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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 immunoassay tests with antifimbria- 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. Substitution of arginine 118 by serine also had a negative effect on the amount of SfaS adhesin proteins isolated from the S fimbrial adhesin complex.

The ability of pathogenic microorganisms to adhere to particular host receptors is of primary importance in several infectious diseases (27, 30). Extraintestinal *Escherichia coli* strains causing urinary tract infections, newborn meningitis, and sepsis express different filamentous appendages of the cell surface, called fimbriae (15). The presence of fimbriae is often associated with the binding capacity of the bacteria. Adhesive fimbriae of extraintestinal *E. coli* are able to recognize different receptor substances. While P and Prs fimbriae bind to glycolipids containing Galα-(1-4)-Gal and GalNAcα-(1-3)-GalNAc, respectively (22, 24), type I fimbriae adhere to mannose-containing glycolipids (15). In contrast, S fimbrial adhesins (Sfa) expressed by *E. coli* causing newborn meningitis or urinary tract infections attach to glycoproteins terminating with NeuNAcα-(2-3)-Gal (18, 31, 33). Each of the fimbrial adhesins is 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% glycerin, 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^r [7]). For the construction of site-specific mutations, the plasmid pANN801-11 (Ap^r) was used. Plasmid pANN801-11 carries a 5.3-kilobase (kb) *Bam*HI-*Eco*RI fragment specific for the distal portion of the *sfa* gene cluster (see also Fig. 1) which was cloned into plasmids pMA254 and pMC254 (Stannsens 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 $CaCl_2$ method (20).

Site-specific mutagenesis. In order to mutagenize the gene *sfaS*, the 5.3-kb *Eco*RI-*Bam*HI fragment of the plasmid pANN801-13 (Fig. 1) was cloned into the vectors pMA254 and pMC254. Both plasmids carry an f1 origin and are

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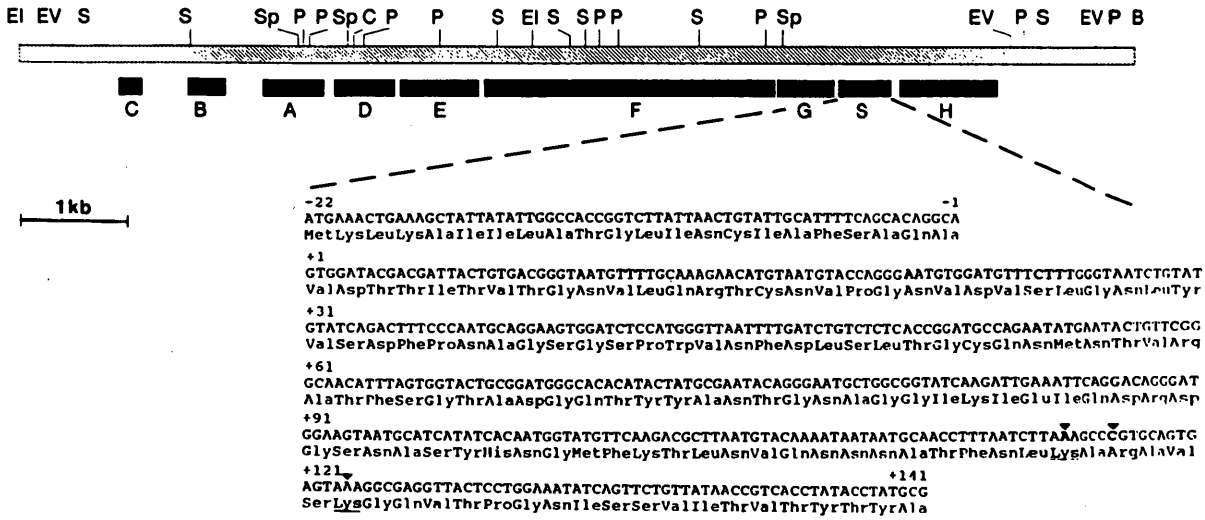

FIG. 1. (Top) Genetic organization of the S fimbrial adhesin (*sfa*) determinant. The black boxes represent the *sfa*-specific genes. The main direction of transcription of the determinant is from left to right (see reference 6). The following restriction sites are indicated: EI, *Eco*RI; EV, *Eco*RV; S, *Sma*I; Sp, *Sph*I; P, *Pst*I; C, *Cla*I; B, *Bam*HI. (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.

identical except that pMA254 carries an amber mutation in the chloramphenicol resistance gene and pMC254 carries an amber mutation in the β-lactamase gene (Stannsens 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 *Sph*I and *Eco*RI, and after elution from an agarose gel, it was annealed to the single-stranded pMC254 derivative. The following mutagenic oligonucleotides, containing one mismatch (*), were used: 5'-CCTTTAATCTTAC*AGCCCGT GCAGTG-3' to change SfaS Lys-116 into SfaS Thr-116; 5'-CCTTTAATCTTAAAGCCA*GTGTGCAGTG-3' to change SfaS Arg-118 into SfaS Ser-118; and 5'-GCAGTGAGTAC*A GGCCAGGTTACTCCTGG-3' to change SfaS Lys-122 into SfaS Thr-122. DNA sequencing was used to confirm the mutations and to ensure that no other mutations had occurred. In order to restore the *sfa* determinants, the *Eco*RI-*Bam*HI inserts (5.3 kb) carrying the different mutations in *sfaS* were ligated into pANN801-13 cleaved with *Eco*RI and *Bam*HI following deletion of the original *sfa*-specific *Eco*RI-*Bam*HI fragments. Restriction analysis confirmed that the constructs pANN801-116 (Thr-116), pANN801-118 (Ser-118), and pANN801-122 (Thr-122) were identical to the wild-type plasmid pANN801-13 except for the three site-specific mutations.

Isolation of the fimbria-adhesin complex and separation of major and minor subunits. The isolation of the fimbria-adhesin complexes and the separation of the subunits were carried out as described previously (8, 36).

Agglutination and adhesion tests. Fimbriated clones were characterized by agglutination with antisera on glass slides. S-specific adhesion was determined after mixing the bacterial cells with human and/or bovine erythrocytes with and without 2% mannose and with erythrocytes treated with neuraminidase (7, 33). In order to quantify hemagglutination, the test was done in microtiter plates as described previously (11). The hemagglutination tests were carried out at pH 7.

Electron microscopy. Bacteria were transferred to copper grids coated with polyvinyl-Formvar. After being soaked in 1% phosphotungstic acid (pH 6.4) for 1 min, the grids were examined under a Zeiss 10A transmission electron microscope (28, 32).

Preparation of monoclonal antisera. The preparation and characterization of the monoclonal antisera used have been described (28).

ELISA. The quantitative enzyme-linked immunosorbent assay (ELISA) was done with whole bacteria by the method of Boylan et al. (3).

Oligonucleotide synthesis. Oligonucleotides were synthesized with an Applied Biosystems 380A DNA synthesizer by the phosphoramidite method of Beaucage and Caruthers (1). Oligonucleotides were purified on a polyacrylamide gel.

DNA sequencing. The sequence of the DNA region coding for the gene *sfaS* was determined with the help of a sequence kit from Boehringer as described by a protocol of the manufacturer.

Computer analysis. The programs used for compiling the nucleotide sequence data were from J. Devereux (University of Wisconsin Genetics Computing Group).

RESULTS

Construction of site-specific mutations in the gene *sfaS*. It was demonstrated previously that positively charged amino acids may play a role in binding of bacterial adhesins to sialic acid-containing receptors (10; for a review, see reference 13). In order to determine whether a certain amino acid domain may be involved in sialic acid-specific binding of the *E. coli* adhesin SfaS, we used synthetic oligonucleotides to direct mutations of codons for the amino acids lysine 116, arginine 118, and lysine 122, which are located very close to each other. A 5.3-kb *Eco*RI-*Bam*HI fragment which carries the gene *sfaS* (Fig. 1) was used for the site-specific mutagenesis, which was done by the method of Kramer et al. (19). DNA sequence studies revealed that the lysine residues at positions 116 and 122 were changed into threonine residues in the clones HB101(pANN801-116) and HB101(pANN801-122), respectively. The arginine residue at position 118 was

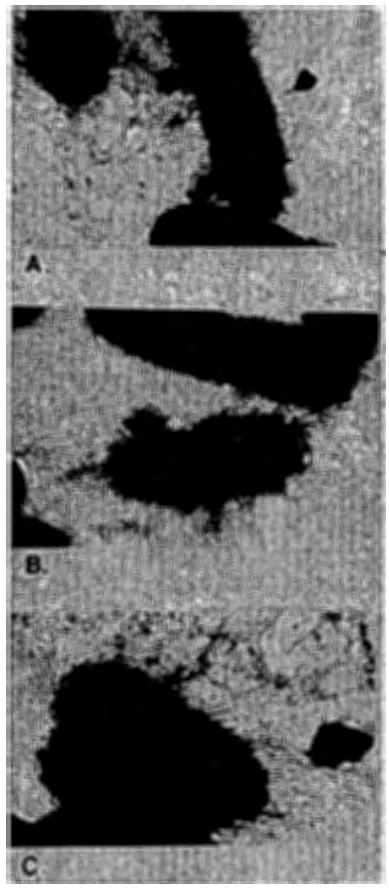

FIG. 2. Electron micrographs of the *sfa* mutant clones. The following strains were used: (A) HB101(pANN801-116); (B) HB101 (pANN801-118); (C) HB101(pANN801-122).

changed into a serine residue in the clone HB101(pANN801-118).

Fimbriation of mutant clones. In order to determine whether the mutations influenced fimbriation, the clones were inspected by electron microscopy. As indicated in Fig. 2, the mutant clones HB101(pANN801-116) (Fig. 2A), HB101(pANN801-118 (Fig. 2B), and HB101(pANN801-122) (Fig. 2C) showed similar degrees of fimbriation, which were comparable to fimbrial production by the *sfa*$^+$ wild-type clone HB101(pANN801-13) (7, 28, 36).

Analysis of S fimbria-adhesin complexes. From the clones HB101(pANN801-13), HB101(pANN801-116), HB101 (pANN801-118), 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.

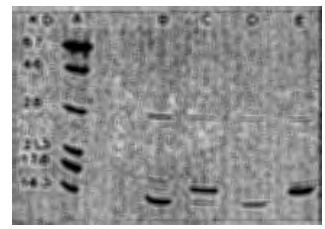

FIG. 3. Electrophoretic analysis of major and minor subunit proteins of the S fimbria-adhesin complexes encoded by *sfa* wild-type and mutant gene clusters. Lanes: B, HB101(pANN801-13), *sfa*$^+$; C, HB101(pANN801-116); D, HB101(pANN801-122); E, HB101(pANN801-118); A, size markers (in kilodaltons).

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 Table 1, only mutant HB101(pANN801-122), which showed a replacement of lysine 122 by threonine, was able to agglutinate erythrocytes. In contrast, the clones HB101(pANN801-116) and HB101(pANN801-118), carrying substitutions of lysine 116 by threonine and arginine 118 by serine, respectively, failed to react in the hemagglutination assay. It therefore seems that replacement of lysine 116 by threonine and arginine 118 by serine influenced the binding properties of the S adhesin protein SfaS.

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

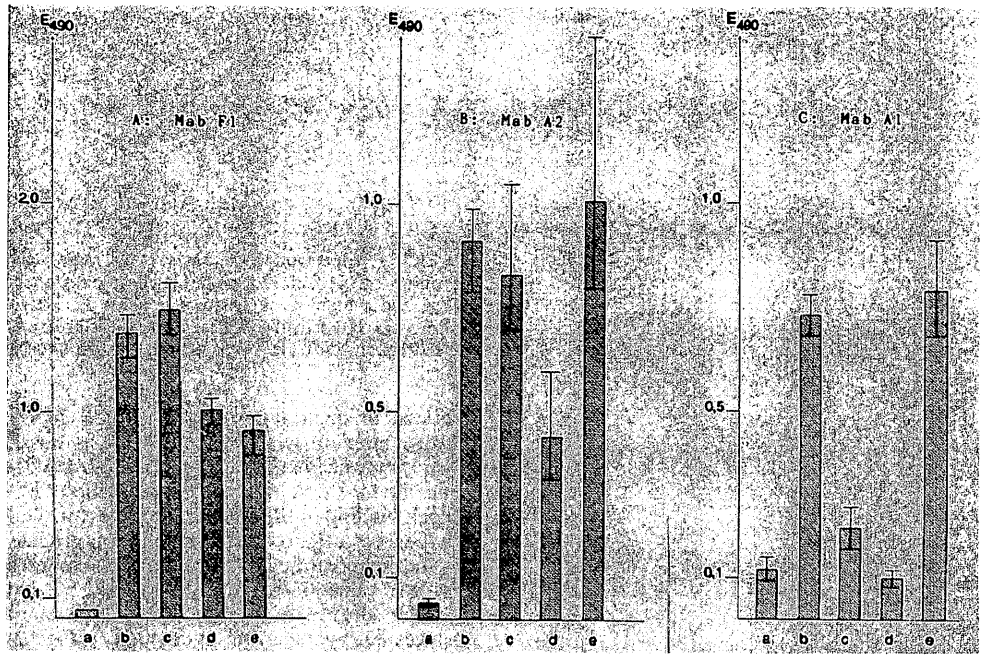

FIG. 4. Quantitative whole-cell ELISAs of the *sfa* wild-type and mutant clones with MAbs specific for the major subunit protein sfaA (A, MAbF1) and the S adhesin SfaS (B, MAbA2; C, MAbA1). Columns: a, HB101(pBR322); b, HB101(pANN801-13), *sfa*$^+$; C, HB101 (pANN801-116), Lys-116; d, HB101(pANN801-118), Arg-118; e, HB101(pANN801-122), Lys-122. Bars indicate standard errors. Trials were repeated two times.

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.

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

Plasmid	Amino acid change	Titer
pANN801-13	None	32
pANN801-116	Lys-116 → Thr-116	0
pANN801-118	Arg-118 → Ser-118	0
pANN801-122	Lys-122 → Thr-122	32
pBR322	Control	0

[a] Hemagglutination was carried out in microtiter plates as described by Jones and Rutter (11).

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

SfaS:	116	Lys	Ala	Arg	Ala	Val	Ser	Lys
K99:	132	Lys	-	Lys	Asp	-	Asp	Lys
CFAI:	56	Lys	-	Lys	Val	Ile	Val	Lys
CT-B:	62	Lys	-	Lys	Ala	Ile	Glu	Arg
LTI-B:	62	Lys	-	Lys	Ala	Ile	Glu	Arg

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.

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 Lys-62 and Arg-67 of the LTI and CT B-subunits in the receptor recognition process. It seems therefore that the positively charged amino acids lysine and arginine have an influence on the interactions of these proteins with the negatively charged sialic acid moieties.

In contrast, neither Arg nor Lys residues belong to the receptor-binding site of the influenza virus hemagglutinin (38). It was reported, however, that the sialic acid-containing oligosaccharides involved in the interaction with the various binding proteins differ from each other. While SfaS recognizes a terminal NeuAcα-2-3-lactose moeity (33), the influenza virus hemagglutinin binds to NeuAcα-2-6-lactose-containing receptors (39). The K99 adhesin, on the other hand, recognizes Neu5Gcα-2-3-lactose (29, 37), and the B subunits of the LTI and CT toxins interact with pentaoligosaccharides carrying a sialyl residue in an intermediate position (5, 13).

These variations in the carbohydrate structures may correspond to the differences in the protein sequences observed for the influenza virus hemagglutinin, the S adhesin SfaS, and the other sialic acid-binding proteins.

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