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Glycosylation of type-IV fimbriae of *Dichelobacter nodosus*

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Abstract

Dichelobacter nodosus is the causative agent of ovine footrot and the type-IV fimbriae on this bacterium are essential for maintaining its virulence. In this study, we reveal that these fimbriae are glycosylated. This was demonstrated in several ways: by the detection of carbohydrate on fimbrial protein using periodic acid Schiff reagent (PAS) staining of SDS-PAGE gels and by demonstrating enzymatic deglycosylation and by analysis of the amino acid sequences derived from the *fimA* gene, whereby the gene from isolates of *D. nodosus* that appeared to be glycosylated had potential glycosylation sites both inside and outside of the variable region of *fimA*. The results would also explain the observation that the calculated molecular weight of *fimA* from some *D. nodosus* serotypes does not correlate with the apparent size determined from electrophoretic mobility.

Keywords: *Dichelobacter nodosus*; Type-IV fimbriae; Glycosylation; *FimA* gene

1. Introduction

Dichelobacter nodosus is a fastidious gram-negative anaerobe that is the primary causative agent of footrot. The virulence of *D. nodosus* isolates varies and depends upon such factors as variation in the fimbrial protein *fimA*, the production of extracellular proteases and the presence of particular gene sequences (*vap* and *vrl* regions) (Billington et al., 1996; Parker et al., 2006).

The type-IV fimbriae found on *D. nodosus* are similar to those found on many gram-negative organisms and are typified by the sequence similarity of the subunit protein, their polar location and the presence of particular N-terminal residues (Craig et al., 2004). They have been shown to be involved in twitching motility (Mattick, 2002) and essential for virulence, protease secretion, and natural competence (Kennan et al., 2001).

Until recently, the glycosylation of bacterial proteins was regarded as uncommon and thought to be limited to special circumstances such as S-layer protein and some archeal outer membrane proteins (Power and Jennings, 2003). However, there are an increasing number of reports focusing on the glycosylation of proteins in various gram-negative bacteria and its importance in microbial pathogenesis (Benz and Schmidt, 2002; Messner, 2004; Ng et al., 2006; Weerapana and Imperiali, 2006). More specifically, it has been shown that the type-IV fimbriae of *Neisseria gonorrhoea* (Virji et al., 1993; Ghosh et al.,

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2004), *Pseuodomonas aeroginosa* 1244 (Comer et al., 2002) and *Neisseria meningitidis* (Virji, 1999; Power et al., 2006) are glycosylated. The overall aim of this study was therefore to investigate the potential for glycosylation of the fimbrial subunit proteins of *D. nodosus*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Reference strain *D. nodosus* 25549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Nine cultures of *D. nodosus* vaccine strains representing serogroups A–M were obtained from Shearing-Plough Animal Health Ltd. (SPAHL), Upper Hutt, New Zealand (NZ). New Zealand field isolates of *D. nodosus* were collected, identified and grown anaerobically on eugon agar (BBL, Cockeysville, MD, USA) at 37 °C or in eugon broth (BBL) medium (Table 1). *FimA* sequences of new NZ isolates of *D. nodosus* were cloned and analysed and their sequence data were submitted to the NCBI GenBank (see Table 1 for accession numbers).

2.2. Purification of fimbrial subunit protein

D. nodosus suspensions in broth cultures were harvested by centrifugation at 12,000 × *g* for 10–30 min. The supernatant containing released fimbrial protein was adjusted to pH 4.5 by the addition of 0.1 M sodium acetate and left to stand at 4 °C overnight to precipitate the fimbriae. The suspension was then centrifuged at 12,000 × *g* for 15 min to pellet the fimbriae and the supernatant retained from this centrifugation step was re-precipitated with 30 mL of 0.1 M sodium acetate, and re-centrifuged.

The total pelleted fimbriae were washed in PBS, centrifuged at 12,000 × *g* for 15 min and stored in aliquots in PBS buffer at 4 °C. These proteins were filtered and concentrated with Amicon Microcon® centrifugal ultra-filtration cells (Millipore Corp., USA). Low binding Ultracell™ YM membrane (Millipore) with 10,000 nominal molecular weight limit (NMWL) cutoff was used to maximize the recovery of the samples. After filtration, the concentrated fimbrial subunit proteins were resuspended in PBS buffer at pH 6.5.

2.3. Electrophoretic analysis of fimbrial proteins

Fimbrial proteins were analysed using SDS-PAGE with 12–15% resolving gels and a 4% stacking gels. Samples were dissolved in denaturing sample buffer containing 25 mM Tris–HCL, pH 6.5, 2.0% (w/v) SDS, 10% (v/v) glycerol, 1.0% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue and boiled for 3–5 min. The resulting gels were stained with Coomassie Brilliant Blue R-250 (BioRad Laboratories, Hercules, CA, USA).

2.4. Carbohydrate staining of fimbrial proteins

Immediately following electrophoresis, the gels were washed in 50% (v/v) ethanol for 30 min and fixed for 1–2 h in fixation solution (10% acetic acid, 35% methanol) with gentle agitation. After 1 h fixing the gels were placed in periodate solution [0.7 g periodic acid (H_5IO_6) in 100 mL of 5% (v/v) acetic acid] for 30 min–1 h. The gels were then washed at least six times in distilled water for 5 min per wash. Fifty millilitres of metabisulphite solution [0.2 g $Na_2S_2O_5$ in 100 mL of 5% (v/v) acetic acid] was added to gels and after 5–10 min, fresh metabisulphite solution was added and left until the gel decolourised (5–10 min). The gels were then placed in Schiff's reagent (BioRad Laboratories, Hercules, CA, USA) and incubated in the dark at room temperature until red or magenta coloured bands appeared (0.5–2 h). Gels were then washed in 0.1% (w/v) $Na_2S_2O_5$ in 10 mM HCl for 1 h in the dark. They were stored in 7.5% (v/v) acetic acid and 5% (v/v) methanol for several days or photographed and dried on a vacuum gel drier.

2.5. Deglycosylation of fimbrial subunit proteins

The fimbrial subunit proteins were deglycosylated with three enzymes (PNGase F, *O*-glycosidase, sialidase-II) using the Glyco® Enzymatic Deglycosylation Kit (Prozyme, California, USA), according to the manufacturer's instructions. Treated and untreated glycoproteins were run in either Ready™ 5–15% gradient gels (BioRad Laboratories, Hercules, CA, USA) or 12% SDS-PAGE gels.

Table 1
List of *D. nodosus* strains used for fimbrial purification and amino acid sequencing

Serotype	Reference and accession no.	Source of isolate
Reference strains		
Serotype A198	Dn25549	American Type Culture Collection (ATCC), USA
Vaccine strains		
Serotype A	Dn6	McMaster Laboratory CSIRO, NSW, Australia
Serotype B	Dn2541	P. Claxton, Sydney University, NSW, Australia
Serotype B2	Dn58	P. Claxton, Sydney University, NSW, Australia
Serotype C	Dn8	McMaster laboratory CSIRO, NSW, Australia
Serotype D	Dn16	P. Claxton, Sydney University, NSW, Australia
Serotype E	Dn5	P. Claxton, Sydney University, NSW, Australia
Serotype F	Dn66	P. Claxton, Sydney University, NSW, Australia
Serotype G	Dn52	P. Claxton, Sydney University, NSW, Australia
Serotype H	Dn340	Sheep isolate from New Zealand
NZ isolates		
Serotype A-NZ3	AY835828	Sheep isolate from New Zealand
Serotype A-NZ4	–	Sheep isolate from New Zealand
Serotype B1-NZ2	–	Sheep isolate from New Zealand
Serotype B_{2541}-NZ1	AY835840	Sheep isolate from New Zealand
Serotype C-NZ4	AY835830	Sheep isolate from New Zealand
Serotype D-NZ1	–	Sheep isolate from New Zealand
Serotype E-NZ5	AY835834	Sheep isolate from New Zealand
Serotype F-NZ2	AY835835	Sheep isolate from New Zealand
Serotype I-NZ2	AY835838	Sheep isolate from New Zealand
Serotype M-NZ2	AY835839	Sheep isolate from New Zealand

2.6. DNA sequence analysis of the *fimA* gene

D. nodosus cells in liquid culture were pelleted by centrifugation at 13,000 × *g* for 10 min then rewashed in 10 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and resuspended in 4 mL of SET solution (75 mM NaCl, 25 mM EDTA and 20 mM Tris, pH 7.5). Lysozyme (Roche, Mannheim, Germany) was added to a final concentration of 4 mg/mL and the cells were incubated on ice for a minimum of 20 min or maximum overnight. Ten percent of SDS and 200 μg/mL of proteinase K were added and the tubes were inverted gently and incubated at 55 °C for at least 2 h with occasional inversion.

Subsequently, 1.2 mL of 5 M NaCl and 4 mL of phenol:chloroform (1:1) was added, and the contents mixed. Tubes were shaken for 50 s to lyse the bacterial cells. The lysates were then centrifuged at 13,000 × *g* for 2 min and the aqueous phase containing the DNA was collected. The crude lysates were extracted with equal volumes of phenol:chloroform (50:50) (twice), phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1). The genomic DNA was then recovered by ethanol precipitation and resuspended in 50–200 μL of TE buffer. The stock DNA was stored at either −20 °C or −80 °C.

Primers for amplification of part of the conserved region and the variable region of the *fimA* gene were as described previously (Zhou and Hickford, 2000; Cagatay and Hickford, 2006) and these primers were synthesised by GIBCO BRL. Extracted *D. nodosus* DNA was amplified in a 20 μL reaction containing 0.25 μM of each primer, 200 μM of each dNTP (Roche, Mannheim, Germany), 1× PCR buffer containing 1.5 mM $MgCl_2$ with 1 U of *Taq* polymerase (Qiagen, Hilden, Germany) and 1 μL of extracted DNA. Reactions were denatured at 94 °C for 60 s, followed by 35 cycles of deneturation at 94 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 7 min.

Electrophoresis of DNA was performed in 1% (w/v) agarose gels containing ethidium bromide (200 ng/mL), visualised on a UV transilluminator at 254 nm (UVP White/UV TMW20, San Gabriel, CA, USA) and then photographed using a UVP ImageStore 5000 System (UltraViolet Products, Cambridge, UK).

Amplimers were cloned using the pGEM®-T Easy Vector System (Promega Corporation, Madison, WI, USA) as per the manufacturer's recommendations and 2 μL of the ligation mixture was used to transform competent *E. coli* cells (Invitrogen™, One Shot™ INVαF′). Several white colonies for each transformation were picked and incubated overnight in Terrific broth (GIBCO BRL, Life Technologies, USA) at 37 °C in a 225 rpm shaking incubator. These cultures were subsequently used for plasmid isolation.

Plasmid DNA from selected colonies was extracted using a Quantum Prep® Plasmid Miniprep Kit (BioRad Laboratories, Hercules, CA, USA) and the concentration of the DNA adjusted to 200 ng/μL. The DNA was sequenced at the Waikato DNA Sequencing Facility, the University of Waikato, Hamilton, New Zealand and sequence alignments; translations and comparisons were performed using DNAMAN (Version 4.0, Lynnon BioSoft, Vaudreuil, Canada).

3. Results

3.1. Fimbrial subunit protein electrophoresis

The fimbrial subunit protein from *D. nodosus* strain ATCC 25549, eight NZ isolates, and nine vaccine strains (SPAHL) were purified by precipitation. Fig. 1a and b shows the Coomassie Brilliant Blue R-250 profiles of these proteins, separated on 12% SDS-PAGE gels. In Fig. 1a the relative molecular weights (M_r) of the fimbrial subunits varies from ∼15.8 kDa to ∼20 kDa. For serogroups A–C, and E, the weight clusters from ∼17 kDa to ∼18 kDa, whereas serogroup G is about 18.5 kDa. In both Fig. 1a and b serogroup F has the largest fimbrial subunits at about 20 kDa.

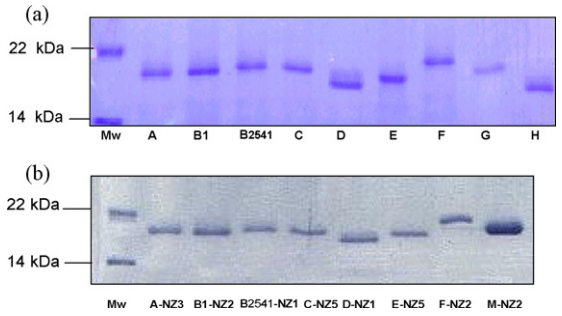

Fig. 1. (a) SDS-PAGE analysis of purified fimbriae from SPAHL vaccine strains. Twelve percent of SDS-PAGE gel stained with Coomassie Brillant Blue R-250. Lane 1, molecular weight standard. (b) SDS-PAGE analysis of purified fimbriae from *D. nodosus* NZ strains. 15% SDS-PAGE gel stained with Coomassie Brillant Blue R-250. Lane 1, molecular weight standard.

3.2. Carbohydrate detection on fimbrial subunit protein

PAS staining suggested that *D. nodosus* fimbrial subunit protein from some NZ strains (A-NZ3, B_1-NZ2, D-NZ1 and F-NZ2) were glycosylated, while other fimbrial subunit proteins did not stain (serotypes C, E, G, I and M), indicating, at least within the detection limits of the test system, the absence of carbohydrate modification (Fig. 2).

3.3. The enzymatic deglycosylation of fimbrial subunit proteins

Of eight NZ serotypes and nine SPAHL serotypes, fimbrial subunit protein of serotypes B_1-NZ2 and F-NZ2 that had been treated with PNGaseF migrated further on the gel than untreated fimbrial subunit protein with PNGaseF treatment, and the difference in M_r was about 10 kDa. The digestion of serotypes B_1-NZ2 and F-NZ2 fimbrial subunit proteins with *O*-glycosidase after sialidase-II pre-treatment did not change the mobility of fimbrial subunit proteins on SDS-PAGE (Fig. 3).

3.4. Putative glycosylation sites in the fimA gene

The *fimA* gene sequence of *D. nodosus* field isolates from NZ (serogroups A, B_1, B_{2541}, C–F, I and M) and SPAHL cultures were amplified with PCR. The resulting amplimers were cloned and then sequenced,

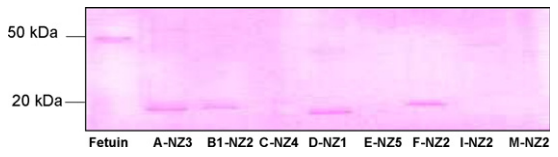

Fig. 2. Detection of *D. nodosus* fibrial subunits from NZ serotypes by periodic acid-Schiff staining. Lane 1 is fetuin as a glycosylated positive control, Lanes 2–9 are serotypes A, B_1, C–F, I, and M from NZ, respectively.

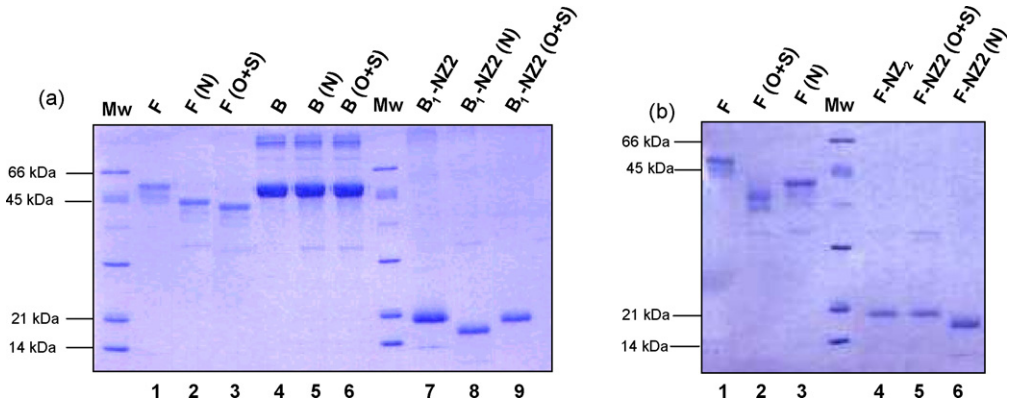

Fig. 3. Deglycosylation of fimbrial subunit proteins from *D. nodosus*. (a) Lane 1, fetuin control; lane 2, *N*-glycosidase F treated fetuin; lane 3, *O*-glycosidase and sialidase-II treated fetuin; lane 4, bovine serum albumin; (B) control, lane 5, *N*-glycosidase F treated B; lane 6, *O*-glycosidase and sialidase treated B; lane 7, untreated B_1-NZ2; lane 8, *N*-glycosidase F treated B_1-NZ2; lane 9, *O*-glycosidase and sialidase-II treated B_1-NZ2. (b) Lane 1, fetuin control; lane 2, *O*-glycosidase and sialidase-II treated Fetuin; lane 3, *N*-glycosidase F treated Fetuin; lane 4, untreated F-NZ2; lane 5, *O*-glycosidase and sialidase-II treated F-NZ2; lane 6, *N*-glycosidase F treated F-NZ2.

and the amino acid sequence of *fimA* from all the strains was predicted.

Amino acid sequence analysis of *D. nodosus fimA* from serotypes A-NZ3, B_1-NZ2, and F-NZ2 suggested that the fimbrial subunit protein molecules potentially had glycosylation sites (Fig. 4). These motifs were at a.a. 47–49 Asn-Asn-Thr (NNT) in serotype A-NZ3 and region 47–49 Asn-Asn-Ser (NNS) in serotype F-NZ2, and in the region 52–54 Asn-Asp-Thr (NNT) in serotype B_1-NZ2. In addition to these regions, there were other potential glycosylation sites in the region 69–71 Asn-Gly-Ser (NGS) and region 181–183 Asn-Asn-Thr (NNT) for serotype F-NZ3 and in the region 155–157 Asn-Gly-Ser (NGS) for both serotypes A-NZ3 and B_1-NZ4.

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          1                                                  40
A-NZ3     VRQLTLNKMI FKCSHS.ENM MKSLQKGFTL IELMIVVAII
F-NZ2     VRQLTLNKMI FKCSHSWENM MKSSQKGFTL IELMIVVAII
B1-NZ2    VRQLTLNKMI FKCSHS.ENM MKSLQKGFTL IELMIVVAII

          41                                                 80
A-NZ3     GILAAFNNTA YNDYIARSQA AEGLTLADGL KVRISDHLES
F-NZ2     GILSAFNNTA YNDYIARSQA AEGLTLADGL KIRIADHLNG
B1-NZ2    GILAAFAIPA YNNTIARSQA AEGVSLADGL KVRIAENLQD

          81                                                 120
A-NZ3     GECKE.DANP AVGSLGNDDK GKYALATIDG DYNKDAKTAD
F-NZ3     SCTEAGAGEK .....GNQDI GKYGLAVISG TYDESKTDAK
B1-NZ2    GECKGPDADP QSGVVGNEDK GKYGLAKIEG DYNASKTEAG

          121                                                160
A-NZ3     EKNGCKVVIT YGQGTAGEKI SKSIVGKKLV LDQFVNGSYK
F-NZ2     DNNTCVVTIT YGSGTAEGKV SKLINGKTLI LHQLLHGSYT
B1-NZ2    DPNGCKVEIT YGQGTAGDKI SNLITGKKLV LDQLVNGSFV

          161                                                198
A-NZ3     YNEGETDLEL KFIPNAVKNL ALKCESL... .......
F-NZ2   QGGGTIDA.. KFVPDAVKKY NNTLLNVKAS .......
B1-NZ2    QGDG.TDLAD KFIPNAVKAK KPKLKNSICK HLTKPLS
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Fig. 4. Alignment of predicted fimbrial subunit sequences from *D. nodosus* A-NZ3, B_1-NZ2, and F-NZ2. Amino acid sequences were determined by translating the DNA sequence determined from the *fimA* gene. Putative glycosylation sites are shown with bold and underlined.

4. Discussion

SDS-PAGE analyses revealed that fimbrial subunits from NZ strains of *D. nodosus* had M_r's in the range of 15–20 kDa. The smallest (M_r ~16.5 kDa) was isolated from samples D-NZ1, D-NZ2 and D-SPAHL. The largest subunit (M_r ~20 kDa) was isolated from samples F-NZ1 and F-SPAHL. All isolates classified within serogroups A–C and E had fimbriae with a similar electrophoretic mobility (M_r ~17–18 kDa). This supports the findings of Lee et al. (1983), who reported the molecular weight of fimbrial subunit from 16 *D. nodosus* isolates from Australia and the United States of America in the range of ~14–20 kDa. Anderson et al. (1986) also showed that there is variation in the mobility of the fimbrial subunits of *D. nodosus* strains. These mobility differences were observed between the fimbrial subunits from strains classified within serotypes A2 (VCS-1133), B2 (VCS-1208), B3 (VCS-1190), B4 (VCS-1125), D

(VCS-1172), and E2 (VCS-1071), whereas no variation was observed between isolates from serotypes A1 (VCS-1001) and F1 (VCS-1017).

DNA sequence data derived from nine NZ isolates of *D. nodosus* and nine SPAHL cultures revealed extensive DNA sequence variation in the fimbrial genes (Cagatay and Hickford, 2006). The majority of this sequence variation occurred in the variable region of the *fimA* gene confirming the findings of Zhou and Hickford (2000). However, the predicted molecular weight (M_w based on the gene sequence) of the *D. nodosus fimA* from the NZ field isolates and SPAHL cultures did not correlate with the M_r determined by electrophoretic mobility. For example, the sequence of the *fimA* gene from F-NZ1 suggests a molecular weight of 15.8 kDa whereas it exhibits the largest apparent size of approximately 20 kDa on SDS-PAGE. The conflict between these results may be explained by migrational aberrations of molecules in SDS-PAGE, compared with their known molecular mass, or potentially by post-translational modifications.

There are a number of recent studies showing similar conflicts between M_r and the calculated M_w with other type-IV fimbriate bacteria such as *P. aeroginosa* PAK (Brett et al., 1994; Brimer and Montie, 1998) *N. meningitides* (Virji et al., 1993; Virji, 1997), *Salmonella typhimurium* (Brett et al., 1994) and *Campylobacter jejuni* 81–176 (Larsen et al., 2004). Sequencing of the fimbrial gene of the *P. aeroginosa* PAK suggested a calculated molecular weight of ~40 kDa, while SDS-PAGE revealed a M_r of ~45.5 kDa, while studies on *N. meningitides* strain C311 have shown that the calculated molecular weights of fimbrial subunits are considerably lower than their M_r on SDS-PAGE (Virji et al., 1993; Virji, 1999).

The results from the DNA sequence analysis of the fimbrial subunit genes from NZ field isolates suggested that *D. nodosus fimA* molecules from serotypes A-NZ3, B_1-NZ2, and F-NZ2 have the potential to be glycosylated. Specifically: in crucial areas, variation in the amino acid sequence of fimbrial subunit proteins may create putative *N*-glycosylation sites (Asn-X-Ser/Thr) based on the known sites of glycosylation in eukaryotes. The presence of this tripeptide pattern is however no guarantee, even in higher eukaryotes, that an asparagine residue will always be glycosylated (Imperiale et al., 1992). However, glycosylation has been reported for the fimbriae of other type-IV bacteria such as *Neisseria* sp. (Marceau and Nassif, 1999; Power and Jennings, 2003; Banerjee and Ghosh, 2003; Warren et al., 2004; Power et al., 2006) and *P. aeroginosa* (Castric et al., 2001; Comer et al., 2002; DiGiandomenico et al., 2002; Smedley et al., 2005). The reported glycosylation generally occurs at serine or threonine residues between residues 63 and 93 in the mature subunit protein.

Positive staining with PAS stain further supports the contention that fimbrial subunit proteins from *D. nodosus* serotypes A-NZ3, B_1-NZ2 and F-NZ2 are glycosylated. PAS staining is not a new technique, but it is considered to be a reliable method. Bedouet et al. (1998) used Schiff staining to reveal the glycosylation of flagellins *Clostridium tyrobuttyricum* ATCC 25755. However, Rhodes and Amlung (1991) could not show glycosylation of the proteases of *Aspergillus flavus* with PAS staining, even though they postulated that unusual appearance of the protein of *A. flavus* in SDS-PAGE might be due to glycosylation. Although the PAS staining method exhibits a relatively high degree of specificity for glycoproteins, it has certain disadvantages as some glycoproteins, particularly non-sialated glycoproteins and those which do not contain vicinal 1,2-diol groups may stain poorly because of resistance to periodate oxidation (Thornton et al., 1996), and hence in this study strains that did not appear to be glycosylated, may in fact have been.

The fimbrial subunit proteins from isolates F-NZ2 and B_1-NZ2 demonstrated a decrease in mobility on SDS-PAGE, when digested with PNGase F, whereas they did not show any mobility difference on SDS-PAGE following digestion with sialidase II and *O*-glycosidase. The mobility differences could be caused by presence of one or more carbohydrate moieties and the result was consistent with both the predicted amino acid sequence which indicated putative glycosylation sites and the result of the PAS staining. Consequently, the glycosylation of *D. nodosus* fimbrial subunit proteins would explain some of the discrepancy observed between the molecular weights deduced from the predicted amino acid sequence of these proteins and the M_r determined from SDS-PAGE.

Despite an increasing awareness of the presence of glycoproteins in bacteria, many questions remain

unanswered as to the mechanism of glycosylation and about the biological–physico-chemical functions of the glycosyl modifications. The literature suggests that fimbrial glycosylation may directly affect protein function, such as subunit interactions and/or the assembly of fimbriae and their adherence to host cells (Virji, 1997; Benz and Schmidt, 2002; Banerjee and Ghosh, 2003), while other researchers have revealed that glycosyl modifications can play a role in adhesion and twitching mobility (Benz and Schmidt, 2002; Comer et al., 2002), protection against proteolytic cleavage (Hermann et al., 1996), solubility (Marceau and Nassif, 1999), antigenic variation (Doig et al., 1996), and protective immunity (Romain et al., 1999).

Overall, it could be concluded that the presence of glycosyl groups on the fimbrial subunit of *D. nodosus* could radically affect interactions between the bacteria and host cells. Alternatively, the glycosylation could also play a role in the antigenicity of fimbriae. This suggests that the glycosylation of fimbrial subunit proteins may need to be considered in designing any new *D. nodosus* fimbrial protein-based vaccine.

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