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Novel Aggregative Adherence Fimbria Variant of Enteroaggregative Escherichia coli

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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 (AAFs), 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 adhesion 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...
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 JMM21, 042, 55989, and C1010-00, each expressing one of the four AAfs, were used in this study (21, 22, 25, 26).

**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, aagA, aafA, aag3A, and aag4A, was performed as previously described (12). Singleplex PCR amplifications were performed by using the Maxima Hot Start PCR master mix (2X; 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 into the cloning vector 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 upstream 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 JMM21ΔaggR 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 Uppagg5D_F and Uppagg5D_R and primers Dagg5A_F and Dagg5A_R, respectively. At their 5′ ends, the primers Uppagg5D_R and Dagg5A_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 Uppagg5D_F and Dagg5A_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 agg5 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, C338-14aggR, and C338-14agg5ABCD 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 DNA polymerase (Invitrogen) using the primers listed in Table 2.
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 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 calf serum (FBS) according to the manufacturer's instructions. Fresh erythrocytes were obtained from a human volunteer (A 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 OD600 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% OsO4 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.

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% CO₂ 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 thalassemia positive), and the HA assay was performed as previously described (34).

**Nucleotide sequence and phylogenetic analyses.** BLAST searches and comparisons were conducted using the databases of the National Center
The adherence AAF-mediated adhesion to HEp-2 cells (19). To investigate the
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 AAF/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 5989 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 peptide) 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 0.07 was significantly higher than the rate of synonymous mutations (dS) at 0.009

### Table 3 Prevalence of agg5A in clinical strains

<table>
<thead>
<tr>
<th>AAF pilin-encoding gene (AAF type)</th>
<th>Danish strain collection (n = 118)</th>
<th>Cases (n = 61)</th>
<th>Controls (n = 56)</th>
<th>Total (n = 121)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aggA (AAF/II)</td>
<td>9.32 (11)</td>
<td>4.3 (3)</td>
<td>5 (3)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>agg3A (AAF/III)</td>
<td>9.32 (11)</td>
<td>1.6 (1)</td>
<td>8.3 (5)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>agg4A (AAF/FIV)</td>
<td>18.6 (22)</td>
<td>8.2 (5)</td>
<td>1.6 (1)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>agg5A (AAF/V)</td>
<td>11.8 (14)</td>
<td>14.75 (9)</td>
<td>13.3 (8)</td>
<td>14 (17)</td>
</tr>
<tr>
<td>None</td>
<td>29.6 (35)</td>
<td>52.45 (32)</td>
<td>36.6 (22)</td>
<td>44.6 (54)</td>
</tr>
</tbody>
</table>

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

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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/msa/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 peptide 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.
indicating that positive selection for adaptive structural changes in \textit{agg5A} seems to occur rather than purifying selection.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{(A) Annotation of the AAF/III and AAF/V biogenesis clusters as determined by nucleotide sequence analysis of the product amplified from strains 59989 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 \textit{agg3A} and \textit{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.}
\end{figure}

\subsection*{The AAF/V gene cluster promotes EAEF-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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{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 \textit{agg5A}. The predicted signal sequence is underlined, and the predicted translation site is indicated by +1.}
\end{figure}
A Novel Adhesin of EAEC

A. A Novel Adhesin of EAEC

Bacterial adhesion and biofilm formation are critical aspects of pathogenesis in Enterobacteriaceae, including Enterohemorrhagic E. coli (EHEC) and Enteroinvasive E. coli (EAEC). In this study, we aimed to identify a novel adhesin of EAEC strain C338-14.

The AAF/V gene cluster is essential 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 Δagg5DCBA 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 Δagg5DCBA 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 C338-14 Δagg5DCBA 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).

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**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 presented 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 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 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.
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 C338-14aggR 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-
A Novel Adhesin of EAEC

The identification 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 ush 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 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 non-synonymous 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 Agg5A compared to that of Agg5A could be speculated to be related to the fact that the allelic exchanges of agg5A 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 Agg5A 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/V). 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.

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We declare that we have no conflicts of interest.

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