Novel Aggregative Adherence Fimbria Variant of Enteroaggregative Escherichia coli

Jønsson, Rie; Struve, Carsten ; Boisen, Nadia; Mateiu, Ramona Valentina; Santiago, Araceli E.; Jenssen, Håvard; Nataro, James P.; Krogfelt, Karen A.

Published in:
Infection and Immunity

Link to article, DOI:
10.1128/IAI.02820-14

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

IAI Journals.ASM.org

Novel Aggregative Adherence Fimbria Variant of Enteroaggregative *Escherichia coli*

Rie Jønsson,[a,b] Carsten Struve,[a] Nadia Boisen,[a] Ramona Valentina Mateiu,[c] Araceli E. Santiago,[d] Håvard Jenssen,[b] James P. Nataro,[d] Karen A. Krogfelt[a]

Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark[a]; Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark[b]; Center for Electron Nanoscopy, Technical University of Denmark, Kgs Lyngby, Denmark[c]; Department of Pediatrics, University of Virginia School of Medicine, University of Virginia, Charlottesville, Virginia, USA[d]

Enteroaggregative *Escherichia coli* (EAEC) organisms belong to a diarrheagenic pathotype known to cause diarrhea and can be characterized by distinct aggregative adherence (AA) in a stacked-brick pattern to cultured epithelial cells. In this study, we investigated 118 EAEC strains isolated from the stools of Danish adults with traveler's diarrhea. We evaluated the presence of the aggregative adherence fimbriae (AAFs) by a multiplex PCR, targeting the four known major subunit variants as well as their usher-encoding genes. Almost one-half (49/118) of the clinical isolates did not possess any known AAF major fimbrial subunit, despite the presence of other AggR-related loci. Further investigation revealed the presence of an AAF-related gene encoding a yet-uncharacterized adhesin, termed *agg5A*. The sequence of the *agg5DCBA* gene cluster shared fimbrial accessory genes (usher, chaperone, and minor pilin subunit genes) with AAF/III, as well as the signal peptide present in the beginning of the *agg3A* gene. The complete *agg5DCBA* gene cluster from a clinical isolate, EAEC strain C338-14, with the typical stacked-brick binding pattern was cloned, and deletion of the cluster was performed. Transformation to a nonadherent *E. coli* HB101 and complementation of the nonadherent C338-14 mutant with the complete gene cluster restored the AA adhesion. Overall, we found the *agg5A* gene in 12% of the 118 strains isolated from Denmark, suggesting that this novel adhesin represents an important variant.

During the past decades, enteroaggregative *Escherichia coli* (EAEC) has emerged as an important pathogen, causing diarrhea in adults and children in both industrialized and nonindustrialized countries (1–5). Moreover, EAEC has also been linked to diarrheal outbreaks (6, 7) including a recent outbreak of foodborne hemorrhagic colitis in Germany affecting more than 4,000 individuals and resulting in a high case fatality rate (8, 9). Additionally, an EAEC urinary tract infection-related outbreak was reported in Denmark (10). Nevertheless, despite EAEC implications in several clinical scenarios, the molecular epidemiology of this pathogen remains unclear. This is mostly due to the heterogeneity of strains, and even though several virulence genes have been identified in EAEC, none have shown to be present in all strains (11–14), making the recognition of truly virulent strains difficult.

Several reports suggest that the key step in EAEC pathogenesis is the ability of the pathogen to adhere to and colonize the intestinal tract, which in EAEC prototype strains is facilitated by aggregative adherence fimbriae (AAF), followed by heavy biofilm formation (15–18). Four variants of the AAF major structural subunit have been described so far: AggA (AAF/I), AafA (AAF/II), Agg3A (AAF/III), and Agg4A (AAF/IV), all regulated by the transcriptional activator AggR, situated on the EAEC virulence plasmid pAA (19–23). AAFs are distantly related to the Dr family of adhesins, whose biogenesis requires a dedicated periplasmic chaperone, an outer membrane usher protein, and two surface-expressed subunits (a major subunit and a putative cap subunit) (22, 24). AAFs and Dr adhesins display a high level of conservation of the usher and chaperone genes and a sequence divergence of the fimbrial subunit genes (24). Previous studies have shown that approximately one-half of clinical EAEC isolates do not express any of the four known AAF variants, despite the presence of the pAA plasmid and/or other EAEC-specific genes (11, 12). In this study, we investigated 118 Danish EAEC strains for the presence of AAF genes. Sixty-nine strains were found positive for one of the four known major pilin subunits (58%), whereas the gene for the most frequent AAF variant found was *aggA* (21%), followed by *agg4A* (19%), *aafA* (9%), and *agg3A* (9%). Forty-one percent of the strains in the collection were negative for a known major pilin subunit, although 80% of these strains harbored the closely related usher gene for AAF/III and AAF/IV variants. Taken together, these findings strongly suggest the presence of uncharacterized adhesins in these EAEC strains. Accordingly, here we report the characterization of a novel adhesin variant (AAF/V) in EAEC related to the Afa/Dr/AAF family. AAF/V was found in 12% of the clinical isolates, suggesting that this adhesin is prevalent among EAEC strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The 118 EAEC strains were isolated from consecutive fecal samples submitted for routine analysis from patients suffering from traveler's diarrhea illness in Denmark

Received 22 October 2014 Returned for modification 5 January 2015
Accepted 16 January 2015
Accepted manuscript posted online 26 January 2015
Citation Jønsson R, Struve C, Boisen N, Mateiu RV, Santiago AE, Jenssen H, Nataro JP, Krogfelt KA. 2015. Novel aggregative adherence fimbria variant of enteroaggregative *Escherichia coli*. Infect Immun 83:1396–1405. doi:10.1128/IAI.02820-14.
Editor: A. J. Bäumler
Address correspondence to Karen A. Krogfelt, kak@ssi.dk.

doi:10.1128/IAI.02820-14

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
226	Wild-type EAEC expressing AAF/V	27
C338-14	Wild-type EAEC expressing AAF/V	This study
C338-14Δ*agg5ABCD*	C338-14 in which a kanamycin cassette was inserted into the *agg5DCBA* gene cluster; Kan^r	This study
C338-14Δ*aggR*	C338-14 in which a kanamycin cassette was inserted into the *aggR* gene; Kan^r	This study
JM221	Wild-type EAEC expressing AAF/I	23
042	Wild-type EAEC expressing AAF/II	25
55989	Wild-type EAEC expressing AAF/III	21
C1010-00	Wild-type EAEC expressing AAF/IV	22
HB101	Nonfimbriated, noncapsulated *E. coli* K-12 laboratory strain	28
HB101(pDKAAF5)	HB101 transformed with pDKAAF5; Cm^r	This study
HB101(pUKAAF5)	HB101 transformed with pUKAAF5; Cm^r	This study
HB101(pAAF/3)	HB101 transformed with pAAF/3; Cm^r	This study
MG1655	Commensal *E. coli* K-12 strain	38
Plasmids		
pDKAAF5	pACYC184, low-copy-no. vector containing *agg5DCBA* from strain C338-14; Cm^r	This study
pUKAAF5	pACYC184, low-copy-no. vector containing *agg5DCBA* from strain 226; Cm^r	This study
pAAF/3	pACYC184, low-copy-no. vector containing *agg3DCBA* from strain 55989; Cm^r	This study
pKOBEGApra	Thermosensitive plasmid encoding the lambda red recombinase; $Apra^r$	32
pBAD*aggR*	pBAD, expression plasmid encoding the transcriptional activator *aggR*; Amp^r	49

[a] Kan, kanamycin; Cm, chloramphenicol; Apra, apramycin; Amp, ampicillin.

during 2011 to 2013. An *E. coli* strain, C338-14, which was isolated from a 69-year-old woman returning from Tanzania, showed high AA binding to cells and was selected for further characterization. Prototype strains JM221, 042, 55989, and C1010-00, each expressing one of the four AAFs, were used in this study (21, 22, 25, 26). *E. coli* strain 226 was isolated from a 3-year-old female with hemolytic-uremic syndrome (HUS) by the Laboratory of Gastrointestinal Pathogens at the Health Protection Agency in Colindale, United Kingdom, in February 2012; it harbored the Shiga toxin (Stx) type 2c-encoding gene stx_{2c} as well as *aggR* and AggR-regulated genes (27).

Stock cultures were frozen at −80°C in Statens Serum Institut (SSI) Luria broth (LB) containing 10% (vol/vol) glycerol. All strains were grown at 37°C. Chloramphenicol (30 μg ml^{-1}), apramycin (30 μg ml^{-1}), ampicillin (100 μg ml^{-1}), and kanamycin (50 μg ml^{-1}) were added appropriately.

Characterization of EAEC strains by PCR. All primers and their corresponding product sizes are listed in Table 2. DNA templates were obtained by using the DNeasy blood and tissue kit (QIAgen, Inc., Valencia, CA) according to the manufacturer's instructions. Detection of the four AAF-encoding genes, *aggA*, *aafA*, *agg3A*, and *agg4A*, was performed as previously described (12). Singleplex PCR amplifications were performed by using the Maxima Hot Start PCR master mix (2×; Thermo Scientific, Inc., MA, USA) according to the manufacturer's instructions. The singleplex PCR cycles comprised (i) denaturation for 2 min at 94°C, (ii) denaturation for 30 s, (iii) annealing for 30 s at the primer-specific temperature, and (iv) extension for 1.5 min for 55°C with 35 cycles of steps 2 to 4. The final extension was for 10 min at 72°C.

Cloning of the AAF/V gene cluster. The AAF/III and the two AAF/V gene clusters were amplified from *E. coli* strains 55989, C338-14, and 226, respectively, using the Expand Long Template PCR system (Roche Applied Science, Penzberg, Germany). XbaI and SalI restriction sites were introduced at the 5′ ends of the PCR primers (Table 2). The PCR products were digested with the respective restriction enzymes and ligated into the corresponding sites of pACYC184. The constructs were verified by Sanger sequencing (Macrogen, Seoul, South Korea). The plasmid was then transformed into the nonfimbriated laboratory *E. coli* strain HB101 (28).

Construction of an isogenic *agg5DCBA* and *aggR* deletion mutant. The AAF/V cluster (*agg5DCBA*) and the *aggR* gene in strain C338-14 were both deleted by allelic exchange with a PCR-synthesized cassette encoding kanamycin resistance flanked by regions homologous to the regions up- and downstream of the AAF/V cluster and the *aggR* gene, respectively. All primers used are listed in Table 2. The *aggR* cassette was generated by PCR amplification from a previously constructed JM221Δ*aggR* mutant strain using primers UpaggR_F and DwaggR_R (29).

The *agg5DCBA* cassette was generated by a three-step PCR procedure, previously described by Struve et al. (30). At the first step, the kanamycin resistance-encoding gene (*kan*) was generated from pKD4 by use of primers Kn1 and Kn2 (31). Second, from C338-14 chromosomal DNA, a 358-bp region and a 343-bp region flanking the *agg5* gene cluster were PCR amplified by use of primers Upagg5D_F and Upagg5D_R and primers Dwagg5A_F and Dwagg5A_R, respectively. At their 5′ ends, the primers Upagg5D_R and Dwagg5A_F contained 20-bp regions homologous to the extremities of the *kan* gene. In the third step, the flanking regions were added on each side of the *kan* gene by mixing 100 ng of each fragment, followed by PCR amplification using primers Upagg5D_F and Dwagg5A_R. The purified PCR products were transformed into strain C338-14 harboring the thermosensitive plasmid pKOBEGApra, which encodes the lambda Red recombinase (32). Both the *agg5A* and *aggR* mutants were selected on LB agar plates containing kanamycin and incubated overnight at 37°C, and the loss of the pKOBEGApra plasmid was verified by the inability of the mutant to grow on LB agar plates containing apramycin. Successful allelic exchange was verified by PCR analysis using the primers listed in Table 2.

RNA extraction and RT-PCR. Overnight cultures of C338-14, C338-14*aggR*, and C338-14*agg5ABCD* were diluted 1:100 in 20 ml of Dulbecco's modified Eagle medium (DMEM) (1%) supplemented with 0.45% glucose (DMEM-HG). The strains were incubated with shaking at 37°C until an optical density at 600 nm (OD_{600}) of 0.8 was reached. RNA was extracted using the RNase Plus minikit with the addition of an optional on-column digestion in order to remove contaminating DNA using the RNase-Free DNase Set (Qiagen, Inc., Valencia, CA). RNA was quantified using a Qubit RNA BR assay (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of RNA by using random hexamer primers and the Thermoscript reverse transcriptase (RT) enzyme (Invitrogen, Carlsbad, CA, USA) for 10 min at 25°C, 1 h at 50°C, and 5 min at 85°C. PCR was performed to standard procedures with Platinum *Taq*

TABLE 2 Primers used in this study

Use	Primer	Sequence (5′–3′)	Reference or source
AAF multiplex	AGGA	TCTATCTRGGGGGGCTAACGCT	12
		ACCTGTTCCCCATAACCAGACC	
	AAFA	CTACTTTATTATCAAGTGGAGCCGCTA	
		GGAGAGGCCAGAGTGAATCCTG	
	AAFC	ACAGCCTGCGGTCAAAAGC	
		GCTTACGGGTACGAGTTTTACGG	
	AGG3A	CCAGTTATTACAGGGTAACAAGGGAA	
		TTGGTCTGGAATAACAACTTGAACG	
	AGG3/4C	TTCTCAGTTAACTGGACACGCAAT	
		TTAATTGGTTACGCAATCGCAAT	
		TCTGACCAAATGTTATACCTTCAYTATG	
	AGG4A	TGAGTTGTGGGGCTAYCTGGA	
		CACCATAAGCCGCCAAATAAGC	
agg5A detection primer	AGG5A	CATGTTCATTATCTATTAGTTCGCCTCCACCGTACGTCGTCATTA	This study
Cloning primers	AAF3/5_XBA_F	GCGCGCTCTAGAAGCGTGGGCAGAGTCAGATTG	This study
	AAF5_SAL_R	GCGCGCGTCGACTTAATTTAAGCTGAAGAATCCAGTC	
	AAF3_SAL_R	GCGCGCGTCGACTTATGGTAAAACCATAACACCATGGG	
Three-step knockout of *agg5DCBA*	UPAGG5D_F	CGTCAATACGGTGCTCTA	
	UPAGG5D_R	GAAGCAGCTCCAGCCTACACGCTGGTGTTTTTTGGTCT	This study
	DWAGG5A_F	GGACCATGGCTAATTCCCATCAGATGCTAAAGATGGAAAGCT	
	DWAGG5A_R	TTCGTTAGCCAGAGCCTG	
	KN_1	CAGTCATAGCCGAATAGCCT	30
	KN_2	CGGTGCCCTGAATGAACTGC	
aggR knockout construction	UPAGGR_F	TCAAGAATTGTTTTGGTGTTATGC	28
	DWAGGR_R	AAAACAAAACATCGAAAAAGAGA	
rpoA for RT-PCR	RPOA_F	TTGATATCGAGCAAGTGAGTTCG	31
	RPOA_R	GCATCGATGAGAGCAGAATACG	
RT-PCR primer for *agg5A*	AGG5A_100BP_F	AGATGGAAAGCTTGTCATG	This study
	AGG5A_100BP_R	GTGGTTACGGATATTATC	

DNA polymerase (Invitrogen, Carlsbad, CA, USA). The constitutively expressed housekeeping gene *rpoA* was used as a control (33). As negative controls, all samples were tested without addition of reverse transcriptase.

HEp-2 cell adherence assays (stacked-brick AA pattern). Cells from the human larynx cancer-derived epithelial cell line HEp-2 (ATCC CCL-23) were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS) according to the manufacturer's instructions.

For detection of the aggregative adherence pattern (19), cells were grown overnight to 50% confluence in Dulbecco's Eagle's minimal essential medium and 10% fetal calf serum on Nunc Lab-Tek Chamber slides at 37°C. Overnight cultures of EAEC were diluted 100-fold, subcultured in DMEM for 4 h, and washed, and then the cells were infected with 10 μl of bacterial suspension (1×10^8 CFU) for 3 h at 37°C. After incubation, the cells were washed with phosphate-buffered saline (PBS), fixed with 10% (vol/vol) formalin for 10 min, and stained with 0.1% crystal violet (Sigma Chemical Co., St. Louis, MO) for 5 min, followed by washing of the slides. The cells were visualized under light microscopy (Olympus BX61).

For the HEp-2 cell adherence assay, 25 μl of bacterial suspension (2×10^6 bacteria) was added to confluent monolayers in a 24-well plate (Nunc Intermed) and incubated at 37°C in 5% CO_2 for 3 h. Cells were washed 3 times with PBS and incubated with 500 μl of 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS for 30 min at room temperature. The medium was serially diluted and plated on LB agar containing antibiotics when appropriate. To calculate the percentage recovery of adherent bacteria, bacteria were enumerated by colony counts before and after the infection period. The data shown are numbers of cell-associated bacteria relative to the numbers of bacteria recovered.

Biofilm assay. The EAEC biofilm assay was performed as previously described by Sheikh et al. (17) and modified as described in reference 29.

HA of red blood cells by whole bacteria. Hemagglutination (HA) has been shown to correlate with AAF adhesin expression by EAEC strains (19, 20). Fresh erythrocytes were obtained from a human volunteer (A rhesus positive), and the HA assay was performed as previously described (34).

SEM. Strains were grown in LB overnight with shaking at 37°C. The next day, strains were diluted 1:100 in 5 ml DMEM-high glucose and incubated with shaking to reach an OD_{600} of 0.8. A 1-ml sample was washed, fixed in 3% glutaraldehyde in PBS (pH 7.3) for 16 h, and washed 3 times in distilled water, followed by staining with 1% OsO_4 at 4°C for 16 h. The next day, the sample was dehydrated in several steps of ethanol, followed by steps of acetone. Next, the samples were pipetted onto a filter disc with pore sizes of 1 to 1.6 μm and further dried in a CPD300 Leica. After the critical point drying (CPD) step, the filter discs with the samples were coated with platinum in a Cressington 208 HR High Resolution Sputter Coater for 4 s at 80 mA, attached to an aluminum stub with a double-sided C tape, and imaged in an FEI Helios Dual Beam scanning electron microscope (SEM). The images were recorded with the Everhart Thornely detector at 2 keV, spot 2.5, with 20-μm aperture.

Nucleotide sequence and phylogenetic analyses. BLAST searches and comparisons were conducted using the databases of the National Center

TABLE 3 Prevalence of *agg5A* in clinical strains

AAF pilin-encoding gene (AAF type)	% Prevalence (no.) of strains carrying *agg5A* in clinical strains from:			
	Danish strain collection	Mali strain collection (12)		
	Cases ($n = 118$)	Controls ($n = 61$)	Cases ($n = 60$)	Total ($n = 121$)
aggA (AAF/I)	21.1 (25)	18 (11)	35 (21)	26.4 (32)
aafA (AAF/II)	9.32 (11)	4.9 (3)	5 (3)	5 (6)
agg3A (AAF/III)	9.32 (11)	1.6 (1)	8.3 (5)	5 (6)
agg4A (AAF/IV)	18.6 (22)	8.2 (5)	1.6 (1)	5 (6)
agg5A (AAF/V)	11.8 (14)	14.75 (9)	13.3 (8)	14 (17)
None	29.6 (35)[a]	52.45 (32)	36.6 (22)	44.6 (54)

[a] Of the 35 strains, only 3 exhibited the stacked-brick pattern on HEp-2 cells.

for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of Agg3A and Agg5A were analyzed using Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) at default settings. The sequences of Agg5A variants were analyzed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) at default settings. The putative signal sequences were predicted by the SignalP algorithm (http://www.cbs.dtu.dk/services/SignalP/).

Average pairwise nucleotide diversity (π) and the rates of synonymous (*dS*) and nonsynonymous (*dN*) mutations between and within the clades were calculated using the MEGA4 program (35). The analysis of statistical significance was performed using a *Z*-test with the π and *dN*/*dS* values (36).

Statistical analyses. Student's *t* test was used for statistical evaluation, and *P* values of <0.05 were considered statistically significant. Statistical analyses and graphs were performed using GraphPad Prism v6.00 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession number. The sequence of the *agg5A* gene from strain C338-14 was deposited in GenBank under accession number KP202151.

RESULTS

AAF characterization of Danish EAEC isolates. The 118 EAEC strains used in this study were isolated from adult travelers with diarrhea. The isolates were classified as EAEC when positive by PCR for at least one of the following genes; *aggR*, *aaiC*, and/or *aatA*. To determine the presence of the four previously described AAFs among the isolates, a multiplex PCR was employed (12). The results are listed in Table 3. Sixty-nine strains were found to be positive for one of the four known major pilin subunits (58%). The most frequent AAF pilin-encoding gene found was *aggA* (AAF/I), found in 25 strains (21%), followed by *agg4A* (AAF/IV), found in 22 strains (19%), *aafA* (AAF/II), found in 11 strains (9%), and *agg3A* (AAF/III), found in 11 strains (9%). Forty-nine (42%) of the strains in the collection were negative for a major pilin subunit, although 39 (80%) of these harbored the closely related usher genes for AAF/III and AAF/IV variants (*agg3C* and *agg4C*). Thus, 10 (8.4%) of the 118 EAEC strains were negative for any known *aaf* fimbrial gene and accessory genes. Therefore, we hypothesized that these isolates harbored either distant homologues of known AAF alleles or novel AAF pilins.

Adherence properties of the AAF-negative strains. EAEC can be distinguished from other diarrheagenic *E. coli* by the formation of an aggregative "stacked-brick" pattern of adherence (AA) by AAF-mediated adhesion to HEp-2 cells (19). To investigate the adherence properties of the 49 isolates negative for a known AAF pilin subunit, we tested their ability to exhibit the aggregative or stacked-brick pattern on HEp-2 cells. We found that among the 49 strains, 32 exhibited the stacked-brick pattern. The 17 strains that did not exhibit the stacked-brick pattern were not characterized further. The AA pattern of the 32 strains varied, ranging from the typical honeycomb formation as exhibited by the EAEC prototype strain 042 to other AA types (37) (data not shown).

As AAF expression has been shown to promote biofilm formation to abiotic surfaces (17), the isolates were also screened for their ability to form biofilm in microtiter plates. The majority of the isolates were found to produce significantly more biofilm than the commensal *E. coli* strain MG1655 (38) (data not shown), indicating expression of AAFs or other biofilm-promoting factors.

Identification of the *agg5A* gene. Further analysis of the AAF-negative strains revealed that 25 of the 32 isolates exhibiting the stacked-brick pattern were positive for the *agg3C* gene encoding the usher protein of AFF/III. In the prototype AAF/III strain 55989, the *aggR* gene is located downstream of *agg3C*, and the region interspacing the two genes includes *agg3A*, the gene encoding the major pilin subunit of AAF/III (21). Therefore, in an effort to reveal unknown AAF-related sequences, we performed PCR with a forward primer targeting *agg3C* and a reverse primer targeting *aggR* in a selected clinical isolate exhibiting high adhesion, C338-14. Also, this isolate harbors *aggR*, *aatA*, and dispersin genes, as well as the chromosomal gene *aaiC*, suggesting that it carries the complete pAA virulence plasmid as well as the AAI island (39). The PCR-amplified region between *aggR* and *agg3C* from C338-14 was sequenced and was found to encode an uncharacterized AAF, termed here AAF/V. The whole AAF/V cluster was subsequently obtained by PCR using primers located in the start of *agg3D* and *agg5A* and sequenced.

Interestingly, upstream of the *agg5A* gene encoding the major fimbrial subunit of AAF/V, three open reading frames (ORFs) identical to the accessory genes (*agg3D*, *agg3C*, *agg3B*) of AAF/III from prototype EAEC strain 55989 were identified (Fig. 1). Both *agg3A* from strain 55989 and *agg5A* from C338-14 were located 174 bp downstream of *agg3B*. Alignment of the two major fimbria subunits, *agg3A* and *agg5A*, revealed that the first 65 bp were 100% identical whereas the following 451 bp of the sequences differed (Fig. 1). Alignment of the two proteins (including the signal peptides) showed that they shared 32.2% identity. Further analysis revealed that the 65 bp shared were the signal peptide.

Comparison of the *agg5A* variants. BLAST analysis of the *agg5A* gene from C338-14 revealed four other *agg5A* sequences; three previously submitted to NCBI (accession numbers AB513347, AB571097, and AB571098) and one *agg5A* sequence found in a Shiga toxin (Stx) type 2c-producing EAEC strain termed 226 (NCBI Short Read Archive accession number SRA055981) (27). Translation of the five nucleotide sequences showed that several of the unconserved regions had both silent point mutations and altered amino acid composition among the sequences. Alignment of the sequences (Fig. 2) revealed several variable regions with percentage identity ranging from 83% to 100% among the isolates, except for the accessory genes and signal sequence, which showed full conservation.

To test if the amino acid changes in the variants are the results of adaptive mutations/selection, we calculated the ratio of nonsynonymous mutations (*dN*) to synonymous mutations (*dS*). This showed that the rate of nonsynonymous mutations (*dN*) at 0.070 was significantly higher than the rate of synonymous mutations (*dS*) at 0.009

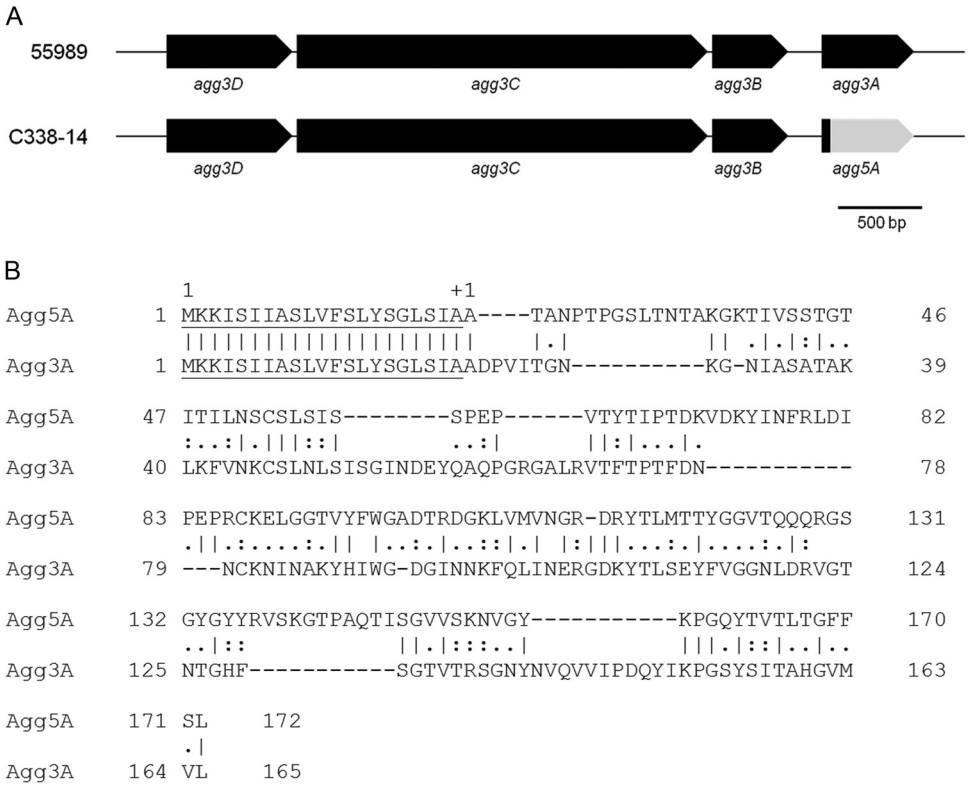

FIG 1 (A) Annotation of the AAF/III and AAF/V biogenesis clusters as determined by nucleotide sequence analysis of the product amplified from strains 55989 and C338-14. Gene designations are from GenBank accession numbers YP002415688 and KP202151. All ORFs encoding >50 predicted amino acids are indicated. (B) Sequence alignment of the two adhesins encoded by *agg3A* and *agg5A*. Gaps, indicated by dashes, have been inserted to optimize the alignment. Asterisks and points represent identical and similar residues, identified with the Needle program. The predicted signal peptide is underlined, and the translational start site is indicated by +1.

($P < 0.05$), indicating that positive selection for adaptive structural changes in *agg5A* seems to occur rather than purifying selection.

The AAF/V gene cluster promotes EAEC-specific phenotypes. Alignment of the Agg5A from strains C338-14 and 226 revealed variations on both nucleotide and protein levels (Fig. 2). Therefore, to assess the phenotypic characteristics of the two AAF/V variants, we cloned the gene cluster from both strains, C338-14(pDKAAF5) and 226(pUKAAF5), into a nonfimbriated

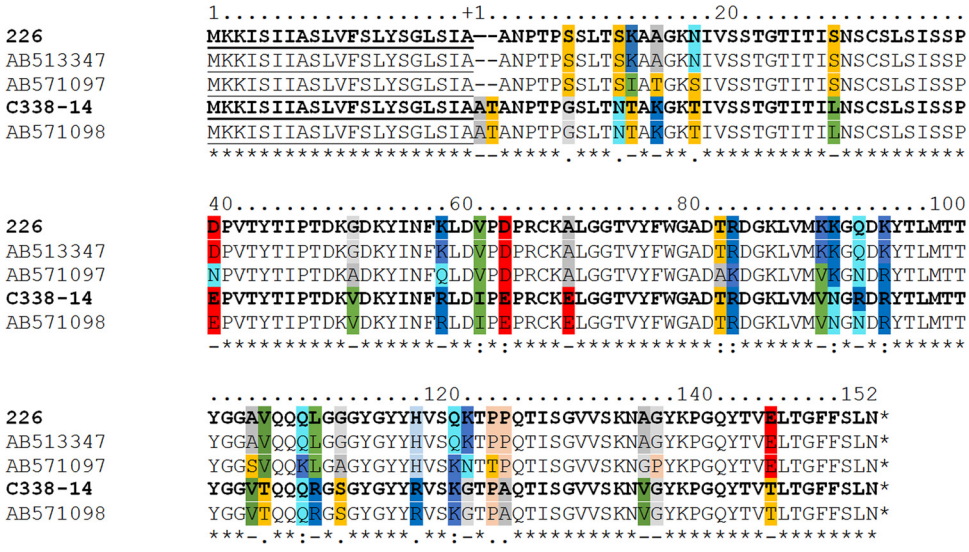

FIG 2 Sequence alignment of the five Agg5A variants with their differences. Gaps, indicated by dashes, have been inserted to optimize the alignment. Asterisks and points represent identical and similar residues, identified with the MAFFT (Multiple Alignment using Fast Fourier Transform) program, and these have been highlighted with colors according to the RasMol amino acid color scheme. Numbers correspond to amino acid positions in the protein encoded by *agg5A*. The predicted signal sequence is underlined, and the predicted translation site is indicated by +1.

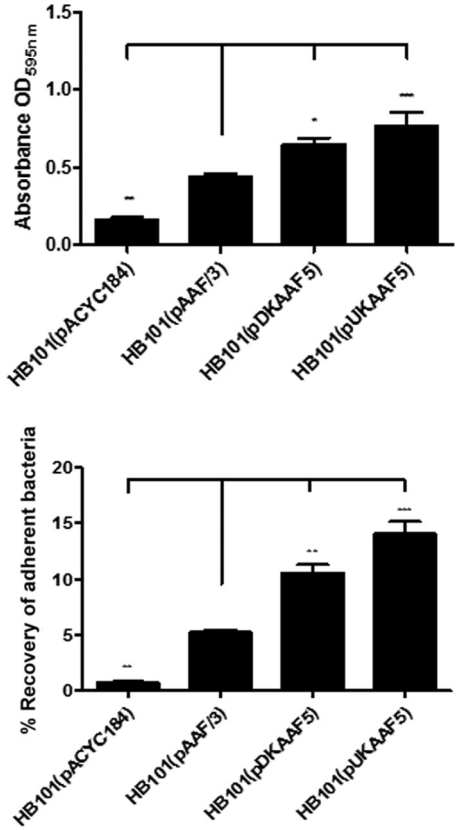

FIG 3 AAF/V-encoding plasmids adhere significantly better than AAF/III. (Top) The AAF/III-encoding plasmid pAAF/3 promotes biofilm in laboratory *E. coli* strain HB101. The two AAF/V variants (pDKAAF5 and pUKAAF5) produce significantly more biofilm than the AAF/III-encoding plasmid. The results are represented as the means ± standard errors of the means for eight replicates and represent one of three independent experiments performed with similar results. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.5$. (Bottom) HEp-2 monolayer cells were infected with HB101 harboring one of the three fimbria-encoding plasmids pDKAAF5, pUKAAF5, and pAAF/3. The number of cell-adhering bacteria was determined 3 h later. The results are presented as the means ± standard errors of the means for at least triplicate samples and represent one of three independent experiments performed with similar results. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.5$.

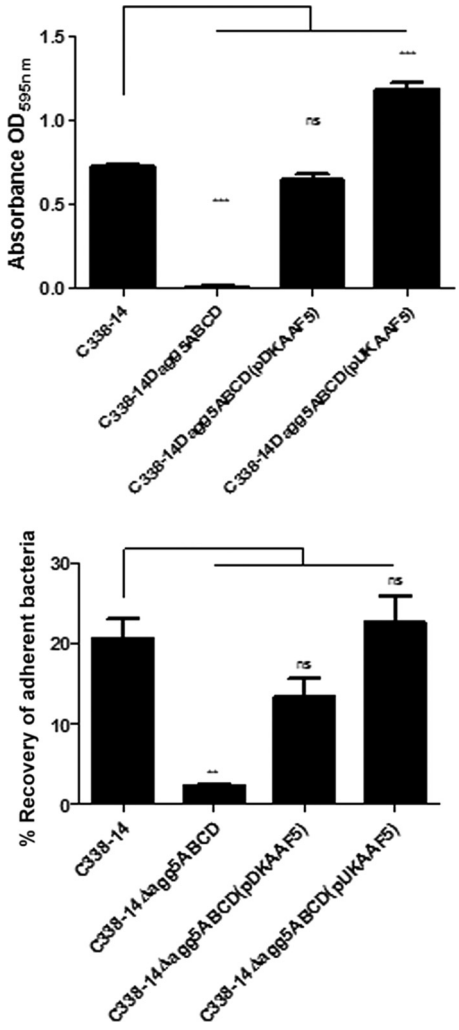

FIG 4 Deletion of the AAF/V cluster attenuates biofilm as well as adhesion to epithelial cells. (Top) C338-14 forms AAF/V-dependent biofilm in microtiter plates. Wild-type (WT) C338-14, the AAF/V mutant, and the strains complemented with pDKAAF5 and pUKAAF5 were grown at 37°C in microtiter plates containing DMEM with 0.45% glucose for 24 h under shaking conditions, after which biofilm formation was quantified as described in Materials and Methods. The results are represented as the means ± standard errors of the means for eight replicates and represent one of three independent experiments performed with similar results. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.5$. (Bottom) HEp-2 monolayers were infected with WT, the AAF/V mutant, and the mutant complemented with pDKAAF5 and pUKAAF5. The numbers of cell-adhering bacteria were determined after 3 h. The results are represented as the means ± standard errors of the means for eight replicates and represent one of three independent experiments performed with similar results. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.5$.

laboratory *E. coli* strain HB101 generating HB101(pDKAAF5) and HB101(pUKAAF5). Furthermore, the AAF/III cluster from the prototype strain 55989(pAAF/3) was also included to compare the phenotypic properties of the two AAF types.

The AAF/V gene clusters from both strains displayed the typical stacked-brick pattern of adherence to HEp-2 cells, as well as biofilm formation on abiotic surfaces (Fig. 3, top). In comparison, the AAF/III-encoding clone showed significantly less biofilm formation than both AAF/V-encoding clones ($P < 0.001$), and the same pattern was also seen for adherence to the HEp-2 cells [$P = 0.05$ for HB101(pDKAAF5) and $P = 0.01$ for HB101(pUKAAF5)] (Fig. 3).

Agg5DCBA is necessary for adherence of EAEC strain C338-14. To investigate if the AAF/V cluster mediates the aggregative phenotype and biofilm formation in C338-14, a Δ*agg5DCBA* mutant of C338-14 was constructed. As expected, deletion of the AAF/V cluster resulted in the significant loss of the AA phenotype to HEp-2 cells ($P = 0.01$) as well as biofilm formation ($P = 0.001$). However, the biofilm formation (Fig. 4, top) and adherence to HEp-2 cells (Fig. 4, bottom) of the C338-14 Δ*agg5DCBA* mutant strain was restored by complementation with plasmid pDKAAF5 encoding its native AAF/V cluster, verifying that AAF/V does confer the aggregative adherence pattern of C338-14.

Interestingly, when we compared the adherent phenotype in the complemented mutant C338-*14agg5ABCD* with the two AAF/V-encoding plasmids (pDKAAF5 and pUKAAF5), the 226 variant produced significantly ($P = 0.001$) more biofilm than the variant from C338-14 (Fig. 4, top).

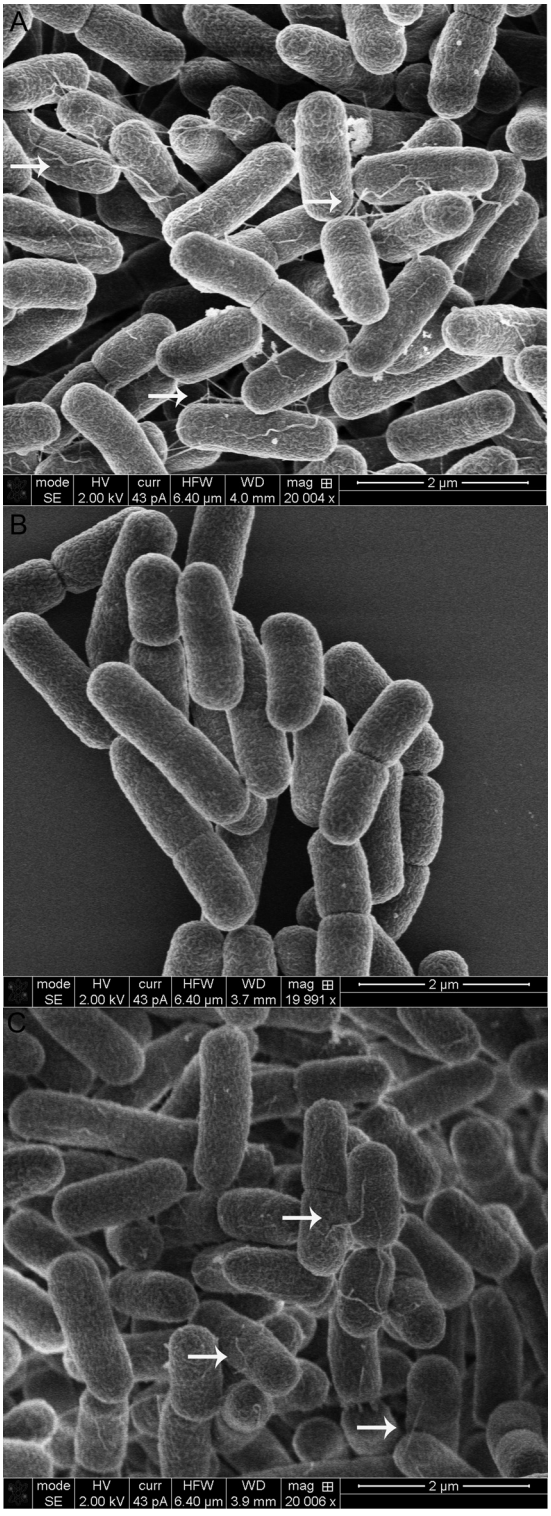

FIG 5 Scanning electron microscopy (SEM) was performed on the following strains; C338-14, C338-14Δ*agg5ABCD*, and the complemented mutant C338-14Δ*agg5ABCD*(pDKAAF5). Overnight cultures of bacteria were inoculated 1:100 in DMEM-HG and grown with shaking until an OD_{600} of 0.8 was reached; then, they were washed, fixed, stained, dehydrated, and examined. (A) Wild-type C338-14 showing fimbriae present; (B) a fimbrial mutant, C338-14Δ*agg5ABCD*; (C) the complemented mutant with its native fimbriae (pDKAAF5) with visible fimbriae similar to those of the wild-type.

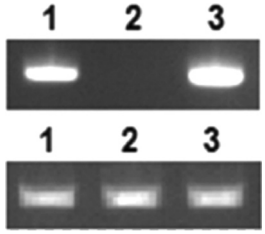

FIG 6 The *agg5A* gene is under the control of AggR. RT-PCR for the *agg5A* transcript was performed. RNA was extracted from EAEC strain C338-14 (lane 1), C338-14Δ*aggR* (lane 2), and C338-14Δ*aggR*(*pBADaggR*) (lane 3) and subjected to reverse transcriptase-PCR and cDNA amplification by PCR for *agg5A* (top) or the constitutive housekeeping gene *rpoA* (bottom).

Phenotypic characterization of AAFV. In order to assess the ultrastructure of the AAF/V fimbriae, we performed scanning electron microscopy (SEM) (Fig. 5). Unlike the afimbriated mutant C338-*14agg5ABCD* (Fig. 5B), SEM of wild-type strain C338-14 revealed the presence of fimbriae in the form of hair-like structures (Fig. 5A). The wild-type fimbriated phenotype was restored when complementing C338-*14agg5ABCD* with its native fimbriae from pDKAAFV (Fig. 5).

Hemagglutination (HA) has previously been shown to correlate with the expression of AAF by several EAEC strains (20). Therefore, the HA profile was also examined for AAF/V, and we observed that like other AAF-producing strains (19), wild-type C338-14 was capable of agglutinating human erythrocytes, whereas the AAF/V mutant failed to do so (data not shown).

AggR is necessary for the transcription of *agg5A*. It is characteristic for AAFs that their expression is promoted by the transcriptional activator AggR (40). To confirm that the *agg5A* gene is regulated by AggR, we deleted the *aggR* gene in C338-14. As expected, the *aggR* mutant lost both the phenotype of aggregation to HEp-2 cells and biofilm formation (data not shown). The effect of AggR on AAF/V expression was also shown at the transcriptional level, as an RT-PCR analysis revealed that the *agg5A* gene was transcribed in the C338-14 wild-type strain, whereas this *agg5A* transcription was undetectable in the *aggR* mutant. The *agg5A* gene transcription was restored when complementing C338-14*aggR* with a plasmid carrying *aggR* (Fig. 6).

Prevalence of the *agg5A* gene. To determine the prevalence of the *agg5A* gene among the 118 Danish EAEC strains, we performed PCR on the AAF-negative strains. Since 32 of the 49 strains exhibited phenotypically a stacked-brick formation, these were the ones tested. Fourteen strains (44%) of the 32 strains tested positive for *agg5A*. Thus, overall, *agg5A* was found in 12% of the 118 EAEC strains (Table 3). In order to investigate if *agg5A* was associated with disease, we tested isolates from a previous EAEC case-control study from Mali (12). In this study, 33 strains from controls (52%) and 22 strains from cases (37.3%) were reported negative for AAF/I-IV. However, we found that 17 strains (9 cases and 8 controls) of the 71 strains were positive for the *agg5A* gene, although overall *agg5A* was not significantly correlated with disease among the EAEC Mali strains ($P = 0.799$) (Table 3).

DISCUSSION

EAEC is a common diarrheal pathogen and has been associated with endemic pediatric diarrhea and implicated in several outbreaks. Yet, apart from those outbreak-associated strains, identi-

fication of true pathogens remains difficult because of the heterogeneity among strains. AAFs are believed to play a key role in EAEC pathogenicity, and since the discovery of AAF/I, three other AAFs have been identified (20–22). Nevertheless, several EAEC strains are negative for any known AAF, suggesting a wide diversity of EAEC adhesive structures, which possibly include uncharacterized nonfimbrial and fimbrial adhesins.

In the search for potential new AAF fimbriae, we investigated 118 EAEC strains isolated from Danish travelers with an AAF multiplex PCR. We found that 58% of the strains were positive for one of the four known AAFs. This is consistent with previous studies that found AAF in one-half of the strain collections studied (11, 12). Among the 49 strains (42%) that were negative for a known AAFs, 39 (80%) of the strains were positive for the usher of AAF/III, suggesting that the strains harbored a variant of AAF/III. The 49 AAF-negative strains in our study were tested for their ability to form the stacked-brick pattern on epithelial cells, and we found that 17 of the 49 strains were not able to form the stacked-brick pattern.

PCR assay with *E coli* clinical isolate C338-14 and BLAST analysis revealed an uncharacterized gene, *agg5A*, whose sequence has been deposited in GenBank (AB571097, AB571098) and which was mentioned in three recent studies (27, 41, 42). However, as of this writing no experimental characterization of this gene is available.

Here, we show that AAF/V is a new adhesin of EAEC that shows a significantly better adherent phenotype than that of AAF/III, with which it shares accessory genes. Our data reveal that like the other four AAFs, AAF/V is required for mediating the aggregative adherence pattern and promotes biofilm production, and its expression requires the activity of the AggR activator, which is a distinct characteristic of AAFs (40). AAF/V was found to be present in 12% of clinical isolates from Danish patients with traveler's diarrhea, suggesting the importance of the fimbrial type. When EAEC strains from a case-control study performed in Mali were tested (12), AAF/V was present in 8 to 9% of strains in both cases and controls, supporting the endemic situation of EAEC in Africa. The facts that AAF/V exhibits high binding capacity to HEp-2 cells and plastic surfaces and that it was present in a recent outbreak strain causing HUS in Northern Ireland (27) suggest that this variant can result in a highly pathogenic combination.

Alignment of the different AAF/V variants revealed a remarkable variability of the adhesin. Furthermore, the high ratio of nonsynonymous to synonymous mutations (*dN/dS*) indicates that AAF/V is under positive selection for structural changes. Interestingly, cloning of two AAF/V variants revealed significantly better biofilm formation in the AAF/V variant from strain 226 than in the variant from C338-14, showing that the amino acid changes between the two variants influence biofilm formation. This reveals that the amino acid changes in Agg5A can markedly influence the binding function of the adhesin as previously described for the FimH adhesin of type 1 fimbriae in *E. coli* and *K. pneumoniae* as well as the MrkD adhesin of *K. pneumoniae* type 3 fimbriae (43–45). Besides the potential to directly influence pathogenicity by providing functional changes, mutational changes of surface structures can play an important role by allowing immune evasion during chronic or recurrent infections and/or the ability to colonize new niches in the host (46–48).

The observed variability of the Agg5A adhesin presents a formidable challenge when it comes to detection of the fimbriae as well as for vaccine development. Comparison of the major pilin subunit from a large subset of different strains representing the other four AAF variants has not yet been performed. However, the fact that a known AAF is detected in only approximately 50% of the isolates in epidemiological studies could reflect difficulties in designing molecular tools able to detect all variants.

To our knowledge, no previous study has described a chimeric fimbrial variant like that in AAF/V, in which all accessory genes are maintained and only the adhesin is replaced. The low prevalence of Agg3A compared to that of Agg5A could be speculated to be related to the fact that the allelic exchanges of *agg3A* with *agg5A* confer a higher overall fitness, as exemplified by the increased biofilm formation as shown in the present study. This hypothesis is supported by the overall low prevalence of Agg3A in our study as well as previous studies (11, 12).

In this study, we present a fifth variant in the AAF family, which exhibits the same aggregative phenotype as the four other variants. This new variant shares the same accessory genes with AAF/III, as well as its signal peptide, but the two adhesins have less than 25% amino acid identity.

We show that Agg5A harbors multiple amino acid substitutions in 5 different Agg5A strains, and by cloning and expression of two Agg5A variants, we show that the amino acid substitutions result in altered biofilm abilities. We tested for the prevalence of this new variant and found that 12% of the Danish collection harbors AAF/V; thus, AAF/V is more prevalent than AAF/II and AAF/III (9% each). The same frequency of prevalence was also observed in the study conducted in Mali (12) (Table 3), suggesting that Agg5A is the second-most-prevalent AAF in Mali (after AAF/I) of the currently five AAFs described and the third-most-prevalent AAF in the Danish collection (after AAF/I and AAF/IV). The identification of a novel prevalent fimbria type in EAEC may be a step in the further understanding of the pathogenicity of this important pathogen.

ACKNOWLEDGMENTS

We thank Betina Hebbelstrup Jensen, Statens Serum Institut, Denmark, for providing the EAEC strain collection as well as for inspiring discussions. We also thank Susanne Jespersen and Flemming Scheutz, SSI, for their assistance with serotyping of C338-14. Lastly, we thank Håvard Jenssen, RUC, for his patience as well as spiritual guidance.

Strains were collected with financial support from Danish Council for Strategic Research, Innovation and Higher Education grant number 2101-07-0023 to Karen A. Krogfelt. Work in the Nataro lab is supported by US PHS grant AI-033096 to James P. Nataro.

We declare that we have no conflicts of interest.

REFERENCES

1. **Huang DB, Nataro JP, DuPont HL, Kamat PP, Mhatre AD, Okhuysen PC, Chiang T.** 2006. Enteroaggregative Escherichia coli is a cause of acute diarrheal illness: a meta-analysis. Clin Infect Dis **43:**556–563. http://dx.doi.org/10.1086/505869.
2. **Nataro JP, Mai V, Johnson J, Blackwelder WC, Heimer R, Tirrell S, Edberg SC, Braden CR, Glenn Morris J, Jr, Hirshon JM.** 2006. Diarrheagenic Escherichia coli infection in Baltimore, Maryland, and New Haven, Connecticut. Clin Infect Dis **43:**402–407. http://dx.doi.org/10.1086/505867.
3. **Nataro JP, Steiner T, Guerrant RL.** 1998. Enteroaggregative Escherichia coli. Emerg Infect Dis **4:**251–261. http://dx.doi.org/10.3201/eid0402.980212.
4. **Okeke IN, Lamikanra A, Czeczulin J, Dubovsky F, Kaper JB, Nataro JP.** 2000. Heterogeneous virulence of enteroaggregative Escherichia coli strains isolated from children in Southwest Nigeria. J Infect Dis **181:**252–260. http://dx.doi.org/10.1086/315204.

5. **Weintraub A.** 2007. Enteroaggregative Escherichia coli: epidemiology, virulence and detection. J Med Microbiol **56:**4–8. http://dx.doi.org/10.1099/jmm.0.46930-0.
6. **Cobeljic M, Miljkovic-Selimovic B, Paunovic-Todosijevic D, Velickovic Z, Lepsanovic Z, Zec N, Savic D, Ilic R, Konstantinovic S, Jovanovic B, Kostic V.** 1996. Enteroaggregative Escherichia coli associated with an outbreak of diarrhoea in a neonatal nursery ward. Epidemiol Infect **117:**11–16. http://dx.doi.org/10.1017/S0950268800001072.
7. **Itoh Y, Nagano I, Kunishima M, Ezaki T.** 1997. Laboratory investigation of enteroaggregative Escherichia coli O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. J Clin Microbiol **35:**2546–2550.
8. **Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M, Bernard H, Fruth A, Prager R, Spode A, Wadl M, Zoufaly A, Jordan S, Kemper MJ, Follin P, Muller L, King LA, Rosner B, Buchholz U, Stark K, Krause G, HUS Investigation Team.** 2011. Epidemic profile of Shiga-toxin-producing Escherichia coli O104:H4 outbreak in Germany. N Engl J Med **365:**1771–1780. http://dx.doi.org/10.1056/NEJMoa1106483.
9. **Scheutz F, Nielsen EM, Frimodt-Moller J, Boisen N, Morabito S, Tozzoli R, Nataro JP, Caprioli A.** 2011. Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing Escherichia coli O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. Euro Surveill **16**(24):pii:19889. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19889.
10. **Olesen B, Scheutz F, Andersen RL, Menard M, Boisen N, Johnston B, Hansen DS, Krogfelt KA, Nataro JP, Johnson JR.** 2012. Enteroaggregative Escherichia coli O78:H10, the cause of an outbreak of urinary tract infection. J Clin Microbiol **50:**3703–3711. http://dx.doi.org/10.1128/JCM.01909-12.
11. **Lima IF, Boisen N, Quetz Jda S, Havt A, de Carvalho EB, Soares AM, Lima NL, Mota RM, Nataro JP, Guerrant RL, Lima AA.** 2013. Prevalence of enteroaggregative Escherichia coli and its virulence-related genes in a case-control study among children from north-eastern Brazil. J Med Microbiol **62:**683–693. http://dx.doi.org/10.1099/jmm.0.054262-0.
12. **Boisen N, Scheutz F, Rasko DA, Redman JC, Persson S, Simon J, Kotloff KL, Levine MM, Sow S, Tamboura B, Toure A, Malle D, Panchalingam S, Krogfelt KA, Nataro JP.** 2012. Genomic characterization of enteroaggregative Escherichia coli from children in Mali. J Infect Dis **205:**431–444. http://dx.doi.org/10.1093/infdis/jir757.
13. **Czeczulin JR, Whittam TS, Henderson IR, Navarro-Garcia F, Nataro JP.** 1999. Phylogenetic analysis of enteroaggregative and diffusely adherent Escherichia coli. Infect Immun **67:**2692–2699.
14. **Nataro JP, Deng Y, Cookson S, Cravioto A, Savarino SJ, Guers LD, Levine MM, Tacket CO.** 1995. Heterogeneity of enteroaggregative Escherichia coli virulence demonstrated in volunteers. J Infect Dis **171:**465–468. http://dx.doi.org/10.1093/infdis/171.2.465.
15. **Hicks S, Candy DC, Phillips AD.** 1996. Adhesion of enteroaggregative Escherichia coli to pediatric intestinal mucosa in vitro. Infect Immun **64:**4751–4760.
16. **Tzipori S, Montanaro J, Robins-Browne RM, Vial P, Gibson R, Levine MM.** 1992. Studies with enteroaggregative Escherichia coli in the gnotobiotic piglet gastroenteritis model. Infect Immun **60:**5302–5306.
17. **Sheikh J, Hicks S, Dall'Agnol M, Phillips AD, Nataro JP.** 2001. Roles for Fis and YafK in biofilm formation by enteroaggregative Escherichia coli. Mol Microbiol **41:**983–997. http://dx.doi.org/10.1046/j.1365-2958.2001.02512.x.
18. **Wakimoto N, Nishi J, Sheikh J, Nataro JP, Sarantuya J, Iwashita M, Manago K, Tokuda K, Yoshinaga M, Kawano Y.** 2004. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative Escherichia coli. Am J Trop Med Hyg **71:**687–690.
19. **Nataro JP, Deng Y, Maneval DR, German AL, Martin WC, Levine MM.** 1992. Aggregative adherence fimbriae I of enteroaggregative Escherichia coli mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect Immun **60:**2297–2304.
20. **Czeczulin JR, Balepur S, Hicks S, Phillips A, Hall R, Kothary MH, Navarro-Garcia F, Nataro JP.** 1997. Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative Escherichia coli. Infect Immun **65:**4135–4145.
21. **Bernier C, Gounon P, Le Bouguenec C.** 2002. Identification of an aggregative adhesion fimbria (AAF) type III-encoding operon in enteroaggregative Escherichia coli as a sensitive probe for detecting the AAF-encoding operon family. Infect Immun **70:**4302–4311. http://dx.doi.org/10.1128/IAI.70.8.4302-4311.2002.
22. **Boisen N, Struve C, Scheutz F, Krogfelt KA, Nataro JP.** 2008. New adhesin of enteroaggregative Escherichia coli related to the Afa/Dr/AAF family. Infect Immun **76:**3281–3292. http://dx.doi.org/10.1128/IAI.01646-07.
23. **Savarino SJ, Fox P, Deng Y, Nataro JP.** 1994. Identification and characterization of a gene cluster mediating enteroaggregative Escherichia coli aggregative adherence fimbria I biogenesis. J Bacteriol **176:**4949–4957.
24. **Servin AL.** 2005. Pathogenesis of Afa/Dr diffusely adhering Escherichia coli. Clin Microbiol Rev **18:**264–292. http://dx.doi.org/10.1128/CMR.18.2.264-292.2005.
25. **Mathewson JJ, Oberhelman RA, Dupont HL, Javier de la Cabada F, Garibay EV.** 1987. Enteroadherent Escherichia coli as a cause of diarrhea among children in Mexico. J Clin Microbiol **25:**1917–1919.
26. **Nataro JP, Baldini MM, Kaper JB, Black RE, Bravo N, Levine MM.** 1985. Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe. J Infect Dis **152:**560–565. http://dx.doi.org/10.1093/infdis/152.3.560.
27. **Dallman T, Smith GP, O'Brien B, Chattaway MA, Finlay D, Grant KA, Jenkins C.** 2012. Characterization of a verocytotoxin-producing enteroaggregative Escherichia coli serogroup O111:H21 strain associated with a household outbreak in Northern Ireland. J Clin Microbiol **50:**4116–4119. http://dx.doi.org/10.1128/JCM.02047-12.
28. **Purcell BK, Clegg S.** 1983. Construction and expression of recombinant plasmids encoding type 1 fimbriae of a urinary Klebsiella pneumoniae isolate. Infect Immun **39:**1122–1127.
29. **Boll EJ, Struve C, Boisen N, Olesen B, Stahlhut SG, Krogfelt KA.** 2013. Role of enteroaggregative Escherichia coli virulence factors in uropathogenesis. Infect Immun **81:**1164–1171. http://dx.doi.org/10.1128/IAI.01376-12.
30. **Struve C, Bojer M, Krogfelt KA.** 2009. Identification of a conserved chromosomal region encoding Klebsiella pneumoniae type 1 and type 3 fimbriae and assessment of the role of fimbriae in pathogenicity. Infect Immun **77:**5016–5024. http://dx.doi.org/10.1128/IAI.00585-09.
31. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
32. **Bojer MS, Struve C, Ingmer H, Hansen DS, Krogfelt KA.** 2010. Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of Klebsiella pneumoniae. PLoS One **5:**e15467. http://dx.doi.org/10.1371/journal.pone.0015467.
33. **Fujimitsu K, Su'etsugu M, Yamaguchi Y, Mazda K, Fu N, Kawakami H, Katayama T.** 2008. Modes of overinitiation, dnaA gene expression, and inhibition of cell division in a novel cold-sensitive hda mutant of Escherichia coli. J Bacteriol **190:**5368–5381. http://dx.doi.org/10.1128/JB.00044-08.
34. **Qadri F, Haque A, Faruque SM, Bettelheim KA, Robins-Browne R, Albert MJ.** 1994. Hemagglutinating properties of enteroaggregative Escherichia coli. J Clin Microbiol **32:**510–514.
35. **Tamura K, Dudley J, Nei M, Kumar S.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24:**1596–1599. http://dx.doi.org/10.1093/molbev/msm092.
36. **Suzuki Y GT.** 2003. The phylogenetic handbook: a practical approach to DNA and protein phylogeny, 1st ed. Cambridge University Press, Cambridge, United Kingdom.
37. **Araujo JM, Tabarelli GF, Aranda KR, Fabbricotti SH, Fagundes-Neto U, Mendes CM, Scaletsky IC.** 2007. Typical enteroaggregative and atypical enteropathogenic types of Escherichia coli are the most prevalent diarrhea-associated pathotypes among Brazilian children. J Clin Microbiol **45:**3396–3399. http://dx.doi.org/10.1128/JCM.00084-07.
38. **Blattner FR, Plunkett G, III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y.** 1997. The complete genome sequence of Escherichia coli K-12. Science **277:**1453–1462. http://dx.doi.org/10.1126/science.277.5331.1453.
39. **Dudley EG, Thomson NR, Parkhill J, Morin NP, Nataro JP.** 2006. Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative Escherichia coli. Mol Microbiol **61:**1267–1282. http://dx.doi.org/10.1111/j.1365-2958.2006.05281.x.
40. **Nataro JP, Yikang D, Yingkang D, Walker K.** 1994. AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative Escherichia coli. J Bacteriol **176:**4691–4699.
41. **Prager R, Lang C, Aurass P, Fruth A, Tietze E, Flieger A.** 2014. Two

novel EHEC/EAEC hybrid strains isolated from human infections. PLoS One **9:**e95379. http://dx.doi.org/10.1371/journal.pone.0095379.

42. **Dallman TJ, Chattaway MA, Cowley LA, Doumith M, Tewolde R, Wooldridge DJ, Underwood A, Ready D, Wain J, Foster K, Grant KA, Jenkins C.** 2014. An investigation of the diversity of strains of enteroaggregative Escherichia coli isolated from cases associated with a large multipathogen foodborne outbreak in the UK. PLoS One **9:**e98103. http://dx.doi.org/10.1371/journal.pone.0098103.
43. **Sokurenko EV, Courtney HS, Maslow J, Siitonen A, Hasty DL.** 1995. Quantitative differences in adhesiveness of type 1 fimbriated Escherichia coli due to structural differences in fimH genes. J Bacteriol **177:**3680–3686.
44. **Stahlhut SG, Tchesnokova V, Struve C, Weissman SJ, Chattopadhyay S, Yakovenko O, Aprikian P, Sokurenko EV, Krogfelt KA.** 2009. Comparative structure-function analysis of mannose-specific FimH adhesins from Klebsiella pneumoniae and Escherichia coli. J Bacteriol **191:**6592–6601. http://dx.doi.org/10.1128/JB.00786-09.
45. **Stahlhut SG, Chattopadhyay S, Kisiela DI, Hvidtfeldt K, Clegg S, Struve C, Sokurenko EV, Krogfelt KA.** 2013. Structural and population characterization of MrkD, the adhesive subunit of type 3 fimbriae. J Bacteriol **195:**5602–5613. http://dx.doi.org/10.1128/JB.00753-13.
46. **Sokurenko EV, Chesnokova V, Dykhuizen DE, Ofek I, Wu XR, Krogfelt KA, Struve C, Schembri MA, Hasty DL.** 1998. Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. Proc Natl Acad Sci U S A **95:**8922–8926. http://dx.doi.org/10.1073/pnas.95.15.8922.
47. **Trinchina EV.** 2003. Structural and functional study of the receptor binding site for FimH adhesin in uropathogenic strains of Escherichia coli. Bull Exp Biol Med **136:**380–384. http://dx.doi.org/10.1023/B:BEBM.0000010958.02737.e7.
48. **Sokurenko EV, Feldgarden M, Trintchina E, Weissman SJ, Avagyan S, Chattopadhyay S, Johnson JR, Dykhuizen DE.** 2004. Selection footprint in the FimH adhesin shows pathoadaptive niche differentiation in Escherichia coli. Mol Biol Evol **21:**1373–1383. http://dx.doi.org/10.1093/molbev/msh136.
49. **Sheikh J, Czeczulin JR, Harrington S, Hicks S, Henderson IR, Bouguénec CL, Gounon P, Phillips A, Nataro JP.** 2002. A novel dispersin protein in enteroaggregative Escherichia coli. J Clin Invest **110:**1329–1337. http://dx.doi.org/10.1172/JCI16172.