Minireview

New molecular microbiology approaches in the study of *Campylobacter fetus*

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Summary

Campylobacter fetus infection is a substantial problem in herds of domestic cattle worldwide and a rising threat in human disease. Application of comparative and functional genomics approaches will be essential to understand the molecular basis of this pathogen's interactions with various hosts. Here we report recent progress in genome analyses of *C. fetus* ssp. *fetus* and *C. fetus* ssp. *venerealis*, and the development of molecular tools to determine the genetic basis of niche-specific adaptations. Campylobacter research has been strengthened by the rapid advancements in imaging technology occurring throughout microbiology. To move forward in understanding the mechanisms underlying *C. fetus* virulence, current efforts focus on developing suitable *in vitro* models to reflect host- and tissue-specific aspects of infection.

Introduction

Pathogenesis of Campylobacter fetus

Campylobacter fetus is traditionally recognized as a significant pathogen of livestock. Its occurrence as an opportunistic human pathogen is uncommon, yet this is believed to be underestimated and is apparently increasing (Blaser, 1998; Blaser *et al.*, 2008). *Campylobacter fetus* belongs to the group of ε-proteobacteria and is highly adapted to mucosal surfaces (Hu and Kopecko, 2000). Two subspecies of *C. fetus* have been designated, *C. fetus* ssp. *fetus* and *C. fetus* ssp. *venerealis*. Nearly all

C. fetus infections in humans are systemic and arise due to *C. fetus* ssp. *fetus*. This subspecies is the predominant *Campylobacter* species isolated from human blood (Blaser, 1998) and is considered to be an emerging pathogen placing infants, elderly, immunocompromised and debilitated persons at risk (Skirrow and Blaser, 2000; Thompson and Blaser, 2000). Both *C. fetus* ssp. *fetus* and *C. fetus* ssp. *venerealis* cause disease in cattle. The subspecies show distinct niche preferences, yet these are not strictly exclusive. In ruminants *C. fetus* ssp. *venerealis* colonizes the genital tract while *C. fetus* ssp. *fetus* is largely confined to the gut. However, both subspecies can be recovered from the genital tract and are a major cause of abortion and infertility causing substantial losses in bovine, ovine and caprine herds worldwide. Despite this pathogen's global economic and rising clinical significance, the molecular mechanisms underlying infection of its human and animal hosts remain largely unknown. Until very recently, the absence of genetic tools to manipulate *C. fetus* (Kienesberger *et al.*, 2007) and the lack of tractable infection models to investigate the molecular basis of host–pathogen interactions has slowed research with this organism. In this review we describe recent progress in molecular approaches applicable in the study of *C. fetus*.

Genome analyses and the mobile gene pool

The first complete genome sequence of a *Campylobacter* species, the *C. jejuni* clinical isolate NCTC 11168, was published in 2000 (Parkhill *et al.*, 2000). Up to now 11 complete and 24 unfinished genome sequences have been deposited in the public databases (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Pearson *et al.*, 2007; Poly *et al.*, 2007; Miller, 2008). Similar to other campylobacters the genome size of *C. fetus* is rather small (~1.8 Mb). Genome sizing by pulsed field gel electrophoresis revealed size variation between strains (Salama *et al.*, 1992). Multilocus sequence typing (MLST) is a procedure for characterizing isolates of bacterial species using the sequences of internal fragments of genes (Maiden *et al.*, 1998; http://pubmlst.org/). Typically, seven housekeeping genes are used in this analysis. The profile of allele

sequences for a given bacterial isolate defines the sequence type. The MLST analysis has shown a clonal population structure within *C. fetus*, wherein *C. fetus* ssp. *venerealis* represents a bovine clone (van Bergen *et al.*, 2005; Dingle *et al.*, 2010). This is in contrast to other *Campylobacter* species, which show extensive genetic variation (e.g. *C. jejuni*). This variability is thought to continuously improve the capacity to colonize and persist in various habitats (de Boer *et al.*, 2002; Wiesner *et al.*, 2003).

Current efforts to obtain complete genome sequences of representatives of both *C. fetus* subspecies are expected to bring rapid advances. The complete sequence of *C. fetus* ssp. *fetus* 82-40 was finished in 2006, revealing that 90% of the genome constitutes coding sequence (GenBank Accession number NC_008599). A draft genome sequence of *C. fetus* ssp. *venerealis* 84-112 has just become available (EBI Project ID: 42511). Access to both genome sequences provides the resources for detailed analysis of *C. fetus* physiology as well as subspecies-specific adaptations, and will stimulate efforts to identify mechanisms contributing to pathogen–host interactions and virulence. The comparative approach will certainly shed light on the niche preferences displayed by the *C. fetus* subspecies. In addition, the *C. fetus* sequences are expected to open new perspectives for subtyping methods as well as for improving or establishing novel diagnostic approaches for this emerging pathogen.

The process of horizontal gene transfer (HGT) in bacteria drives genetic diversity and evolution providing also a basis for variation in the virulence repertoire as well as resistance to antibiotics and host defences (Hacker *et al.*, 1997; Gogarten *et al.*, 2002; Koonin and Wolf, 2008; Boto, 2010; Juhas *et al.*, 2009). DNA acquired by these mechanisms apparently accounts for 5–6% of the genome of certain campylobacters (Eppinger *et al.*, 2004). Comparative genomic analysis is expected to reveal further species- or strain-variation due to evolutionary gene mobility. An initial comparison of the *C. fetus* genomes confirmed that *C. fetus* ssp. *venerealis* DNA presumably acquired by horizontal mechanisms indeed represents the major difference between the two subspecies.

An important technique in functional and comparative genomics is representational difference analysis (RDA). The methodology was developed to compare the differences in complex genomes as well as to obtain clones of those differentiating genes (Lisitsyn and Wigler, 1993). Before the availability of the *C. fetus* genome sequences RDA was applied to reveal genes uniquely or predominantly present in just one *C. fetus* subspecies (Gorkiewicz *et al.*, 2010). Consistent with the hypothesis that distinguishing physiological and virulence properties of the subspecies relate to the existence of different sets of genes,

homology searches revealed that multiple genes uniquely or predominantly associated with a given subspecies indeed encode virulence-related attributes ranging from motility to lipopolysaccharide production to bacterial secretion. A large genomic island unique to *C. fetus* ssp. *venerealis* was shown to encode a conjugation-related type IVa macromolecular secretion system (T4SS) (for system classifications see Christie and Vogel, 2000). Mutational analyses confirmed that the secretion machinery is involved in the ability of *C. fetus* ssp. *venerealis* to infect and induce cytopathic effects in cultured human epithelial and placenta cells (see below; Gorkiewicz *et al.*, 2010). Moreover, the T4SS was shown to be active in intra- and interspecies conjugative mobilization of plasmid DNA (S. Kienesberger, G. Gorkiewicz, A. Fauster and E.L. Zechner, unpublished) derived from the cryptic *C. coli* plasmid pIP1455 (Lambert *et al.*, 1985). This finding marks the first experimental demonstration of HGT in *C. fetus*. Evidence for natural transformation is still lacking (Blaser *et al.*, 2008). Characterization of gene transfer by conjugation will certainly lead to the application of tools for DNA delivery among campylobacters (S. Kienesberger, G. Gorkiewicz, A. Fauster and E.L. Zechner, unpublished).

Knowledge of gene mobilization via conjugative mechanisms is also important in assessing the contribution of HGT to the evolution of the *C. fetus* subspecies and campylobacters generally. Conversely analysis of the genomes will provide insights to the presence and activities of clustered, regularly interspaced, short palindromic repeat (CRISPR) loci, which have been identified in a variety of different bacteria including campylobacters (Miller, 2008). These hypervariable genetic loci capture incoming DNA acquired by multiple routes of HGT and provide sequence-directed immunity to invasive phage and plasmids (Marraffini and Sontheimer, 2008; Horvath and Barrangou, 2010). The CRISPR interference thus limits HGT. It is reasonable to predict that future studies will demonstrate a continued proficiency for mobility for some of the horizontally acquired elements in *C. fetus* as well as direct evidence for CRISPR-directed counteraction of lateral gene spread.

Molecular genetics

Genetic manipulation of *C. fetus* is becoming easier as new molecular tools are developed. The available *E. coli* – Campylobacter shuttle vectors suitable for applications in *C. fetus* are all derived from small cryptic plasmids endogenous to campylobacter (Kienesberger *et al.*, 2007). Figure 1 illustrates a selection of shuttle vectors and their salient features. Currently three distinct replicons are known to be functional in *C. fetus*, namely derivatives of the *C. coli* plasmid pIP1455 (Lambert *et al.*, 1985), a cryptic *C. hyointestinalis* plasmid present in
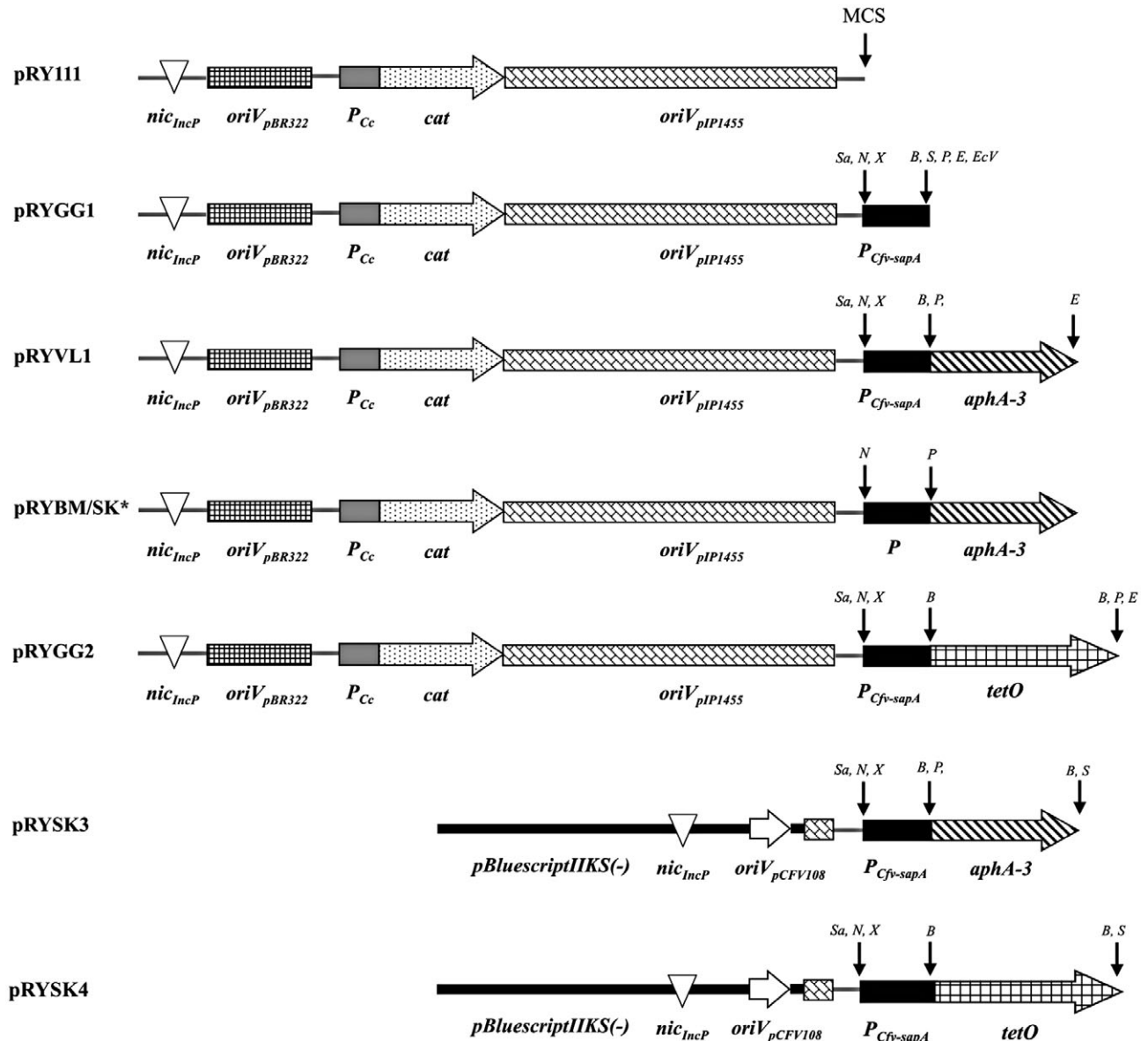
Fig. 1. Selected *E. coli-C. fetus* shuttle vectors based either on the pIP1455 or on the pCFV108 replication origin (*oriV*). Functional modules are identified as follows: mobilization from *E. coli* to *Campylobacter* (*nic*$_{IncP}$); replication in *E. coli* (*oriV*$_{pBR322}$, *pBluescriptIIKS(-)*), *Campylobacter* (*oriV*$_{pIP1455}$), or *C. fetus* (*oriV*$_{pCFV108}$); antibiotic selection for chloramphenicol (*cat*), kanamycin (*aphA-3*), tetracycline (*tetO*); promoters for gene expression in *E. coli* P_{Cc} (*C. coli* promoter) and *C. fetus* ($P_{sapA-Cfv}$, P: *gatC, rpsJ, glnA, gltA, gyrA*). Restriction enzymes for cloning are indicated. Abbreviations: Sa, SacI; N, NotI; X, XbaI; B, BamHI; S; SmaI; P, PstI; E, EcoRI; EcV, EcoRV.

strain 45104 (Waterman *et al.*, 1993), and the *C. fetus* ssp. *venerealis* plasmid pCFV108 (Kienesberger *et al.*, 2007). The pIP1455 derivatives are universally used for *C. jejuni* and *C. coli* applications. The pCFV108 replicon is restricted to *C. fetus*. A 4.5 kb fragment of pIP1455 DNA is required for vector derivatives to be efficiently replicated in campylobacter. By contrast, the minimal replicon of pCFV108 is comprised of only 82 bp. These vector lineages are stably maintained in all the *C. fetus* strains we have tested thus far. Importantly, both replicons remain stable in the absence of antibiotic selection. That property

is advantageous for *in vitro* experiments involving co-cultivation of plasmid-carrying bacteria and cultured mammalian cells over days (> 6 days), or in reviving frozen stocks of plasmid-carrying *C. fetus* on blood agar without selection. It is also significant that the pIP1455 and the pCFV108 vector lineages are compatible for stable maintenance in the same host cell, thereby diversifying options for expression of molecular reporters and genes of interest.

Campylobacter fetus acquires plasmid DNA efficiently only via conjugative mobilization (Kienesberger *et al.*,

2007). MOB_P conjugation functions expressed by *E. coli* [e.g. *E. coli* S17-1lpir (de Lorenzo and Timmis, 1994) harbouring RP4 transfer genes on its chromosome] work well for shuttle vector mobilization to *C. fetus* and other campylobacters as long as these carry a P-like *nic* site for conjugative DNA processing (Pansegrau and Lanka, 1991; Francia *et al.*, 2004) (Fig. 1). The type strains *C. fetus* ssp. *venerealis* ATCC 19438 and *C. fetus* ssp. *fetus* ATCC 27374 take up DNA readily, but *C. fetus* field isolates are somewhat harder to manipulate genetically. Nonetheless, only one of eight *C. fetus* field isolates, *C. fetus* ssp. *venerealis* strain 4111/108 (Hum *et al.*, 1997), proved completely resistant to DNA uptake and plasmid vector maintenance thus far in our experiments. This strain already carries at least three natural plasmids including the original cryptic plasmid pCFV108, thus the limiting factor in this case may be stable maintenance or physiological stress of the host cell due to the high plasmid load. Little is known concerning naturally occurring plasmids in *C. fetus*. We surveyed the plasmid profiles of 34 *C. fetus* isolates. From 17 *C. fetus* ssp. *fetus* isolates four carried large ($>$ 25 kb) plasmids and one small plasmid. Among the *C. fetus* ssp. *venerealis* isolates 15 of 17 harbour large plasmids and another three were found to carry a small plasmid. The presence of large plasmid genomes does not generally limit shuttle vector uptake.

Over 90% of all the *C. fetus* ssp. *venerealis* strains tested thus far ($n = 67$) harbour genes carried by a subspecies-specific genomic island on the chromosome which encode a bacterial T4SS (Gorkiewicz *et al.*, 2010). The T4SS of *C. fetus* ssp. *venerealis* exhibits the most apparent synteny and homology with the resistance plasmids pCC31 of *C. coli* and pTet of *C. jejuni* (Batchelor *et al.*, 2004; Friis *et al.*, 2007). As described in the previous section, this T4SS supports intra- and interspecies mobilization of plasmids based on pIP1455 (S. Kienesberger, G. Gorkiewicz, A. Fauster and E.L. Zechner, unpublished) and is therefore useful for disseminating plasmids from *C. fetus* ssp. *venerealis* donor strains to other campylobacters and *E. coli* recipients. On the other hand, in some experiments, plasmid spread from a *C. fetus* ssp. *venerealis* strain to other (plasmid free) strains may be undesirable. In such cases, pCFV108-derived vectors are preferable as these are not mobilized by the *C. fetus* ssp. *venerealis* T4SS at detectable frequencies.

Plasmid DNA isolation from *C. fetus* is typically inefficient resulting in low yields and frequent nuclease degradation. Verification that a shuttle vector is maintained extrachromosomally in a *C. fetus* transconjugant is most efficient using a plasmid rescue strategy. To this end, *E. coli* cells are transformed with DNA preparations obtained from the putative *C. fetus* transconjugants. The emergence of *E. coli* transformants and subsequent plasmid isolation from the surrogate host proves the plasmid's extrachromosomal maintenance in *C. fetus*. Once confirmed, routine checks for plasmid maintenance in *C. fetus* are possible via positive selection or PCR amplification of plasmid genes. Spontaneous mutations to antibiotic resistance in *C. fetus* colonies occur very rarely. Moreover, we have never observed that shuttle vectors become integrated in the chromosome.

Gene expression

Unlike *C. jejuni* and *C. coli*, which tolerate exogenous promoters derived from non-related bacterial species (e.g. *E. coli*), *C. fetus* shows a higher stringency and efficient gene expression depends on the presence of a *C. fetus*-specific promoter. Until recently, the *sapA* promoter, which is present on the chromosomes of both subspecies, was the only characterized promoter known to be active in *C. fetus* (Dworkin *et al.*, 1997; Tu *et al.*, 2003). Two important characteristics of this promoter limit its use as an efficient genetic tool. First, gene-specific inactivation using a suicide vector that depends on the *sapA* promoter is subject to a high frequency of integration at alternative sites (see below). Second, the activity of the *sapA* promoter is temperature dependent. The optimal temperature appears to be around 37°C. Low gene expression was observed at 32°C (S. Kienesberger, G. Gorkiewicz, A. Fauster and E.L. Zechner, unpublished).

Options for controlled gene expression in *C. fetus* were improved by a recent screening for promoter fragments active in *C. fetus* ssp. *venerealis* under laboratory conditions. The 5′ regulatory regions of putative housekeeping genes (Dingle *et al.*, 2001) predicted to support constitutive expression (*gatC, rpsJ, glnA, gltA, gyrA*) were identified on the *C. fetus* ssp. *fetus* genome sequence and test constructions for promoter activity were prepared by exchange of the *sapA* promoter from pRYVL1 (Fig. 1). The putative regulatory regions were amplified via PCR and introduced upstream of a *aphA-3* gene from the *C. coli* plasmid pIP1433 (Trieu-Cuot *et al.*, 1985). *aphA-3* encodes 3′-amino-glycoside phosphotransferase, thus expression of this reporter confers kanamycin resistance. These recombinant plasmids were introduced into *C. fetus* ssp. *venerealis* and promoter activity was verified by antibiotic selection. The availability of alternative promoters provides us with a larger range of tractable conditions for gene expression and mutational analysis as described below. The temperature dependence of the *sapA* promoter provides an option for controlled gene expression. By contrast, the temperature independence of the *gatC* promoter is compatible with complementation studies performed at lower temperatures (S. Kienesberger, G. Gorkiewicz, A. Fauster and E.L. Zechner, unpublished). Phenotypic expression of resistance or molecular

reporter genes in *C. fetus* has been demonstrated using *aphA-3*, the chloramphenicol acetyltransferase (*cat*) gene obtained from the *C. coli* plasmid pNR9589 (Wang and Taylor, 1990), the tetracycline resistance (*tetO*) gene originating from the *C. jejuni* plasmid pUA466 (Manavathu *et al.*, 1988), and *gfp* (Miller *et al.*, 2000; Kienesberger *et al.*, 2007).

Mutational analyses

Molecular genetics approaches require tractable general and site-specific gene inactivation. Suitable strategies based on transposon mutagenesis and homologous recombination were developed for *C. jejuni* and *C. coli* (Labigne-Roussel *et al.*, 1988; Yao *et al.*, 1993; Golden *et al.*, 2000; Colegio *et al.*, 2001; Karlyshev and Wren, 2005), but are much less advanced in *C. fetus* applications. The current lack of tools for transposition continues to be a disadvantage. On the other hand, sequence-specific gene disruptions are now more readily obtained via recombination. *Campylobacter fetus* supports *recA*-mediated homologous recombination as well as *recA*-independent mechanisms (Ray *et al.*, 2000). A facile approach for recombinant engineering of *C. fetus* ssp. *fetus* (Dworkin and Blaser, 1997) and *C. fetus* ssp. *venerealis* strains (Gorkiewicz *et al.*, 2010) replaces the gene of interest with a selectable phenotypic marker via flanking homologous sequences. The targeting DNA for gene insertion is delivered conjugatively to *C. fetus* via a suicide plasmid not proficient for replication. Although 400 bp of homologous DNA on each side of the selectable marker can be sufficient for targeted gene insertion, the frequency of obtaining the desired gene disruption is enhanced substantially when the length of each homologous flank is extended to 1 kb. The selectable gene cassette, e.g. antibiotic resistance genes *aphA* or *tetO*, requires a *C. fetus* promoter. Constitutively expressed examples include *rpsJ* or *gatC*.

Application of cassette-specific PCR primers in combination with chromosome sequence outside of the targeting DNA is sufficient to verify the specificity of the gene disruption. Southern analyses of chromosomal DNA obtained from mutants routinely indicate single cassette insertions in our experiments. We have noted a pronounced strain variation in the efficiency of mutagenesis of *C. fetus*. In strains with a high frequency of site-specific insertions, e.g. *C. fetus* ssp. *venerealis* 84-112, complementation of the gene defect *in trans* is sometimes hard to achieve. Further analysis revealed that in these cases, wild-type levels of gene function are not obtained because the complementing gene on the vector and the chromosome undergo recombination ultimately disrupting expression. Thus for efficiently recombinogenic strains, limiting the length of the experiment worked best for effective complementation. A

promising alternative for constitutive expression may be integration of the complementation cassette in a non-essential chromosomal gene, as shown previously for *C. jejuni* (Watson *et al.*, 2007).

In vitro virulence assays

The biology of *C. fetus* infection is poorly defined. Molecular studies analysing *Campylobacter*–host interactions have focused predominantly on the species *C. jejuni* as this organism is the main cause of bacterial diarrhoea worldwide (Allos, 2001). No evidence is available to indicate whether *C. fetus* employs similar virulence strategies. In addition to colonizing mucosal tissues, which leads to enteritis and genital tract infections, both subspecies cause invasive extraintestinal infections and bacteraemia (Blaser *et al.*, 2008). The molecular determinants underlying the difference in host and tissue preference of *C. fetus* in relation to the mainly 'enteric' *C. jejuni* are not known. General features like the spiral morphology and the pronounced corkscrew-like motion are typical for all *Campylobacters* and prerequisites for the effective colonization of mucous membranes. These attributes have been directly correlated to gut colonization potential and severity of disease (Nachamkin *et al.*, 1993; Yao *et al.*, 1994; Chang and Miller, 2006; Malik-Kale *et al.*, 2007). Mucosal infection also requires motility for the organism to gain access to exposed host cells (e.g. endometrium).

Epithelial cell attachment and invasion are virulence traits employed by several pathogens including *Campylobacter*. Several lines of evidence demonstrate that *C. jejuni* invades intestinal epithelial cells including histological examinations of intestinal biopsies from patients with enteritis (van Spreeuwel *et al.*, 1985), animal models (Newell and Pearson, 1984; Babakhani *et al.*, 1993; Russell *et al.*, 1993; Yao *et al.*, 1997) and *in vitro* experiments with cultured human intestinal epithelial cells (De Melo *et al.*, 1989; Konkel and Joens, 1989; Oelschlaeger *et al.*, 1993). According to current understanding, *C. jejuni* invades nonphagocytic intestinal epithelial cells via a microtubule and caveolae-dependent process, persists in the so-called *Campylobacter*-containing vacuole (Watson and Galan, 2008) and is later found in a close association with the Golgi apparatus. Several molecular determinants involved in adhesion and invasion have been identified so far (Hu and Kopecko, 2008; Larson *et al.*, 2008). Among them a unique fibronectin-dependent attachment to the basolateral surface of epithelial cells via the CadF protein have been described for *C. jejuni* (Konkel *et al.*, 2000; Kopecko *et al.*, 2001). Mutagenesis applied to hyper-invasive clinical isolates offer a promising strategy to understand mechanisms contributing to invasion. The heightened activity in such isolates provide a wider range

to detect reduced phenotypes and thus identify larger gene sets contributing to this activity (Javed *et al.*, 2010).

A sheep model of *C. fetus* infection investigating ovine abortion was used to establish the role of the S-layer in pathogenesis (Grogono-Thomas *et al.*, 2000; 2003; Tu *et al.*, 2005). Although the *C. fetus* S-layer is essential for establishment and persistence of a systemic infection, it was clearly not the contributing factor of the feto-pathogenic effects resulting in abortion (Grogono-Thomas *et al.*, 2000). As *sap* expression is shared by both subspecies, the divergent nature of the virulence attributes of both subspecies remains unknown. A *C. fetus* – SCID mouse model of chronic gastric infection was also reported, wherein chronic atrophic gastritis develops with a marked inflammatory response mimicking chronic gastritis caused by *H. pylori* (Young *et al.*, 2000). Strikingly, no evidence of physical contact between bacteria and host, nor direct bacterial invasion of epithelial cells was observed microscopically in this study.

In vitro systems that adequately model the niche preferences of *C. fetus* are lacking. Fortunately, tractable cell culture-based approaches are emerging to support an initial investigation of host–pathogen interactions and analysis of the molecular determinants involved. *Campylobacter fetus* ssp. *fetus* adheres, invades and replicates within the epithelial cell line Int407 (Graham, 2002). Moreover, fibronectin was shown to enhance *C. fetus* interaction with extracellular matrix components and Int407 cells (Graham *et al.*, 2008). The two sequenced *C. fetus* genomes carry homologues to *C. jejuni cadF* (G. Gorkiewicz and E. Zechner, unpublished data), thus an adherence mechanism similar to *C. jejuni* may represent a shared virulence attribute. Current efforts with *C. fetus* focus on assay development relying on well-documented *C. jejuni* properties and reference strains. Subsequent extension to less well-characterized endothelial and placental cells of human and bovine origin will be important to more closely model natural niche conditions for *C. fetus*. In the section below we summarize progress made thus far in studying the shared and divergent virulence properties of *C. fetus* subspecies manifest *in vitro* in combination with a functional genomics approach.

Mammalian cell invasion

Commonly used gentamicin protection assays (Elsinghorst, 1994) were performed using semi-confluent or confluent monolayers of mammalian epithelial cell lines. Thus far all lines we have tested (Caco-2, Int407, HeLa, CHO-K1, ACH-3P) were invaded by *C. fetus in vitro* with human colonic Caco-2 cells reproducibly supporting the highest level of invasion (Gorkiewicz *et al.*, 2010). Mammalian cell lines are cultivated *in vitro* and *Campylobacter* are introduced at a moi of 100–500. Over a time-course up to 24 h,

the maximum of cell invasion occurs within the first 2 h of co-cultivation. The subsequent gentamicin treatment (400 μg ml^{-1}) is necessary for at least 2 h to kill all extracellular bacteria. Bacteria more efficiently invade semi-confluent monolayers than confluent monolayers. Invasion is also strain dependent and clinical isolates are generally more invasive than the *C. fetus* type strains (100-fold) (Fig. 2). The sequenced strains *C. fetus* ssp. *fetus* 82-40 and *C. fetus* ssp. *venerealis* 84-112 achieve invasion levels of about one bacterium per Caco-2 cell after 2 h of co-cultivation, comparable to that of *C. jejuni* (Fig. 2). Additionally, both subspecies invade the human chorioncarcinoma cell line ACH-3P (Hiden *et al.*, 2007).

The molecular mechanisms involved in *C. fetus* host cell invasion are largely unknown. However, analyses of the bacterial invasion phenotype are readily combined with mutational inactivation of putative virulence genes. The gentamicin protection assay can be easily performed and its application will facilitate a functional characterization of *C. fetus* virulence attributes similarly to previous investigations of host–pathogen interaction in other systems. For example disruption of genes carried by a *C. fetus* pathogenicity island and complementation of the mutant strain in *trans* was reported recently. In this study mutation of *virD4*, encoding an essential component of a bacterial Type IV secretion apparatus reduced invasiveness of strain *C. fetus* ssp. *venerealis* 84-112 by 50% compared with that of the wild-type parent strain (Gorkiewicz *et al.*, 2010). The extent of attenuation due to the *virD4* disruption is in line with invasion phenotypes observed in a mutational analysis of the pVir encoded T4SS components of *C. jejuni* (Bacon *et al.*, 2000; 2002). By contrast mutational inactivation of the *cdtB* gene, encoding the active subunit of the cytolethal distending toxin, did not result in an attenuated invasion phenotype (Gorkiewicz *et al.*, 2010).

Differential immunofluorescence staining

Intracellular *C. fetus* can be visualized using differential immunofluorescence staining (Fig. 3) as previously established for other pathogens (Elsinghorst, 1994; Dehio *et al.*, 1997; Pentecost *et al.*, 2006). A standard staining procedure for labelling intracellular bacteria (Srivastava and Isberg, 2002) was adapted for *C. fetus* using monoclonal anti-*C. fetus* serotype A antibodies (Brooks *et al.*, 2002) and Alexa Dye-conjugated secondary antibodies (Invitrogen). *Campylobacter fetus*-infected Caco-2 cells were treated sequentially with gentamicin, then with primary and secondary antibodies. To distinguish intracellular from extracellular bacteria the host cells were then permeabilized with methanol followed by restaining with the same primary but a different secondary antibody. Microscopy readily localizes intracellular bacteria within the infected mammalian cells when clinical or field iso-
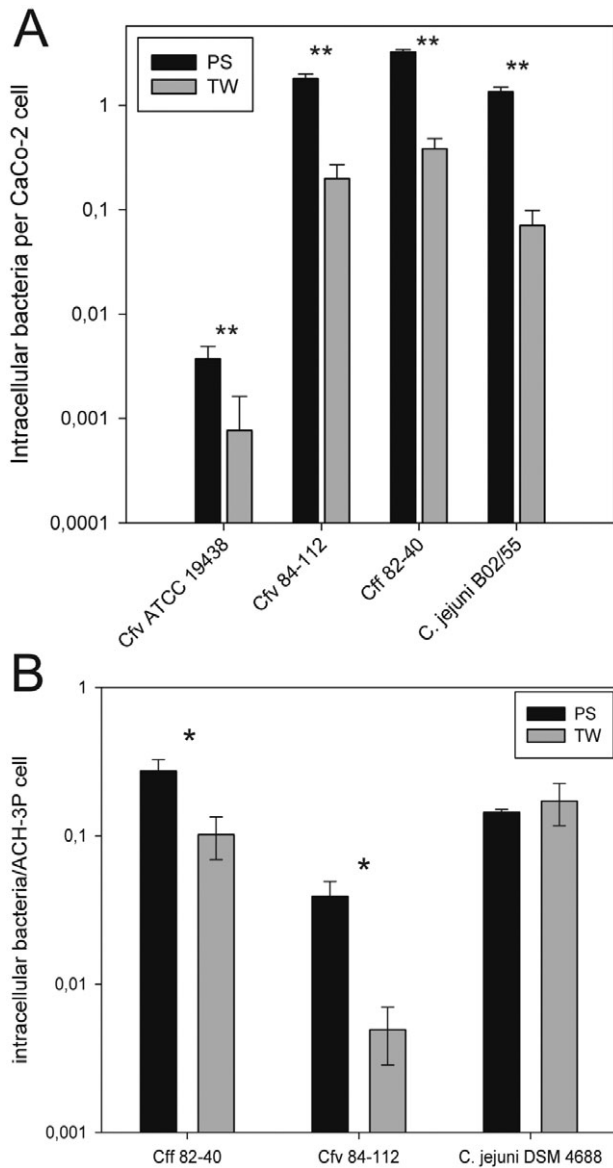
Fig. 2. Quantitative comparison of intracellular *Campylobacter* when Caco-2 cells or the human placental cell line ACH-3P were cultivated on a typical plastic substratum (PS) or on transwell filters (TW).

A. Values for invasion per Caco-2 cell reflect the number of bacteria surviving 2 h of 400 μg ml^{-1} gentamicin treatment relative to the number of exposed host cells after 2 h invasion time. Intracellular bacteria/Caco-2 cell: *C. fetus* ssp. *venerealis* ATCC19438 PS: 0004 ($\pm$0001); TW 0001 ($\pm$0001); *C. fetus* ssp. *venerealis* 84-112: PS 1,80 ($\pm$0,20); TW 0,20 ($\pm$0,07); *C. fetus* ssp. *fetus* 82-40: PS 3,23 ($\pm$0,17); TW 0,38 ($\pm$0,10); *C. jejuni* B02/55: PS 1,34 ($\pm$0,14); TW 0,07 ($\pm$0,03); **$P < 0.005$;
B. Values for invasion per ACH-3P cell reflect the number of bacteria surviving 2 h of 400 μg ml^{-1} gentamicin treatment relative to the number of exposed host cells after 3 h invasion time; *C. fetus* ssp. *fetus* 82-40: PS 0,27 ($\pm$0,05); TW 0,10 ($\pm$0,03); *C. fetus* ssp. *venerealis* 84-112 PS 0,04 ($\pm$0,01); TW 0,01 ($\pm$0,00); *C. jejuni* DSM 4688: PS 0,14 ($\pm$0,01); TW 0,17 ($\pm$0,05); *$P < 0.05$.

lates of either *C. fetus* subspecies are investigated. By contrast the type strains *C. fetus* ssp. *venerealis* ATCC 19438 and *C. fetus* ssp. *fetus* ATCC 27374 are non-invasive and serve as negative controls for intracellular visualisation of bacteria. An example of Caco-2 cells infected with the clinical isolate *C. fetus* ssp. *fetus* 82-40 is shown in Fig. 3. Intracellular bacteria are stained with red only. Solely green or yellow (red plus green) dually labelled bacteria (circle) identify cells in extracellular locations. This immunofluorescence approach combined with confocal microscopy is at an early stage yet it is proving informative for visualizing *C. fetus*–host cell interactions. The development of techniques including differential staining of cell compartments or structures will offer clearer, more detailed localization data for bacteria within infected cells (Watson and Galan, 2008). Moreover, the capacity to differentiate intracellular and extracellular bacteria is important to defining the phenotypes of mutants functionally deficient for host cell entry versus those defective in intracellular viability.

Bacterial migration: detection and quantification

In developing the intracellular imaging protocols we routinely observed foci of bacteria (Fig. 3, yellow arrowhead) appearing in the red channel, despite the fact that the location of these foci was actually extracellular in repeated experiments (verified by cross sectioning with the confocal microscope). This phenomenon revealed an unexpected proficiency of *C. fetus* for migration underneath the cell monolayer and raised the question of whether this ability is important for *C. fetus* virulence. Moreover, the observed migration was very likely to yield artificially high values for intracellular bacteria in conventional gentamicin protection assays. To quantitatively distinguish intracellular bacteria from those in the protected compartment underneath the mammalian monolayer we altered the cell culture substrate used in the cell invasion assays. To permit gentamicin penetration from both the apical and basal sides, transwell membrane inserts were used. Host cells were grown to confluence either in typical 24-well plastic tissue culture plates or on membranes (pore size 3 μm) inserted into such plates to allow application of gentamicin apically and basally to kill extracellular bacteria. The relative difference in surviving *C. fetus* as determined with standard culture dishes versus membrane inserts allows an assessment of the percentage of migrating cells. Remarkably we find clear evidence for gentamicin protection via bacterial migration not only for all the invasive *C. fetus* isolates tested thus far ($n = 4$) but also for the *C. jejuni* strain B02/55 (Fig. 2). Significantly higher numbers of bacteria (10–20-fold) survive gentamicin treatment when host cells were grown on a standard plastic substratum compared with the transwell system
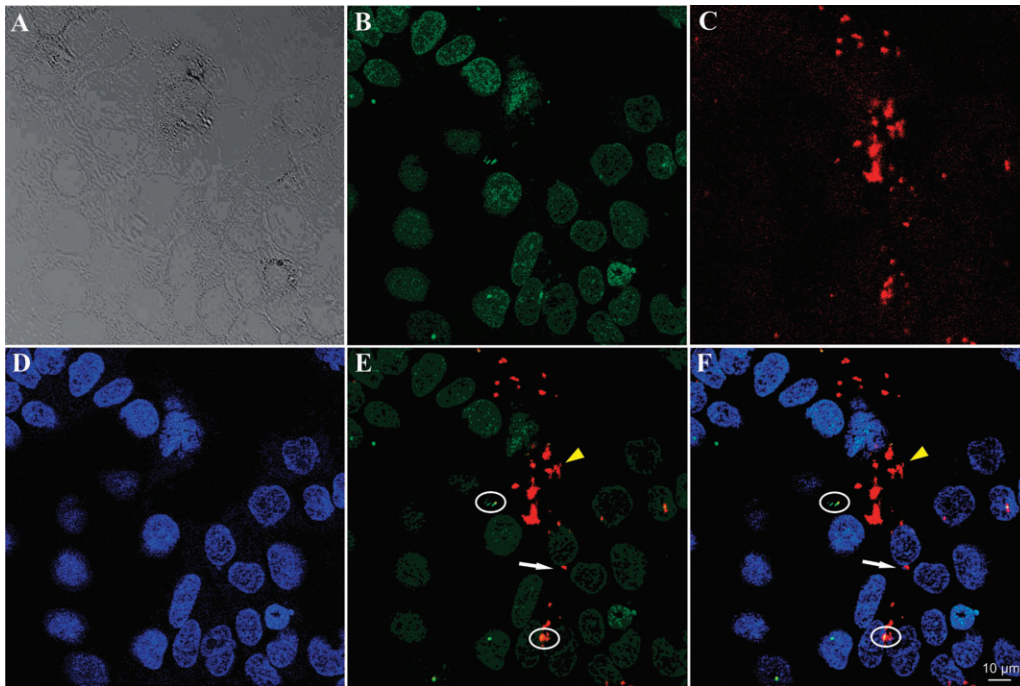
Fig. 3. Confocal microscopic visualization of intracellular bacteria. Caco-2 cells were cultured to confluency in chamber slides or on coverslips placed in 12-well tissue culture dishes. *Campylobacter fetus* ssp. *fetus* 82-40 (*Cff*) were added at moi = 500 for 14 h, treated with gentamicin for 2 h, then washed and fixed with 3% paraformaldehyde. Extracellular *Cff* cells were then labelled with a monoclonal mouse anti-*C. fetus* serotype A antibody (42), followed by an Alexa-488 conjugated goat-anti-mouse-antibody (B, green channel). Eukaryotic cells were permeabilized with 100% methanol for 10 s and *Cff* was again labelled with the monoclonal mouse anti-*C. fetus* serotype A antibody followed by an Alexa-633-conjugated goat-anti-mouse antibody (C, red channel). Caco-2 nuclei were stained with DAPI (D, blue channel). In this differential immunostaining approach intracellular *C. fetus* appear solely red, whereas extracellular bacteria are doubly stained (red and green), the combination appears yellow when superimposed (E and F).
A. Differential interference contrast.
B–D. Green, red and blue channels respectively.
E and F. Overlay of the green and red channels (E) and the green, red and blue channels (F). Intracellular bacteria localized in the vicinity of the nucleus (white arrow), putative bacterial subvasion (yellow arrowhead) and extracellular, doubly labelled bacteria (circles) are indicated (E, F). Scale bar, 10 µm. Negative controls were provided by the non invasive *C. fetus ssp. venerealis* ATCC 19438 and samples treated the same way, except for permeabilization, to confirm the integrity of the fixed cell membranes (not shown).

(Fig. 2). This clearly implies that artificially high numbers of bacteria are assigned an intracellular localization when standard gentamicin protection assays are applied to campylobacters. The migration underneath the host cells, also termed subvasion, has been observed with *C. jejuni*, which subsequently migrates to the basolateral cell side prior to cell invasion (Van alphen *et al.*, 2008). Taken together it follows that studies using a polarized cell layer on appropriate transwell membrane inserts combined with microscopic techniques may provide mechanistic details concerning bacterial migration, attachment and invasion, which in turn will facilitate our understanding of *C. fetus* virulence.

Concluding remarks

Molecular microbiology has witnessed dramatic breakthroughs over the last decade in understanding the molecular basis of host interactions with bacterial pathogens. That in part is due to tremendous progress in

imaging technologies applied to bacteria/host cell interactions (e.g. high-resolution fluorescence microscopy) and to advances in functional genomics as well as in the capacity to decipher complex gene regulatory networks. Research aims with *Campylobacters* remain at a comparatively early stage, but are certain to benefit widely from these rapid technical developments in the field, and intensified efforts with *Campylobacters* specifically.

The small-sized genomes of Campylobacter (about 1.5 Mb) are in a process of gene decay (Fouts *et al.*, 2005) and nearly devoid of non-coding sequence, and thus represent a very attractive model for comparative genomic analyses. Moreover campylobacter genome sequences determined thus far reveal relatively little overlap with the virulence repertoire of other bacterial pathogens. An important prospective for the field therefore depends on an expanded database of genomic information to support intra- and inter-species comparisons among camplyobacters, as well as with other pathogens (e.g. *Campy*DB; Chaudhuri and Pallen, 2006). Those data provide the

resources to support a detailed analysis of their pathogenic potential. Based on our current knowledge priorities within such surveys are genes contributing to motility, cell surface structures and systems capable of modifying these, gene sets involved in host cell invasion and intracellular survival, as well as metabolic systems important to growth *in vitro* and in human and animal hosts. Comparative analyses provide the rationale for mutagenesis of interesting candidate genes selected because of their conservation, their uniqueness, their genome context, or their probable acquisition through HGT. Conversely, the genomic data vastly facilitate interpretation of the results of random mutagenesis in studies of multifaceted phenotypes such as pathogen- host cell interactions.

The host and tissue tropism of *C. fetus* subspecies offers a unique opportunity to understand the molecular bases of habitat selection of bacterial pathogens and consequently to understand the different disease entities arising from this adaptation. Much is still to be learned about the phenotypic differences distinguishing the subspecies. The availability of the genome sequence of *C. fetus* ssp. *venerealis* 84-112 in conjunction with *C. fetus* ssp. *fetus* 82-40 reveals that the major genotypic difference consists of horizontally acquired DNA. The remaining core genomes are nearly identical, consistent with the early proposal that *C. fetus* ssp. *fetus* and *C. fetus* ssp. *venerealis* are genetically clonal, wherein *C. fetus* ssp. *venerealis* represents a bovine clone of *C. fetus* ssp. *fetus* (van Bergen *et al.*, 2005). By contrast, *C. fetus* strains adapted to reptilian hosts appear to represent a more ancient clade (Dingle *et al.*, 2010). Exploration of the genetic basis underlying gut mucosal tropism versus tropism to the genital mucosa is expected to rapidly advance using in-depth comparative genomic analyses of the two genomes. A logical extension of the genome comparison would pursue the potential for cross-complementation of *C. fetus* structures and functions. This strategy is feasible for mutated single genes as well as in linked gene sets or functional modules specific to a given subspecies. For example, the recently reported subspecies-specific pathogenicity island of *C. fetus* ssp. *venerealis* harbours multiple genes potentially involved in mucosal infection (Gorkiewicz *et al.*, 2010).

To fully capitalize on the insights offered by the genomic comparison, an important immediate challenge for the field is the need to establish *in vitro* infection models reflecting *C. fetus* pathogenicity *in vivo* including an expansion into bovine cells of genital origin. A subsequent long-term goal must be to develop simple animal models of colonization and disease. Existing infection models in cattle and sheep are limiting in a number of practical ways. Successful adaptation of comparatively powerful systems such as *C. elegans* or rodent models would mark a true breakthrough towards a mechanistic understanding of *C. fetus* infection.

Acknowledgements

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