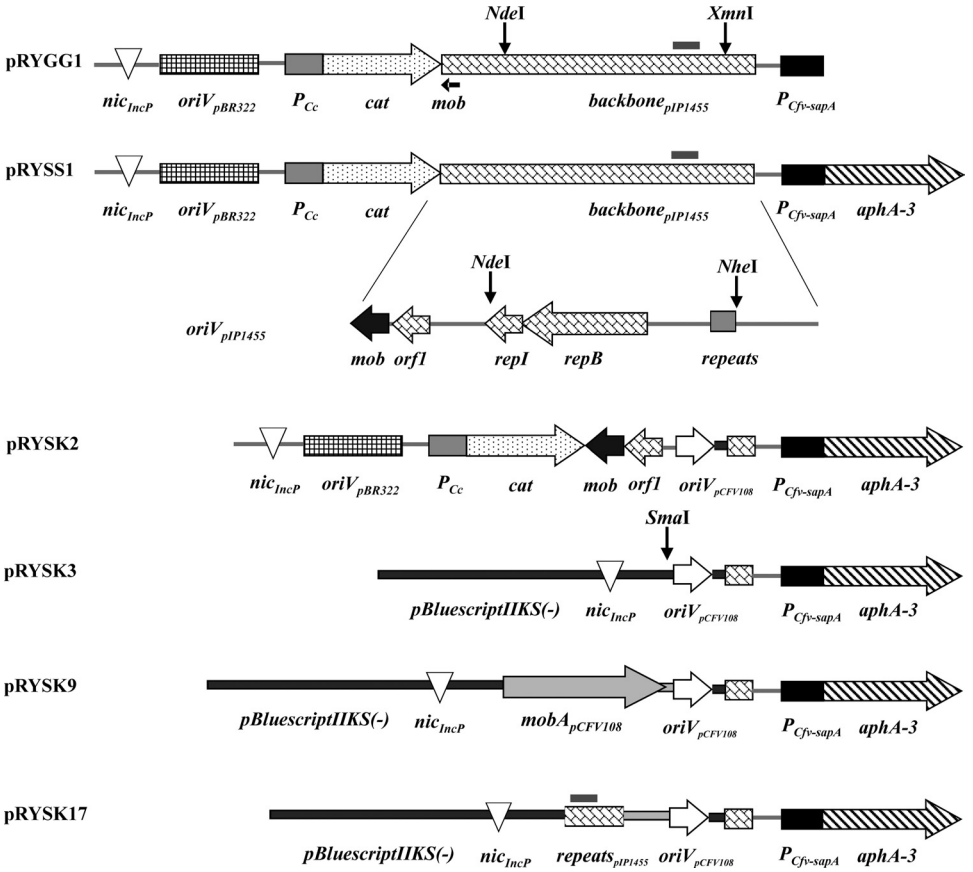

FIG. 2. Constructions defining requirements for plasmid mobilization by the *C. fetus* subsp. *venerealis* T4SS. *C. fetus* cannot be readily transformed; thus, all vectors carry a P-type *nic* site to introduce the plasmid to donor cells. Selection in *Campylobacter* relies on a kanamycin resistance cassette (*aphA-3*) expressed from the *C. fetus sapA* promoter. Regions of the *C. coli* plasmid pIP1455 (backbone) are shown with functional modules identified in the expanded view below, including a putative mob gene (*mob*) and a potential P-type *nic* site (repeats). The *C. fetus* replicon ($oriV_{pCFV108}$) and putative relaxase gene *mobA* from pCFV108 (gray arrow) are shown.

transfer of a different plasmid with replication functions of the *C. fetus* plasmid pCFV108 (33). No transfer from *C. fetus* subsp. *venerealis* ATCC 19438 or 84-112 to *E. coli* MT102 was observed for this replicon (pRYSK3, Fig. 2), even with the additional presence of the putative relaxase gene *mobA* from pCFV108 on the construction (pRYSK9) (not shown). In an effort to identify the genetic elements present in pIP1455 that support mobilization from *C. fetus* subsp. *venerealis* donors, we eliminated all pIP1455 DNA in the test plasmid except the putative *mob* gene and the adjacent *orf1* of unknown function resulting in pRYSK2 (Fig. 2). A 427-bp fragment of pIP1455 carrying a putative *oriT* was excised to create pRYSK17 (Fig. 2). We found that division of the pIP1455 DNA into distinct putative mobilization modules failed to support detectable mobilization of pRYSK2 and pRYSK17 under our conditions (not shown). In summary, interspecies transfer from *C. fetus* subsp. *venerealis* to *E. coli* MT102 occurred at detectable levels only when the mobilization substrate carried all of the *C. coli* plasmid pIP1455 sequences provided in pRYSS1. The mechanism of plasmid DNA transmission was unaffected by externally applied nuclease, supporting a conclusion of direct cell transfer.

Interspecies plasmid DNA transfer is attenuated in *vir*-deficient *C. fetus* subsp. *venerealis* mutants. To establish a functional link between the T4SS and DNA transfer, mutants carrying gene disruptions in *virB9* or *virD4* were investigated. The mutant *vir* derivatives of *C. fetus* subsp. *venerealis* ATCC 19438 strains were shown in our earlier work to attenuate virulence during infection of cultured human cells (28). Plasmid transfer efficiencies were compared when the wild-type parental strains and mutants *C. fetus* subsp. *venerealis* 3-18 (Δ*virB9*) or *C. fetus* subsp. *venerealis* SK1 (Δ*virD4*) served as plasmid donors. Test plasmid pRYGG1 contained the same pIP1455 backbone elements as pRYSS1 but lacked the kanamycin expression cassette also used for insertion disruption of the *vir* genes in the mutant donors. To complement the mutant phenotypes *in trans*, the mobilizable (pRYGG1-based) vector carried also a wild-type copy of the affected *vir* gene(s). Each experiment was performed in triplicate with recipient *E. coli* MT102 at both 32 and 37°C. For technical reasons, each experiment compared a maximum of four donor strains. The results are summarized in Fig. 3 and Table 5. The plasmid transfer frequencies were expressed relative to the parental strain at one temperature (100%), as indicated. For the wild-type strains, the frequencies

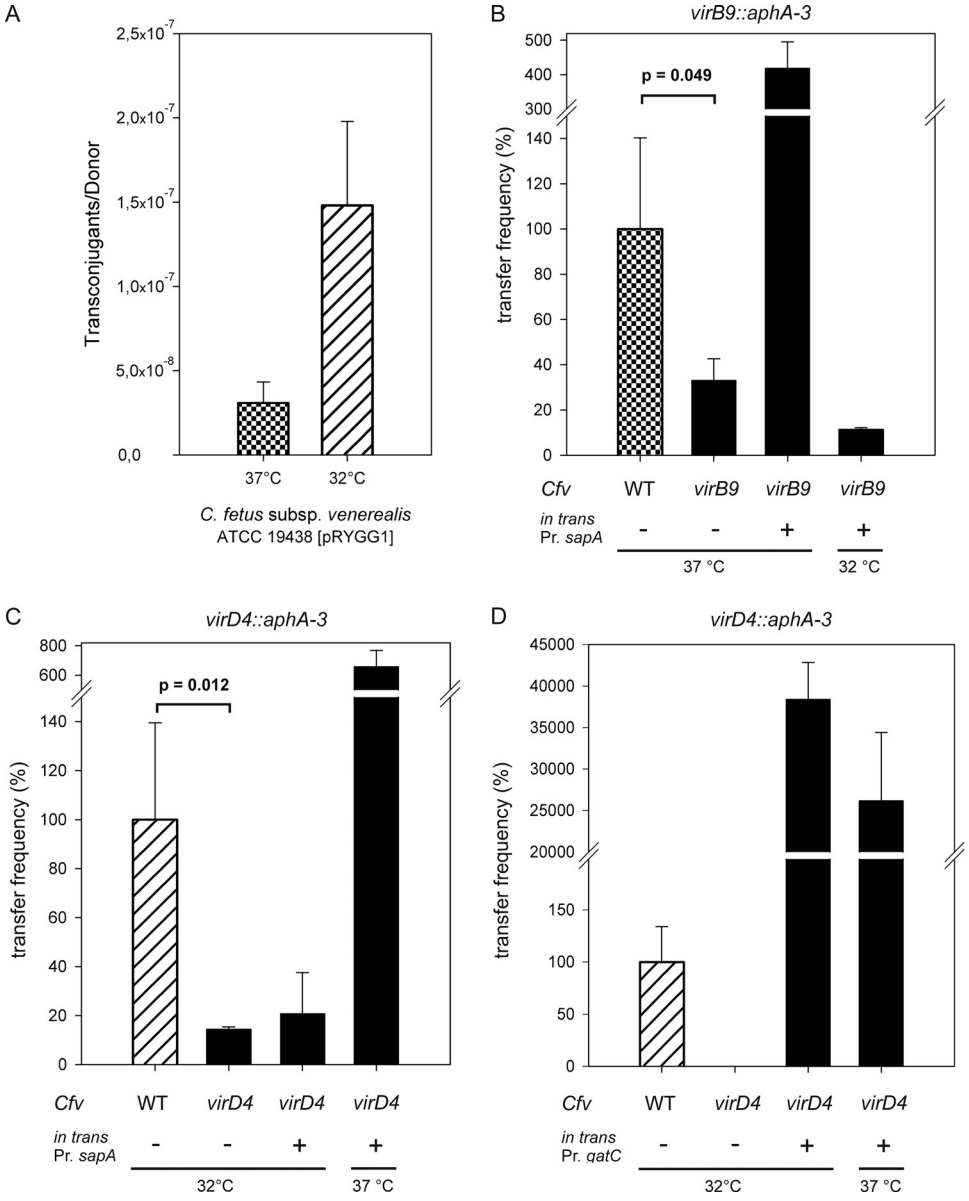

FIG. 3. Efficient plasmid DNA translocation from *C. fetus* subsp. *venerealis* to *E. coli* requires the T4SS components VirB9 and VirD4 and moderate temperature. (A) Transfer of pRYGG1 DNA from *C. fetus* subsp. *venerealis* ATCC 19438 to *E. coli* MT102 was measured at 37 and 32°C (expressed as transconjugants relative to donor cells). (B to D) Comparison of the transfer frequencies of pRYGG1 from wild-type *C. fetus* subsp. *venerealis* ATCC 19438 (expressed as 100%) and either the isogenic *virB9* mutant 3-18 (B) or that of pRYSS1 from the *virD4* mutant SK1 (C and D). For complementation wild-type *virB9* was expressed in 3-18 (B) and *virD4* in mutant SK1 (C and D) from the promoter indicated (below) and at the experimental temperatures shown. The frequencies shown are expressed relative to the wild-type strain at the temperature indicated. The data are from at least three experiments. Statistically significant differences (unpaired Student *t* test) between wild-type and mutant conjugation frequencies are indicated.

were higher at 32°C than at 37°C (as illustrated generally in Fig. 3A). In the absence of *virB9*, transfer of the plasmid from *C. fetus* subsp. *venerealis* was reduced significantly to one third of the wild-type activity (Fig. 3B and Table 5). The mobilizable complementation vector pRYMJ2 (33) provided *in trans virB9* plus all downstream genes from the operon: *virB10*, *virB11*, and *virD4*, under the control of the *sapA* promoter. Interestingly, although a higher transfer efficiency was expected at 32°C, maximal complementation could only be achieved at 37°C. Similar results were obtained when the *C. fetus* subsp. *venerealis* host deficient in *virD4* was used as a donor. Inactivation of *virD4* reduced plasmid transfer significantly to 15% of the wild-type level. Again, complementation using the *sapA* promoter-dependent vector pRYVL2 was only successful at 37°C (Fig. 3C and Table 5). Since conjugation frequencies were generally higher at the lower temperature (Fig. 3A), we hypothesized that the failure of complementation at 32°C was most likely due to low expression levels from the complementation constructions. In the *C. fetus* chromosome, the *sapA* promoter controls synthesis of the surface layer proteins, which

TABLE 5. *virB9* and *virD4* are required for efficient DNA mobilization by *C. fetus* subsp. *venerealis*

C. fetus subsp. *venerealis* donor strain	Plasmid	Conjugation temp (°C)	Frequency (transconjugants/donor)		Conjugation frequency (%)[a]		*P*[b]
			Mean	SD	Mean	SD	
ATCC 19438	pRYGG1	37	3.09×10^{-8}	1.24×10^{-8}	100	40	0.049
3-18	pRYGG1	37	1.02×10^{-8}	3.0×10^{-9}	33	10	
3-18	pRYMJ2	37	1.29×10^{-7}	2.4×10^{-8}	418	77	
3-18	pRYMJ2	32	3.54×10^{-9}	2.2×10^{-10}	11	1	
ATCC 19438	pRYGG1	32	1.48×10^{-7}	5.0×10^{-8}	100	40	0.012
Cfv_SK1	pRYSS1	32	2.23×10^{-8}	1.4×10^{-9}	15	1	
Cfv_SK1	pRYVL2	32	3.21×10^{-8}	2.59×10^{-8}	21	17	
Cfv_SK1	pRYVL2	37	1.01×10^{-6}	1.7×10^{-7}	657	112	
ATCC 19438	pRYGG1	32	1.43×10^{-7}	4.9×10^{-8}	100	34	
Cfv_SK1	pRYSS1	32	ND[c]	ND	ND	ND	
Cfv_SK1	pRYSK12	32	5.49×10^{-5}	6.4×10^{-6}	38,376	4,458	
Cfv_SK1	pRYSK12	37	3.75×10^{-5}	1.18×10^{-5}	36,157	8,268	
84-112	pRYSS1	37	1.91×10^{-7}	1.9×10^{-8}	100	10	0.009
V81_SK1	pRYSS1	37	3.56×10^{-8}	6.2×10^{-9}	19	3	
84-112	pRYSS1	32	1.90×10^{-6}	3.2×10^{-7}	100	17	0.001
V81_SK1	pRYSS1	32	5.67×10^{-7}	3.58×10^{-6}	30	19	

[a] Conjugation frequency is expressed as the mean percent relative to the corresponding wild-type strain at the indicated temperature.
[b] The statistical significance (*P*) between wild-type and *vir* mutant strains (unpaired *t* test) is given.
[c] ND, not detected.

mediate serum resistance and antigenic variation in the mammalian host (64). It is conceivable that expression of this locus is temperature dependent. To confirm this, we isolated total RNA from *C. fetus* subsp. *venerealis* ATCC 19438 carrying plasmid pRYSS1 after cultivation at 32, 37, or 39°C. pRYSS1 carries *aphA-3* under the control of the *sapA* promoter. RT-PCR was used to convert *aphA-3* transcripts to cDNA for a quantitative comparison of *sapA* promoter activity as a function of temperature. For each preparation of RNA, cDNA was also generated from transcripts of the housekeeping gene *glnA*. Amplification of the initial yield of *glnA* cDNA templates resulted in equivalent amounts of product regardless of the cultivation temperature. In contrast the yield of *aphA-3* product was consistently weaker from RNA obtained after 32°C growth than at the higher temperatures (Fig. 4). This significant difference implies that the *sapA* promoter is less active at 32°C.

To eliminate the observed Pr_{sapA} temperature dependence in complementation experiments, housekeeping genes were identified in the *C. fetus* subsp. *fetus* 82-40 genome sequence (GenBank accession number NC_008599) and used to amplify putative constitutively expressed promoter elements from the chromosome. The *C. fetus* gene *gatC* is predicted to encode an aspartyl/glutamyl-tRNA amidotransferase. We replaced the

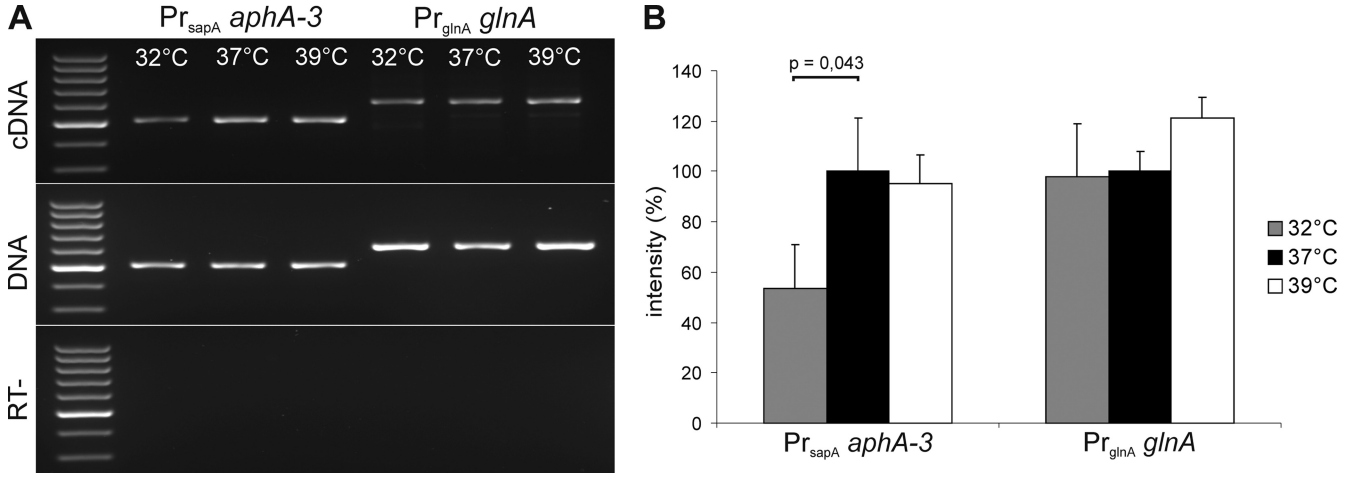

FIG. 4. Expression of *aphA-3* controlled by Pr_{sapA} is significantly reduced at 32°C. (A) RNA of *Campylobacter fetus* subsp. *venerealis* ATCC 19438(pRYSS1) was isolated after cultivation at the temperatures shown. cDNA of *aphA-3* and *glnA* was produced with reverse transcriptase (RT) from an equivalent portion of total RNA and amplified, and the products were analyzed on a 2% agarose TAE gel (upper panel). Plasmid DNA (*aphA-3*) and genomic DNA (*glnA*) templates served as positive controls for the amplification conditions (middle panel). Negative controls for DNA contamination (RT−) lacked reverse transcriptase (lower panel). (B) The intensities of products of amplified cDNAs were measured with ImageJ 1.43 (http://rsbweb.nih.gov/ij/), and the yield for each gene is expressed relative to the value obtained after cultivation at 37°C. Significantly lower expression of the *aphA-3* marker controlled by Pr_{sapA} was measured at 32°C compared to 37°C growth, as indicated (unpaired *t* test). Standard deviations from three independent experiments at each growth temperature are shown.

TABLE 6. Exclusion index values for prototypic incompatibility groups in *C. fetus* subsp. *venerealis* conjugative mobilization

E. coli recipient strain	Inc group	Frequency (transconjugants/donor)		Conjugation frequency (%)		P^a	EI[b]
		Mean	SD	Mean	SD		
MT102	None	1.26×10^{-5}	1.6×10^{-6}	100	13		
MT102(RP4)	IncP	3.96×10^{-7}	4.2×10^{-8}	3	0	0.006	31.7
MT102(pSU2007)	IncW	2.36×10^{-5}	8.9×10^{-6}	188	70	0.158	0.5
MT102(pAR119)	IncF	7.41×10^{-6}	3.4×10^{-7}	59	3	0.028	1.7
MT102(pLG211)	IncI	1.11×10^{-5}	1.0×10^{-6}	89	8	0.278	1.1

[a] *P* values refer to the statistical significance distinguishing transfer frequencies to plasmid-free recipients and plasmid-carrying *E. coli* (unpaired *t* test).
[b] The exclusion index (EI) expresses the fold difference in transfer frequency to plasmid-free recipients versus plasmid-carrying *E. coli.*

sapA promoter on pRYVL2 with the *gatC* promoter to generate the complementation vector pRYSK12. Gene transfer experiments using the *virD4* mutant were then repeated. In this set of experiments (Fig. 3D), the mutant's ability to mobilize plasmid DNA was reduced below the detection limit at 32°C. The expression of wild-type *virD4* under the control of the *gatC* promoter not only restored plasmid mobilization from this host at 37 and 32°C but actually led to >100-fold enhancement of transfer compared to the wild type. For our analysis of the virulence attributes of *C. fetus* subsp. *venerealis*, we additionally created a gene disruption in *virD4* of a particularly virulent field isolate *C. fetus* subsp. *venerealis* 84-112 (28). Comparison of the DNA mobilization capacity of this mutant and the parent strain verified the importance of *virD4* in gene transfer (Table 5). In summary, we conclude that transmission of plasmid DNA from *C. fetus* subsp. *venerealis* to *E. coli* involves at least two functional components of the T4SS: VirB9 and VirD4. The data also show that the *sapA* promoter was more active at 37°C than at 32°C. Replacement of this regulatory element with a more constitutively expressed promoter led to dramatically enhanced DNA translocation frequencies. This finding implies that the wild-type level of expression of the chromosomally encoded T4SS under these laboratory conditions is low. As a result, the level of secretion activity observed thus far is apparently well below the capacity of the system.

IncPα plasmid-mediated surface exclusion disrupts DNA delivery by the *C. fetus* subsp. *venerealis* T4SS. Conjugation systems typically express one or more entry or surface exclusion functions, which minimize the likelihood that the host cell participates in mating with a second bacterium harboring the same or a closely related plasmid (25, 77). Restriction of lateral DNA transmission to a plasmid-carrying host due to surface exclusion is indicative, therefore, of functional relatedness between the competing transfer machineries. To functionally classify the *C. fetus* subsp. *venerealis* T4SS components, we tested whether the efficiency of plasmid mobility from a wild-type strain was measurably reduced by the established presence of alternative T4SS in the recipient population. Paradigm conjugation systems drawn from plasmid incompatibility groups Inc-P, -W, -I, and -F were introduced to the *E. coli* recipient strain. No variation in the frequency of plasmid transmission to *E. coli* MT102 was observed when the strain additionally harbored Inc-W, -I, or -F conjugative prototypes (Table 6). In contrast, transfer from *C. fetus* subsp. *venerealis* was substantially diminished when the recipient population carried the IncPα plasmid RP4. The observed exclusion index (EI) (~32-fold) indicates that the *C. fetus* subsp. *venerealis* T4 pathway is functionally related to P-like conjugation proteins.

Intraspecies mobilization of plasmid DNA in *C. fetus*. Determination of the *C. fetus* subsp. *venerealis* T4SS host range is cumbersome due to the lack of suitable shuttle vectors that can be maintained in both *C. fetus* donors and a diversity of potential hosts. At present, vectors adapted for *E. coli-C. fetus* applications have proven to be largely species specific among *Campylobacters* (33). Moreover, *C. jejuni* and *C. coli* are poor recipients due to the presence of closely related conjugative plasmids pTet and pCC31, respectively, and due to natural competence. Alternatively, the *C. fetus* subsp. *venerealis* cryptic plasmid pCFV108 that we have used for vector development carried a *repE* homologue most closely related to replication initiation proteins of enterococcal plasmids pS68, pEF47, and pAMα1 (33). Attempts to stably transform *Enterococcus faecalis* with shuttle vectors based on the pCFV108 *repE* gene with or without the replicative iterons in *cis* proved negative, however (K. Weaver, unpublished data). In the absence of more universally applicable plasmid substrates, the testable range of mobilization by the *C. fetus* subsp. *venerealis* T4SS was limited to intraspecies transfer. Selection for *C. fetus* subsp. *fetus* transconjugants was facilitated by the ciprofloxacin-resistant phenotype of the human blood isolate F12 (37). Triplicate experiments revealed that pRYSS1 was mobilized from *C. fetus* subsp. *venerealis* 84-112 to *C. fetus* subsp. *fetus* F12 with a frequency of $2.84 \times 10^{-6} \pm 5.02 \times 10^{-7}$ in the absence of DNase I and $2.89 \times 10^{-6} \pm 4.02 \times 10^{-7}$ with DNase I. *C. fetus* subsp. *fetus* strains in our collection ($n = 43$) lack detectable homologues to the *C. fetus* subsp. *venerealis* T4SS (28). Accordingly, a subsequent transmission of the plasmid from *C. fetus* subsp. *fetus* F12 transconjugants was not observed. Further experiments combining *C. fetus* subsp. *fetus* ATCC 27374(pRYGG1) or *C. fetus* subsp. *fetus* 82-40(pRYEL1) as potential plasmid donors with *E. coli* MT102 did not yield transconjugants. Finally, to assess whether DNA can be exchanged within the subspecies we inserted the resistance marker *aphA-3* into the *C. fetus* subsp. *venerealis cdtB* gene (outside of the PAI) to obtain the selectable recipient strain JL1. In the subsequent mating experiments, either *C. fetus* subsp. *venerealis* ATCC 19438 harboring pRYGG1 or strain 84-112 harboring pRYEL1 served as donors. A strong exclusion phenotype was expected and, indeed, the obtained frequencies were lower than 3×10^{-9} transconjugants per donor. Nonetheless, plasmid uptake was confirmed in all colonies we tested. Taken together, these results demonstrate that *C. fetus*

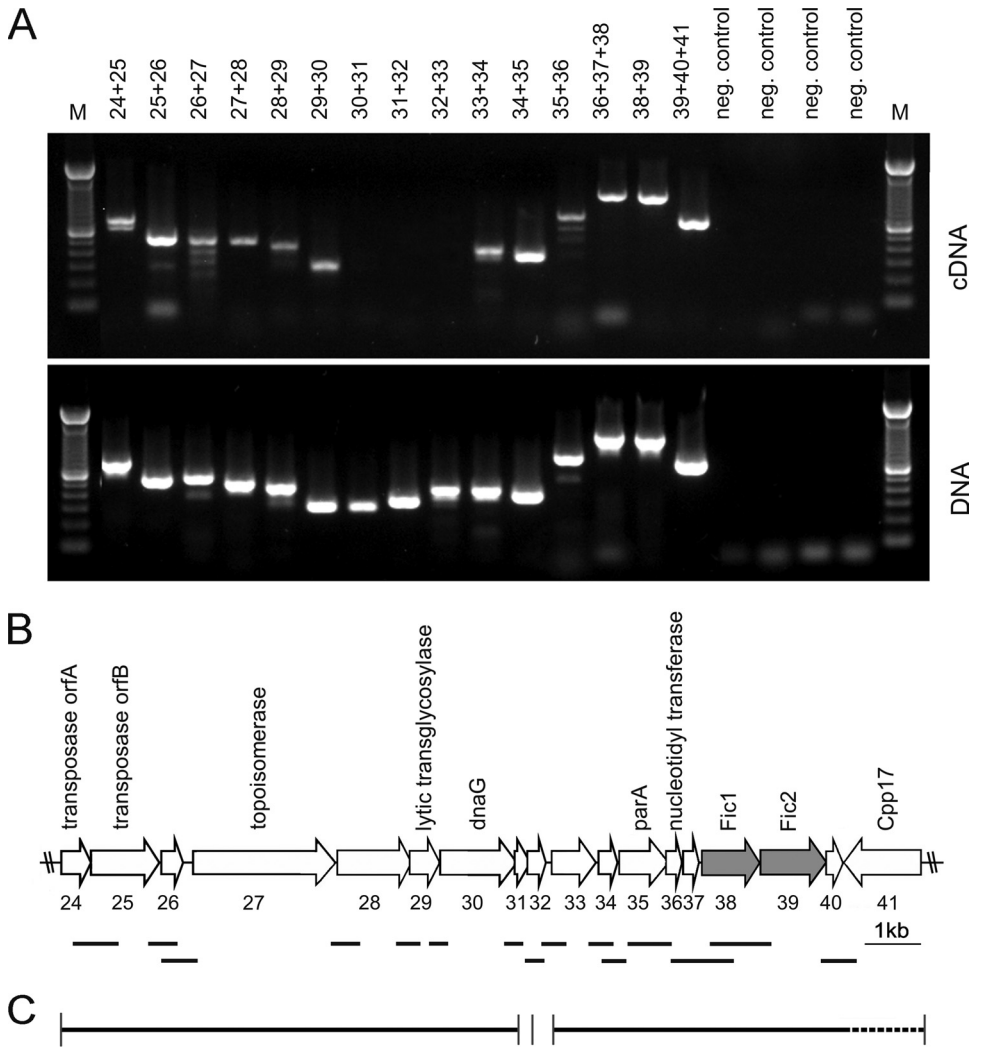

FIG. 5. Transcriptional organization of *C. fetus* subsp. *venerealis* PAI region harboring the *fic* genes. (A) Gene organization of the region extending from transposase *orfA* to the putative nickase gene downstream of the *fic* genes was analyzed in strain ATCC 19438 by ORF-spanning amplification of cDNA produced with reverse transcriptase PCR (upper panel). Genomic DNA served as positive controls for each primer combination (lower panel). Numbers above the lanes indicate the ORFs common to the amplified cDNA according to the gene designations shown in panel B. Negative controls lacked reverse transcriptase (upper panel) or template (lower panel). (B) The 3′ region of the genomic island of *C. fetus* subsp. *venerealis* ATCC 19438 is illustrated. Gene numbers assigned previously (28) (below) and putative functional assignments are shown (above). *fic* genes are shaded gray. (C) Transcription map depicts two distinct mRNA fragments encoding ORFs 24 to 30 and ORFs 33 to 40, respectively (solid lines). ORFs 31 and 32 of unknown function are not transcribed with the other genes nor each other. Transcripts initiated within units 33 to 40 extend into the putative relaxase gene *cpp17* in reverse orientation (dotted line). The separately amplified cDNA regions shown in panel A are indicated schematically (above).

subsp. *venerealis* supports intra- and interspecies gene transfer via a mechanism that is independent of transformation, requires the T4SS, and is sensitive to the surface or entry exclusion functions of RP4, as well as *C. fetus* subsp. *venerealis* strains expressing an identical system.

Transcriptional organization of *C. fetus* subsp. *venerealis* PAI region harboring the *fic* genes. The genome region distal to the *virB*/*virD4* module contains two ORFs carrying the FIC signature motif common to secreted effector proteins of several bacterial pathogens. We investigated the transcriptional organization of this genome region using RT-PCR. Primers were placed to detect readthrough of transcripts into the adjacent or further downstream genes. One large transcriptional unit spanning 7.5 kb was mapped from the transposase orfA, or gene 24 of the PAI sequence (28), and the putative primase gene *dnaG* (Fig. 5). No transcripts were detected for the small ORFs 31 and 32 depicted in Fig. 5. A second large transcript spanning 6 kb, including the *fic1* and *fic2* genes, was delineated. Transcription originating in this region is not effectively terminated, since readthrough into the downstream, oppositely oriented gene *cpp17* was observed.

Test for interbacterial protein translocation. Currently, the most promising approach available to identify potential effector proteins recognized and secreted by T4SS is the Cre recombinase assay for translocation (CRAfT) developed by Vergunst et al. (67, 68). The assay enables translocation events to

be detected via fusion of the candidate protein to the Cre recombinase. Cre alone is not transferable, but when fused to a true T4 substrate, it gains access to the T4 pathway via the T4CP. Target cells are engineered to indicator strains with a reporter gene flanked by *loxP* recombination sites. Uptake of the T4 substrate-Cre fusion protein by recipients is detected via recombination of the reporter cassette and the emergence of a heritable phenotypic difference.

As described above, the Fic1 and Fic2 proteins encoded by the *C. fetus* subsp. *venerealis* PAI are possible secretion substrates. We have shown that *E. coli* is a suitable recipient for *C. fetus* subsp. *venerealis* T4-mediated secretion of nucleoprotein substrates. It is conceivable, therefore, that if the function of the *Campylobacter* Fic proteins normally requires recognition by the T4CP, then those interactions should also enable the putative effectors to be transferred via this pathway to *E. coli.* To test this hypothesis, fusions of Fic1 and Fic2 to Cre recombinase were cloned in the *C. fetus* expression vector, pRYSK3, which cannot be mobilized by the T4SS. *C. fetus* subsp. *venerealis* strains ATCC 19438 and 84-112, each expressing Cre alone (pRYSK15), or the fusions Cre-Fic1 (pRYSK13), or Cre-Fic2 (pRYSK14) were combined with *E. coli* indicator strains under conditions that maximally supported DNA mobilization via the *C. fetus* T4SS. The *E. coli* recipient cells were plated onto chloramphenicol containing LB agar to detect recombinant progeny. The frequency of recombination was measurably higher in every experiment involving the Cre-Fic2 fusion protein compared to Cre alone ($n = 4$), but the difference was not statistically significant (data not shown). Drawing on our results with the T4-mediated plasmid transmission from *C. fetus* to *E. coli*, we varied the temperature, the species ratio, and overall cell density. Again, Cre-Fic2 expressing cells led to 2-fold-higher recombination frequencies in *E. coli* than in cultures expressing Cre alone. Despite these steps toward optimization, the low frequencies we observed were not sufficiently distinct from background levels to be statistically significant. We have used CRAfT to map the protein translocation signals present on the TraI protein recognized by the F-like T4SS in *E. coli* (39). In our experience with F-like systems, even under conditions that support very efficient nucleoprotein transfer (>1 transfer events per donor cell), the frequency of concomitant protein translocation leading to productive recombination is typically limited to 3 to 0.3% of the transconjugant cells. It follows that the frequency of DNA transfer we are currently able to obtain in *C. fetus-E. coli* mating (10^{-6} to 10^{-7} transconjugants/donor) is too low for the subsequent Cre-based recombination events to occur at readily detectable levels.

The *C. fetus* subsp. *venerealis* Fic proteins display conserved motifs shared by diverse T4S substrates, suggesting that heterologous T4SS might support productive interactions with the Fic proteins. To test this possibility, we chose the F and R1 conjugation systems since we have investigated protein translocation by these paradigms in detail (39). The T4CPs of the broad-host-range plasmids R388 and RP4 are more permissive for interactions with heterologous plasmids, and, importantly, RP4 is apparently most functionally related to the *C. fetus* components (Table 6). To provide positive controls for translocation of the Cre-protein fusions, relaxase genes were fused 3′ to *cre* in the CRAfT reporter vector CFP B (49). Protein translocation was measured indirectly by the frequency of Cre-catalyzed recombination in the recipient cell population. Simultaneous monitoring of plasmid transfer provided a measure for the secretion activity of the system in every experiment. A summary of the translocation data is shown in Fig. 6. High frequencies of DNA (self) transfer were detected for the F-like systems R1-16 (1/donor) and pOX38 (10^{-1}/donor), as well as R388 (1/donor). By contrast, interbacterial transfer of the *C. fetus* Fic proteins was not detectable with any conjugation system. The high exclusion index mediated by IncP plasmid RP4 (Table 6) against the *C. fetus* transfer machinery implies functional relatedness for these systems. Nevertheless, the RP4 transfer proteins expressed by the S17 λ *pir* donor did not support detectable Fic protein transfer. The RP4 conjugation genes are integrated in the S17 λ *pir* chromosome, and

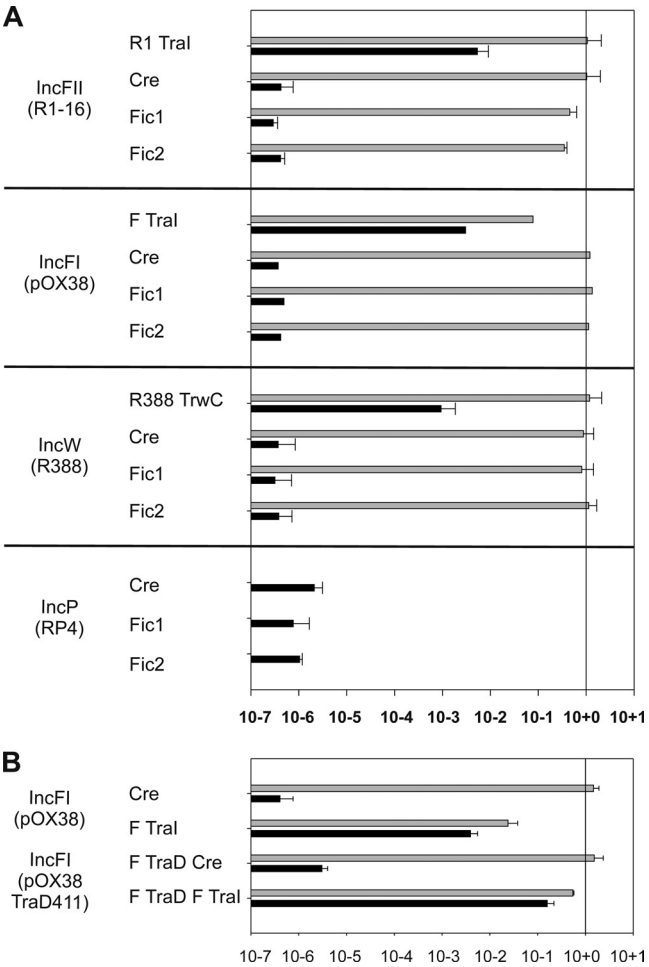

FIG. 6. Efficient T4 secretion is sensitive to the relative concentration of T4CP and substrate protein. (A) Heterologous conjugation systems were tested for recognition and transfer of the *C. fetus* Fic proteins. The protein translocation frequencies (recombination events per donor) supported by R1-16, pOX38, R388, and RP4 conjugation systems are shown (black bars, right) for Cre alone or Cre fused to the full-length proteins indicated (left). DNA self-transfer frequencies for each plasmid system are indicated with gray bars. Standard deviations are shown. (B) DNA self-transfer of pOX38 is inhibited by *cre-traI* overexpression in *trans* (gray bars). By comparison, frequencies of gene and protein transfer are markedly increased when F *traD* and the *cre-traI* are both overexpressed in *trans* to *traD*-null derivative pOX38*traD*411.

thus an internal standard for conjugation proficiency is lacking. Given that we used this strain routinely for delivery of all shuttle vectors into *C. fetus* strains throughout the study, we propose that the negative results for Fic1 and Fic2 translocation indicate a true lack of productive interactions with the RP4 substrate receptor, TraG.

Relative concentrations of a substrate protein and the T4CP alters secretion efficiency. The *C. fetus* subsp. *venerealis virB*/*virD4* genes are organized as an operon, and in an earlier study we demonstrated that the operon is expressed under laboratory conditions (28). The dramatic stimulation in gene transfer obtained when *virB9* and the downstream genes *virB10*, *virB11*, and *virD4*—or *virD4* alone—were expressed in *trans* from plasmid vectors on the *virB9* and *virD4* mutant backgrounds (Fig. 3 and Table 5) suggests that controlled gene regulation is limiting the translocation activity observed. Nonetheless, *virD4* is cotranscribed with upstream *virB* genes (data not shown); thus, the intracellular concentration of this protein would be expected to be similar to the other coregulated T4 components. Regulation of the *virB*/*virD4* operon promoter has not been studied. We sought to determine whether in *trans* expression of *virD4* might be part of a positive-feedback loop for operon upregulation. Expression of *virB* genes upstream of *virD4* and downstream PAI genes in *C. fetus* subsp. *venerealis* V81_SK1 was compared with or without the *virD4* expression vector pRYSK12 *in trans*. No significant change in mRNA levels was apparent based on the yield of amplified cDNA (data not shown). A VirD4-dependent positive regulation of transporter components was therefore ruled out under these conditions.

Balanced intracellular concentrations of the T4CP VirD4 and secretion substrates may be important to transporter activity. The identity of the conjugative relaxase involved in DNA mobilization by *C. fetus* subsp. *venerealis* is not yet known. To evaluate the principle of how imbalanced expression of substrate and receptor genes might affect T4 secretion, we instead returned to the well-characterized F system. Overexpression of the F *traI* gene in *trans* to the wild-type conjugation genes causes a negative dominant phenotype for conjugation (30). That negative effect is also visible in our experiments where pOX38 transfer was reduced by 66-fold in the presence of a plasmid expressing Cre-$TraI_F$ compared to Cre alone (Fig. 6A). We tested the effect of simultaneous overexpression of both F *traD* and *traI* from plasmids in *trans* (Fig. 6B). Under these conditions, the efficiencies of conjugative DNA transfer increased by 22-fold, and protein translocation was raised 40-fold in comparison to *traI* expression alone. We conclude that in the F system the relative concentrations of the T4CP and substrate protein are indeed decisive for efficient transfer. *C. fetus* gene transfer was markedly increased when *virD4* was expressed in *trans* from plasmid vectors (Fig. 3 and Table 5). Based on these observations, we propose that a lack of coordinate regulation of the conjugative relaxase with the *virB*/*virD4* components under our laboratory conditions may lead to the low levels of conjugative transfer observed with this organism thus far.

DISCUSSION

This study demonstrates that the *C. fetus* subsp. *venerealis* VirB/VirD4 T4SS supports inter- and intraspecies mobilization of DNA. The frequencies of plasmid transfer observed under these conditions were low. The VirB/VirD4 T4SS is also necessary for *C. fetus* subsp. *venerealis* to efficiently invade and induce cytolethal effects in cultured human epithelial cells *in vitro* (28). The question then arises whether the capacity to translocate DNA represents an important contribution to the fitness and pathogenicity of *C. fetus* or whether the (remaining) capacity for gene transfer observed here represents a vestige of the system's evolutionary origin as a conjugation system. In any case, this report is the first description of conjugative plasmid transfer in *C. fetus*.

We currently know little about regulation of the *vir* genes. Conjugation was measured over the temperature range from 25 to 39°C with a modest (5-fold) gain in the activity maximum observed at 32°C. Whether this reflects a temperature dependence in *vir* gene regulation or the activity optima of the proteins involved is unknown. Controlled synthesis of conjugative pili on the bacterial cell surface probably alters motility and would be expected to initiate formation of stabilizing multicellular structures such as microcolonies and biofilm similarly to many Gram-negative bacteria (6, 26, 46, 51, 71). Thus, the elaboration of pili may provide an advantage in the successful colonization of the bovine genital tract also in the absence of macromolecular transport. Given this diversity of potential functions, the *virB*/*virD4* gene region is probably subject to complex patterns of gene regulation that respond to both environmental and host-dependent cues.

Current knowledge of genome composition and dynamics for this pathogen is rudimentary, but laterally acquired genes are predicted to contribute to the distinct niche preferences exhibited by this taxon (2, 34). Experimental evidence for natural transformation is still lacking (8); thus, knowledge of gene mobilization via conjugative mechanisms is important in analyzing the emerging genome sequences of the *C. fetus* subspecies. Moreover, knowledge that lateral gene transfer occurs between the *C. fetus* subspecies has significant implications for the design of genotyping schemes that reliably differentiate the subspecies (1). *C. fetus* subsp. *venerealis* is the causative agent of a contagious venereal disease causing severe reproductive problems in cattle. Bovine genital campylobacteriosis is a notifiable disease of the World Organization for Animal Health (OIE) and is a substantial burden for the international trade of animals and animal products. The microbiological and molecular differentiation of *C. fetus* subsp. *venerealis* from *C. fetus* subsp. *fetus* is extremely difficult (72). A single phenotypic test is recommended by the OIE for typing the subspecies (66); thus, international efforts currently focus on optimizing genotypic tests. The present study demonstrated that genes are exchanged between the *C. fetus* subspecies via conjugation. Clearly, knowledge of lateral DNA transfer should factor into the selection of diagnostic targets.

The FIC domain-containing proteins of *C. fetus* are potential effector proteins translocated by the T4 machinery to mammalian cells. Transmission of bacterial Fic proteins to the eukaryotic cytosol regulates host processes important to pathogen survival and replication. AnkX protein of *Legionella pneumo-*

phila alters the microtubule-dependent transport of vesicles (47). The FIC domain of VopS covalently modifies Rho GTPase threonine with AMP disrupting downstream signal transduction in the host cell (76). The AMPylation domain is shared by doc toxins, FIC, and the type III effector AvrB (35). IbpA protein of *Histophilus somni* adenylylates and inactivates Rho GTPases of epithelial target cells ultimately leading to collapse of the cellular cytoskeleton (74, 78). The VirB/VirD4-translocated substrates of *Bartonella henselae*, BepA-BepG, are required for invasion, proinflammatory activation and antiapoptotic protection of vascular endothelial cells (56, 60). BepA, BepB, and BepC share an FIC domain. In light of the genetic organization of the virB/virD4/bep PAI of *Bartonella* (57), the distal proximity of the *fic1* and *fic2* genes to the *C. fetus vir* operon supports the hypothesis that these proteins are delivered as effector molecules to target cells. Experimental approaches to demonstrate translocation of a putative bacterial secretion substrate to a recipient cell have been vastly improved by molecular reporter systems based on Cre recombinase or the calmodulin-dependent adenylate cyclase (Cya) (45, 56, 62). Application of the Cre recombinase reporter assay to the Fic proteins of *C. fetus* was chosen to evaluate whether the cognate or heterologous systems would support detectable levels of secretion to bacterial target cells. The current results neither support nor rule out the potential for Fic proteins to perform relevant pathogenic functions in animal cell infection. Our efforts now focus on applications of the Cya reporter in cultured human cells invaded by *C. fetus*.

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REFERENCES

1. **Abril, C., I. Brodard, and V. Perreten.** 2010. Two novel antibiotic resistance genes, *tet*(44) and *ant(6)-Ib*, are located within a transferable pathogenicity island in *Campylobacter fetus* subsp. *fetus.* Antimicrob. Agents Chemother. **54:**3052–3055.
2. **Abril, C., et al.** 2007. Discovery of insertion element ISCfe1: a new tool for *Campylobacter fetus* subspecies differentiation. Clin. Microbiol. Infect. **13:** 993–1000.
3. **Alvarez-Martinez, C. E., and P. J. Christie.** 2009. Biological diversity of prokaryotic type IV secretion systems. Microbiol. Mol. Biol. Rev. **73:**775–808.
4. **Andersen, J. B., et al.** 2001. gfp-based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. Appl. Environ. Microbiol. **67:**575–585.
5. **Backert, S., and T. F. Meyer.** 2006. Type IV secretion systems and their effectors in bacterial pathogenesis. Curr. Opin. Microbiol. **9:**207–217.
6. **Barrios, A. F., R. Zuo, D. Ren, and T. K. Wood.** 2006. Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. Biotechnol. Bioeng. **93:**188–200.
7. **Blaser, M. J.** 1998. *Campylobacter fetus*–emerging infection and model system for bacterial pathogenesis at mucosal surfaces. Clin. Infect. Dis. **27:**256–258.
8. **Blaser, M. J., D. G. Newell, S. A. Thompson, and E. L. Zechner.** 2008. Pathogenesis of *Campylobacter fetus* infections, p. 401–428. *In* I. Nachamkin, C. M. Szymanski, and M. J. Blaser (ed.), *Campylobacter*, 3rd ed. ASM Press, Washington, D.C.
9. **Boschiroli, M. L., et al.** 2002. Type IV secretion and *Brucella* virulence. Vet. Microbiol. **90:**341–348.
10. **Buchanan-Wollaston, V., J. E. Passiatore, and F. Cannon.** 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature **328:**172–175.
11. **Cambronne, E. D., and C. R. Roy.** 2006. Recognition and delivery of effector proteins into eukaryotic cells by bacterial secretion systems. Traffic **7:**929–939.
12. **Cascales, E., and P. J. Christie.** 2003. The versatile bacterial type IV secretion systems. Nat. Rev. Microbiol. **1:**137–149.
13. **Chandler, M., and D. J. Galas.** 1983. Cointegrate formation mediated by Tn9. II. Activity of IS1 is modulated by external DNA sequences. J. Mol. Biol. **170:**61–91.
14. **Chatfield, L. K., E. Orr, G. J. Boulnois, and B. M. Wilkins.** 1982. DNA primase of plasmid ColIb is involved in conjugal DNA synthesis in donor and recipient bacteria. J. Bacteriol. **152:**1188–1195.
15. **Chen, I., P. J. Christie, and D. Dubnau.** 2005. The ins and outs of DNA transfer in bacteria. Science **310:**1456–1460.
16. **Christie, P. J., K. Atmakuri, V. Krishnamoorthy, S. Jakubowski, and E. Cascales.** 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu. Rev. Microbiol. **59:**451–485.
17. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
18. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol. **235:**386–405.
19. **Ding, Z., K. Atmakuri, and P. J. Christie.** 2003. The outs and ins of bacterial type IV secretion substrates. Trends Microbiol. **11:**527–535.
20. **Fernandez-Lopez, R., et al.** 2006. Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. FEMS Microbiol. Rev. **30:**942–966.
21. **Fischer, W., R. Haas, and S. Odenbreit.** 2002. Type IV secretion systems in pathogenic bacteria. Int. J. Med. Microbiol. **292:**159–168.
22. **Fischer, W., et al.** 2001. Systematic mutagenesis of the *Helicobacter pylori cag* pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol. Microbiol. **42:**1337–1348.
23. **Franco, I. S., H. A. Shuman, and X. Charpentier.** 2009. The perplexing functions and surprising origins of *Legionella pneumophila* type IV secretion effectors. Cell Microbiol. **11:**1435–1443.
24. **Frank, A. C., C. M. Alsmark, M. Thollesson, and S. G. Andersson.** 2005. Functional divergence and horizontal transfer of type IV secretion systems. Mol. Biol. Evol. **22:**1325–1336.
25. **Garcillan-Barcia, M. P., and F. de la Cruz.** 2008. Why is entry exclusion an essential feature of conjugative plasmids? Plasmid **60:**1–18.
26. **Ghigo, J. M.** 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature **412:**442–445.
27. **Goebel, W., W. Lindenmaier, H. Schrempf, R. Kollek, and D. Blohm.** 1977. Dissociation and recombination of fragments with defined functions of the antibiotic resistance factor R1, p. 261–275. *In* J. Drews and G. Högenauer (ed.), Topics in infectious diseases, vol. 2. Springer-Verlag, New York, NY.
28. **Gorkiewicz, G., et al.** 2010. A genomic island defines subspecies-specific virulence features of the host-adapted pathogen *Campylobacter fetus* subsp. *venerealis.* J. Bacteriol. **192:**502–517.
29. **Grandoso, G., M. Llosa, J. C. Zabala, and F. de la Cruz.** 1994. Purification and biochemical characterization of TrwC, the helicase involved in plasmid R388 conjugal DNA transfer. Eur. J. Biochem. **226:**403–412.
30. **Haft, R. J., et al.** 2006. General mutagenesis of F plasmid TraI reveals its role in conjugative regulation. J. Bacteriol. **188:**6346–6353.
31. **Hu, L., and D. J. Kopecko.** 2000. Interactions of *Campylobacter* with eukaryotic cells: gut luminal colonization and mucosal invasion mechanisms, p. 191–215. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, DC.
32. **Juhas, M., D. W. Crook, and D. W. Hood.** 2008. Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. Cell Microbiol. **10:**2377–2386.
33. **Kienesberger, S., et al.** 2007. Development of experimental genetic tools for *Campylobacter fetus*. Appl. Environ. Microbiol. **73:**4619–4630.
34. **Kienesberger, S., G. Gorkiewicz, H. Wolinski, and E. L. Zechner.** 2010. New molecular microbiology approaches in the study of *Campylobacter fetus.* Microb. Biotechnol., in press. doi:10.1111/j.1751–7915.2010.00173.x.
35. **Kinch, L. N., M. L. Yarbrough, K. Orth, and N. V. Grishin.** 2009. Fido, a novel AMPylation domain common to *fic*, *doc*, and AvrB. PLoS One **4:**e5818.
36. **Komano, T., R. Utsumi, and M. Kawamukai.** 1991. Functional analysis of the *fic* gene involved in regulation of cell division. Res. Microbiol. **142:**269–277.
37. **Krause, R., et al.** 2002. Recurrent septicemia due to *Campylobacter fetus* and *Campylobacter lari* in an immunocompetent patient. Infection **30:**171–174.
38. **Lambert, T., G. Gerbaud, P. Trieu-Cuot, and P. Courvalin.** 1985. Structural relationship between the genes encoding 3′-aminoglycoside phosphotransferases in *Campylobacter* and in gram-positive cocci. Ann. Inst. Pasteur Microbiol. **136B:**135–150.
39. **Lang, S., et al.** 2010. Molecular recognition determinants for type IV secretion of diverse families of conjugative relaxases. Mol. Microbiol., *in press.*
40. **Lawley, T. D., W. A. Klimke, M. J. Gubbins, and L. S. Frost.** 2003. F factor conjugation is a true type IV secretion system. FEMS Microbiol. Lett. **224:** 1–15.
41. **Lin, T. S., and C. I. Kado.** 1993. The *virD4* gene is required for virulence

while *virD3* and *orf5* are not required for virulence of *Agrobacterium tumefaciens*. Mol. Microbiol. **9:**803–812.
42. **Lu, J., et al.** 2008. Structural basis of specific TraD-TraM recognition during F plasmid-mediated bacterial conjugation. Mol. Microbiol. **70:**89–99.
43. **Maneewannakul, K., et al.** 1996. Construction of derivatives of the F plasmid pOX-tra715: characterization of *traY* and *traD* mutants that can be complemented in trans. Mol. Microbiol. **22:**197–205.
44. **Martinez, E., and F. de la Cruz.** 1988. Transposon *Tn21* encodes a RecA-independent site-specific integration system. Mol. Gen. Genet. **211:**320–325.
45. **Nagai, H., et al.** 2005. A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. Proc. Natl. Acad. Sci. U. S. A. **102:**826–831.
46. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
47. **Pan, X., A. Luhrmann, A. Satoh, M. A. Laskowski-Arce, and C. R. Roy.** 2008. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. Science **320:**1651–1654.
48. **Pansegrau, W., et al.** 1994. Complete nucleotide sequence of Birmingham IncP alpha plasmids: compilation and comparative analysis. J. Mol. Biol. **239:**623–663.
49. **Parker, C., and R. J. Meyer.** 2007. The R1162 relaxase/primase contains two, type IV transport signals that require the small plasmid protein MobB. Mol. Microbiol. **66:**252–261.
50. **Perez-Perez, G. I., M. J. Blaser, and J. H. Bryner.** 1986. Lipopolysaccharide structures of *Campylobacter fetus* are related to heat-stable serogroups. Infect. Immun. **51:**209–212.
51. **Reisner, A., J. A. Haagensen, M. A. Schembri, E. L. Zechner, and S. Molin.** 2003. Development and maturation of *Escherichia coli* K-12 biofilms. Mol. Microbiol. **48:**933–946.
52. **Reisner, A., S. Molin, and E. L. Zechner.** 2002. Recombinogenic engineering of conjugative plasmids with fluorescent marker cassettes. FEMS Microbiol. Ecol. **42:**251–259.
53. **Roy, C. R., and S. Mukherjee.** 2009. Bacterial FIC proteins AMP up infection. Sci. Signal **2:**pe14.
54. **Salgado Pabon, W., et al.** 2010. Increased expression of the type IV secretion system in piliated *Neisseria gonorrhoeae* variants. J. Bacteriol. **192:**1912–1920.
55. **Sastre, J. I., E. Cabezon, and F. de la Cruz.** 1998. The carboxyl terminus of protein TraD adds specificity and efficiency to F-plasmid conjugative transfer. J. Bacteriol. **180:**6039–6042.
56. **Schmid, M. C., et al.** 2006. A translocated bacterial protein protects vascular endothelial cells from apoptosis. PLoS Pathog. **2:**e115.
57. **Schroder, G., and C. Dehio.** 2005. Virulence-associated type IV secretion systems of *Bartonella*. Trends Microbiol. **13:**336–342.
58. **Schroder, G., and E. Lanka.** 2005. The mating pair formation system of conjugative plasmids: a versatile secretion machinery for transfer of proteins and DNA. Plasmid **54:**1–25.
59. **Schulein, R., and C. Dehio.** 2002. The VirB/VirD4 type IV secretion system of *Bartonella* is essential for establishing intraerythrocytic infection. Mol. Microbiol. **46:**1053–1067.
60. **Schulein, R., et al.** 2005. A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells. Proc. Natl. Acad. Sci. U. S. A. **102:**856–861.
61. **Skirrow, M. B., and M. J. Blaser.** 2000. Clinical aspects of *Campylobacter* infections, p. 69–88. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. American Society for Microbiology, Washington, DC.
62. **Sory, M. P., and G. R. Cornelis.** 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. **14:**583–594.
63. **Thompson, S. A., and M. J. Blaser.** 2000. Pathogenesis of *Campylobacter fetus* infections, p. 321–347. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, DC.
64. **Tummuru, M. K., and M. J. Blaser.** 1993. Rearrangement of *sapA* homologs with conserved and variable regions in *Campylobacter fetus*. Proc. Natl. Acad. Sci. U. S. A. **90:**7265–7269.
65. **van Bergen, M. A., et al.** 2005. Clonal nature of *Campylobacter fetus* as defined by multilocus sequence typing. J. Clin. Microbiol. **43:**5888–5898.
66. **van Bergen, M. A., et al.** 2005. Amplified fragment length polymorphism based identification of genetic markers and novel PCR assay for differentiation of *Campylobacter fetus* subspecies. J. Med. Microbiol. **54:**1217–1224.
67. **Vergunst, A. C., et al.** 2000. VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science **290:**979–982.
68. **Vergunst, A. C., et al.** 2005. Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of Agrobacterium. Proc. Natl. Acad. Sci. U. S. A. **102:**832–837.
69. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
70. **Voth, D. E., and R. A. Heinzen.** 2009. *Coxiella* type IV secretion and cellular microbiology. Curr. Opin. Microbiol. **12:**74–80.
71. **Watnick, P. I., and R. Kolter.** 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Mol. Microbiol. **34:**586–595.
72. **Willoughby, K., et al.** 2005. A multiplex polymerase chain reaction to detect and differentiate *Campylobacter fetus* subspecies *fetus* and *Campylobacter fetus* subspecies *venerealis*: use on UK isolates of *C. fetus* and other *Campylobacter* spp. J. Appl. Microbiol. **99:**758–766.
73. **Woodcock, D. M., et al.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res. **17:**3469–3478.
74. **Worby, C. A., et al.** 2009. The *fic* domain: regulation of cell signaling by adenylylation. Mol. Cell **34:**93–103.
75. **Yao, R., R. A. Alm, T. J. Trust, and P. Guerry.** 1993. Construction of new *Campylobacter* cloning vectors and a new mutational *cat* cassette. Gene **130:**127–130.
76. **Yarbrough, M. L., et al.** 2009. AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. Science **323:**269–272.
77. **Zechner, E. L., et al.** 2000. Conjugative-DNA transfer processes, p. 87–174. *In* C. M. Thomas (ed.), The horizontal gene pool: bacterial plasmids and gene spread. Harwood Academic Publishers, Amsterdam, Netherlands.
78. **Zekarias, B., et al.** *Histophilus somni* IbpA DR2/Fic in virulence and immunoprotection at the natural host alveolar epithelial barrier. Infect. Immun. **78:**1850–1858.