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Comparison of the localization and post-translational modification of *Campylobacter coli* CjaC and its homolog from *Campylobacter jejuni*, Cj0734c/HisJ★

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Campylobacter **is an asaccharolytic microorganism which uses amino acids as a source of carbon and energy. CjaC/HisJ is a ligand-binding protein, a component of the ABC transport system.** ***Campylobacter*** **CjaC/HisJ is post-translationally modified by glycosylation. The number of glycosylation motifs present in the CjaC protein is species-specific.** ***C. coli*** **CjaC has two and** ***C. jejuni*** **one motif (E/DXNYS/T) which serves as a glycan acceptor. Although the two** ***C. coli*** **CjaC motifs have identical amino-acid sequences they are not glycosylated with the same efficiency. The efficacy of CjaC glycosylation in** ***Escherichia coli*** **containing the** ***Campylobacter pgl*** **locus is also rather low compared to that observed in the native host. The CjaC localization is host-dependent. Despite being a lipoprotein, CjaC is recovered in** ***E. coli*** **from the periplasmic space whereas in** ***Campylobacter*** **it is anchored to the inner membrane.**

Keywords: *Campylobacter*, protein localization, glycosylation

INTRODUCTION

Campylobacter spp., Gram-negative microorganisms, members of the *ε-proteobacteria*, are considered to be one of the emerging pathogens (Woolhouse, 2002; Moore *et al.*, 2005). They are presently recognized as a leading bacterial cause of food-borne illnesses in Europe and the USA, and a major agent of bacterial diarrhoea worldwide. *Campylobacter* infections have been increasing steadily over the past decade. In the USA, an estimated 1.2 to 2.4 million cases of human campylobacteriosis occur every year. The disease symptoms of *Campylobacter*-mediated enteritis range from mild, watery diarrhoea to severe inflammatory diarrhoea. Infection with *C. jejuni* has been also associated with GBS (Guillain-Barre syndrome), an autoimmune disorder of the peripheral nervous system that may lead to respiratory muscle compromise or even death. The majority of cases of human enteritis caused by *Campylobacter* species are due to *C. jejuni* (80–85% of all enteric *Campylobacter* infections) and *C. coli* infections (10–15% of all enteric *Campylobacter* infections) (Taylor, 1992; Nachamkin *et al.*, 1998; Skirrow & Blaser, 2000; Swartz, 2002).

Experimental documentation and data confirmed by sequencing have determined that *C. jejuni* NCTC 11168 lacks the phosphofructokinase gene necessary for the activity of the glycolytic pathway. This finding confirmed that *C. jejuni* NCTC 11168 is an asaccharolytic organism (Parkhill *et al.*, 2000; Kelly, 2001; Velayudhan & Kelly, 2002). No phosphofructokinase gene has been identified in the genomes of other *Campylobacter* species sequenced so far or in the genomes of two closely related microorganisms:

★The nucleotide sequence of the *cjaC* gene from *Campylobacter coli* 72Dz/92 has been deposited in the EMBL Nucleotide Sequence Database under accession no. Y10872.

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Abbreviations: IM, inner membrane; LB, Luria–Bertani; MH, Müller-Hinton medium; OM, outer membrane; PBS, phosphate-buffered saline; r, recombinant.

Helicobacter pylori and *H. hepaticus.* Among the members of the *ε-proteobacteria* only *Wolinella succinogenes,* which is a cattle commensal, possesses a complete set of the glycolytic pathway genes (Fouts *et al*., 2005). Thus, *Campylobacter* relies on amino acids as a source of carbon and energy and its genome contains many genes responsible for amino acid uptake, mainly components of the ABC transport system. Consistent with this notion, inactivation of some genes potentially involved in amino acid transport resulted in attenuation of growth in the bird gut as documented by STM mutagenesis (Hendrixson & DiRita, 2004). Additionally, removal of single amino acids from the defined growth medium decreased the microorganism growth rate *in vitro* (Velayudhan *et al*., 2004).

In this report we analyzed the localization and post-translational modification of *C. coli* CjaC in comparison to its ortholog from *C. jejuni* 81176. Both genes encode 28 kDa immunopositive proteins, homologues of the solute binding components of the ABC transport system. For *C. coli* 72Dz/92 the protein was designated as CjaC, its ortholog from *C. coli* M275 was named HisJ and the one derived from the first *Campylobacter* strain to be sequenced (*C. jejuni* NCTC 11168) was annotated as Cj0734c. As it has been previously shown, the orthologs from *C. jejuni* and *C. coli* have only 90% of nucleotide sequence identity, whereas those from different *C. coli* clinical isolates (CjaC from *C. coli* 72Dz/92 and M275) display 100% identity. It has also been documented that specific primers which were designed based on the nucleotide sequence of the *C. coli cjaC* gene do not amplify the gene from *C. jejuni* (Garvis *et al*., 1996; Pawelec *et al*., 1998; 2000).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The *C. coli* 72Dz/92 strain (formerly classified by biochemical tests as *C. jejuni*) was obtained from Child Health Centre (Warszawa, Poland), from a patient with diarrhoea. The strain belongs to the most commonly isolated serotype in Poland, Lior 71. The *C. jejuni* 81–176 strain, isolated from an outbreak of *Campylobacter* diarrhoea associated with raw milk consumption, and widely used in pathogenesis studies, was a gift of M. J. Blaser (Korlath *et al*., 1985). *Campylobacter* and *E. coli* strains were cultured as previously described (Pawelec *et al*., 2000). Antibiotics (ampicillin (100 μg mL^{-1}), kanamycin (40 μg mL^{-1}) or chloramphenicol (20 μg mL^{-1})) were added to the media when appropriate.

Recombinant DNA techniques. Procedures for plasmid DNA isolation and DNA analysis (digestion with restriction enzymes, T4 ligation), agarose gel electrophoresis and transformation of *E. coli* competent cells were carried out as described by Sambrook and Russel (2001). Preparations of plasmid DNA for electroporation as well as isolation of DNA from agarose gels were performed according to manufacturer's instructions (A&A Biotechnology). Polymerase chain reactions (PCR) were performed with *Taq* polymerase (Qiagen) on a Mastercycler Personal (Eppendorf) under standard conditions. Oligonucleotide primers used in this work were synthesized by Sigma-Ark GmbH. Sequences of the primers are given in Table 2.

Recombinant plasmid construction. Inverse PCR was employed to disrupt the *cjaC* gene from *C. coli* 72Dz/92. PUWM410, a derivative of pBluescript II KS carrying a 0.84 kb DNA fragment containing the *cjaC* gene, was used as a template. Deletion of 300 bp and a unique restriction *Eco*RI site were introduced into the *cjaC* gene by inverse PCR with primers CL and CR. The resulting plasmid was designated pUWM411. A 1.4-kb *Eco*RI restriction DNA fragment of the pBF14 plasmid containing a gene encoding resistance to kanamycin was cloned into the unique *Eco*RI site of pUWM411 generating pUWM416.

Transformation of *Campylobacter*. Inactivated genes were introduced into the *Campylobacter* genome by allelic exchange as described earlier (Wassenaar *et al*., 1993). *C. coli* 72Dz/92, in which the *cjaC* gene was disrupted, was designated AW6. The expected disruption of the chromosomal locus as a result of double cross-over recombination event was verified by PCR amplification. The loss of CjaC in *C. coli* AW6 was also demonstrated by Western blotting of whole-cell proteins with specific rCjaC antibodies. The mutated strain had normal colony morphology and exhibited normal growth rate when cultured on blood agar plates or MH medium.

Site-directed mutagenesis. Point mutations were generated using the Quick-Change site-directed mutagenesis kit by following the procedures recommended by the supplier (Stratagene). Plasmid pUWM77 (derivative of pBluescript II SK containing *cjaC* gene from *C. coli* 72Dz/92 (Pawelec *et al*., 1998)) was used as a template for PCR-mediated mutagenesis. Point mutations: N28A, N34A, N132A were introduced with primers: CNAS1/CNAS2, CNTT1/CNTT2, CNDS1/CNDS2, respectively. The mutagenic oligonucleotide primers are summarized in Table 2. Plasmids containing the *cjaC* gene with various point mutations were transformed into *E. coli* DH5α and the presence of the desired mutations was verified by DNA sequencing. Fragments containing the *cjaC* gene with various point mutations were cloned into the pRY111 shuttle vector. Resulting plasmids were named pUWM758 (*cjaC* with N28A

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains	Relevant characteristics	Origin
Strains		
E. coli XL1-Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A1 *lac*$^-$F′[*pro*AB *lac*I^{q}ZΔM15 Tn10 (tet^R)]	Stratagene
E. coli DH5α	F′ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*1 *hsd*R17 (r_K^-, M_K^-) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Gibco BRL
C. coli 72Dz/92	Lior 71; isolated in Poland from child with bloody diarrhoea	Child Health Centre, Warsaw, Poland
C. jejuni 81176	Lior 5; isolated in Minnesota, USA, from child with bloody diarrhoea	M.J. Blaser, NYU, USA
C. coli AW6	*C. coli* 72Dz/92 *cjaC*::Km	This study
Plasmids		
pBluescript II KS	Ap^R, LacZα	Stratagene
pBluescript II SK	Ap^R, LacZα	Stratagene
pBF14	Km^R	University of Utrecht, The Netherlands
pRY111	Cm^R, shuttle vector	(Yao *et al*., 1993)
pACYC184/*pgl*	pACYC184/ *Campylobacter pgl* gene cluster	(Wacker *et al*., 2002)
pUWM77	pBluescript II SK/*cjaC C. coli*	(Pawelec *et al*., 1998)
pUWM410	pBluescript II KS/*cjaC C. coli*	This study
pUWM411	pBluescript II KS/*cjaC C. coli* with deletion of 300 bp	This study
pUWM416	pBluescript II KS/*cjaC*::Km *C. coli*	This study
pUWM758	pRY111/ *C. coli cjaC* gene with N28A mutation	This study
pUWM764	pRY111/ *C. coli cjaC* gene with N34A mutation	This study
pUWM763	pRY111/ *C. coli cjaC* gene with N132A mutation	This study
pUWM771	pRY111/ *cjaC C. coli*	This study

mutation), pUWM764 (*cjaC* with N34A mutation), pUWM763 (*cjaC* with N132A mutation). The shuttle plasmid containing a wild-type copy of the *C. jejuni cjaC* gene was named pUWM771. All derivatives of pRY111 were introduced into *C. coli* AW6 by electroporation.

Preparation of cellular fractions. Proteins from the periplasmic space were released by chloroform as described by Ames and coworkers (1984). The cell envelope was fractionated into inner and outer membranes by selective solubilization of the inner membrane (IM) with sarcosyl detergent (*N*-lauroyl sarcosine sodium salt). Preparation of *Campylobacter* membrane fractions was performed according to the method of Blaser and coworkers (1983) and *E. coli* membrane fractions were prepared using the procedure described by Filip and coworkers (1973). CjaCx6His protein was purified by affinity chromatography (Qiagen) under non-denaturing conditions, following cloning of the mature protein-encoding nucleotide sequence amplified by PCR into the pQE31 plasmid.

Labeling of CjaC with [^{3}H]palmitate. *E. coli* XL1Blue cells harboring pUWM77 (pBluescript II SK containing the *cjaC* gene expressed from its own promoter) were grown in LB medium at 37°C to an

Table 2. Oligonucleotides used in this study

Name	**Primer sequence (5′→3′)***	Orientation	Restriction enzyme/point mutations
CR	ACTGAATTC**AACAGATGGAGGAGAAGG**	forward	*Eco*RI
CL	ACTGAATTC**CATCAGTTGCGCTCATAG**	reverse	*Eco*RI
	Mutagenic oligonucleotide primers		
CNAS1	**CACTAAAACAGAAAATGCTGCAAGCAATGAAGCTAATACTAC**	forward	N28A
CNAS2	**GTAGTATTAGCTTCATTGCTTGCAGCATTTTCTGTTTTAGTG**	reverse	N28A
CNTT1	**GCAAGCAATGAAGCTGCTACTACACTCACTTTAAAGG**	forward	N34A
CNTT2	**CCTTTAAAGTGAGTGTAGTAGCAGCTTCATTGCTTGC**	reverse	N34A
CNDS1	**CTTAAGCTAAAAAACGCCGACTCTCTTCAAAC**	forward	N132A
CNDS2	**GTTTGAAGAGAGTCGGCGTTTTTTAGCTTAAG**	reverse	N132A

*Bold letters indicate *C. coli* sequences; restriction recognition sequences introduced for cloning purposes are underlined; mismatches are double underlined

OD_{600} of 0.4. Afterwards, [^{3}H]palmitic acid (25 μCi mL^{-1} culture; DuPont-NEN) was added to the culture which was further incubated for 3 h. *E. coli* cells were then harvested, washed twice with 100% ethanol and the pellet air-dried. Bacteria were resuspended in PBS buffer and lysed with sample buffer. The released proteins were separated on a Tricine/SDS/PAGE gel (TSDS/PAGE, 16.5% T, 3% C) and the radiolabeled lipoproteins detected by fluorography.

Protein immunoblot analysis. Preparation of bacterial protein extracts, SDS/PAGE and blotting were done by standard methods. Total proteins expressed by *Campylobacter* or *E. coli* containing *C. coli* DNA, and proteins from cellular fractions (periplasm, cell envelope, outer-membrane) were separated by electrophoresis in 12% polyacrylamide gels containing SDS or by TSDS/PAGE and electrotransferred onto nitrocellulose membrane. Blots were developed using rabbit anti-*Campylobacter* or anti-rCjaC antibodies. To eliminate nonspecific reactivity, rabbit sera were absorbed first with heat-killed *E. coli* cells, and then with an *E. coli* cell lysate obtained by sonication. Afterwards, the sera were sterilized by filtration and kept frozen at –20°C. The serum against rCjaC was raised in rabbits immunized with rCjaCx6His as described earlier (Pawelec *et al*., 2000). Omp50 serum, obtained from J. M. Bolla, was raised in rabbit by three successive subcutaneous injections of the purified protein (Bolla *et al*., 2000).

RESULTS AND DISCUSSION

The *Campylobacter cjaC* gene (*cj0734c*) was identified in a *C. coli* 72Dz/92 cosmid genomic library by immunoscreening with rabbit anti-*Campylobacter* antibodies. Its product, of a predicted molecular mass of 28.6 kDa, exhibits an overall homology to solute-binding proteins (family 3) of the ABC transport system (Pawelec *et al*., 1998). Its homolog from *C. jejuni/coli* M275 complements a *hisJ* deletion in *Salmonella*. Originally designated as *C. jejuni*, the *Campylobacter* M275 strain is now classified as *C. coli* (Konkel *et al*., 1999).

The *Campylobacter cjaC* gene was mutagenized by gene replacement as described in the methods section. Proteins isolated from the *cjaC* mutant and wild-type strain were separated by SDS/PAGE, transferred onto nitrocellulose and reacted with a specific rabbit anti-rCjaC serum. Three immunoreactive bands of approx. 30, 33 and 35 kDa were observed when proteins derived from the wild-type strain were examined, whereas knockout of *cjaC* resulted in a loss of all immunoreactive bands (Fig. 1, lanes 4 and 5). The CjaC molecular mass (30, 33 and 35 kDa) calculated from the migration rate are all higher than the predicted one. The discrepancy probably reflects protein post-translational modification (see below). These results encouraged us to study CjaC derived from different *Campylobacter* clinical isolates in regard to its post-translational modification. Western immunoblot analysis with anti-rCjaC antibodies was carried out using whole-cell extracts of fourteen human clinical isolates belonging to two species: *C. jejuni* and *C. coli*. The *Campylobacter* isolates were classified into species based on PCR amplification with primers complementary to 23S rRNA (Pawelec *et al*., 2000). Two or three forms of CjaC were recognized by anti-rCjaC antibodies. The number of CjaC forms detected was strain-dependent (Fig. 2A and B). In

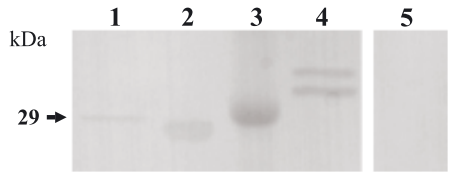


Figure 1. Immunoblot analysis of proteins isolated from wild-type *C. coli* 72Dz/92 and from the mutant AW6.
Campylobacter protein extracts were separated electrophoretically on a 12% polyacrylamide gel, blotted onto nitrocellulose membrane and probed with rabbit polyclonal anti-rCjaC antibodies. The position of molecular mass marker (29 kDa) is indicated on the left. Lanes: 1, *E. coli/cjaC*; 2, rCjaCx6His; 3, molecular size standard; 4, *C. coli* 72Dz/92 wt; 5, *C. coli* 72Dz/92 AW6 (*cjaC*$^{-}$).

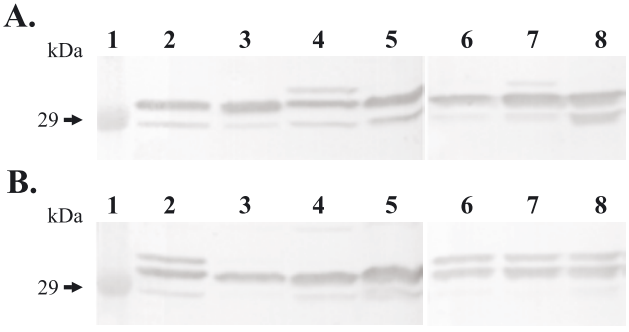
the majority of cases, two forms of the protein were recognized with anti-CjaC antibodies in lysates obtained from *C. jejuni* cells whereas three CjaC forms were detected when *C. coli* lysates were analyzed.

In silico analysis of the CjaC orthologs from *C. jejuni* (251 amino acids), *C. coli* (256 amino acids), *C. upsaliensis* (252 amino acids) and *C. lari* (252 amino acids) revealed differences in the amino-acid sequence of the N-terminal fragment of the protein (Fig. 2C).

In trans complementation of the *cjaC:Km* disruption with pUWM771 carrying the *cjaC* gene resulted in three forms of CjaC whose molecular masses corresponded to those present in wild-type cells (Fig. 3B, lane 4). These data suggested that *C. coli* CjaC is present in three forms of different molecular masses. The two protein bands of lower mobility were much more intense than the one of the highest migration rate. The product of the *cjaC* gene expressed in *E. coli* from pUWM77 is visible mainly as a band that corresponds in mass to the lowest band seen for *C. coli* (Fig. 1, lane 1; Fig. 3A, lane 2). Recombinant CjaC (rCjaCx6His) was obtained as a cytoplasmic protein of a molecular mass lower than that of mature CjaC (Fig. 1, lane 2).

Post-translational modification of *C. coli* CjaC

Nita-Lazar *et al*. (2005) demonstrated that *C. jejuni* 81176 HisJ/CjaC, which contains two potential motifs of glycosylation, exists in two forms, an unglycosylated and a monoglycosylated one. That



C.

C. jejuni NCTC11168	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. jejuni 84-25	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. jejuni CF93-6	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. jejuni HB93-13	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. jejuni RM1221	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. jejuni 260.94	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. jejuni 81-176	MKKILSIALVALVGLFLGACSDSKNK-----ESNASVELK
C. coli RM2228	MKKFLTAFLVAFTGLFLVACQNTKTENNASNEANTTLTLK
C. coli **72Dz92**	**MKKFLTAFLVAFTGLFLVACQNTKTENNASNEANTTLTLK**
C. upsaliensis RM3195	MRKIF-AFLVAFLSLFVVACSDTSVTNN---DSNASLSLR
C. lari RM2100	MKKILYVVLALFGMLVLGACSSDKNQ----ASASSEKVYK

Figure 2. CjaC post-translational modification is strain-dependent.
A and **B.** Immunoblot analysis of proteins isolated from various *Campylobacter* spp. clinical isolates with rabbit antiserum against recombinant CjaCx6His. *Campylobacter* protein extracts were separated electrophoretically on a 12% polyacrylamide gel, blotted onto a nitrocellulose membrane and probed with antibodies. The position of molecular mass marker (29 kDa) is indicated on the left. Lanes: **A.** 1, molecular size standard; 2, *C. jejuni* 11Dz/94; 3, *C. jejuni* 199/89; 4, *C. jejuni/coli* 4Dz/88; 5, *C. jejuni/coli* 38Dz/89; 6, *C. jejuni* 81176; 7, *C. jejuni* 52N/90; 8, *C. jejuni* 202/95. **B.** 1, molecular size standard; 2, *C. coli* 4Dz/96; 3, *C. jejuni/coli* 129108; 4, *C. jejuni* 404/96; 5, *C. jejuni/coli* 332/93; 6, *C. coli* 72Dz/92; 7, *C. coli* 569Ba; 8, *C. coli* 48Dz/92. **C.** Amino-acid sequences of the N-terminal fragments of the CjaC protein derived from different *Campylobacter* strains. The potential N-glycosylation motifs are underlined.

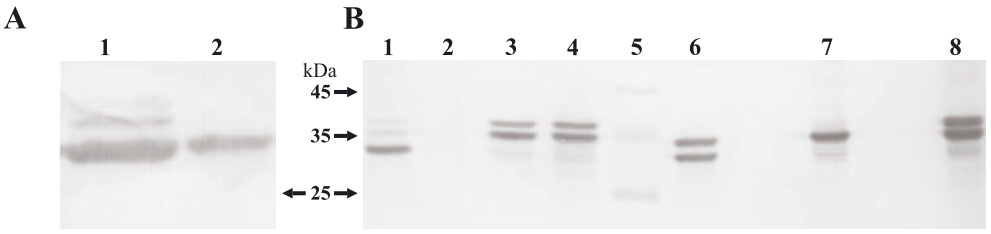


Figure 3. Post-translational modification of *C. coli* CjaC.
A. Analysis of *E. coli* overexpressing CjaC in the presence or absence of the *C. jejuni pgl* locus. **B.** Analysis of *C. coli* AW6 overexpressing CjaC or CjaC with asparagine residues replaced by alanine in one of the three motifs for N-glycosylation. Protein extracts were separated electrophoretically on a 12% polyacrylamide gel, blotted onto a nitrocellulose membrane and probed with polyclonal anti-*Campylobacter* (**A**) or anti-rCjaC (**B**) antibodies. **A**. Lanes: 1, *E. coli*/pUWM77 /pACYC184/*pgl*; 2, *E. coli*/pUWM77. **B**. Lanes: 1, *E. coli*/pUWM77; 2, *C. coli* AW6; 3, *C. coli* 72Dz/92 (wild type); 4, *C. coli* AW6/pUWM771; 5, molecular size standard; 6, *C. coli* AW6/pUWM758 (CjaC N28A); 7, *C. coli* AW6/pUWM764 (CjaC N34A); 8, *C. coli* AW6/pUWM763 (CjaC N132A).

finding, consistent with data presented in this work (see below), proved that only one NXS/T motif is modified by the glycan binding. In contrast to *C. jejuni* CjaC the amino-acid sequence of *C. coli* 72Dz/92 CjaC includes three potential N-glycosylation sites (28-NAS-30, 34-NTT-36 and 132-NDS-134). The first possible *C. coli* CjaC glycosylation motif is encoded by a nucleotide sequence which is missing from the *cj0734c/hisJ* genes in all *C. jejuni* strains sequenced so far (Fig. 2C). The second motif has different amino-acid sequences: NTT and NAS for the *C. coli* and *C. jejuni* protein, respectively. The third motif is identical in CjaC from both species.

To examine the potential post-translational modifications of *C. coli* CjaC, the pACYC184/*pgl* plasmid (which carries the *Campylobacter pgl* (protein glycosylation) gene cluster functioning in *E. coli*) was introduced into *E. coli* expressing *cjaC* from its own promoter. Next, whole-cell extracts were examined for reactivity with rabbit anti-*Campylobacter* serum. The activity of *pgl* gene product resulted in two extra forms of CjaC of a decreased mobility, proving that CjaC is its target (Fig. 3A). The intensities of the two glycosylated protein bands were much weaker than of the band corresponding to the unglycosylated CjaC, indicating that the glycosylation process is not as efficient as in the native host. It should be pointed out that in *E. coli* most of CjaC is periplasmic, whereas in *Campylobacter* the protein is anchored in the inner membrane (see below). A silmilar difference between the efficiency of glycosylation mediated by the *pgl* locus in *Campylobacter* and *E. coli* cells was also observed for the *C. jejuni* VirB10 (Cjp3) protein, which is a pVir-encoded component of TFSS. VirB10 was isolated as a component of a glycine-acid extract fraction but its localization in either *C. jejuni* or *E. coli* has not been determined by fractionation (Bacon *et al.*, 2002; Larsen *et al.*, 2004).

Further analysis using site-directed mutagenesis was employed to study which of the three NXS/T motifs of CjaC is really modified by glycan binding. All N's from the three NXS/T motifs of CjaC were replaced by the neutral A. In effect, three derivative proteins with single amino acid substitutions were constructed as described in the methods section. The mutated versions of the proteins produced in *C. coli* AW6 were examined for their mobility by SDS/PAGE. Among the derivative proteins, two were impaired in glycosylation. Replacement of N by A at positions 28 or 34 resulted in proteins visible on the gel as two bands. The first band contained a protein of an apparent mass that corresponded to the nonglycosylated protein present in *E. coli.* The migration rate of the second band was like that of the middle band observed for wild-type CjaC from *C. coli*. Presumably, it contained a monoglycosylated form of the protein. Recently, it has been documented that the *Campylobacter* N-glycosylation system, unlike the eukaryotic one, requires a negatively charged amino acid at position –2 (motif D/EXNXS/T) (Kowarik *et al.*, 2006). Although the two analyzed motifs (28-NAS-30 and 34-NTT-36) are preceded by an E residue in position –2 they are not equivalent substrates for glycosylation. Alanine substitution of N28 resulted in obtaining almost equal amounts of nonglycosylated and monoglycosylated forms of CjaC, whereas the mutated derivative with alanine substitution of N34 appeared on the gel as a band corresponding to the monoglycosylated form with a molecular mass equivalent to the middle band of the wild-type CjaC (Fig. 3 B, lanes 6 and 7). The same observation was made for the VirB10 protein (Larsen *et al.*, 2004). Interestingly, the effectively glycosylated NAS motif is missing in the *C. jejuni* CjaC protein. In contrast to the NAS and NTT motifs, substitution of the NDS (position 132–134) amino-acid sequence by an ADS motif did not influence the number of CjaC forms. The latter motif, which is not preceded by E/D (position –2), is apparently not glycosylated in the *C. jejuni* CjaC protein.

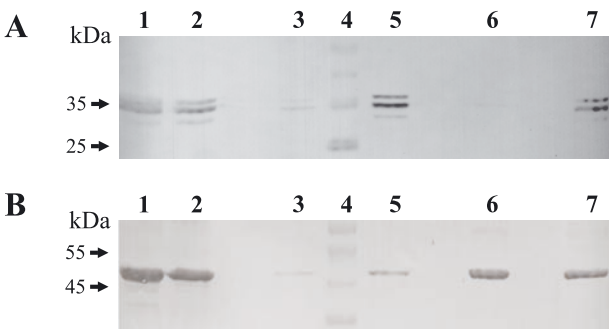


Figure 4. Subcellular localization of CjaC (A) and Omp 50 (B) in *C. coli.*
Proteins derived from different cell fractions were separated electrophoretically on a 12% polyacrylamide gel, blotted onto a nitrocellulose membrane and probed with polyclonal anti-rCjaC (**A**) or anti-Omp50 (**B**) antibodies. The positions of molecular mass markers are indicated on the left. Lanes: 1, whole cell lysate; 2, osmotically shocked cells; 3, osmotic shock fluid-fraction of periplasmic proteins; 4, molecular size standard; 5, sarcosyl-soluble fraction (proteins of the inner membrane); 6, sarcosyl-insoluble fraction (proteins of the outer membrane); 7, proteins of both membranes.

CjaC localization

Analysis of the amino-acid sequence of CjaC showed that it is equipped with a putative signal sequence containing the LVAC motif that can be processed by signal peptidase II specific for lipoprotein precursors. The lipoprotein nature of CjaC was examined by growing *E. coli* XL-Blue harboring pUWM77 (pBluescript II SK containing the *cjaC* gene expressed from its own promoter) in the presence of [^{3}H]palmitic acid. The experiment suggested that CjaC is modified with palmitic acid, at least when expressed in *E. coli*. The data obtained was not completely clear, mainly because *E. coli* carrying the empty vector expressed a lipoprotein of a molecular mass comparable to that of CjaC (not shown).

The subcellular localization of CjaC in *C. jejuni* and *C. coli* was further examined by cell fractionation. Proteins obtained from different cell compartments were separated by SDS/PAGE, electrotransferred onto nitrocellulose membrane and detected with anti-rCjaC antibodies. Localization of the outer membrane protein Omp50 was also determined (Bolla *et al*., 2000). The CjaC localization was host-dependent. In both *Campylobacter* species the protein was recovered in the inner-membrane fraction (Fig. 4A, lane 5). Omp50 was mainly recovered from outer membrane, as expected (Fig. 4B, lane 6). In contrast most of CjaC expressed in *E. coli* cells was recovered as periplasmic (not shown). Also experiments carried out by Garvis *et al*. (1996) indicated that the CjaC/HisJ derived from the *C. coli* M275 strain in *E. coli* localizes in the periplasm.

Lipoproteins of Gram-negative bacteria are anchored in the inner or outer membrane *via* fatty acids attached to the N-terminal cysteine. Destination of *E. coli* lipoproteins to either the inner or outer membrane is dependent on the lipoprotein sorting signal, the amino-acid residue localized next to the lipid-modified cysteine, and on the activity of the LolA–LolB system (Tokuda & Matsuyama, 2004). According to the +2 rule, the presence of a G residue next to the lipid-modified cysteine in *C. coli* 72Dz/92 CjaC and an S residue in the same position in the sequence of the CjaC homologue from *C. jejuni* 81176, suggests that these proteins should be located in the outer membrane. The amino-acid sequences of *E. coli* Lol system proteins were used to search the non-redundant database at the National Center for Biotechnology Information (NCBI) with the PSI-BLAST algorithm to look for Lol proteins in *C. jejuni* NCTC 11168. Homologs of all of them but one (LolB) were found: LolA–Cj0943c, LolC–Cj0941c, LolD–Cj1277c, LolE–Cj1662c. The defectiveness of the *C. jejuni* Lol transport system may explain why some lipoproteins such as CjaC, CjaA or JlpA are partially released from the inner membrane and transported to the cell surface instead of being incorporated into the outer membrane (Jin *et al*., 2001).

CONCLUSIONS

In summary, we have shown that localization of CjaC is host-dependent, as was also observed for *Campylobacter* CjaA lipoprotein (unpublished). The CjaC protein is probably not recognized by the *E. coli* Lol system and is released into the periplasm. Although the lipid-modified cysteine of CjaC is followed by the G/S residue, the protein was recovered in the inner-membrane fraction of the native host. The inner-membrane localization of CjaC is consistent with its physiological function. The post-translational modification of CjaC is species-dependent. *C. jejuni* CjaC exists in two forms, unglycosylated and monoglycosylated, whereas the protein from *C. coli* is present as three forms. Based on the predicted amino-acid sequences of CjaC from *C. upsaliensis* RM3195 and *C. lari* RM2100, which do not contain a potential glycosylation motif (E/DXNXS/T), one can suspect that these proteins are not glycosylated. Further experiments will be necessary to determine the physiological role of the post-translational modification of *C. jejuni* and *C. coli* CjaC. A simple PCR assay or Western blot analysis with anti-CjaC antibodies can be used to distinguish between two *Campylobacter* species: *C. jejuni* and *C. coli.*

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REFERENCES

Ames GF, Prody C, Kustu S (1984) Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol* **160**: 1181–1183.

Bacon DJ, Alm RA, Hu L, Hickey TE, Ewing CP, Batchelor RA, Trust TJ, Guerry P (2002) DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81–176. *Infect Immun* **70**: 6242–6250.

Blaser MJ, Hopkins JA, Berka RM, Vasil ML, Wang WL (1983) Identification and characterization of *Campylobacter jejuni* outer membrane proteins. *Infect Immun* **42**: 276–284.

Bolla JM, De E, Dorez A, Pages JM (2000) Purification, characterization and sequence analysis of Omp50, a new porin isolated from *Campylobacter jejuni*. *Biochem J* **352** (Pt 3): 637–643.

Filip C, Fletcher G, Wulff JL, Earhart CF (1973) Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* **115**: 717–722.

Fouts DE, Mongodin EF, Mandrell RE *et al.* (2005) Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. *PLoS Biol* **3**: e15. Epub 2005 Jan 2004.

Garvis SG, Puzon GJ, Konkel ME (1996) Molecular characterization of a *Campylobacter* jejuni 29-kilodalton periplasmic binding protein. *Infect Immun* **64**: 3537–3543.

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

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.

Kelly DJ (2001) The physiology and metabolism of *Campylobacter jejuni* and *Helicobacter pylori*. *Symp Ser Soc Appl Microbiol* 16S–24S.

Konkel ME, Gray SA, Kim BJ, Garvis SG, Yoon J (1999) Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the *cadF* virulence gene and its product. *J Clin Microbiol.* **37**: 510–517.

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.

Kowarik M, Young NM, Numao S *et al.* (2006) Definition of the bacterial N-glycosylation site consensus sequence. *EMBO J* **25**: 1957–1966.

Larsen JC, Szymanski C, Guerry P (2004) N-linked protein glycosylation is required for full competence in *Campylobacter jejuni* 81–176. *J Bacteriol* **186**: 6508–6514.

Moore JE, Corcoran D, Dooley JS *et al.* (2005) *Campylobacter Vet Res* **36**: 351–382.

Nachamkin I, Allos BM, Ho T (1998) *Campylobacter* species and Guillain-Barre syndrome. *Clin Microbiol Rev* **11**: 555–567.

Nita-Lazar M, Wacker M, Schegg B, Amber S, Aebi M (2005) The N-X-S/T consensus sequence is required but not sufficient for bacterial N-linked protein glycosylation. *Glycobiology* **15**: 361–367.

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

Pawelec D, Jakubowska-Mroz J, Jagusztyn-Krynicka EK (1998) *Campylobacter jejuni* 72Dz/92 *cjaC* gene coding 28 kDa immunopositive protein, a homologue of the solute-binding components of the ABC transport system. *Lett Appl Microbiol* **26**: 69–76.

Pawelec DP, Korsak D, Wyszynska AK, Rozynek E, Popowski J, Jagusztyn-Krynicka EK (2000) Genetic diversity of the *Campylobacter* genes coding immunodominant proteins. *FEMS Microbiol Lett* **185**: 43–49.

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

Skirrow MB, Blaser MJ (2000) *Clinical aspects of Campylobacter infection. Campylobacter* (Nachamkin I, Blaser MJ, Tompkins LS, eds) 2nd edn, pp 69–89. ASM Press, Washington.

Swartz MN (2002) Human diseases caused by foodborne pathogens of animal origin. *Clin Infect Dis* **34** (Suppl 3): S111–122.

Taylor DE (1992) Genetics of *Campylobacter* and *Helicobacter*. *Annu Rev Microbiol* **46**: 35–64.

Tokuda H, Matsuyama S (2004) Sorting of lipoproteins to the outer membrane in *E. coli*. *Biochim Biophys Acta* **1693**: 5–13.

Velayudhan J, Kelly DJ (2002) Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. *Microbiology* **148**: 685–694.

Velayudhan J, Jones MA, Barrow PA, Kelly DJ (2004) L-Serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni*. *Infect Immun* **72**: 260–268.

Wacker M, Linton D, Hitchen PG *et al.* (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science* **298**: 1790–1793.

Wassenaar TM, Fry BN, van der Zeijst BA (1993) Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation. *Gene* **132**: 131–135.

Woolhouse ME (2002) Population biology of emerging and re-emerging pathogens. *Trends Microbiol* **10**: S3–7.

Yao R, Alm RA, Trust TJ, Guerry P (1993) Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. *Gene* **130**: 127–130.