New Fimbrial Gene Cluster of S-Fimbrial Adhesin Family

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Fimbrial adhesins that mediate attachment to host cells are produced by most virulent Escherichia coli isolates. These virulence factors play an important role in the initial stages of bacterial colonization and also in determination of the host and tissue specificity. Isolates belonging to serotype O78 are known to cause a large variety of clinical syndromes in farm animals and humans and have been shown to produce several types of adherence fimbriae. We studied the fimbrial adhesin from an avian septicemic E. coli isolate of serotype O78. Analysis of the genetic organization of the fac (fimbria of avian E. coli) gene cluster indicates that it belongs to the S-fimbrial adhesin family. Seven open reading frames coding for major and minor structural subunits were identified, and most of them showed a high degree of homology to the corresponding Sfa and Foc determinants. The least-conserved open reading frame was facS, encoding a protein known to play an important role in determining adherence specificity in other S-fimbrial gene clusters.

Avian colisepticemia is a major disease of poultry caused by virulent strains of Escherichia coli. This septicemic disease of chickens and turkeys starts as a respiratory tract infection that extends to the air sacs and then to vital organs. The disease is economically important as it brings about heavy economic losses due to mortality and morbidity. In addition, there are also massive indirect losses due to intensification of other respiratory diseases, such as Newcastle disease and mycoplasma infections (35, 36).

Avian colisepticemia is similar in many respects to human extraintestinal diseases (urinary tract infections [UTI] and newborn meningitis [NBMI]) caused by virulent E. coli strains (2). These diseases can be contained within the initially infected organ (such as the trachea in poultry or urinary tract in humans) but can develop into septicemia, usually under conditions of stress or immune deficiency. The bacteria involved in both kinds of diseases are similar in terms of the virulence factors that they contain and the organization of the genes coding for them. Bacteria isolated from persons with these diseases are usually nontoxigenic but carry genes encoding serum resistance and an iron-binding protein (6, 10, 28, 47).

One important virulence factor that has been intensively studied in pathogenic extraintestinal E. coli strains is adherence fimbriae. Fimbrial adhesins are fiber-like structures, visible by electron microscopy on the bacterial cell surface, that are composed of major and minor subunit proteins. These fimbriae enable attachment of pathogenic bacteria to moieties of eukaryotic cells, therefore mediating host colonization, which is often an essential step in bacterial infection. Furthermore, the adhesion properties determine the host and tissue specificity (9, 14, 41, 42). In the case of fimbrial adhesins produced by extraintestinal E. coli strains, the minor subunits that facilitate adhesion are preferentially located at the tip of the fimbriae (10, 14, 21, 25, 46). Fimbrial adhesins are classified by their receptor specificity. Strains associated with UTI usually produce P-fimbrial adhesins, which interact with glycolipids containing α-D-Gal-1,4-β-D-Gal (7, 20, 22), and FIC fimbriae, which were recently shown to bind β-GalNac-1,4-β-Gal (13, 15, 34, 44, 45). E. coli strains causing sepsis or NBMI in most cases produce S-fimbrial adhesins, which interact with glycoproteins containing sialic acid (12, 25, 30).

We have been studying the adherence fimbriae of an avian E. coli O78 strain (36) that is frequently involved in severe epidemics of colisepticemia. These fimbriae do not hemagglutinate red blood cells but were shown to adhere to epithelial cells, preferentially avian tracheal cells. The fimbriae were purified and named by us AC/I (avian E. coli I) fimbriae (27, 48). Preliminary experiments using long-range mapping with specific DNA probes suggested that AC/I fimbriae may be related to S fimbriae (2).

In this study we describe the molecular cloning and characterization of the fac (fimbriae of avian E. coli strains) gene cluster coding for AC/I fimbriae in avian septicemic E. coli O78. Molecular analysis of this gene cluster reveals a high degree of homology to other S-fimbrial gene clusters in the nature of the subunits and their organization and indicates that AC/I fimbriae constitute a new member of the group of S-fimbriae.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Wild-type E. coli O78 strain 781 was isolated from a chicken with acute septicaemia (2, 48). E. coli K-12 strains VCS257 (ton33 dagD8 lacY1 glnV44) (Stratagene Cloning Systems, San Diego, Calif.) and VLS54 [Δαlac-pro staR thi ara] (kind gift from B. I. Eisenstein) were used for construction and screening of the genomic DNA libraries. E. coli K-12 strain DH5α was used for plasmid manipulation and propagation. Agglutination assays were performed with the E. coli K-12 host strain HB101 (24). The plasmids used are listed in Table 1. Luria-Bertani medium (liquid or solid) was used for bacterial cultivation, and antibiotics were added when required, as described by Sambrook et al. (37).

Recombinant DNA techniques. Agarose gel electrophoresis and bacterial transformations (CaCl2 method) were performed as previously described (37). The QIAprep spin miniprep kit (Qiagen GmbH) was used for plasmid and cosmid preparations. QIAquick gel extraction kit (Qiagen GmbH) was used for isolation of DNA fragments from the agarose gel. Restriction endonucleases and DNA ligase were obtained from Boehringer Mannheim GmbH and used according to the manufacturer’s recommendations.

Construction of genomic library. Total genomic DNA from strain 781 was isolated and partially digested with Sau3A. DNA fragments of 30 to 40 kb were isolated from a 10 to 40% sucrose gradient and ligated into the BamHI sites of the cosmid vector pMMP33 (8, 37). After in vitro packaging using the Gigapack Gold kit (Stratagene Cloning Systems), 5,000 recombinant E. coli K-12 VCS257 clones were selected. E. coli K-12 strain VLS54, which has no fimbriae, was later...
used as a recipient to detect the positive AC/I-fimbriae clone by DNA-DNA dot-blot colony hybridization and agglutination with monoclonal antibodies.

Preparation of monoclonal antibodies. The preparation and characterization of the monoclonal antibodies have been described previously (48).

Colony dot-blot and Southern hybridization. Rapid detection of DNA-DNA hybridization by colony dot-blot and Southern analysis with specific probes were performed as previously described (2).

Subcloning of fac determinant. Cloning of the fac cluster was done in two steps. Cosmid pRBS15 was cleaved with restriction enzymes EcoRI and both EcoRI and BamHI. The EcoRI-BamHI 5-kb fragment was cloned into vector pBR322 cut with EcoRI and BamHI to obtain plasmid pBRAC3-3. The 6.6-kb EcoRI fragment from pRBS15 was then ligated to pBRAC3-3 linearized by EcoRI. E. coli DH5α cells transformed with the resulting plasmid, pRAC221, were agglutinated by monoclonal antibody 875 (see Results), while these transformed with pBRAC3-3 were not.

Agglutination tests. Agglutination tests with monoclonal antifimbria antibodies were performed in 24-well microtiter plates as a rapid indication for the production of fimbriae. Cells carrying the recombinant plasmids listed in Table 1 were grown in liquid medium at 37°C for 18 h with agitation, 200 μl of the cell suspension was incubated with the antiseraum, and agglutination was determined after 20 min.

DNA sequencing. The sequence of the DNA region coding for the AC/I fimbrial genes facA, facD, facE, facF, facG, facS, and facH in strain 781 was determined by using the ABI Prism 377XL sequencer and the BigDye terminator cycle sequencing kit (PE Biosystems).

Computerized sequence analysis. Comparison of different subunit proteins with their related proteins was performed by the multiple sequence alignment program CLUSTAL W (43) at the Network Protein Sequence Analysis net (http://www.ch.embnet.org). Pretty printing and shading of the multiple alignment sequences were done by BOXSHADE program, version 3.21 (http://www.ch.embnet.org). Sequence similarity search was performed with the BLAST program at the NCBI server (http://www.ncbi.nlm.nih.gov) (1) or FASTA program at the European Bioinformatics Institute server (http://www2.ebi.ac.uk) (32, 33).

RESULTS

Characterization of fimbriae of E. coli 781. Most characterized fimbrial adhesins are able to agglutinate red blood cells of different organisms; this phenotype allows the characterization and identification of different fimbriae. Cells of E. coli strain 781 (serotype O78) expressing AC/I fimbriae adhere preferentially to avian epithelial cells. However, the bacteria failed to agglutinate red blood cells from calves, chickens, turkeys, rabbits, sheep, horses, mice, guinea pigs, and humans. In order to further characterize the AC/I fimbriae and study their genetic organization, monoclonal antibodies were raised. Several monoclonal clones were examined (clones 82, 92, 863, 921, and 1421), and all of them agglutinated the pilated 781 strain but failed to agglutinate the negative control strains E. coli K-12 DH5α and HB101.

Cloning and subcloning of fac gene cluster of E. coli 781. The fac gene cluster coding for AC/I of septicemic avian E. coli O78 was obtained from a cosmid genomic DNA library. To identify clones that contain the fac determinants, the library was screened by the use of monoclonal antibody 875 (48). Several positively reacting clones were obtained, and one of them (pRBS15) was studied further.

Since preliminary results suggested that the AC/I fimbriae are closely related to other S-fimbriae, we examined the homology between the cosmid pRBS15 and other S-fimbrial adhesin clusters by DNA-DNA hybridization. Southern colony dot-blot analysis of pRBS15 was performed using specific sfa gene probes. Positive hybridization under stringent conditions was observed for sfaA1 (the major subunit gene of the S-fimbrial adhesin II complex) and sfaG14, but that with the sfaS gene probe was seen only under less stringent conditions (data not shown). To further characterize cosmid pRBS15, it was cleaved with restriction enzymes EcoRI and both EcoRI and BamHI. Following Southern hybridization with the sfaA1 gene probe, an EcoRI-BamHI fragment of 5 kb was observed. When sfaS was used as a probe, a 6.6-kb positive EcoRI fragment was obtained. The sfaA1- and sfaS-specific gene probes are located at the two ends of the sfa determinant; therefore, we specu...
labeled that the DNA stretch containing the two positively hybridizing fragments will include the whole fac determinant. The EcoRI-BamHI 5-kb fragment was cloned in vector pBR322 to obtain plasmid pBRAC3-3. After cleavage of pBRAC3-3 with EcoRI, the 6.6-kb EcoRI fragment from pRBS15 was inserted to form plasmid pRAC221. As expected, recipient cells transformed with plasmid pRAC221 were agglutinated with monoclonal anti-fimbrial antibodies (31).

**TABLE 2. Identity and similarity of amino acid sequences of the seven open reading frames found in the AC/I fimbrial gene cluster compared with other clusters in the S-fimbrial family (11, 13, 18, 38–40, 44, 45)**

<table>
<thead>
<tr>
<th>Fac protein</th>
<th>Coding sequence (nucleotides)</th>
<th>Calculated size (kDa)</th>
<th>% Identity (% similarity) to Fac-specific proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>FacA</td>
<td>14–559</td>
<td>18.4</td>
<td>66 (78)</td>
</tr>
<tr>
<td>FacD</td>
<td>630–1169</td>
<td>19.7</td>
<td>98 (98)</td>
</tr>
<tr>
<td>FacE</td>
<td>1210–1905</td>
<td>25.7</td>
<td>99 (99)</td>
</tr>
<tr>
<td>FacF</td>
<td>1975–4605</td>
<td>96.3</td>
<td>98 (98)</td>
</tr>
<tr>
<td>FacG</td>
<td>4618–5145</td>
<td>18.6</td>
<td>100 (100)</td>
</tr>
<tr>
<td>FacS</td>
<td>5167–5658</td>
<td>17.1</td>
<td>72 (81)</td>
</tr>
<tr>
<td>FacH</td>
<td>5794–6693</td>
<td>30.0</td>
<td>80 (89)</td>
</tr>
</tbody>
</table>

DNA sequence of genes coding for major and minor subunits of fac determinant. In order to identify the genes involved in the biogenesis of AC/I, the DNA sequence of most of the insert was determined. Sequence analysis performed on a fragment of 6.9 kb revealed seven open reading frames in the same orientation. The cluster was named fac (fimbriae of avian E. coli), and the organization of these genes, named facA, facD, facE, facF, facG, facS, and facH, resembled the gene organization found for fimbriae belonging to the S-fimbrial family (Fig. 2).

A similarity search using the BLAST program and multiple sequence alignments using the CLUSTAL W program were performed on these open reading frames. The results are depicted in Table 2 and Fig. 3.

In order to trace fac-related sequences in the E. coli genome, we performed a FASTA search with the 6.9-kb DNA sequence. Sequences that showed more than 50% identity to fragments of the fac cluster are presented in Fig. 4. None of these sequences showed homology to the whole AC/I 6.9-kb fragment sequenced.

**DISCUSSION**

Although classified as enteric microorganisms, many strains of E. coli are capable of colonizing extraintestinal tissues. In contrast to intestinal colonization, the E. coli strains that colonize other organs often generate systemic infection. The strains involved in extraintestinal diseases do not carry genes for production of enterotoxins but usually express polysaccharide capsules such as K1. Another difference between intestinal and extraintestinal strains involves adherence to epithelial cells. Usually, adherence of intestinal E. coli is mediated via adherence fimbriae, such as K99 and K88, that are coded for by plasmid genes, and adhesion is mediated by the major subunit. In strains involved in human extraintestinal infection, the fimbriae are encoded by chromosomal genes and composed of a major subunit and several minor subunits, which influence binding abilities and are responsible for specific adhesion.

**TABLE 3. Identity of major and minor subunit open reading frames of AC/I to other clusters that belong to the S-fimbrial family (11, 13, 38, 39, 44, 45)**

<table>
<thead>
<tr>
<th>fac gene</th>
<th>% Identity to fac-specific open reading frames</th>
</tr>
</thead>
<tbody>
<tr>
<td>facA</td>
<td>72.8</td>
</tr>
<tr>
<td>facG</td>
<td>100</td>
</tr>
<tr>
<td>facD</td>
<td>100</td>
</tr>
<tr>
<td>facE</td>
<td>99.8</td>
</tr>
<tr>
<td>facF</td>
<td>63.6</td>
</tr>
<tr>
<td>facS</td>
<td>82.6</td>
</tr>
<tr>
<td>facH</td>
<td>98.4</td>
</tr>
</tbody>
</table>

FIG. 2. Genetic organization of fac, sfa, and foc gene clusters (11, 19, 34).
The best-studied extraintestinal human E. coli strains are those involved in UTI, and the majority of them produce P-fimbriae, which bind to the digalactoside part of glycolipids (7, 11, 22, 26). Other extraintestinal strains were shown to produce fimbriae that do not have the same adhesin specificity as P-fimbriae. This includes type F1C fimbriae, Sfr fimbriae, and Sfa-I fimbriae, which recognize receptors containing α-sialyl-2,3-β-Gal (11, 22, 25, 29–31, 34). Recently, a new type of adherence fimbriae was identified in E. coli strains isolated from NBM (11). These fimbriae, named Sfa-II fimbriae, differ from Sfa-I fimbriae in the major subunit and in two minor subunits. However, they also bind sialylgalactosides, and their gene coding for the fimbral adhesin, sfaS, is identical to the equivalent gene of the sfaI cluster from UTI bacteria (11, 16).

We have studied the AC/I fimbriae produced by an avian E. coli O78 strain. These fimbriae are an important virulence factor, as they mediate preferential binding of this extraintestinal pathogen to avian epithelial tissues. The results presented here indicate that the AC/I fimbriae constitute a new member of the S-fimbrial family. Several observations demonstrate the relatedness between AC/I and other S-fimbrial gene clusters. (i) The various gene clusters coding for S-fimbriae (fac, sfr, sfa, and foc) have related restriction patterns. (ii) Monoclonal antibodies that were

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**FIG. 3.** Multiple sequence alignment to different proteins encoded by the fac gene cluster. (A) FacA (major subunit). (B) FacG (minor subunit). (C) FacS (minor subunit). (D) FacH (minor subunit) (5, 11, 13, 17, 38, 39, 44, 45). Black shading, identity; grey shading, similarity.
raised against AC/I were also able to bind to another S-fimbrial

group member, showing immunological cross-reactivity. (iii)

The genetic organization of the \textit{fac} gene cluster, as determined

by DNA sequencing, was in good agreement with that of other

clusters belonging to the S-fimbrial family (Fig. 2 and Table 2).

Comparison of the predicted AC/I protein subunits to the

corresponding subunits belonging to the S-fimbrial family re-

vealed an interesting insight into the evolution and function of

the fimbriae—the AC/I major subunit protein, FacA, is 100%

similar to the major subunit protein SfaA-II of the

\textit{sfa} II gene

cluster. This finding is unexpected, since previous studies com-

paring genes from different adhesin clusters have shown that

the major subunit genes are the most heterogeneous, probably

reflecting better adaptation to different environments encoun-

tered by the bacteria in their host (5, 11). The unexpected high

degree of identity found in the major subunit proteins SfaA-II

and FacA may be a result of the evolutionary process of cluster

formation and could perhaps be due to horizontal gene trans-

fer in recent times. This high degree of homology can also

explain the immunological cross-reactivity of the monoclonal

antibodies that were raised against the AC/I fimbriae.

Despite the similarity between the major subunits of the

\textit{sfa} II and \textit{fac} clusters, the minor subunit proteins of AC/I show a

higher degree of homology to other fimbrial gene clusters, like

\textit{sfa} I and \textit{foc}.

These proteins are preferentially found at the

fimbrial tip, and they play an important role in determining

adhesin specificity (16, 46).

The most conserved minor subunit is FacG, which is iden-

tical to SfaG-I and closely related to SfaG-II and FocF (99 and

98% identity, respectively). It was shown that \textit{sfaG} contributes

to the attachment of the bacteria to tubular cells as well as to

plasminogen. The conservation of the FacG protein found in

FIG 3—Continued.
this study supports previous suggestions that this subunit might contribute to the adherence process (11, 23). Based on this conservation and the complete identity to SfaG-I, we propose a similar function for this protein in the avian-viral strain. The least conserved minor subunit was FacS, in agreement with its adhesion properties, which are different from those of other fimbriae of the S-group. For example, AC/I fimbriae do not agglutinate red blood cells, in contrast to SfaS-I and SfaS-II, which are identical and responsible for the sialic acid-specific binding of the S-fimbriae. Furthermore, while AC/I fimbriae preferentially bind avian tracheal cells, FIC fimbriae attach to uroepithelial cells of human origin (16, 29).

The data presented indicate that the AC/I cluster contains one gene that is highly homologous to the Sfa-II cluster (the sfaAII gene), while other genes are more homologous to other gene clusters. FacG and FacS are homologues of SfaG-I and SfaS-I, respectively, while FacH is a homologue of the FocH protein. This is also shown at the DNA level, as depicted in Figure 3. We propose that the observed phenomenon constitutes a combinatorial shuffling of fimbrial genes, which could be of ecological and functional importance. This gene shuffling could account for the diversity seen in the fimbrial loci and be of ecological and functional importance. This gene shuffling could be achieved by horizontal gene transfer or by recombination events with other genomic fimbrial clusters, such as the fimI-like genes (Fig. 4) (3). Evidence for the existence of horizontal gene transfer was obtained from the finding that in the 3’ end of the fac cluster, there is an insertion of 74 nucleotides in a stretch of 100 nucleotides between the end of facS and the start of facH. These 100 nucleotides have a GC content of 30%, compared to the 47.8% GC found in the whole group of S-fimbriae. The group of S-fimbriae is interesting because it contains several types of fimbriae with different adherence specificities that are produced by bacteria that cause a variety of clinical symptoms in mammals and poultry. The molecular evolution of the gene clusters coding for these fimbriae can serve as a model system for studying combinatorial gene shuffling as an adaptive process in host-pathogen interactions.

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