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The pilin *O*-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase

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ABSTRACT

O-Glycosylation is emerging as a common posttranslational modification of surface exposed proteins in bacterial mucosal pathogens. In pathogenic *Neisseria* an *O*-glycosylation pathway modifies a single abundant protein, pilin, the subunit protein that forms pili. Here, we identify an additional outer membrane glycoprotein in pathogenic *Neisseria*, the nitrite reductase AniA, that is glycosylated in its C-terminal repeat region by the pilin glycosylation pathway. To our knowledge, this is the first report of a general *O*-glycosylation pathway in a prokaryote. We also show that AniA displays polymorphisms in residues that map to the surface of the protein. A frame-shift mutation abolishes AniA expression in 34% of *Neisseria meningitidis* strains surveyed, however, all *Neisseria gonorrhoeae* strains examined are predicted to express AniA, implying a crucial role for AniA in gonococcal biology.

There are now many reports of glycosylation of proteins in bacteria, particularly in bacterial pathogens. In most of these reports a specific glycoprotein has been identified using structural, immunological or genetic approaches, and in some cases key genes involved in the glycosylation process have been identified (reviewed in [1,2]). Recently there have been key advances in understanding the physiology and molecular mechanisms of glycosylation in the two best characterised bacterial systems; *N*-glycosylation in *Campylobacterjejuni* [3–5] and *O*-glycosylation in pathogenic *Neisseria*. Pilin of pathogenic *Neisseria* was one of the first examples of an *O*-glycosylated glycoprotein in a bacterial pathogen [6]. A series of studies have identified the genes encoding glycosyltransferases required for the biosynthesis of the pilin glycan [7–9]; and of alternate structures than can be synthesised in different strains [1,10,11] (Fig. 1A). Recent studies in *Neisseria meningitidis* have proposed that the addition of *O*-linked glycan to pilin is performed by an *O*-oligosaccharyltransferase, PglL, which is homologous to *O*-antigen ligases that catalyse addition of *O*-antigen to lipopolysaccahride [12]. This is supported by subsequent work in *Neisseria gonorrhoeae* [13], and by the transfer of the *N. meningitidis* and *Pseudomonas aeruginosa* pilin *O*-glycosylation systems into *Escherichia coli* [14]. In this report we demonstrate that the pilin *O*-glycosylation system in pathogenic *Neisseria* is a general pathway for protein *O*-glycosylation.

Materials and methods

Bacterial strains and culture conditions. Neisseria meningitidis and *Neisseria gonorrhoeae* strains are listed in are Supplementary Tables 1 and 2 see [15]), and were grown on brain heart infusion medium (BHI) supplemented with Levinthal's base (and IsoVitaleX For *N. gonorrhoeae*).

Immunological analysis. Western blotting was performed essentially as previously described [8]. Primary antibodies used were rabbit anti-trisaccharide sera [8], rabbit anti-pilin sera [8], mouse anti-AniA monoclonal antibodies (mAb) [16], or mouse anti-FLAG mAb (Sigma-Aldrich). Secondary antibodies used were anti-rabbit IgG and anti-mouse IgG (Sigma-Aldrich). Whole cell ELISA was performed as described [17], with mouse anti-AniA mAb and HRP-conjugated anti-mouse IgG (Dako). Colony-immunoblotting was performed as previously described [18], using mouse anti-AniA mAb and anti-mouse IgG.

Enrichment of novel glycoproteins from outer membrane subcellular fractions. Outer membrane proteins were enriched essentially as described [19]. Solubilised proteins were enriched by anion exchange Q Sepharose™ Fast Flow (GE Healthcare) column.

In vivo nitrite utilisation assays. Cells were grown to OD 0.5 A_{600}, washed once in PBS containing 27.8 mM glucose, then resuspended to an OD of 1 in 10 ml of the same solution. After equilibration at 37 °C for 30 min, $NaNO_2$ was added to the cultures to a final

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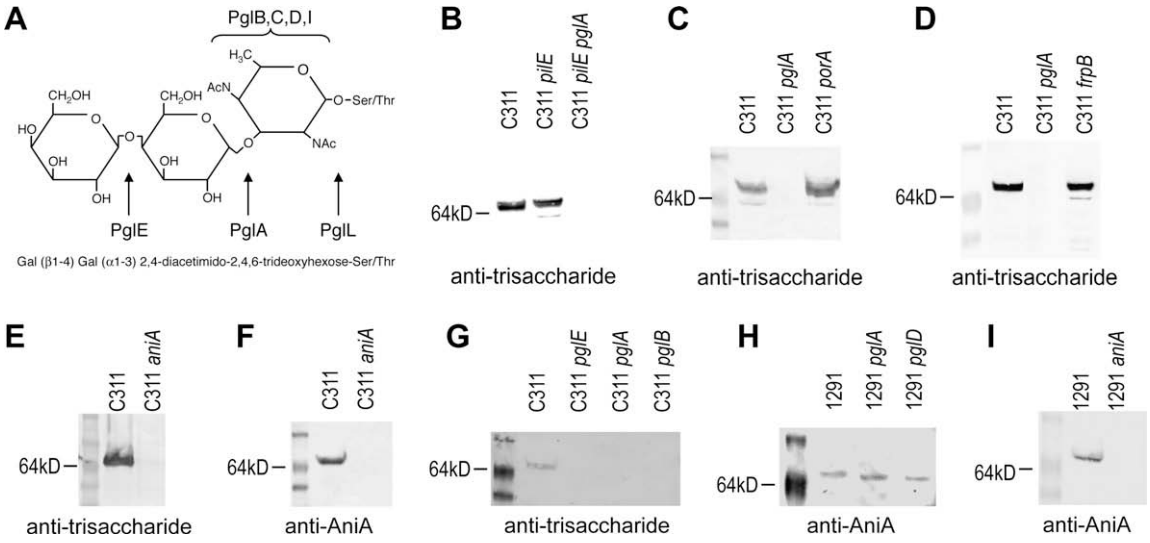

Fig. 1. Identification of AniA as a glycoprotein in *N. meningitidis* and *N. gonorrhoeae*. (A) The *N. meningitidis* trisaccharide and biosynthetic enzymes. Outer membrane proteins reactive with (B–E) anti-trisaccharide sera or (F) anti-AniA mAb. (G) Glycosylation of AniA in glycosylation pathway mutant strains. (H) *N. gonorrhoeae* strain 1291, glycosylation pathway mutant strains 1291*pglA* and 1291*pglD*; (I) strains 1291 and 1291*aniA* (anti-AniA mAb).

concentration of 1 mM. Nitrite utilisation was assayed colorimetrically with the Griess reagent [20] essentially as described [21].

Nucleotide sequence analysis of aniA from clinical isolates. The *aniA* gene was amplified and sequenced using primers aniA_upstm and aniA_downstm or primers aniA_F and aniA_R (Table 1). Multiple sequence alignment was performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and a phylogenetic tree built with WorkBench (http://workbench.sdsc.edu/).

Construction of porA, frpB and aniA mutant strain in C311. The plasmid pIP52 (Dr. Ian Peak, unpublished) was used to inactivate *porA* in strain C311. The *frpB* gene was amplified using frpB14R and frpB11F, cloned into pGEM-T Easy (Promega), generating pGEM-*frpB*. The *aniA* gene in two fragments, using primers aniA_F and aniA5′XhoI_R and primers aniA_R and aniA5′XhoI_F, introducing a *XhoI* restriction site and cloned into pGEM-T Easy, generating pGEM-*aniA*(*XhoI*). pGEM-*aniA*(*XhoI*) and pGEM-*frpB* were digested with *XhoI* and ligated together with the isolated *SalI* digested fragment from pUC4Kan (Amersham Biosciences) containing the kanaymcin cassette. The *porA*, *frpB* and *aniA* mutant constructs were linearised and transformed into *N. meningitidis* strain C311 and *N. gonorrhoeae* strain 1291 as described previously [22]. The pUC4kan kanamycin cassette has no promoter or terminator that is active in *Neisseria* and does not affect transcription nor have a polar effect on expression of adjacent genes [22,23].

Construction and purification of FLAG-tagged AniA from N. meningitidis strain C311. Sequence encoding a FLAG-tag (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) was fused to the C-terminus of aniA of C311. Vector pT7blueaniA::flagtetMB was constructed by overlap PCR using the primers aniA_F, NMB1624_R, 3′flag_XhoI_ds and NMB1624_F_XhoI. The 3′flag_XhoI_ds primer encoded a FLAG-tag extension followed by a stop codon to allow incorporation of the tag in frame with aniA. The aniA::flag construct with NMB1624 (downstream of aniA) was initially cloned into pT7blue (Novagen) to create pT7blueaniA::flag. A tetracycline antibiotic resistant cassette (TetMB) was digested with SalI and cloned into XhoI linearised pT7blueaniA::flag to create pT7blueaniA::flag tetMB, which was transformed to the chromosome of C311 by homologous recombination as described above. Cells expressing AniA-FLAG were grown on BHI agar containing 1 μg/mL tetracycline and 1 mM of sodium nitrite at 37 °C for 24 h. AniA-FLAG protein was purified according to the manufacturer's instructions.

Mass spectrometry. Protein was reduced/alkylated with dithiothreitol/iodoacetamide. AniA-FLAG was precipitated by addition of 4 volumes of 1:1 acetone:methanol, incubation at −20 °C

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
frpB14R	CGCATCGTGTTGAGCCACATGAAAG
frpB11F	TTGCGGCAGGTTTTGCCCACGC
aniA_F	ATGAAACGCCAAGCCTTAG
NMB1624_R	TTATCGGCTTGTGCAACGGAAGCCC
3′flag_XhoI_ds	AATAACCGGACATACTTCATCTCGAGTCACTTGTCGTCATCGTCCTTGTAGTCATAAACGCTTTTTTCGGATGCAGAGGC
NMB1624_F_XhoI	CTCGAGATGAAGTATGTCCGGTTATTTTTCC
aniA5′XhoI_R	TGAATGTGGAAGTACGGCCTCGAGCGGT
aniA_R	TAAACGCTTTTTTCGGATGCAG
aniA5′XhoI_F	ACCGCTCGAGGCCGTACTTCCACATTCA
aniA_R500	CCACATTCAGCTTCAAAGCCCTGCA
aniA_upstm	AACTACCCTGCCTTTGCCTGATT
aniA_downstm	CCGAGGAAAAATAACCGGACATAC