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

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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.

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(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

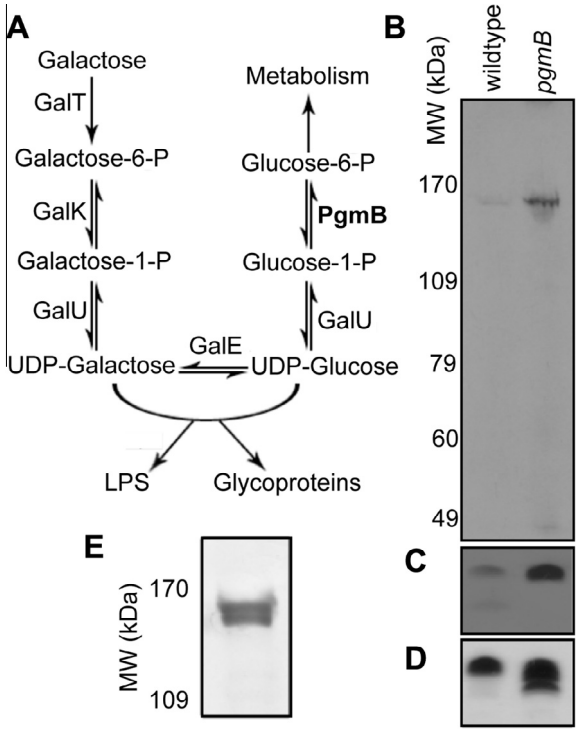

Fig. 1. Metabolic engineering of the Leloir pathway for glycoprotein detection. (A) The Leloir pathway. PgmB was inactivated in NTHi 86-028NP. (B) Autoradiograph detection of glycoproteins modified with ^{14}C-galactose (C) autoradiograph detection of LPS and (D) silver stain detection of LPS in 86-028NP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

whole cell extracts of wild type (86–028NP) NTHi grown on media supplemented with ^{14}C-galactose showed faint bands corresponding to HMWA (Fig. 1B) and LPS (Fig. 1C). Analysis of replicate gels using immunoblotting with monoclonal antibody AD6 [29] confirmed the identity of HMWA (Fig. 1E), while silver staining confirmed the presence of LPS (Fig. 1D). To improve the sensitivity of glycoconjugate detection, we engineered the Leloir pathway of NTHi. If there is a large influx of galactose in NTHi, then galactose-4-epimerase (GalE) can easily convert UDP-galactose to UDP-glucose, preventing galactose toxicity (Fig. 1A). UDP-glucose can be used in macromolecule biosynthesis or converted to glucose-1-phosphate by GalU and finally to glucose-6-phosphate by phosphoglucomutase (PgmB) where it can then be used in other metabolic processes [16]. We chose to inactivate PgmB, as this would still allow the interconversion of galactose and glucose by GalE, allowing both sugars to become available for macromolecule biosynthesis, but prevent their loss to other metabolic processes by trapping them within the Leloir pathway as nucleotide sugars [16]. Autoradiography of SDS–PAGE gels of whole cell extracts of NTHi with inactive pgmB (86-028NPpgmB::kan) grown with ^{14}C-galactose showed intense bands corresponding to HMWA (Fig. 1B) and LPS (Fig. 1C). However, no bands corresponding to additional NTHi glycoproteins were detected. This approach is a novel unbiased method of detecting glycoproteins and has the potential to be applied to other bacterial species.

3.2. Ex vivo HMW1C glycoproteomics

As no further glycoproteins were identified in NTHi in addition to the known HMWA glycoprotein adhesins, we tested if HMW1C was potentially capable of general N-glycosyltransferase activity, using a glycoproteomic approach to characterise its activity in an *ex vivo E. coli* expression system [30]. We expressed the HMW1ABC locus from a plasmid in *E. coli*, and performed untargeted LC–ESI–MS/MS proteomic analysis of whole cell extracts from these cells. This analysis detected twelve used glycosylation sites on HMW1A (Supplementary Table 1). Although it has been reported that HMW1C can glycosylate asparagines with multiple hexose monosaccharides [6], we did not observe modification with more than one hexose. We detected one peptide that contained three sites of modification that have not been previously reported (Asn1388, Asn1393 and Asn1398 in peptide Asp1378-Arg1410). We also detected two peptides with Asn residues in glycosylation sequons that were not modified (Asn1107 in peptide Val1100-Lys1124 and Asn1491 in peptide Phe1487-Arg1509).

In addition to these glycosylation sites in the known HMW1A glycoprotein substrate of HMW1C, we also detected glycopeptides with hexose-asparagine modifications from the HMW1C glycosyltransferase protein itself and from an *E. coli* protein, the 30S ribosomal protein S5 (Supplementary Table 1). This modification of *E. coli* S5 had not been previously reported in the UniProtKB protein knowledgebase (Accession number P0A7W1). To explicitly test if *E. coli* S5 and NTHi HMW1C were glycosylated by the NTHi HMW1C glycosyltransferase and not an endogenous *E. coli* enzyme, we subcloned the HMW1C gene into the pBAD expression vector, and also created an inactive HMW1C variant through Lys467Ala point mutation at its active site [31]. We then performed untargeted proteomic analyses of whole cell extracts from *E. coli* cells with the pBAD-HMW1C or pBAD-HMW1C$_{K467A}$. These analyses showed that both Asn78 in *E. coli* S5 and Asn12 in HMW1C were only glycosylated in the presence of active NTHi HMW1C glycosyltransferase, and remained unglycosylated with inactive HMW1C$_{K467A}$ (Fig. 2). With expression of active HMW1C, both non-glycosylated and hexose-modified forms of the tryptic peptides could be identified (Fig. 2A and E). However, with expression of inactive HMW1C$_{K467A}$, only the non-glycosylated forms of the peptides were detected (Fig. 2B and F). Both the glycosylated and non-glycosylated forms of both peptides were identified by MS/MS (Fig 2C, D, G and H). These experiments indicated that *E. coli* S5 is not natively glycosylated by another enzyme, but rather that NTHi HMW1C can partially glycosylate Asn78 in *E. coli* S5 and Asn12 in HMW1C itself.

We performed several additional targeted and non-targeted proteomic discovery approaches to attempt to detect additional glycosylation sites in *E. coli* proteins modified by the NTHi HMW1C glycosyltransferase. These approaches included long gradient LC–MS analysis with untargeted detection and with neutral loss scanning. However, we did not detect any additional glycosylated Asn residues. It is possible that other proteins may have been glycosylated by HMW1C *ex vivo* in *E. coli*, but at sites that did not allow efficient protein folding or multiprotein complex integration. Any such proteins would be subject to degradation if glycosylated, would not be present at high levels, and would therefore not be detected by proteomic surveys.

3.3. Bioinformatic analyses

To investigate the potential consequences of modification of Asn78 in *E. coli* S5 we examined its structural environment in the *E. coli* ribosome [32]. This showed that modification was not likely to severely affect S5 activity as this residue in S5 is not directly involved in protein–protein interactions with other ribosomal subunits (Fig. 3C), and is not conserved amongst the Enterobacteriaceae (Fig. 3A). However, the position corresponding to Asn78 in *E. coli* S5 is never Asn in sequenced Pasteurellaceae genomes (Fig. 3B). Statistical comparison showed that the absence of asparagine at this position in the Pasteurellaceae compared with the Enterobacteriaceae is highly significant ($P < 0.001$, 2-sided Fisher's exact test). The difference is particularly striking given that the remainder of the S5 protein sequence is highly similar between *H. influenzae* and *E. coli*, with overall 99% similarity and 92% identity.

We performed a bioinformatic survey to test for evidence of selection against HMW1C glycosylation sites in *H. influenzae* in addition to Asn78 in *E. coli* S5. We used the 30S ribosomal proteins in our analysis as these are highly conserved and 30S ribosomal protein S5 was a substrate of HMW1C (Supplementary Table 1, Fig. 1). We first performed alignments of the protein sequences of each 30S ribosomal protein homologue from *E. coli* and *H. influenzae*. There were 117 Asn residues in this set of *E. coli* proteins, of which 13 were present in sequons. Of the Asn residues not in sequons, 86% were also conserved in *H. influenzae*, such as Asn32 and Asn42 in 30S ribosomal protein S3 (Fig. 3D). However, for Asn residues located in a sequon only 54% were also sequons in the corresponding *H. influenzae* protein, such as N19 and N185 in 30S ribosomal protein S3 (Fig. 3D and E). Some of these non-conserved sequons possessed a mutation at the Asn, while others were mutated at the +2 Ser/Thr (Fig. 3D). The difference in the conservation of Asn residues located in sequons and those not in sequons was statistically significant ($P < 0.01$, 2-sided Fisher's exact test).

3.4. HMW1C dependent growth inhibition

Our proteomic and bioinformatic analyses (Figs. 2 and 3) suggested that there was a selective pressure against glycosylation sites modifiable by HMW1C in the Pasteurellaceae. *E. coli* proteins contain more Asn in sequons than the Pasteurellaceae, and glycosylation at these sites may impair protein folding or function. To experimentally test for the presence of a selective pressure against glycosylation by HMW1C, we compared the growth rate of *E. coli* expressing active or inactive HMW1C. Cells expressing either active or inactive HMW1C showed growth defects compared with cells carrying the empty vector, presumably due to the high metabolic load of protein over-expression (Fig. 4). Nonetheless, expres-

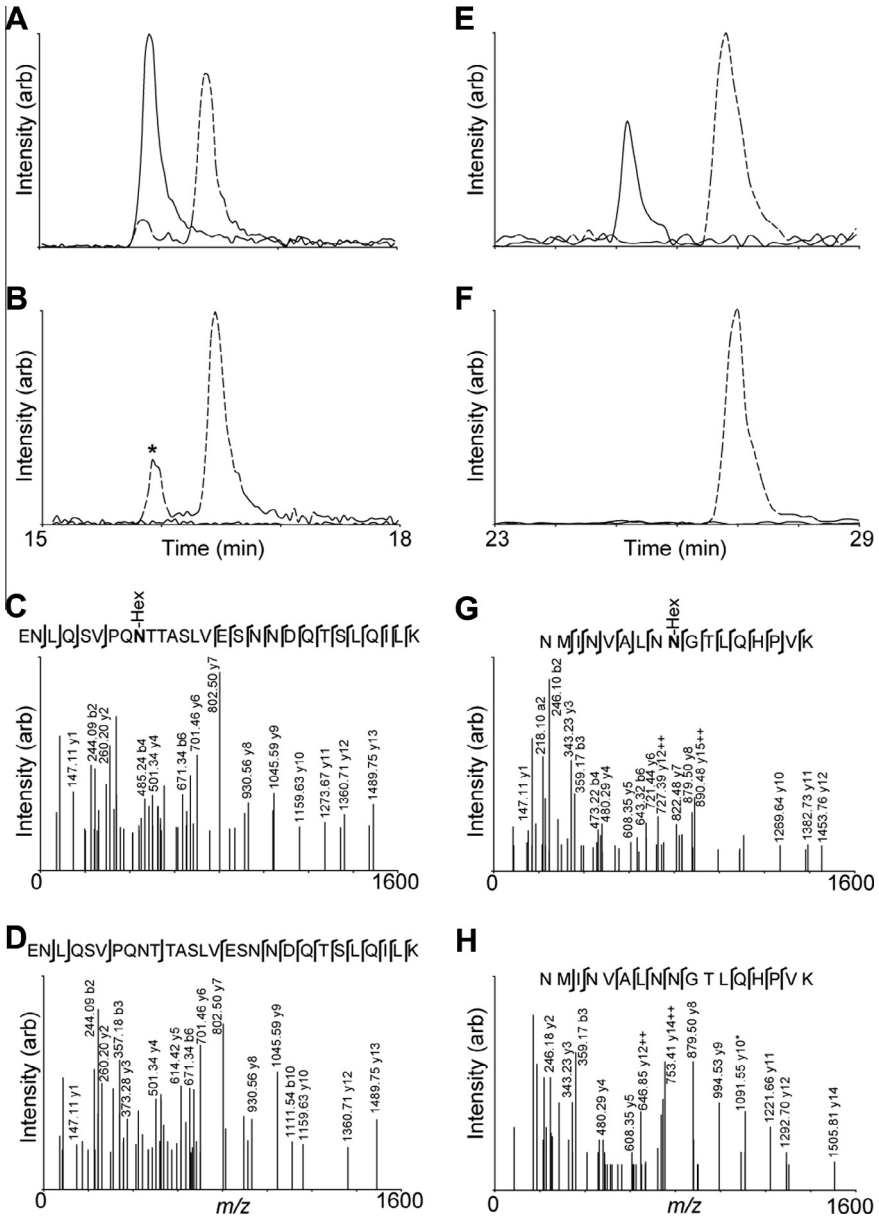

Fig. 2. HMW1C-dependent glycosylation of Asn12 in HMW1C and Asn78 in *E. coli* S5. Extracted ion chromatograms from cells with (A/E) HMW1C or (B/F) HMW1C-K467A corresponding to glycosylated (solid) and non-glycosylated (dashed) forms of tryptic peptide HMW1C Glu4-Lys31 (*m/z* of 768.65^{4+} and 809.16^{4+}, A/B) or S5 Asn70-Lys86 (*m/z* of 931.99^{2+} and 1013.03^{2+}, E/F). *, peak corresponds to non-monoisotopic peak from peptide with *m/z* of 768.41^{4+} from *E. coli* ClpX. MS/MS of peptides from (A/B/E/F) identifying (C/G) glycosylated and (D/H) non-glycosylated HMW1C Glu4-Lys31 peptide (C/D) or S5 Asn70-Lys86 peptide (G/H).

sion of active HMW1C resulted in a further significant growth defect compared to expression of inactive $HMW1C_{K467A}$, with a large and significant reduction in the final cell density reached (Fig. 4A). When protein expression was not induced there was no difference between these cells in growth rate or final cell density (Fig. 4B). *E. coli* grown on agar showed similar phenotypes, with cells expressing active HMW1C forming colonies that were distinctly smaller than cells expressing inactive $HMW1C_{K467A}$ (Fig. 4C). No difference was observed without arabinose-induction of protein expression (Fig. 4D).

4. Discussion

The Pasteurellaceae lineage commonly encode HMW1C-like enzymes, and these enzymes show a high degree of sequence identity and similarity. Furthermore, they have been shown to be functionally equivalent, as the *A. pleuropneumoniae* HMW1C-like homologue is capable of glycosylating the NTHi HMW1A glycoprotein adhesin [33]. These enzymes have similar protein substrate specificities and would likely be capable of glycosylating S5 at Asn78 and other target proteins, as we report here for NTHi HMW1C in *E. coli*. Together, this is consistent with selection against glycosylation sites in ribosomal proteins in the Pasteurellaceae. This is consistent with a recent report which detected glycosylation only of HMW-like autotransporter adhesins in *A. pleuropneumoniae* [34]. Although some NTHi strains lack HMWABC, these strains are likely to be recently evolved [3]. The selection pressure may be due to pleiotropic systems-level effects of glycosylation on the folding, dynamics or multiprotein complex assembly of many proteins. Point mutation of such glycosylation sites in the Pasteurellaceae could therefore remove the possibility of glycosylation by HMW1C while otherwise retaining protein function.

We detected a clear growth inhibition caused by HMW1C glycosyltransferase activity in *E. coli* cells (Fig. 4). Even a small reduction

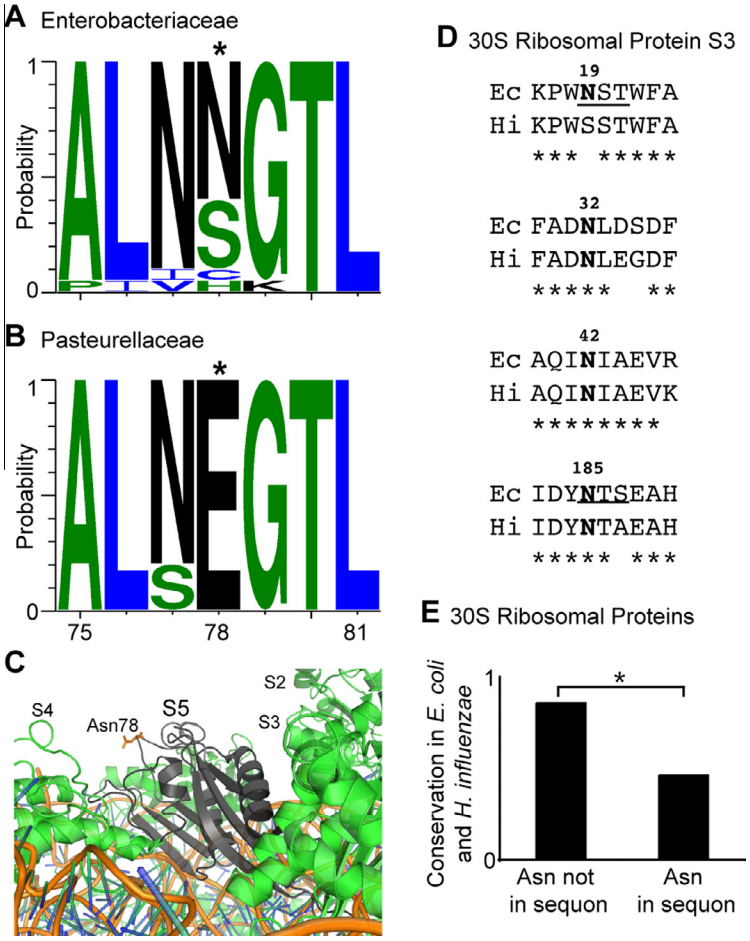

Fig. 3. Selection against asparagines in sequons in 30S ribosomal proteins in the Pasteurellaceae. Weblogo representations of aligned sequences of S5 proteins from (A) Enterobacteriaceae and (B) Pasteurellaceae. The glycosylated Asn78 in *E. coli* S5 and the corresponding Glu78 in S5 in the Pasteurellaceae are asterisked. (C) Cartoon representation of the structural vicinity within the ribosome (PDB code 3I1M) [32] of Asn78 in *E. coli* S5. Ribosomal proteins S2, S3 and S4 are green; S5 is grey; glycosylated Asn78 in S5 is orange. (D) Alignment of 30S ribosomal protein S3 from *E. coli* (Ec) and *H. influenzae* (Hi) showing conservation of selected Asn not in sequons (Asn32 and Asm42), but not of those located in sequons (Asn19 and Asn185). Bold, Asn; underlined, sequon; *, conserved; Asn positions are numbered. (E) Proportion of Asn, in sequons or not in sequons, conserved in 30S ribosomal proteins in *E. coli* and *H. influenzae*. *, $P < 0.01$, 2-sided Fisher's exact test.

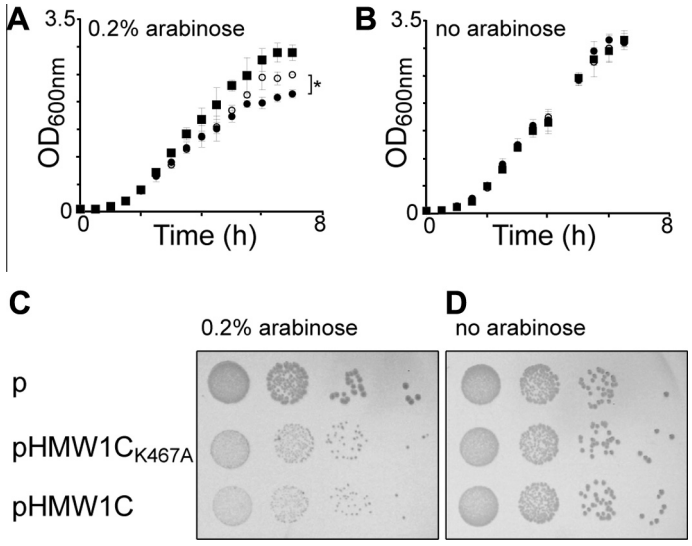

Fig. 4. Growth of *E. coli* expressing active and inactive HMW1C. Growth of *E. coli* cells with empty plasmid (■), inactive pHMW1C$_{K467A}$ (○) or pHMW1C (●) in liquid culture with (A) 0.2% arabinose or (B) no arabinose or on agar with (C) 0.2% arabinose or (D) no arabinose. Error bars show standard deviation. *, $P < 0.05$, 2-sided students *T*-test.

in growth rate or final cell density can exert a large selection pressure, and the measurable growth defect we detect would therefore be strongly selective against glycosylation by HMW1C (Fig. 4). However, NTHi require HMW1C activity, as HMW1C is required for glycosylation of the HMW1A adhesin glycoprotein, glycosylation of HMW1A is required for protein stability and secretion, and efficient adherence of NTHi to epithelial cells requires functional and correctly secreted HMW1A [14,30,35,36]. There is therefore a selection pressure in NTHi to maintain expression of active HMW1C to allow efficient infectivity, but a counteracting pressure against glycosylation at some other asparagines due to the general glycosyltransferase activity of HMW1C. Our results suggest that a mechanism to escape the general glycosyltransferase activity of HMW1C is mutation of selected sequons that would otherwise be glycosylated by the HMW1C enzyme. A consequence of this is the absence of glycoproteins in NTHi in addition to the HMWA glycoprotein adhesins (Fig. 1). Selection for and against glycosylation sites may therefore be a significant factor in the evolution of proteins in the many organisms now reported to contain general glycosylation systems [22,37–40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.044.

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