J Appl Genet 51(3), 2010, pp. 383–393

Original article

Campylobacter protein oxidation influences epithelial cell invasion or intracellular survival as well as intestinal tract colonization in chickens

A. M. Łasica[1*], A. Wyszyńska[1*], K. Szymanek[1,3], P. Majewski[2], E. K. Jagusztyn-Krynicka[1]

[1]Department of Bacterial Genetics, Institute of Microbiology, Biology Faculty, University of Warsaw, Warsaw, Poland
[2]Department of Vertebrate Physiology, Institute of Zoology, Biology Faculty, University of Warsaw, Warsaw, Poland
[3]Department of Medical Microbiology, Medical University of Warsaw, Warsaw, Poland

Abstract. The Dsb family of redox proteins catalyzes disulfide bond formation and isomerization. Since mutations in *dsb* genes change the conformation and stability of many extracytoplasmic proteins, and since many virulence factors of pathogenic bacteria are extracytoplasmic, inactivation of *dsb* genes often results in pathogen attenuation. This study investigated the role of 2 membrane-bound oxidoreductases, DsbB and DsbI, in the *Campylobacter jejuni* oxidative Dsb pathway. *Campylobacter* mutants, lacking DsbB or DsbI or both, were constructed by allelic replacement and used in the human intestinal epithelial T84 cell line for the gentamicin protection assay (invasion assay) and chicken colonization experiments. In *C. coli* strain 23/1, the inactivation of the *dsbB* or *dsbI* gene separately did not significantly affect the colonization process. However, simultaneous disruption of both membrane-bound oxidoreductase genes significantly decreased the strain's ability to colonize chicken intestines. Moreover, *C. jejuni* strain 81-176 with mutated *dsbB* or *dsbI* genes showed reduced invasion/intracellular survival abilities. No cells of the double mutants (*dsbB*$^-$ *dsbI*$^-$) of *C. jejuni* 81-176 were recovered from human cells after 3 h of invasion.

Keywords: *Campylobacter*, colonization, disulfide bonds, Dsb proteins, invasion, oxidoreductase.

Introduction

Campylobacter strains are a major cause of food-borne gastrointestinal diseases. Two *Campylobacter* species (*C. jejuni* and *C. coli*) are of particular interest since they together account for over 95% of human *Campylobacter* infections. The majority of sporadic human cases of campylobacteriosis are associated with the consumption of undercooked poultry. However, *Campylobacter* asymptomatically colonizes a variety of warm-blooded animals.

The elucidation of host and microbial factors that influence its pathogenicity and commensalism has undergone enormous progress due to recently advanced technologies that allow global analysis of this bacterium; yet we are still far from its complete understanding (Humphrey et al. 2007; Young et al. 2007; Janssen et al. 2008; Zilbauer et al. 2008).

In Gram-negative microorganisms, many periplasmic, membrane-bound or secreted proteins that contain 2 or more cysteine residues acquire their proper structure as a result of disulfide bridge formation or their rearrangement. This process is catalyzed by the Dsb (disulfide bond) family of redox proteins and takes place in the oxidative environment of the bacterial periplasm.

*A. Łasica and A. Wyszyńska contributed equally to this work

Received: October 28, 2009. Revised: February 26, 2010. Accepted: March 1, 2010.
Correspondence: E.K. Jagusztyn-Krynicka, Department of Bacterial Genetics, Institute of Microbiology, Biology Faculty, University of Warsaw, Miecznikowa 1, 02–096 Warsaw, Poland; e-mail: kjkryn@biol.uw.edu.pl

The most extensively studied disulfide oxidoreductases include those of *E. coli*, which are involved in 2 pathways: the oxidative pathway (DsbA, DsbB) and the isomerization pathway (DsbC, DsbD). The periplasmic DsbA protein is an enzyme capable of direct transfer of its disulfide bond onto proteins just after their translocation through the cytoplasmic membrane. DsbA is maintained in its oxidized, active form by a membrane-bound electron acceptor, the DsbB protein. DsbB, in turn, is reoxidized by a mechanism that involves components of the respiratory pathway. The function and structure of *E. coli* Dsb proteins have been thoroughly reviewed by Masip et al. (2004) and Messens and Collet (2006).

Proteins of the Dsb family are crucial for the correct folding and activity of numerous pathogenic determinants (Lasica and Jagusztyn-Krynicka 2007). Detailed research shows that the Dsb oxidative pathways in many microorganisms are diverse and do not resemble the one described for the laboratory *E. coli* strain. Genomes of some bacterial species encode several DsbB and/or DsbA homologues, whereas genomes of other species encode either DsbA or DsbB but not both. Such differences have been seen in *E. coli*, *Salmonella enterica*, *Neisseia meningitides*, *Listeria monocytogenes*, *Staphylococcus aureus* or *Helicobacter pylori* (Lasica and Jagusztyn-Krynicka 2007; Heras et al. 2009). Additionally, there are some microorganisms that do not have any component of the Dsb system (Dutton et al. 2008).

We still do not understand fully why some bacterial species need the activity of only one DsbA periplasmic oxidoreductase for the correct folding of various proteins, while other species require 2 or more DsbA homologues. Elucidation of the cellular network of interactions between DsbA and DsbB in many pathogenic bacteria also requires further investigation.

The *Campylobacter jejuni* Dsb oxidative system is relatively complex, as it comprises, depending on the strain, 4–5 proteins: 3 membrane-bound proteins (accessory protein Dba and oxidoreductases DsbB and DsbI), and 1–2 periplasmic proteins (DsbA1 and DsbA2). So far, the DsbI protein has been identified in the proteomes of 24 microbial species. Fifteen of those species are pathogenic to humans or animals and 13 are members of Epsilonproteobacteria (Table 1). Insertional inactivation of the *H. pylori* SS1 *dsbI* gene results in reduced capacity for colonization of the mouse stomach (Godlewska et al. 2006).

The nomenclature of Dsb proteins is inconsistent and possibly confusing. We have previously characterized and described a paralog of DsbB from *H. pylori* and *C. jejuni*, which we named DsbI. However, no DsbA-like partner has so far been found for DsbI. The protein AAN82231 from a pathogenic *E. coli* strain CFT073 has recently been described as a member of the DsbI family (Grimshaw et al. 2008), but we have found that AAN82231 shares no characteristic features with DsbI proteins (Pawlowski et al. 2009).

In this report, we present evidence that disruption of the *Campylobacter jejuni/coli* Dsb oxidative pathway significantly impairs both colonization of the chicken intestinal tract and the interaction of the pathogen with the human intestinal cell line T84.

Materials and methods

Bacterial strains, plasmids, cell lines, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2. The *C. jejuni* strain 81–176 was a gift from M. J. Blaser (Korlath et al. 1985). The *C. coli* strain 23/1, used in the colonization experiment, was a broiler-isolated strain carrying the pUOA18 plasmid containing the *cat* gene (Wyszynska et al. 2004). *Campylobacter* strains were routinely cultured in microaerobic conditions on Blood Agar Base No2 (BA) containing 5% horse blood or on Mueller-Hinton agar supplemented with appropriate antibiotics: kanamycin (25 μg mL^{-1}) and/or chloramphenicol (15 μg mL^{-1}) under microaerobic conditions at 37°C or 42°C. The *E. coli* strains used for recombinant plasmid construction were cultured on LB agar plates or in LB broth supplemented with antibiotics: ampicillin (100 μg mL^{-1}), kanamycin (25 μg mL^{-1}) or chloramphenicol (20 μg mL^{-1}).

The human intestinal epithelial T84 cell line was obtained from the European Collection of Cell Culture (ECACC No. 8802101) and cultivated in DMEM (Dulbecco's Modified Eagle's Medium) and F12 (1:1) supplemented with 10% fetal bovine serum containing penicillin (60 μg mL^{-1}) and streptomycin (10 μg mL^{-1}). Cultures were maintained at 37°C in a humidified atmosphere with 6% CO_2.

Table 1. Proteomes in which the DsbI protein is present (updated in October 2009)

Strain	Pathogenic in	DsbI paralogs
Epsilonproteobacteria		
Campylobacter		
C. coli RM2228***	animals	1
C. concisus 13826	humans	1*
C. curvus 525.92	humans	1
C. fetus subsp. *fetus* 82–40	humans, animals	1
C. jejuni RM1221	humans	1
C. jejuni subsp. *doylei* 269.97	humans	1
C. jejuni subsp. *jejuni* 260.94***	humans	1
C. jejuni subsp. *jejuni* 81116	humans	1
C. jejuni subsp. jejuni 81-176	humans	1
C. jejuni subsp. *jejuni* 84-25***	humans	1
C. jejuni subsp. *jejuni* CF93-6***	humans	1
C. jejuni subsp. *jejuni* CG8421***	humans	1
C. jejuni subsp. *jejuni* CG8486***	humans	1
C. jejuni subsp. *jejuni* HB93-13***	humans	1
C. jejuni subsp. *jejuni* NCTC 11168	humans	1
C. lari RM2100	animals	2
C. rectus RM3267***	humans	4
C. showae RM3277***	humans	1
C. upsaliensis RM3195***	animals	1
Helicobacter		
H. acinonychis str. *Sheeba*	animals (cats)	1
H. canadensis MIT 98-5491***	humans	1
H. pullorum MIT 98-5489***	humans	1
H. pylori 26695	humans	1
H. pylori 98-10***	humans	1
H. pylori B128***	humans	1
H. pylori G27	humans	1
H. pylori HPAG1	humans	1
H. pylori HPKX_438_AG0C1***	humans	1**
H. pylori J99	humans	1
H. pylori P12	humans	1
H. pylori Shi470	humans	1
Actinobacteria		
Corynebacterium		
C. diphteriae NCTC 13129	humans	1
Gammaproteobacteria		
Aggregatibacter		
A. actinomycetemcomitans HK1651***	humans	1
Photobacterium		
Photobacterium sp. SKA34***	nonpathogenic	1*
Shewanella		
S. baltica OS155	nonpathogenic	1
S. halifaxensis HAW-EB4	nonpathogenic	2
S. pealeana ATCC 700345	nonpathogenic	1
S. woodyi ATCC 51908	nonpathogenic	1
Shewanella sp. MR-4	nonpathogenic	1
Shewanella sp. MR-7	nonpathogenic	1
Vibrio		
V. angustum S14***	nonpathogenic	1*

*Proteins that are nonfunctional or devoid of N-terminal fragment containing CXXC motif, which may result from unfinished assembly of this part of genome (proteins denoted as putative); **Protein divided into N-terminal domain, with CXXC motif, and C-terminal domain; may be nonfunctional or needs additional assembly (protein denoted as putative); ***Unfinished genomes (http://www.ncbi.nlm.nih. gov/genomes/lproks.cgi)

Table 2. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Origin
Strains		
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1lac* [*F' proAB lacIqZ*Δ *M15* Tn*10* (*tetr*)]	Stratagene
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17* (*rK- mK+*)*supE44* Δ*lacU169* F' (Φ*80dlacZ*M15)	Gibco BRL
Campylobacter jejuni AR1	*C. jejuni* 81-176 *dsbI*::Cm	(Raczko et al. 2005)
C. jejuni AR2	*C. jejuni* 81-176 *dsbB*::Km	(Raczko et al. 2005)
C. jejuni AR3	*C. jejuni* 81-176 *dsbI*::Cm; *dsbB*::Km	(Raczko et al. 2005)
Campylobacter coli 23	wild type, obtained from chicken carcass rinse	(Wyszynska et al. 2004)
C. coli 23/1	*C. coli* 23 harbouring pUOA18 (Cm^r)	(Wyszynska et al. 2004)
C. coli AR1	*C. coli* 23 *dsbI*::Cm	This study
C. coli AR2	*C. coli* 23 *dsbB*::Km	This study
C. coli AR3	*C. coli* 23 *dsbI*::Cm; *dsbB*::Km	This study
Plasmids		
pUOA18	Cm^r Ap^r; shuttle vector *C. coli*/*C. jejuni*/*E. coli*	(Wang and Taylor 1990)
pUWM466	pBluescript II SK/*dsbI*::Cm *C. jejuni*	(Raczko et al. 2005)
pUWM607	pGEM-T Easy/*dsbB*::Km *C. jejuni*	(Raczko et al. 2005)

Protein analysis

The total proteins expressed by *C. jejuni* were analyzed by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot techniques. Proteins were resolved by electrophoresis in 12% polyacrylamide gel containing SDS and were electrotransferred onto nitrocellulose membrane. The blot was developed using rabbit anti-CadF serum kindly provided by Prof. J.M. Bolla or rabbit anti-rDsbI as primary antibodies and anti-rabbit IgG conjugated to AP (Sigma) as secondary antibodies.

Invasion assay

The gentamicin protection assay (or invasion assay) was performed using human intestinal epithelial T84 cell line grown in 24-well tissue culture trays. Semiconfluent monolayers of about 2×10^5 cells/well were washed 3 times with DMEM/F12 supplemented with 1% fetal bovine serum (FBS), resuspended in the same medium, and then infected with *C. jejuni* cells to achieve multiplicity of infection (MOI) of 100–200. After the invasion period (3 h incubation at 37°C in 6% CO_2) the monolayers were rinsed 3 times with DMEM and incubated for additional 2 h with DMEM/F12 (1:1) supplemented with 1% FBS containing gentamicin (250 μg mL^{-1}) to kill extracellular bacteria. After incubation, the cell monolayer was rinsed 3 times with phosphate buffered saline (PBS). Epithelial cells were lysed for 30 min with saponin (0.1%, w/v). The number of internalized bacterial cells was enumerated by plating serial lysate dilutions on BA with 5% horse blood.

Colonization assay

Groups of commercial chickens (N = 5; Hy Line breed) obtained from a local hatchery on the day of hatch, were individually inoculated orally at day 1 of age with *C. coli* wild-type strain 23/1, which harbors pUOA18, and its mutants: *dsbB*$^-$, *dsbI*$^-$, and *dsbB*$^-$*dsbI*$^-$ (10^5 cfu mL^{-1} in 0.1 mL of PBS with 0.01% gelatin). Inocula were prepared by harvesting bacteria grown overnight at 42°C on BA plates. Cells were washed once with PBS with 0.01% gelatin and finally resuspended in the same buffer. Optical density was determined (A_{650}=0.6; 10^9 cfu mL^{-1}) and dilutions were made to achieve the density of about 10^6 cfu mL^{-1}. The number of cells was verified by plating. The level of colonization was determined on day 7 after inoculation. Chicks were euthanized by cervical dislocation, the intestine was excised, and *C. coli* cells present in chicken cecal contents were enumerated by plating serial dilutions on BA plates as described previously (Wyszynska et al. 2004). This procedure permitted detection of 10^2 cfu per g of cecal content. Before the challenge, 2 chicks from each experimental group were examined for *Campylobacter* colonization.

All studies involving animals were performed in accordance with ethical standards, after approval from the Local Ethic Committee No.1 Warsaw (Poland)(no. 338/2004).

Additional recombinant DNA techniques

Procedures for plasmid DNA isolation and DNA analysis were carried out as described by Sambrook and Russel (2001). Insertional inactivation of *C. coli* 23/1 *dsbB* and *dsbI* genes by allelic exchange was performed as previously described (Raczko et al. 2005). The expected disruptions of the chromosomal *loci* were verified by PCR amplification (primers: Cj16RS, Cj18LM, Cj864RX, Cj865RS, kanL, kanR, CM-L, CM-R). The loss of the wild-type *dsbI* gene product was also demonstrated by Western blotting of whole-cell proteins with specific anti-rDsbI antibodies. Specific anti-rDsbI serum was obtained by rabbit immunization with rDsbI purified from *E. coli* using affinity chromatography (unpublished).

Sequences of primers are given in Table 3.

Table 3. Oligonucleotide primers used in this study

Name	Primer sequence (5'→3')	Orientation	Restriction enzyme*	Origin
Cj16RS	GCAgtcgacTCAATGAAGGTACGAGTA	reverse	SalI	(Raczko et al. 2005)
Cj18LM	TATggatccCAGGAGCACTATTAACAATA	forward	BamHI	(Raczko et al. 2005)
Cj864RX	CGCtctagaAAGCAATGAATGTAAGTAA	forward	XbaI	(Raczko et al. 2005)
Cj865RS	CAGgtcgacCAATTATTTAAGACATCCTA	reverse	SalI	(Raczko et al. 2005)
Cj865RM	ATAggatcccccgggACgATTCCTATTTATCCATTTA	forward	BamHI, SmaI	(Raczko et al. 2005)
Cj883X2	AGTtctagaGCTAATGCAAAACTTGAATA	reverse	XbaI	this study
Cj18Nco	GGTAccatggAGTTTCTTGAACTTTTA	forward	NcoI	this study
Cc_dba_X	TATtctagaCATCGGATATGTCTTAGAGG	forward	XbaI	this study
Cc_dsbBR	GAAtctagaGAATATGGCTACTTGCTG	forward	XbaI	this study
Cc_dsbAL	TCCgtcgacAAGGCTTACAGGATAGAG	reverse	SalI	this study
kanL	TATCACCTCAAATGGTTCGCTGGG	reverse	–	this study
kanR	GGGGATCAAGCCTGATTGGGAGA	forward	–	this study
CM-L	ATATCACGCAATTAACTTGG	reverse	–	this study
CM-R	GGATGAATTACAAGACTTGC	forward	–	this study
CC1 (CC18F)	GGTATGATTTCTACAAAGCGAG	forward	–	(Linton et al. 1997)
CC2 (CC519R)	ATAAAAAGACTATCGTCGCGTG	reverse	–	(Linton et al. 1997)

*Marked in primer sequence with lower-case letters

Results

In silico analysis of *C. jejuni* and *C. coli* Dsb oxidative pathway regions

The analysis revealed strain-dependent differences in *dsb* genes organization. As a first step in studying the influence of the Dsb system on the *Campylobacter coli/jejuni* adaptation process, we scanned their genomes for presence of Dsb oxidative pathway genes and examined the organization of the surrounding region. The genome sequence of the first *Campylobacter* strain, a clinical isolate from the United Kingdom, NCTC 11168, was published in 2000 (Parkhill et al. 2000) and re-annotated in 2007 (Gundogdu et al. 2007). Currently genome sequences are known for 28 *Campylobacter* spp. (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). This number includes both completed genomes and genomes in progress. Most of the information is public. Among these genomes, there are 12 *C. jejuni* subsp. *jejuni* strains of clinical and veterinary origin and one genome of *C. coli*. Exploration for DsbA, DsbB and DsbI orthologs in *C. jejuni* and *C. coli* proteomes (Table 4) has shown a correlation in the occurrence of DsbA, DsbB, and DsbI. Two exceptions are *C. jejuni* CG8486 and *C. jejuni* BH-01-0142, both apparently lacking the DsbB protein, but their genomes are still being assembled. Moreover, genomes of *C. jejuni* strains have two *dsbA* genes. The first one, *dsbA1*, always encodes 213 amino acids (aa) and is a homolog of *dsbA* found in many other genomes. The product of the second one, *dsbA2,* is located before the 5' end of the *dsbB* gene and shares 51% amino acid identity with the DsbA1 protein sequence. However, it may occur in 2 forms – one functional and longer (usually 220aa) and the other nonfunctional and shorter (119aa) (Lasica and Jagusztyn-Krynicka 2007). In *C. jejuni* 81-176, DsbA2 is 28% identical to EcDsbA and has a CTHC motif, whereas DsbA1 is 23% identical to EcDsbA and has a CIHC motif (Heras et al. 2009). In the only *C. coli* genome sequenced so far (strain RM2228), a single *dsbA* gene is present. All *C. jejuni* and *C. coli* strains (apart from strain BH-01-0142) have single copies of the *dba* and *dsbI* genes (Table 4), which differentiates them from some other *Campylobacter* species. Figure 1

Table 4. Distribution of DsbI, DsbB, DsbA1 and DsbA2 proteins in *Campylobacter jejuni* and *C. coli* proteomes

Campylobacter strain	DsbI	DsbB	DsbA1	DsbA2
C. coli RM2228*	+	+	+	–
C. jejuni RM1221	+	+	+	+L
C. jejuni subsp. *doylei* 269.97	+	+	+	+L
C. jejuni subsp. *jejuni* 260.94*	+	+	+	+L
C. jejuni subsp. *jejuni* 81116	+	+	+	+L
C. jejuni subsp. *jejuni* 81-176	+	+	+	+L
C. jejuni subsp. *jejun*i 84-25*	+	+	+	+L
C. jejuni subsp. *jejuni* BH-01-0142*	–	–	–	–
C. jejuni subsp. *jejuni* CF93-6*	+	+	+	+S
C. jejuni subsp. *jejuni* CG8421*	+	+	+	+L
C. jejuni subsp. *jejuni* CG8486*	+	–	+	+L
C. jejuni subsp. *jejuni* HB93-13*	+	+	+	+S
C. jejuni subsp. *jejuni* NCTC 11168	+	+	+	+S

+/– protein is present/absent in *Campylobacter* strain; DsbA1 = classical DsbA, homolog of Cj0872 of *C. jejuni* NCTC 11168 or CJJ81176_0883 of *C. jejuni* 81-176; DsbA2 = additional copy of DsbA, with 2 forms: L= usually 220aa; homolog of CJJ81176_0883 of *C. jejuni* 81-176; S = 119aa; without CXXC motif; homolog of Cj0864 of *C. jejuni* NCTC 11168; *Unfinished genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi)

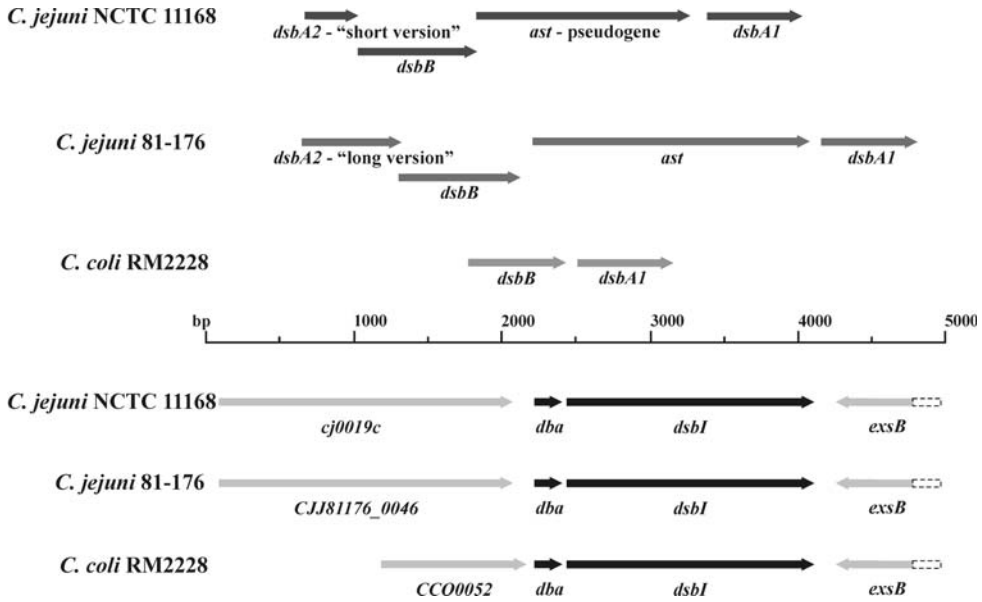

Figure 1. *Campylobacter* genome organization within the regions containing *dsbA*, *dsbB*, and *dsbI* genes: comparison of *C. jejuni* NCTC11168, *C. jejuni* 81–176, and *C. coli* RM2228. Both *C. jejuni* genomes contain two *dsbA* genes: *dsbA1*, common to all strains (213 aa), and *dsbA2*, differing in length (119 or 220 aa) in various strains. The single *dsbB* gene is preceded by the *ast* gene*. *C. coli* has only the *dsbA1* and *dsbB* genes in one short operon. Regions surrounding the *dsbA-B* genes differ significantly between strains. The regions containing the *dba-dsbI* genes are identical in all 3 strains . The gene *CCO0052* preceding *dba* in *C. coli* is not homologous to *CJJ81176_0046* and *cj0019c*.
*The arylsulfate sulfotransferase gene (*ast*) is annotated (NCBI genome data base) as "degenerate" in the *C. jejuni* 81–176 genome, but it was experimentally proved to be functional.

shows the organization of *C. jejuni* NCTC 11168, 81-176, and *C. coli* RM2228 genome regions containing the *dsbA1*, *dsbA2*, *dsbB*, *dba*, and *dsbI* genes.

Gentamicin protection assay

To investigate the influence of disulfide bond formation on *Campylobacter* cells, a standard gentamicin protection assay (T84 cell line) was performed. The invasion efficiency of *dsbI* and *dsbB* single mutants was reduced to 61% and 29%, respectively, compared to the wild-type strain. In contrast, the double mutant strain, in which both Dsb oxidative pathway membrane-bound proteins are inactivated, showed complete lack of ability to invade and/or survive inside T84 cells (Figure 2).

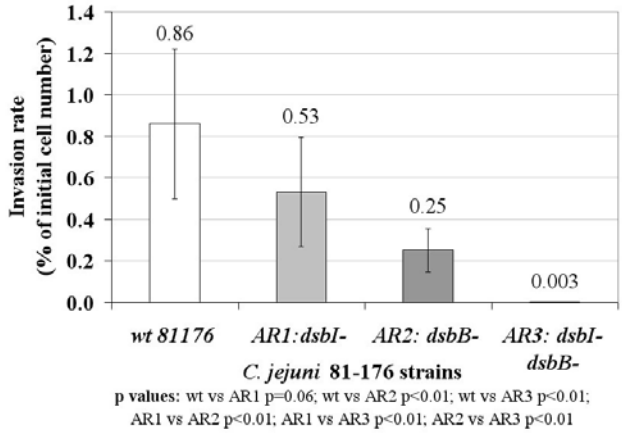

Figure 2. Effect of *dsbB* and *dsbI* gene inactivation on invasion of the human T84 cell line by *Campylobacter jejuni* 81–176 wild-type (wt) and mutant strains: AR1 = *dsbI*$^-$; AR2 = *dsbB*$^-$; AR3 = *dsbI*$^-$*dsbB*$^-$. (Data from 2 independent experiments, each performed in triplicate. *P* values of $P < 0.05$ were considered to be statistically significant and are given at the bottom of the diagram. Error bars indicate standard deviations)

Three *Campylobacter* proteins (JlpA, Peb1 and CadF) have been implicated to play an essential role in the adherence/invasion process (Pei et al. 1998; Jin et al. 2001; Monteville et al. 2003). As Peb1 contains only one cysteine residue (position 229), the activity of the Dsb system most likely does not affect its structure. The JlpA amino acid sequence contains 3 cysteine residues, so it can be a potential target of the Dsb proteins (positions 18, 60, and 69). The nucleotide sequence of the *C. jejuni* 81-176 *cadF* gene also revealed the presence of 3 cysteine residues in its product. The first cysteine (position 7) is cleaved by a signal peptidase during the processing of the protein, while 2 others (positions 191 and 206) can potentially form a disulfide bond. Thus the CadF-fold can also be affected by the activity of the Dsb proteins. To test this, we examined the presence of the fibronectin-binding protein in the defective (*dsbI*$^-$, *dsbB*$^-$, and *dsbI*$^-$*dsbB*$^-$) and wild-type cells by Western blotting with rabbit polyclonal anti-CadF antibodies. In all mutated strains, a 37-kDa protein reacting with anti-CadF was observed (Figure 3).

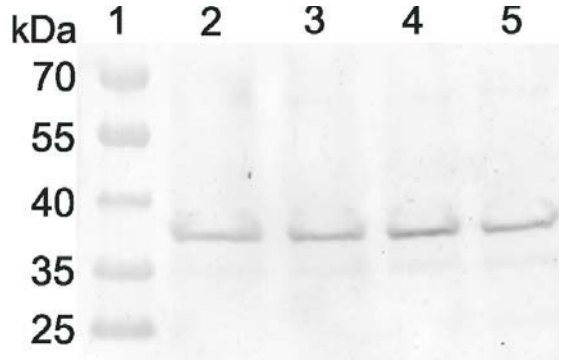

Figure 3. Detection of CadF protein in *Campylobacter jejuni* 81–176 wild-type and mutant strains. Protein extracts were resolved by SDS-PAGE (30 μg of the proteins per line), transferred to nitrocellulose, and reacted with specific anti-CadF serum. Lanes: 1 = molecular weight standard; 2 = wild-type; 3 = *dsbI*$^-$ mutant; 4 = *dsbB*$^-$ mutant; 5 = *dsbI*$^-$*dsbB*$^-$ mutant.

Colonization experiments

Colonization experiments were performed to test the influence of the Dsb oxidative pathway on adaptation of *Campylobacter* to the chicken intestinal tract. In contrast to the results presented by Hendrixon and DiRita (2004), we were not able to establish *C. jejuni* 81–176 colonization in chickens. We instead used the *C. coli* strain 23/1 for colonization experiments. It was obtained from a carcass rinse and has a high colonization potential in one-day-old chicks (Wyszynska et al. 2004). The presence of *dsbI* and *dsbB* genes in the *C. coli* 23/1 genome was confirmed by PCR experiments. Moreover, using PCR and primers complementary to *C. jejuni* and/or *C. coli* DNA sequences, we analyzed the genomic organization of the potential *C. coli* 23/1 operons encoding either *dba-dsbI* genes or *dsbA-dsbB* genes. Results indicated that the organization was identical to that encountered in *C. coli* RM2228 (data not shown). Insertional inactivation of *C. coli* 23/1 *dsbI* and *dsbB* genes was performed with recombinant plasmids (pUWM466 and pUWM607) previously employed for *C. jejuni* 81-176 mutagenesis (Raczko et al. 2005).

The colonization potential of wild-type and mutated *C. coli* strains was tested using a one-day-old chicken infection model. The experiment was preceded by studies showing that chicks

were not colonized by *Campylobacter.* The levels of colonization were assessed 7 days after the challenge. All chickens infected with the wild-type bacteria were colonized to high levels. By contrast, inactivation of *dsbB* and *dsbI* genes resulted in about 100-fold reduction of cecal colonization. Single mutants with only one gene disrupted, encoding the membrane-bound oxidoreductase, exhibited only a slightly reduced colonization ability (3 to 5-fold) relative to the wild type (Figure 4). Chicks gavaged with PBS with gelatin (negative control) did not have *C. coli* in their ceca 7 days after infection. Chromosomal (total) DNA from 5 isolates taken from every experiment was isolated and used for PCR amplification using 2 pairs of primers. The first pair, identical to the primers used for checking the accuracy of the mutagenesis process, was complementary to the nucleotide sequence of either the *dsbI* or *dsbB* gene. The second primer pair was specific for *C. coli* species (CC1 and CC2). All PCR tests were positive and resulted in generation of DNA fragments of expected sizes, confirming that the isolates were the progeny of the *C. coli* strains used for infection.

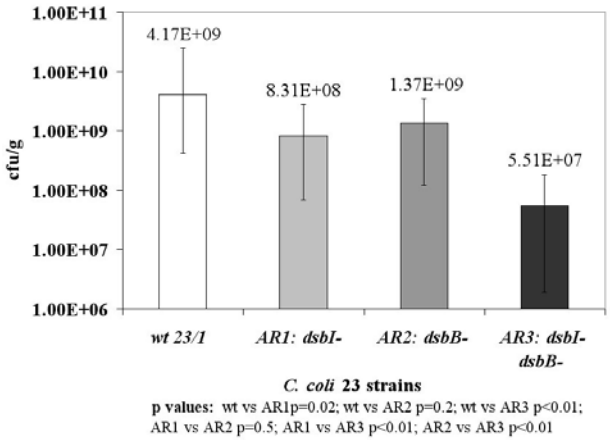

Figure 4. Colonization of chickens by *Campylobacter coli* 23/1 wild-type (wt) and mutant strains: AR1 = *dsbI*$^-$; AR2 = *dsbB*$^-$; AR3 = *dsbI*$^-$ *dsbB*$^-$. (Data from 2 independent experiments, each with 5 chicks for every *C. coli* strain. Data are given as the mean log cfu/g of cecal contents calculated from both experiments. *P* values of $P < 0.05$ were considered to be statistically significant and are given at the bottom of the diagram. Error bars indicate minimum and maximum values)

Discussion

Since post-translational protein modifications are often essential for structure and activity of many extracytoplasmic proteins, and since most of the virulence determinants are extracytoplasmic, disruption of the metabolic pathways involved in post-translational protein modification often results in pathogen attenuation. Recently, protein N-glycosylation has been shown to be an essential factor in pathogen adaptation. *Campylobacter* strains mutated in the *pgl* locus have a reduced capacity for invasion of human epithelial cells and are impaired in colonizing both mouse and chicken intestinal tracts (Karlyshev et al. 2004). In this study, we showed that disulfide bond formation is another post-translational protein modification that plays a key role in adaptation of *Campylobacter* to different ecological niches.

Epithelial cell invasion, a multifactorial process, is thought to be an essential step in *Campylobacter* infection. The internalization efficiency (i.e. efficiency of invasion) observed for *C. jejuni* strains differs significantly depending on the number of bacteria in the inoculum, the multiplicity of infection, and the cell line used. Even data from experiments performed on the same cell line and with the same *C. jejuni* strain vary significantly between laboratories. For example, the internalization efficiency of *C. jejuni* 81-176 by T84 cells as measured by Watson and Galan (2005) was 2.7%, compared to 0.05% reported by Monteville and Konkel (2002). The internalization efficiency in the present study was 0.8%, which is intermediate between those values. Differences in the efficiency of invasion observed between the wild-type and mutant strains resembled those observed with the Ellman test (Raczko et al. 2005), which assesses the level of periplasmic proteins containing –SH groups. The decrease in invasion was higher for the *dsbB*$^-$ mutant than for the *dsbI*$^-$ mutant; yet, only inactivation of both genes resulted in complete loss of invasion ability. The data indicate that only a small number of invasion factors are directly or indirectly DsbI-dependent or, alternatively, that the lack of DsbI activity can be, in most cases, complemented

by DsbB. The gentamicin protection assay was performed under standard conditions. After 3 h of infection and 2 h of gentamicin treatment, monolayers were lysed and total viable intracellular (invasion) bacteria were determined. The number of viable *Campylobacter* wild-type cells recovered from eukaryotic cells was similar to that observed by others (Watson and Galan 2008; Svensson et al. 2009). However, no intracellular bacteria were recovered in the case of the *dsbB*$^-$ *dsbI*$^-$ mutant, which suggests that the invasion process was disturbed. Alternatively, the lack of 2 membrane oxidoreductases may have quickly impaired the intracellular survival of *Campylobacter*.

In addition to invasion, inactivation of both membrane oxidoreductases (DsbB and DsbI) resulted in significant attenuation of the colonization process (about 100-fold reduction), at least in *C. coli* strain 23/1. Its genome has not been sequenced yet, but PCR experiments conducted in this study suggested the absence of the *dsbA2* gene. Thus, in contrast to *C. jejuni* 81-176, *C. coli* 23/1 contains only one functional DsbA protein, and its oxidative Dsb pathway can function differently from the Dsb oxidative pathway of *C. jejuni* 81-176. Our results indicate that colonization factors containing disulfide bridges might be dependent on the activity of both DsbI and DsbB. An alternative explanation might be that both membrane oxidoreductases cooperating with one DsbA can complement each other.

Motility is an important factor in *Campylobacter* pathogenesis and commensalism (Hendrixson and DiRita 2004; Kalmokoff et al. 2006). It has been shown previously that a mutated *C. jejuni* strain 81-176, which lacked the 2 membrane-bound oxidoreductases of the oxidative pathway, was completely immotile due to the absence of flagella, whereas its *dsbB*$^-$ mutant was motile. The *Campylobacter* flagellin A does not contain cysteine residues. Thus the lack of flagella, which potentially might result in decreased colonization and invasion abilities of *dsbI*$^-$ *dsbB*$^-$ mutants might be an indirect effect of the inactivation of the protein(s) taking part in flagellum biogenesis. A similar observation has been reported for *S. enterica* or *E. coli*, when proteomes of *dsbA*$^-$ mutant cells were analyzed (Agudo et al. 2004; Hiniker and Bardwell 2004). An analysis of amino-acid sequences of proteins participating in *Campylobacter* flagellum biogenesis for the presence and position of cysteine residues revealed 2 proteins (PflA and FlhA) that could potentially require Dsb protein activity to adopt a proper tertiary structure. The paralyzed flagellar (PflA) protein, due to the presence of a signal sequence, is assumed to be located outside the cytoplasm. The protein contains 6 cysteine residues. Mutants with a defective *pflA* gene are immotile but have a flagellum; cells with both genes mutated (*dsbI*$^-$ and *dsbB*$^-$) do not develop this organellum (Bleumink-Pluym et al. 1999; Raczko et al. 2005). This suggests that an additional protein is involved in flagellum assembly, which is post- translationally modified by the Dsb system. The FlhA protein belongs to the FHIPEP protein family (flagellar/Hr/invasion protein export pore), which is part of the protein transport system that translocates proteins participating in flagellum biogenesis, and components of the type III transport system with strongly conserved C- and N-terminal domains (Macnab 2003). FlhA contains 4 cysteine residues. It was reported that *flhA* mutant strains are devoid of flagella and are seriously impaired in respect of chicken colonization, invasion, and autoagglutination (Carrillo et al. 2004). Further studies are necessary to confirm the hypothesis that Dsb proteins influence the FlhA protein fold.

Our experiments indicate that motility is not the main invasion factor affected by *dsb* mutations, because the *dsbB* mutated strain showed a significantly reduced invasion capability, even though its motility was not different from the wild-type strain. Moreover, two *C. jejuni* strains isolated in Thailand (CG84486 and BH-01-0142) are not invasive, even though at least one of them potentially has an active flagellar apparatus. Interestingly, genomes of both strains probably lack the *dsbB* gene.

We found that disruption of the *dsbB* gene resulted in a significant reduction of invasion but did not affect the colonization process. This suggests that other proteins, which are targets of the Dsb oxidative pathway, might play a crucial role in *Campylobacter* pathogenesis and chicken intestinal tract colonization. Since different *Campylobacter* strains were used for invasion and colonization assays, this conclusion should be treated with caution. Preliminary results of comparative proteomic experiments (comparison of periplasmic subproteomes derived from wild-type and mutated strains) conducted in our lab indicate that the abundance of a dozen proteins is impeded by inactivation of the Dsb oxidative pathway. Some proteins that are involved in the respiratory process, nutrient supply or biofilm formation can

be crucial factors for pathogen interaction with human epithelial cells, and/or for the colonization process (manuscript in preparation). Additionally, transcriptomic experiments showed that *dsb* gene expression is regulated by environmental stimuli, such as oxygen tension, iron-concentration or sodium deoxycholate concentration (Woodall et al. 2005; Holmes et al. 2005; Malik-Kale et al. 2008). Thus environmental factors may also regulate the abundance of extracellular proteins that are targets of the Dsb system.

Conclusions

The data presented here document that disulfide bond formation is an important factor determining both pathogenicity and commensalism of *Campylobacter*. This finding is similar to previously described effects of glycosylation. Further studies will be carried out to clarify the interaction between membrane-bound and periplasmic *dsb* gene products in the oxidative Dsb pathway of *Campylobacter* species. Moreover, proteomic studies should help identify the targets of the Dsb system that influence pathogenicity and commensalism.

Campylobacter Dsb proteins, both membrane-bound and periplasmic, may be considered as anti-*Campylobacter* drug targets. Development of new anti-*Campylobacter* drugs that are inhibitors of the Dsb system will require solving the proteins' 3-dimensional structures, since the structure of *C. jejuni* membrane oxidoreductases seems to differ from the *E. coli* DsbB structure, which was recently solved (Inaba et al. 2009). In contrast to EcDsbB, which has 4 transmembrane domains (TMs), *Campylobacter* DsbB contains 5 TMs. The DsbI C-terminal domain is predicted to achieve a -propeller structure and to localize it in the periplasm. Gaining knowledge about Dsb protein structures might help in designing specific inhibitors.

Acknowledgements. The work was supported by grants from the Polish Ministry of Scientific Research and Information Technology (No. 6 P06K 043 21) and Ministry of Science and Higher Education (No. 401 183 31/3968). We are grateful to Prof. M.J. Blaser for providing the *C. jejuni* strain and to Prof. J.M. Bolla for providing anti-CadF serum. We thank Dr J. Hansen for critical reading of the manuscript.

REFERENCES

Agudo D, Mendoza MT, Castanares C, Nombela C, Rotger R, 2004. A proteomic approach to study *Salmonella typhi* periplasmic proteins altered by a lack of the DsbA thiol: disulfide isomerase. Proteomics 4: 355–363.

Bleumink-Pluym NM, Verschoor F, Gaastra W, van der Zeijst BA, Fry BN, 1999. A novel approach for the construction of a *Campylobacter* mutant library. Microbiology 145: 2145–2151.

Carrillo CD, Taboada E, Nash JH, Lanthier P, Kelly J, Lau PC, et al. 2004. Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. J Biol Chem 279: 20327–20338.

Dutton RJ, Boyd D, Berkmen M, Beckwith J, 2008. Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. PNAS USA 105: 11933–11938.

Godlewska R, Dzwonek A, Mikula M, Ostrowski J, Pawlowski M, Bujnicki JM, et al. 2006. *Helicobacter pylori* protein oxidation influences the colonization process. Int J Med Microbiol 296: 321–324.

Grimshaw JP, Stirnimann CU, Brozzo MS, Malojcic G, Grutter MG, Capitani G, et al. 2008. DsbL and DsbI form a specific dithiol oxidase system for periplasmic arylsulfate sulfotransferase in uropathogenic *Escherichia coli*. J Mol Biol 380: 667–680.

Gundogdu O, Bentley SD, Holden MT, Parkhill J, Dorrell N, Wren BW, 2007. Re-annotation and re-analysis of the *Campylobacter jejuni* NCTC11168 genome sequence. BMC Genomics 8: 162.

Hendrixson DR, DiRita VJ, 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol 52: 471–484.

Heras B, Shouldice SR, Totsika M, Scanlon MJ, Schembri MA, Martin JL, 2009. DSB proteins and bacterial pathogenicity. Nat Rev 7: 215–225.

Hiniker A, Bardwell JC, 2004. *In vivo* substrate specificity of periplasmic disulfide oxidoreductases. J Biol Chem 279: 12967–12973.

Holmes K, Mulholland F, Pearson BM, Pin C, McNicholl-Kennedy J, Ketley JM, et al. 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology 151: 243–257.

Humphrey T, O'Brien S, Madsen M, 2007. Campylobacters as zoonotic pathogens: a food production perspective. Int J Food Microbiol 117: 237–257.

Inaba K, Murakami S, Nakagawa A, Iida H, Kinjo M, Ito K, et al. 2009. Dynamic nature of disulphide bond formation catalysts revealed by crystal structures of DsbB. EMBO J 28: 779–791.

Janssen R, Krogfelt KA, Cawthraw SA, van Pelt W, Wagenaar JA, Owen RJ, 2008. Host-pathogen in-

teractions in *Campylobacter* infections: the host perspective. Clin Microbiol Rev 21: 505–518.

Jin S, Joe A, Lynett J, Hani EK, Sherman P, Chan VL, 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol Microbiol 39: 1225–1236.

Kalmokoff M, Lanthier P, Tremblay TL, Foss M, Lau PC, Sanders G, et al. 2006. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. J Bact 188: 4312–4320.

Karlyshev AV, Everest P, Linton D, Cawthraw S, Newell DG, Wren BW, 2004. The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. Microbiology 150: 1957–1964.

Korlath JA, Osterholm MT, Judy LA, Forfang JC, Robinson RA, 1985. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. J Infect Dis 152: 592–596.

Lasica AM, Jagusztyn-Krynicka EK, 2007. The role of Dsb proteins of Gram-negative bacteria in the process of pathogenesis. FEMS Microbiol Rev 31: 626–636.

Linton D, Lawson AJ, Owen RJ, Stanley J, 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J Clin Microbiol 35: 2568–2572.

Macnab RM, 2003. How bacteria assemble flagella. Annu Rev Microbiol 57: 77–100.

Malik-Kale P, Parker CT, Konkel ME, 2008. Culture of *Campylobacter jejuni* with sodium deoxycholate induces virulence gene expression. J Bact 190: 2286–2297.

Masip L, Pan JL, Haldar S, Penner-Hahn JE, DeLisa MP, Georgiou G, et al. 2004. An engineered pathway for the formation of protein disulfide bonds. Science 303: 1185–1189.

Messens J, Collet JF, 2006. Pathways of disulfide bond formation in *E. coli*. Int J Biochem Cell Biol 38: 1050–1062.

Monteville MR, Konkel ME, 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect Immun 70: 6665–6671.

Monteville MR, Yoon JE, Konkel ME, 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. Microbiology 149: 153–165.

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, et al. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403: 665–668.

Pawlowski M, Łasica AM, Jagusztyn-Krynicka EK, Bujnicki JM, 2009. AAN82231 protein from uropathogenic *E. coli* CFT073 is a close paralog of DsbB enzymes and does not belong to the DsbI family. Pol J Microbiol 58: 181–184.

Pei Z, Burucoa C, Grignon B, Baqar S, Huang XZ, Kopecko DJ, et al. 1998. Mutation in the *peb1A* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect Immun 66: 938–943.

Raczko AM, Bujnicki JM, Pawlowski M, Godlewska R, Lewandowska M, Jagusztyn-Krynicka EK, 2005. Characterization of new DsbB-like thiol-oxidoreductases of *Campylobacter jejuni* and *Helicobacter pylori* and classification of the DsbB family based on phylogenomic, structural and functional criteria. Microbiology 151: 219–231.

Sambrook J, Russel DW, 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Svensson SL, Davis LM, MacKichan JK, Allan BJ, Pajaniappan M, Thompson SA, et al. 2009. The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. Mol Microbiol 71: 253–272.

Wang Y, Taylor DE, 1990. Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector construction. Gene 94: 23–28.

Watson RO, Galan JE, 2005. Signal transduction in *Campylobacter jejuni*-induced cytokine production. Cell Microbiol 7: 655–665.

Watson RO, Galan JE, 2008. *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. PLoS Pathogens 4: e14.

Woodall CA, Jones MA, Barrow PA, Hinds J, Marsden GL, Kelly DJ, et al. 2005. *Campylobacter jejuni* gene expression in the chick cecum: evidence for adaptation to a low-oxygen environment. Infect Immun 73: 5278–5285.

Wyszynska A, Raczko A, Lis M, Jagusztyn-Krynicka EK, 2004. Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 *cjaA* gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*. Vaccine 22: 1379–1389.

Young KT, Davis LM, Dirita VJ, 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat Rev 5: 665–679.

Zilbauer M, Dorrell N, Wren BW, Bajaj-Elliott M, 2008. *Campylobacter jejuni*-mediated disease pathogenesis: an update. Trans R Soc Trop Med Hyg 102: 123–129.