Functional Analysis of the Sialic Acid-Binding Adhesin SfaS of Pathogenic Escherichia coli by Site-Specific Mutagenesis

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The gene coding for the sialic acid-specific adhesin SfaS produced by the S fimbrial adhesin (sfa) determinant of Escherichia coli has been modified by oligonucleotide-directed, site-specific mutagenesis. Lysine 116, arginine 118, and lysine 122 were replaced by threonine, serine, and threonine, respectively. The mutagenized gene clusters were able to produce S fimbrial adhesin complexes consisting of the S-specific subunit proteins including the adhesin SfaS. The mutant clones were further characterized by hemagglutination and by enzyme-linked immunosassay tests with anti-fimbria- and anti-adhesin-specific monoclonal antibodies, one of which is able to block S-specific binding (Moch et al., Proc. Natl. Acad. Sci. USA 84:3462-3466, 1987). The lysine-122 mutant clone was indistinguishable from the wild-type clone in these assays. Replacement of lysine 116 and arginine 118, however, abolished hemagglutination and resulted in clones which showed a weak (lysine 116) or a negative (arginine 118) reaction with the antiadhesin-specific antibody A1. We therefore suggest that lysine 116 and arginine 118 have an influence on binding of SfaS to the sialic acid residue of the receptor molecule.

Small subunit proteins represent the adhesin molecules and are composed of major protein subunits and minor components. It has been shown that minor subunit proteins represent the adhesin molecules of P, type I, and S fimbriae (6, 16, 22, 24, 26; for a review, see reference 6).

We have identified the minor subunit of S fimbriae, SfaS, a 14-kilodalton (kDa) protein, as the sialic acid-binding adhesin. The adhesin was isolated, and monoclonal antibodies (MAbs) directed against SfaS were selected (28). In addition, the sfaS-specific gene was localized in the distal part of the sfa gene cluster (Fig. 1) and its DNA sequence was determined (36). In order to identify regions of the SfaS protein which have an influence on sialic acid-specific binding, three amino acids of the C-terminal half of the adhesin were changed by site-specific mutagenesis. The data show that lysine 116 and arginine 118 play a role in the interaction between SfaS and its receptor molecule.

MATERIALS AND METHODS

Media, enzymes, and chemicals. Bacteria were grown in enriched nutrient broth or in L broth. For isolation of fimbrial subunits, the clones were grown in a medium containing 1% Bacto-Peptone (Difco Laboratories), 1% yeast extract, 0.25% glycerol, and 50 mM sodium phosphate buffer (PH 7). Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass.; antibiotics were a gift from Bayer, Leverkusen, Federal Republic of Germany (FRG). All other chemicals were obtained from E. Merck AG, Darmstadt, FRG. Restriction enzymes, T4 polynucleotide kinase, and T4 ligase were purchased from Bio-Rad Laboratories, Richmond, Calif. DNA polymerase I was obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Bacterial strains and plasmids. All recombinant plasmids were transformed into the E. coli K-12 HB101. The different S fimbrial adhesin (sfa)-specific recombinant DNAs were derived from the plasmid pANN801-13 (sfa* Ap') (7). For the construction of site-specific mutations, the plasmid pANN801-11 (Ap') was used. Plasmid pANN801-11 carries a 5-kilobase (kb) BamHI-EcoRI fragment specific for the distal portion of the sfa gene cluster (see also Fig. 1) which was cloned into plasmids pMA254 and pMC254 (Strannsens et al., unpublished data). The recombinant DNAs used for site-specific mutagenesis were introduced into the E. coli strains WK6 and BMH71-18 mutS (19). Recombinant DNA techniques. Plasmid DNA was isolated as described previously (2, 17). For restriction enzyme analysis, DNA was treated with appropriate enzymes and the resulting fragments were separated by gel electrophoresis on 0.7 to 1.0% agarose gels (25). DNA fragments were isolated after agarose gel electrophoresis by electroelution. For cloning, DNA fragments were ligated into suitable vector molecules after heat inactivation of the restriction endonucleases at 65°C for 6 min. E. coli K-12 strains were transformed by the CaCl2 method (20).

Site-specific mutagenesis. In order to mutagenize the gene sfaS, the 5.3-kb EcoRI-BamHI fragment of the plasmid pANN801-13 (Fig. 1) was cloned into the vectors pMA254 and pMC254. Both plasmids carry an fl origin and are
identical except that pMA254 carries an amber mutation in the chloramphenicol resistance gene and pMC254 carries an amber mutation in the β-lactamase gene (Stannens et al., unpublished data). The mutagenization was done by the gap-duplex method of Kramer et al. (19). The pMA254 derivative was cleaved with the restriction enzymes SpIh and EcoRI, and after elution from an agaro gel, it was annealed to the single-stranded pMC254 derivative. The following mutagenic oligonucleotides, containing one mismatch (5′), were used: 5′-CCTTTAATCTTAC*AGCCCGTGACAGTG-3′ to change Arg-118 into Thr-118. DNA sequencing was used to confirm the specific mutations. The nucleotide used for site-specific mutagenesis are marked by triangles.

**FIG. 1.** (Top) Genetic organization of the S fimbral adhesin (sfa) determinant. The black boxes represent the sfa-specific genes. The main direction of transcription of the determinant is from left to right (see reference 6). The following restriction sites are indicated: EI, EcoRI; EV, EcoRV; S, Smal; Sp, SpIh; P, PstI; C, CiaI; B, BamHI. (Bottom) Nucleotide sequence of the gene sfaS and the corresponding primary protein sequence of the S-specific adhesin SfaS. The nucleotides used for site-specific mutagenesis are marked by triangles.
amino acid exchanges affected the relative amounts of the major subunit protein SfaH. The agreement with the data obtained recently (36) demonstrated that the proteins of 31, 17, 16, and 14 kDa. These results are in agreement with the data obtained recently (36). It was suggested that the major fimbrial subunit SfaA, while MAbA2 and MAbA1 are specific for the S adhesin SfaS. Antibody MAbA1 is able to block binding of SfaS to erythrocytes, while MAbA2 has no influence on the receptor recognition process.

As demonstrated in Fig. 4A, the clones showed similar ELISA values in reactions with MAbF1, indicating a comparable degree of fimbriation of the clones, as already suggested by electron microscopy (Fig. 2). The mutant clones HB101(pANN801-116) and HB101(pANN801-122) gave values in the ELISA with MAbA2 identical to that with the wild-type clone HB101(pANN801-13), while the Arg-118 mutant clone HB101(pANN801-118) showed a reduced value, indicating a reduction of the amount of SfaS in HB101(pANN801-118) compared with the amounts of S adhesin proteins in the other clones. As demonstrated in Fig. 4C, the anti-SfaS antibody MAbA1, which is able to block binding, did not react with the Arg-118 mutant clone HB101(pANN801-118). The clone HB101(pANN801-116), which carries a substitution of lysine 116 by threonine, showed a very low value in the ELISA with MAbA1. Only the Lys-122 mutant HB101(pANN801-122) gave a reaction with MAbA1, similar to the wild-type clone.

Hemagglutination tests with mutant clones. To analyze the influence of the mutations in the gene sfaS on sialic acid-specific binding, the mutant clones were characterized in an S-specific quantitative hemagglutination test. As demonstrated in Fig. 4A, and HB101(pANN801-122), the major and minor subunit proteins were isolated. As shown in Fig. 3, the S fimbria-adhesin complexes of the clones consisted of four proteins of 31, 17, 16, and 14 kDa. These results are in agreement with the data obtained recently (36) demonstrating that the Sfa complex is formed by the subunit proteins SfaH (31 kDa), SfAG (17 kDa), SfAA (16 kDa), and SfAS (14 kDa). Thus, the mutant clones still express the whole set of subunit proteins.

ELISA studies with mutant clones. To see whether the amino acid exchanges affected the relative amounts of the major subunit protein SfaA and the S-specific adhesin SfaS of the S fimbria-adhesin complexes, the clones were characterized in quantitative ELISAs with MAbS. It has been shown previously (28) that MAbF1 is directed against the major fimbrial subunit SfaA, while MAbA2 and MAbA1 are specific for the S adhesin SfaS. Antibody MAbA1 is able to block binding of SfaS to erythrocytes, while MAbA2 has no influence on the receptor recognition process.

DISCUSSION

Recent evidence indicates that bacterial fimbrial adhesins are composed of major and minor subunit proteins (6-10, 16, 24, 26, 28). It was suggested that the major subunits of the plasmid-encoded K99 adherence factor of intestinal E. coli strains represent the adhesive entity (10). In contrast, minor subunits are the adhesins in the cases of type I, P, Prs, and S fimbriae (16, 22, 24, 26, 36). The oligosaccharide receptor structures recognized by these chromosomally encoded minor subunit adhesins have been characterized (13, 24, 27, 33), but little is known about the regions of the adhesive...
proteins which are involved in binding to eucaryotic recognition sites.

In order to initiate studies on the influence of certain protein domains of the bacterial adhesin SfaS in sialic acid-specific binding, we used oligonucleotide directed site-specific mutagenesis. Lysine residues at positions 116 and 122 were substituted with threonine residues, and an arginine residue at position 118 was replaced by a serine residue. The three mutant clones HB101(pANN801-116) (Lys-116), HB101(pANN801-118) (Arg-118), and HB101 (pANN801-122) (Lys-122) still produced fimbriae and S-specific adhesins, as demonstrated by electron microscopy (Fig. 2), by the isolation of the S fimbria-adhesin complexes (Fig. 3), and by ELISAs with antifimbria- and antiadhesin-specific MAbs (Fig. 4).

Two lines of evidence suggest that the exchange of Lysine 116 with threonine and arginine 118 with serine influences binding of SfaS to the receptor. The mutant clones Lys-116 and Arg-118 were negative in S-specific hemagglutination tests. In addition, the anti-SfaS-specific antibody MAbA1, which in contrast to MAbA2 inhibits S-mediated hemagglutination (28), did not recognize mutant Arg-118 and showed only a weak reaction with mutant Lys-116. The influence of the substitution of the two positively charged amino acids on sialic acid-specific binding may be due to a change of the recognition domain, which may interact with the oligosaccharide receptor by ionic interactions. On the other hand, the exchange of the two amino acids may also have an effect on the conformation of SfaS. The modification in the conformation of SfaS may cause loss of its binding properties. It is therefore possible that the changes in the primary amino acid sequence of SfaS do not have a direct effect on the sialic acid-binding site but have an indirect influence on receptor recognition.

It is demonstrated in Fig. 2 and 4 that the degree of fimbriation and the production of the major subunit protein SfaA are similar in the case of the wild-type and mutant clones. The adhesin SfaS, however, was detected in reduced amounts in the mutant Arg-118 compared with the wild-type clone and the mutants Lys-116 and Lys-122 (Fig. 4). This result may be because the stability of the SfaS protein was affected by the replacement of Arg-118 by Ser-118. It is also possible that the amino acid exchange had a negative effect on the incorporation of SfaS into the S fimbria-adhesin complex. As a third possibility, it can be speculated that the Arg-118 mutant molecule is not as efficiently transported across the periplasmic space as the other S-specific adhesins. Such an effect was shown recently for the P-specific adhesin PapG which carried a mutation in its C-terminal half (9).

The amino acid sequence of the SfaS segment between the two lysine residues at positions 116 and 122 was compared with the sequences of other sialic acid-binding proteins, such as the influenza virus hemagglutinin (38, 39) and toxins and

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**TABLE 1. Hemagglutination of E. coli clones carrying sfa-specific sequences**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amino acid change</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pANN801-13</td>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td>pANN801-116</td>
<td>Lys-116 → Thr-116</td>
<td>0</td>
</tr>
<tr>
<td>pANN801-118</td>
<td>Arg-118 → Ser-118</td>
<td>0</td>
</tr>
<tr>
<td>pANN801-122</td>
<td>Lys-122 → Thr-122</td>
<td>32</td>
</tr>
<tr>
<td>pBR322</td>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

* Hemagglutination was carried out in microtiter plates as described by Jones and Rutter (11).
adhesins of different bacterial species (4, 12, 14, 21, 34). As demonstrated in Fig. 5, homologies were found between SfaS and domains of the K99 (34) and the CFAI (12, 14) adhesins of E. coli and of the B subunits of the cholera toxin (4) and the E. coli LTI toxin (21). In a recent study (10), it was shown by site-specific mutagenesis that lysine 132 and arginine 136 influence binding of the K99 major subunit adhesin to its sialic acid-containing receptor. Biochemical analysis (23) and binding inhibition assays with synthetic peptides (4) argue for a contribution of a segment between lysine 116 and lysine 122 of the sialic acid-binding proteins K99 adhesin (34), CFAI adhesin (12, 14), cholera B subunit (4), and the other sialic acid-binding proteins.

FIG. 5. Comparison of the amino acid sequence segment between lysine 116 and lysine 122 of SfaS to amino acid sequences of the sialic acid-binding proteins K99 adhesin (34), CFAI adhesin (12, 14), cholera B subunit (4), and E. coli LTI B subunit (21). Identical or functionally identical amino acids are boxed. Dashes represent gaps introduced for optimal alignment.

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LITERATURE CITED


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