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The *Campylobacter jejuni/coli cjaA* (*cj0982c*) Gene Encodes an N-Glycosylated Lipoprotein Localized in the Inner Membrane

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Abstract The *Campylobacter coli* 72Dz/92 *cjaA* gene (orthologue of *cj0982c* of *C. jejuni* NCTC 11168) product is a highly immunogenic, amino acid–binding protein. CjaA was palmitic acid-modified when processed in *E. coli*. In addition, site-directed mutagenesis of the Cys residue of the LAAC motif of its signal sequence confirmed that CjaA is a lipoprotein when processed in *Campylobacter*. Localization of the protein appeared to be host dependent. In *Campylobacter*, CjaA was recovered mainly as an inner-membrane protein, whereas in *E. coli* most of the protein was present in the periplasmic space. Interestingly, antiserum raised against *Campylobacter* glycine-extracted material also recognized CjaA produced by *Campylobacter* and *Escherichia coli*, indicating that at least part of the protein may be surface exposed. Site-directed mutagenesis of the Asn residues of two putative N-linked glycosylation sites (NIS and NFT) showed that CjaA is glycosylated and that only the first N-X-S/T sequeon serves as a glycan acceptor.

Introduction

Campylobacter spp. are now among the world's most common enteropathogens. The clinical spectrum of enteric disease caused by *Campylobacter* infection ranges from generally mild noninflammatory diarrhea to severe inflammatory diarrhea with fecal blood and leukocytes. *C. jejuni* is also considered to be the second most common cause of traveller's diarrhea after *Escherichia coli* ETEC strains [5, 31]. In addition to causing acute gastrointestinal disease, *C. jejuni* infection has been shown to be associated with Guillain-Barré syndrome, a neurologic disease that may lead to respiratory muscle compromise and death. In addition, *C. jejuni* has been increasingly identified as a cause of bacteremia. Systemic infections do occur, especially in patients at the extremes of age or in those who are immunocompromised, such as human immunodeficiency virus (HIV)–infected individuals [1, 22, 31].

Campylobacter cannot catabolize glucose because it lacks the gene for a 6-phosphofructokinase. The comparative analysis of the five sequenced *Campylobacter* genomes showed that the phosphofructokinase gene is missing in all of them. However, biochemical data and in silico analysis indicate that the Embden-Meyerhof pathway functions in gluconeogenesis. The major noncarbohydrate precursors of glucose are lactate, amino acids, and glycerol [7, 13, 34]. The genome of *C. jejuni* NCTC 11168, as well as the sequenced genomes of four strains of other *Campylobacter* species (*C. jejuni*, *C. lari*, *C. coli*, and *C. upsaliensis*), contain many genes that are potentially involved in amino-acid binding, which is consistent with the limited biosynthetic capacity of the microorganism [7]. *Cj0982c* (named *cjaA*), the product of which exhibits a significant overall homology to solute-binding proteins (family 3), which are components of the ABC transport system, is the first gene of the *cjaAB* operon, which consists of two genes that encode proteins belonging to different transporter families [26]. Muller et al. [21] demonstrated that recombinant Cj0982c produced in *E. coli* is the component of the ABC cysteine transport system.

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Amino acids are the fundamental source of carbon, nitrogen, and energy for *Campylobacter*, which implies that CjaA may be an essential protein involved in many aspects of bacterial physiology and pathogenesis. In support of that hypothesis, our previous study demonstrated that immunization of chickens with avirulent *Salmonella* expressing CjaA protects birds against colonization with wild-type *Campylobacter* [37].

The aim of the work presented here was to investigate posttranslational modification of *C. coli* CjaA (Cj0982c), a highly immunogenic *Campylobacter* protein. Because in silico analysis showed that CjaA contains a signal sequence with the LAAC motif attributed to lipoprotein precursors, its localization was also examined.

Materials and Methods

Bacterial Strains and Plasmids and Growth Conditions

C. coli 72Dz/92 strain was obtained at the Child Health Centre from a patient with diarrhea. The strain was originally classified as *C. jejuni* by biochemical testing. However, it was classified as *C. coli* by polymerase chain reaction (PCR) [24]. The strain belongs to the most commonly serotype isolated in Poland: Lior 71. *C. coli* 72Dz/92, which easily accepts foreign DNA, was used in all complementation experiments. The *C. jejuni* 81176 strain, which was isolated during an outbreak of *Campylobacter* diarrhea associated with raw milk consumption and widely used in pathogenesis studies, was a gift of M. J. Blaser [14]. *Campylobacter* strains were cultured at 42°C on blood agar base no. 2 medium supplemented with 7.5% horse blood and *Campylobacter*-selective supplement (Oxoid) under microaerobic conditions. All *E. coli* strains were routinely cultured in Luria-Bertani broth (LB) or on 1.5% agar LB plates. Antibiotics (100 gmg/ml ampicillin plus 40 gmg/ml kanamycin or 20 gmg/ml chloramphenicol) were added to the media when appropriate.

Recombinant DNA Techniques

Procedures for plasmid DNA isolation and DNA analysis, agarose gel electrophoresis, and transformation of *E. coli* competent cells were carried out according to Sambrook and Russel [30]. Preparations of plasmid DNA for electroporation, as well as isolation of DNA from agarose gel, were performed according to the manufacturer's instructions (A & A Biotechnology). Polymerase chain reactions (PCRs) 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. DNA sequencing was performed with an ABI Prism 373 automated DNA sequencer (Perkin Elmer). The nucleotide sequences of the *cjaA* gene from *C. coli* 72Dz/92 and *C. jejuni* 81176 were deposited in the European Molecular Biology Laboratory Nucleotide Sequence Database under accession numbers Y10872 and AM055719, respectively.

Recombinant Plasmid Construction

Based on vector pBluescript II KS (Stratagene), which is nonreplicable in *Campylobacter*, two suicide plasmids were designed to inactivate the *cjaA* gene in *C. coli* 72Dz/92 and *C. jejuni* 81176 by inverse PCR methodology. First, the 765-bp fragment of the *cjaA* gene, amplified from the total DNA of *C. coli* 72Dz/92, using the oligonucleotide pairs 5′ ACAGGATCCGAATTCAGATTCTGGTGCTTC 3′ and 5′ ACAGGATCCTTACCGCCTTCAATAACTAC 3′, was inserted into pBluescript II KS with an inactivated EcoRI site. The resulting plasmid, named pUWM294, was employed as the template in an inverted PCR with the oligonucleotide primers 5′ ACTGAATTCTATCTTGAGGCACAGCCA 3′ and 5′ ACTGAATTCAG GAACTACT GCTGATGC 3′ containing EcoRI restriction site overhangs (underlined text). The primers' orientations resulted in amplification of template DNA in opposite directions around the cloning vector. The resultant DNA product was digested with EcoRI, self ligated, and electroporated into competent *E. coli* XL1-Blue. The construct, with a deletion of 53 bp introduced within the *cjaA* open reading frame, was designated pUWM408. Next, the Km cassette, which lacked a transcriptional termination signal, was inserted in the orientation corresponding to the *cjaA* transcription, which was confirmed by PCR. The resulting plasmid was named pUWM409.

The plasmid pUWM415 (4.6 kb) was used as the template in an inverse PCR with the oligonucleotide primers 5′ ATCGGATCCTGGAGATGATGTTAAGGCTG 3′ and 5′ ATCGGATCCCCAATCCTAACAACTCCATT 3′ to generate a deletion within the *C. jejuni* 81176 *cjaA* gene, which includes a unique BamHI site. The resulting 4-kb PCR product was gel purified, digested with BamHI, and self ligated to yield the plasmid pUWM423. This plasmid, after digestion with BamHI, was ligated to the BamHI-BamHI Km^R cassette (approximately 1.4 kb) excised from pBF14. The resulting plasmid was named pUWM428.

A *pglB* knockout mutant was constructed in *C. jejuni* 81176. The construct for *pglB* mutagenesis was created by insertion of a chloramphenicol cassette derived from pRY109 [41] into the *pglB*-coding sequence cloned in pGEM-T Easy (Promega). Orientation of the Cm cassette (corresponding to the *pglB* transcription) was determined through PCR reaction with primers complementary to the *pglB* and *cat* gene DNA sequences.

The inactivated genes were introduced into the *Campylobacter* genome by allelic exchange. *Campylobacter* electrocompetent cells were prepared as described by Wassenaar et al. [36]. The *C. jejuni* and *C. coli cjaA* mutant strains were named AW1 and AW5, respectively. The *C. jejuni* 81176 *pglB* mutant was designated WW1. The expected disruptions of the chromosomal loci as a result of double-crossover recombination events were verified by PCR amplification. Loss of the wild-type *cjaA* gene product in *C. jejuni* AW1 and *C. coli* AW5 was also demonstrated by Western blotting whole-cell proteins with specific anti-rCjaA antibodies. A generated mutant displayed normal viability, indicating that the product of the *cjaA* and *pglB* genes belongs to the class of nonessential proteins.

Site-Directed Mutagenesis

Point mutations were generated using a Quick-Change site-directed mutagenesis kit according to the procedures recommended by the supplier (Stratagene). Plasmid pUWM60 (a derivative of pBluescript II KS containing the *cjaA* gene from *C. coli* 72Dz/92 [26]) was used as a template for PCR-mediated mutagenesis. Point mutations C20A, N108A, and N139A were introduced with primers GTATTTTTGGCTGCTGCTGGAGGAAATTCAGATTCT GG and CCAGAATCTGAATTTCCTCCAGCAGCAGCC AAAAATAC, GTTGATATTATTTTAGCTGCTTTTACT CAAACACCTGAAAG and CTTTCAGGTGTTTGAGT AAAAGCAGCTAAAATAATATCAAC, and GGCTGTG CCTCAAGATAGCGCTATCAGTAGCATAGAAG and CTTCTATGCTACTGATAGCGCTATCTTGAGGCACA GCC, respectively (mismatches are double underlined). Plasmids containing the *cjaA* gene with various point mutations were transformed into *E. coli* DH5ga, and the presence of the desired mutations were verified by DNA sequencing. After digestion of the resulting recombinant plasmids with NotI and SalI, fragments containing the *cjaA* gene were cloned into shuttle vector pRY111 [41] and digested with the same enzymes. The resulting plasmids were named pUWM645 (*cjaA* with C20A mutation), pUWM703 (*cjaA* with N139A mutation), and pUWM704 (*cjaA* with N108A mutation). Shuttle plasmids containing a wild copy of the *C. coli cjaA* gene were named pUWM638. All derivatives of pRY111 were introduced into *C. coli* AW5 by electroporation.

Preparation of Cellular Fractions

Proteins from periplasmic space were released using chloroform as described by Ames et al. [2] or using the cold-shock osmotic procedure as described by Pittman et al. [29]. The cell envelope was fractionated into inner (IM) and outer (OM) membranes by selective solubilization of IM with N-lauroylsarcosine sodium salt. Preparation of *Campylobacter* membrane fractions was performed according to the method of Blaser et al. [3], and *E. coli* membrane fractions were prepared using the procedure described by Filip et al. [6]. Glycine-acid extract was prepared by the procedure of Garvis [8]. CjaAx6His protein was purified by affinity chromatography (Qiagen) under nondenaturing conditions after cloning the mature protein-coding nucleotide sequence amplified by PCR into pQE31 plasmid.

Labeling CjaA with [^{3}H]-palmitate

E. coli XL1-Blue, which harbored pUWM60 (pBluescript II KS containing the *cjaA* gene expressed from its own promoter) and *E. coli* XL1-Blue/pBluescript II KS, were grown in LB medium at 37°C to an optical density $(OD)_{600}$ of 0.4. Afterward, [^{3}H]-palmitic acid (25 gmCi/ml; DuPont-NEN) was added and incubated for 3 h incubation. *E. coli* cells were then harvested and washed twice in 100% methanol, and the pellet was air dried. Bacteria were resuspended in phosphate-buffered saline (PBS) and lysed with the sample buffer. The released proteins were separated by tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 16.5% T and 3% C), and radiolabeled lipoproteins were detected by fluorography.

Protein Immunoblot Analysis

Preparation of bacterial protein extracts, SDS-PAGE, and blotting procedures were done by standard methodologies [30]. The blots were developed using rabbit anti-rCjaA antibodies. To eliminate nonspecific reactivity, rabbit serum was absorbed first with heat-killed *E. coli* cells and then with an *E. coli* cell lysate obtained by sonication. Serum against rCjaA was raised in rabbits immunized with rCjaAx6His as described earlier [27]. Serum from rabbits immunized with *Campylobacter* glycine-extracted proteins was a gift from R. Curtiss III. Anti-Omp50 and anti-CadF antibodies were a gift from J. M. Bolla.

SBA Affinity Assay

Preparations of membrane proteins were mixed with soybean agglutinin (SBA) agarose (Vector Labs) and incubated at room temperature with gentle shaking for 2 h. The supernatant was removed, and beads were washed 15 times with 1 ml PBS. *Campylobacter* SBA-binding proteins were eluted from the beads with three washings with 0.1 ml 0.5 M galactose [17].

Results

Our earlier studies presented the preliminary characterization of the *C. coli cjaA* gene product. CjaA belongs to

family 3, solute-binding proteins, which are a component of the ABC transport system [26] Based on the known *C. jejuni* NCTC 11168 *cj0982c* (*cjaA* orthologue) nucleotide sequence, the *C. jejuni* 81176 *cjaA* gene has been also cloned and sequenced. Comparison of these two nucleotide sequences showed 98.9% identity.

The *C. cjaA* gene was mutagenized by insertion of a kanamycin cassette as described in "Materials and Methods". Proteins isolated from *cjaA* mutant and wild-type strains were resolved by SDS-PAGE and reacted with specific rabbit anti-rCjaA serum. The results of the experiment are presented in Fig. 1. (lanes 3 and 4). Two immunoreactive bands of approximately 30 and 32 kDa were observed when proteins derived from the wild-type strain were examined. Developing the blot that contained proteins from *cjaA* :: Km mutant using anti-rCjaA resulted in loss of both immunoreactive bands, indicating that CjaA consists of two forms of different Mr. In addition, Western immunoblot analysis with anti-rCjaA antibodies was carried out using whole-cell extracts of 10 human clinical isolates that were members of 2 species: *C. jejuni* and *C. coli*. The species of the *Campylobacter* strains were determined through PCR amplification using primers complementary to 23S rRNA [27]. All examined strains produced CjaA protein that existed in 2 forms having different mobility (data not shown).

Posttranslational Modification of CjaA

N- and O-linked protein glycosylation pathways of *Campylobacter* have recently been characterized. The genes of the N-glycosylation 16-kb locus responsible for posttranslational modification of >20 proteins were named *pgl*

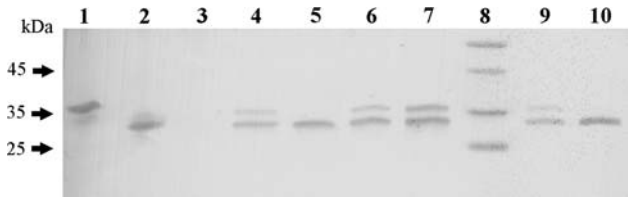

Fig. 1 Immunoblot analysis of proteins isolated from wild-type *C. coli* 72Dz/92, from the mutant AW5 and from *C. coli* AW5 overexpressing CjaA, with replacement of the asparagine residues to alanine in one of the two putative consensus sequences for N-linked glycosylation. *Campylobacter* protein extracts were electrophoretically separated on a 12% polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with polyclonal anti-rCjaA antibodies. The relative positions of molecular weight markers are listed on the left. Lane 1 = CjaAx6His protein purified by affinity chromatography. Lane 2 = *E. coli* /pUWM60 (CjaA). Lane 3 = *C. coli* 72Dz/92 AW5 (*cjaA*$^-$). Lane 4 = *C. coli* 72Dz/92 wild-type. Lane 5 = *C. coli* AW5/pUWM703 (CjaA N139A). Lane 6 = *C. coli* AW5/pUWM704 (CjaA N108A). Lane 7 = *C. coli* AW5/pUWM638 (CjaA wild-type). Lane 8 = molecular size standard. Lane 9 = *C. jejuni* 81176 wild-type. Lane 10 = *C. jejuni* 81176 WW1 (*pglB*$^-$)

[12, 32]. The CjaA amino-acid sequence contains two motifs (108-NFT-110 and 139-NIS-141), whose asparagines potentially can be N-glycosylated. Only 139-NIS-141 motif contains a negatively charged amino acid at the –2 position. To determine whether CjaA is glycosylated, a *pglB*-knockout mutant was constructed in *C. jejuni* 81176. PgIB is a homologue of the Stt3p subunit of the eukaryotic oligosaccharyl transferase complex and plays a key role in N-linked glycosylation. PglB is involved in the transfer of an undecaprenyl-linked heptasaccharide to the asparagine residue of the conserved glycosylation motif. Loss of the *pglB* gene product resulted in one CjaA form with increased mobility, proving that CjaA is its target (Fig. 1, lanes 9 and 10).

The N-glycosylation process is absent in *E. coli* and *Salmonella enterica sv. Typhimurium* cells. To examine further the potential posttranslational modifications of CjaA, the pACYC184/*pgl* plasmid, which carries a *Campylobacter pgl* gene cluster functioning in *E. coli* [35], was introduced into *E. coli/S. enterica* expressing CjaA. Next, whole-cell extracts were examined for reactivity with rabbit anti-*Campylobacter* serum. The lack of presence of the *pgl* gene in *E. coli* and *S. enterica* did not change the amount of CjaA formed (data not shown).

To more fully investigate the posttranslational modification of CjaA using site-specific mutagenesis, either N of two NXS/T motifs of CjaA was replaced by A. Plasmids containing mutated forms of the *cjaA* gene were electroporated into the *C. coli* AW5 strain. The mobility of the mutated versions of the proteins was examined using SDS-PAGE. It was documented that only one NXS/T motif within CjaA is modified by glycan binding. Replacement of N with A at position 139 resulted in only a nonglycosylated form of the protein, whereas changing the NFT (position 108 to 110) sequence to AFT did not affect CjaA mobility. The experiment confirmed that CjaA is modified by glycan binding (Fig. 1, lanes 5 through 7).

CjaA affinity for SBA was also tested. *C. jejuni* membrane proteins were mixed with SBA agarose, and elution fractions were analyzed by Western blot. As shown in Fig. 2A, the experiment did not provide unequivocal data. Antiserum against CjaA recognized both CjaA forms among eluted proteins. However, antiserum against nonglicosylated CadF did not detect this protein (data not shown). In addition, when *C. jejuni pglB* mutant was employed for the test, none of the CjaA forms were detected by specific serum among proteins eluted by 0.5 M galactose (Fig. 2B).

CjaA Localization

Analysis of the amino-acid sequence of CjaA showed that it contains a putative signal sequence that can be processed by signal peptidase I as well as by signal peptidase II, which are specific for lipoprotein precursors. In silico

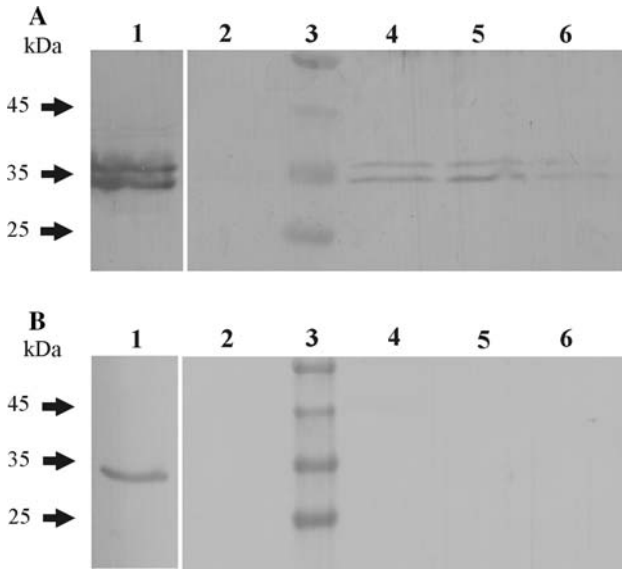

Fig. 2 CjaA affinity for SBA. Protein extracts from wild-type *C. coli* 72Dz/92 (**A**) and *C. jejuni* 81176 WW1 (*pglB*$^{-}$) (**B**) were electrophoretically separated on a 12% polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with polyclonal anti-rCjaA antibodies. The relative positions of molecular weight markers are listed on the left. Lane 1 = membrane proteins applied to SBA agarose. Lane 2 = wash fraction. Lane 3 = molecular size standard. Lanes 4 through 6 = fractions containing proteins eluted from SBA agarose with 0.5 M galactose

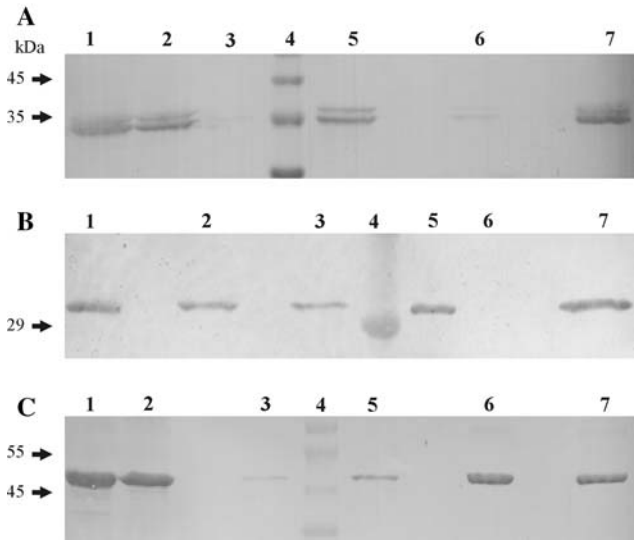

Fig. 3 Subcellular localization of CjaA in *C. coli* (**A**) and *E. coli* XL Blue/pBluescript containing the *cjaA* gene expressed from its own promoter (**B**). Subcellular localization of Omp50 in *C. coli* (**C**). The proteins derived from cell fractions were electrophoretically separated on a 12% polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with polyclonal anti-rCjaA (**A** and **B**) or anti-Omp50 (**C**) antibodies. The relative positions of molecular weight markers are listed on the left. Lane 1 = whole cell lysate. Lane 2 = chloroform-shocked cells. Lane 3 = fraction of periplasmic proteins released by chloroform. Lane 4 = molecular size standard. Lane 5 = sarcosyl-soluble fraction (IM proteins). Lane 6 = sarcosyl-insoluble fraction (OM proteins). Lane 7 = proteins of both membranes

analysis suggested that CjaA is instead processed by signal peptidase II (LipoP v1.0 log odds score 20.3685; signalP v3.0 probability 0.271). The subcellular localization of CjaA in *C. coli*, *E. coli*, and *S. enterica sv. Typhimurium* was analyzed by cell fractionation. Proteins derived from different cell compartments were separated by SDS-PAGE, electrotransferred onto nitrocellulose membrane, and probed with anti-rCjaA antibodies (Fig. 3). The experiment demonstrated that CjaA localization is host dependent. In the native host, the protein was found primarily in the IM (Fig. 3A, lane 5), and no CjaA was present among the periplasmic proteins (Fig. 3A, lane 3). In contrast, CjaA was overexpressed in *E. coli* or in *S. enterica* was detected both as an IM (Fig. 3B, lane 5) and as a periplasmic protein (Fig. 3B, lane 3). The subcellular localization of Cj0982c of *C. jejuni* 81176 was identical to CjaA from *C. coli* 72Dz/92 (data not shown). To verify the employed method, subcellular fractions of *C. coli* were also used to determine Omp50 localization. As expected, Omp50 was found as an OM protein (Fig. 3C, lane 6) [4].

To further examine CjaA localization, we investigated the reactivity of CjaA produced by *Campylobacter* and *E. coli* strains with rabbit antiserum to *Campylobacter* glycine-extracted proteins. The results showed that this antiserum reacted with CjaA produced by *C. coli* and *E. coli* cells, indicating that CjaA is rather loosely connected with the IM and transported into the cell surface (data not shown).

The lipoprotein nature of CjaA was confirmed by growing *E. coli* XL1-Blue harboring pUWM60 (pBluescript II KS containing the *cjaA* gene expressed from its own promoter) in the presence of [^{3}H] palmitic acid. The experiment showed that CjaA was modified with palmitic acid when processed in *E. coli* (Fig. 4A, lane 1). To provide further insight into the role of the LAAC motif of the CjaA signal sequence, we carried out alanine mutagenesis of C20. Proteins derived from different cell compartments of the *Campylobacter cjaA*$^{-}$ containing a plasmid that expressed C20-mutated CjaA were examined for the presence of CjaA. Specific anti-rCjaA antibody recovered the protein, mainly from the *Campylobacter* periplasmic space, thereby confirming the role of the cystein in the processing the protein (Fig. 4B, lane 3). The intensity of the band representing the more slowly migrating form of mutated CjaA is much weaker than in the case of wild-type protein. Periplasmic proteins were also obtained using the cold-osmotic shock method. Changing the procedure did not influence CjaA localization. CjaA expressed in weight *C. jejuni* was absent among periplasmic proteins compared with the mutated form (C20A), which was detected as being located in the periplasm (data not shown).

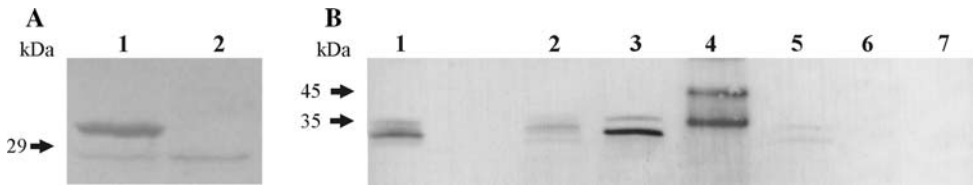

Fig. 4 (**A**) [^{3}H]-palmitate lipoproteins in *E. coli*/pUWM60 (CjaA). Radioactive palmitate was added to label proteins. Total cellular proteins were separated on a 12% polyacrylamide gel, and radiolabelled polypeptides were visualized by fluorography. Lane 1 = *E. coli*/pUWM60 (CjaA). Lane 2 = *E. coli*/pBluescript II KS. (**B**) Subcellular localization of CjaA in *C. coli cjaA*$^-$/pUWM645 (pRY111 containing *C. coli cjaA* gene with the C20A mutation). The proteins derived from cell fractions were electrophoretically separated on a 12% polyacrylamide gel, blotted onto nitrocellulose membrane, and probed with polyclonal anti-rCjaA antibodies. The relative positions of molecular weight markers are listed on the left. Lane 1 = whole cell lysate. Lane 2 = chloroform-shocked cells. Lane 3 = fraction of periplasmic proteins released by chloroform. Lane 4 = molecular size standard. Lane 5 = proteins of both membranes. Lane 6 = sarcosyl-insoluble fraction (OM proteins). Lane 7 = sarcosyl-soluble fraction (IM proteins)

Discussion

All of the polar amino acid–binding proteins of Gram-negative bacteria are located in the periplasm, whereas those of Gram-positive microorganisms are attached to the membrane [9, 10]. CjaA, unlike other solute-recognizing (family 3) proteins of Gram-negative bacteria, are lipid modified. Moreover, other *Campylobacter* proteins that play a role in the uptake process are lipid modified, whereas their analogues from *E. coli* are periplasm located. Further investigation of CjaA localization showed that the protein is recognized by rabbit serum raised against glycine-extracted proteins. Acid-glycine treatment of Gram-negative bacteria is a mild method that preferentially releases surface-located proteins. Thus, CjaA is probably easily released from the cell membrane. This is also seen with two others *Campylobacter* proteins: JlpA and CjaC (HisJ). JlpA and CjaC were recovered as IM proteins by cell fractionation, and CjaA was also present in glycine-extracted material [11, 38]. Peb1 is bifunctional *Campylobacter* protein playing a role in adhesion as well as L-aspartate and L-glutamate transport. It was originally isolated from a glycine-extracted *Campylobacter* cell fraction [28]. Peb1 is equipped with a signal sequence similar to that of CjaA. However, compared with CjaA, Peb1 is localized in the periplasmic space of *Campylobacter* cells but not in the IM or OM [20]. Moreover, we demonstrated that the CjaA localization is host dependent. Lipoproteins of Gram-negative bacteria are anchored into the IM and the OM by way of fatty acids attached to N-terminal cysteine. The destination of *E. coli* lipoproteins to either the IM or OM is dependent of the lipoprotein-sorting signal, the amino-acid residue next to the lipid modified cysteine. Generally, an Asp at this position results in retention of the lipoprotein in the IM, whereas others residues direct lipoproteins to the OM [40]. The mechanisms underlying the signal-dependent localization of the lipoproteins were examined in detail by Tokuda's Laboratory. In *E. coli*, the LolA-LolB system, consisting of five proteins (LolABCDE), is the general mechanism by which OM-directed lipoproteins are efficiently released from the cytoplasmic membrane and transported into the OM [19, 39, 42]. According to the +2 rule, CjaA having a Gly at the position next to lipid-modified cystein should be OM located, but a comparable amount of the protein was found in the IM and in the periplasmic space of *E. coli*. Thus, the CjaA sorting signal functions as a rather weak retention signal in *E. coli*. It is possible that CjaA is recognized by the *E. coli* LolA transporter, but the created complexes are unstable and do not interact with LolB. In addition, other *Campylobacter* ligand-binding lipoprotein, CjaC (HisJ), is detectable in the *Campylobacter* IM but overexpressed in *E. coli*, where it is localized mainly in the periplasmic space [38]. Alternatively, CjaA can be processed successively by two signal peptidases, which results in release of part of the protein into the periplasmic space. Defectiveness of the *C. jejuni* Lol transport system (lack of LolB protein) may explain why some lipoproteins are partially released from the IM and transported to the cell surface instead of being incorporated into the OM.

In contrast, several observations indicate that the ge-Proteobacteria (*C. jejuni* and *H. pylori*) cell envelope structure has some unique properties. The IMs and OMs of these species interact tightly. The observed number of fusions between IM and OM is much higher than that seen in *E. coli* [25]. This might be the second alternative explanation of untypical localization observed for *Campylobacter/Helicobacter* lipoproteins and may suggest that cell-fractionation methods developed for *E. coli* are inappropriate for *Campylobacter*.

N-linked glycosylation of *Campylobacter* proteins has been recently identified. Many details of the process have been examined [18, 33, 43]. It is widely accepted that the process plays an important role in many aspects of bacterial physiology and pathogenesis. More than 30 *Campylobacter* proteins are targets of *pgl* gene products [43]. CjaA is a novel characterized *Campylobacter* N-glycosylated protein. Although the *pgl* locus activity in *E. coli/Salmonella*

enterica did not result in CjaA glycosylation, site-specific mutagenesis and CjaA processing in *C. jejuni pglB*$^{-}$ cells proved its glycosylation in *Campylobacter*. In wild-type *Campylobacter*, CjaA is present in two forms of different mobility as determined by SDS-PAGE, whereas only one form has been observed in *C. jejuni pglB*$^{-}$. Replacement of the N of the NIS sequeon resulted in a nonglycosylated form. The Asn residue of the 108-NFT-110 motif is located in the predicted amino acid–binding pocket, and it seems that a potential glycosylation would inhibit ligand binding. In contrast, the Asn residue of the 139-NIS-141 motif is located on the protein surface and is accessible to the solvent. Our results also confirm Kowarik et al's observation that *Campylobacter* N-glycosylation requires the presence of -D/E-X1-N-X2-S/T, where X1 and X2 can be any amino acid except proline [15]. The weak intensity of the more slowly migrating form of mutated CjaA (C20A) might suggest that membrane attachment of the protein is required for its efficient glycosylation and explain why CjaA, whose localization is host dependent, was not glycosylated in *E. coli* containing the *pgl* locus. However, as documented by Nita-Lazar et al. [23], the AcrA protein was glycan modified both as a membrane-attached and a soluble periplasmic protein. The soybean lectin affinity assay did not provide an unequivocal answer to the problem. Anti-CjaA antibodies recognized two CjaA forms among eluted proteins. However, antiserum against nonglicosylated CadF did not react with any protein, thus indicating accuracy of the assay. It is worth noting that when VirB10 was analyzed for lectin affinity, a small amount of nonglycosylated form was also eluted from the column [16]. We hypostatize that under native conditions, glycosylated and nonglycosylated CjaA forms, which are present among membrane proteins, interact and that the complex is recognized by SBA. Two forms can be separated on SDS-PAGE, and both can be detected by antiserum against CjaA. A similar assay was performed using *C. jejuni pglB* mutant. Anti-CjaA antibodies did not detect CjaA among proteins present in the elution fraction, which supports our hypothesis. Therefore, further experiments are required to clarify the correlation of CjaA localization and its posttranslational modification.

In summary, our results showed that CjaA is a glycosylated lipoprotein in *Campylobacter* spp. Localization of the protein appears to be host dependent. In *Campylobacter*, CjaA was recovered mainly as an IM protein, whereas in *E. coli* most of the protein was present in the periplasmic space. *Campylobacter* contains some homologues of the *E. coli* Lol proteins, but the sorting of its lipoproteins still remains unclear. Such learned knowledge would substantially increase the possibilities for manipulation of *Campylobacter* lipoproteins in *E. coli*/*S. enterica* strains.

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