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# Campylobacter Protein Glycosylation Affects Host Cell Interactions

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***Campylobacter jejuni* 81-176 *pgl* mutants impaired in general protein glycosylation showed reduced ability to adhere to and invade INT407 cells and to colonize intestinal tracts of mice.**

There is an increasing awareness of the existence of prokaryotic glycoproteins (36), often in complex surface structures such as pili (7, 8, 28, 39), S layers (37), and flagella (6, 10, 11, 12, 19, 23, 44). Among glycosylated flagellins, those of *Campylobacter* spp. are the best characterized (11, 16, 42). The nature and extent of flagellin glycosylation have been determined for strain 81-176, one of the best-characterized strains of *Campylobacter jejuni* (2, 3, 5, 21, 29, 41, 45, 46) and one which has been documented to cause diarrheal disease in two volunteer feeding studies 5; D. T. Tribble, unpublished data). Flagellin from 81-176 contains 19 sites of O-linked glycosylation to the monosaccharide pseudaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid) and analogs of pseudaminic acid (42). Additionally, *C. jejuni* 81-176 has been shown to contain a general protein glycosylation (*pgl*) system affecting many other soluble and membrane-associated proteins (41). The only reported phenotype of *pgl* mutants has been the loss of immunogenicity of multiple proteins as detected by Western blot analyses using polyclonal, hyperimmune rabbit antisera, changes that were identical to those seen following chemical deglycosylation of the same protein preparations (42). However, neither the identity of the proteins glycosylated by the *pgl* system nor the chemical nature of the attached carbohydrate(s) has been reported. This study describes additional phenotypes of 81-176 *pglB* and *pglE* mutants. The predicted protein encoded by *pglB* shows significant similarity to domains of an oligosaccharide transferase of *Saccharomyces cerevisiae* (48) and an ortholog in *Methanobacterium* spp. (38). PglE shows highest similarity to a putative aminotransferase involved in lipopolysaccharide synthesis in *Bacteroides fragilis* (9). The protein also shows homology to proteins involved in glycosylation of pilin in *Neisseria* spp. (20, 31) and flagellin in *Caulobacter crescentus* (23) and *Aeromonas caviae* (13, 32).

**Growth comparisons.** Cell morphology, as determined by transmission electron microscopy, was similar for 81-176 and *pglB* and *pglE* mutants (results not shown). Bacterial growth curves (Fig. 1) indicated that both mutants had slightly faster doubling times relative to 81-176. However, only the *pglE* mutant demonstrated a statistically significant increase in growth rate ($P < 0.05$) compared to the wild type by paired *t*-test analysis. Complementation of the *pglE* mutation in *trans* with plasmid pCS101, an *Escherichia coli-Campylobacter* shuttle vector containing an intact copy of *pglE* and its putative promoter (41), restored the wild-type doubling time.

Since numerous soluble and membrane proteins appear to be glycosylated by the *pgl* system, it was possible that the mutants would display increased sensitivity to growth inhibitors. The sensitivity of wild-type 81-176 and the *pglE* mutant to a variety of agents was determined by the method of Yethon et al. (47). Cultures were adjusted to an optical density at 600 nm ($OD_{600}$) of 0.1 in Mueller-Hinton (MH) broth supplemented with inhibitors. Growth was compared following incubation at 37°C under microaerophilic conditions with overnight shaking for 14 h. Growth was considered positive if the $OD_{600}$ was greater than 0.2 (47). No differences between the wild type and the *pglE* mutant were observed for growth in 0.05 mg of sodium dodecyl sulfate per ml (40, 47), 0.1 and 0.2% (wt/vol) sodium deoxycholate (34), or 0.0625 and 0.125 M NaCl (1, 33) (data not shown). In addition, no differences between the wild type and either *pgl* mutant were observed for growth in MH broth at pH 7.2 versus MH broth adjusted to pH 5.0 or 6.0 (data not shown).

TABLE 1. *C. jejuni* adherence to and invasion of INT407 cells[a]

| Strain | % (mean ± SE) | |
|---|---|---|
| | Adherence | Invasion |
| 81-176 | 5.91 ± 0.85 | 2.93 ± 0.19 |
| 81-176 *pglB* | 2.26 ± 0.74[b] | 0.13 ± 0.06[b] |
| 81-176 *pglE* | 3.49 ± 0.97 | 0.27 ± 0.12[b] |
| 81-176 *pglE*(pCS101) | 6.32 ± 0.89 | 1.85 ± 0.41 |

[a] Approximately $3 \times 10^6$ bacteria were added to a layer of approximately $4 \times 10^5$ cells (multiplicity of infection of 8) and incubated at 37°C. For determination of adherence, after 2 h of incubation with bacteria, INT407 cells were washed four times in Hanks' balanced salt solution with strong agitation for 2 min prior to lysing of the monolayer with 0.01% Triton X-100. For determination of invasion, the monolayer was incubated with 100 mg of gentamicin per ml in minimal essential medium (Gibco) for an additional 2 h prior to lysis with Triton X-100. Bacteria were enumerated by plate count, and the data are the percentages of the inoculum which adhered to or invaded INT407 cells in four or five independent experiments.

[b] $P < 0.05$ by paired *t*-test analyses.

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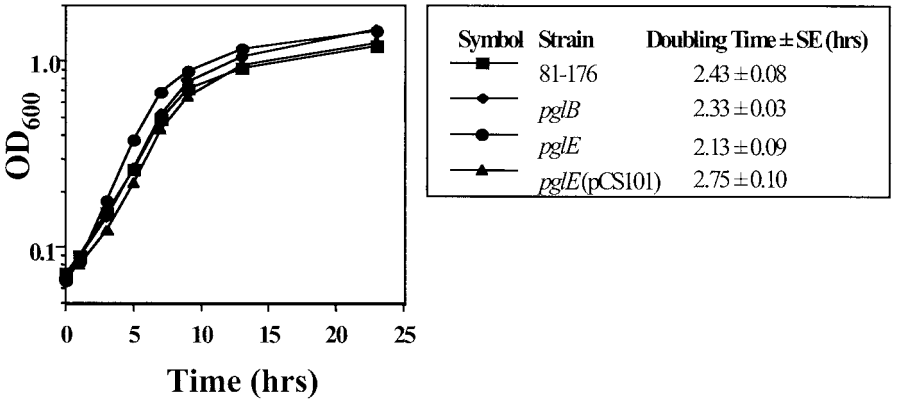


FIG. 1. Growth curve of 81-176 and *pgl* mutants. Bacterial cultures were adjusted to an $OD_{600}$ of 0.1 and grown in MH broth under microaerophilic conditions with shaking at 37°C. The relationship of $OD_{600}$ to viable count was equivalent for all strains examined. The mean doubling time of each strain from two or three experiments is shown. The growth curve shown is an example of one experiment.

**Adherence to and invasion of INT407 cells.** Motility has been shown to be required for *C. jejuni* adherence to and invasion of intestinal epithelial cells. Since *pglB* and *pglE* mutants show wild-type levels of motility (41), adherence and invasion assays using a human intestinal epithelial cell line (INT407) were done as previously described (27, 46, 47). The *pglB* mutant adhered at 38% and invaded at 4.4% of the level of the wild-type strain, while the *pglE* mutant adhered at 59% and invaded at 9.2% of wild-type levels (Table 1). When the *pglE* mutant was complemented in *trans* with pCS101, the strain adhered and invaded at levels comparable to those of the wild type.

**Mouse colonization.** Experiments reported herein were conducted according to the principles set forth in reference 26a. Hsd:ICR mice were fed $6 \times 10^9$ to $9 \times 10^9$ organisms and monitored for colonization for 3 weeks as described previously (46). Four freshly passed fecal pellets per animal were homogenized in phosphate-buffered saline (Sigma) daily and plated on *C. jejuni* selective agar (Remel). An animal was considered to be no longer colonized by *C. jejuni* after three consecutive negative cultures. As shown in Fig. 2, mice were colonized with wild-type 81-176 for 21 days. Both *pglB* and *pglE* strains demonstrated a significant reduction in percent colonization ($P <$ 0.001, using paired *t*-test analysis) as early as day 7 postinfection, and colonization remained significantly low through 21 days (Fig. 2). The presence of pCS101 in the *pglE* mutant in *trans* restored wild-type levels of colonization at all time points examined.

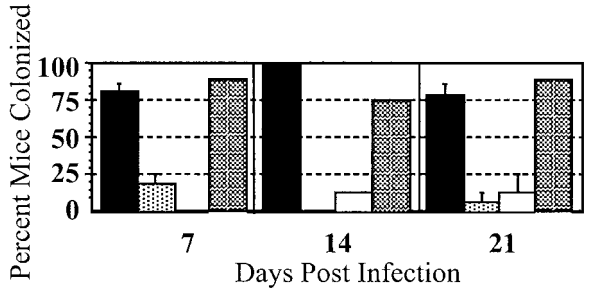


FIG. 2. Colonization of Hsd:ICR mice by *C. jejuni*. Each group contained 12 to 16 mice in two separate experiments except the group fed *pglE*(pCS101), which contained eight mice in a single experiment. Data are means ± standard errors. Black bar, 81-176; lightly stippled bar, *pglB* strain; white bar, *pglE* strain; heavily stippled bar, *pglE* strain carrying pCS101. The *pglB* and *pglE* mutants showed a statistically significant reduction in colonization ($P < 0.001$) compared to the wild type at all time points.

**Conclusions.** Despite an increasing awareness of the presence of glycoproteins in bacteria, little is understood about the biological significance of these modifications. Recent studies in bacteria have suggested that carbohydrate modifications on proteins can play a role in adhesion (4, 22, 24, 26), protection against proteolytic cleavage (18), solubility (25), antigenic variation (11, 15, 17), and protective immunity (16, 35). The glycosyl modifications on campylobacter flagellin are immunogenic and surface exposed in the flagellar filament (17, 30), suggesting that they may protect the flagellin protein from the immune system. The glycosyl modifications synthesized by the *pgl* genes on other campylobacter proteins have also been shown to be highly immunogenic (41). The observation that mutations in either *pglB* or *pglE* in 81-176 resulted in a significant reduction in adherence to and invasion of INT407 cells in vitro, and a reduced ability to colonize the intestinal tract of mice suggests a role for the general protein glycosylation system in virulence. Adherence to and invasion of *C. jejuni* 81-176 have been shown to be multifactorial, requiring motility (45, 46), the capsular polysaccharide (2), particular ganglioside mimicries in the lipooligosaccharide (14), a plasmid-encoded type IV secretion system (3), protein adhesins (29), and potentially other undetermined factors. It remains to be determined which glycoprotein(s) is responsible for the observed changes in virulence in the *pgl* mutants. Given the extent of general protein glycosylation in *C. jejuni* (41), the responsible proteins could be either soluble glycoproteins affecting key pathogenic processes or surface exposed glycoproteins that, like their eukaryotic counterparts (43), play a direct role in cellular interactions.

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