The *Haemophilus* Cryptic Genospecies Cha Adhesin Has at Least Two Variants That Differ in Host Cell Binding, Bacterial Aggregation, and Biofilm Formation Properties

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The *Haemophilus* cryptic genospecies (HCG) causes genital tract infections in pregnant and postpartum women and respiratory infections in neonates. The major surface adhesin in HCG is called Cha, which mediates bacterial adherence to cultured human epithelial cells. In this study, we report that there are two antigenically distinct variants of Cha, dubbed Cha1 and Cha2. These variants are encoded by the same genetic locus in diverse strains and have nearly identical N-terminal export and C-terminal surface anchoring domains but significantly different internal adhesive domains. Based on the comparison of derivatives of a laboratory strain of *Haemophilus influenzae* expressing either surface-associated Cha1 or surface-associated Cha2, Cha1 mediates a higher level of adherence to cultured human epithelial cells and Cha2 mediates a higher level of adherence to abiotic surfaces. We hypothesize that variation in the Cha1 and Cha2 internal region results in changes in binding specificity or binding affinity and may be associated with adaptation to different host environments during colonization and disease.

Haemophilus is an important cause of genital tract infections in pregnant and postpartum women and of maternally acquired pneumonia and sepsis in newborn infants, resulting in significant morbidity and mortality (1–4). Up to one-half of *Haemophilus* isolates associated with maternal and neonatal infections belong to an unnamed species, currently designated the *Haemophilus* cryptic genospecies, or HCG (5, 6). HCG has several features that distinguish it from *Haemophilus influenzae*, including a distinct outer membrane protein profile, a unique P6 outer membrane protein variant, a specific 16S rRNA gene sequence, and a unique periplasmic Cu-superoxide dismutase (7–9).

Most bacterial diseases begin with colonization of a host epithelial surface. HCG is thought to initiate infection by colonizing the genital tract in women and the respiratory tract in newborn infants, leading to urethritis, vaginitis, chorioamnionitis, and endometritis in pregnant or postpartum women and to pneumonia and sepsis in neonates. Notably, HCG is rarely isolated from the respiratory tract in older children or adults, suggesting a particular predilection for infection of the neonatal respiratory tract.

In previous work on HCG strain 1595, we identified an adhesin called Cha that mediates HCG adherence to respiratory tract and genital tract epithelial cells (10). Cha is a member of the trimeric autotransporter adhesin (TAA) family, a group of surface-localized proteins that are characterized by an N-terminal signal sequence, an internal passenger domain, a C-terminal β -barrel outer membrane anchor domain, and a trimeric architecture (11). TAA proteins transit the inner membrane via the Sec secretion system and undergo cleavage of the N-terminal signal peptide. Subsequently, the short outer membrane anchor trimerizes to create a 12-stranded β -barrel pore that facilitates export of the trimERIC passenger domain across the outer membrane, resulting in attachment of the passenger domain to the membrane anchor on the bacterial surface. Many TAA family members appear to have more than one role in pathogenesis and can be involved in adherence to host cells, the formation of biofilms, and evasion of the immune system (12–15). While the β -barrel domain is the only

conserved portion among the TAA family, all members described to date have adhesive activity and share a repetitive structural domain organization, typically a coiled-coil stalk and a spherical adhesive tip (16). The prototypic TAA adhesive domain is the YadA-like head (Ylh) domain, which forms left-handed β -roll structures and is found in several TAA proteins in different bacterial species (11, 17). A single cluster of repetitive Ylh amino acid motifs forms the globular Ylh domain head of the *Yersinia enterocolitica* YadA adhesin (18) and appears to be responsible for YadA-mediated adherence to collagen, as mutation of conserved amino acids in the Ylh domain eliminates adherence (18, 19). The Cha adhesin of HCG 1595 includes 4 clusters of Ylh motifs spaced throughout the passenger domain, as well as 2 coiled-coil stalks, 4 neck or connector motifs, and a nearly 350-amino-acid stretch with no predicted structure.

We previously determined that the unstructured N-terminal 404 amino acids in the mature Cha passenger domain (amino acids 70 to 473) harbor the binding domain and mediate adherence to at least four different human cell lines. This region of the protein also mediates Cha-Cha interaction that results in bacterial aggregation, an activity associated with other autotransporters (20–23). In addition, the Cha passenger domain contains 28-amino-acid tandem peptide repeats that vary in number within a strain and between strains. While these repeats are not directly responsible for Cha-mediated binding, the number of repeats modulates adherence and aggregation. An increase in repeat number extends the length of the Cha fiber and is inversely correlated with the level of adherence and bacterial aggregation (22).

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TABLE 1 Bacterial strains used in this study

Strain	Description or genotype	Reference or source
<i>Haemophilus influenzae</i> Rd	Nonadherent, capsule-deficient serotype d laboratory strain	41
<i>H. influenzae</i> Rd-derived strains		
Rd1	Strain Rd expressing chromosomally encoded Cha1 protein in <i>hap</i> locus, Cha construct lacks repeat region and amino acids 1138 to 1297	22
Rd2	Like strain Rd1, except Cha construct lacks amino acids 1094 to 1297	22
Rd3	Like strain Rd1, except Cha construct lacks amino acids 804 to 1297	22
Rd4	Like strain Rd1, except Cha construct lacks amino acids 474 to 1297	22
Rd5	Like strain Rd1, except Cha construct lacks amino acids 382 to 1297	22
Rd/Cha1/1-473F	Like strain Rd4, except Cha construct has a FLAG tag fused to the C-terminal end of the passenger domain	
Rd/Cha2	Strain Rd expressing chromosomally encoded Cha2 protein in <i>hap</i> locus, Cha2 construct lacks repeat region and amino acids 807 to 1003 and has a FLAG epitope fused to the C-terminal end of the passenger domain	This work
Rd/Cha2/1-426F	Like strain Rd/Cha2, except Cha2 construct lacks amino acids 427 to 1003	This work
Rd/Cha2/1-402F	Like strain Rd/Cha2, except Cha2 construct lacks amino acids 403 to 1003	This work
<i>Haemophilus cryptic</i> genospecies-derived strains		
420	Neonatal blood isolate, contains untranscribed <i>cha-1</i>	J. Musser
421	Neonatal blood isolate, contains <i>cha-1</i>	6
422	Neonatal blood isolate, contains <i>cha-1</i> with mixed repeat length	6
427	Amniotic fluid isolate, contains untranscribed <i>cha-1</i>	6
1595	Neonatal blood isolate, contains <i>cha-1</i> with mixed repeat length	5
1595-A9	Derivative of strain 1595, contains <i>cha-1</i> with zero repeats	22
1595 <i>cha-1</i> ::Tn	Nonadherent transposon mutant of 1595 with insertional inactivation of <i>cha-1</i>	10
1610	Neonatal blood isolate, contains <i>cha-2</i> , ~60 repeats	5
1610 <i>cha-2</i> ::Tn	Nonadherent transposon mutant of 1610 with insertional inactivation of <i>cha-2</i>	This study
1673	Neonatal gastric isolate, contains <i>cha-1</i> with mixed repeat length	5
2446	Clinical isolate, contains untranscribed <i>cha-2</i>	J. Musser
2452	Clinical isolate, contains <i>cha-1</i> , ~95 repeats	J. Musser
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE441 thi-1 gyrA96 relA</i>	24

In this study, we determined that there are at least two variants of the Cha adhesin, now designated Cha1 and Cha2. By sequencing adherent clinical isolates expressing a Cha variant not recognized by an antiserum against Cha1, we determined that the Cha2 variant is encoded by a gene at the same locus as the *cha-1* gene and also possesses adhesive activity. Cha1 and Cha2 have nearly identical C-terminal regions but differ at their N termini beyond the signal peptide and have different binding domains. Comparison of the adhesive properties of Cha1 and Cha2 revealed differences

in the magnitudes of adherence to human genital and respiratory epithelial cells *in vitro*, the magnitudes of bacterial aggregation based on tube settling assays, and the magnitudes of biofilm formation based on binding to plastic.

MATERIALS AND METHODS

Plasmid and bacterial strain construction. The strains used in this study are described in Table 1, the plasmids are described in Table 2, and the primers are described in Table 3. DNA ligations, restriction endonuclease

TABLE 2 Plasmids used in this study

Plasmid	Genotype	Description	Reference or source
pXLhap	<i>aph cat hap</i> flanking <i>cha</i> CTD ^a	Cloning vector used for Cha passenger domain expression in the <i>H. influenzae</i> Rd <i>hap</i> locus	22
pFLAG	pXLhap FLAG- <i>cha</i> -CTD	Plasmid for FLAG-tagged Cha passenger domain expression in <i>H. influenzae</i> Rd <i>hap</i> locus	This study
p109FLAG	pFLAG+Cha1 AA1-473	Plasmid for recombination of Cha1 AA1-473-FLAG-CTD into <i>hap</i> locus of Rd	This study
p161	pFLAG+Cha1 AA1-806	Plasmid for recombination of Cha1 AA1-1531-FLAG-CTD into <i>hap</i> locus of Rd	This study
p163	pFLAG+Cha2 AA1-802	Plasmid for recombination of Cha2 AA1-802-FLAG-CTD into <i>hap</i> locus of Rd	This study
p165	pFLAG+Cha2 AA1-426	Plasmid for recombination of Cha2 AA1-426-FLAG-CTD into <i>hap</i> locus of Rd	This study
p166	pFLAG+Cha2 AA1-402	Plasmid for recombination of Cha1 AA1-402-FLAG-CTD into <i>hap</i> locus of Rd	This study

^a CTD, C-terminal domain.

TABLE 3 Oligonucleotides used in this study

Primer	Sequence	Restriction site(s)
M13F	GTAAACGACGGCCAG	
M13R	CAGGAAACAGCTATGAC	
24F	CAATGAAACGAATTTACAAAGCTACTCTATTCTC	
33F	GATTGTATATTTATCATCATTTTATGGC	
17F	GCAGTAGCTGACTCTAAAGCAGAAGCAGTG	
24F	CAATGAAACGAATTTACAAAGCTACTCTATTCTC	
28R	AGGATCCTTTACCAAAATAATGACAAAAAT	BamHI
	ACCGCTC	
69F	AGGATCCGCCTCTTTTACAGATAACTACACT	BamHI
	GAGGGT	
87R	TGCTGCAGTTTGGCATCTGT	
101	GGAATAAACGACCCACGATTGGAAG	
107R	CTACGCGTATCATTTCTACCAACAGAAAC	MluI
	AACCC	
109Cla	CGATCGATTACATGGAAGTACTCTGAGCT	ClaI
	ACTTTCTCC	
118F	GAACAACTCTCCATTGGTATTGGTAATG	
119F	CATTCCAATACCAATGGAGAGTTGTC	
120F	GGAGTCGTTTCAGTAGGTAGTTCTAGTGTTAG	
121F	GAATGGGCTGACAATATTAGATGGAATAAC	
122F	CGTGCTGCATTAAATGAGACAGCG	
126F	GCGCGTGACGTATAGGCTCAGGACG	
127R	GTTGCAGACAATCCGATAACGCAC	
130c	GGATCC-CCGCTGAAAAATCAGCATTGGGAC	BamHI
161R	ATCGATTACCGCATTGACTTGACCAACATT	ClaI
163R	ATCGATTGACGTTTAAATCTGGTTTATCATTTTC	ClaI
165R	ATCGATAGCTGCTACCGCTGTAGTTTGGC	ClaI
166R	ATCGATTGGCGCTAACACTAGAACTA	ClaI
	CCTACTG	
167	GGATCCAAAATCGATATGGACTACAAAGACGATG	BamHI-ClaI-MluI
	ACGACAAGACGCGT	
168	ACGCGTCTTGTGCTCATCGTCTTTGTAGTCCAT	MluI-ClaI-BamHI
	ATCGATTTTGGATCC	

digestions, and gel electrophoresis was performed according to standard techniques (24). In *H. influenzae*, transformation was performed using the MII/MIV method of Herriott et al. (25). In the *Haemophilus* cryptic genospecies, transformation was accomplished by incubating a 500-μl suspension of bacteria in Schaedler broth (Becton, Dickinson and Co., Sparks, MD) with approximately 1 μg of transforming DNA at room temperature for 30 min and then supplementing the suspension with clarified horse blood and 2 μg/ml NAD and incubating for 1 h at 37°C with aeration. Transformation reaction mixtures were plated on chocolate agar containing 2 μg/ml chloramphenicol or 50 μg/ml kanamycin to select for transformants. *Escherichia coli* strains were grown with 50 μg/ml kanamycin or 100 μg/ml ampicillin to select for plasmids.

To sequence the *cha* locus from HCG strain 1610, the coding sequence was amplified using three primer sets (24F/107R, 122F/87R, and 17F/28R) and ligated into pCR-XL-TOPO (Invitrogen). The 24F/107R and 122F/87R primer sets amplified the coding sequence upstream from the 84-nucleotide repeats, and the 17F/28R primer set amplified the sequence 3' to the 84-nucleotide repeats. Subsequently, sequence walking was performed using the following primers: M13F, M13R, 101F, 119F, 118F, 120F, 121F, 122F, 24F, 33F, 108R, and 87R.

To express Cha1 and Cha2 truncations on the surface of *H. influenzae* strain Rd under the control of the native promoter, we generated a cloning vector designated pFLAG that allowed us to fuse fragments of the Cha passenger domain with a FLAG epitope (M-DYDDDDK) between the end of the passenger domain and the beginning of the Cha β-barrel domain. The pFLAG plasmid is based on the pXLhap vector described by Sheets and St. Geme (22), which contains the coding region for the Cha beta-barrel domain and a chloramphenicol resistance cassette surrounded by a flanking sequence derived from the *H. influenzae* hap locus, allowing gene fusions to be recombined into the nonfunctional hap locus in *H. influenzae* Rd. The FLAG tag was created using the 167 and 168 oligomers (see Table 3), which were annealed, digested with BamHI and MluI, and ligated into BamHI-MluI-digested pXLhap. Portions of each *cha* variant gene were amplified using forward primer 130c, which starts with a BamHI restriction overhang and recognizes a promoter sequence that is

identical in the upstream regions of *cha-1* and *cha-2*, combined with one of the following reverse primers with a ClaI overhang: 161R for the Cha1 fragment that ends at amino acid 1521, 109Cla for the Cha1 fragment ending at amino acid 473, 163R for the Cha2 fragment that ends at amino acid 806, 165R for the Cha2 fragment that ends at amino acid 426, and 166R for the Cha2 fragment that ends at amino acid 402 (Table 3). In each case, pFLAG was digested with BamHI and ClaI and ligated with BamHI- and ClaI-digested *cha-1* or *cha-2* PCR-amplified fragments, resulting in constructs that encode a Cha1 or Cha2 passenger domain fragment fused to a FLAG tag and Cha β-barrel domain adjacent to a chloramphenicol resistance cassette, all flanked by a sequence homologous to the *hap* locus. Primers 126F and 127R were then used to amplify the entire fusion construct. The resulting amplified DNA was transformed into *H. influenzae* Rd, and chloramphenicol-resistant transformants were screened by dot immunoblotting with an antibody against the FLAG epitope for surface expression of Cha.

Protein analysis. Outer membrane protein fractions from equal numbers of bacterial cells of the strains (based on OD measurements) of *H. influenzae* Rd were prepared, and the presence of Cha fusions was confirmed by Western blotting as described previously (26). Surface localization of FLAG-tagged Cha protein was qualitatively measured for the presence or absence of protein using whole-cell dot immunoblotting and anti-FLAG antiserum (Sigma) diluted to 1:1,000.

Adherence assays. Quantitative adherence assays were performed essentially as described previously (27). Briefly, 1.8×10^5 tissue culture cells/well were seeded into 24-well plates and incubated for 24 h. Bacteria were resuspended directly from agar plates into brain heart infusion (BHI) to an optical density at 600 nm (OD₆₀₀) of 0.8, and 10-μl volumes were added to cells in triplicate wells. Plates were centrifuged at $165 \times g$ for 5 min and incubated at 37°C and 5% CO₂ for 25 min. Subsequently, monolayers were washed 4 times with phosphate-buffered saline (PBS) to remove nonadherent bacteria, treated briefly with 0.05% trypsin–0.5 mM EDTA (Gibco), and resuspended in BHI broth. Aliquots from each well were then diluted and plated on chocolate agar to enumerate adherent bacteria.

Aggregation assays. Aggregation (settling) assays were performed as described previously (22). Briefly, bacteria were resuspended directly into BHI broth in borosilicate tubes to an OD₆₀₀ of 0.8, and the tubes were allowed to stand at room temperature. The OD₆₀₀ of these standing cultures was measured every 30 min for a 2.5- to 4-h period.

Biofilm assays. Bacteria were resuspended to an OD₆₀₀ of 0.01 in BHI broth supplemented with hemin and 2 μg/ml NAD for *H. influenzae* or to an OD₆₀₀ of 0.4 in Schaedler broth supplemented with hemin and 2 μg/ml NAD for HCG and then aliquoted into quadruplicate wells of flat-bottom polystyrene 96-well plates (Fisher Scientific). Subsequently, the plates were incubated without shaking at 37°C and 5% CO₂ for 48 to 72 h. Medium was then aspirated out of each well, and the wells were washed with PBS and fixed with 100% methanol. Following a 5-min staining with crystal violet, the wells were washed with deionized water, and the remaining crystal violet was solubilized with 33% acetic acid. The acetic acid mixture was moved to fresh wells, and the absorbance of each well was read at 590 nm in a plate-reading spectrophotometer.

Statistical analyses. Adherence data were analyzed using paired Student's *t* tests in GraphPad Prism version 6.0 software.

Nucleotide sequence accession number. The *cha-2* gene sequence in HCG strain 1610 has been deposited in the GenBank database and assigned accession number [GU171279](#).

RESULTS

HCG strain 1610 produces a variant Cha protein called Cha2. In previous work, Southern analysis demonstrated that the *cha* gene is present in all of the *Haemophilus* cryptic genospecies (HCG) clinical isolates in our strain collection (10). With this information in mind, we examined the correlation between the production of Cha and adherence to human genital and respiratory epithelial

FIG 1 HCG strain 1610 expresses a variant Cha adhesin called Cha2. (A) Whole bacteria from the indicated HCG strains were spotted onto a nitrocellulose membrane and examined by immunoblot assay for surface expression of Cha1. The indicated strains were also tested for adherence to Chang respiratory epithelial cells. Adherence levels below 5% of the inoculum were scored as negative. Strains 420 and 427 contain untranscribed *cha-1* alleles. Strain 2446 contains untranscribed *cha-2*. Strain 1595 *cha-1::Tn* has a transposon insertion in the *cha-1* allele, does not produce Cha1, and is nonadherent. Strain 2452 contains a *cha-1* allele containing ~95 tandem repeat domains, rendering it nonadherent. (B) Domain comparison of Cha in HCG 1595 (Cha1) and the variant Cha in HCG strain 1610 (Cha2). Domain annotations were determined by daTAA algorithm analysis (<http://toolkit.tuebingen.mpg.de/dataa>). The dashed line indicates domains in Cha1 that are not present in Cha2. The bracket indicates the region of greatest diversity between Cha1 and Cha2, and the shaded block indicates the region of greatest homology between Cha1 and Cha2. Amino acid numbers are indicated above and below protein depictions.

cells. As shown in Fig. 1A, by using a dot immunoblot technique and an antiserum raised against the N terminus of Cha from HCG 1595, Cha was detected on the surface of adherent strains 421, 422, 1595, and 1673 but was not detected on the surface of nonadherent strains 420, 427, and 2446. Notably, Cha was not detected in adherent strain 1610 (Fig. 1A).

Sequencing of the *cha* locus in HCG strain 1610 revealed a variant gene that we designated *cha-2*, encoding a distinct Cha protein that we called Cha2. Based on nucleotide sequencing of upstream and downstream DNA, *cha-1* and *cha-2* are located at the same position in the chromosome, flanked upstream by the predicted cytochrome oxidase gene and downstream by two homologous genes of unknown function.

The predicted Cha2 protein in HCG strain 1610 is 1,092 amino acids in length when one 28-amino-acid repeat sequence is included. The 28-amino-acid repeat sequence is rich in alanines, serines, and threonines and is identical in Cha1 and Cha2 (22). While the native Cha2 from strain 1610 contains approximately 60 repeats based on Southern analysis (data not shown), Fig. 1 shows a comparison of Cha1 and Cha2 with a single repeat included for simplicity. The C-terminal 666 amino acids of Cha1 and Cha2 are 98% identical, and the C-terminal 90 amino acids of Cha1 and Cha2 that form the β -barrel anchoring domain are 100% identical. In contrast, the 404-amino-acid binding domain at the N terminus of Cha1 shares only 35% identity and 56% similarity with the corresponding region in Cha2. The majority of this divergent region contains a structurally undefined sequence in both Cha variants and includes a putative head insert motif (HIN2) in Cha2 (28). Both Cha1 and Cha2 contain several clusters of YadA-like head (Yhl) motifs throughout their passenger domains, with 4 clusters in Cha1 and 3 clusters in Cha2. Interestingly, the sequence between the first and second neck/HIM domains of Cha1 is absent in Cha2, suggesting a deletion event via homologous recombination at the repetitive HIM sequences. Sequencing of the *cha* locus in the strains in our collection revealed the *cha-1* allele in strains 420, 421, 422, 427, 2452, and 1673 and

the *cha-2* allele in strain 2446, indicating that *cha-1* may be more common than *cha-2* in HCG isolates (Table 1). While our strain collection is small, there was no obvious correlation between the site of strain isolation and the Cha variant expressed by a given strain. Besides predicted homology to specific structural domains within other TAA, Cha1 and Cha2 are most similar at the amino acid level to the uncharacterized BaaA2 trimeric autotransporter in the Brazilian purpuric fever clone of *H. influenzae* biogroup *aegyptius* (29), with 50% identity and 63% similarity across a large portion of the passenger domains (30).

Cha2 has adhesive activity. To determine if the Cha2 protein in HCG strain 1610 is functional, we disrupted *cha-2* by transforming strain 1610 with DNA from strain 1595*cha-1::Tn*, which has a *solo* transposon cassette that includes a kanamycin resistance gene inserted in the *cha-1* locus (10). As shown by the data in Fig. 2A, the resulting 1610*cha-2::Tn* strain was no longer adherent, suggesting that strain 1610 expresses functional Cha2 on the surface that is not recognized by the antiserum raised against the Cha1 protein from strain 1595.

To address whether Cha1 and Cha2 have the same cellular binding properties, we compared HCG 1595-A9 (Cha1 positive [Cha1⁺]), HCG strain 1610 (Cha2⁺), and HCG strain 1610*cha-2::Tn* (lacking Cha2 expression) in adherence assays with human genital and respiratory epithelial cell lines. As shown by the data in Fig. 2A, Cha2 mediated appreciable adherence to Chang, Detroit 562, HeLa cells, and HecIB cells, similar to Cha1-mediated adherence in assays with Detroit 562 and HeLa cells and significantly lower than the Cha1-mediated adherence in assays with Chang and HecIB cells.

In previous work, we found that Cha1-mediated adherence decreases as the number of repeat sequences increases (22). HCG strain 1595-A9 is a variant that produces Cha1 molecules with no repeat sequences in the passenger domain, while HCG strain 1610 produces Cha2 molecules with approximately 60 tandem repeat sequences in the passenger domain. To eliminate the possible influence of the tandem repeats in our comparison of the cellular

FIG 2 Cha2 is an adhesin that binds to respiratory and cervical epithelial cells but does not mediate aggregation. (A) HCG 1595 clone A9 (produces Cha1 with 0 repeats), wild-type HCG1610 (produces Cha2 with ~60 repeats), and 1610cha-2::Tn (produces no Cha2) were inoculated onto monolayers of Chang conjunctival, Detroit-562 pharyngeal, HeLa cervical, and HEC-1-B endometrial epithelial cells. (B and C) *H. influenzae* Rd derivatives expressing nearly full-length Cha1 and Cha2 passenger domains were used to inoculate Chang respiratory epithelial cells (B) or HeLa cervical epithelial cells (C). In all panels, adherence is expressed as the percentage of the result for the inoculum. Error bars indicate standard deviations. Data shown are representative of three replicate assays. *, $P \leq 0.05$.

binding properties of Cha1 and Cha2, we inserted Cha1 and Cha2 expression constructs into the nonfunctional *hap* locus in the nonadherent laboratory strain *H. influenzae* Rd. We designed these constructs to place a FLAG-tagged Cha passenger domain lacking the repeat region on the surface of the bacterium. The *cha-1* promoter sequence from strain 1595 was used to drive the expression of each construct, as this sequence is identical to the *cha-2* promoter sequence in strain 1610. We confirmed that *cha-1* in Rd/Cha1 and *cha-2* in Rd/Cha2 were transcribed at similar levels (*cha-1* transcripts at 1.31 ± 0.84 -fold [mean $\pm$ standard deviation] versus *cha-2* transcripts at 1.35 ± 0.78 -fold compared to *rpoE* transcripts). Consistent with the observed transcript levels, the amounts of surface-associated Cha1 and Cha2 were similar as assessed by dot immunoblot assays with an antibody against the FLAG epitope (data not shown). As shown by the data in Fig. 2B and C, Rd/Cha1 was significantly more adherent than Rd/Cha2 in assays with Chang cells (Fig. 2B) and HeLa cells (Fig. 2C), suggesting interaction with different receptors or adherence to the same receptor but with different affinities.

Cha2 domains beyond the N-terminal 337 amino acids contribute to adherence. Previous work established that the N-terminal 404 amino acids in the mature Cha1 protein (following

cleavage of the signal sequence) are responsible for Cha1-mediated adherence to epithelial cells (22). To determine if the corresponding N-terminal region of Cha2 is responsible for Cha2-mediated adherence, we generated a series of FLAG-tagged Cha2 passenger domain truncation mutants (Fig. 3A), introduced these constructs into *H. influenzae* Rd, and tested the resulting recombinant strains for the ability to adhere to Chang epithelial cells. In performing these assays, we used a construct encoding the nearly full-length Cha2 passenger domain fused to the FLAG epitope and the Cha β -barrel domain as a control (Rd/Cha2). As shown by the data in Fig. 3B, the signal sequence and the N-terminal 337 amino acids of mature Cha2 (amino acids 65 to 402 of the passenger domain, corresponding to amino acids 70 to 473 in Cha1) were not able to mediate adherence *in vitro*, despite presentation as a trimer on the surface of *H. influenzae* Rd (data not shown). In comparison, the equivalent FLAG-tagged Cha1 fragment ending in amino acid 473 was hyperadherent compared to the full-length passenger domain of Cha1 (Fig. 3B) (22). A slightly larger fragment of the Cha2 passenger domain ending at amino acid 426 was only partially adherent and could not recapitulate the binding seen with Rd/Cha2. These data indicate that Cha2 requires amino

FIG 3 Binding domain in passenger domain is longer in Cha2 than in Cha1. (A) Cha2 domains expressed in *H. influenzae* Rd fused with a FLAG epitope and the Cha membrane anchor are depicted, and numbers represent amino acid positions. (B) FLAG-tagged Cha1 and Cha2 partial passenger domains expressed on the surface of *H. influenzae* Rd were tested for adherence to Chang cells. Error bars represent standard deviations of the means. Data shown are representative of three replicate experiments. *, $P \leq 0.02$.

FIG 4 A high number of tandem repeats do not interfere with Cha1- and Cha2-mediated adherence to plastic. (A) The indicated *H. influenzae* Rd derivatives expressing Cha1 or Cha2 constructs were resuspended in BHI broth to an optical density at 600 nm (OD_{600}) of 0.8 to 1. Bacterial aggregation and settling were assessed by measuring absorbance every 30 min for 3.5 h. (B to E) The indicated HCG strains (B and E) and *H. influenzae* Rd strains (C and D) were incubated in 96-well plates for 48 to 72 h. Wells were washed repeatedly, stained with crystal violet, and washed again, and the remaining stain was solubilized in dilute acetic acid. Plates were then read in a spectrophotometer, reflecting the fact that absorbance is positively correlated with the number of bacteria adherent to each well. Error bars represent standard deviations of absorbance from quadruplicate wells. Each graph is representative of three replicate experiments. *, $P \leq 0.04$.

acids beyond those at positions 65 to 426 to efficiently interact with host cells, thus differing from Cha1.

Cha2 is unable to mediate bacterial aggregation but promotes efficient biofilm formation. Previous work demonstrated that the production of Cha1 on the surface of *H. influenzae* strain Rd is associated with efficient bacterial aggregation, resulting in bacterial settling in tube settling assays (22). To address whether Cha2 mediates bacterial aggregation, we examined Rd/Cha2 by phase-contrast microscopy and in tube settling assays and observed no evidence of bacterial aggregation (Fig. 4A and data not shown).

To extend this result, we examined the abilities of strains 1595, 1595cha-1, 1610, and 1610cha-2 to form biofilms, as assessed by adherence to untreated plastic 96-well plates. As shown by the data in Fig. 4B, strain 1595 adhered at very low levels, only slightly above the levels of adherence by strains 1595cha-1 and 1610cha-2. In contrast, strain 1610 adhered very efficiently.

To eliminate the influence of the tandem repeats on the ability of Cha1 and Cha2 to mediate biofilm formation, we examined Rd/Cha1 and Rd/Cha2. As shown by the data in Fig. 4C, Rd/Cha1 adhered appreciably to 96-well plates but still at lower levels than Rd/Cha2. Examination of Rd producing Cha2 passenger domain truncation mutants (Rd/Cha2/1-402 [with N-terminal amino ac-

ids 1 to 402] and Rd/Cha2/1-426) revealed that Cha2 amino acids 65 to 402 and 65 to 426 mediated moderate adherence to plastic but at levels significantly lower than the adherence by the nearly full-length Cha2 passenger domain (Rd/Cha2), indicating that amino acids between positions 426 and 802 are required for adherence to plastic, analogous to observations with genital and respiratory epithelial cells.

As shown in Fig. 4D, comparison of Cha1 truncation mutants demonstrated that the N-terminal 404 amino acids of the mature Cha1 protein were not sufficient for adherence to plastic, distinct from observations in assays with human genital and respiratory epithelial cells and in tube settling assays. We also tested whether the number of tandem repeats in the passenger domain of Cha1 would have an effect on adherence to plastic. As shown by the data in Fig. 4E, we found that strain 1595 expressing Cha1 with approximately 95 tandem repeats in the passenger domain adhered at a significantly higher density than did strain 1595 expressing Cha1 with no repeats.

DISCUSSION

Colonization is an important first step in the pathogenesis of bacterial disease, and adherence to the host epithelium generally contributes to colonization. In the *Haemophilus* cryptic genospecies,

the Cha TAA is the predominant adhesin in assays with cultured human epithelial cells. In this study, we identified a second Cha variant in a subset of clinical HCG isolates and designated this variant Cha2. Both Cha1 and Cha2 have the conserved C-terminal β -barrel domain that defines the TAA family (31). On the other hand, Cha1 and Cha2 are unique among TAAs in that they both have a motif at the N-terminal end of the passenger domain with no structural similarity to any crystallized protein. Furthermore, the Cha passenger domains appear to have amino acid sequence homology only to each other and to predicted TAA proteins in the genus *Haemophilus*.

Cha1 and Cha2 have the greatest amino acid homology to the uncharacterized BaaA2 TAA in a recently sequenced strain of *H. influenzae* biogroup aegyptius associated with Brazilian purpuric fever (29). Similar to Cha1 and Cha2, BaaA2 contains repetitive amino acid sequences within its passenger domain. Also similar to Cha1 and Cha2, BaaA2 and trimeric autotransporters in *Burkholderia* spp. are encoded in an apparent operon with a conserved gene (Pfam accession number DUF2827) for a predicted cytoplasmic protein with unknown function. It is unlikely that the product of this gene plays an essential role in the production or export of Cha for the following reasons. First, we did not identify this gene product as being required for adherence in our original transposon screen for mutants with reduced adherence. Second, this gene is predicted to make a cytoplasmic protein and is therefore unlikely to help stabilize or interact with Cha following export across the inner membrane or at the bacterial outer membrane. Finally, this gene is absent in *H. influenzae* Rd, which produces functional, surface-associated Cha1 or Cha2 under the control of the *cha* native promoter.

There is precedent for the expression of multiple variants of the same TAA in other organisms. For example, *Moraxella catarrhalis* produces at least three major variants of the UspA TAA. These variants share significant sequence similarity but exhibit differential binding to host proteins (32). However, in contrast to the Cha variants, the UspA variants are encoded at different genetic loci within the same strain of *M. catarrhalis* and thus are potentially expressed by the same organism (33). Another example of TAA variants is found among the pathogenic yersiniae. In particular, *Y. enterocolitica* produces a YadA variant that has lost a 30-amino-acid insert and is unable to mediate bacterial aggregation but promotes binding to collagen I and laminin. In contrast, *Y. pseudotuberculosis* produces a YadA variant that carries the 30-amino-acid insert and binds poorly to collagen I and laminin but promotes bacterial aggregation, biofilm formation, and host cell uptake (34).

In this study, we found that Cha2 exhibited less robust adherence to host cells than did Cha1. In prior work, we showed that organisms expressing the N-terminal portion of mature Cha1 ending in amino acid 473 are hyperadherent to host cells and hyperaggregative compared with organisms expressing the full-length passenger domain. We determined that the N-terminal 402 amino acids of Cha2 form the equivalent of this Cha1 binding domain. However, the Cha2 N terminus does not confer full adherence to *H. influenzae* Rd when heterologously expressed. In addition, while Cha1 mutants with truncations up to amino acid 473 confer aggregation (this work and reference 22), none of the Cha2 constructs promoted the formation of aggregates in our settling assays (Fig. 4A and data not shown). The N-terminal regions of both Cha1 and Cha2 consist of a signal sequence, an unstruc-

tured domain, and a single Ylh domain with 4 Ylh motif repeats in Cha1 and 3 Ylh motif repeats in Cha2. In addition, Cha2 has a poorly conserved predicted head insert (HIN2) sequence that is absent in Cha1, and Cha1 has a neck domain that is absent in Cha2. Therefore, the lack of full adhesive activity in the Cha2 N-terminal region could be due to differences in the unstructured sequence, the decreased number of Ylh motifs in the first Ylh domain, or the shortened length of the amino acid sequence prior to the first neck domain shared by both Cha variants. The structural differences between the N-terminal regions of Cha1 and Cha2 are an ongoing focus of study in our laboratory.

Nontypeable *H. influenzae* forms robust and complex biofilms on host tissue (35) and abiotic surfaces (36), with no clear relationship to TAA proteins based on published information. In this study, we found that Cha was required for adherence to plastic and that Cha2 mediated significantly greater adherence to plastic than did Cha1. The explanation for this difference may be that Cha1 is more efficient than Cha2 at self-association (Fig. 4) (22), causing strains with Cha1 to settle out of suspension and leaving fewer available Cha surfaces for adherence to plastic. This possibility may be especially true for strain Rd/Cha1/1-473, which aggregates and adheres to host cells more efficiently but has reduced adherence to plastic compared to the results for Rd expressing full-length Cha1. This result suggests either that the Cha1 sequence between amino acids 473 and 804 contributes to adherence to an abiotic surface but is dispensable for bacterial aggregation and bacterial adherence to host cells or that more efficient aggregation leaves less ability to form biofilmlike adherence to abiotic surfaces. Interestingly, this hypothesis contradicts the idea that bacterial aggregation promotes biofilm formation in Cha1 (37–39).

In previous work, we demonstrated that strains with a high number of repeats in the Cha1 passenger domain are less adherent to epithelial cells (22). The data in this study indicate that a high number of peptide repeats do not block Cha1-mediated adherence to plastic. We hypothesize that the longer Cha fibers enable more Cha-Cha interaction and potential entanglement at the bacterial cell surface and less adhesive-surface availability for Cha-host interaction. Interestingly, the number of repeats appears to have no effect on adherence to abiotic surfaces.

We have not yet investigated whether HCG is able to secrete exopolysaccharide and form true, complex biofilms on host tissue or an abiotic surface. However, a scan of the HCG 1595 genome in draft form (E. Mardis, A. J. Sheets, and J. W. St. Geme III, unpublished data) reveals two genes that encode predicted sialyltransferase enzymes required for biofilm formation in the nontypeable *H. influenzae* strain 2019 (36). Further study will help to elucidate the differential roles that Cha1 and Cha2 play in adherence to abiotic surfaces and their potential to contribute to the formation of complex biofilms.

Variation in surface proteins is a common mechanism used by bacterial pathogens to colonize their respective niches while evading the host immune system (40). Differences in the ability of Cha variants to adhere to host tissues may help the pathogen to negotiate the line between host colonization, invasion, and recognition and subsequent elimination by the host. An animal model that may be used to test these hypotheses does not yet exist, but the definition of Cha receptors may help us to genetically engineer a suitable model for this human-restricted pathogen.

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