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The N-X-S/T consensus sequence is required but not sufficient for bacterial N-linked protein glycosylation

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In the Gram-negative bacterium *Campylobacter jejuni* there is a *pgl* (protein glycosylation) locus–dependent general N-glycosylation system of proteins. One of the proteins encoded by *pgl* locus, PglB, a homolog of the eukaryotic oligosaccharyltransferase component Stt3p, is proposed to function as an oligosaccharyltransferase in this prokaryotic system. The sequence requirements of the acceptor polypeptide for N-glycosylation were analyzed by reverse genetics using the reconstituted glycosylation of the model protein AcrA in *Escherichia coli*. As in eukaryotes, the N-X-S/T sequon is an essential but not a sufficient determinant for N-linked protein glycosylation. This conclusion was supported by the analysis of a novel *C. jejuni* glycoprotein, HisJ. Export of the polypeptide to the periplasm was required for glycosylation. Our data support the hypothesis that eukaryotic and bacterial N-linked protein glycosylation are homologous processes.

Key words: AcrA/*Campylobacter jejuni*/glycoproteins/N-glycosylation

Introduction

N-linked protein glycosylation is the most frequent protein modification in eukaryotic cells. It occurs in the lumen of the endoplasmic reticulum (ER) through a complex pathway that is conserved in most of the eukaryotes (Helenius and Aebi, 2004; Kornfeld and Kornfeld, 1985). This pathway can be divided into two distinct processes: (1) the assembly of the lipid-linked oligosaccharide at the membrane of the ER, and (2) the transfer of the preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) from the lipid anchor dolichyl pyrophosphate to nascent polypeptides with the consensus sequence N-X-S/T (Helenius and Aebi, 2004). The transfer of the oligosaccharide to protein is catalyzed by the oligosaccharyltransferase (OST), a protein complex that is composed of multiple subunits. In the best studied yeast enzyme, eight different components, all transmembrane proteins, have been identified (Knauer and Lehle, 1999). Stt3p is the most highly conserved protein in this complex. Genetic and biochemical studies in both yeast and mammalian cells suggest that this protein plays a central role in the enzymatic activity of OST (Kelleher *et al*., 2003; Nilsson *et al*., 2003; Yan and Lennarz, 2002).

N-linked protein glycosylation, for a long time considered specific for eukaryotes and archaea, was recently detected in the Gram-negative bacterium *Campylobacter jejuni*. The N-glycosylation machinery of *C. jejuni* is encoded by the *pgl* locus (Szymanski *et al*., 2003; Wacker *et al*., 2002; Young *et al*., 2002). Based on the sequence analysis of *pgl* encoded polypeptides, it was concluded that bacterial and eukaryotic N-glycosylation represent homologous processes (Wacker *et al*., 2002). We proposed that in the bacterial N-glycosylation, an oligosaccharide is assembled on a lipid carrier at the cytoplasmic side of the plasma membrane, translocated into the periplasm, and transferred to selected asparagine residues of polypeptide chains. As in eukaryotes, the glycosylation sites would be characterized by the N-X-S/T sequon (Gavel and Von Heijne, 1990), where X can be any amino acid except proline (Bause, 1983; Bause and Legler, 1981; Gavel and Von Heijne, 1990; Imperiali *et al*., 1992). Limited structural data of *C. jejuni* glycoproteins support this hypothesis (Wacker *et al*., 2002). Bacterial N-linked protein glycosylation is dependent on the PglB protein, a transmembrane protein with significant sequence similarity to the eukaryotic Stt3p protein family. In particular, PglB contains the highly conserved sequence motif WWDYG in the C-terminal part essential for enzymatic activity in vivo (Wacker *et al*., 2002; Yan and Lennarz, 2002). For that reason it was suggested that the PglB protein is the bacterial OST (Wacker *et al*., 2002). Importantly, the oligosaccharide structure transferred in the *C. jejuni* system differs significantly from the oligosaccharide used in eukaryotic cells (Wacker *et al*., 2002; Young *et al*., 2002).

In this article, we aimed at testing experimentally two of the predictions based on the hypothesis that bacterial and eukaryotic N-linked protein glycosylation are homologous processes. (1) N-X-S/T is the acceptor sequence of bacterial N-linked glycosylation, and (2) N-linked protein glycosylation occurs in the periplasm. For the experimental setup, we took advantage of the finding that protein glycosylation can be functionally transferred from *C. jejuni* to *Escherichia coli* by expressing the *C. jejuni pgl* locus and glycosylatable proteins in this host (Wacker *et al*., 2002).

Results

Analysis of point mutations in the N-X-S/T sequon

Two out of five N-X-S/T sequons serve as glycan acceptor in AcrA. AcrA, the best characterized *C. jejuni* glycoprotein, contains five potential N-glycosylation sites that follow the

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canonical N-X-S/T sequence. We have shown previously that one of these sites, 123-N-R-S-125, was glycosylated by the *C. jejuni* glycosylation system (Wacker *et al.*, 2002). We speculated that in addition to this glycosylation site, one other site of AcrA protein was glycosylated, leading to the diglycosylated form. The other potential glycosylation sites are located at positions 117-N-A-S-119, 147-N-N-S-149, and 273-N-N-S-T-276. The latter sequence represents two potential glycosylation sites with overlapping consensus sequences. To test our hypothesis that the second glycosylation site of AcrA also conforms the N-X-S/T rule, mutant forms of the AcrA protein containing N to L replacements at each of the putative glycosylation sites were generated and coexpressed with the *pgl* locus in *E. coli*. To detect AcrA glycosylation, membrane protein extracts of different *E. coli* strains were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to membranes, and probed with the R12 serum. This serum, raised against whole *C. jejuni* cells, recognizes the glycosylated *C. jejuni* AcrA protein but that does not react with the nonglycosylated protein (Wacker *et al.*, 2002). It is therefore an ideal tool to detect glycosylated AcrA when expressed in *E. coli.* In addition a serum raised against recombinant *C. jejuni* AcrA protein was used. This serum reacts with AcrA protein irrespective of its glycosylation status.

Expression of *C. jejuni* AcrA in *E. coli* yielded unglycosylated AcrA (Figure 1, lane 1), whereas coexpression of AcrA with the *C. jejuni pgl* locus revealed glycosylated AcrA, visualized by the R12 serum (Figure 1, lane 2), demonstrating the specificity of the R12 serum. The two glycosylation specific bands represent monoglycosylated (g1AcrA) and diglycosylated (g2AcrA) AcrA protein. The majority of the AcrA protein remains unglycosylated, as shown with the serum raised against AcrA (Figure 1, lane 2), indicating partial glycosylation under the conditions used.

As expected, the N to L replacement at position 123, the previously identified glycosylation site, resulted in only monoglycosylated AcrA (Figure 1, lane 4). In contrast, the N to L mutations at position 117 and 147, respectively, did not alter AcrA glycosylation (Figure 1, lanes 3 and 5). On the contrary, the N273-274L double replacement NN to LL resulted in monoglycosylated AcrA, as did the N273L mutation (Figure 1, lanes 6 and 7), whereas the N274L mutation did not affect glycosylation (Figure 1, lane 8). We concluded that also the second glycosylation site of AcrA was found among the putative glycosylation sites (position 273N-N-S275) as deduced from the eukaryotic glycosylation system.

Serine or threonine can serve as hydroxyl amino acid in the acceptor sequence, proline as the X residue prevents glycosylation. The asparagine residue of the N-X-S/T sequence is directly modified by the glycosylation machinery. In the eukaryotic glycosylation system, the hydroxyl amino acid serine or threonine within the consensus sequence is an essential component of the acceptor sequence (Bause and Legler, 1981; Gavel and Von Heijne, 1990; Imperiali *et al.*, 1992). To assess whether the bacterial N-glycosylation system imposes the same sequence requirements, we replaced the serine residue of the 123-N-R-S-125 sequon by an alanine or a threonine residue. As visualized in Figure 2, lane

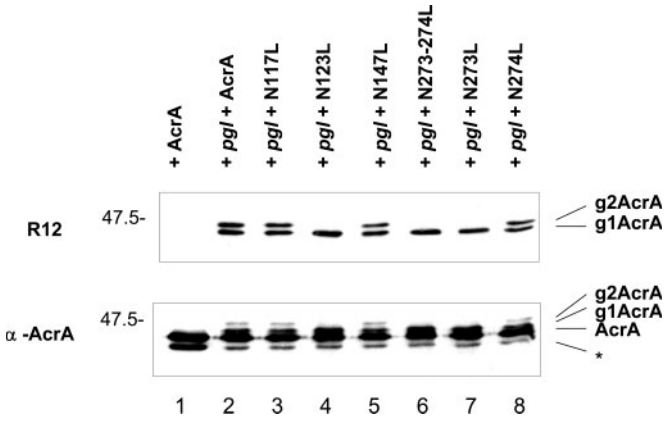

Fig. 1. Analysis of membrane proteins of *E. coli* BL21(DE3) overexpressing AcrA or AcrA with replacement of the asparagine residues of the five putative consensus sequences for N-linked glycosylation. Membrane proteins were separated by SDS–PAGE and, after transfer to nitrocellulose, immunodetected with the *C. jejuni* antiserum R12 (top) or with the antiserum raised against recombinant AcrA produced in *E. coli* (bottom). The presence of the AcrA-expressing plasmid and the *pgl* locus containg plasmid is indicated above the lanes. Extracts of cells expressing the proteins encoded by the *pgl* locus (lanes 2–8) and the AcrA protein in its wild-type (lanes 1 and 2) or mutant forms (lanes 3–8) were analyzed. The following amino acid exchanges were studied: N117L (lane 3), N123L (lane 4), N147L (lane 5), N273–274L (lane 6), N273L (lane 7), N274L (lane 8). The migrating positions of nonglycosylated AcrA (AcrA), monoglycosylated AcrA (g1AcrA), and diglycosylated AcrA (g2AcrA) are indicated. The position of an AcrA degradation product is marked with an asterisk.

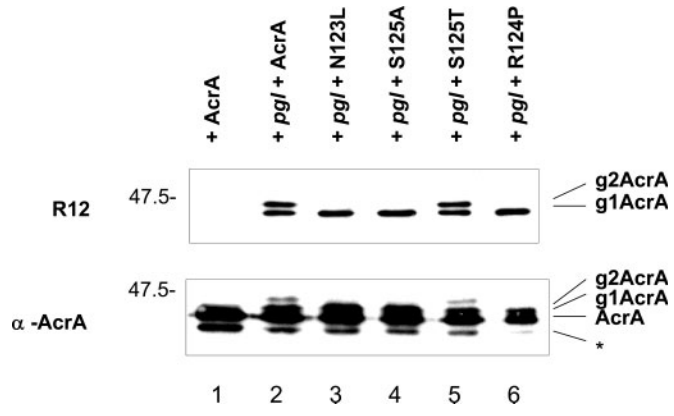

Fig. 2. Analysis of membrane proteins of *E. coli* BL21(DE3) overexpressing AcrA or AcrA with amino acid replacements in the 123NRS125 acceptor sequence. Membrane proteins were separated by SDS–PAGE and, after transfer to nitrocellulose, immunodetected with the *C. jejuni* antiserum R12 (top) or with the antiserum raised against recombinant AcrA produced in *E. coli* (bottom). The presence of the AcrA-expressing plasmid and the *pgl* locus containg plasmid is indicated above the lanes. Extracts of cells expressing the proteins encoded by the *pgl* locus (lanes 2–6) and the AcrA protein in its wild-type (lanes 1 and 2) or mutant forms (lanes 3–6) were analyzed. The following amino acid exchanges were studied: N123L (lane 3), S125A (lane 4), S125T (lane 5), R124P (lane 6). The migrating positions of nonglycosylated AcrA (AcrA), monoglycosylated AcrA (g1AcrA), and diglycosylated AcrA (g2AcrA) are indicated. The position of an AcrA degradation product is marked with an asterisk.

4, the S to A mutation inactivated the glycosylation site, only monoglycosylated AcrA was detected. In contrast, the replacement of the serine with a threonine residue did not alter the glycosylation state of AcrA protein (Figure 2, lane 5). As was the case for the eukaryotic glycosylation sites (Bause, 1983), a proline separating the acceptor asparagines from the hydroxyl amino acid serine inactivated the acceptor sequence (Figure 2, lane 6).

We concluded that the N-glycosylation site requirements of the bacterial glycosylation system were the same as for the eukaryotic system. Importantly, of the five potential glycosylation sites within the AcrA sequence, only two were utilized, indicating that the N-X-S/T sequon was necessary but not sufficient to act as an acceptor sequence in the bacterial system.

The N-X-S/T sequon is required but not sufficient for glycosylation of the HisJ protein

Identification of HisJ as a glycoprotein. To support this conclusion, we identified an additional *C. jejuni* protein that reacted with the R12 antiserum. This protein appears as a band of 32 kDa on SDS–PAGE of wild-type membrane extracts that are probed with the R12 serum (Figure 3, lane 1). This band was no longer observed when a *pglB* mutant strain of *C. jejuni* was analyzed the same way (Figure 3, lane 2). This putative glycoprotein was purified by affinity chromatography using an soybean agglutinin–lectin column (Linton *et al*., 2002) and tryptic fragments were analyzed by mass spectrometry (MS). Comparison with the deduced proteome of *C. jejuni* identified the glycoprotein as the HisJ protein, a component of the histidine uptake system located in the periplasm (Garvis *et al*., 1996, 1997; Pawelec *et al*., 1998, 2000) and encoded by the Cj0734c gene. Site-directed deletion of the Cj0734c gene and complementation by a plasmid-borne *hisJ* copy verified that the 32-kDa protein was encoded by the *hisJ* (Cj0734c) locus (Figure 3, lanes 3 and 4). The primary amino acid sequence of HisJ suggested an N-terminal lipid anchor of the mature protein (Sankaran *et al*., 1995), supported by strong membrane association of the HisJ protein during purification (data not shown).

One out of two N-X-S/T sequons serve as an acceptor for glycosylation. Two potential N-linked glycosylation sites (29-N-A-S-31 and 127-N-D-S-129) were identified in the primary HisJ coding sequence. To confirm that HisJ was indeed a glycoprotein, the *C. jejuni* HisJ protein containing a C-terminal His_6-tag was expressed in *E. coli* in the presence and absence of the *C. jejuni pgl* locus. The R12 serum recognized HisJ in its nonglycosylated form (Figure 4, lane 2). Co-expression of the *pgl* glycosylation locus in HisJ-expressing *E. coli* cells yielded an additional, slower-moving protein in SDS–PAGE (Figure 4, lane 1) that was absent when the locus encoded the mutant, inactive form of PglB (Figure 4, lane 5; Wacker *et al*., 2002). These data suggested a single oligosaccharide chain attached to the HisJ protein. Indeed, we found that the mutation N29L within the first potential N-glycosylation site resulted in a disappearance of the glycosylated HisJ, whereas the N127L mutation within the second glycosylation sequon did not affect glycosylation (Figure 4, lanes 3 and 4).

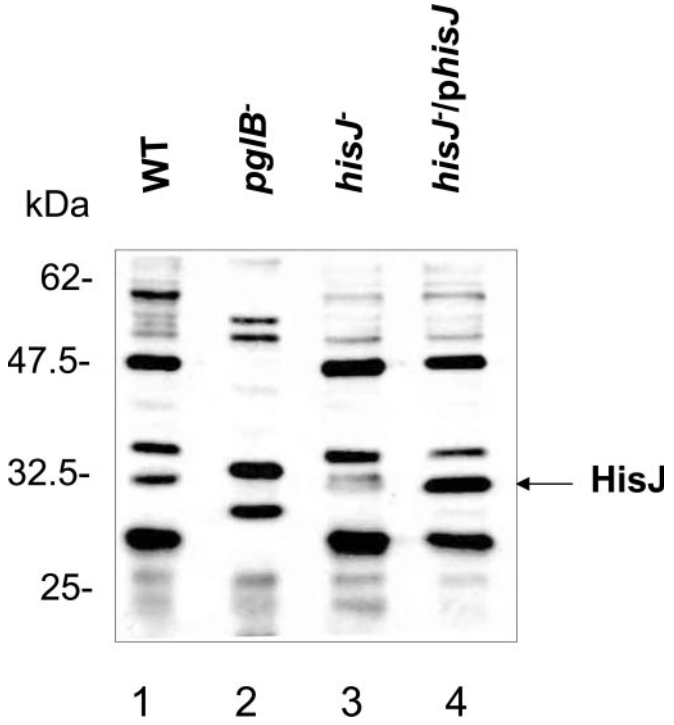

Fig. 3. Analysis of *C. jejuni* membrane proteins of wild type (WT), *pglB* and *hisJ* mutants, and complementation *in trans*. Membrane proteins were separated by SDS–PAGE and, after transfer to nitrocellulose, immunodetected with *C. jejuni* antiserum R12. Lane 1, WT; lane 2, *pglB* mutant; lane 3, *hisJ* mutant; lane 4, *hisJ* mutant containing expressing HisJ in *trans.* Molecular weight markers are indicated on the left. The arrow indicates the position of the HisJ glycoprotein (gHisJ) absent in the mutant strains but present when complemented in *trans* by introduction of the specific plasmids.

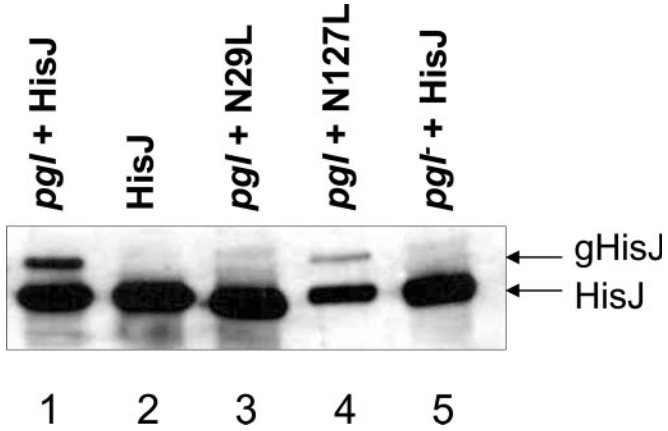

Fig. 4. Analysis of whole-cell extracts of *E. coli* BL21(DE3) overexpressing HisJ or HisJ with replacement of the Asn residues of the two putative consensus sequences for N-linked glycosylation. Whole-cell extracts were precipitated by trichloracetic acid and separated by SDS–PAGE and, after transfer to nitrocellulose, immunodetected with the *C. jejuni* antiserum R12. Lanes 1, 3, and 4, cells expressing the proteins encoded by the *pgl* cluster and HisJ (lane 1) or HisJ with amino acid exchanges: N29L (lane 3), N127L (lane 4). Lane 5, cells expressing the proteins encoded by the *pgl* cluster, where PglB contains amino acid substitutions (W458A and Y459A) in the highly conserved domain, and HisJ. The migrating position of unglycosylated HisJ (HisJ) and glycosylated His J (gHisJ) is indicated.

N-glycosylation occurs in the periplasm

Both AcrA and HisJ protein are periplasmic proteins with a specific signal sequence containing the information for the attachment of the protein to the membrane via an N-terminal lipid anchor (Sankaran *et al.*, 1995). To assess whether periplasmic location and lipid anchor were required for glycosylation, mutant forms of the AcrA protein were expressed in glycosylation-competent *E. coli* cells. First, the endogenous AcrA signal sequence was replaced by that of PelB from *Erwinia carotovora* (Lei *et al.*, 1987). This resulted in a secreted but soluble periplasmic protein that did not contain the lipid anchor. Importantly, this exchange of the signal sequence did not alter glycosylation of AcrA in *E. coli* (Figure 5, lane 2). However, deletion of the signal sequence, resulting in cytoplasmic expression of the protein, did completely abolish glycosylation (Figure 5, lane 3). The proposed localization of the different forms of AcrA was confirmed by subcellular fractionation (Figure 6). AcrA with its endogenous signal sequence was mainly localized in the membrane fraction, whereas the AcrA with the PelB signal sequence was in the periplasmic fraction, and both these forms were glycosylated. AcrA lacking a signal sequence was mainly found in the cytosolic but also in the other fractions. This was most likely due to the formation of cytoplasmic inclusion bodies and suebsequent copurification with periplasmic and membrane fractions. SurA, a periplasmic protein, and OmpF, a membrane protein, served as controls for proper fractionation. We concluded that periplasmic location of the protein was required for N-linked protein glycosylation, but attachment of the protein to the membrane by a lipid anchor was not necessary for this post-translational modification.

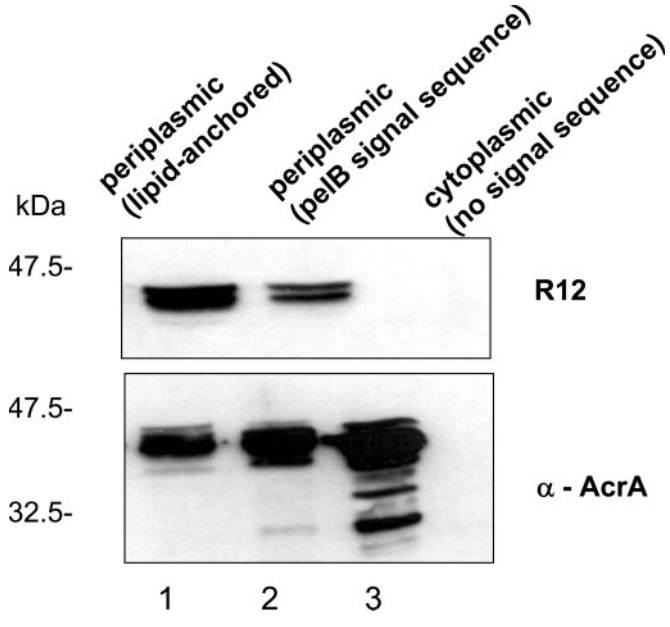

Fig. 5. Analysis of whole-cell extracts of *E. coli* BL21(DE3) overexpressing membrane-attached (lane 1), periplasmic soluble (lane 2), and cytosolic (lane 3) forms of AcrA protein. All cells coexpress the *pgl* locus. Proteins were separated by SDS–PAGE and, after transfer to nitrocellulose, immunodetected with the *C. jejuni* antiserum R12 (top) or with the antiserum raised against recombinant AcrA produced in *E. coli* (bottom).

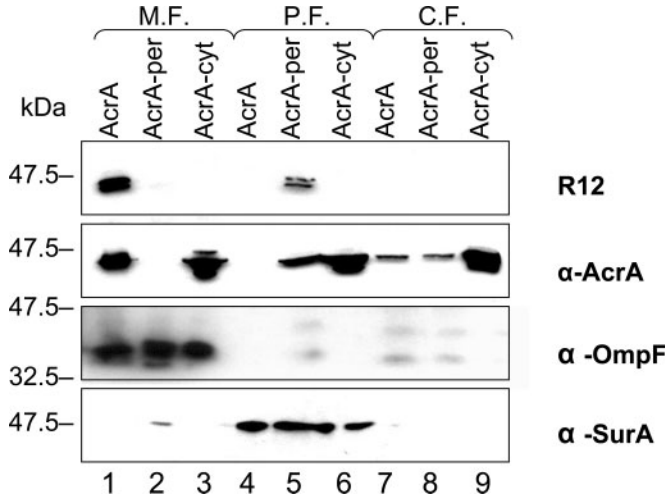

Fig. 6. Intracellular localization of the three forms of AcrA. Subcelluar fractions of *E. coli* BL21(DE3) overexpressing membrane-attached (lanes 1, 4, and 7), periplasmic soluble (lanes 2, 5, and 8) and cytosolic (lanes 3, 6, and 9) forms of AcrA protein in the presence of the *pgl* locus were separated by SDS–PAGE and, after transfer to nitrocellulose, immunodetected with the *C. jejuni* antiserum R12, with the antiserum raised against recombinant AcrA produced in *E. coli* and with an antiserum raised against OmpF and SurA from *E. coli*. Lanes 1–3 represent the membrane fraction (MF), lanes 4–6 the periplasmic fraction (PF), and lanes 7–9 the cytoplasmic fraction (CF). Constitutive proteins of *E. coli*, SurA, a periplasmic protein, and OmpF, a membrane protein, were used as controls for the fractionation.

Discussion

We analyzed the requirements for the polypeptide substrate in the bacterial N-linked protein glycosylation system. As an experimental system, we used *E. coli* cells transformed with the *C. jejuni pgl* locus. In these glycosylation competent cells, two different periplasmic *C. jejuni* proteins (AcrA and HisJ) were expressed. Both of these substrates were modified in a PglB-dependent manner. Site-directed mutagenesis of putative N-X-S/T glycosylation sites revealed that utilization of a glycosylation site was impaired by exchanging the asparagine acceptor residue with a leucine residue, by exchanging the serine residue with alanine, or by placing a proline as the X residue in between the two functionally important sites. Importantly, exchanging the serine residue of one glycosylation site by threonine did not affect utilization of this site. These findings follow the peptide substrate requirements for eukaryotic N-glycosylation with the acceptor sequence N-X-S/T, where X can be any amino acid except proline. As in eukaryotic systems, where about 60% of the sequons in secretory proteins are glycosylated (Petrescu *et al.*, 2004), the N-X-S/T sequon was necessary but not sufficient to define an N-glycosylation acceptor sequence. Three out of five and one out of two potential glycosylation sites were not utilized in the proteins we analyzed. This selected utilization was not an artifact due to the heterologous *E. coli* test system but was also observed in *C. jejuni* (see Figure 3 for HisJ; Wacker *et al.*, 2002, for AcrA).

Interestingly, a recent study of sequence requirements in the archaeal N-glycosylation system revealed two distinct

N-glycosylation processes, one of them independent of the hydroxyl amino acid residue (Zeitler *et al.*, 1998).

In eukaryotes, N-linked protein glycosylation occurs in the lumen of the ER. Our present findings with signal sequences directing translocation of the polypeptide into the periplasm suggest that N-linked protein glycosylation occurs in the periplasm, the functionally equivalent compartment to the ER lumen of eukaryotes.

In summary, we have shown that with respect to localization and peptide acceptor sequence, eukaryotic and bacterial N-glycosylation are homologous processes. Similar conclusions were obtained by analyzing the requirements for the oligosaccharide substrate and the role of the putative PglB OST in this process (Feldman *et al.*, unpublished data). The bacterial N-glycosylation process is therefore an ideal experimental system to address the molecular mechanism of N-linked protein glycosylation, a ubiquitous protein modification.

Materials and methods

Bacterial strains and plasmids

C. jejuni 81–176 (Black *et al.*, 1988) was routinely grown on Mueller Hinton agar (Difco, Sparks, MD) under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) at 42°C. *E. coli* strains were grown on Luria Bertani agar at 37°C. Antibiotics kanamycin (Km), 50 μg/ml; chloramphenicol, 10 μg/ml; and ampicillin, 100 μg/ml were added to the media for selection. *E. coli* DH5α was the host for cloning experiments and *E. coli* BL21(DE3) the host for high-level expression of AcrA, HisJ, and proteins encoded by *pgl* locus. *E. coli* DH5α(RK212.1) was used as a donor in conjugation experiments (Figurski and Helinski, 1979). Plasmids pACYC184 (New England Biolabs, Beverly, MA), pET22b, pET24b (Novagen, Madison, WI), and pRY111 (Yao *et al.*, 1993) were used as cloning vectors. A full list of plasmids used in this work is given in Table I.

Purification of C. jejuni *glycoproteins and identification by MS*

C. jejuni glycoproteins were purified as described elsewhere (Wacker *et al.*, 2002). The purified glycoproteins were separated by 12% SDS–PAGE and stained with GELCODE blue stain reagent (Pierce Biotechnology, Rockford, IL). The protein of ~ 32 kDa was cut from the gel using a scalpel and reduced with 10 mM dithiothreitol; the free cysteine thiols were alkylated with 50 mM iodoacetamide and the protein was trypsin-digested (sequencing grade, Promega, Madison, WI). The peptide fragments were analyzed by matrix-assisted laser desorption/ionization MS and the protein identified by mass fingerprinting and database searching.

Mutagenesis of hisJ *in* C. jejuni

Mutagenesis of *hisJ* was carried out by replacing most of the coding sequence with a kanamycin resistance cassette. The following primer pairs were used: 5′ GGGAGTACTCTAGGAACGGCTTGAGTATC 3′ and 5′ TATCGATCGCCAACTAA-AGCAACTAGAGC 3′ (for amplification of 5′ fragment), 5′ GGATTAATGCAAAAACTGATGGATTTATG 3′ and 5′ GGATTAATGAAGCAGGTGATAAAATCGC 3′ (for amplification of 3′ fragment). The restriction sites are underlined. The 778-bp 3′ fragment was amplified by polymerase chain reaction (PCR) using *C. jejuni* 81–176 genomic DNA as template, cut with *Vsp*I, and cloned into pILL600 cleaved with the same enzyme, located downstream of the Km^r cassette (Labigne-Roussel *et al.*, 1988). Next, the 776-bp 5′ fragment was amplified by PCR using *C. jejuni* 81–176 genomic DNA as template; the fragment was cut with *Sca*I and *Pvu*I and cloned into the resulting plasmid cleaved with the same enzymes. These restriction sites are located upstream of the Km^r cassette. The resulting plasmid pILL600 (hisJ) containing *aphA* inserted in the same transcriptional orientation as *hisJ* was used to transform *C. jejuni* 81–176 by natural transformation (Guerry *et al.*, 1994) and mutants were selected on MH agar containing kanamycin. The mutants were characterized by PCR to confirm that the incoming plasmid DNA had integrated by a double crossover event and in the same transcriptional orientation as the deleted gene, minimizing the risk of polar effects.

Table I. Plasmids used in these studies

Plasmid	Description	Reference
pILL600	Km resistance cassette without transcription terminator, can be used for gene deletions in *C. jejuni*, Tet^R and Km^R	Labigne-Roussel *et al.* (1988)
pILL600 (hisJ)	Km resistance cassette between 5′ and 3′ fragment of *hisJ* for deletion of *hisJ* of *C: jejuni*, Tet^R and Km^R	This work
pRY111	Shuttle plasmid for *C. jejuni*, Cm^R	Yao *et al.* (1993)
pRY111 (hisJ)	*HisJ* under control of its own promoter, Cm^R	This work
pET24b	Cloning vector, T7 promoter, Km^R	Novagen
pET24 (AcrA)	AcrA with His_6-tag cloned into pET24b, Km^R	This work
pET24 (AcrA-cyt)	AcrA without signal sequence and with His_6-tag cloned into pET24b, Km^R	This work
pET24 (AcrA-per)	Soluble AcrA with pelB signal sequence and His_6-tag cloned into pET24b, Km^R	This work
pET24 (HisJ)	HisJ with His_6-tag cloned into pET24b, Km^R	This work

Complementation of hisJ *mutant in trans*

HisJ including promoter and transcription terminator was amplified by PCR with the following primers using *C. jejuni* 81–176 genomic DNA as template: 5′ CAATCCCCTCTGCAGGGTTTGC 3′ and 5′ CGAGCGTGCGGCCGCTAAATTTAGTGG 3′. The restriction sites are underscored. The 1.75-kb PCR product was cut with *Pst*I and *Not*I and cloned into the chloramphenicol-resistant *Campylobacter* shuttle plasmid pRY111 cut with the same enzymes. The resulting plasmid pRY111(hisJ) was mobilized from *E. coli* DH5α containing pRK211.1 into *C. jejuni hisJ* mutant cells

(Guerry *et al.*, 1994). Transconjugants were selected on MH agar containing kanamycin and chloramphenicol.

Expression of AcrA and HisJ in E. coli

AcrA and *hisJ* were amplified by PCR using *C. jejuni* 81–176 genomic DNA as template with the following primers: 5′ GGAATTCCATATGAAATTATTTCAAAAAAATACTATTTTA-GC 3′ and 5′ CCGCTCGAGTTGTGCTCCAATTTCTTTAACTTCGCTACC 3′ (*acrA*), 5′ GGAATTCCATATGAGCAAAGAAGAAGCACCAAAAATAC 3′ and 5′ CCGCTCGA-GTTGTGCTCCAATTTCTTTAACTTCGCTACC 3′ (*acrA*, no signal sequence), 5′ GAGCT-CCGTCGACAAGCTATGAAAAAAATATTAAGCATTGCTCTAGTT 3′and 5′ TGCTCG-AGTGCGGCCGCAAGCTTGTCGTTCTAATTCATATTTT-TTAATTAAAG 3′ (*hisJ*). The restriction sites are underscored. The PCR products were cut with *Nde*I and *Xho*I or *Sal*I and *Not*I and cloned into pET24b cut with the same enzymes. Under the control of the T7 promoter, this leads to the expression of membrane-attached AcrA (pET24 [AcrA]), soluble cytoplasmic AcrA (pET24 [AcrA-cyt]) and membrane-attached HisJ (pET24 [HisJ]), all of them with a His_6 tag at the C-terminus. To express AcrA as a soluble protein in the periplasm, the nucleotides encoding for the pelB leader sequence were amplified using pET22b as template and the following primers: 5′ GGAATTCCATATGCATGGCCATCGCCG-GCTG-GG 3′ and 5′ CTTCCGGGCGCTATCATGCCATACCGCGAAAGG 3′. The restriction site is underscored. The PCR product was cut with *Nde*I and cloned into the plasmid expressing AcrA in the cytoplasm cut with the same enzyme, resulting in pET24 (AcrA-per). Correct orientation of the pelB leader sequence was verified by PCR.

Introduction of point mutations in AcrA and HisJ

Introduction of point mutations in AcrA and HisJ was performed using the QuikChange mutagenesis kit (Statagene, La Jolla, CA), pET24b (AcrA) and pET24 (HisJ) as template and the primers noted in Table II.

SDS–PAGE and detection

Expression of AcrA and HisJ was induced by addition of 1 mM ispropylthiogalactoside. Membrane proteins from BL21 (DE3) expressing AcrA and the proteins encoded by the *pgl* cluster were prepared as described (Wacker *et al.*, 2002). Whole-cell extracts from BL21 (DE3) expressing HisJ and the proteins encoded by the *pgl* cluster were pelleted by trichloracetic acid. The protein expression was analyzed by 10% and 12% SDS–PAGE, respectively, and detection by western blot using antiserum raised against whole cell extracts of *C. jejuni* (R12) and antiserum raised against recombinant AcrA produced in *E. coli*.

Preparation of subcellular fractions

AcrA and proteins encoded by the *pgl* locus were expressed in *E. coli* BL21 cells. Cells were pelleted in the mid-log phase and incubated in lysis buffer (1 mg/ml lysozyme, 20% w/v sucrose, 30 mM Tris-HCl [pH 8.5], 1 mM ethylenediamine tetra-acetic acid [pH 8.0]) for 30 min at 4°C. The spheroblasts were pelleted at 14,000 × *g* for 2 min at 4°C. The supernatant contains the periplasmic fraction. The pellet was resuspended in 0.1 M Tris-HCl, pH 8.5. Spheroblasts were broken by freeze and thaw method with alternating liquid nitrogen and 37°C incubation cycles. Cell debris was pelleted at 5000 × *g* for 5 min and membranes at 250,000 × *g* for 30 min at 4°C. The supernatant contains the cytoplasmic proteins.

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Table II. Primers used in PCR for site-directed mutagenesis

Mutations in AcrA	Mutagenic oligonucleotide	Restriction site introduced
N117L	CCTTGCT**AGC**C**AG**TTCGAAAGTTGC	*Nhe*I
N123L	GAGCTTTAGA**T**C**T**A**AG**AAAATCCTTGC	*Bgl*II
N147L	GCTTTTGAATT**CAG**AAATGTAGC	*Eco*RI
N273L,N274L	GGTAAAAGTGTTGA**CAGCAG**GTTATCAAATACG	*Hinc*II
N273L	GGTAAAAGTGTTGA**ATTCAG**GTTATCAAATACG	*Eco*RI
N274L	GGTAAAAGTGTTGA**CAG**ATTGTTATCAAATACG	*Hinc*II
S125A	GCTAAAAAGAGC**C**TT**GGCT**CGATTAAAATCC	*Eco*130I
S125T	GAGCTTT**CGT**ACGATTAAAATCCTTGCTTGC	
R124P	GAGCTTTAGAA**G**GATTAAAATCCTTGC	
Mutations in HisJ		
N29L	GCAGTTCCAACCTTTAATTCTACACTTGC**CAG**TGATTCTTTATTTTTAGAATC	*Bse*NI
N127L	GAAGAGAGTC**CAG**GTTTTTTAG	*Bst*NI

Only the forward primers complementary to the coding strand are shown. Primer bases in boldface note substituted nucleotides.
Presence of the mutations in the final constructs was verified by restriction analysis and by DNA sequencing

Abbreviations

ER, endoplasmic reticulum; MS, mass spectrometry; OST, oligosaccharyltransferase; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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