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The role of lysine-132 and arginine-136 in the receptor-binding domain of the K99 fibrillar subunit

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The gene encoding the K99 fibrillar adhesin of *Escherichia coli* has been modified by oligonucleotide-directed, site-specific, mutagenesis. The tryptophan-67, lysine-132, lysine-133 or arginine-136 were replaced by leucine, threonine, threonine and serine, respectively. The threonine-133 mutant fibrillae were indistinguishable from wild-type fibrillae. In contrast, replacement of lysine-132 or arginine-136 by threonine or serine, respectively, resulted in mutant fibrillae which had completely lost adhesive capacity, suggesting that the positive charges of these residues are essential for the interaction with the negatively charged sialic acid residue of the receptor molecules. After the replacement of tryptophan-67 with leucine neither fibrillae nor subunits were detectable, indicating that the mutant product is unstable and that tryptophan-67 has an essential structural role in the K99 subunit.

Key words: fimbriae/adhesin/K99/receptor-binding domain

Introduction

The ability of the pathogenic bacteria to adhere to certain host epithelia is of primary importance in diseases such as diarrhoea, gonorrhoea and urinary tract infections (Beachey, 1981). The enterotoxigenic *Escherichia coli* strains isolated from calves, lambs and piglets frequently express filamentous appendages, called K99 fibrillae. These K99 fibrillae are composed of identical repeating protein subunits with a mol. wt of 16 545 and were implicated in the binding of the bacteria to intestinal epithelial cells and some species of erythrocytes (Gaastra and de Graaf, 1982). The amino acid sequence of the K99 subunit has been deduced from the nucleotide sequence of its corresponding gene (Roosendaal *et al.*, 1984). A glycolipid, identified as Neu5Gc-α (2 → 3)−Gal*p*-β (1 → 4)−Glc*p*-β (1 → 1)−Ceramide (Smit *et al.*, 1984) has been found to function as K99 receptor on horse erythrocytes.

The role of specific amino acid residues in the recognition of receptor substances by K99 fibrillae has been examined by using chemical modification (Jacobs *et al.*, 1985). Modification of arginine residues with 2,3-butanedione or lysine residues with 4-chloro-3,5-dinitrobenzoate destroyed the adhesive capacity of the fibrillae at low levels of incorporation (< 1 mol incorporation/mol subunit). The modified lysine residues were mapped and it appeared that after the incorporation of 0.7 mol dinitrobenzoate per mol subunit, lysine-132 and lysine-133 had incorporated 27.5 and 52.5% of the totally incorporated label, respectively. Furthermore, both lysine residues were partially protected from modification in the presence of the ligand (Jacobs *et al.*, 1986).

Comparison of the primary structure of functionally related proteins may provide indications about structure function relationships for these proteins. Recently, we reported about the homology between the segment Lys-132−Arg-136 in the K99 subunit and segments of three other sialic acid-specific lectins; cholera toxin B subunit, heat-labile toxin B subunit of *E. coli* and the CFA1 fimbrial subunit, suggesting a common function in receptor binding (Jacobs *et al.*, 1986). Furthermore, biochemical studies on cholera toxin B subunit implicated lysine, arginine and tryptophane residues in the receptor-binding domain of this protein (Ludwig *et al.*, 1985).

In this study we used oligonucleotide-directed, site-specific, mutagenesis to change the codons for Trp-67, Lys-132, Lys-133 and Arg-136 in the primary structure of the K99 fimbrial subunit, to establish the role of these amino acid residues in the receptor-binding event.

Results

Synthetic oligonucleotides have been used to direct the mutation of the codons for Trp-67, Lys-132, Lys-133 or Arg-136 into codons for Leu-67, Thr-132, Thr-133 and Ser-136, respectively. Single-stranded DNA from a recombinant M13 mp11 bacteriophage containing the 1646-bp *Xba*I/*Sma*I fragment (Figure 1), served as a template for the synthesis of DNA primed with one of the mutagenic oligonucleotides and the universal M13 sequence primer as described in Materials and methods. Phages containing the desired mutations within the cloned K99 sequence were initially identified by differential hybridization to the appropriate mutagenic oligonucleotide and their identities were confirmed by DNA sequencing. The mutants selected were sequenced entirely, i.e. that part of the K99 gene that was used to reconstruct the mutant pAJ99, to check that no other mutations had occurred. In the case of the Leu-67 mutation the 192-bp *Bgl*II/*Bss*HII fragment was used to reconstruct the mutant pAJ99, in all other cases the 1276-bp *Bss*HII/*Sma*I fragment was used to reconstruct the mutant pAJ99 (Figure 1). The effects of the different amino acid substitutions on the antigenic, adhesive and structural characteristics of the K99 fibrillae were analyzed.

The Thr-133 mutant fibrillae were indistinguishable from wild-type fibrillae as judged by ELISA, haemagglutination assay (Table I), or SDS−PAGE (Figure 2). Furthermore, the mutant strain appeared to be normally fimbriated when examined under the electron microscope (not shown). In contrast, the Leu-67 mutant was unable to produce fibrillae using the same criteria. Moreover, the antigen was not detectable in ultrasonic extracts of the cells or in the culture medium.

Replacement of Lys-132 by threonine resulted in a total loss of adhesive capacity of the K99 mutant fibrillae (Table I). These mutant fibrillae were expressed to a normal extent and were immunologically indistinguishable from wild-type fibrillae (Table I, Figure 2). The mutant strains appeared to be normally fimbriated when examined under the electron microscope (not shown). A similar phenotype was observed for the Ser-136 mutant except that in this case some (< 1%) residual adhesive capacity was observed (Table I, Figure 2).

Fig. 1. Genetic map of cloned K99 DNA. The thick lines and thin line represent the cloning vehicle pACYC and the cloned DNA, respectively. The location of the various structural genes are indicated by boxes (A–H). The black ends of the boxes indicate the part of the genes coding for a signal peptide. The number beneath the boxes refer to the mol. wts ($\times 10^3$) of the corresponding polypeptides. The gene encoding the K99 subunit is shaded. The horizontal arrow indicates the direction of transcription. The cleavage sites for *Xba*I, *Bgl*II, *Bss*HII and *Sma*I are indicated. P means promoter sequence.

Discussion

Mutation of Lys-132 and Lys-133

In a previous paper we reported that modification of lysine residues with 4-chloro-3,5-dinitrobenzoate affects the adhesive capacity of the K99 fibrillae (Jacobs *et al.*, 1985). About 75% of the activity disappeared when 0.7 mol dinitrobenzoate was incorporated per mol subunit. At this stage of the modification reaction the modified residues were mapped and it appeared that Lys-132 and Lys-133 incorporated 27.5% and 52.5% of the total incorporated label (Jacobs *et al.*, 1986). To establish the specific role of Lys-132 and/or Lys-133 we used site-directed *in vitro* mutagenesis to change the codons of these lysine residues into a codon for a threonine residue. The small, indifferent amino acid residue threonine was chosen to minimalize effects on secondary structure. Replacement of Lys-133 by threonine had no apparent effect on the biosynthesis of the fibrillae nor on their ability to recognize receptor substances or antibodies. In contrast, substitution of Lys-132 for threonine resulted in a total loss of binding capacity, although in other respects these mutant fibrillae were indistinguishable from wild-type fibrillae. This result strongly suggests that the positive charge of the ϵ-NH_2 group of Lys-132 is essential for the interaction of the fibrillar subunit with the negative charge of the terminal sialic acid residue in the receptor molecule. In the chemical modification studies Lys-133 was shown to be twice as reactive as Lys-132 (Jacobs *et al.*, 1986) indicating that Lys-133 is more exposed to the external environment. We suppose that the receptor-binding site forms a cleft in the K99 subunit and that modification of Lys-133 with the bulky, negatively charged dinitrobenzoate group contributes to the observed effect on adhesive capacity of the fibrillae by steric hinderance and/or charge repulsion of the receptor.

Mutation of Arg-136

Chemical modification of arginine residues with 2,3-butanedione resulted in the loss of the binding capacity of the fibrillae (Jacobs *et al.*, 1985). Mapping of the modified residues is difficult because of the absence of a spectral probe and the strong reversibility of this modification (Riordan *et al.*, 1973). In a previous paper we reported about the homology between the segment Lys-132–Arg-136 of K99 and segments of three other sialic acid-specific lectins – cholera toxin B subunit, heat labile toxin B subunit of *E. coli* and the CFA1 fimbrial subunit – suggesting a common function in receptor binding (Jacobs *et al.*, 1986). Further support for this assumption is given by biochemical studies on the cholera toxin B subunit which implicated the presence of lysine and arginine residues in the receptor-binding domain of this protein (Ludwig *et al.*, 1985). For these reasons we chose to mutate Arg-136 into Ser-136. This mutation resulted in a dramatic decrease in the adhesive capacity of the fibrillae as was found for the Thr-132 mutant fibrillae. Both mutants have a similar phenotype although in the case of the Ser-136 mutant fibrillae some (< 1%) residual adhesive activity was observed. This could mean that Lys-132 is directly involved in a charge interaction with the negatively charged sialic acid residue of the receptor and that Arg-136 has a function in the initial attraction of the receptor and/or in stabilizing this interaction.

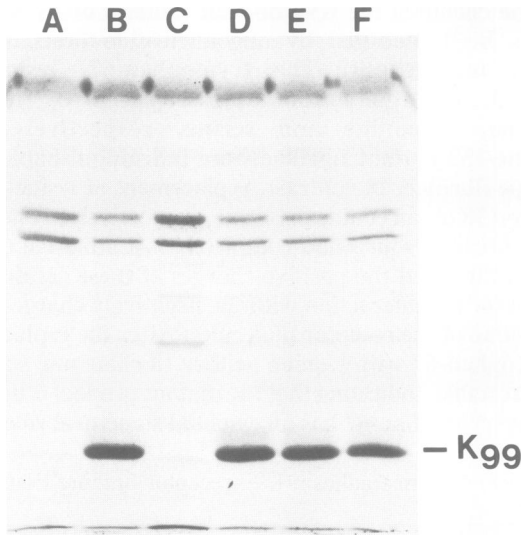

Fig. 2. SDS–PAGE of heat extracts of *E. coli* K12 without plasmid (**A**), or with pAJ99 or its mutant derivatives (**B–F**). (**B**) pAJ99, (**C**) pAJ99–Leu-67, (**D**) pAJ99–Thr-132, (**E**) pAJ99–Thr-133, (**F**) pAJ99–Ser-136.

Table I. Properties of pAJ99 and its mutant derivatives

Plasmid	ELISA[a]	Haemagglutination[b]
pAJ99	2^{21}	2^{15}
pAJ99–Leu-67	2^{0}	2^{0}
pAJ99–Thr-132	2^{21}	2^{0}
pAJ99–Thr-133	2^{21}	2^{15}
pAJ99–Ser-136	2^{21}	2^{5}
Without	2^{0}	2^{0}

[a]The amount of K99 fibrillae was quantitated in an enzyme-linked immunosorbant assay.
[b]The ability of the fibrillae to adhere to the receptors present on horse erythrocytes was determined in a quantitative haemaggutination assay. In both tests preparations of cell-free K99 were used. Comparable results were found when whole cells or ultrasonic extracts were used (not shown).

Mutation of Trp-67

Biochemical studies on cholera toxin B subunit also implicated, besides lysine and arginine, functional tryptophan residues in the receptor-binding domain of this protein (Ludwig *et al.*, 1985). Because of these data, and because the receptors for K99 and cholera toxin are very related, we changed the single tryptophan residue at position 67 into a leucine residue. The Leu-67 mutant was unable to express fibrillae as determined by SDS−PAGE, ELISA, haemaggutination assay and electron microscopy. Furthermore, the antigen was not detectable in the culture medium nor in ultrasonic extract of the cells. These data suggest that Trp-67 has an essential role in maintaining a stable conformation of the K99 protein subunit.

The K99 adhesin

Recent genetic evidence obtained with PAP, Type I and X-fimbriae, suggests that although these fimbriae function as carriers of adhesiveness, the adhesive capacity is not represented by the major fimbrial subunit but by specific adhesin molecules which consitute a minor component of the respective fimbriae (Lindberg *et al.*, 1984; Norgren *et al.*, 1984; Hacker *et al.*, 1985; Maurer and Orndorff, 1985).

The primary structure of some of these minor components has been deduced from the nucleotide sequence of the corresponding genes and shows the characteristic features of fimbrial subunit proteins (Lindberg *et al.*, 1986). Probably, these adhesin molecules co-polymerize with the major fimbrial subunits during their assembly into fimbriae. In this context, it should be noted that the amino acid sequence at the N-terminal site of residue Arg-136 is the primary structure of the K99 subunit shows a certain homology to a small stretch of amino acid residues located in the primary structure of P fimbrial subunits at a corresponding position. It cannot be excluded that this part of the structure is involved in a common feature of fimbrial subunits, i.e. their translocation through the outer membrane or their polymerization, including the co-polymerization of minor components essential for adhesion. Although we have no indication that also K99 fibrillae contain hitherto undetected minor components it seems likely that the K99 subunit itself represents the adhesive entity of the K99 fibrillae. First, the existence of homology between the K99 subunit and three other sialic acid-specific lectins: cholera toxin B subunit, heat labile toxin B subunit of *E. coli* and the CFA1 fimbrial subunit (Jacobs *et al.*, 1986). Second, chemical modification studies implicated both lysine and arginine residues in the receptor-binding domain of K99 and cholera toxin (Jacobs *et al.*, 1985; Ludwig *et al.*, 1985). Furthermore, the modified lysine residues were mapped and shown to be located in the homologous segment of K99 (Jacobs *et al.*, 1986). Third, the importance of Lys-132 and Arg-136 in the K99 fibrillar subunit was shown by site-directed *in vitro* mutagenesis (this paper). Fourth, solubilized K99 fibrillae show a strong haemagglutination whereas PAP, Type I or X-fimbriae do not. In the latter cases only aggregated fimbriae suspensions show significant haemagglutination (Lindberg *et al.*, 1984; A.A.C.Jacobs, unpublished). This suggests that K99 fibrillae are polyvalent. In this context it is interesting to note that solubilized K88 fibrillae also show a strong haemagglutinating activity and it has been shown that the major fimbrial component constitutes the adhesive entity (Mooi *et al.*, 1984).

It is tempting to speculate that during the course of the evolution the enteropathogen *E. coli* has developed the capacity to produce other adhesin molecules as additional minor fimbrial constituents to obtain the ability to colonize extra-intestinal epithelia.

Materials and methods

Materials

T4 polynucleotide kinase, T4 DNA ligase and T4 DNA polymerase I (Klenow fragment), were obtained from Pharmacia. The restriction enzymes *Sma*I, *Xba*I and *Bgl*II and the universal M13 sequence primer (15-mer) were from Boehringer-Mannheim. *Bss*HII was purchased from New England Biolabs. The mutagenic oligonucleotides (17-mers) were generously donated by Dr J.H.van Boom, Gorlaeus Laboratory, Leiden. [γ-^{32}P]ATP was obtained from Radiochemical Center, Amersham. All chemicals used were of analytical grade.

Plasmids, strains and phage

The plasmid pAJ99 is composed of a 6.7-kb *Bam*HI DNA fragment, carrying all genes involved in the biosynthesis of the K99 fibrillae as well as the vector pACYC. *E. coli* K12 C-600 (λ^-, *ton A* 21, *thr*$^-$, *leu*-6$^-$, *thi*-1$^-$, *sup E* 44, *Lac* $y-1^{stable}$) was used as a host for pAJ99 and its mutant derivatives. Restriction fragments were cloned into double-stranded DNA of phage M13 mp11 (Messing and Vieira, 1982) and single-stranded DNA was isolated after propagation in *E. coli* JM103 (Messing *et al.*, 1981).

Isolation of plasmid and M13 double-stranded DNA

For the isolation of plasmid and M13 double-stranded DNA the alkaline method of Birnboim and Doly (1979) was used.

Isolation of DNA restriction fragments

DNA restriction fragments were separated on horizontal 1.0% agarose slab gels and extracted from the agarose by electro-elution with an electrophoretic sample concentrator (ISCO).

Transformation

Transformation of $CaCl_2$-treated cells was performed according to the method of Dagert and Ehrlich (1979).

Oligonucleotide-directed in vitro mutagenesis

A 1646-bp DNA fragment, encompassing the gene encoding the K99 protein subunit (Figure 1), was cloned into the M13 mp11 bacteriophage and mutagenized by the double primer method as described by Zoller and Smith (1984). The following mutagenic oligonucleotides, 17-mers containing one mismatch (*) were employed: 5'AACCAGACA*AGTCAATA3' to change K99−Trp-67 into K99−Leu-67; 5'CATCTTTTG*TCAGCTGG3' to change K99−Lys-132 into K99−Thr-132; 5'GGTCATCTG*TTTTCAGC3' to change K99−Lys-133 into K99−Thr-133 and 5'ACGGAGCGCT*GTCATCT3' to change K99−Arg-136 into K99−Ser-136. Putative mutants were initially identified by colony probing, using the 5'-^{32}P-mutagenic oligonucleotide and successive washing at increasing temperatures (Zoller and Smith, 1984). DNA sequencing using the dideoxy termination procedure of Sanger *et al.* (1977) was used to confirm the mutations and to sequence that part of the K99 gene that was used to reconstruct the mutant pFK99, as control that no other mutations had occurred.

Preparation of cell-free K99 fibrillae and ultrasonic extract

The wild-type and mutant strains were cultured in Minca medium (Guinee *et al.*, 1976) supplemented with 0.1% yeast extract and ampicillin (200 μg/ml), and harvested by centrifugation at the end of the exponential growth phase. The cell pellets were suspended to an optical density of 10 at 660 nm in 50 mM sodium phosphate pH 7.2 supplemented with 0.9% w/v NaCl. The fibrillae were removed from the cells by heating the suspensions for 10 min at 70°C. Subsequently, the cells were spun down and the supernatant fractions were used for haemagglutination assays, ELISA and SDS−PAGE. For the preparation of ultrasonic extracts, the heat treatment was omitted and the cells were disrupted by ultrasonic treatment.

ELISA

The wild-type and mutant fibrillae were detected and quantitated by an ELISA as described by Mooi *et al.* (1979), using disposable polystyrene microtiter trays (Cooke). The trays were read with a micro ELISA-reader (Organon Teknica) at 492 nm.

Haemaggutination assay

The ability of the K99 fibrillae to adhere to horse erythrocytes was determined by quantitative haemagglutination assays. Serial 2-fold dilutions (50 μl) of cell-free K99, ultrasonic extracts or culture supernatant were made in KRTM buffer, followed by the addition of 50 μl of a 1% v/v suspension of washed horse erythrocytes in KRTM. The trays were examined after 3 h of incubation at 4°C. KRTM buffer is a 10 mM Tris−HCl buffer pH 7.4 containing 7.5 g NaCl; 0.383 g KCl; 0.318 g $MgSO_4.7H_2O$; 0.404 g $CaCl_2 \cdot 2H_2O$ and 5 g D-mannose per liter.

SDS–PAGE

SDS–PAGE was performed in 12% slab gels using the buffer systems of Laemmli (1970).

Electron microscopy

For negative staining, cells were suspended in 50 mM sodium phosphate pH 7.2 supplemented with 0.9% NaCl to a concentration of $\sim 10^{10}$ cells/ml. Droplets of this suspension were applied to Formvar-coated 400 mesh-grids. Attached cells were stained for 20 s with phosphotungstic acid (1%). The grids were examined in a Philips EM 300 electron microscope operating at 60 kV.

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