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The Native 67-Kilodalton Minor Fimbria of *Porphyromonas gingivalis* Is a Novel Glycoprotein with DC-SIGN-Targeting Motifs[▽]

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We recently reported that the oral mucosal pathogen *Porphyromonas gingivalis*, through its 67-kDa Mfa1 (minor) fimbria, targets the C-type lectin receptor DC-SIGN for invasion and persistence within human monocyte-derived dendritic cells (DCs). The DCs respond by inducing an immunosuppressive and Th2-biased CD4^{+} T-cell response. We have now purified the native minor fimbria by ion-exchange chromatography and sequenced the fimbria by tandem mass spectrometry (MS/MS), confirming its identity and revealing two putative N-glycosylation motifs as well as numerous putative O-glycosylation sites. We further show that the minor fimbria is glycosylated by ProQ staining and that glycosylation is partially removed by treatment with β(1–4)-galactosidase, but not by classic N- and O-linked deglycosidases. Further monosaccharide analysis by gas chromatography-mass spectrometry (GC-MS) confirmed that the minor fimbria contains the DC-SIGN-targeting carbohydrates fucose (1.35 nmol/mg), mannose (2.68 nmol/mg), *N*-acetylglucosamine (2.27 nmol/mg), and *N*-acetylgalactosamine (0.652 nmol/mg). Analysis by transmission electron microscopy revealed that the minor fimbria forms fibers approximately 200 nm in length that could be involved in targeting or cross-linking DC-SIGN. These findings shed further light on molecular mechanisms of invasion and immunosuppression by this unique mucosal pathogen.

Porphyromonas gingivalis is one of several mucosal pathogens that have been implicated in chronic periodontitis (CP), a common oral disease that may affect 40 to 60% of the U.S. population (7). *P. gingivalis* utilizes a myriad of virulence factors that contribute to chronic periodontitis. Among these are a polysaccharide capsule, fimbriae, proteases for opsonins C3 and IgG, gingipains (21, 30, 43, 52), bacterial lipopolysaccharides (LPS) (22, 44), and toxins and hemagglutinins (10, 25).

The fimbriae of *P. gingivalis* play a crucial role in adhesion to and invasion of host cells. We have shown that optimum entry of *P. gingivalis* into human dendritic cells (DCs) requires the presence of two fimbriae, termed the major and minor fimbriae. The major fimbria is composed of a 41-kDa protein termed fimbrillin, encoded by the *fimA* gene (65). Much less is known about the minor fimbria, the focus of this paper. The minor fimbria is comprised of a 67-kDa protein (19) that is encoded by the *mfa1* gene. The major and minor fimbriae are antigenically distinct, and they also differ based on amino acid composition and size (5, 19). Very little is understood about the formation and secretion of the minor fimbriae and about possible posttranslational modifications of these fimbriae. Formation and secretion of the major fimbriae is a complex reaction consisting of numerous steps required for transfer of prefimbrillin proteins from the cytoplasm to the periplasm, cleavage of the N-terminal signal peptide (24, 50), transport of prefimbrillin to the outer face of the outer membrane, and assembly into fimbria structures (23, 24, 34).

Deciphering the cellular receptors for the fimbriae is an active area of research. Evidence suggests that the cellular targets of the major fimbriae are the β-1 integrins (CD29) (32, 66). Others have proposed a role for β-2 integrins (CD18) (17, 18, 55) in the cellular response to major fimbriae. In contrast, little is known of the cellular receptors for the minor fimbriae. Lamont et al. in 2002 showed that the minor fimbria of *P. gingivalis* intimately interacts with the SspB protein of *Streptococcus gordonii* (26). This interaction might aid in *P. gingivalis* colonization of plaque biofilm before it invades gingival tissue (26, 41). We recently showed that the minor fimbria targets DC-SIGN on DCs for entry into DCs and that this targeting has the immunological consequence of dampening the immune response (68).

DC-SIGN is a type II membrane protein on DCs in which the extracellular domain consists of a stalk that promotes tetramerization (13). DC-SIGN contains a C-terminal carbohydrate-recognizing domain (CRD) that belongs to the C-type lectin superfamily (13). Early studies by Feinberg et al. in 2001 showed that the DC-SIGN CRD preferentially binds to the high-mannose N-linked oligosaccharides GlcNAc (*N*-acetylglucosamine) and Manα1–3[Manα1–6] Man (mannose) (13). Furthermore, Appelmelk et al. showed that DC-SIGN also binds to fucose-containing Lewis blood antigens (4). Guo et al. utilized an extensive glycan array and showed that DC-SIGN will bind high-mannose-containing glycans or glycans that contain terminal fucose residues (16). Previous studies showed that DC-SIGN on DCs is used by microorganisms such as *Neisseria gonorrhoeae*, *Mycobacterium tuberculosis*, *Mycobacte-*

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rium leprae, HIV, and *Helicobacter pylori* for entry into DCs and induction of immunosuppression (4, 27, 42, 51, 69). Like *P. gingivalis*, many of these pathogens can induce chronic life-long infections.

Our previously published work established that the minor fimbria is necessary for targeting DC-SIGN, resulting in entry of *P. gingivalis* into DCs (68). We were able to abrogate minor fimbria-mediated DC-SIGN ligation by using DC-SIGN-blocking agents or agonists, including fucose, mannose, and mannan (68). Additionally, we described that the minor fimbria is able to induce immunosuppression of DCs via its interaction with DC-SIGN, which was blocked by sugars (68). Further, we demonstrated that minor fimbriated strains of *P. gingivalis* inhibited DC maturation and suppressed proinflammatory cytokine secretion (68). Moreover, DCs that were pulsed with minor fimbriated strains of *P. gingivalis* and then cocultured with autologous T cells shifted the T-cell effector phenotype to a Th2 effector phenotype, as evidenced by high interleukin-4 (IL-4) production (68).

Our previous results, described above, suggested that the minor fimbria–DC-SIGN interaction was mediated by glycosylated proteins. We therefore set out to identify the carbohydrate moieties on the minor fimbria that could account for its DC-SIGN-targeting function. The intact native minor fimbria was purified and analyzed for glycosylation and for the presence of relevant monosaccharides. We show here by a combination of ProQ gel staining and gas chromatography-mass spectrometry (GC-MS) analysis that the minor fimbria is glycosylated and expresses the DC-SIGN ligands fucose, mannose, GlcNAc, and GalNAc. Use of classic N- and O-linked deglycosidases on the native minor fimbria revealed a novel glycoprotein structure. Overall, these results indicate that the minor fimbria is glycosylated with DC-SIGN-binding motifs that likely account for the reported ability of *P. gingivalis* to bind to and invade DCs, resulting in an immunosuppressive DC response.

MATERIALS AND METHODS

Bacterial growth conditions and minor fimbria purification. The isogenic, major fimbria-deficient mutant DPG3, which expresses only the minor fimbria (Pg min$^+$/maj$^-$), was maintained anaerobically (10% H_2, 10% CO_2, 80% N_2) in a Forma Scientific anaerobic system glove box, model 1025/1029, at 37°C in Difco anaerobe broth MIC. Erythromycin (5 μg/ml) was added according to the selection requirements of the strain (11, 20, 54, 68). Fimbriae were purified as described by Davey et al. (11). Briefly, bacterial pellets of *P. gingivalis* DPG3 were shattered by ultrasonication for 5 min, pulsing at 50% power, on ice. The cellular debris was removed by centrifugation, and the remaining supernatant was combined with saturated ammonium sulfate (40%) to precipitate the fimbriae. After centrifugation the resulting pellets were dialyzed in 20 mM Tris buffer (pH 7.8). The dialysate was further purified by multiple runs on a DEAE-Sepharose CL-6B column (Amersham Biosciences) equilibrated with 20 mM Tris buffer (pH 7.6 to 8.0) and eluted with a linear gradient of 0 to 1.0 M NaCl. Fractions were analyzed by 12% SDS-PAGE and silver staining (Bio-Rad) to ensure purity and quantified by Bradford assay. Fimbria preparations underwent further screening to confirm lack of LPS contamination via silver staining (20). Samples were then analyzed by tandem MS (MS/MS) to verify identity and to ensure no other protein contaminants were present.

Limulus amebocyte lysate assay. Lack of endotoxin was verified by using the limulus amebocyte lysate (LAL) Pyrogent 03 Plus gel clot assay (catalog no. N294-03; Lonza) following the manufacturer's instructions. Briefly, an *Escherichia coli* LPS standard as well as 100-μg/ml, 50-μg/ml, and 25-μg/ml replicates of purified minor fimbriae were incubated with limulus amebocyte lysate for 1 h at 37°C. After 1 h the glass test tubes were inverted. A positive test was characterized by momentary formation of a firm gel when the tube was inverted. A negative test result was the absence of a solid clot. The sensitivity of this test is 0.03 endotoxin units (EU)/ml. An EU is defined by the manufacturer as the endotoxin activity of 0.2 ng of reference endotoxin standard.

Tandem mass spectrometry. To confirm the identity of the minor fimbria, purified proteins were run on an SDS-PAGE gel and analyzed by the Proteomics Center at Stony Brook University. Gel bands were cut out, destained, reduced, alkylated, and digested with trypsin (Promega Gold, mass spectrometry grade) as described by Shevchenko et al. with minor modifications (49). The resulting concentrated peptide extract was diluted into a solution of 2% acetonitrile (ACN)–0.1% formic acid (FA) (buffer A) for analysis. For solution digest, 10 μl of purified protein was diluted in 40 μl of 50 mM ammonium bicarbonate. The proteins were reduced with 2 mM dithiothreitol and alkylated with 4 mM iodoacetamide for 30 min each. A 0.25-μg aliquot of trypsin was added, and digests were incubated overnight at 37°C. Protease reactions were stopped with 100% formic acid (final concentration, 5%). Ten microliters of the peptide mixture was analyzed by automated microcapillary liquid chromatography-tandem mass spectrometry. Fused silica capillaries (100 μm inside diameter [i.d.]) were pulled using a P-2000 CO_2 laser puller (Sutter Instruments, Novato, CA) to a 5 μm i.d. tip and packed with 10 cm of 5-μm Magic C_{18} material (Agilent, Santa Clara, CA) by using a pressure bomb. This column was then placed in line with a Dionex 3000 high-performance liquid chromatograph (HPLC) equipped with an autosampler. The column was equilibrated in buffer A, and the peptide mixture was loaded onto the column using the autosampler. The HPLC separation at a flow rate of 300 nl/min was provided by a gradient between buffer A and buffer B (98% acetonitrile, 0.1% formic acid). The HPLC gradient was held constant at 100% buffer A for 5 min after peptide loading, followed by a 30-min gradient from 5% buffer B to 40% buffer B. Then, the gradient was switched from 40% to 80% buffer B over 5 min and held constant for 3 min. Finally, the gradient was changed from 80% buffer B to 100% buffer A over 1 min and then held constant at 100% buffer A for 15 more minutes. The application of a 1.8-kV distal voltage electrosprayed the eluted peptides directly into a Thermo LTQ ion trap mass spectrometer equipped with a custom nanoLC electrospray ionization source. Full MS spectra were recorded on the peptides over a 400 to 2,000 *m/z* range, followed by five MS/MS events sequentially generated in a data-dependent manner on the first, second, third, fourth, and fifth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan, San Jose, CA). MS/MS spectra were extracted from the RAW file with ReAdW.exe (http://sourceforge.net/projects/sashimi). The resulting mzXML file with all the data for all MS/MS spectra was read by using the analysis software. The MS/MS data were searched with Inspect (56) against a *Porphyromonas gingivalis* database containing 4,251 proteins, in addition to an *Escherichia coli* database plus common contaminants, with the following modifications: +16 on methionine, +57 on cysteine, and +1 on asparagine and glutamine. Only peptides with a *P* value of ≤0.01 were analyzed further.

Detection of glycosylation. To detect the presence of glycosylation, purified native minor fimbriae were run on SDS-PAGE and carbohydrates were stained using the ProQ Emerald glycoprotein stain kit (Molecular Probes) following the manufacturer's instructions. All ProQ gels were run with CandyCane glycoprotein molecular weight standards (Molecular Probes) provided in the ProQ Emerald glycoprotein stain kit, i.e., as positive controls for staining. Further verification of glycosylation on the minor fimbriae included treatment with the native protein deglycosylation kit (NDEGLY; Sigma) following the manufacturer's instructions. This kit is specific for N-glycosylation and utilizes three different endoglycosidase F (endo F) enzymes. According to the manufacturer's instructions, endo F1 cleaves all asparagine-linked hybrid or high-mannose oligosaccharides but not complex oligosaccharides. Endo F2 cleaves biantennary complex and to a lesser extent high-mannose oligosaccharides. Fucosylation has little effect on endo F2 cleavage of biantennary structures. Endo F2 will not cleave hybrid structures. Endo F3 cleaves biantennary and triantennary complex oligosaccharides. However, nonfucosylated biantennary and triantennary structures are hydrolyzed at a slow rate by endo F3. Core fucosylated biantennary structures are efficient substrates for endo F3 oligosaccharides. Core fucosylation of biantennary structures increases activity up to 400-fold. Endo F3 has no activity on oligomannose and hybrid molecules. The untreated and treated minor fimbriae were run under native nonreducing conditions and reducing conditions with boiling on SDS-PAGE and probed with ProQ to detect loss of glycosylation. Further glycosylation analysis was performed using the enzymatic protein deglycosylation kit (E-DEGLY; Sigma) following the manufacturer's instructions. This kit utilizes PNGase F (which cleaves N-glycosylation), α-2(3,6,8,9)-neuraminidase (which removes sialic acids), O-glycosidase (endo-α-*N*-acetylgalactosaminidase removes core structure with no modifications to serine or threonine residues), β(1–4)-galactosidase, and β-*N*-acetylglucosaminidase. Samples were

then run on 12% SDS-PAGE gels and stained for ProQ. Additionally, we preincubated the minor fimbriae with α-L-fucosidase (from bovine kidney; Sigma) following the manufacturer's instructions, prior to treatment with E-DEGLY.

Monosaccharide analysis by GC-MS. To identify the carbohydrate motifs, 2 mg of purified minor fimbriae was sent to a commercial laboratory (M-SCAN, Inc., West Chester, PA) for analysis by GC-MS. An aliquot of purified minor fimbriae (60 μl) was spiked with 10 μg arabitol (Ara) as an internal standard (IS) and lyophilized. The dried sample was hydrolyzed, re-N-acetylated, derivatized, and analyzed by GC-MS. A standard mixture containing 10 μg each of fucose (Fuc), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc), *N*-acetylgalactosamine (GalNAc), and *N*-acetylglucosamine (GlcNAc) plus arabitol (Ara) and a tube/reagent blank containing 10 μg Ara were also hydrolyzed, re-N-acetylated, derivatized, and analyzed by GC-MS (as described below) alongside the carbohydrate sample. An aliquot (1 μl) of each derivatized carbohydrate sample dissolved in hexane (2 ml) was analyzed by GC-MS using a Perkin-Elmer Turbomass quadrupole mass spectrometer with integrated gas chromatograph under the following conditions. Samples were injected onto a DB5 column at 95°C using helium as a carrier gas. The program was run as follows: 1 min at 90°C, then 25°C/minute to 140°C, then 5°C/minute to 220°C, then 10°C/min to 300°C, and finally holding at 300°C for 5 min. The mass spectrometry ionization voltage was 70 eV, the acquisition mode was set to scanning, and mass range was 50 to 500 Da. Monitored ions were 173 for *N*-acetylhexosamines, 204 for hexoses, deoxyhexoses, and pentoses, and 217 for arabitol. On comparison of the data with those obtained from the standard mixtures containing known amounts of the expected monosaccharides, the sugars hydrolyzed from the sample were identified and the quantity of each monosaccharide present was estimated.

Transmission electron microscopy of native minor fimbriae. For transmission electron microscopy (TEM), a 0.8-μg/μl solution of purified native Mfa1 protein in 20 mM Tris-HCl (pH 7.8) was adsorbed onto polyvinyl formal carbon-coated grids (Ernest F. Fullam, Latham, NY) for 2 min, washed twice with phosphate-buffered saline and twice with water, and then negatively stained with 0.5% phosphotungstic acid (Ted Pella, Inc., Redding, CA) for 30 s. All grids were viewed in a transmission electron microscope (FEI TECNAI 12 BioTwin G02) at an 80-kV accelerating voltage, and images were obtained by using an AMT XR-60 charge-coupled-device digital camera system. Direct magnification was at $\times$98,000.

RESULTS

Purification of native minor fimbriae. After growth of *P. gingivalis* DPG-3 under anaerobic conditions, the bacteria were disrupted by sonication, and minor fimbriae were purified by ion-exchange chromatography using a DEAE-Sepharose column. In Fig. 1A we show a representative elution profile of the minor fimbria on a DEAE-Sepharose column. The minor fimbria eluted at 0.3 M NaCl, whereas other proteins were still bound until 0.5 M NaCl. An aliquot of the peak corresponding to the putative minor fimbria was analyzed by SDS-PAGE and rerun on the DEAE-Sepharose column multiple times, changing either the steepness of the elution gradient or the pH of the buffers. Protein purification was continued until minor fimbria samples showed no contaminating bands (i.e., for LPS or other contaminants) by Coomassie staining and silver staining (Fig. 1B) (20). Absence of endotoxin was further confirmed by LAL assay (Lonza). None of the dilutions tested generated a positive LAL reaction at an assay sensitivity of 0.03 EU/ml (data not shown) (6, 11, 48). The native fimbria was further analyzed by TEM, demonstrating the presence of oligomeric strands approximately 100 to 200 nm in length, which is similar to previous observations (41) (Fig. 1C). The single band corresponding to the correct 67-kDa size of the minor fimbria protein was analyzed for protein purity and peptide sequence by MS/MS (Fig. 2A). MS/MS confirmed the purity and correct peptide sequence (Fig. 2A, bold letters). Examination of the peptide sequence also revealed two conserved Asn-Xaa-Ser/

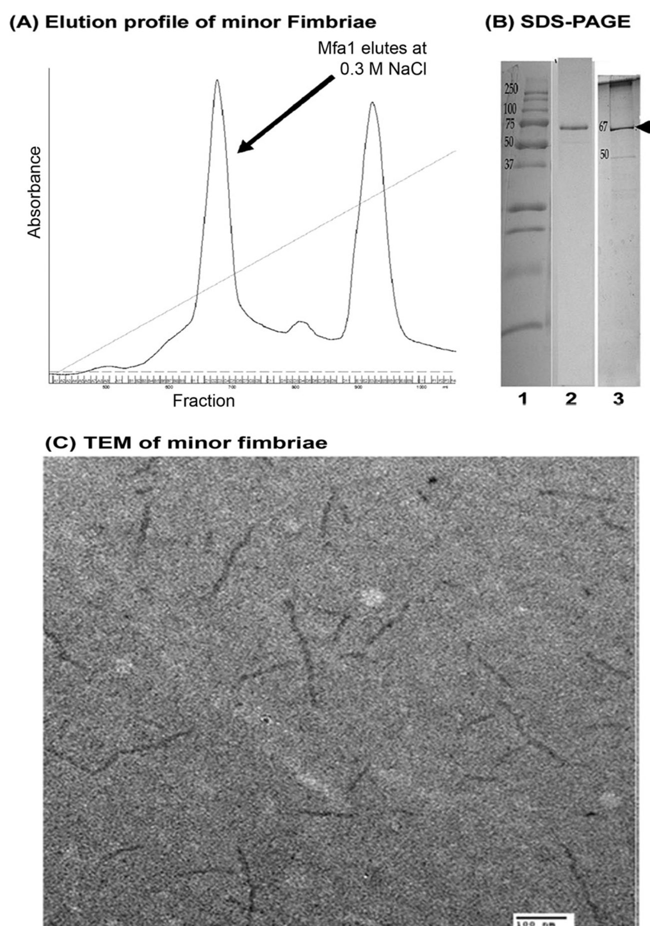

FIG. 1. Purification and characterization of the minor (Mfa1) fimbria. (A) Elution profile of the 67-kDa fimbria on DEAE-Sepharose CL-6B, showing a peak that eluted with 0.3 M NaCl. (B) SDS-PAGE analysis of the minor fimbria. Lane 1, MW standard; lane 2, Coomassie blue stain showing the 67-kDa minor fimbria; lane 3, silver stain showing the single band of the minor fimbria (arrow). (C) Transmission electron micrograph of the purified minor fimbria showing 100- to 200-nm fibers. Bar, 100 nm.

Thr asparagine-linked (N-linked) (putative) glycosylation motifs (Fig. 2A, gray boxes) (29, 53). The Mfa1 amino acid sequence also contained numerous serines and threonines, which can function as putative O-linked glycosylation sites (1, 53). We further analyzed the purified native minor fimbria for glycosylation by SDS-PAGE and ProQ staining (Fig. 2B), which revealed a positive staining reaction.

The native minor fimbria is susceptible to some N- and O-linked enzymatic deglycosylation. Based on the amino acid sequence, N-linked glycosylation was a distinct possibility. We therefore subjected the purified minor fimbria to treatment with endoglycosidases F1, F2, and F3. These enzymes cleave N-linked glycoproteins but differ significantly in their oligosaccharide specificity, as previously reported (37, 57–59). Briefly, endoglycosidase F1 cleaves oligomannose and hybrid oligosaccharides, but this activity is greatly reduced by core fucosylation. Endoglycosidase F1 will also not cleave any complex oligosaccharides. Endoglycosidase F2 does not cleave hybrid or triantennary complex oligosaccharide structures, but it does

(A) Peptide sequence of 67 kDa (minor) fimbrillin

1 MKLNKMFLVG ALLSLGFASC SK**EGNGPDPD NAAK**SYMSMT LSMPMGSAR**A**
51 **GDGQDQANPD YHYVGEWAG**K DKIEKVSIYM VPQGGPGLVE SAEDLDFGTY
101 YENPTIDPAT HNAILKPKKG IKVNSAVGKT VK**VYVVLNDI AGK**AK**ALLAN**
151 **VNAADFDAK**F KKIIELSTQA QALGTVADGP NPATAAGKIA KKNGTTDETI
201 MMTCLQPSDA LTIEAAVSEA NAIAGIKNQA KVTVERSVAR AMVSTKAQSY
251 EIK**ATTQIGE IAAGSVLATI TDIRWVVAQG ER**RQYLSKKR GTVPENTWVT
301 PGSGFVPTSS TFYTNATEYY DYAGLWEDHN TNEAVISGTQ VPTLADYQLQ
351 DVTGELANAL SGKFLLPNTH K**SGANAASSD YKRGNTAYVL VR**AKFTPKKE
401 AFIDRGKTYS DNTAVPEYVA GEDFFVGENG QFYVSMKSVT DPKVGGVAGM
451 KAHKYVKGKV LYYAWLNPST TSPDSWWNSP VVRNNIYHIH IKSIKKLGFN
501 WNPLVPDPDP SNPENPNNPD PNPDEPGTPV PTDPENPLPD QDTFMSVEVT
551 VLPWK**VHSYE VDL**

(B) ProQ staining of minor fimbriae

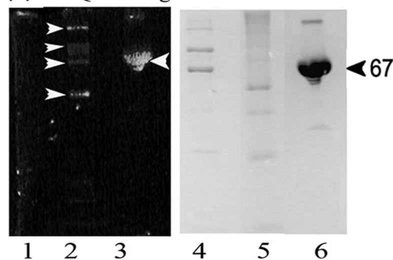

FIG. 2. Glycosylation of the minor fimbriae. (A) Peptide sequence obtained by MS/MS (shown in bold), which confirmed the identity of the minor fimbria. Boxed are putative N-X-S/T asparagine-linkage motifs. (B) Confirmation of glycosylation on the minor fimbria by ProQ (glycosylation stain). Minor fimbriae samples were run on SDS-PAGE gels and stained with ProQ (lanes 1 to 3), and then the same gel was stained with Coomassie (lanes 4 to 6). Lanes 1 and 4, nonglycosylated MW standard; lanes 2 and 5, CandyCane glycoprotein standard; lanes 3 and 6, the minor fimbria. White arrowheads highlight the CandyCane (Molecular Probes) glycoprotein standard.

cleave oligomannose and biantennary complex oligosaccharides. Core fucosylation has little to no effect on endoglycosidase F2 activity. Endoglycosidase F3 can cleave biantennary and triantennary complex oligosaccharides with a preference for those oligosaccharides with core fucosylation. Endoglycosidase F3 will also cleave fucosylated trimannosyl core structures on oligosaccharides but has no activity on oligomannose or hybrid structures (37, 57–59).

The results under nonreducing (native) conditions (Fig. 3A and B, lanes 3 and 4) showed that endoglycosidases F2 and F3 have moderate effects on the native minor fimbria, as revealed by either by ProQ staining or an apparent molecular mass shift. This suggested one of several possibilities. First, the minor fimbria contains N-linked complex biantennary complex oligosaccharide glycosylation, but the glycosylation site might be inaccessible to the endoglycosidases under its oligomeric native configuration (Fig. 1C), resulting in the step ladder pattern in lanes 3 and 4 (Fig. 3A and B). Second, it is possible that the minor fimbriae are O-linked glycoproteins or contain both N- and O-linked motifs. Finally, the minor fimbria may contain a novel glycosylation structure that is resistant to classic deglycosylation. To address the first two possibilities, we employed an additional N-linked endoglycosidase, PNGase F, and denatured the minor fimbria prior to enzymatic treatment (Fig. 3C to F). PNGase F is a more potent N-linked deglycosidase that optimally works on non-core-fucosylated denatured proteins, as reported previously (37). It works by specifically recognizing an Asp-GlcNAc-oligosaccharide complex. Under these conditions we observed that the protein retained its glycosylation even when denatured and treated with PNGase F (Fig. 3C and D). To address the possibility that fucose residues interfere with PNGase F, as previously reported (37), we pretreated the minor fimbria with α-L-fucosidase. Again, pretreatment had no affect on PNGase F, ruling out classic Asp-GlcNAc-oligosaccharide linkages or the presence of other blocking carbohydrates.

To examine O-linked glycosylation, the purified minor fimbriae were treated with α-2(3,6,8,9)-neuraminidase (which removes sialic acids), O-glycosidase, which cleaves serine- or threonine-linked unsubstituted Gal-β(1-3)-GalNAc-α,β(1-4)-galactosidase, which releases the terminal β(1-4)-galactose provided that it is nonreducing, and β-*N*-acetylglucosaminidase, which cleaves terminal nonreducing β-linked *N*-acetylglucosamine residues (Fig. 3C to F) (37). Intriguingly, we did observe a reduction in ProQ staining of the 67-kDa band in response to treatment with β(1-4)-galactosidase and β-*N*-acetylglucosaminidase (Fig. 3C and 3D, lanes 6 and 7), suggesting the presence of O-linked galactose and *N*-acetylglucosamine motifs in the minor fimbria. Furthermore, the samples treated with β(1-4)-galactosidase (Fig. 3E and F, lanes 4 and 7) were partially deglycosylated and contained a band with an approximate molecular mass of 55 kDa, suggesting a modest amount of β(1-4)-galactose on the minor fimbria. The 55-kDa band does not correlate to any of the known molecular masses of the enzymes we tested. It should be mentioned in this context that the predicted molecular mass of the minor fimbria is 61 kDa (8, 41), based on the complete amino acid sequence as analyzed with the ExPASy proteomics server (Expert Protein Analysis System; http://au.expasy.org/cgi-bin/protparam; Swiss Institute of Bioinformatics, Geneva, Switzerland). However, Mfa1 has two predicted signal peptidase cleavage sites. We propose that the 55-kDa band is a cleaved form of Mfa1 that has been tethered by carbohydrates, as further discussed below. The 55-kDa bands were confirmed to be the minor fimbria (lacking the first 50 amino acids) by MS/MS analysis (data not shown). Overall, these results suggest a novel pattern of glycosylation of the minor fimbria that is resistant to common methods of enzymatic deglycosylation.

Monosaccharide analysis of the minor fimbria by GC-MS. Samples of the purified native minor fimbria were analyzed by GC-MS as described in Materials and Methods. A representative chromatograph is shown (Fig. 4), and results are summarized in Table 1. We confirmed that the monosaccharides fucose, mannose, and *N*-acetylglucosamine were present on the purified minor fimbria (1.35 nmol/mg, 2.68 nmol/mg, and 2.27 nmol/mg, respectively) (Table 1). These carbohydrates have been implicated in ligation of DC-SIGN on dendritic cells, which promotes an immunosuppressive response (4, 68, 70). Also present in large quantities were xylose, galactose, and glucose (3.76 nmol/mg, 5.71 nmol/mg, and 14.1 nmol/mg, respectively) (Table 1). *N*-Acetylgalactosamine was also present in low concentrations (0.65 nmol/mg) (Table 1).

DISCUSSION

Our results demonstrate that the purified native minor fimbria is present as strands of 100 to 200 nm in length and is glycosylated (Fig. 1). The glycosylated minor fimbria, while susceptible to endoglycosidases F2 and F3 as well as β(1–4)-galactosidase (Fig. 3), was resistant to other classical N-linked

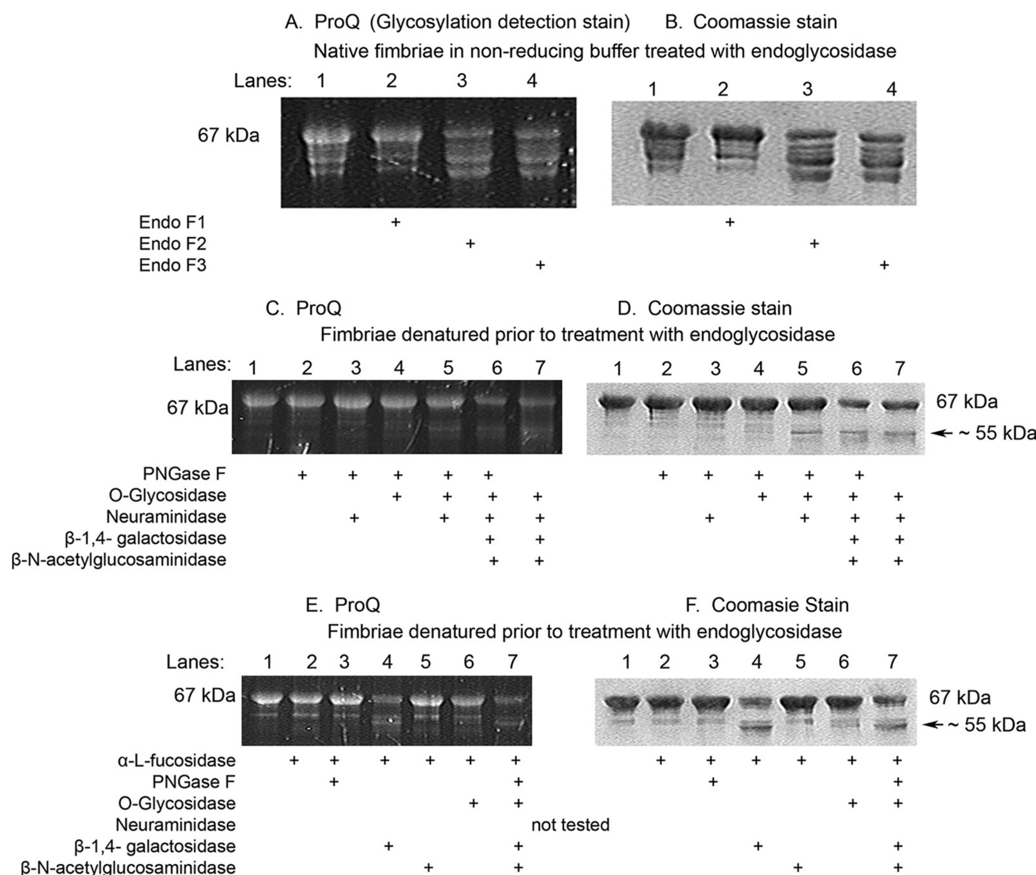

FIG. 3. Enzymatic deglycosylation of the minor fimbria observed in the presence of endoglycosidase F2, endoglycosidase F3, and β(1–4)-galactosidase. Enzymatic deglycosylation treatment on purified minor fimbria (Mfa1), as verified by lack of shift or the loss of ProQ (glycosylation detection) signal, is shown. (A, C, E) ProQ gels; (B, D, and F) the same gel after Coomassie blue staining. (A and B) Nonreduced native fimbria treated with endoglycosidase. All lanes were loaded with 5 μg of Mfa1 and digested with the indicated endoglycosidase(s). (C and D) Fimbriae denatured prior to treatment with endoglycosidase. All lanes were loaded with 7 μg of Mfa1 and digested with the indicated endoglycosidase(s). (E and F) Minor fimbria pretreated with α-L-fucosidase, then denatured and treated with endoglycosidase. All lanes were loaded with 7 μg Mfa1 and digested with the indicated endoglycosidase(s).

and O-linked deglycosylation enzymes, suggesting a novel structural linkage. Finally, we showed using GC-MS that the monosaccharide composition of the minor fimbria contains moderate amounts of the DC-SIGN ligands fucose, mannose, and *N*-acetylglucosamine and large amounts of xylose, galactose, and glucose (Fig. 4 and Table 1).

Many mucosal pathogens exhibit glycosylation motifs on their flagella, pili, and fimbriae (53). Glycosylation reportedly

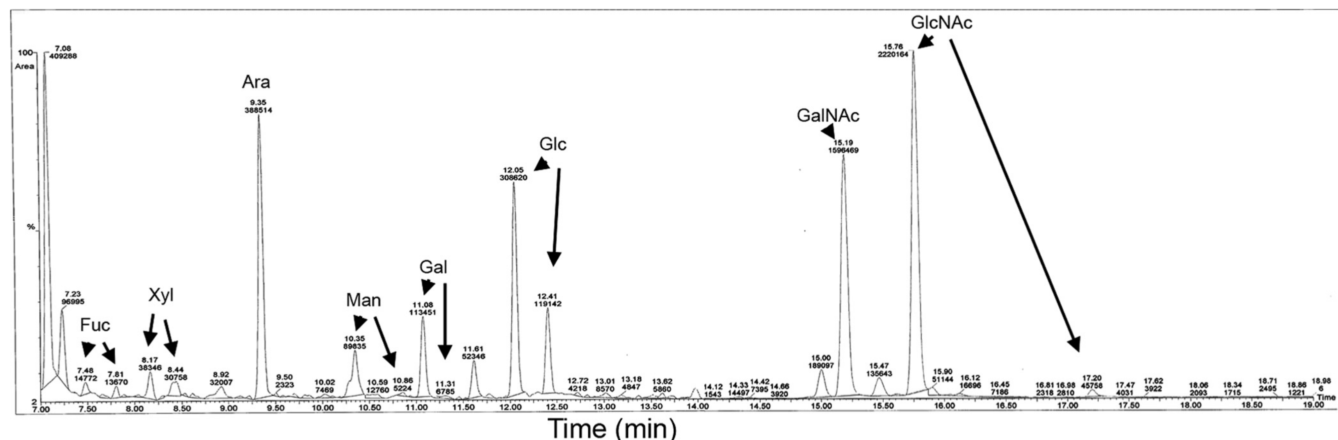

FIG. 4. Representative chromatograph from GC-MS analysis of the purified minor fimbria. The purified minor fimbria was analyzed by GC-MS for monosaccharide content relative to monosaccharide standards for Fuc, Xyl, Man, Gal, Glc, GalNac, and GlcNAc.

TABLE 1. Summary of monosaccharide compositional analyses by GC-MS of purified minor fimbriae

Monosaccharide	Mfa1 (nmol/mg)	Ratio to GalNAc[a]
Fuc	1.35	2.07
Xyl	3.76	5.77
Man	2.68	4.11
Gal	5.71	8.76
Glc	14.1	21.6
GalNAc	0.652	1.00
GlcNAc	2.27	3.48

[a] Shown are the ratios of monosaccharides found on the minor fimbriae relative to GalNAc (set as 1.0).

plays a role in maintenance of the protein structure, protection from proteolytic degradation, immune evasion, host cell adhesion, and surface recognition (53). A role for glycosylation of the major fimbria in *P. gingivalis* has been previously suggested. Knockouts of *gftA* (a *wcaE* glycotransferase homolog of *E. coli*) in *P. gingivalis* fail to make mature fimbriae (35). The gingipains of *P. gingivalis* are apparently glycosylated, with different isoforms being differentially glycosylated (9, 15). The glycosylation activity is regulated by the *vimF*, *vimA*, and *vimE* glycotransferase genes (60, 61). Knocking out these genes causes a failure to glycosylate these gingipains, leading to their inactivation (9, 60, 61). Recently, it was discovered that the OMP85 protein of *P. gingivalis* is glycosylated (33). All of these outer membrane protein genes encode a signal peptide that gets cleaved before it exits the periplasm (31, 36, 50). Given that all of the elements for glycosylation are present in *P. gingivalis* and that the minor fimbria apparently targets DC-SIGN (68), this study confirms our suspicion that the minor fimbria is glycosylated.

We have shown that the minor fimbria does not contain LPS by LAL test and silver staining (Fig. 1B). Although the LPS structures of different strains of *P. gingivalis* may not be conserved (9, 38, 39, 47), it is worth mentioning a study by Curtis et al. in 1999 which characterized the LPS of *P. gingivalis* (9). This study determined that the LPS does not contain mannose or fucose, while our minor fimbria does. Furthermore, the Curtis study determined that the core region of *P. gingivalis* LPS lacked *N*-acetylglucosamine and *N*-acetylgalactosamine (9), while our minor fimbria contains these sugars. The Curtis study was also the first to characterize the glycosylation of the gingipains, which contain arabinose, rhamnose, fucose, mannose, galactose, glucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid (9). Again, our samples lacked the gingipains as determined by both silver staining (Fig. 1B) and tandem MS (Fig. 2A). However, the molar ratios of monosaccharides that we detected on the minor fimbria (Table 1) differed from those previously published for the gingipains and LPS (9, 15, 38, 45, 47). Finally, the fimbria was purified from a nonencapsulated *P. gingivalis* strain, ruling out the possibility that the monosaccharides originated from the capsule (2, 11).

The Mfa1 protein component of the minor fimbria has a predicted size of 61 kDa, but reported sizes vary starkly from 67 to 75 kDa (41, 67). These size discrepancies could be attributed to glycosylation, the extent of which may depend on purification protocols and growth conditions (3, 28, 40, 63, 64).

Although the predicted molecular mass for Mfa1 is approximately 61 kDa (8, 41), we showed that the purified native minor fimbria migrated at 67 kDa. Shoji et al. in 2004 demonstrated that the minor fimbria is processed by a lipoprotein signal peptidase (signal peptidase II), as evidenced by improper processing in the presence of globomycin (50). They also described that the precursor proteins of the minor fimbria are lipidated (50). Moreover, they described that the minor fimbria gets processed twice, first by the lipoprotein signal peptide and then again by the gingipains (50). This led us to search for signal peptide cleavage sites on Mfa1 by using the SignalP server (http://www.cbs.dtu.dk/services/SignalP) (12). Interestingly, this analysis revealed two potential signal peptide cleavage sites, at amino acid (aa) positions 21 and 22 and at 50 and 51 (12, 14). Removal of the first signal peptide (aa 21–22) by signal peptidase I would result in a protein of approximately 58 kDa (based on the ExPASy prediction). However, removal of the second signal peptide (aa 50–51) by lipoprotein signal peptidase would result in a protein of approximately 55 kDa (based on ExPASy prediction). We demonstrated that treatment with β(1–4)-galactosidase resulted in the minor fimbria migrating at 55 kDa, corresponding to the second cleavage site. Therefore, it is possible that Mfa1 is processed at this site but that the N-terminal piece is tethered to the mature protein by carbohydrates with a β(1–4)-galactose linkage.

Additionally, the minor fimbria is not the only fimbria expressed by *P. gingivalis* that exhibits sizes in the expected range. The major fimbria is also reported to vary in size (41 to 43 kDa) (67). Efforts are under way to determine if the major fimbria is also glycosylated. Since both fimbriae and gingipains undergo similar mechanisms of translocation to the outer membrane, it is feasible that during this process they might become glycosylated.

While nonpathogenic *E. coli* normally does not express the glycosylation machinery necessary to modify proteins, recent studies have transferred the *pgl* gene cluster of *Campylobacter jejuni*, enabling *E. coli* to perform N-linked glycosylation (1, 62). Also, Fleckenstein et al. (14) reported that EptA from a naturally occurring strain of enterotoxigenic *E. coli* (ETEC) is glycosylated. The report went on to demonstrate that the entire *eptBAC* locus is necessary for production of glycosylated EptA and that the loss of EptC results in nonglycosylated EptA (14). Moreover, they demonstrated that the *eptBAC* gene locus was restricted to some ETEC strains but was absent in other pathogenic and nonpathogenic strains of *E. coli*, confirming that *E. coli* can glycosylate proteins when provided with the proper genes (14). Recently Sartain and Belisle showed that expression of recombinant SodC (of *M. tuberculosis*) resulted in proteins that were not processed correctly, nor were they glycosylated (46). These studies suggest that *E. coli* normally does not possess the necessary glycosylation machinery. Our finding that the native minor fimbria is glycosylated suggests that caution should be used in interpretation of studies that use recombinant minor fimbria expressed in *E. coli* (41).

It is important to note that bacterial O-glycosylation makes use of unusual sugars (1). Also, the sugars are not always added in a sequential manner to the protein. There are reports that sugars are preassembled and added to a lipid carrier

before being added to the protein acceptor (1). Understanding how the *P. gingivalis* minor fimbria becomes glycosylated and translocated would expand our understanding of this organism and how it eludes host immunity.

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