RESEARCH ARTICLE

# Unconventional N-Linked Glycosylation Promotes Trimeric Autotransporter Function in *Kingella kingae* and *Aggregatibacter aphrophilus*

**Katherine A. Rempe,[a,b] Lynn A. Spruce,[b] Eric A. Porsch,[b] Steven H. Seeholzer,[b] Niels Nørskov-Lauritsen,[d] Joseph W. St. Geme III[b,c]**

Department of Molecular Genetics and Microbiology, Duke University, Durham, North Carolina, USA[a]; The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA[b]; University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA[c]; Department of Clinical Microbiology, Aarhus University Hospital, Aarhus, Denmark[d]

**ABSTRACT** **Glycosylation is a widespread mechanism employed by both eukaryotes and bacteria to increase the functional diversity of their proteomes. The nontypeable *Haemophilus influenzae* glycosyltransferase HMW1C mediates unconventional N-linked glycosylation of the adhesive protein HMW1, which is encoded in a two-partner secretion system gene cluster that also encodes HMW1C. In this system, HMW1 is modified in the cytoplasm by sequential transfer of hexose residues. In the present study, we examined *Kingella kingae* and *Aggregatibacter aphrophilus* homologues of HMW1C that are not encoded near a gene encoding an obvious acceptor protein. We found both homologues to be functional glycosyltransferases and identified their substrates as the *K. kingae* Knh and the *A. aphrophilus* EmaA trimeric autotransporter proteins. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed multiple sites of N-linked glycosylation on Knh and EmaA. Without glycosylation, Knh and EmaA failed to facilitate wild-type levels of bacterial autoaggregation or adherence to human epithelial cells, establishing that glycosylation is essential for proper protein function.**

**IMPORTANCE** **This work emphasizes the importance of glycosylation for proper function of bacterial proteins. Here we show that the *Kingella kingae* Knh and the *Aggregatibacter aphrophilus* EmaA trimeric autotransporter proteins are N-glycosylated by novel homologues of the *Haemophilus influenzae* HMW1C glycosyltransferase, highlighting the first examples of trimeric autotransporters that are modified by HMW1C-like enzymes. In the absence of glycosylation, Knh and EmaA lack adhesive activity. This work has relevance to our understanding of bacterial pathogenicity and expression of potential vaccine antigens.**

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Address correspondence to Joseph W. St. Geme III, stgemeiiij@email.chop.edu.

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Glycosylation provides a means by which a cell can further specify a particular function for a protein. Once thought to be unique to eukaryotic organisms, glycosylation of proteins is now recognized to be common in prokaryotes (1). Modification of bacterial proteins promotes proper folding (2), adhesive activity (3), solubility (4), antigenic variation (5), and protection against proteases (6). There are two major forms of protein glycosylation: O-linked, in which the modification is attached to the side chain oxygen of a serine or threonine residue, and N-linked, in which the modification is attached to the amide nitrogen of an asparagine residue. Rarely, proteins can be glycosylated on residues other than serine, threonine, or asparagine (7).

O-linked glycosylation in bacteria generally takes place in the cytoplasm and involves a series of glycosyltransferases that sequentially build polysaccharide chains on the acceptor protein. These glycosyltransferases are not membrane bound and are commonly able to transfer only a single specific activated saccharide molecule (8). In a limited number of cases, O-glycosylation has been shown to occur in the periplasm. Specifically, the O-linked modification of pili in *Pseudomonas aeruginosa* and *Neisseria meningitidis* involves an inner membrane bound oligosaccharyltransferase (9). At present, there is no recognized consensus sequence for modification by O-linked glycosylation, though existing evidence suggests that there may be a structural element in the acceptor peptide that directs glycosylation (2).

The best-studied mechanism of bacterial protein N-linked glycosylation is the *pgl* system used by the Gram-negative pathogen *Campylobacter jejuni*. In this system, undecaprenyl-linked sugars are used to assemble branching heptasaccharide chains attached to a lipid anchor on the cytoplasmic face of the bacterial inner membrane. Once assembled, the chains are flipped across the membrane into the periplasm and are transferred to proteins by an inner membrane-bound oligosaccharyltransferase. Glycans are attached to the substrate protein at the consensus site Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except proline (10). The *pgl* system mimics the N-linked glycosylation system found in eukaryotic cells, in which branching sugar chains are built on the cytoplasmic face of the endoplasmic reticulum, flipped into the


**FIG 1** HMW1C$_{Kk}$ and HMW1C$_{Aa}$ are highly homologous to HMW1C. The amino acid sequences of the *K. kingae* strain 269-492 and *A. aphrophilus* strain NJ8700 HMW1C homologues are aligned with the HMW1C sequence from nontypeable *H. influenzae* strain R2846. The plus signs below the sequences denote conserved residues. The three conserved residues involved in binding UDP are highlighted in gray.

lumen, and then transferred to substrate proteins by a membrane-bound oligosaccharyltransferase at the same Asn-Xaa-Ser/Thr consensus site (10).

Nontypeable *Haemophilus influenzae* (NTHi) uses a glycosylation system with elements of both the N-linked and O-linked processes. In this system, the N-linking glycosyltransferase HMW1C transfers glucose and galactose to the acceptor protein HMW1 using the activated UDP forms of the sugars (11). This transfer occurs in the cytoplasm, and sugar chains are built on HMW1, similar to O-linked glycosylation. However, the sugars are added at the Asn-Xaa-Ser/Thr N-linked glycosylation consensus site (12). HMW1 is modified at at least 31 sites with either mono- or dihexoses, and HMW1C is responsible for this modification (3, 11, 12).

HMW1 is an adhesive protein that mediates interaction between bacterial cells and human epithelial cells and is encoded by a two-partner secretion system gene cluster that also encodes HMW1B (an outer membrane pore-forming protein that facilitates the translocation of HMW1 to the bacterial cell surface) and HMW1C (13, 14). Without glycosylation, HMW1 is released from the bacterial cell surface and is unable to facilitate adherence (3). A two-partner secretion system genetic locus with similar organization is found in enterotoxigenic *Escherichia coli* (ETEC). This locus contains the *etpB*, *etpA*, and *etpC* genes, with the HMW1C homologue EtpC being responsible for modifying the adhesive protein EtpA in a manner similar to HMW1C modification of HMW1 (15).

Due to the unique nature of HMW1C-mediated glycosylation, we wondered if this system is used by Gram-negative pathogenic species other than NTHi and ETEC. In this study, we identified a number of HMW1C homologues in other species using bioinformatic approaches. Interestingly, these HMW1C homologues were not always encoded by a genetic locus that also encodes a potential acceptor protein as in NTHi and ETEC. To look specifically at HMW1C homologues that are not encoded near a gene for a potential acceptor protein, we examined *Kingella kingae* and *Aggregatibacter aphrophilus*. Our results demonstrate that the HMW1C homologues in these species modify adhesive proteins with mono- and dihexoses and establish that modification is needed for normal function of the acceptor protein.

## RESULTS

***Kingella kingae* and *Aggregatibacter aphrophilus* have HMW1C homologues.** An NCBI standard protein BLAST search using the HMW1C polypeptide sequence as the query sequence and default parameters revealed 21 bacterial species encoding an HMW1C homologue with identity over 50%. From these results, we chose to investigate the homologues in *K. kingae* and *A. aphrophilus*, in particular because of their high homology to HMW1C (Fig. 1), the fact that they are encoded in the genome away from any gene encoding an obvious acceptor protein, and the relevance of *K. kingae* and *A. aphrophilus* to human health. The *K. kingae* homologue is encoded by a gene located between an upstream gene encoding a putative protein-disulfide reductase and a downstream gene encoding a putative 2-*C*-methyl-D-erythritol 4-phosphate cytidylytransferase, gene products that are predicted cytoplasmic proteins and are not suspected to be targets of glycosylation. Similarly, the *A. aphrophilus* homologue is encoded between genes encoding predicted cytoplasmic proteins, namely, an upstream gene encoding the putative ribosomal protein S12 methyltransferase and a

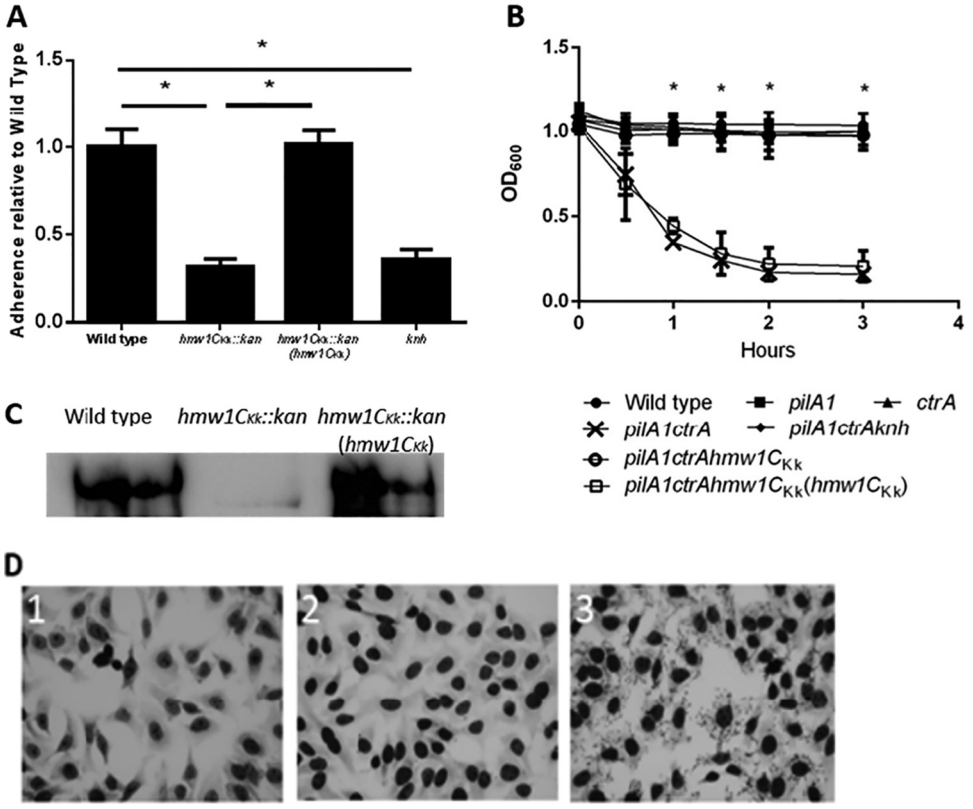


**FIG 2** Expression of $HMW1C_{Kk}$ is required for adherence and autoaggregation. (A) Mean adherence of *K. kingae* with the wild-type $hmw1C_{Kk}$ gene, an interrupted $hmw1C_{Kk}$ gene, and the interrupted $hmw1C_{Kk}$ gene reverted to the wild type. (B) Mean autoaggregation of *K. kingae*. The *pilA1* mutant lacks type IV pili, the *ctrA* mutant lacks the polysaccharide capsule, the *knh* mutant lacks the trimeric autotransporter protein Knh, and the $hmw1C_{Kk}$ mutant lacks $HMW1C_{Kk}$. Graphs in panels A and B show data from biological replicates performed in triplicate. Error bars represent standard errors. An asterisk denotes a *P* value of $<0.05$ obtained from a paired *t* test. (C) Western blot of the formic acid-treated outer membrane fraction of wild-type *K. kingae* (lane 1), *K. kingae* with an interrupted $hmw1C_{Kk}$ gene (lane 2), and *K. kingae* with the interrupted $hmw1C_{Kk}$ gene reverted to the wild type (lane 3) using an antiserum raised against the Knh protein. (D) Qualitative adherence assay using *E. coli* strain BL21 expressing $HMW1C_{Kk}$ (frame 1), Knh (frame 2), or $HMW1C_{Kk}$ and Knh (frame 3).

downstream gene encoding a putative low-molecular-weight protein tyrosine phosphatase. The fact that the flanking genes do not encode proteins that are targets of glycosylation or are involved in glycosylation suggests that any phenotypes observed when the *hmw1C* homologue genes are interrupted are not due to polar effects. The *K. kingae* and *A. aphrophilus* HMW1C homologues are referred to here as $HMW1C_{Kk}$, encoded by $hmw1C_{Kk}$, and $HMW1C_{Aa}$, encoded by $hmw1C_{Aa}$.

Previous work identified residues in HMW1C that are needed to bind UDP-hexose, including lysine 467, asparagine 547, and aspartic acid 551. Aligning HMW1C to $HMW1C_{Kk}$ and $HMW1C_{Aa}$ revealed that these residues are conserved, suggesting that the homologues may be capable of binding and transferring UDP-hexose (Fig. 1).

**$HMW1C_{Kk}$ is required for *K. kingae* adherence to human epithelial cells and autoaggregation.** *K. kingae* derivatives with an interruption of $hmw1C_{Kk}$ or reversion of the interrupted $hmw1C_{Kk}$ gene were compared with the wild-type strain for their ability to adhere to cultured human epithelial cells. The interruption of $hmw1C_{Kk}$ resulted in a marked reduction in adherence (Fig. 2). Wild-type levels of adherence were restored when the interrupted $hmw1C_{Kk}$ was reverted to the wild-type sequence (Fig. 2A).

A number of bacterial species are capable of autoaggregation (adherence of bacteria to themselves). To assess whether *K. kingae* autoaggregates, we performed tube settling assays, measuring optical density at 600 nm ($OD_{600}$) over time. We found that *K. kingae* is capable of autoaggregation when the polysaccharide capsule (CtrA) and type IV pili (PilA1) are lacking, suggesting that these two surface factors mask one or more other surface factors responsible for autoaggregation. Interruption of $hmw1C_{Kk}$ in a capsule-deficient, type IV pilus-deficient mutant abolished autoaggregation, and reversion of the interrupted $hmw1C_{Kk}$ gene reversed this phenotype. Interestingly, elimination of the trimeric autotransporter Knh by interruption of the *knh* gene abolished the ability of *K. kingae* to autoaggregate, suggesting that Knh may be a substrate of $HMW1C_{Kk}$. These results are illustrated in Fig. 2B, where it can be seen that the curves for the wild type and the *pilA1*, the *ctrA*, the *pilA1 ctrA knh*, and the *pilA1 ctrA* $hmw1C_{Kk}$ mutants overlap and the curves for the *pilA1 ctrA* and *pilA1 ctrA* $hmw1C_{Kk}(hmw1C_{Kk})$ mutants are indistinguishable.

**$HMW1C_{Kk}$ glycosylates the trimeric autotransporter Knh.** In previous work on *K. kingae*, we established that type IV pili and the trimeric autotransporter Knh are responsible for mediating bacterial adherence to human epithelial cells (16, 17). In an effort to determine the mechanism by which interruption of $hmw1C_{Kk}$ interferes with *K. kingae* adherence, we examined the PilA1 major subunit of type IV pili and the Knh protein in the wild-type, mu-

**TABLE 1** Glycopeptides identified with single hexose modification in Knh

| Modified sequence[a] | Asparagine position(s) | Calculated *m/z* |
|---|---|---|
| STDGKPNTTNTTDADINK | 218 | 685.6502 |
| STDGKPNTTNTTDADINK | 218, 221 | 739.6678 |
| FIAGDNLNLTQTGSNFTYSLNK | 603, 610 | 2,741.2953 |
| DGINAGNATISNVK | 640, 643 | 930.4338 |
| DTLTPNGDPSNVGNPVTK | 665 | 1,986.9384 |
| DTLTPNGDPSNVGNPVTK | 670 | 1,986.9376 |
| DGSGSPVNASNVTSQISAIK | 785, 788 | 1,128.54 |
| GVDNVTLLTNGTTTTVTNK | 801, 807 | 1,056.039 |
| VNVTTTPTNTPDANNVTINEAGK | 844, 845 | 899.0979 |
| KVNVTTTPTNTPDANNVTINEAGK | 845, 849 | 941.7963 |
| VVAPTNSTVAGNTFLTAK | 859, 865 | 705.695 |
| NINITQTGSTITVATK | 912, 914 | 1,985.0025 |
| DNVTFNNVNTTTMTVGSKPDNAVNFK | 929 | 3,151.4489 |
| TNAPTTALNITSADGK | 967, 974 | 949.9575 |
| GTLENTTSVANADGKGNAATGIGTEVTK | 1171 | 2,838.3720 |
| SNVSYNVAVDNK | 1267 | 898.402 |
| TTTLDVSTEPMTANNNTPAGK | 1329, 1330 | 2,486.1227 |
| AQGENASVVNPGGTVDMK | 1371, 1376 | 1,049.478 |
| INVSTNTTTGANIYDVSVNTGK | 1497 | 865.0836 |
| INVSTNTTTGANIYDVSVNTGK | 1501 | 865.0836 |
| SLTINSTTGAIDVK | 1522 | 1,742.8663 |
| GDLTVAGNTTVK | 1638 | 669.3434 |
| NFTVNPNSTVNMGGNK | 1649 | 936.4256 |

[a] Underlined residues represent hexosylated sites.

tant, and revertant strains by Western analysis. There was no difference in mobility of the PilA1 protein (data not shown). However, Western analysis using Knh antiserum demonstrated that Knh migrated at a lower molecular mass and was less abundant in the *hmw1C*$_{Kk}$ mutant than in the wild-type strain (Fig. 2C). Both species of Knh were excised from a Coomassie blue-stained gel of outer membrane fractions from *K. kingae* and *K. kingae* lacking HMW1C$_{Kk}$ and were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a hybrid LTQ Orbitrap Elite mass spectrometer.

We obtained 57% coverage of the Knh protein from the wild-type strain. With this coverage, we identified 32 sites of glycosylation (Table 1). All 32 of these sites were modified with at least a single hexose, and four of the 32 sites were modified with either a single hexose or a dihexose (Table 2). Potential sites of glycosylation were sorted by comparing the expected and observed *m/z* ratios. Sites for which the expected and observed *m/z* ratios were within 1 ppm of each other were considered true sites of modification. All hexose modifications were on asparagine residues, though only 81% of these sites were in the recognized Asn-Xaa-Ser/Thr consensus sequence for N-linked glycosylation. In contrast, there was no evidence of glycosylation of the Knh protein from the *hmw1C*$_{Kk}$ mutant (for which we obtained 50% coverage).

For samples from the wild-type and *hmw1C*$_{Kk}$ mutant strains, control database searching for O-linked glycosylation on serine or threonine residues showed nothing above the false discovery rate.

**TABLE 2** Glycopeptides identified with dihexose modification in Knh

| Modified sequence[a] | Asparagine position | Calculated *m/z* |
|---|---|---|
| SNVSYNVAVDNK | 1267 | 979.4284 |
| AQGENASVVNPGGTVDMK | 1371 | 705.3196 |
| GDLTVAGNTTVK | 1638 | 750.3698 |
| NFTVNPNSTVNMGGNK | 1649 | 1,017.452 |

[a] Underlined residues represent dihexosylated sites.

**Coexpression of Knh and HMW1C$_{Kk}$ in *E. coli* is sufficient to facilitate bacterial adherence to epithelial cells.** To further demonstrate the dependence of Knh function on glycosylation, we expressed both Knh and HMW1C$_{Kk}$ in the nonadherent *E. coli* strain BL21. When Knh or HMW1C$_{Kk}$ was expressed alone, the bacteria remained nonadherent (Fig. 2D). However, when Knh and HMW1C$_{Kk}$ were coexpressed, the bacteria were capable adhering to human epithelial cells, establishing that Knh is capable of facilitating adherence only when glycosylated.

**Mutating *hmw1C*$_{Kk}$ decreases the amount of Knh on the bacterial cell surface.** In an effort to assess whether HMW1C$_{Kk}$ influences surface structures on *K. kingae*, we immunogold labeled Knh on the the wild-type and *hmw1C*$_{Kk}$ mutant strains and examined them by transmission electron microscopy. In order to avoid interference by type IV pili, we used strains lacking type IV pili. As shown in Fig. 3, we saw labeling on the surface of the type IV pili mutant (Fig. 3A) that disappeared when the *knh* gene was interrupted (Fig. 3B). Labeling was less abundant in the *hmw1C*$_{Kk}$ mutant strain (Fig. 3C) and became more abundant when *hmw1C*$_{Kk}$ was reverted to the wild type (Fig. 3D). The average number of gold particles on the type IV pili mutant strain was 19.1. When the *knh* gene was interrupted, there was an average of 1.1 particles per cell; when *hmw1C*$_{Kk}$ was interrupted, there was an average of 6.7 particles per cell; and when *hmw1C*$_{Kk}$ was reverted to the wild type, there was an average of 11.1 particles per cell. Bacteria were also examined without labeling, as Knh fibers can be detected on the surface. Observations made in this way corresponded with the immunogold-labeling experiments, as fewer fibers were seen on the surface of the *hmw1C*$_{Kk}$ mutant (data not shown).

**HMW1C$_{Aa}$ is necessary for *A. aphrophilus* autoaggregation and adherence to human epithelial cells.** To expand our knowledge of species that use HMW1C-mediated glycosylation, the

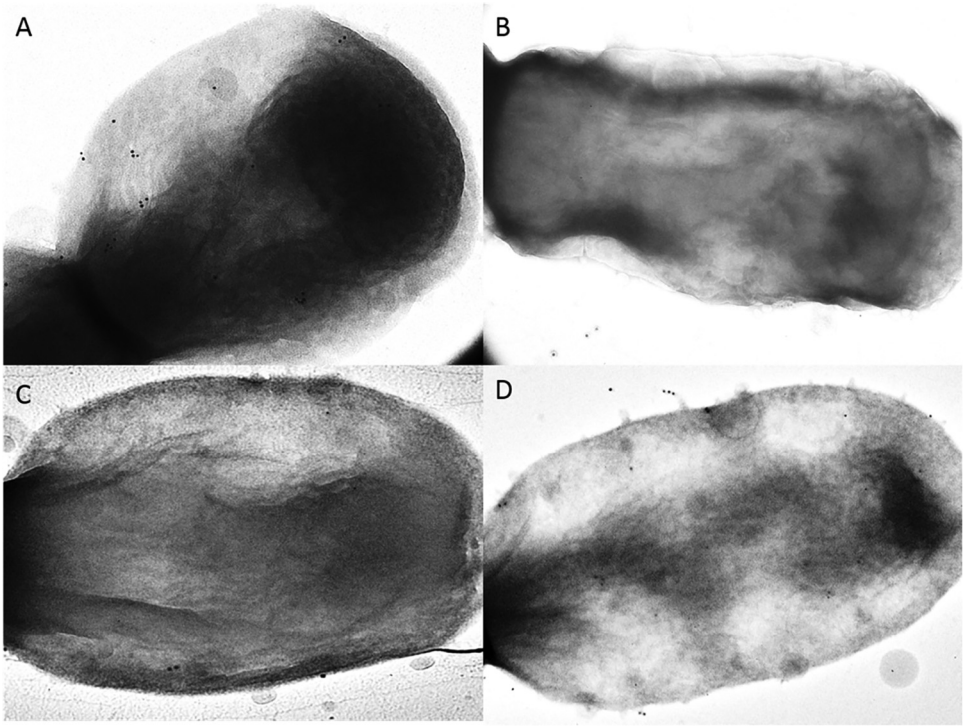


**FIG 3** HMW1C$_{Kk}$ is required for Knh expression on the surface of *K. kingae*. Images show immunogold-labeled Knh on the surface of *K. kingae* Δ*pilA1* Δ*ctrA* (A), *K. kingae knh*::*kan* (B), *K. kingae hmw1C*$_{Kk}$::*kan* (C), and *K. kingae hmw1C*$_{Kk}$::*kan* (*hmw1C*$_{Kk}$) (D). All strains lacked polysaccharide capsule and type IV pili.

*hmw1C*$_{Aa}$ gene in *A. aphrophilus* was interrupted, and adherence by this strain was compared with adherence by the wild-type strain. The interruption of *hmw1C*$_{Aa}$ resulted in a statistically significant reduction in adherence (Fig. 4A). Wild-type levels of adherence were restored when the interrupted *hmw1C*$_{Aa}$ was reverted to the wild-type sequence (Fig. 4A).

Experiments with *A. aphrophilus* have demonstrated that this species autoaggregates rapidly in liquid culture in the absence of agitation (18). To determine if interruption of *hmw1C*$_{Aa}$ affects the ability of *A. aphrophilus* to autoaggregate, the OD$_{600}$ was measured over the course of 8 h for isogenic strains containing the wild-type, interrupted, and reverted forms of the *hmw1C*$_{Aa}$ gene. As shown in Fig. 4, the *hmw1C*$_{Aa}$ mutant initially autoaggregated at a lower rate but was able to attain the same degree of autoaggregation as the wild-type strain over the course of the experiment. Reversion of the interrupted *hmw1C*$_{Aa}$ to the wild type restored the rate of autoaggregation to wild-type levels (Fig. 4B).

**HMW1C$_{Aa}$ glycosylates the trimeric autotransporter EmaA.** Adherence factors have not been described previously for *A. aphrophilus*. To determine the mechanism by which interruption of *hmw1C*$_{Aa}$ interferes with adherence in this species, we examined outer membrane fractions from isogenic strains of *A. aphrophilus* with wild-type, interrupted, or reverted *hmw1C*$_{Aa}$ on a Coomassie blue-stained SDS-PAGE gel (Fig. 4C). We noticed a band at approximately 180 kDa that had increased mobility in the strain with an interrupted *hmw1C*$_{Aa}$ and a band larger than 300 kDa that disappeared in the strain with an interrupted *hmw1C*$_{Aa}$ (Fig. 4C). These bands were excised and analyzed by LC-MS/MS on a hybrid LTQ Orbitrap Elite mass spectrometer.

In the samples from *A. aphrophilus*, both the 180-kDa band and the >300-kDa band were identified as the trimeric autotransporter protein EmaA, with the 180-kDa band corresponding to the monomer form and the >300-kDa band likely representing a multimer. Coverage of 82% of the EmaA sequence in the sample from wild-type *A. aphrophilus* revealed six sites of glycosylation with a single hexose (Table 3). All six of the identified sites were asparagine residues and were in the Asn-Xaa-Ser/Thr consensus sequence for N-linked glycosylation. We obtained 23% coverage of the EmaA protein from the strain with an interrupted *hmw1C*$_{Aa}$ gene and detected no modifications. Peptides obtained from the mutant corresponded to the peptides that were modified in the wild-type sample.

**EmaA facilitates autoaggregation and bacterial adherence to host cells.** The identification of EmaA as the substrate of HMW1C$_{Aa}$ and the phenotypes demonstrated by an *hmw1C*$_{Aa}$ mutant suggested that EmaA is involved in *A. aphrophilus* adherence to human epithelial cells and autoaggregation. To determine if EmaA does indeed mediate these functions, we insertionally inactivated the *emaA* gene with a kanamycin resistance cassette. The resulting mutant was unable to adhere to human epithelial cells or autoaggreate (Fig. 5). The dependence of bacterial adherence and autoaggreation on EmaA was confirmed by reverting the *emaA* gene to the wild type by removing the kanamycin resistance cassette (Fig. 5).

## DISCUSSION

In this study, we describe two homologues of the *H. influenzae* HMW1C protein that are functional glycosyltransferases. Unlike HMW1C, HMW1C$_{Kk}$ in *K. kingae* and HMW1C$_{Aa}$ in *A. aphrophilus* are not encoded in the same genetic locus as a two-partner secretion system. Rather, these enzymes modify type V secretion proteins encoded by genes at distant locations in the genome. The

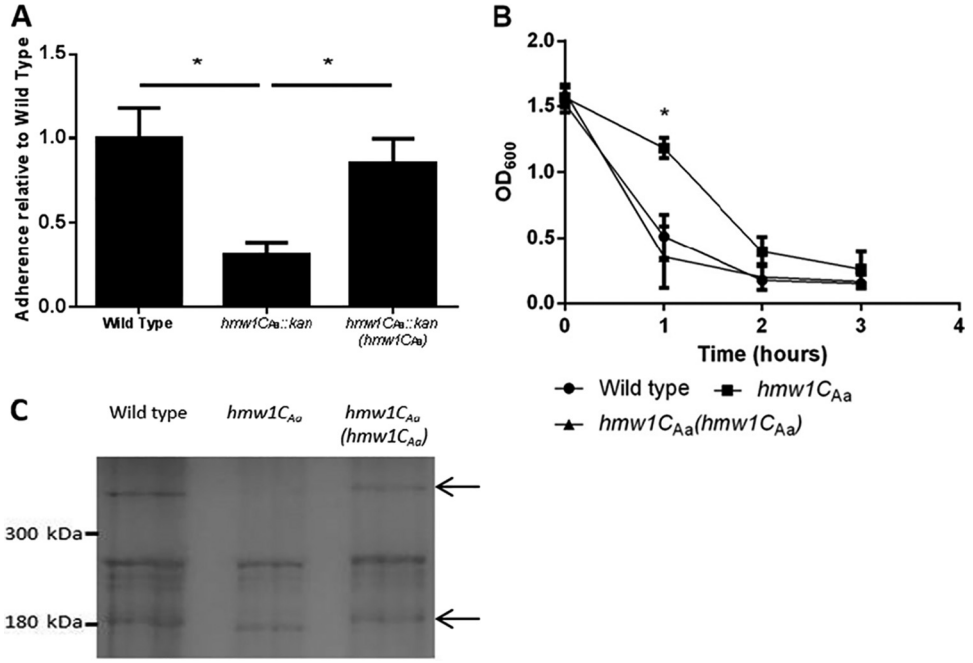


**FIG 4** HMW1C$_{Aa}$ expression is required for *A. aphrophilus* adherence and autoaggregation. (A) Mean adherence of *A. aphrophilus* with the wild-type *hmw1C*$_{Aa}$ gene, an interrupted *hmw1C*$_{Aa}$ gene, and the interrupted *hmw1C*$_{Aa}$ gene reverted to the wild type. (B) Mean autoaggregation of *A. aphrophilus* with the wild-type *hmw1C*$_{Aa}$ gene, an interrupted *hmw1C*$_{Aa}$ gene, and the interrupted *hmw1C*$_{Aa}$ gene reverted to the wild type. The graphs in panels A and B show data from biological replicates performed in triplicate. Error bars represent standard errors. An asterisk denotes a *P* value of $<0.05$ obtained from a paired *t* test. (C) Commassie blue-stained 7.5% SDS-PAGE gel of the outer membrane fractions of *A. aphrophilus* with the wild-type *hmw1C*$_{Aa}$ gene, an interrupted *hmw1C*$_{Aa}$ gene, and the interrupted *hmw1C*$_{Aa}$ gene reverted to the wild type.

acceptor protein for HMW1C$_{Kk}$ was identified as the trimeric autotransporter protein Knh, and the acceptor protein for HMW1C$_{Aa}$ was found to be EmaA, also a trimeric autotransporter.

Autotransporters are a family of adhesive proteins characterized by a C-terminal domain that forms an outer membrane beta-barrel pore and facilitates surface localization of the N-terminal passenger domain of the protein. A number of autotransporters are modified by O-glycosylation, including Ag43, AIDA-I, and TibA of *E. coli*. Glycosylation of these proteins affects stability (AIDA-I and TibA), oligomerization (TibA), and adhesive activity (AIDA-I and TibA) (19–21). In the case of trimeric autotransporters, the protein must trimerize in order to form a fully functional pore. To date all known trimeric autotransporters have adhesive properties, and only one has been identified as being glycosylated, namely, the *Aggregatibacter actinomycetemcomitans* EmaA protein (22).

Knh is a trimeric autotransporter adhesin expressed by the pediatric pathogen *Kingella kingae* and has been shown in previous work to be essential for bacterial adherence to human epithelial cells (17). Our data in this study show that the ability of Knh to mediate adherence and autoaggregation is dependent on expression of *hmw1C*$_{Kk}$.

Interestingly, only 81% of the identified modified residues in Knh were in the N-linked glycosylation consensus sequence. This lack of sequence specificity is reminiscent of O-linking glycosyltransferases, for which no glycosylation consensus sequence has been identified, leading to the hypothesis that O-linked glycosylation sites are specified by structural cues (2). It is possible that there is a structural component to the selection of modification sites on Knh. Recent work showed that the HMW1C homologue from *Actinobacillus pleuropneumoniae* is able to modify asparagine residues outside the NXS/T consensus sequence, including NXA and NXV, albeit with low efficiency (23). This variation is much more limited than the sites identified in Knh, which included NXG, NXV, NXF, NXP, and NXN.

Glycosylation can protect against protease activity, stabilize protein structure, and facilitate interaction between a protein and

**TABLE 3** Glycopeptides identified in EmaA

| Peptide sequence[a] | Asparagine position | Calculated *m/z* |
|---|---|---|
| INLNNTLDLGSSGSIK | 687 | 904.96 |
| VSGTSPITVNK | 819 | 632.83 |
| TTNNGVDDYAVSFNGTEAAK | 834 | 1,118.49 |
| AAVAGTPVNGANGTDGKDGVATVQNVVDALNK | 907 | 1,062.53 |
| VSLGGDNGNTTEK | 1140 | 727.33 |
| DGANASITVAQGK | 1592 | 697.34 |

[a] Underlined residues represent hexosylated sites.

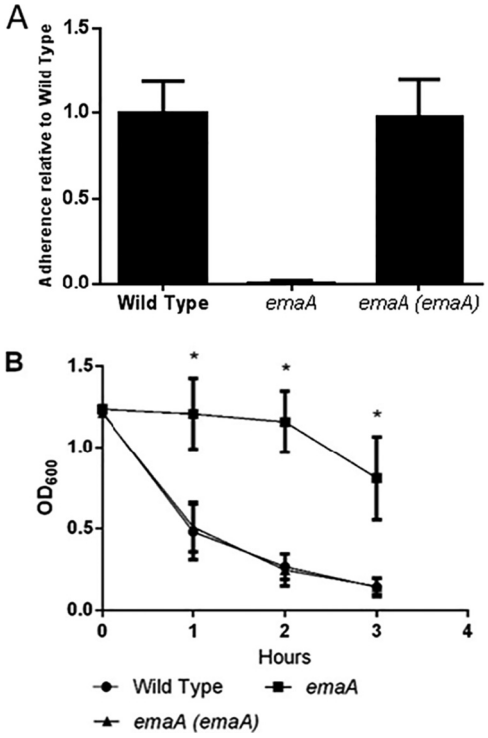


**FIG 5** EmaA is an adhesin and involved in autoaggregation. (A) Mean adherence to human epithelial cells by *A. aphrophilus* with the wild-type *emaA* gene, an interrupted *emaA* gene, and the interrupted *emaA* gene reverted to the wild type. (B) Mean autoaggregation of *A. aphrophilus* with the wild-type *emaA* gene, an interrupted *emaA* gene, and the interrupted *emaA* gene reverted to the wild type. Each graph shows data from biological replicates performed in triplicate. Error bars represent standard errors. An asterisk denotes a *P* value of $<0.05$ obtained from a paired *t* test.

a binding partner. Any of these mechanisms could explain the phenotypes that we observed when Knh was not glycosylated. The Western blot of outer membrane fractions revealed that Knh was present in the outer membrane of the strain with the interrupted *hmw1C*$_{Kk}$ gene, albeit at reduced levels. To further investigate the relationship between the amount of Knh on the bacterial surface and glycosylation, we used transmission electron microscopy. We observed surface fibers on wild-type *K. kingae* and significantly fewer fibers on a derivative with an interrupted *hmw1C*$_{Kk}$ gene. When the *knh* gene was deleted, we observed no surface fibers. This result is consistent with the observation that the *hmw1C*$_{Kk}$ mutant is deficient in adherence and autoaggregation, similar to a *knh* mutant (17). The observed decrease of Knh on the surface of the *hmw1C*$_{Kk}$ mutant could be a result of either a decrease in efficiency in trafficking to the outer membrane or a change in protein stability.

The *A. aphrophilus* EmaA protein is also a trimeric autotransporter adhesin. The contribution of EmaA to bacterial adherence and/or autoaggregation had not been characterized prior to this study. By interrupting the *emaA* gene, we showed that both adherence to human epithelial cells and autoaggregation are dependent on expression of EmaA. In the presence of HMW1C$_{Aa}$, EmaA is modified with a monosaccharide at at least six sites. All six of these sites were at the NXS/T N-linked glycosylation consensus motif, similar to observations with HMW1C and HMW1 and different from HMW1C$_{Kk}$ and Knh. The strain lacking HMW1C$_{Aa}$ displayed reduced adherence to cultured epithelial cells and delayed autoaggregation. Unlike the strain lacking EmaA, the strain lacking HMW1C$_{Aa}$ was still capable of a low level of adherence to human epithelial cells, suggesting that unmodified EmaA is still functional, though to a lesser extent than glycosylated EmaA.

The EmaA protein in *A. aphrophilus* is 69% identical to the EmaA protein in *Aggregatibacter actinomycetemcomitans*, a protein that has been studied extensively and facilitates adherence to collagen (24). Interestingly, *A. actinomycetemcomitans* EmaA is O-glycosylated in a process involving enzymes used in the biosynthesis of the O-polysaccharide of LPS (23). The proposed modification is a nonasaccharide rather than the monosaccharide that we identified on *A. aphrophilus* EmaA. It is interesting that *A. actinomycetemcomitans* does not contain an HMW1C homologue.

In summary, this work describes the first examples of an HMW1C-like enzyme that glycosylates a protein that is not encoded in the same gene cluster as the enzyme itself and is not a member of a two-partner secretion system. Furthermore, Knh and EmaA are the first examples of trimeric autotransporters that are N-glycosylated. To date, all known targets of HMW1C-facilitated modification are members of type V secretion systems and function as adhesins, with adhesive activity being dependent on HMW1C-mediated glycosylation. As adherence to host tissue is a critical first step in infection, the ability to interrupt HMW1C-mediated glycosylation may have therapeutic potential.

## MATERIALS AND METHODS

**Strains and culture conditions.** The bacterial strains are listed in Table 4. *K. kingae* strains were cultured at 37°C with 5% $CO_2$ on chocolate agar supplemented with 50 μg/ml kanamycin, 1 μg/ml erythromycin, or 0.75 μg/ml chloramphenicol, as needed. *A. aphrophilus* strains were cultured at 37°C with agitation (225 rpm) in tryptic soy broth (TSB) supplemented with NAD (3.5 μg/ml) and lysed horse blood (1:1,000 dilution), using 50 μg/ml kanamycin for selection as necessary.

**Strain construction.** Disruption of *hmw1C*$_{Kk}$ (the *K. kingae hmw1C* homologue) was achieved via random transposon mutagenesis as described earlier and was identified by nucleotide sequencing (16). All other gene disruptions in *K. kingae* and *A. aphrophilus* were generated as described previously (17). Briefly, plasmid-based gene disruption constructs were made in *E. coli*, linearized, and introduced into the appropriate species via natural transformation. Transformants were recovered after plating on medium containing the appropriate antibiotic. Correct localization of the interruption was confirmed by PCR and nucleotide sequencing.

Reversions of gene disruptions were generated by PCR amplifying a wild-type copy of the gene and using the wild-type gene to replace the disrupted copy via natural transformation. Transformants were screened for loss of resistance to the appropriate antibiotic, and sequence integrity was confirmed by nucleotide sequencing.

**Homologue identification.** Homologues of HMW1C were identified by using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the nontypeable *Haemophilus influenzae* HMW1C protein (GenBank no. ADO96126.1) as the query sequence. Results were organized by percent similarity and were assessed for relevance of the host organism to human health. Identity and similarity were determined using the EMBOSS Needle program (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

**Adherence assays.** Quantitative adherence assays were performed as previously described using Chang epithelial cells (ATCC CCL 20.2) (16). Briefly, approximately $6.5 \times 10^6$ CFU of bacteria were inoculated onto a monolayer of epithelial cells in a 24-well tissue culture plate. Bacteria were allowed to adhere to the cells for 30 min. Subsequently, monolayers were rinsed with phosphate-buffered saline (PBS) to remove nonadherent bac-

**TABLE 4** Bacterial strains

| Strain | Description | Reference |
|---|---|---|
| DH5$\alpha$ | F$^-$ $\phi$80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *phoA supE441 thi-1 gyrA96 relA* | |
| 269–492 (KK03) | Clinical isolate, spontaneous spreading, corroding colony variant of 269-492 | 16 |
| KK03 *hmw1C*$_{Kk}$::*kan* | Interruption of *hmw1C*$_{Kk}$ with kanamycin resistance cassette | This study |
| KK03 *hmw1C*$_{Kk}$::*kan*(*hmw1C*$_{Kk}$) | Reversion of interrupted *hmw1C*$_{Kk}$ to wild type | This study |
| KK03 *crtA*::*erm* | Interruption of *ctrA* with erythromycin resistance cassette | This study |
| KK03 *pilA1*::*cm* | Interruption of *pilA1* with chloramphenicol resistance cassette | This study |
| KK03 *ctrA*::*erm pilA1*::*cm* | Interruption of *ctrA* with erythromycin resistance cassette and *pilA1* with chloramphenicol resistance cassette | This study |
| KK03 *ctrA*::*erm pilA1*::*cm hmw1C*$_{Kk}$::*kan* | Interruption of *ctrA* with erythromycin resistance cassette, *pilA1* with chloramphenicol resistance cassette, and *hmw1C*$_{Kk}$ with kanamycin resistance cassette | This study |
| KK03 *ctrA*::*erm pilA1*::*cm knh*::*kan* | Interruption of *ctrA* with erythromycin resistance cassette, *pilA1* with chloramphenicol resistance cassette, and *knh* with kanamycin resistance cassette | 17 |
| KK03 *ctrA*::*erm pilA1*::*cm hmw1C*$_{Kk}$::*kan*(*hmw1C*$_{Kk}$) | Interruption of *ctrA* with erythromycin resistance cassette and *pilA1* with chloramphenicol resistance cassette and reversion of interrupted *hmw1C*$_{Kk}$ to wild type | This study |
| CCUG 11575 | Clinical isolate of *A. aphrophilus* | Culture collection of the University of Gothenburg |
| CCUG 11575 *hmw1C*$_{Aa}$::*kan* | Interruption of *hmw1C*$_{Aa}$ with kanamycin resistance cassette | This study |
| CCUG 11575 *hmw1C*$_{Aa}$::*kan*(*hmw1C*$_{Aa}$) | Reversion of interrupted *hmw1C*$_{Aa}$ to wild type | This study |
| CCUG 11575 *emaA*::*kan* | Interruption of *emaA* with kanamycin resistance cassette | This study |
| CCUG 11575 *emaA*::*kan*(*emaA*) | Reversion of the interrupted *emaA* to wild type | This study |
| *E. coli* BL21 pACYC HMW1C$_{Kk}$ + pBAD | *E. coli* expressing HMW1C$_{Kk}$ | This study |
| *E. coli* BL21 pACYC + pBAD Knh | *E. coli* expressing Knh | This study |
| *E. coli* BL21 pACYC HMW1C$_{Kk}$ + pBAD Knh | *E. coli* expressing HMW1C$_{Kk}$ and Knh | This study |

teria. Adherent bacteria were released from the monolayer using trypsin-EDTA and were quantitated by plating dilutions on chocolate agar plates. CFU counts of adherent bacteria were compared to CFU counts of the original inoculum to determine the percentage of bacteria that were adherent.

Qualitative adherence assays were performed as quantitative adherence assays with the following modifications: human epithelial cells were seeded onto glass coverslips in a 24-well tissue culture plate; coverslips were removed and stained with Giemsa stain; stained coverslips were observed at a magnfication of ×400 with a light microscope.

For adherence assays with *K. kingae*, human epithelial cells were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer prior to the assay to prevent disruption of the monolayer by the *K. kingae* RTX toxin.

**Autoaggregation assays.** For autoaggregation assays with *K. kingae*, bacteria were grown overnight on chocolate agar at 37°C with 5% $CO_2$. The next day, the bacteria were resuspended in tubes containing brain heart infusion broth to an $OD_{600}$ of 1.0. Tubes were allowed to stand at room temperature, and $OD_{600}$ was measured after 30, 60, 90, 120, and 180 min.

For autoaggregation assays with *A. aphrophilus*, bacteria were grown overnight with agitation in TSB supplemented with NAD and heme. The following day, cultures were removed from agitation and allowed to stand at room temperature, and the $OD_{600}$ was measured hourly for 3 h.

**Statistical analysis.** Statistical analyses were done using the GraphPad Prism 6 software. Values were compared using the unpaired *t* test. *P* values of less than 0.05 were considered statistically significant.

**Isolation of outer membrane proteins.** Bacteria were resuspended in 10 mM HEPES (pH 7.5) and sonicated until the suspension cleared. Suspensions were then centrifuged for 2 min at 21,000 × *g* and 4°C to remove any intact cells. The supernatant was centrifuged again in a Beckman Coulter Optima MAX-TL ultracentrifuge using a TLA-5 rotor for 1 h at 100,000 × *g* and 4°C to pellet membranes. The membrane pellet was treated with 1% Sarkosyl to solubilize the inner membrane fraction, and the solution was centrifuged again for 1 h at 100,000 × *g* and 4°C to pellet the outer membrane fraction.

**Formic acid treatment of outer membrane proteins.** Outer membrane fractions were incubated with 75% formic acid overnight, at 25°C in the dark. Samples were then lyophilized three times to remove the formic acid, and the dried protein was resuspended in 1.5 M Tris (pH 8.8).

**SDS-PAGE.** Samples were boiled with Laemmli SDS-PAGE loading buffer for 5 min and were then loaded and resolved on 7.5% SDS-PAGE gels.

**In-gel digestion.** Coomassie-stained samples were excised from gels, cut into 1-mm cubes, destained with 50% methanol–1.25% acetic acid, reduced with 5 mM dithiothreitol (Thermo), and alkylated with 20 mM iodoacetamide (Sigma). Gel pieces were then washed with 20 mM ammonium bicarbonate (Sigma) and dehydrated with acetonitrile (Fisher). Trypsin (Promega) (5 ng/ml in 20 mM ammonium bicarbonate) was added to the gel pieces, and proteolysis was allowed to proceed overnight at 37°C. Peptides were extracted with 0.3% trifluoroacetic acid (J.T. Baker) and then 50% acetonitrile. Extracts were combined, and the volume was reduced by vacuum centrifugation.

**Mass spectrometry analysis.** Tryptic digests were analyzed by LC-MS/MS on a hybrid LTQ Orbitrap Elite mass spectrometer (Thermo, Fisher Scientific San Jose, CA) coupled with a nanoLC Ultra (Eksigent). Peptides were separated by reverse phase high-performance liquid chromatography (RP-HPLC) on a nanocapillary column (75 $\mu$m [inside diameter] by 15 cm; Reprosil-pur; 3 $\mu$m silica, 120 Å pore diameter; Dr. Maisch, Germany) in a nanoflex chip system (Eksigent). Mobile phase A consisted of 1% methanol (Fisher)–0.1% formic acid (Thermo), and mobile phase B consisted of 1% methanol–0.1% formic acid–80% acetonitrile. Peptides were eluted into the mass spectrometer at 300 nl/min, with each RP-LC run comprising a 90-min gradient from 10 to 25% B in 65 min and 25 to 40% B in 25 min. The mass spectrometer was set to repetitively scan *m*/*z* from 300 to 1,800 ($R$ = 240,000 for LTQ-Orbitrap Elite) followed by data-dependent MS/MS scans on the twenty most

abundant ions, with a minimum signal of 1,500, dynamic exclusion with a repeat count of 1, repeat duration of 30 s, exclusion size of 500, duration of 60 s, isolation width of 2.0, normalized collision energy of 33, and waveform injection and dynamic exclusion enabled. The Fourier transform MS (FTMS) full-scan AGC (automatic gain control) target value was 1e6, and the MSn (multistage mass spectrometry) AGC was 1e4. The FTMS full-scan maximum fill time was 500 ms, and the ion trap MSn fill time was 50 ms; microscans were set at one. FT preview mode, charge state screening, and monoisotopic precursor selection were all enabled, with rejection of unassigned and 1+ charge states.

**Database searching.** The tandem mass spectra were extracted using ProteoWizard (v3.0.5047). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using SEQUEST (Thermo, Fisher Scientific; version 1.0). SEQUEST was set up to search the UniProt *Kingella* sp. complete proteome database (2014-03-06; 10,238 entries) or *Aggregatibacter aphrophilus* complete proteome database (2014-07-01; 2,407 entries) appended with common contaminants, assuming a full tryptic digestion with the possibility of two missed cleavages. SEQUEST was searched with a fragment ion mass tolerance of 1 Da, and a parent ion tolerance of 15 ppm *S*-carbamamidomethyl of cysteine was specified in SEQUEST as a fixed modification. Oxidation of methionine and hexose on asparagine were specified in SEQUEST as variable modifications.

**Immunogold labeling and transmission electron microscopy.** Bacteria were grown overnight on chocolate agar at 37°C and resuspended in PBS to on $OD_{600}$ of 0.8. Samples were then fixed using 1% paraformaldyhde at room temperature for 30 min. Following fixing, samples were washed twice with Tris-buffered saline (TBS). Samples were blocked for 30 min using 2% naive guinea pig serum and 0.1% bovine serum albumin (BSA) in PBS. Following blocking, samples were incubated with guinea pig serum raised against Knh (1:250) for 1 h. Samples were then washed with PBS and incubated with goat polyclonal antibody raised against guinea pig IgG and conjugated to 10-nm gold particles for 1 h. Following washing in PBS, bacteria were resuspended in 0.2 M ammonium acetate. Samples were negatively stained with uranyl acetate, and transmission electron microscopy was performed using a FEI-Technai 12 microscope.

**GenBank Accession number.** The sequence of *hmw1C*$_{Aa}$ has been deposited in GenBank under accession no. KR131724.

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