Polish Journal of Microbiology
2006, Vol. 55, No 2, 85–94

Genetic Characterisation of the *cjaAB* Operon of *Campylobacter coli*

AGNIESZKA WYSZYŃSKA[a], MARCIN PAWŁOWSKI[b], JANUSZ BUJNICKI[b], DARIUSZ PAWELEC[a,e], JOS P. M. VAN PUTTEN, ELŻBIETA BRZUSZKIEWICZ[a,d] and ELŻBIETA K. JAGUSZTYN-KRYNICKA[a,*]

[a] Department of Bacterial Genetics, Institute of Microbiology, Warsaw University, 02-096 Warsaw, Miecznikowa 1, Poland;
[b] Laboratory of Bioinformatics, International Institute of Molecular and Cell Biology in Warsaw, Trojdena 4, 02-109 Warsaw, Poland;
[c] Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;
[d] Current address: University of Göttingen, Institute of Microbiology and Genetics, Germany;
[e] Current address: Adamed Sp z o.o., Pienków 149, 05-152 Czosnów, Poland

Received 6 January 2006, revised 30 January 2006, accepted 1 February 2006

Abstract

We investigated the regulation of the *cjaA* and *cjaB* genes of *Campylobacter coli*. These genes are seemingly arranged into one operon but appear to encode functionally different proteins *i.e.* an extracytoplasmic solute receptor and a MHS – metabolite: H^+ symporter transport protein. Analysis of various transcriptional *cjaA* and/or *cjaB lacZ* fusion constructs revealed that both genes are arranged in an operon. RACE analysis located the transcription start site of the *cjaAB* operon 46 bp upstream of the translation start point. β-galactosidase reporter assays yielded much higher activity for the *cjaA* than the *cjaB* gene fusion products. RT-PCR showed unequal amounts of mRNA, indicating differential post-transcriptional processing of *cjaA* and *cjaB* mRNA possibly related to the presence of inverted repeats in the intergenic region. Phylogenetic analysis grouped CjaB into a new MHS sub-family together with potential transporters with uncharacterised functions of *Campylobacter* and *Helicobacter*. Notably, no CjaB family members were identified in ε-Proteobacteria from different ecological niches, such as *H. hepaticus* and *Wolinella succinogenes*.

Key words: *Campylobacter*, *cjaAB* operon, gene expression, phylogenetic analysis

Introduction

The Gram-negative bacteria *Campylobacter coli* and *Campylobacter jejuni* are commensal bacteria of warm-blooded animals and a major cause of human enteritis all over the world (Coker *et al.*, 2002). Among the sequenced bacterial genomes, that of *C. jejuni* is one of the most compact one; 94.3% of the genome is occupied by protein-coding regions. Most of *C. jejuni* genes are possibly expressed as polycistronic operons as judged from the genome organisation (Parkhill *et al.*, 2000). In contrast to many prokaryotic genomes, the majority of *C. jejuni* genes are not functionally grouped. This fact might suggest separate transcription of the majority of *Campylobacter* genes. On the other hand, short intergenic DNA fragments present in the genome and the high number of overlapping genes (more than 26% of all of the genes) rather contradicts this hypothesis (Meinersmann and Wassenaar, 2003). Such an untypical genome organisation is also an attribute of the genome of *Aquifex aeolicus*, a thermophilic, chemolithoautotrophic bacterium (Deckert *et al.*, 1998) and methanogenic archaeon *Methanococcus jannaschii* (Bult *et al.*, 1996). This unusual organisation of the genetic material raises an important question concerning the regulation of gene expression at the transcriptional level.

Campylobacter, with a medium genome size of 1.64 Mb, like the closely related *Helicobacter*, contains three sigma factors, σ^{28}, σ^{54} and σ^{70} encoded by *fliA*, *rpoN* and *rpoD* genes, respectively (Parkhill *et al.*, 2000; Tomb *et al.*, 1997). The hitherto characterised *Campylobacter* promoters are unusual compared to

* Corresponding author: E.K. Jagusztyn-Krynicka, Department of Bacterial Genetics, Institute of Microbiology, Warsaw University, 02-096 Warsaw, Miecznikowa 1, Poland; Phone (48) 22-5541216; Fax (48) 22-5541402; 35; e-mail: kjkryn@biol.uw.edu.pl

other bacteria. They have two consensus sequences TATAATT and TTTTTTG located in –10 and –16 regions but appear to lack a conserved –35 motif. The absence of a –35 region seems to be compensated by a specific periodic signal located upstream of the –16 region (Petersen *et al.*, 2003; Wosten *et al.*, 1998).

The present study was designed to learn more about the regulation of *Campylobacter cjaA* and the downstream *cjaB* gene expression. These genes are separated by a 41 nucleotides long DNA fragment and encode proteins that belong to different transporter superfamilies. CjaA encodes a putative amino acid binding protein of the ABC (ATP-binding cassette) transport system, while CjaB encodes a putative integral membrane protein of the MFS (major facilitator superfamily) family six (MHS – metabolite: H^+ symporter). Preliminary sequence analysis suggested that the genes may be located in an operon. In the present work, we further investigated the regulation of the *cjaA* and *cjaB* genes. Since CjaA is highly immunogenic protective *Campylobacter* antigen the better understanding of the *cjaA* gene expression is crucial for elucidation its role in pathogenesis and may facilitate its use as a vaccine component.

Experimental

Materials and Methods

Bacterial strains, plasmids, media and growth conditions. Bacterial strains and plasmids used in this work are listed in Table I. *C. coli* 72Dz/92 (Lior 71) was cultured as described earlier (Pawelec *et al.*, 1997). Minimal essential medium (MEM) from GibcoBRL was used as a defined minimal medium. *E. coli* strains were grown in LB medium at 37°C. Antibiotics: ampicillin (50 μg ml^{-1}), kanamycin (40 μg ml^{-1}) chloramphenicol (20 mg μl^{-1}), were added to the media when appropriate.

Table I
Bacterial strains and plasmids used in this study

	Relevant genotype or phenotype	Source of reference
Bacterial strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi1 hsdR17 supE4 4 relA1lac* [F' *proAB lacIqZ* Δ*M15* Tn*10* (*tet*^{r})]	Stratagene
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44* Δ*lacU169* F' (Φ*80dlacZ*M15)	Institute of Microbiology, Warsaw University, Poland
E. coli WG350	F^- *trp rpsL thi* Δ(*putPA*)*101* Δ(*proP mel*)*212*	Culham *et al.*, 1993
C. coli 72Dz/92	serotype Lior 71, biotype 1	Child Health Centre, Warsaw, Poland
C. jejuni 81176	Lior 5; isolated in Canada from a child with a bloody diarrhoea	M. Blaser UV, Nashville, USA
C. coli AW4	*C. coli* 72Dz/92 *cjaB*::Kan^R	This study
Plasmids		
pBluescript II KS	Ap^r, LacZα	Stratagene
pBluescript II SK	Ap^r, LacZα	Stratagene
pBGS18	Km^r, LacZα	(Spratt *et al.*, 1986)
pGEM-T	TA cloning vector, Ap^r	Promega
pBF14	Km^r	University of Utrecht, The Netherlands
pMW10	Km^r	(Wosten *et al.*, 1998)
pUWM76	pBGS18 containing SalI-SspI 1.8 kb DNA fragment carrying *cjaA* gene transcribed from own promoter	(Pawelec *et al.*, 1997)
pUWM201	pBluescript II SK containing 5.8 kb EcoRV-EcoRV DNA fragment carrying *cjaAB* genes transcribed from the opposite DNA strand to *lacZ*	(Pawelec *et al.*, 1997)
pUWM272	pMW10 containing *cjaA* gene and 5' fragment of *cjaB* gene (545 bp)	This study
pUWM273	pMW10 containing 3' fragment of *cjaA* gene (80 bp) and 5' fragment of *cjaB* gene (545 bp)	This study
pUWM472	pMW10 containing *cjaA* upstream region –591 to +34	This study
pUWM471	pMW10 containing 5' fragment of *cjaA* gene (679 bp)	This study
pUWM477	pMW10 containing 5' fragment of *cjaA* gene (361 bp)	This study
pUWM421	pGEM-T containing *cjaB* gene without 418 bp central region	This study
pUWM422	Km^R cassette of pBF14 cloned in the unique BamHI site in pUWM421 in *cjaB*	This study

Table II
Oligonucleotides used in this study

Primer	sequence[a] (5' to 3')	Orientation
***cjaA* gene**		
H3	AcccgggatcgatggatccG**AATCCACTTGCTCTGCTCTT**	Reverse
H1	**GCTTATGATGAAACTTTAAAAAGTC**	Forward
327–3	**TTAACAGCAGGAGCAATTAC**	Reverse
AL	ACTgaattc**TATCTTGAGGCACAGCCA**	Reverse
***cjaB* gene**		
H5B	Acccgggtagctaggatccg**CTATACTCTCATAGCTTGGC**	Reverse
CB1B	Cggatcctagctacccggg**TAACCAGCGGTGTAGCGACT**	Forward
CB2	**GAGCAGCATCCTACCACCAT**	Reverse
***lacZ* gene**		
lacZ	**AGGTTACGTTGGTGTAGATG**	Reverse
lacZ1	**GGAATTCACTGGCCGTCGTT**	Forward
***aph* gene**		
KmL1	**GAGAATATCACCGGAATTGA**	Forward
KmR1	**CTTCATACTCTTCCGAGCAA**	Reverse

[a] Capital bold letters indicate *C. jejuni* sequence, small letters – restriction recognition sequences introduced for cloning purposes

DNA sequencing and analysis. The sequence of the *cjaB* gene was determined in the DNA Sequencing and Oligonucleotides Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The complete nucleotide sequence of *cjaB* was submitted to the EMBL Nucleotide Sequence Database (accession number Y17971). The sequence of the *cjaA* gene is available in the EMBL under accession number Y10872.

DNA manipulation. DNA techniques including plasmid purification, ligation and transformation into *E. coli* were done according to standard procedures (Sambrook and Russell, 2001). Restriction endonucleases and DNA – modifying enzymes were obtained from Promega and used according to the manufacturer's instruction. Polymerase chain reactions (PCR) were performed with Taq polymerase (Qiagen) in a Mastercycler Personal (Eppendorf). Oligonucleotide primers for PCR were from Sigma ARK (Table II).

Mutagenesis of *cjaB* gene. A *cjaB* mutant of *C. coli* 72Dz/92 was constructed by a two-step PCR and insertion of kanamycin resistance cassette (Km) derived from pBF14. First the 5' and 3' ends of *cjaB* were PCR amplified using the primer combinations H1 and H5B, and CB1B and CB2. CB1B and H5B were designed with complementary protruding 5' tails with a BamHI restriction site. The obtained fragments of 432 and 447 bp were gel-purified, mixed and used as a template in a second PCR with the "outward" oriented primers H1 and CB2. The obtained PCR product that lacked the 418 bp central region of *cjaB* was cloned into the pGEM-T cloning vector. The resulting plasmid pUWM421 (*cjaB*) was digested with BamHI and the BamHI-BamHI kanamycin cassette (*aph*) was inserted to obtain the final suicide plasmid pUWM422. Plasmid was introduced into *Campylobacter* by electroporation (with a Bio-Rad Gene Pulser set at 0.7 kV/cm, 25 μF and 600 Ω) and transformants were selected for kanamycin resistance. *C. coli* 72Dz/92 with disrupted *cjaB* was designated AW4. The disruption of the *cjaB* gene was verified by PCR amplification.

Transcription and translation *in vitro* assay. The *in vitro* transcription and translation reaction was done with the *E. coli* Extraction System for Circular DNA kit (Promega).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA from *C. coli* containing pMW10 derivatives was extracted using TRIzol Reagent (Molecular Research Center). RNA concentration was determined spectrophotometrically in 10 mM of Tris-HCl, pH 7.2 at 260 nm as described (Sambrook and Russell, 2001). RNA preparations (1 μg of each sample) were rendered DNA free by incubation with RNase-free DNase (Roche). RT-PCR analysis was carried out by incubating equal amounts of total RNA first with reverse transcriptase (48°C, 45 min, followed by 2 min at 95°C) and then with *Taq* DNA polymerase. The RNA used as a template in RT-PCR was reverse transcribed with the primer lacZ and the obtained cDNA was subsequently amplified with primers lacZ1 and lacZ. PCR was performed with reagents purchased from Qiagen at the concentration recommended by the supplier. The conditions for amplification were 20 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The amplified DNA fragments were analysed by 1.5% agarose gel electrophoresis.

β-galactosidase assay. β-galactosidase activity in *C. coli* and *E. coli* cell extracts was measured by the conversion of o-nitrophenyl-β-D-galactopyranoside (ONPG) to nitrophenol as described by Miller (1972), with the modification that *C. coli* transformants were grown for 20 h on BA plates before being harvested in LB medium and diluted until the absorbance at 600 nm was between 0.3–0.7. β-galactosidase activity assays were carried out in triplicate.

Rapid amplification of cDNA ends (RACE) analysis. To determine the start site of transcription the commercial "5' RACE system for rapid amplification of cDNA ends, version 2.0" (Gibco BRL, Life Technologies) was used according to the manufacturer's directions. RACE analysis was carried out using the primers provided by the manufacturer and *cjaA* – specific primers (327–3, AL, H3) indicated in Table II.

Database searches and sequence alignment. The amino-acid sequence of *C. coli* 72Dz/92 CjaB was used as a query in PSI-BLAST (Altschul *et al.*, 1997) searches of the non-redundant database (NCBI, Bethesda, USA) to identify homologous proteins. Sequences reported with the expectation (e)-value above the threshold of 10^{-8} were downloaded and used to build the multiple sequence alignments using ClustalX (Thompson *et al.*, 1997).

Phylogenetic analysis. Phylogenetic inference was carried out using the conserved regions of the sequence alignment of CjaB homologues based on the minimum-evolution method implemented in the Mega2 software package (Kumar *et al.*, 2001). The stability of all branches in the tree was validated using the interior branch test and the bootstrap method. The majority-rule consensus tree was visualised using Mega2.

Results

Transcription and translation *in vitro* assay. Examination of the nucleotide sequence downstream of the *C. coli* 72Dz/92 *cjaA* gene (orthologue of *C. jejuni* NCTC11168 *cj0982c*) revealed the presence of an open reading frame designated *cjaB*. To study *cjaB* gene expression the plasmid pUWM201 was applied. It carries a 5.8 kb DNA fragment of *C. coli* 72Dz/92 including a 2.9 kb region located downstream of the *cjaA* gene (Pawelec *et al.*, 1997). *CjaA* gene present in pUWM201 is transcribed in opposite orientation relative to *lacZ* gene. The size of the proteins specified by pUWM201 was determined in an *in vitro* coupled transcription-translation system. As shown in Fig. 1, pUWM201 encoded several proteins with molecular masses of 18, 20, 30, 50 and 55 kDa that were not expressed from the parental vector pBluescript II SK. Based on the gene sizes, the 30 kDa proteins likely represented CjaA and β-lactamase. The 18 kDa protein could be a truncated product of a gene located upstream of *cjaA*. The predicted amino acid sequence of this protein showed 46.9% identity to the *C. jejuni* JlpA (Cj0983) (Jin *et al.*, 2001). This gene together with *hipO* is part of 15 kb DNA fragment that has been by now recognised as *C. jejuni* specific (Chan *et al.*, 2000). Our results showed that there are *Campylobacter* strains containing, at least, part of this DNA region and classified by PCR assays as *C. coli*. The remaining proteins were likely to be encoded by the DNA fragment downstream of *cjaA*. The sizes of the proteins were comparable to those predicted for the products of *C. jejuni* NCTC11168 genes surrounding *cj0982c*, suggesting a similarity of the genetic organisation of the analysed region in the genomes of two clinical isolates of different species, *C. coli* and *C. jejuni*. The results obtained suggest that the majority, if not all, of the promoters located within this DNA region were active in *E. coli.*

Sequence analysis of *cjaA* downstream region. Sequencing of the DNA region downstream of *cjaA* yielded 1347 bp starting at the last position in the sequence previously obtained from pUWM76 carrying truncated *cjaB* (Pawelec *et al.*, 1997). The *cjaB* gene consists of 1248 nucleotides and carries an ATG (Met) start codon and TAA stop codon. Six nucleotides upstream of the proposed translation initiation codon a putative ribosome binding site (AGGA) was observed. Downstream of *cjaB* an orthologue of *cj0980* was found as deduced from the amino acid sequence of the C – terminus of the coding region. *CjaB* and the

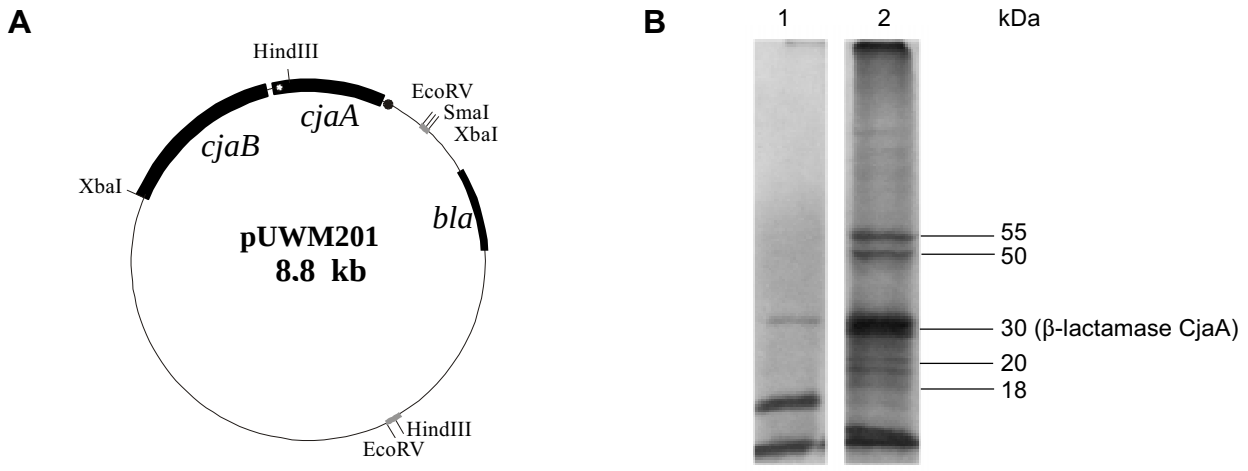


Fig. 1. Analysis of proteins encoded by genes located within the *C. jejuni* DNA fragment cloned in pUWM201 by *in vitro* transcription and translation method.

The plasmid pUWM201 was isolated out of the overnight bacterial cultures of *E. coli* strain. The proteins were separated on 12% poliacrylamide gel, dried and exposed against Kodak X-OMAT AR film; a. pUWM201 restriction map, b. Autoradiogram: Lines: 1. pBluescript II KS, 2. pUWM201.

C. coli 72Dz/92 **TAA** CAAA**AAAGGGC**· TTTT**GCCCTTT**AGTTGATTTAGGATAAAAT **ATG**
C. jejuni 81176 **TAA T**· · · **AAAGGGC**TTTTT**GCCCTTTA**GTTGATTTAGGATAAAAT **ATG**
C. jejuni NCTC11168 **TAA T**· · · **AAAGGGC**TTTTT**GCCCTTTA**GTTGATTTAGGATAAAAT **ATG**

Fig. 2. The comparison of the nucleotide sequence of *C. coli* 72Dz/92 *cjaA- cjaB* intergenic region with its counterparts from *C. jejuni* NCTC11168 and *C. jejuni* 81176

downstream gene are convergently transcribed and overlap by 18 bp. Their orthologues from *C. jejuni* NCTC11168 *(cj0980* and *cj0981c*) also overlap by 18 bp.

Comparison of the nucleotide sequence of the *cjaB* gene from *C. coli* 72Dz/92 with its orthologue from *C. jejuni* NCTC11168 revealed 84% identity. Part of the *C. jejuni* 81176 *cj0981c* orthologue was sequenced (330 bp of the 5' end of the gene) and showed 100% identity with the corresponding fragment of the *C. jejuni* NCTC11168 *cj0981c.* The start codon ATG of *cjaB* is located 41 bp downstream of the *cjaA* stop codon (TAA). Further inspection of the intergenic region revealed a lack of a putative promoter sequence for the *cjaB* gene. On the other hand, an inverted repeat (**AAAGGGC**TTTT**GCCCTTT**) which potentially might regulate mRNA stability, was found in that region. The features described above are also characteristic for the intergenic region between *cj0981c* and *cj0982c* of *C. jejuni* NCTC11168 and their orthologues in *C. jejuni* 81176 (Fig. 2).

Identification of the transcription start site and the promoter region. The transcription start site for the *cjaAB* was determined by rapid amplification of cDNA ends (RACE). Sequencing of the resulting amplicon showed that the first base of the transcript was located 46 bp upstream from the ATG start codon. Examination of the sequence located upstream of this point revealed putative –10 (TATAAT), –16 (TTTTTaaag) and –35 (TTGAaA) promoter sequences. It also contains periodically repeated T-stretches located upstream of the TATA box (thymines are predominant in regions – 18 to –24 and –29 to –35). The transcription is initiated at a pyrimidine (cytydine) located 7 nucleotides from the first nucleotide of the TATA box. The similarity between the nucleotide sequence of the –35 region and that recognised by σ^{70} *E. coli* RNA polymerase probably accounted for the expression of this operon both in *Campylobacter* and *E. coli.*

Characteristics of the putative CjaB protein. The deduced amino acid sequence of the 49.8 kDa (415 aa) CjaB exhibits a significant overall similarity to several prokaryotic transmembrane polypeptides that belong to the MFS group, family 6 (MHS – metabolite: H^+ symporter). Members of this family include transporters of citrate, β-ketoglutarate and osmoprotectants (proline and betaine) as well as some permeases of unknown transport function (Pao *et al.*, 1998). The CjaB protein contains three signature sequences specific for the family. The MFS superfamily groups inner membrane proteins with 12 to 14 transmembrane helices (TMs). *In silico* analysis of the CjaB amino acid sequence revealed the presence of 12 hypothetical TMs, each about 20 amino acids long. The N-terminus of the protein is predicted to be cytoplasmic.

The organisation into one operon of two genes, *cjaA* and *cjaB*, with seemingly unrelated functions, led us to assess the function of the *C. coli cjaB* gene product. For this purpose, the *cjaB* gene was disrupted by insertion of an antibiotic cassette (see Materials and Methods). The mutant showed growth characteristics, at least on rich media, similar to those of their parental strains. Because the deduced amino acid sequence of the CjaB exhibits a significant overall similarity to prokaryotic transmembrane transporters of citrate and osmoprotectants (proline and betaine), its function was further investigated by complementation of the *E. coli* strain WG350. This mutant is deficient in the transport of osmoprotectants (Culham *et al.*, 1993). Introduction of pUWM201 carrying the *cjaAB* operon into *E. coli* WG350 did not restore its ability to grow in minimal medium (MOPS) supplemented with 0.6 M NaCl and 1 mM proline. PUWM 201 was also introduced into *E. coli* DH5α and the growth of transformants was monitored on minimal citrate medium. In neither case the CjaB protein produced by pUWM201 complements the defect of *E. coli* indicating that it is involved neither in osmoprotection nor citrate transport.

To learn more about the CjaB protein, its evolutionary relationship to other transporters was investigated. According to the analysis reported by Saier *et al.* (1999), the MHS family comprises four subfamilies, represented by CitA Eco (Citrate transporter), PcaT Eco (dicarboxylic transporter), ProP Eco (proline/betaine transporter) and MopB Bce (4-methyl-O-phthalate transporter), respectively. Fig. 3 shows the phylogenetic tree based on the multiple sequence alignment of the MHS proteins (family metabolite: H^+ symporter), comprising the new members identified in the course of our analysis, including CjaB, as well as the SHS and SP families, used as an outgroup of the MHS family. The topology of the tree with respect to the location of the MHS, SHS, and SP families as well as the distribution of the CitA, PcaT, MopB and ProP subfamilies, are in accordance with the results of the earlier analysis of the transporter family reported by

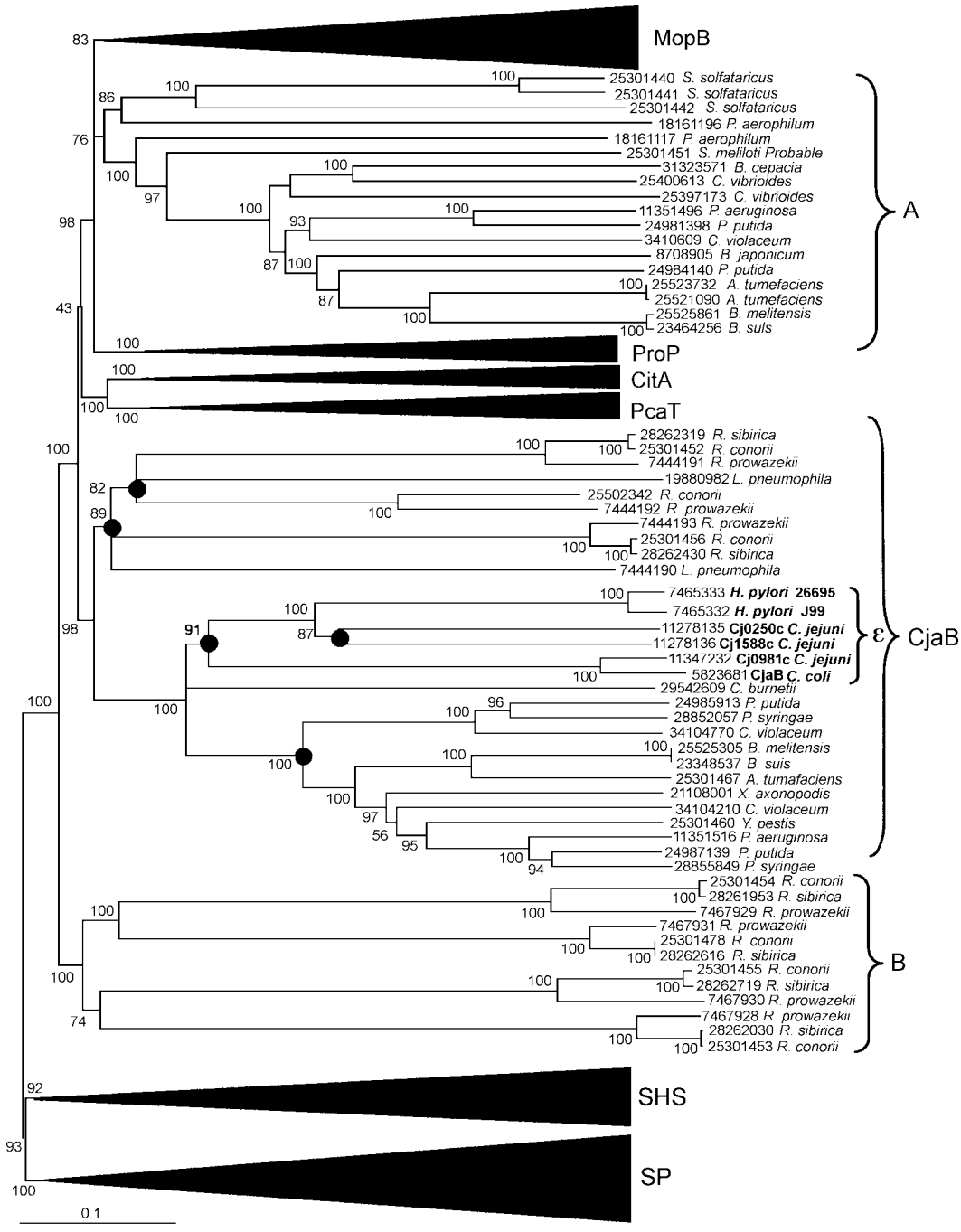
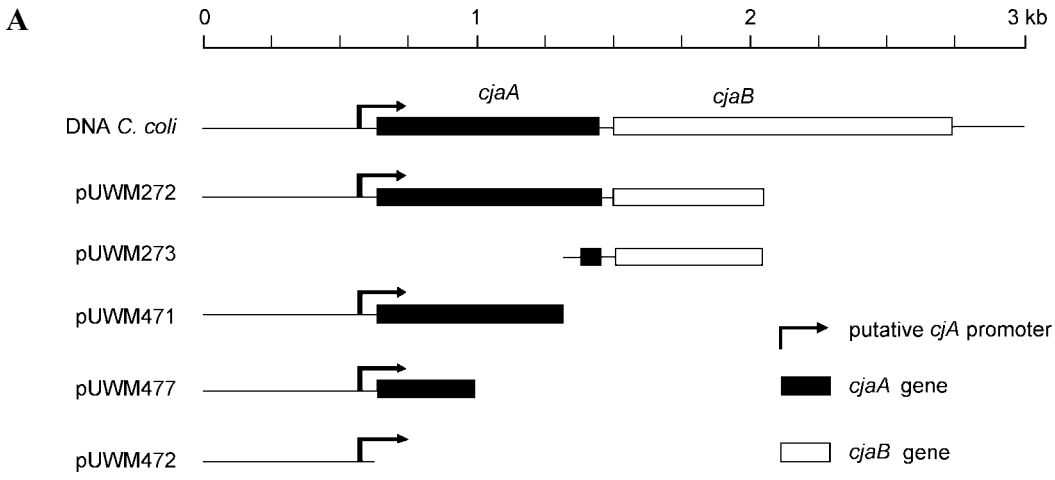


Fig. 3. The phylogenetic tree of the MHS, SHS and SP protein families.

Major subfamilies reported and analyzed previously (Saier *et al.*, 1999) have been shown in simplified representation, as black triangles. The newly identified subfamilies have been shown in detail. Proteins are indicated by the Gene Identification number and the species name. Numbers at the nodes indicate the % bootstrap support (calculated based on 1000 pseudoreplications). Nodes with bootstrap support <50% have been collapsed and are shown as unresolved. Gene duplications in the evolution of the CjaB subfamily are indicated by circles at the nodes corresponding to the duplication events.

Saier *et al.* (1999). Strikingly, our analysis reveals that CjaB and its close homologues do not group together with any of the previously defined subfamilies, but instead form a completely new subfamily. In addition to the CjaB subfamily, our analysis has led to the identification of additional two MHS subfamilies (indicated as A and B in Fig. 3), which form clearly distinct branches in the MHS family tree, with strong bootstrap support. All three new subfamilies delineated by our analysis comprise functionally uncharacterised proteins.

Detailed analysis of the phylogeny of the CjaB subfamily reveals several duplications. The CjaB protein from *C. coli* has an orthologue in the completely sequenced genome of the *C. jejuni* strain NCTC11168, but no orthologues in the completely sequenced genomes of *Helicobacter pylori* strains J99 and 26695. Inter-



B

plasmid	β-galactosidase activity (Miller Units) (± SD)	
	C. coli 72Dz/92	*E. coli* DH5α
pUWM272	316.7 (± 95)	27.6 (± 6)
pUWM273	0.1 (± 0.2)	1.3 (± 1.3)
pUWM471	1827.6 (± 444)	62.4 (± 12)
pUWM472	3 (± 1.6)	248 (± 8)
pUWM477	1720.7 (± 188)	29.8 (± 5)

Fig. 4. Transcriptional analysis of *cjaAB – lacZ* reporter fusions.

A. The scheme of the plasmids, derivatives of pMW10, containing fragments of *cjaAB* operon used in expression studies. Names of the recombinant plasmids are indicated on the left. Promoter of the *cjaAB* operon is indicated by arrow.The result of expression studies of *cjaAB* operon in both *E. coli* DH5á and *C. coli* 72Dz/92.

B. The β-galactosidase activity (Miller Units) in *E. coli* and *C. coli* harbouring plasmids containing fragments of *cjaAB* operon. Strains were grown on rich media: BA base No 2 (*C. coli*) and LB (*E. coli*). The assays of β-galactosidase activity were carried out in triplicate.

estingly, CjaB is an out-paralogue of a lineage grouping orthologous proteins from both strains of *H. pylori* (present in their genomes as single copies) and two in-paralogous copies in the genome of *C. jejuni* NCTC11168. The proliferation of CjaB paralogues in *C. jejuni* suggests that these proteins may be functionally diversified and for instance exhibit different preference for the transported substrates.

Transcriptional analysis of *cjaAB-lacZ* reporter fusions. The arrangement of the *C. coli* DNA region containing the *cjaA* and *cjaB* genes suggested that the two genes are co-transcribed. *CjaAB*::*lacZ* reporter gene fusions were used to study the regulation of the putative *cjaAB* operon expression. A set of *cjaAB-lacZ* operon fusion were constructed in which progressively longer fragments of the *cjaAB* coding sequence were fused to the promotorless *lacZ* gene in the shuttle vector pMW10 (Wosten *et al.*, 1998). It is equipped with translational stop codons in the three reading frames present between polylinker and *lacZ* gene.

Recombinant plasmids (pUWM472, pUWM477, pUWM471, pUWM272 and pUWM273) were obtained by insertion of appropriate PCR amplified *Campylobacter* DNA fragments into pMW10 previously cut with BamHI and XbaI, or by subcloning. All of them but one (pUWM273) contained the –591 to +1 *cjaA* upstream region (+1 is the experimentally determined transcription start point, see above). Details of the vector structures are depicted in Fig. 4A. β-galactosidase activity indicated that the fusion present in pUWM273 was not expressed in *C. coli* and the level of β-galactosidase from this fusion in *E. coli* was very low (Fig. 4B). This suggested that *cjaA* and *cjaB* are co-transcribed. Maximum activity of β-galactosidase (~2000 units) was reached in cultures of *C. coli* carrying pUWM477 and pUWM471, that harbour the reporter gene inserted into *cjaA*. The level of β-galactosidase from the fusion with *lacZ* downstream of the 5' end of *cjaB* (pUWM272) was approximately six times lower than that from the fusion with *lacZ* within *cjaA*. Unexpectedly, the fusion carried by pUWM472, containing only the *cjaA* upstream region, was not expressed in *Campylobacter*.

Generally, the activities observed in *E. coli* were significantly lower than in *Campylobacter*. This was most pronounced for the fusion with the reporter gene inserted into *cjaA*. Interestingly, the operon fusion present on pUWM472 was expressed in *E. coli*, in contrast to the results determined for *C. coli.*

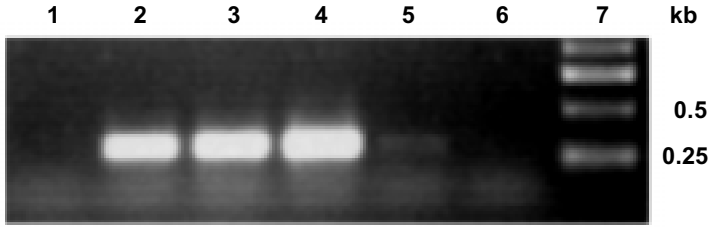


Fig. 5. RT-PCR analysis of the *cjaAB* operon transcription.

The reaction was carried out with two pairs of oligonucleotide primers: complementary to *lacZ* gene. The primers for the *lacZ* gene amplified a 307 bp product. There was no product when reverse transcriptase was omitted. Total RNA was from *C. coli* containing the following recombinant plasmid: Line: 1. pUWM273; 2. pUWM471; 3. pUWM472; 4. pUWM477; 5. pUWM272; 6. pMW10; 7. markers.

Transcriptional analysis of the putative *cjaAB* operon by RT-PCR. The differences in β-galactosidase activity observed for *C. coli* carrying different operon fusions could be due to different mRNA stability or its secondary structure influencing the level of translation. To distinguish between these two possibilities we measured the amount of *lacZ* gene transcript using RT-PCR. All the products obtained were of expected size and the absence of products in the reactions without RT (data not shown) clearly demonstrated that the bands observed with reverse transcriptase were derived from mRNA and not from contaminating DNA (Fig. 5). Two primers complementary to the *aph* vector gene were employed to standardise the assay (data not shown). No RT-PCR product was observed for bacteria carrying pUWM273. This confirmed that the lack of β-galactosidase activity in these cells was due to the lack of a promoter region, and thus proved that *cjaA* and *cjaB* are co-transcribed. The differences in the amount of the amplified product from bacteria carrying constructs with the reporter gene in different regions of the operon (pUWM477 *vs.* pUWM272) clearly correlated with the level of β-galactosidase activity. Contrary to expectations, RT-PCR showed the presence of specific mRNA transcribed from pUWM472. The nucleotide sequence of the insert carried by pUWM472 was checked by sequencing and was shown to be correct. The nucleotide sequence of the *cjaA* upstream region present in *Campylobacter* genome differ from that of pUWM472 by the length of the 5' untranslated region. Apparently, the secondary structure of this mRNA region completely abolished translation in *C. coli*, while it did not influence the interaction of the mRNA with *E. coli* ribosomes.

Discussion

CjaA encoded by the first gene of the *cjaAB* operon is an extracytoplasmic solute receptor (Wyszyńska *et al.*, 2003), component of the ABC transport system. In most bacterial genomes the genes for proteins of the ABC-type uptake system are organised in operons containing three, four or five genes (Boos and Lucht, 1994). Thus, when compared to other Gram-negative bacteria, the *C. coli cjaA* gene is unique. The second gene of the operon, *cjaB*, encodes a protein belonging to the MFS group (family 6). Genes coding for MFS permeases generally are separate transcription units. However, in the light of known facts concerning the organisation of the *Campylobacter* genetic material the *cjaAB* operon reflects unusual and still mysterious *Campylobacter* gene arrangement. To date only a few *Campylobacter* operons have been investigated experimentally. One of them contains the *fur* gene involved in the ferric uptake system. It has been shown that *fur* is co-transcribed with two housekeeping genes, *lysS* and *glyA* (van Vliet *et al.*, 2000).

Expression of the reporter gene *lacZ* placed in *cjaA* leads to a very high level of β-galactosidase (about 2000 Miller units) ranking the *cjaA* promoter among the strongest so far described *Campylobacter* promoters (Wosten *et al.*, 1998). Given that *Campylobacter* spp. are unable to use carbohydrates as a primary carbon and energy source and that amino acids can serve not only as a nitrogen but also as a carbon/energy source, the high expression of *cjaAB* could be expected. The protein encoded by the first gene, CjaA, is potentially present in a much higher amount compared to the product of the second gene of the operon, CjaB, as was indicated by β-galactosidase activity. High level of CjaB, an integral membrane transporter of an unknown substrate, might be toxic for cells. The RT-PCR analysis showed that the unequal amounts of the products of the two genes resulted from a modulation process that influences mRNA stability. Two inverted repeats (IR) located downstream of *cjaA* are possibly responsible for the protection of mRNA from 3'→5' exonucleolitic degradation, which causes accumulation of *cjaA* mRNA (Grunberg-Manago, 1999).

Similar IR sequences were found downstream of the first genes of several ABC-type transporter operons (Boos and Lucht, 1994).

The *C. jejuni* genome is AT rich. Due to the specific nucleotide sequence of the region located upstream of the TATA box, we postulate that the *cjaAB* promoter is recognised by *C. coli* RpoD-RNAP. Transcription is initiated at a cytidine, which is uncommon for RNAP with the main sigma factor, at least in *E. coli* (Barrios *et al.*, 1999). Although the –35 region of the promoter is almost identical to that recognised by *E. coli* σ^{70} RNAP the level of the reporter gene product was much lower in *E. coli* than in *C. coli*. This suggests that the *cjaAB* promoter specific for *Campylobacter* RpoD RNAP shows a rather low affinity for *E. coli* σ^{70} RNAP.

Taken together, the reporter gene experiments and RT-PCR studies showed that the *cjaA* and *cjaB* genes, belonging to different transporter superfamilies, are co-transcribed and that the stability of mRNA is responsible for varying amounts of the products of the two co-transcribed genes. In addition, unlike in other Gram-negative bacteria, but similar to some Gram-positive bacteria, the *cjaA* gene is possibly constitutively transcribed by RpoD RNAP. Unfortunately, the CjaB substrate still remains unknown. It is noteworthy that CjaB family members are absent from other ε-Proteobacteria with complete genomes, namely *H. hepaticus*, which occupies a different ecological niche than *C. coli*, *C. jejuni* or *H. pylori*, and non-pathogenic *Wolinella succinogenes*. It suggests that the members of the CjaB subfamily may be involved in the transport of substrates present only in stomach or small intestine.

Acknowledgements. This study received partial financial support from State Committee for Scientific Research (grant No 6P06K 043 21) and University of Warsaw (grant No. 140-501/68-1561-69). D. Pawelec was supported in part by a short-term fellowship from FEMS. J.M. Bujnicki was supported by the EMBO/HHMI Young Investigator Programme and by a fellowship from the Foundation for Polish Science. We thanks A. Zarębska for technical assistance. *E. coli* WG350 was kindly provided by Prof. Janet M. Wood (Department of Microbiology, University of Guelph, Canada).

Literature

Altschul S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389–3402.

Barrios H., B. Valderrama and E. Morett. 1999. Compilation and analysis of sigma(54)-dependent promoter sequences. *Nucleic Acids Res.* **27**: 4305–4313.

Boos W. and J.M. Lucht. 1994. Periplasmic binding protein dependent ABC transporters, p. 1175–1210. In: H.E. Umbarger (eds), *Escherichia coli* and *Salmonella.* Cellular and Molecular Biology. American Society for Microbiology, Washington, DC.

Bult C.J., O. White, G.J. Olsen, L. Zhou, R.D. Fleischmann, G.G. Sutton, J.A. Blake, L.M. Fitz Gerald, R.A. Clayton, J.D. Gocayne, A.R. Kerlavage, B.A. Dougherty, J.F. Tomb, M.D. Adams, C.I. Reich, R. Overbeek, E.F. Kirkness, K.G. Weinstock, J.M. Merrick, A. Glodek, J.L. Scott, N.S. Geoghagen and J.C. Venter. 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**: 1058–1073.

Chan V.L., E.K. Hani, A. Joe, J. Lynett, D. Ng and M. Steele. 2000. The hippurate hydrolase gene and other unique genes of *Campylobacter jejuni*, p. 455–463. In: M.J. Blaser (eds), *Campylobacter,* 2nd edition. ASM Press, Washington, DC.

Coker A.O., R.D. Isokpehi, B.N. Thomas, K.O. Amisu and C.L. Obi. 2002. Human campylobacteriosis in developing countries. *Emerg. Infect. Dis.* **8**: 237–244.

Culham D.E., B. Lasby, A.G. Marangoni, J.L. Milner, B.A. Steer, R.W. van Nues and J.M. Wood. 1993. Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. *J. Mol. Biol.* **229**: 268–276.

Deckert G., P.V. Warren, T. Gaasterland, W.G. Young, A.L. Lenox, D.E. Graham, R. Overbeek, M.A. Snead, M. Keller, M. Aujay, R. Huber, R.A. Feldman, J.M. Short, G.J. Olsen and R.V. Swanson. 1998. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**: 353–358.

Grunberg-Manago M. 1999. Messenger RNA stability and its role in control of gene expression in bacteria and phages. *Annu. Rev. Genet.* **33**: 193–227.

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

Kumar S., K. Tamura, I.B. Jakobsen and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**: 1244–1245.

Meinersmann R.J. and T.M. Wassenaar. 2003. Patterns in the intergenic regions of *Campylobacter jejuni*. *Genome Letters* **2**: 28–33.

Miller J.H. 1972. Experiments in Molecular Genetics. New York: Cold Spring Harbor.

Pao S.S., I.T. Paulsen and M.H. Saier, Jr. 1998. Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **62**: 1–34.

Parkhill J., B.W. Wren, K. Mungall, J.M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R.M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A.V. Karlyshev, S. Moule, M.J. Pallen, C.W. Penn, M.A. Quail, M.A. Rajandream, K.M. Rutherford, A.H. van Vliet, S. Whitehead and B.G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**: 665–668.

Pawelec D., E. Rozynek, J. Popowski and E.K. Jagusztyn-Krynicka. 1997. Cloning and characterization of a *Campylobacter jejuni* 72Dz/92 gene encoding a 30 kDa immunopositive protein, component of the ABC transport system; expression of the gene in avirulent *Salmonella typhimurium*. *FEMS Immunol. Med. Microbiol.* **19**: 137–150.

Petersen L., T.S. Larsen, D.W. Ussery, S.L. On and A. Krogh. 2003. RpoD promoters in *Campylobacter jejuni* exhibit a strong periodic signal instead of a –35 box. *J. Mol. Biol.* **326**: 1361–1372.

Saier M.H., Jr., J.T. Beatty, A. Goffeau, K.T. Harley, W.H. Heijne, S.C. Huang, D.L. Jack, P.S. Jahn, K. Lew, J. Liu, S.S. Pao, I.T. Paulsen, T.T. Tseng and P.S. Virk. 1999. The major facilitator superfamily. *J. Mol. Microbiol. Biotechnol.* **1**: 257–279.

Sambrook J. and D.W. Russell. 2001. Molecular Cloning. A Laboratory Manual. New York: Cold Spring Harbor.

Spratt B.G., P.J. Hedge, S. te Heesen, A. Edelman and J.K. Broome-Smith. 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene* **41**: 337–342.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**: 4876–4882.

Tomb J.F., O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischmann, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E.F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H.G. Khalak, A. Glodek, K. McKenney, L.M. Fitzegerald, N. Lee, M.D. Adams and J.C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539–547.

van Vliet A.H., J.D. Rock, L.N. Madeleine and J.M. Ketley. 2000. The iron-responsive regulator Fur of *Campylobacter jejuni* is expressed from two separate promoters. *FEMS Microbiol. Lett.* **188**: 115–118.

Wosten M.M., M. Boeve, M.G. Koot, A.C. van Nuene and B.A. van der Zeijst. 1998. Identification of *Campylobacter jejuni* promoter sequences. *J. Bacteriol.* **180**: 594–599.

Wyszyńska A., R. Godlewska and E.K. Jagusztyn-Krynicka. 2003. Product of the *cjaA* (*cj0982c*) *Campylobacter jejuni* gene is bifunctional protein. p. 71. In: XIIth International Workshop on *Campylobacter*, *Helicobacter* and related microrganisms. Aarhus, Danmark.