Biochemical and Biophysical Research Communications 445 (2014) 633–638

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Selection against glycosylation sites in potential target proteins of the general HMWC N-glycosyltransferase in *Haemophilus influenzae*

Jayde A. Gawthorne [a,b], Nikki Y. Tan [a], Ulla-Maja Bailey [a], Margaret R. Davis [a], Linette W. Wong [a], Ranjitha Naidu [a], Kate L. Fox [a], Michael P. Jennings [b], Benjamin L. Schulz [a,*]

[a] Australian Infectious Disease Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia
[b] Institute for Glycomics, Griffith University, Gold Coast Campus, Gold Coast, QLD 4222, Australia

ARTICLE INFO

*Article history:*
Received 4 February 2014
Available online 22 February 2014

*Keywords:*
Glycobiology
Metabolic engineering
Mass spectrometry
Proteomics
Protein evolution
Leloir pathway

ABSTRACT

The HMWABC system of non-typeable *Haemophilus influenzae* (NTHi) encodes the HMWA adhesin glycoprotein, which is glycosylated by the HMWC glycosyltransferase. HMWC is a cytoplasmic N-glycosyltransferase, homologues of which are widespread in the Pasteurellaceae. We developed an assay for nonbiased detection of glycoproteins in NTHi based on metabolic engineering of the Leloir pathway and growth in media containing radiolabelled monosaccharides. The only glycoprotein identified in NTHi by this assay was HMWA. However, glycoproteomic analyses *ex vivo* in *Escherichia coli* showed that HMWC of NTHi was a general glycosyltransferase capable of glycosylating selected asparagines in proteins other than its HMWA substrate, including Asn78 in *E. coli* 30S ribosomal protein S5. The equivalent residue in S5 homologues in *H. influenzae* or other sequenced Pasteurellaceae genomes is not asparagine, and these organisms also showed significantly fewer than expected potential sites of glycosylation in general. Expression of active HMWC in *E. coli* resulted in growth inhibition compared with expression of inactive enzyme, consistent with glycosylation by HMWC detrimentally affecting the function of some *E. coli* proteins. Together, this supports the presence of a selective pressure in the Pasteurellaceae against glycosylation sites that would be modified by the general N-glycosyltransferase activity of HMWC.


## 1. Introduction

*Haemophilus influenzae* is a pathogen of the respiratory tract that causes a severe burden of disease in children in both developed and developing countries. Six different capsular serotypes have been identified, as well as unencapsulated (non-typeable) *H. influenzae* (NTHi). Infection by NTHi is the most common cause of exacerbations in chronic obstructive pulmonary disease, a major and growing global health problem in aging populations [1]. NTHi, often in mixed infection with *Streptococcus pneumoniae* and *Moraxella catarrhalis*, is also a leading cause of middle ear infections, which in developed countries is the most common reason for children to visit doctors and for the prescription of antibiotics [2].

The initial stage of infection by NTHi is colonisation of the upper respiratory tract. A key step in this colonisation is binding to the underlying epithelia, a process mediated by adhesin proteins on the bacterial cell surface. Up to 80% of NTHi clinical isolates contain genes encoding the related adhesins HMW1 and HMW2 [3]. These high molecular weight (HMW) adhesins are typical of a family of two-partner secreted glycoprotein adhesins common in Gram-negative pathogens. The NTHi HMW genes are present in gene clusters that include the genes encoding the glycosylated adhesin protein (HMWA), an outer membrane protein required for secretion of the adhesin (HMWB), and a cytoplasmic glycosyltransferase (HMWC) [4].

The HMWA adhesins of NTHi are N-glycoproteins, with many asparagine residues variably modified with 0, 1 or 2 hexoses by the HMWC glycosyltransferase [5,6]. Strikingly, the modification of HMWA requires two distinct enzymatic activities: the formation of an N-glycosidic bond between the asparagine and the first hexose; and an O-glycosidic bond between the two hexoses. These two different chemistries have been reported to be performed by the single enzyme, HMWC [5]. However, not all HMWC homologues possess this dual O- and N-glycosyltransferase activity [7]. N-Glycosylation catalysed by HMWC is therefore distinct from traditional N-glycosylation in eukaryotes, archaea and some bacteria, where N-glycosylation is catalysed by an integral membrane enzyme, oligosaccharyltransferase [8–13]. HMW1A purified from NTHi is modified by a mixture of galactose (55–70%), glucose

*Abbreviations:* NTHi, non-typeable *Haemophilus influenzae*; HMW, high molecular weight; LPS, lipopolysaccharide.

* Corresponding author. Fax: +61 7 3365 4273.
*E-mail address:* b.schulz@uq.edu.au (B.L. Schulz).

http://dx.doi.org/10.1016/j.bbrc.2014.02.044
0006-291X/

(30–45%) and mannose (2%) [14]. *In vitro*, HMW1C rapidly transfers glucose, and transfers galactose at a reduced rate, from UDP-activated substrate to HMW1A [5]. It is not clear if the mannose detected on HMW1A purified from NTHi is covalently linked to HMW1A or is a contaminant. The Leloir pathway is the main enzymatic pathway for galactose metabolism *in vivo* in *H. influenzae* [15], from where it is incorporated into lipopolysaccharide and glycoproteins [16].

General glycosylation systems, in which a single oligosaccharyltransferase or glycosyltransferase modifies many glycoprotein substrates, have been recently reported in bacteria including *Neisseria meningitidis* [17], *Neisseria gonorrhoeae* [18], *Campylobacter jejuni* [11,19], *Bacteroides fragilis* [20] and *Acinetobacter baumannii* [21], and predicted in diverse other organisms [22]. The HMW1C-like enzyme from *Actinobacillus pleuropneumoniae* can glycosylate Asn residues in glycosylation sequons in a range of non-native peptides and flexible stretches of proteins [7]. This prompted us to investigate the range of HMW1C glycoprotein substrates in NTHi.

## 2. Materials and methods

### 2.1. Plasmid construct

*H. influenzae pgmB* was inactivated by insertion of a kanamycin resistance cassette transformed as described [23]. DNA encoding the HMW1C glycosyltransferase was cloned from the pHMW1ABC plasmid [24] to create plasmid pBAD-HMW1C. Site-directed mutagenesis [25] created plasmid pBAD-HMW1C$_{K467A}$.

### 2.2. Growth conditions

NTHi strains were grown at 37 °C in brain heart infusion (BHI) supplemented with 10 mg/L hemin and 2 mg/L NAD in liquid medium, or Levinthal supplement in solid medium. 10 μg/mL Kanamycin was included where required. *E. coli* Top 10 cells were grown in LB media supplemented with 100 μg/mL ampicillin (bearing the pHMW1ABC plasmid) and 0.2% arabinose (bearing the pBAD-HMW1C or pBAD-HMW1C$_{K467A}$ plasmids) until they reached an $OD_{600nm}$ of 1 and were harvested by centrifugation. Biological triplicates were analysed.

### 2.3. SDS–PAGE, autoradiography and immunoblotting

Radiolabelling of NTHi macromolecules was performed using BHI plates supplemented with 0.2% $^{14}$C-galactose (Sigma–Aldrich, St Louis, Missouri, USA). Equal cell numbers were separated by SDS–PAGE with either 16% Tricine or 4–12% Bis-Tris gels and proteins transferred to either nitrocellulose or PVDF. Radiolabelled glycoproteins were detected by exposure of the membrane to autoradiograph film. Antibodies 4G4 and AD6 were used to identify the HMW adhesins [26].

### 2.4. MS sample preparation

Cell pellets were resuspended in 50 mM Tris HCl pH 8, 6 M guanidine hydrochloride with 10 mM DTT and incubated for 30 min at 37 °C. Cysteines were alkylated by addition of acrylamide to 25 mM and incubation for 1 h at 25 °C. Protein corresponding to 50 μL final culture media was precipitated and digested with trypsin or AspN as described [17].

### 2.5. MS and data analysis

Peptides were analysed by LC–ESI–MS/MS as described [27]. For neutral loss scanning, loss of hexose (162.1 or 180.1 Da) was monitored. Data was searched with MASCOT V2.3 at the Australian Proteomics Computational Facility (http://www.apcf.edu.au/). Protein sequences were aligned using ClustalW, and displayed using WebLogo [28]. Protein structural cartoon representations were prepared using MacPymol.

### 2.6. Growth assays

*E. coli* Top 10 cells bearing the empty pBAD vector, pBAD-HMW1C or pBAD-HMW1C$_{K467A}$ plasmids were grown at 37 °C in liquid LB media or solid LB media with 1% agar, supplemented with 100 μg/mL ampicillin, in the presence or absence of 0.2% arabinose. Liquid culture growth was measured by the optical density at 600 nm. Biological triplicates were analysed.

## 3. Results

### 3.1. Leloir pathway metabolic engineering

To identify novel glycoproteins in NTHi in a nonbiased fashion we developed an assay based on metabolic engineering of the Leloir pathway and growth in media containing radiolabelled monosaccharides. In NTHi, galactose is taken up by galactose permease (GalT), converted to galactose-6-phosphate and converted to galactose-1-phosphate by galactokinase (GalK) (Fig. 1A). Uridine diphosphate (UDP) is then added by galactose-1-phosphate uridyltransferase (GalU) to form UDP-galactose. The galactose is then in a conformation that can be directly used in macromolecule biosynthesis [16]. As galactose taken up by cells is directly incorporated into macromolecules, we aimed to selectively label NTHi glycoproteins containing galactose by growing cells in media containing $^{14}$C-galactose. The two known macromolecules that contain galactose in NTHi are lipopolysaccharide (LPS) and the HMW1 and HMW2 glycoproteins [14]. Autoradiography of SDS–PAGE gels of

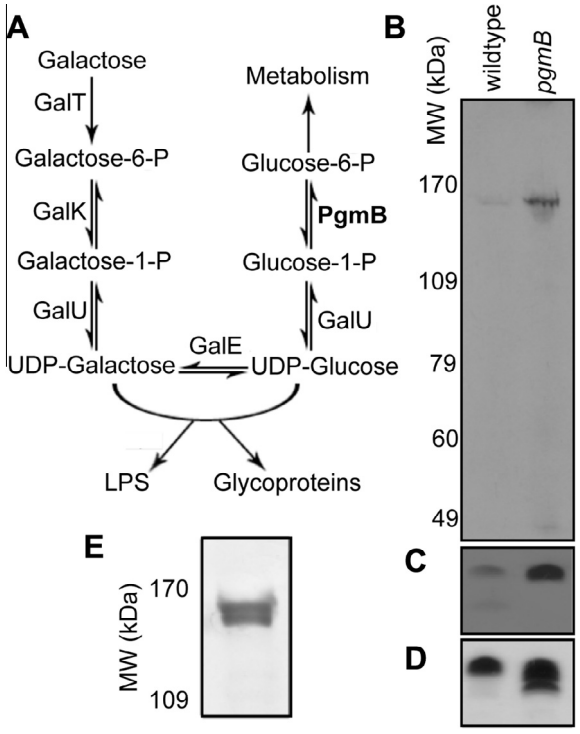


**Fig. 1.** Metabolic engineering of the Leloir pathway for glycoprotein detection. (A) The Leloir pathway. PgmB was inactivated in NTHi 86-028NP. (B) Autoradiograph detection of glycoproteins modified with $^{14}$C-galactose (C) autoradiograph detection of LPS and (D) silver stain detection of LPS in 86-028NP (wildtype) and 86-028NP*pgmB::kan* (*pgmB*). (E) Immunoblot identification of HMWA.